Investigation of acute clinical and chronic anaplasmosis on breeding soundness in beef bulls

by

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B.S., Murray State University, 2015

DVM, Texas A&M University, 2019

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine & Pathobiology

College of Veterinary Medicine

KANSAS STATE UNIVERSITY

Manhattan, Kansas

2023

Approved by:
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Abstract

The causative agent of bovine anaplasmosis, *Anaplasma marginale*, costs the U.S. cattle industry an estimated $300 million per year. Natural service breeding by bulls is common in U.S. beef cow-calf operations. Anemia and fever associated with clinical anaplasmosis may reduce bull breeding soundness and affect testicular tissue beyond resolution of clinical disease. Cattle that recover from acute clinical anaplasmosis transition to a chronic disease state and serve as transmission reservoirs of *A. marginale*. The impact of chronic *A. marginale* infection on reproductive performance parameters in carrier bulls is unknown.

The impact of anaplasmosis on bull breeding soundness was investigated among bulls with clinical anaplasmosis (Objective 1) and among bulls with chronic anaplasmosis (Objective 2). In Objective 1, breeding soundness examination (BSE) outcomes and clinical changes were evaluated in beef bulls over the course of clinical anaplasmosis and after recovery. In Objective 2, the prevalence of chronic *A. marginale* infection in bulls from eastern Kansas was investigated and breeding soundness parameters and overall satisfactory breeding potential rates were compared between *A. marginale* infected and uninfected bulls.

To investigate the effect of clinical anaplasmosis on bull breeding soundness, six healthy, *Anaplasma*-negative, mature, Angus bulls of satisfactory breeding status were included. Fresh blood from an *A. marginale*-infected donor cow was used to challenge three bulls, the other three remaining as unchallenged controls. All bulls were observed for disease progression and soundness of breeding. Fever, anemia via packed cell volume (PCV), pallor, and icterus were monitored weekly. Progression of anaplasmosis was evaluated via quantitative

PCR and percent parasitized erythrocytes (PPE). Seroconversion was monitored by cELISA. Injectable oxytetracycline was given to bulls with a PCV <15% or a temperature >105°F. Weekly BSEs using electroejaculation were performed on all bulls for 16 weeks. Breeding soundness parameters included sperm morphology and motility, external and internal genitalia exam, and physical exam. All *A. marginale*-challenged bulls were PCR-positive, seropositive, and showed clinical signs by 3-, 17-, and 24-days post-challenge, respectively. Common clinical signs of acute anaplasmosis included weight loss, pallor, icterus and fever (≥104.3°F). Acute anemia was observed in all challenged bulls with PCV nadirs ≤18% and peak PPEs ≥50%. Reduced breeding soundness outcomes were observed starting at the onset of clinical signs and continued for weeks beyond resolution of clinical anaplasmosis. Bulls in the control group remained negative for *A. marginale* by PCR and cELISA, and never developed dramatic reductions in breeding soundness outcomes.

To investigate the impact of chronic *A. marginale* infection on bull breeding soundness, 537 Eastern Kansas, client-owned beef bulls undergoing a routine breeding soundness examination (BSE) were enrolled. Complete BSEs were performed by recruited local veterinarians according to the Society for Theriogenology Manual for BSE of Bulls, 2nd edition. Breeding soundness examination parameters included sperm morphology and motility, palpation of external genitalia and internal accessory sex glands, and overall physical soundness. Blood samples were collected for packed cell volume determination and analysis of *A. marginale* infection status via quantitative PCR and cELISA. Logistic and linear regression methods were used to evaluate factors associated with *A. marginale* infection status and BSE parameters.

Prevalence of chronic *A. marginale* was 45.7% (245/537) among the sampled eastern Kansas bulls. Of the bulls with unsatisfactory BSE results, 53.6% were chronically-infected with *A. marginale*. Common reasons for unsatisfactory BSE results were poor sperm motility or increased abnormal sperm morphology.

Collectively, results from this program of work suggest acute anaplasmosis is a driver of reduced breeding soundness in beef bulls and prevalence of *A. marginale* infection among bulls tested is common and similar to eastern Kansas cow infection rates. Bulls that undergo acute disease can recover from or have prolonged evidence of reduced breeding soundness. Many bulls with chronic *A. marginale* infection retain overall satisfactory breeding potential. Outcomes from this research expanded information on the pathogenesis of anaplasmosis in bulls, regional prevalence, and the reproductive and production consequences of bulls exposed to *A. marginale*. Further research pursuits should include additional data collection on the *A. marginale* prevalence status of bulls and females in other areas of the U.S. and a larger clinical trial investigating the effects on testicular tissue in bulls during and after clinical anaplasmosis using advanced diagnostics and imaging. Studies investigating the inter-observer agreement of veterinarians performing BSEs on a specific subset of bulls are also warranted.

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Acknowledgements

There are many individuals and groups who helped make this work a reality. I would like to start with thanking the regional producers and veterinarians that participated in this study. This work is essential for the advancement of providing quality care to livestock and ultimately to agriculture. Thank you to the members of my committee who have provided guidance and support through the entirety of this project and continue to be valuable mentors. They have offered much of their time, knowledge, wisdom, suggestions, and encouragement throughout the project and are a significant reason for this project being one I am incredibly proud of. Thank you to those staff members involved at the Large Animal Research Center at Kansas State University that came through to help with care of the animals in rain, snow, and shine, 7 days a week. Thank you to the other faculty and staff members at Kansas State University that provided their assistance and time in animal care, sample processing and study design. Thank you to the members of the Reif lab that walked me through many of the laboratory protocols and worked equally as hard to prepare and process samples and report the data effectively. Thank you to my friends and family who supported me in all aspects of this project and the many challenges and victories along the way—and the versions of me that came with those seasons. Thank you to all of those involved who have taken the time to read my written reports; given feedback on writings, posters, and oral presentations; and attended out of support for those presentations that have emerged from this work. Funding for this project was provided by the State of Kansas Global Food Systems Seed Grant (0190 80040) and USDA-NIFA (2008-68003-27463, 2020-67015-31873). The funding agencies had no role in study design or collection, data analysis or interpretation, or manuscript preparation or review. Again, I

sincerely thank all producers that participated in this study and am so blessed to have a research project that greatly impacts the veterinary and agricultural sectors.

Dedication

This work is dedicated to the men in my life who paved the way for me by shaping my love for agriculture, people, animals, and growth in knowledge: Dad, Grandpa, Papaw, Robert, Joel, Philip, Zachary, “Doc” Davis, Mark Jackson, Brad Abraham, Michael Glade, David Gibson, Matt, Bryan, and Mark. And to the women who showed me we can: Mom, Grandma, Memaw, Olivia, Meredith, Kathy, Martha, Terra, Jennifer, Dusty, Jennie, Lindsey, Allyson, Leslie, Gina, Natalie, Shyanne, Kayla, Kathryn, Macy, Naemi, Shannon, Tip, and Emily.

Chapter 1 – Literature Review

Anaplasma marginale, a tick-borne blood pathogen of bovids, is a widely investigated infectious pathogen in the United States (U.S.) and has been for decades. Infection of cattle with this rickettsial pathogen that causes the disease anaplasmosis has lasting economic, herd, and epidemiologic implications, costing the U.S. cattle industry upwards of $300 million annually[1].

In U.S. endemic areas, natural transmission occurs when *Dermacentor* ticks attach to the bovine host for a bloodmeal and inoculate *A. marginale* via tick saliva into the host[2]. Other methods of transmission beyond biological tick transmission include mechanical transmission via biting flies and fomites such as needles or surgical instruments contaminated with blood from infected animals[3-5]. Transmission via biting insects is considered less efficient because these routes do not support *A. marginale* replication. Producers re-using needles in pre-clinical animals or missed clinical animals with high parasitemia risk the crisis of an outbreak. In the bovine host, *A. marginale* infects the red blood cells, and the percent parasitized erythrocytes (PPE) can reach up to 75%[5]. The incubation period can range from 7 to 60 days with an average of 28 days[2], depending primarily on the infectious dose, but may also be influenced by the *A. marginale* strain or immune status of the host[6]. The variation in incubation is an important consideration when animals may develop clinical signs and can make accurate diagnosis a challenge. There are multiple strains of *A. marginale* in the U.S., and they vary in morphology, antigen sequence, and transmissibility[6-8]. Independent transmission of multiple strains is possible and superinfection within an individual is possible. While it has been investigated as to whether or not preferential transmission occurs of a single strain over another due to

antigenicity of the strain, the evidence for this is lacking[8]. Peak transmission season for *A. marginale* is generally considered to occur in the late summer to early fall in the U.S. Cattle with depressed immune systems such as those with comorbidities may be predisposed to the development of more severe clinical disease. This may also be true for those animals in peak levels of production such as lactating cows or bulls on pasture in the breeding season which are stressed reproductively and physically, particularly when asked to service up to 60 females at a time[9]. Regardless of immune status, the bovine host experiences clinical signs associated with increasing levels of *A. marginale* bacteremia (≥10^7 bacteria/mL) and phagocytosis of infected erythrocytes by reticuloendothelial cells leading to extravascular hemolysis[10,11]. Classic signs of acute clinical anaplasmosis include anemia and icterus associated with phagocytized erythrocytes, and fever, associated with the developing systemic parasitemia. Infected animals can develop weight loss and lethargy, and occasionally behavior changes, the latter associated with systemic illness, reduced feed intake, and the reduced oxygen carrying capacity associated with the anemia[11]. Cattle older than three years of age typically succumb to more severe disease and even death; mortality rates in this age group can reach up to 49% while younger animals are less likely to experience overt clinical disease (though they are still susceptible to infection)[2,12]. Convalescence can last from weeks to months corresponding with increasing erythropoiesis[12]. Younger cattle are more likely to recover and most recovered cattle (regardless of age) become lifelong, chronic carriers of the organism. Chronic carriers are considered to have concomitant immunity from developing clinical disease upon re-challenge with the original infecting strain of *A. marginale* or antigenically similar strains. These carriers experience waxing and waning levels of parasitemia with the emergence of antigenic variants

re-infecting erythrocytes. This cyclical nature of the rickettsemia occurs about every 5 weeks in the persistently infected carrier[10,13,14]. Persistently infected carriers also serve as a reservoir of *A. marginale* for transmission to the rest of the herd via biological or mechanical transmission, and can be infected with multiple strains[3,10]. A common scenario in which this may occur is during annual vaccinations when needles are reused between different cattle. Carrier animals are also at risk for recrudescence especially during times of stress or immunosuppression[15]. Cattle can experience stress throughout their productive life, such as during transport, comingling groups of animals, and at vaccination. In females this can occur around parturition, during high yield lactation and surrounding weaning and in natural service bulls particularly in production settings where they are asked to service higher volumes of females.

There are multiple considerations in the diagnostic approach to bovine anaplasmosis. A characteristic of *A. marginale* is the presence of major surface proteins (MSPs), with MSPs that are both highly conserved across strains and those that demonstrate significant variation. These MSPs play a role in evasion of the host immune response, persistence of infected carriers, and are used as a targets for diagnostic assays and vaccine development[16,17]. There are both serological and molecular assays available for diagnosis of bovine anaplasmosis. The serologic assay currently, and most commonly, used is the competitive ELISA (cELISA) that detects host antibody against *A. marginale* major surface protein (MSP) 5, a highly conserved protein across *A. marginale* strains[18]. The cELISA is a useful, relatively inexpensive tool to identify cattle likely actively infected with *A. marginale*[19]. The sensitivity and specificity of the assay vary by population as well as the percent inhibition cut-off value used to consider an animal seropositive for seronegative for *A. marginale*[6,20]. In early acute disease, false negative

outcomes can occur prior to seroconversion. Though less important in the U.S., the cELISA can cross react with other species of *Anaplasma*, so results should be interpreted with caution in areas where other *Anaplasma* species are prevalent. There are other serologic options that have been used historically used to detect exposure to *A. marginale* such as the card agglutination and complement fixation test, however the accuracy of these earlier assays are highly variable and the interpretation of the card agglutination test in particular is subjective[6]. While the gold standard for detecting infected animals is via the inoculation of splenectomized calves, this presents several issues logistically, financially, and isn't the most judicious use of animals[21,22]. The primary molecular assay strategy used in diagnosis of anaplasmosis is the quantitative PCR (qPCR). The qPCR targets conserved genes like the 16sRNA gene or *msp5* and is considered to be 1,000 times more sensitive than end-point PCR. This diagnostic approach is generally more expensive[21,23]. The qPCR is an important component of diagnosis of animals in acute clinical disease due to the detection of *A. marginale* in animals that haven't seroconverted, and its ability to evaluate active *A. marginale* infection status in animals at any stage of disease. The possibility for false negatives outcomes exists for qPCR assays if parasitemia is very low (below the limit of detection) during cyclic rickettsemia. Other ancillary diagnostics particularly during acute clinical disease include packed cell volume (PCV) to detect anemia and light microscopy to identify the percent parasitized erythrocytes (PPE) with Giemsa staining[24]. Investigating the PPE has the greatest utility during peak levels of parasitemia, as chronically infected carriers have numbers of infected red blood cells as low as 0.000001%, well-below the limit of detection via light microscopy[11,25].

Management of anaplasmosis among cattle in endemic and naive herds pose many challenges, with management options including vaccination, injectable antimicrobials, and antimicrobial-medicated feed and mineral[8,26]. During clinical disease, injectable antimicrobials are commonly utilized therapeutics. One of the most widely used injectable antimicrobials for clinical signs associated with anaplasmosis is oxytetracycline (200 mg/mL), which is administered under the skin (20 mg/kg SQ q24-48 hrs). More recently, another injectable product that was conditionally labelled for anaplasmosis in cattle—enrofloxacin (100 mg/mL, 12.5 mg/kg SQ once)— has proven comparable to oxytetracycline for treatment of acute anaplasmosis[21,27], however this product is no longer on the market and is now illegal to administer to treat anaplasmosis per label indications. Occasionally but infrequently, anti-inflammatory products are included in the treatment of individual animals. While flunixin meglumine is an appropriate medication for pyrexia and inflammation associated with systemic inflammation, the intravenous product can be a challenge to administer to bulls, especially when needed on a daily basis. Despite the transdermal flunixin meglumine product on the market providing ease of administration, it is not labelled for use in breeding bulls.

Feeding of chlortetracycline (CTC, 1.1 mg/kg) in mineral or a mixed ration is commonly used in endemic areas especially during peak tick season in the U.S. (May – September)[26]. The aim of feeding CTC-medicated feed products in endemic populations is mitigating clinical signs rather than complete clearance of the organism[28]. The option to feed CTC to animals such as purchased bulls in endemic areas should be strongly considered to control active anaplasmosis. Antimicrobials, in summary, have a place in the management of bovine anaplasmosis but do not guarantee complete protection from infection nor clinical disease.

Vaccination would be an ideal management strategy, however, no fully licensed anaplasmosis vaccine is currently available in the U.S., highlighting the need for further research in this area[2,29]. The conditionally-labelled killed vaccine product[30] on the market was formulated from an individual strain of *A. marginale*, and no data is available on the efficacy of this vaccine. Control of anaplasmosis via vaccination remains a challenge as animals can carry and be exposed to multiple strains of *A. marginale*. Important and practical considerations when developing an anaplasmosis vaccine would be cross protection against heterologous strains and duration of immunity. Some have considered "vaccinating" naive animals with blood of infected animals in endemic herds, though there are ethical implications that make this strongly unadvised. Particularly, injected blood from one individual animal to another does not control for transmission of other infectious organisms.

Topical acaricides and insecticides are included in anaplasmosis management strategies, though similarly to antimicrobials, their efficacy is not complete, and these products pose concerns for environmental contamination and tissue residues[4].

Producers moving or introducing new cattle into their herd should consider within-herd endemicity and individual disease status prior to selling cattle or bringing in new introductions to the herd. Producers selling bulls living in endemic areas should have bulls tested for their *A. marginale* status by cELISA or qPCR prior to sale. Regardless of implemented anaplasmosis control methods, any bull positive for *A. marginale* by diagnostic test should be considered infected (and likely will be infected for life) and considered a transmission reservoir and risk for the rest of the herd. Use of artificial insemination during peak vector season to minimize vector-associated transmission risk could be considered. In general, adherence to the mainstays

of anaplasmosis control in cow-calf herds including minimizing vector exposure, avoiding the re-use of needles and blood-contaminated equipment, treating clinical animals, and control of active anaplasmosis via the feeding of CTC should all be important considerations for managing bulls as well.

Depending on necessary management strategies, the economics and labor involved in mitigation, prevention, and treatment of anaplasmosis among herds in the U.S.—particularly in endemic areas such as Kansas— can be extensive. Short- and long-term production losses from anaplasmosis such as abortion and fetal loss, and weight loss in female beef cattle have been reported[31,32]. Other studies have identified altered production parameters such as decreased milk production and undifferentiated fever associated with carrier status in dairy and feedlot cattle[33,34]. However, studies investigating the clinical and reproductive effects of acute clinical anaplasmosis and chronic anaplasmosis among breeding bulls are few[35,36]. Swift et. al[36] investigated the impacts of clinical anaplasmosis on bull reproduction and libido in 1979 with promising initial results, including the observation of reduced libido, testicular degeneration, and abnormal sperm morphology, though much of the morphologic abnormalities of sperm were not quantified in detail. Camejo et. al[35] investigated the prevalence of various hemotropic organisms—namely *A. marginale*, *Trypanosoma vivax*, and *Babesia* spp. – in asymptomatic or carrier bulls and their effects specifically on testosterone levels in bulls. Results from that study demonstrated high prevalence in the Venezuelan-sampled bull population and reduced testosterone levels, most significant in bulls with *T. vivax* in this population. Multiple studies have been performed investigating anaplasmosis prevalence in female cattle[37-39], but minimal data is present for bulls, particularly in the U.S. Investigations are needed to establish

prevalence data among U.S. bull populations and to identify specific alterations to breeding soundness in bulls undergoing clinical anaplasmosis.

Bulls represent a significant source of profitability and investment to the cow-calf producer. In the U.S., there are approximately 2.03 million head of bulls over 500 pounds, with 85,000 (4.24%) of those in the state of Kansas, which accommodates the second largest seedstock producer in the country[40,41]. The importance of bulls to the genetics and vitality of a cow-calf herd continues to be emphasized within the U.S. cattle industry, with average sale prices nearing $5,000 each season[42]. Bulls also comprise the most common class of cattle brought into beef cow-calf operations as a new addition in the U.S., nearing 52,000 beef bulls sold each year[42,43]. Bull fertility is essential to the profitability of a cow-calf operation in which bulls are expected to service large numbers of cows in their productive life. Many factors can affect bull fertility such as nutrition, stocking density and bull-to-cow ratio, climate, herd health and management practices. There is an estimated 20-40% of bulls in U.S. cow-calf operations that are considered to be sub-fertile[44]. Sub-fertile bulls detrimentally impact a cow-calf operation in several ways. They can reduce the overall calf crop in volume and alter the calving season with delayed or no conception, incurring cost to the producer from losses in the calf crop with reports up to $3,000, which can be even higher if replacement bulls are required. Sub-fertile bulls can perpetuate substandard genetics in many ways. Bulls producing ejaculate with high number of sperm abnormalities capable of fertilizing the ovum can lead to lower quality or unsuccessful embryos. Bulls can perpetuate certain abnormal sperm morphologies to male offspring, along with factors leading to decreased fertility such as reduced libido or poor foot and body conformation[45-47]. The reproductive losses, morbidity, and mortality of bulls due

to disease can be devastating to a cow-calf producer. The anticipated marketing and movement of bulls to various geographic locations poses a risk for disease transmission such as anaplasmosis, particularly to naïve bulls moving from non-endemic to endemic areas. A thorough understanding of infectious diseases in bulls, and assessment of the marketed breeding bull in the U.S. via breeding soundness examination (BSE) are thus imperative to the industry, particularly seedstock and cow-calf producers.

The BSE of the beef bull is a commonly utilized tool for the veterinary practitioner to identify reproductive soundness and potential areas of reduced fertility, with the exception of assessing libido. According to Oko and Barth (1984), "Ultimately, the objective of semen evaluation is to select bulls with the potential for high fertility, whether for use in natural service or in artificial insemination (p. 278)"[48]. The BSE consists of a general physical examination, evaluation of external and internal genitalia including scrotal circumference, and collection of a semen sample via electroejaculation for evaluation of semen quality per sperm progressive motility and morphology. Minimum requirements for passing the BSE are listed in Table 1. The main component not included in the standard bull BSE is assessment of libido, ability to perform the act of copulation, or to service a given volume of females, which is best evaluated on farm with the herd of origin[46]. Other factors not commonly investigated on routine BSE include diagnosis of diseases in which bulls can be asymptomatic carriers, such as *A. marginale*, among others such as Bluetongue virus (BTV), bovine venereal campylobacteriosis, trichomoniasis, Bovine Leukemia Virus (BLV), and Bovine Viral Diarrhea Virus (BVDV).

Table 1. Minimum Breeding Soundness Examination (BSE) parameters required for passing a BSE. Based on the standard Society for Theriogenology BSE form[49], included are a basic overview of the minimum requirements for a bull to receive satisfactory breeding status on a routine BSE.

Parameter	Minimum requirements for passing
Physical exam	No obvious lameness, ocular disease, or other signs of systemic illness
Body condition score (BCS)	≥5/9
Scrotal circumference	≥34 cm in ≥24 months of age
Internal and external genitalia	No visible abnormalities on palpation, visualization, etc.
Sperm normal morphology	≥70% Normal morphology
Sperm progressive motility	≥30% Progressive motility

Major components considered in the general physical exam include a nine-point body condition score (BCS), ocular health, skin, and condition of the feet and legs[49]. These are all particularly important considerations for the natural service beef bull covering multiple females on pastures. Normally, bulls cover around 30 females per season, though some operations manage their herd with bulls that are expected to cover 50-60 cattle during a breeding season. Bulls must not have any signs of lameness, eye lesions, or poor BCS in order to pass a BSE[46]. Scrotal circumference (SC) has been directly linked to fertility in mature bulls, with SC reaching peak size around six years in the reproductively healthy beef bull[49]. Minimum SC in bulls over two years of age is 34 cm (Figure 1).

Figure 1. Measuring scrotal circumference (SC) in cm. Scrotal tape is used around the widest girth of the scrotum to obtain the most accurate SC measurement.

The penis, testicular texture, sheath, and prepuce are all assessed on the BSE, and the bull is expected to achieve full extension of the penis via electroejaculation. Internal genitalia are assessed via rectal palpation in addition to the inguinal rings and include the paired ampullae, seminal vesicles, and the prostate. These are palpated for presence of symmetry, lack of discomfort upon manipulation, or presence of abnormalities impairing reproductive

soundness. These abnormalities may include unilateral enlargement or firmness consistent with active infection, inflammation, or more chronic lesions such as fibrosis. The accessory sex glands lubricate the sperm cells during ejaculation and provide energy, stimulate metabolism, and assist in sperm transport among other functions[48], so optimum health of these glands are essential to fertility. Sperm progressive motility is typically evaluated via light microscopy chute-side, immediately after obtaining ejaculate. Various factors can influence assessment of motility and include both patient and environment factors, including timing of evaluating the sample after ejaculation, extreme ambient temperatures, sperm concentration of the sample, sperm morphology, experience of the veterinarian, and microscope quality[46]. Sperm morphology is typically evaluated with a live/dead semen stain such as Eosin-nigrosin stain via light microscopy and can be performed chute-side or in clinic.

The benefits to chute-side assessment of morphology include timelier recommendations regarding bull breeding soundness to the producer. However, analysis of sperm morphology is the most time-consuming component of the examination. Factors that affect assessment of sperm morphology include slide preparation, experience of the veterinarian, and integrity of reproductive tissue—especially testicular tissue[50]. Sperm morphology is perhaps the primary patient factor impacting progressive sperm motility[46,51]. The fertile breeding bull is expected to have superior numbers in sperm morphology and motility, to enhance chances of successful fertilization in the post-coitus female, where peak numbers of sperm cells do not appear at the oviduct until 18 hours after copulation[48].

The testicular tissue is the epicenter for sperm production. Spermatogenesis begins in the testicular tissue and is under the influence of several hormones, including gonadotropin-

releasing hormone (GnRH), testosterone, and estrogens[52]. Multiplication and division of spermatocytes occurs in the seminiferous tubules[48]. At any given point in the cycle, the seminiferous epithelium contains sperm cells of varying levels of maturity (eight general classifications of maturity, Figure 2)[52,53].

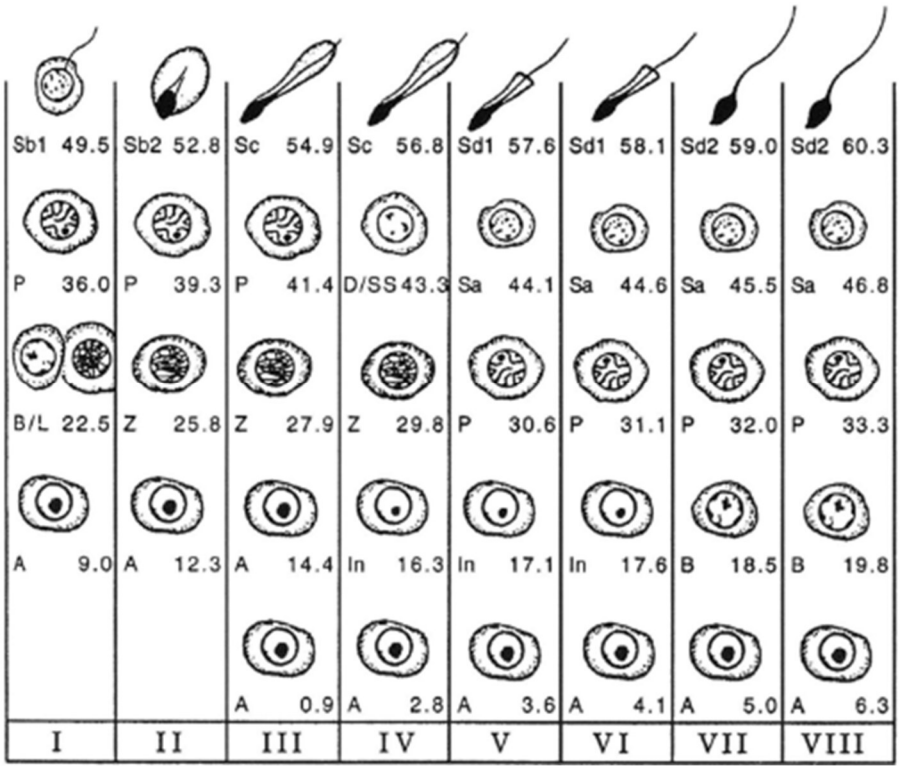

Figure 2 . Expected sperm cells at each of the eight stages of the cycle of seminiferous epithelium. The above figure was adapted from Staub & Johnson (2018). Capital letters on the left of the columns represent each of the types of sperm cells. A, B, and In represent spermatogonia; L, Z, P and D represent various stages of primary spermatocytes; SS represent secondary spermatocytes; Sa, Sb1, Sb2, Sc, Sd1, Sd2 represent spermatids[53].

The two main cells of the testicular tissue include the Sertoli cell and the Leydig cell (Figure 3 and Figure 4). The Sertoli cell is the main cellular component of the blood-testis barrier. It also provides a physically and nutritionally supportive framework for developing

spermatocytes allowing for optimum growth and movement during spermatogenesis and maturation[48,49]. The Leydig cell is under the influence of luteinizing hormone and produces androgen, primarily testosterone, in the testicular tissue to support spermatogenesis and libido[54]. After development in the testes, in the seminiferous tubule, sperm cells travel to the convoluted rete testis and finally to the epididymis, in which final maturation, travel, concentration, and storage of fertile sperm cells occur[55-57]. This final maturation process through the epididymis before entering the ejaculate typically requires 8-11 days[48,49,52], and can be an important location for insult related to environmental changes such as extreme ambient temperature due to its externally located position within the scrotum. The entire spermatogenic cycle for producing a fully mature, fertile sperm cell is approximately 63 days. The normal duration of the spermatogenic cycle is why many practitioners recommend a 60-day waiting period for bulls to be retested after an unsatisfactory BSE.

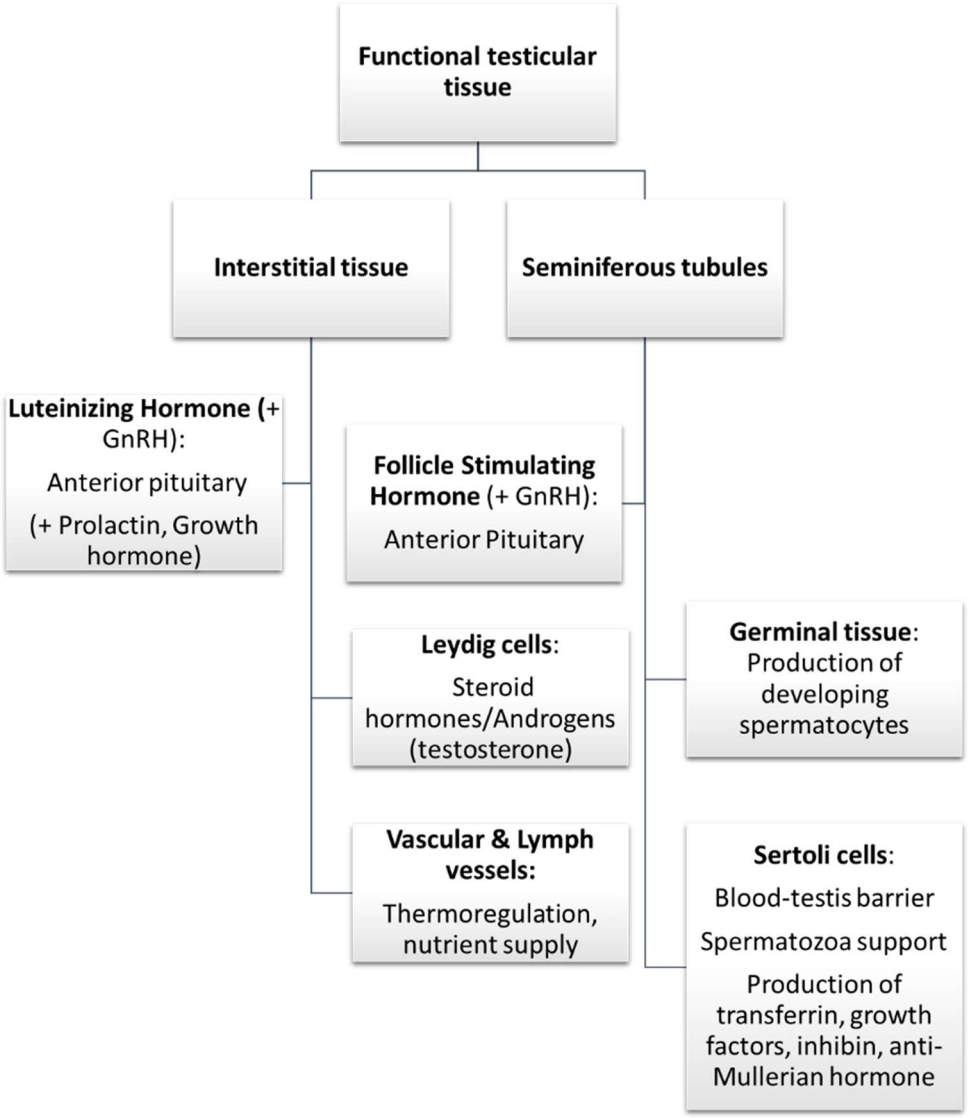

Figure 3. Components of functional testicular tissue. The interstitial tissue is composed of Leydig cells and vascular and lymph supply. The Leydig cells are under the influence of luteinizing hormone (LH) and produce steroid hormones, namely testosterone, in the bull. The seminiferous tubules contain the germinal tissue and Sertoli cell. The germinal tissues support the developing spermatozoa. The Sertoli cells are under the influence of follicle stimulating

hormone (FSH) and are responsible for their role in the blood-testis barrier and support of the developing spermatocyte.

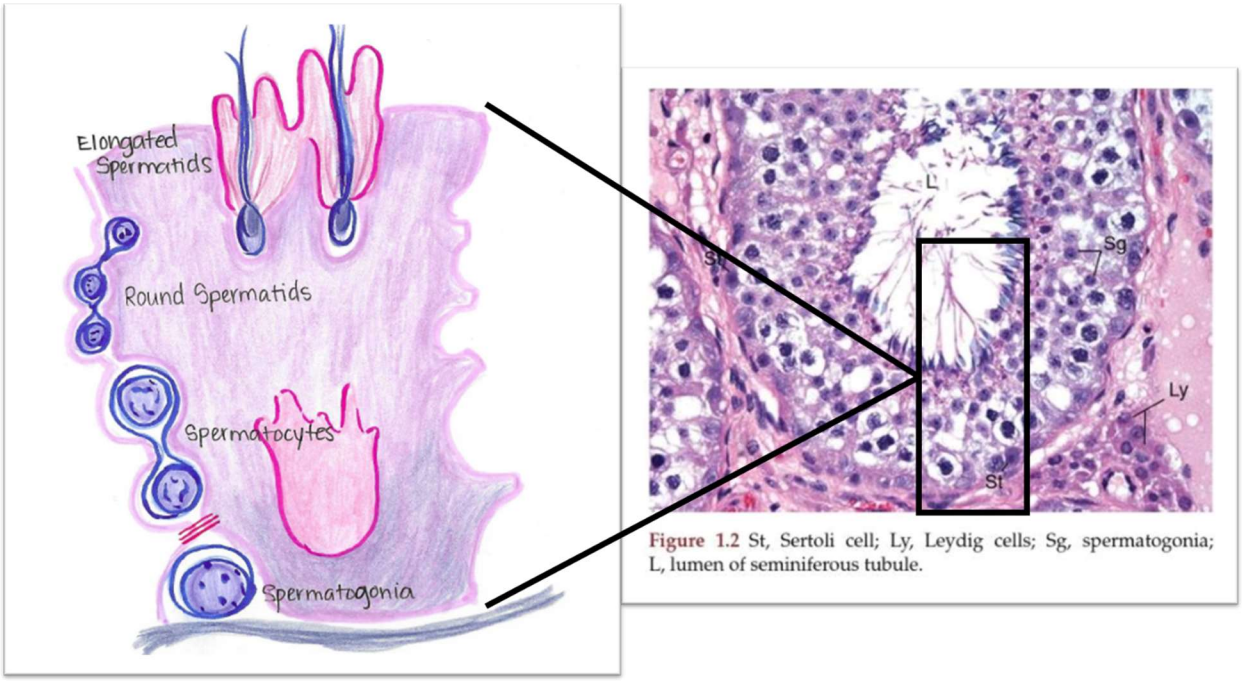

Figure 4. Simplified diagram of spermatocyte maturation and histology of the seminiferous epithelium. The above figure was adapted from a personal drawing and Hopper (2021). The image on the right demonstrates histology of the seminiferous tubule and adjacent Leydig cells[46]. The image on the left is a simplified schematic of the maturation of the spermatocyte from the spermatogonia to the elongated spermatid at the lumen of the seminiferous tubule (personal drawing).

The basic anatomy of the sperm cell includes the head and tail components. The sperm head includes the anterior-most acrosome and the post-acrosomal sheath. The tail includes the proximal-most neck, midpiece, principal piece, and end piece, which are all incorporated by a surrounding membrane[54]. Bulls can experience reductions in fertility at the level of the

testicular tissue, sperm cell, other genitalia, and by anything causing lethargy or reduced libido. Bull testicular tissue is notably thermolabile, and alterations in thermoregulation of the testicles can strongly influence successful spermatogenesis and overall fertility.

The scrotum acts as a "thermoregulatory device" to testicular tissue[54]. Any factor that impairs thermoregulation of the testicles such as body condition, environment, trauma, or systemic illness can impair spermatogenesis[58]; thus, insult due to factors such as fever and anemia—including those experienced during clinical anaplasmosis—at any point of spermatogenesis can lead to impaired production of sperm and bull sub- or infertility[59-61]. Testicular hypoxia brought on by thermal dysregulation is an important driver of these unsatisfactory BSE outcomes, which impacts the spermatogenic endocrine pathways, bull libido, and semen quality[46]. Whether thermal stress on testicular tissue is internal from fever or external from the environment, there are multiple mechanistic causes of testicular hypoxia and subsequent reduced fertility. First, the highly coiled testicular vasculature creates a counter-current heat exchange between arterial and venous blood, which even in the increased metabolic demand of the diseased state leads to an overall reduction or lack of change in blood flow in the presence of increased testicular heat[51,54,62]. In considering the mechanisms of testicular hypoxia in the bull, it should be emphasized that in the presence of scrotal heating whether by fever or environmental causes, blood flow remains low despite drastic increases—up to 67%— in oxygen consumption by the tissues[54,63]. This stress to the testicles is not only exacerbated by fever, which increases testicular temperature and metabolic demand, but also in instances where syndromes such as anemia further contribute to low blood flow to testicular tissues. Second, thermal insult to the germinal epithelium can impair spermatogenesis, with the

potential for degeneration of the tissue and direct insult to the sperm cell[54,64-66]. Much of the damage to testicular tissue caused by hypoxia is a product of increased free radical formation with subsequent suppression of antioxidant enzymes such as superoxide dismutase[51]. Free radicals, when present in excess, are particularly harmful to sperm cells, and can lead to oxidative stress and impaired thermoregulation of testicular tissue[65,67-69]. At the level of the sperm cell, excess free radicals from hypoxia can lead to membrane damage and DNA fragmentation within the sperm head, leading to increased numbers of abnormal sperm cells, decreased total sperm cell count, and impaired fertilization[67]. In addition, excess reactive oxygen species (ROS) can exacerbate the immune response, leading to increased phagocytosis of cells and inflammation of reproductive tissue and germinal epithelium[70]. Finally, in the presence of heat stress, the nutrient metabolism, energy production, and steroidogenesis of substances such as testosterone in the Leydig cell is suppressed and impairs processes such as the uptake of oxygen by glucose in the testis and stimulation of secretions by the accessory sex glands[51,54,71].

Impaired thermoregulation of bull testicles can also be exacerbated in ongoing systemic illness like anaplasmosis, beyond the driving factors of anemia and fever. Bulls may experience loss of body condition, which is particularly concerning for testicular tissue in colder weather, when scrotal fat insulation from extreme cold ambient temperature is essential[72]. A bull in the diseased state also has an increase in cortisol production, and glucocorticoids have been shown to suppress luteinizing hormone (LH) and subsequently testosterone in bulls[58,73]. While this endocrinologic suppression by cortisol can transiently impair spermatogenesis, it can also lead to testicular degeneration and impaired spermatogenesis[66]. Additionally, lethargic bulls may

have a greater tendency to lay down, where scrotal thermoregulation can be impaired by proximity to the body and impaired blood flow from the pressure of recumbency[54]. There is normally a 2-7°C difference between the testicular tissue and the body, dictated by internal body temperature and scrotal temperature[54]. Much of the work investigating fertility in bulls related to this testicular heat stress is in regard to environmental heat and humidity[74-77], with temperature humidity index (THI) used to measure the degree of environmental thermal stress on the testicles by accounting for both air temperature and relative humidity[77,78]. Cattle are generally thought to experience heat stress at THI indices ≥70, and bull fertility can certainly be impaired by the insult of heat stress[65,76,77,79].

Abnormal sperm morphology can be hereditary or acquired. Many potential sperm morphological abnormalities are demonstrated in Figure 5. In general, acquired causes of abnormal morphology such as systemic illness—the focus of this work—as well as changes to body condition and environmental insult carry a better prognosis for fertility as these can often be overcome depending on duration and severity of insult[48,54].

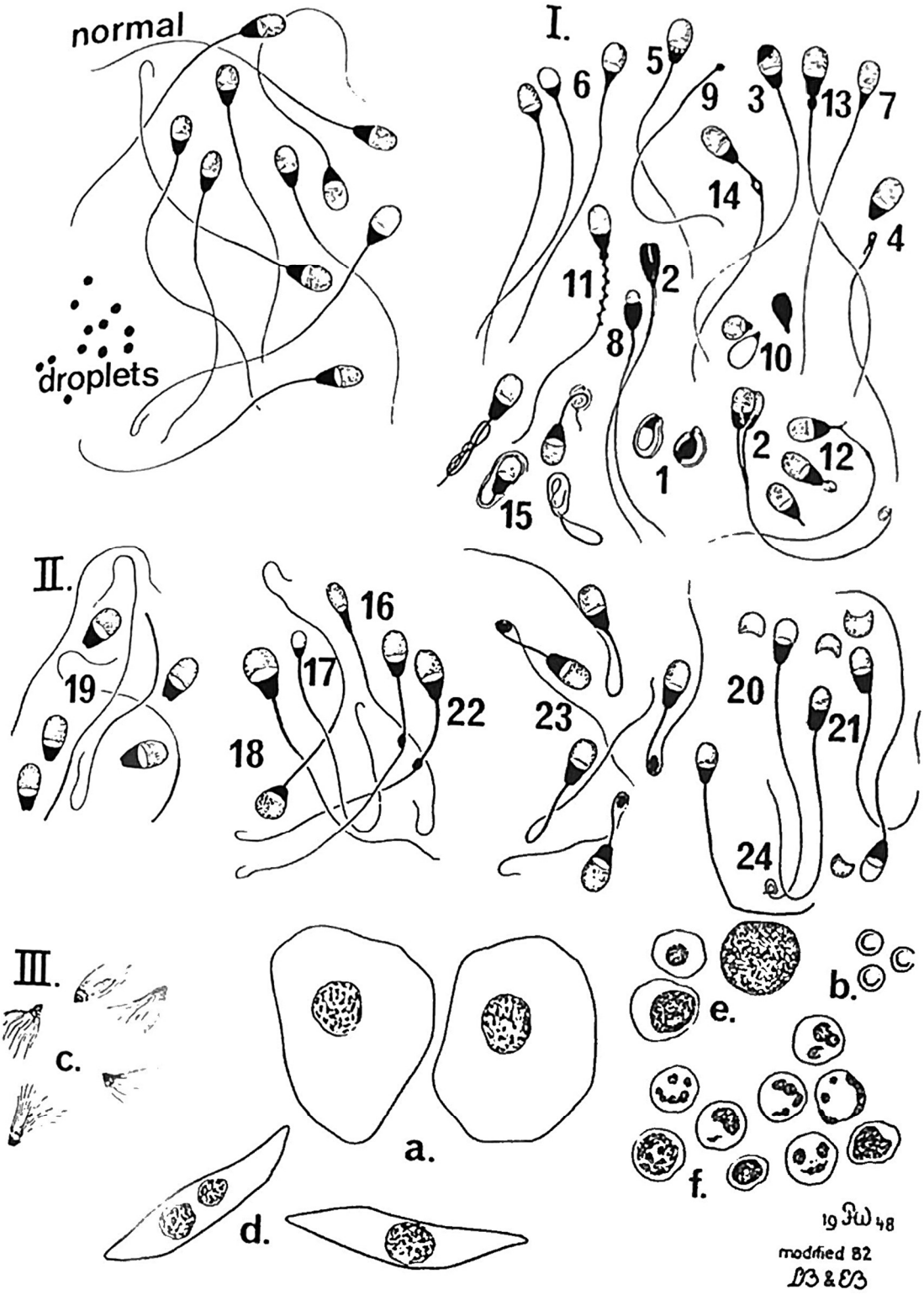

FIG. 8.1. Survey of the formed elements that may be found in bull semen. (Courtesy of E. Blom)

Figure 5. Survey of formed elements that may be found in bull semen. Image from Barth & Oko (1989)[48]. Examples of abnormal sperm morphology of mature spermatocytes (I, II) and examples of immature spermatocytes (III).

Abnormal morphologic changes of the sperm cell can be further categorized into compensable and non-compensable defects to better determine fertility at the time of BSE[46,50,80]. Compensable defects are those sperm morphological abnormalities which allow the bull to achieve maximum fertilization by increasing the sperm dose, such as coiled tails. Non-compensable defects are those sperm morphological abnormalities unable to achieve fertilization regardless of sperm dose, such as acrosomal defects[80]. These defects are typically associated with abnormalities in the DNA and interference with chromatin within the sperm head and can be induced by elevated testicular temperature. The importance of these defects are highlighted during the fertilization process in the female. Abnormal sperm cells with non-compensable defects are capable of reaching the ovum in the female, penetrating the outer zona pellucida, and causing a reaction in the ovum which blocks polyspermy, or the ability of other normal sperm cells to achieve fertilization by ovum penetration[47]. Abnormal sperm cells with compensable defects are considered very unlikely to reach the ovum due to abnormalities impairing motility in particular, which allow the potential for normal spermatocytes to reach the ovum and achieve fertilization. Thus, it is essential to differentiate between these defects during a BSE. Most bulls will have some collection of abnormalities during any given sampling period, and those bulls with non-compensable defects are considered of lower fertility than those with compensable defects[80]. Estimation of prognosis for fertility in bulls with sperm abnormalities depend primarily on the reason for the observed sperm defects and the volume

of observed sperm abnormalities. If the suspected insult was temporary or could be explained from external circumstances, many sperm abnormalities are transient and prognosis for fertility is good. Bulls with high numbers of specific abnormalities in the semen, particularly abnormalities of the head or non-compensable defects, or consistent abnormalities in subsequent ejaculations, should be considered to have poorer prognosis for overall motility[81]. In addition to categorizing these defects, an experienced practitioner utilizes the observed abnormalities in a bovine semen sample to make a conjecture about the approximate timing of insult within the spermatogenic cycle[48,58,65,79]. The primary spermatocyte is particularly sensitive to insult due to high levels of polyunsaturated lipid content in the cell, with sequential changes appearing beginning with the cytoplasm of the sperm cell[48,51,66].

The analysis of the described abnormalities in bull semen samples is variable among veterinary observers. There is a paucity of data regarding the variability among veterinary observers performing BSEs[82,83]. This is likely related to the fact that most bulls will be evaluated by a single veterinary observer and any bull has fluctuations in semen quality during a given sampling period. However, it remains to be considered that discrimination of the bull BSE varies not only on environmental circumstances and resources utilized, but also in the skill, comfort, technique and experience of the veterinarian[46,84]. Particularly, if a novice veterinarian is not trained in identifying more subtle non-compensable defects of the sperm head, there is risk in making positive recommendations about a beef sire to a cow-calf producer that otherwise would require re-evaluation due to sub-fertility at best[50]. On the contrary, a bull that has high numbers of compensable defects, or say, defects related to low ambient temperatures, would best be given a deferred status or retested on the notion that the observed abnormalities were

related to temporary and minor insult. External insult to the testicles is commonly associated with extremes in ambient temperatures and first affect the sperm cells in the epididymis, which is most externally located within the scrotum. Defects associated with minor insult, or those not detrimentally impacting the testicular tissue and seminiferous epithelium, would be associated with insult to sperm cells being stored in the epididymis. These would include potential abnormalities such as acrosomal defects, free normal heads, and altered membrane integrity like a fragmented tail[79]. Mature sperm cells stored in the epididymis are much less likely to experience DNA damage in the presence of insult, compared to those cells still undergoing spermatogenesis. Additionally, the presence of observed sperm abnormalities consistent with DNA damage should exclude the consideration for minor insult. Additionally, it should be noted that even compensable defects, if present in high enough numbers, cannot achieve a high enough semen dose to overcome the abnormalities and improve fertility.

Anaplasmosis causes production losses seen at both the herd and individual level. Breeding bulls are a vital asset to cow-calf operations in the U.S. and management to optimize fertility is essential. Given the endemicity of *A. marginale* in several areas throughout the U.S., and the asset a breeding bull is to a cow-calf operation, further research is warranted into the short and long-term effects of anaplasmosis on reproductive soundness in bulls.

Chapter 2 – Satisfactory breeding potential is transiently eliminated in beef bulls with clinical anaplasmosis

Bull fertility is a key component to success and profitability in commercial cow-calf operations. In July 2021, the United States Department of Agriculture's National Agricultural Statistics Services reported an estimated 2.1 million beef bulls in the United States (U.S.) and 40.9 million beef cows of calving age [85]. In the U.S., 92.9% of commercial beef cows are naturally bred by bulls [43,86], highlighting the importance of sound bull reproductive performance to the cow-calf industry. On average, an individual commercial Angus bull over 3 years of age in the U.S. costs $2,500-5,000 and is expected to service up to 60 head of cows and heifers in a breeding season. Fertile bulls positively contribute to a herd through a variety of avenues including exceptional genetics, calf crops, and annual profits [87,88]. Investing in routine breeding soundness examinations (BSEs) is one way to assess breeding potential in bulls and improve chances for breeding success [89], with a potential benefit-to-cost ratio of approximately 7 for every dollar spent on a BSE [90].

Fertility is five times more valuable than production traits such as beef merit or average daily gain in a beef cow-calf herd [91]. Sub-fertile bulls are detrimental to a cow-calf producer's profitability, from feed costs of non-productive animals to a prolonged calving season [45,92,93]. Some diseases can negatively affect bull reproductive parameters and fertility, causing an adverse chain reaction for herd and producer success, often going unnoticed until assessment of cow pregnancy status [88,94]. One disease with a potentially underappreciated, negative impact on bull breeding soundness is anaplasmosis, caused by the tick-borne rickettsial pathogen,

Anaplasma marginale. Anaplasmosis in cattle causes anemia, fever, weight loss, lethargy, and death in severe acute cases [3,10,35]. Few direct studies have been performed assessing the impact of clinical anaplasmosis on beef bull fertility [35,36]; but, anemia, fever, and weight loss are all associated with unsatisfactory breeding outcomes in bulls [60,86,95]. Anemia leads to exacerbated hypoxic conditions in testicular tissue, ultimately affecting spermatogenesis and thermoregulation [46,59,96,97]. Fever or increased body temperature increases the metabolic rate in tissues, and prolonged fever can result in testicular degeneration, impairments in spermatogenesis, and direct damage to sperm cells [69,81,86,98]. Weight loss and lethargy negatively affect normal sperm production, sperm viability, and bull libido [60,72,91], all of which are essential for natural-service breeding bulls [92]. Finally, systemic illness can cause reductions in breeding soundness from a heightened inflammatory response and increased cortisol production [58,86,99].

The distribution of *A. marginale* is expansive, present in tropical, subtropical, and temperate regions worldwide [6,100]. In the U.S., *A. marginale* has been reported in most continental states, and is most prevalent in the Gulf Coast, Midwest and Western regions—some of the highest cattle-producing regions in the country [3,6,100]. Statewide anaplasmosis seroprevalence studies demonstrate beef cattle seroprevalence rates of 4.4% to 29.0% [37-39]. Further, a 2016-2017 study in Kansas identified 52.5% of beef cattle herds seropositive for *A. marginale* [101]. The expansive distribution of anaplasmosis in endemic areas is in part facilitated by the pathogen establishing persistent infection in its primary reservoir host, cattle. Persistently infected (PI) carrier cattle serve as a reservoir and source of infection to naïve cattle through arthropod vector or iatrogenic transmission. Considering disease prevalence and

PI carriers, a large proportion of bulls in the U.S. are at risk of exposure to *A. marginale* and developing clinical anaplasmosis. Therefore, the objective of this study was to determine the effects of acute *A. marginale* infection on overall breeding soundness examination (BSE) pass rate, including sperm morphology and progressive motility, and scrotal circumference in commercial beef bulls during and upon resolution of clinical anaplasmosis. We hypothesized that bulls with clinical anaplasmosis, including disease-associated anemia and fever, would have reduced semen quality and, subsequently, lower BSE pass rates. Information on how clinical anaplasmosis negatively affects bull breeding soundness will inform strategic anaplasmosis and herd management decisions, including BSE recommendations for bulls in anaplasmosis-endemic areas.

Methods

Study animals

Six crossbred beef bulls from the Kansas State University Western Kansas Agricultural Research and Extension Center herd (Hays, Kansas) were enrolled in this pilot study. Requirements for bull enrollment included: healthy on initial physical examination; ≥24-months of age; sound breeding potential (met minimum standards of a complete BSE on two separates dates within 3 months of study start date); negative for *A. marginale* via PCR; negative for *A. marginale*-specific antibodies via serum cELISA testing. At the time of the study, bull weights ranged from 454 to 799 kg (mean = 619 kg, median = 562 kg). Bull ages ranged from 24- to 48-months old (median = 24-months old).

Bulls in the same treatment group were co-housed in an outdoor dry lot pen for approximately six months. During that time, bulls had *ad libitum* access to fresh water and were fed a standard ration of grain at 0.5% body weight per day with *ad libitum* access to hay.

As the bulls originated from a working herd of cattle, the potential for other common comorbidities in study bulls was evaluated. All bulls were tested for potential co-infection of Bluetongue virus (BTV), Bovine Leukemia Virus (BLV), and Bovine Viral Diarrhea Virus (BVDV). No bulls demonstrated clinical signs associated with any of these potential co-infections at study enrollment. At study enrollment, all bulls were seropositive by ELISA but PCR negative for BTV; bulls 8553, 9431, 9600, and 7532 were seropositive for BLV; and all bulls were negative for BVDV by antigen capture ELISA. Bulls that were subsequently challenged with *A. marginale* were re-tested for these possible co-infections at peak fever and all maintained their pre-study status for BTV, BLV and BVDV, indicating that these co-infections had minimal to no contribution on study outcomes. Furthermore, previous work has demonstrated that positive serologic status for these infections in bulls has not demonstrated unsatisfactory semen quality [102-104].

Study Design

All animal studies were carried out under an approved Institutional Animal Care and Use protocol (IACUC 4476) and an approved Institutional Biosafety Committee protocol (IBC 1495) on file at the University Research Compliance Office at Kansas State University, Manhattan, KS. All methods were carried out in accordance with relevant guidelines and regulations in compliance with ARRIVE guidelines.

Enrolled bulls were blocked by weight and allocated randomly into two equally sized groups using the RAND function in a spread-sheet program (Excel, Microsoft Office, Richmond, WA). Three bulls (animal IDs: 8553, 9528, 9431) were included in the *A. marginale* challenge group (*Ana*) and were inoculated on Day 0 with blood from a persistently infected donor cow (*see 'Anaplasma marginale infection challenge'*). The other three bulls (animal IDs: 7532, 9550, 9600) were included in the unchallenged control group (CON). Breeding soundness examinations were performed weekly on all bulls starting two weeks prior to Day 0 and weekly through to Day 84. Two additional BSEs were performed on all bulls on Day 98 and Day 112, 14- and 16-weeks post-inoculation, respectively.

Blood samples were collected from all bulls (*Ana* and CON) once weekly for 3 weeks prior to inoculation; twice weekly post-inoculation (Day 0 to Day 41); once weekly from Day 42 to 84; and on Day 98 and 112. At each blood collection time point, approximately 20 mL of blood was obtained from the coccygeal vein and distributed between evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing EDTA (1.8 mg/mL whole blood) and tubes with no anticoagulant. Anemia was assessed by quantifying packed cell volume (PCV) from whole blood collected into EDTA tubes and then centrifuged in microhematocrit capillary tubes for 5 min (Micro-Hematocrit centrifuge CMH30, UNICO, Dayton, NJ). A bull was considered anemic if their PCV was reduced by $\geq$25% of their respective baseline value (baseline determined from the mean of three time points prior to inoculation) [105,106]. A rectal temperature was obtained on each bull (Kroger® Comfort Flex Tip Digital Thermometer, Cincinnati, OH) at every blood sample time point. A bull was considered febrile if its rectal temperature was ≥39.2°C, and hypothermic if its rectal temperature was ≤38.0°C [105]. All bulls

were monitored pen-side daily for signs of clinical anaplasmosis (e.g., tachypnea, anorexia, pallor, lethargy, and icterus) and overall health status. Animals were recorded as "healthy" unless clinical signs were noted.

Severe clinical anaplasmosis included a decrease in PCV of 7% or greater in <48 hours, a PCV ≤15%, fever (≥40.8°C), and any combination of pale or icteric mucous membranes, lethargy, anorexia, or loss of body condition. If two or more of these clinical signs were appreciated on daily health checks or at time of blood draw, the bull was administered oxytetracycline subcutaneously (Bio-Mycin® 200, Boehringer Ingelheim Vetmedica Inc, Duluth, GA) at a dose rate of 20 mg/kg of body weight for up to 3 doses given once every 48 hours. The number of oxytetracycline doses administered depended on an individual bull's clinical status.

All bulls were treated every two weeks with 30 mL of a topical permethrin (Ultra Boss®, Merck Animal Health, NJ) applied across the topline to control pre-existing lice and for the control of any potential arthropod vectors.

***Anaplasma marginale* infection challenge**

The *A. marginale* inoculate used as challenge material was obtained from an apparently healthy, persistently infected, mature donor cow (animal ID: 6473) from the Kansas State University Western Kansas Agricultural Research and Extension Center Cow-Calf Unit in Hays, Kansas. The donor was confirmed positive for *A. marginale* via PCR prior to the beginning of the study. This cow was also seropositive but PCR negative for BTV and seropositive for BLV. To collect blood, the donor jugular site was clipped and aseptically prepped with povidone iodine scrub and alcohol. A 14-gauge needle was placed in the right jugular vein and attached to a blood collection set, and approximately 400 mL of blood was collected into a heparinized, 450-

mL blood collection bag. The blood was placed on ice for transport and maintained at 4°C until the time of inoculation the next morning. The three bulls in the *Ana* group were inoculated with 40 mL of donor cow whole blood by direct venipuncture into the right jugular vein. Bulls were monitored for 15 min after inoculation for any signs of hypersensitivity reaction or anaphylaxis.

The strain of *A. marginale* in the donor cow was identified by amplifying, cloning, and sequencing the variable region of the Major Surface Protein 1a gene to determine the Msp1a genotype(s) as previously described [7]. The *A. marginale* challenge strain was confirmed in each experimentally infected bull. Sequences from this study were submitted to GenBank and are available at the following accession numbers: ON597854-ON97859.

Breeding soundness examination (BSE)

All BSEs were performed by the same veterinarian using the *Society for Theriogenology Manual for Breeding Soundness Examination of Bulls*, 2nd Edition guidelines [49]. To perform BSEs, bulls were restrained in a squeeze chute. Semen samples were collected via electroejaculation (Pulsator V, Lane Manufacturing Company, Denver, CO). As part of a routine, complete BSE, the following were evaluated and are described in detail below: general physical health including external and internal genitalia, scrotal circumference measurement, response to electroejaculation, sperm morphology, and sperm progressive motility.

General physical examination included an overall health assessment with specific evaluation of foot and leg soundness, evaluation of vision, palpation of external genitalia, and rectal palpation of internal genitalia. Body condition score (BCS) assessment was considered part of the general physical exam, and was assigned based on a 9-point scoring system [107]. All examination findings were reported on a standardized BSE form [49]. External genitalia

assessment included palpation of penile sheath and prepuce, penis, scrotum and testes for any changes in texture. Abnormalities of the external genitalia, including changes in scrotal size and shape, testicular texture, or penile abrasions, were recorded on the examination form. Scrotal circumference was determined using a scrotal measuring tape (cm) around the widest girth of the scrotum, with a minimum of 34 cm required to pass based on the age of enrolled bulls. Internal genitalia assessment was performed by rectal palpation of seminal vesicles, ampullae, prostate, and inguinal rings. Abnormalities of the internal genitalia, including enlarged seminal vesicles, were recorded. Response to electroejaculation was based on presence or absence of protrusion, erection, and ejaculation.

Sperm morphology was evaluated from a minimum of 100 sperm cells via microscopy using eosin-nigrosin stain (Live/Dead Semen Stain, Jorvet, Loveland, CO) under 1,000X magnification in oil. Each sperm cell was categorized into “normal”, “head abnormality”, “midpiece abnormality”, or “tail abnormality,” with a minimum of 70% morphologically normal sperm cells required for passing. Examples of sperm head abnormalities include pyriform heads and nuclear vacuolations. Examples of sperm midpiece abnormalities include proximal droplets and distal midpiece reflections (DMRs). Examples of sperm tail abnormalities include coiled tails. Presence or absence of other abnormal cells in the ejaculate, such as white cells (i.e. neutrophils, macrophages) or immature sperm cells — spermatocytes — was assessed with Wright-Giemsa-stained semen smears (HEMA-3, Fisher HealthCare, Pittsburgh, PA) under 1,000X magnification in oil. Minimal to no white blood cells or spermatocytes in the semen were required for satisfactory breeding potential.

Sperm motility was assessed chute-side using a compound microscope at 100X magnification (Amscope, Irvine, CA), and scored on a percentage basis, with a minimum of 30% sperm moving in a forward linear motion per high powered field required to pass this metric. Warm, sterile, phosphate-buffered saline (PBS) was incorporated into a drop of neat semen to dilute the sample for more accurate evaluation of sperm cell progressive motility.

Collectively, bulls were considered of satisfactory breeding potential if they had: i) no abnormalities on general physical examination; ii) no abnormalities on assessment of internal and external genitalia; iii) $\geq$34 cm scrotal circumference; iv) $\geq$70% sperm cells of normal morphology; and v) $\geq$30% of sperm with progressive motility. Bulls were considered of unsatisfactory breeding potential if they did not meet minimum requirements in any of the above categories.

DNA extraction and quantitative PCR (qPCR) for bacteremia determination.

Total genomic DNA (gDNA) was extracted from 100 µL of whole blood collected into EDTA tubes using the Quick-DNA Miniprep Kit (Zymo Research, Irvine, CA) according to manufacturer instructions, and the resulting gDNA was eluted in 35 µL of DNA Elution Buffer. Genomic DNA was stored at -20°C. A quantitative, real-time PCR (qPCR) assay targeting a portion of the single-copy *A. marginale* Msp5 gene [29] was used to quantify *A. marginale* bacteremia. PCR mastermix preparations were set up in 20 µL reaction volumes, each containing 0.2 µM of each primer (*Am* msp5 F: ATA CCT GCC TTT CCC ATT GAT GAG GTA CAT and *Am* msp5R AGG CGA AGA AGC AGA CAT AAA GAG CGT), 10 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), and 2 µL gDNA. Reaction cycling was performed using a CFX Connect Real-Time PCR System (Bio-Rad), using the following cycling parameters:

one cycle of 98°C for 2 min; followed by 40 cycles at 98°C for 5 sec, 60°C for 5 sec and 74°C for 15 sec; and a final melt curve cycle of 65-95°C with increasing 0.5°C temperature steps at 10 sec/step. Real-time qPCR data was visualized and analyzed using CFX Maestro Software v1.1 (Bio-Rad).

Percent parasitized erythrocyte (PPE) determination

The percentage of red blood cells infected with *A. marginale* was assessed via microscopic examination of Wright-Giemsa-stained blood smears (HEMA-3, Fisher HealthCare, Pittsburgh, PA). Total red blood cells in a monolayer were counted microscopically under 1,000X magnification. The number of *A. marginale*-infected red blood cells were divided by the total number of evaluated red blood cells, and that value multiplied by 100 to determine the PPE. A minimum of 200 red blood cells were evaluated per sample.

Competitive ELISA

Blood samples collected into tubes without anticoagulant were centrifuged at 3,000 rpm for 10 min at 20°C to separate and collect serum. Serum samples were aliquoted and stored at -80°C before testing. Serum samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA) for *A. marginale* serological screening using a commercial cELISA that detects host antibody produced against *A. marginale* Msp5 (*Anaplasma* Antibody Test Kit, cELISA v2, VMRD, Pullman, WA). Animals with a percent inhibition score $\geq$30% were considered seropositive for *A. marginale*.

Statistical analysis

Packed cell volume percent change and scrotal circumference percent change from baseline were analyzed separately at each post-challenge study day under the linear model

with treatment being the fixed effect. Percent normal morphology and percent progressive motility were analyzed separately at each post-challenge study day, under the linear model with treatment being the fixed effect and baseline average being the covariate. All hypothesis tests were 2-sided tests. Statistical analysis was performed using Statistical Analysis Software (SAS 9.4; Cary, NC) MIXED procedure.

Results

Donor animal anaplasmosis characteristics

Blood from a healthy, 4-year-old, Angus-cross cow persistently infected with *A. marginale* was used to challenge *Ana* group bulls. The PCV and *A. marginale* bacteremia of the donor was 32% and 1.15E+06 *A. marginale*/mL blood, respectively. The *A. marginale* Msp1a genotype identified in the donor cow was: A B B B.

Progression of clinical anaplasmosis in experimentally-infected bulls

All *Ana* bulls developed clinical anaplasmosis and the infecting strain was confirmed to be Msp1a genotype A B B B. All *A. marginale* inoculated bulls developed anemia and fever. Time to initial signs of clinical anaplasmosis (i.e. anemia, fever) ranged from 21 to 24 days post-challenge (mean = 23 days, median = 24 days) (Figure 6, Table 2, Table 3). Signs of clinical anaplasmosis lasted 16 to 17 days (mean = 16.7 days, median = 17 days).

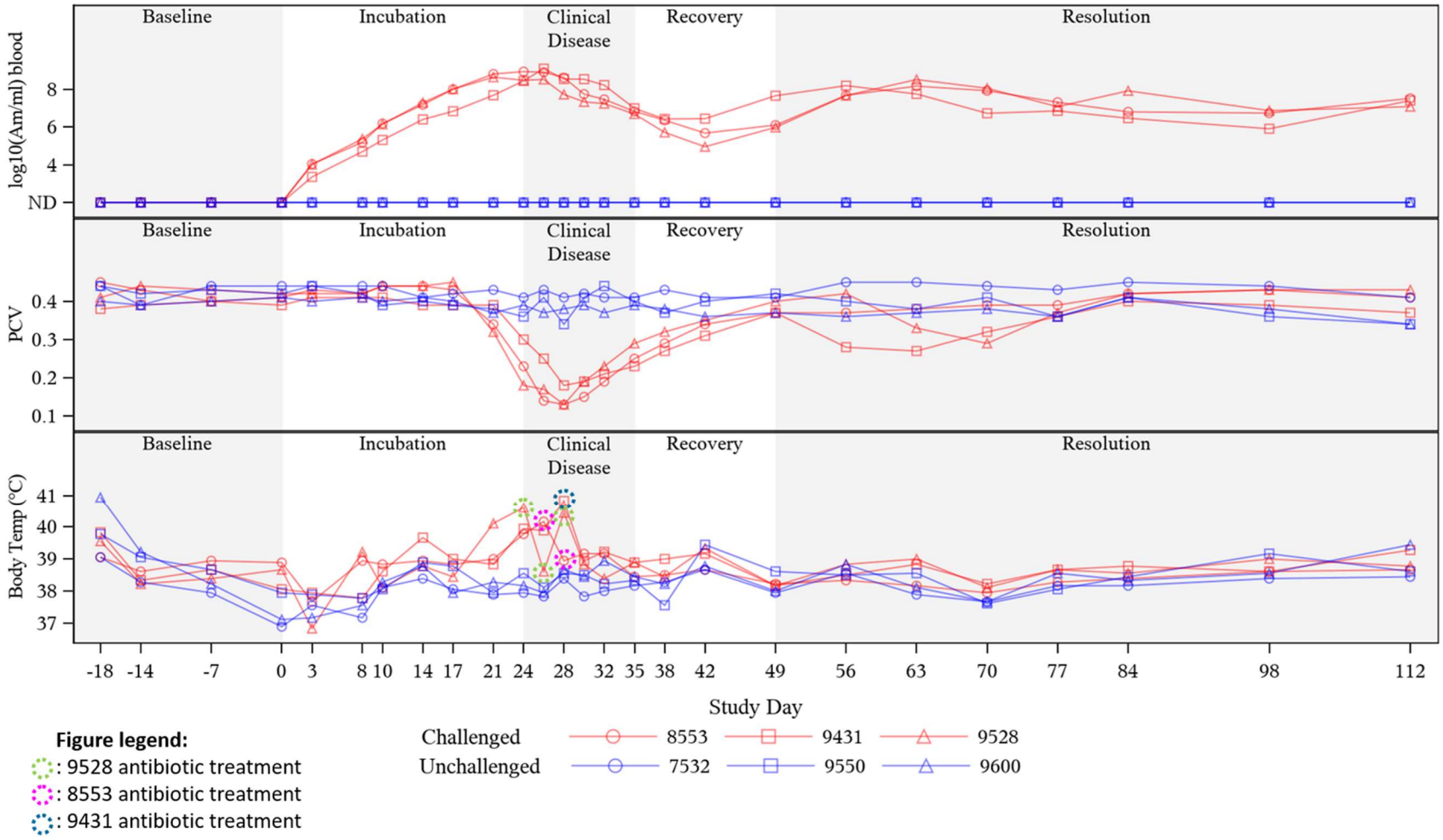

Figure 6. Progression of clinical anaplasmosis in experimentally-challenged beef bulls. Top: Progression of *A. marginale* bacteremia (*A. marginale* (*Am*)/mL blood) evaluated using a quantitative PCR assay targeting the single-copy Msp5 gene. Middle: Changes in packed cell volume (PCV) before, during, and after resolution of clinical anaplasmosis. Bottom: Changes in body temperatures (°C)

prior to, during, and after resolution of clinical anaplasmosis. Figure background shading represents phases of clinical anaplasmosis with Day 0 representing day of *A. marginale* inoculation in challenged bulls.

Table 2. Summary statistics for blood parameters associated with *A. marginale* infection. Mean, minimum and maximum packed cell volume (PCV), bacteremia (*A. marginale* (*Am*)/mL blood), and percent parasitized erythrocytes (PPE) among *A. marginale*-challenged (*Ana*) and unchallenged bulls (CON).

		PCV						***Am*/ml blood**						**PPE**					
		Ana			**CON**			***Ana***			**CON**			***Ana***			**CON**		
		Mean	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**
Phase	**Day**																		
Base-line	**-18**	41%	38%	45%	43%	40%	44%	0	0	0	0	0	0	.	.	.	.	.	.
	-14	42%	39%	44%	40%	39%	42%	0	0	0	0	0	0	.	.	.	.	.	.
	-7	41%	40%	43%	42%	40%	44%	0	0	0	0	0	0	.	.	.	.	.	.
	0	41%	39%	42%	42%	41%	44%	0	0	0	0	0	0	.	.	.	.	.	.
Incuba-tion	**3**	42%	41%	43%	43%	40%	44%	7.90E3	2.27E3	1.11E4	0	0	0	.	.	.	.	.	.
	8	42%	41%	42%	42%	41%	44%	1.49E5	5.05E4	2.40E5	0	0	0	.	.	.	.	.	.
	10	43%	41%	44%	41%	39%	44%	1.07E6	2.09E5	1.56E6	0	0	0	.	.	.	.	.	.
	14	42%	39%	44%	41%	40%	41%	1.28E7	2.57E6	2.02E7	0	0	0	.	.	.	.	.	.
	17	42%	39%	45%	40%	39%	42%	7.18E7	7.03E6	1.05E8	0	0	0	.	.	.	.	.	.
	21	35%	32%	39%	39%	37%	43%	3.9E8	4.94E7	6.68E8	0	0	0	.	.	.	.	.	.

		PCV						*Am*/ml blood						PPE					
		Ana			CON			*Ana*			CON			*Ana*			CON		
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Clinical Disease	24	24%	18%	30%	39%	36%	41%	4.97E8	2.87E8	8.95E8	0	0	0	.	.	.	.	.	.
	26	19%	14%	25%	40%	37%	43%	8.19E8	3.44E8	1.28E9	0	0	0	33%	24%	41%	0%	0%	0%
	28	15%	13%	18%	38%	34%	41%	2.81E8	5.48E7	4.30E8	0	0	0	41%	34%	45%	0%	0%	0%
	30	18%	15%	19%	41%	39%	42%	1.44E8	2.22E7	3.53E8	0	0	0	51%	35%	72%	0%	0%	0%
	32	21%	19%	23%	41%	37%	44%	7.30E7	1.79E7	1.71E8	0	0	0	58%	49%	72%	0%	0%	0%
Recov-ery	35	26%	23%	29%	40%	39%	41%	7.28E6	5.00E6	1.00E7	0	0	0	50%	43%	55%	0%	0%	0%
	38	29%	27%	32%	39%	37%	43%	1.86E6	5.29E5	2.71E6	0	0	0	47%	45%	48%	0%	0%	0%
	42	33%	31%	35%	39%	36%	41%	1.13E6	9.17E4	2.82E6	0	0	0	39%	36%	45%	0%	0%	0%
Resolu-tion	49	38%	37%	40%	40%	37%	42%	1.64E7	9.77E5	4.68E7	0	0	0	15%	8%	20%	0%	0%	0%
	56	36%	28%	42%	40%	36%	45%	8.58E7	4.77E7	1.60E8	0	0	0	10%	8%	12%	0%	0%	0%
	63	33%	27%	38%	40%	37%	45%	1.83E8	5.80E7	3.40E8	0	0	0	15%	13%	18%	0%	0%	0%
	70	33%	29%	39%	41%	38%	44%	6.94E7	5.46E6	1.16E8	0	0	0	29%	14%	54%	0%	0%	0%
	77	37%	36%	39%	38%	36%	43%	1.36E7	7.33E6	2.11E7	0	0	0	26%	16%	38%	0%	0%	0%
	84	41%	40%	42%	42%	41%	45%	3.16E7	2.99E6	8.53E7	0	0	0	14%	9%	20%	0%	0%	0%
	98	42%	39%	43%	39%	36%	44%	4.67E6	8.24E5	7.58E6	0	0	0	18%	13%	27%	0%	0%	0%
	112	40%	37%	43%	36%	34%	41%	2.40E7	1.23E7	3.40E7	0	0	0	19%	12%	25%	0%	0%	0%

Table 3. Summary statistics for bull rectal temperatures. Mean, minimum and maximum body temperatures (°F) among *A. marginale* (*Ana*)-challenged and unchallenged bulls (CON). Ambient outdoor temperature (°F) is also included.

		Ambient Temp. (°F)	Rectal Temperature (°F)					
			Ana			CON		
Phase	Study Day		Mean	Min	Max	Mean	Min	Max
Baseline	-18	35.0	103.1	102.3	103.7	103.9	102.3	105.7
	-14	25.0	101.1	100.8	101.5	101.9	100.9	102.6
	-7	39.0	101.6	101.1	102.1	100.9	100.3	101.6
	0	9.0	101.4	100.5	102.0	99.2	98.4	100.3
Incubation	3	0.0	99.5	98.3	100.3	99.6	98.9	100.2
	8	3.0	101.6	100.0	102.6	99.5	98.9	100.0
	10	18.0	101.3	100.6	101.9	100.7	100.5	100.9
	14	32.0	102.4	101.8	103.4	101.6	101.1	102.0
	17	26.0	101.8	101.2	102.2	100.9	100.3	101.8
	21	39.0	102.8	101.9	104.2	100.5	100.2	100.9
Clinical Disease	24	32.0	104.2	103.6	105.1	100.8	100.3	101.4
	26	46.0	103.2	101.5	104.3	100.3	100.1	100.6
	28	59.0	104.1	102.1	105.5	101.4	101.1	101.6
	30	30.0	102.2	101.9	102.5	100.9	100.1	101.3
	32	43.0	102.1	101.1	102.6	101.1	100.4	102.1
Recovery	35	45.0	101.7	101.2	102.0	101.0	100.7	101.2
	38	37.0	101.6	101.2	102.2	100.4	99.6	100.9
	42	50.0	102.3	101.6	102.8	102.1	101.6	103.0
	49	46.0	100.7	100.7	100.8	100.7	100.3	101.5

Resolution		Ambient Temp. (°F)	Rectal Temperature (°F)					
			Ana			CON		
			Mean	Min	Max	Mean	Min	Max
	56	60.0	101.4	101.0	101.9	101.5	101.3	101.9
	63	46.0	101.6	100.7	102.2	100.7	100.2	101.4
	70	34.0	100.6	100.3	100.8	99.8	99.7	99.8
	77	70.0	101.4	100.9	101.6	100.9	100.5	101.4
	84	52.0	101.4	101.1	101.8	101.0	100.7	101.2
	98	72.0	101.7	101.5	102.2	101.7	101.1	102.5
	112	64.0	102.0	101.6	102.7	101.9	101.2	103.0

Bull PCV was evaluated to track progression of anemia (Figure 6, Table 2). Packed cell volume nadirs were reached in all *Ana* bulls 28 days after inoculation and the average PCV nadir was 15% (median = 13%, range: 13 - 18%), an averaged 64.1% (median = 69.2%, range: 53.8% - 69.4%) loss of red blood cell from baseline (Table 4). All *Ana* bulls had pale or icteric mucous membranes including pallor of the prepuce during peak clinical disease (28 days after inoculation). Individual bull PCV loss compared to their respective baseline value was significant (P = <0.001, Table 4). Compared to unchallenged, time-matched CON bulls which experienced no appreciable loss in PCV throughout the study, *Ana* bulls had significantly lower PCVs on Day 28 (54% lower; P = 0.002) through at least Day 42 (14 days beyond *Ana* PCV nadir). All bulls in the *Ana* group developed fevers (mean = 40.6°C, median = 40.6°C, range: 40.2°C - 40.8°C) which peaked 24 to 28 days after inoculation (Figure 6, Table 3). Fevers were intermittent around peak infection and lasted 7 to 8 days (mean = 7.3 days, median = 7 days), with improvements coinciding with antibiotic treatment (Figure 6).

Table 4. Changes in bull packed cell volume (PCV) during a course of clinical anaplasmosis.

Mean percent (%) PCV change in *A. marginale*-infected (*Ana*) bulls compared to baseline and unchallenged control (CON) bulls over time

						Compared to Unchallenged	
Endpoint	**Phase**	**Day**	**Treatment**	**Mean % Change**	**P-value for Testing %change[1]0**	**Difference in Mean % Change**	**P-value for Testing Diff.[1]0**
PCV	Incubation	3	*Ana*	2%	0.261	0%	0.985
			CON	2%	0.253	.	.
		8	*Ana*	1%	0.562	0%	0.969
			CON	1%	0.528	.	.
		10	*Ana*	4%	0.164	6%	0.154
			CON	-2%	0.48	.	.
		14	*Ana*	3%	0.29	5%	0.153
			CON	-3%	0.272	.	.
		17	*Ana*	3%	0.322	6%	0.131
			CON	-4%	0.196	.	.
		21	*Ana*	-15%	0.065	-9%	0.351
			CON	-6%	0.363	.	.
	Clinical Disease	24	*Ana*	-42%	0.006	-35%	0.034
			CON	-7%	0.39	.	.
		26	*Ana*	-54%	0.001	-51%	0.006
			CON	-4%	0.62	.	.
		28	*Ana*	-64%	<.001	-54%	0.002
			CON	-10%	0.133	.	.
		30	*Ana*	-57%	<.001	-54%	<.001
			CON	-3%	0.378	.	.

		32	*Ana*	-49%	<.001	-46%	<.001
			CON	-3%	0.395	.	.
	Recovery	35	*Ana*	-38%	<.001	-34%	<.001
			CON	-4%	0.133	.	.
		38	*Ana*	-29%	<.001	-23%	0.008
			CON	-6%	0.141	.	.
		42	*Ana*	-19%	<.001	-12%	0.003
			CON	-7%	0.007	.	.
	Resolution	49	*Ana*	-8%	0.018	-3%	0.304
			CON	-4%	0.092	.	.
		56	*Ana*	-14%	0.096	-10%	0.324
			CON	-4%	0.594	.	.
		63	*Ana*	-21%	0.019	-17%	0.1
			CON	-4%	0.465	.	.
		70	*Ana*	-19%	0.022	-17%	0.083
			CON	-2%	0.715	.	.
		77	*Ana*	-9%	0.059	-1%	0.848
			CON	-8%	0.08	.	.
		84	*Ana*	0%	0.907	-1%	0.766
			CON	1%	0.596	.	.
		98	*Ana*	1%	0.811	7%	0.272
			CON	-6%	0.197	.	.
		112	*Ana*	-2%	0.562	11%	0.102
			CON	-13%	0.022	.	.

All CON bulls remained within a PCV range of 34% to 45% (mean = 40%, median = 41%) for the duration of the study (Figure 6, Table 2). CON bulls maintained normal body

temperatures (range: 37.8°C - 38.9°C); however, their temperatures occasionally fluctuated outside of that range when ambient temperature was <37.2°C (Figure 6, Table 3).

Progression of *A. marginale* infection and bull seroconversion

Bacteremia was first detected via qPCR in all *Ana* bulls by 3 days post-inoculation. Bacteremia peaked 21 to 26 days post-inoculation. The average bacteremia peak was 8.75E+08 *A. marginale*/mL blood (median = 8.95E+08, range: 4.51E+08 - 1.28E+09 *A. marginale*/mL blood). All *Ana* bulls transitioned into the PI disease state with resolution of clinical signs. On the last day of the study, average bacteremia was 2.40E+07 *A. marginale*/mL blood (median = 2.58E+07, range: 1.23E+07 - 3.4E+07 *A. marginale*/mL). All bulls remained infected with *A. marginale*, despite treatment with oxytetracycline (8553- two treatments; 9431- one treatment; 9528- three treatments). All CON bulls remained qPCR negative for *A. marginale* throughout the study.

Percent parasitized erythrocytes evaluation began 26 days after inoculation (Table 2, Figure 7). The highest PPE detected was 72.3%, 32 days after inoculation (8553). Average peak PPE was 57.6% (median = 51.4%, range: 49.0% - 72.3%). All *Ana* bulls experienced descending, occasional waves of PPE levels after resolution of clinical signs, never again reaching the peaks observed during the height of clinical disease. On the last day of the study, the average PPE level was 19.3% (median = 20.5%, range: 12.3% - 25.2%). No *A. marginale* parasitized erythrocytes were ever observed in CON bulls at any point in the study.

Earliest seroconversion was detected 10 days post-inoculation (9528). By 17 days-post inoculation, all bulls in the *Ana* group (8553, 9431) had seroconverted and remained seropositive for the remainder of the study. All CON bulls remained negative for *A. marginale* via PCR and visual examination of stained blood smears (Figure 6, Table 2). CON bulls remained seronegative throughout the study.

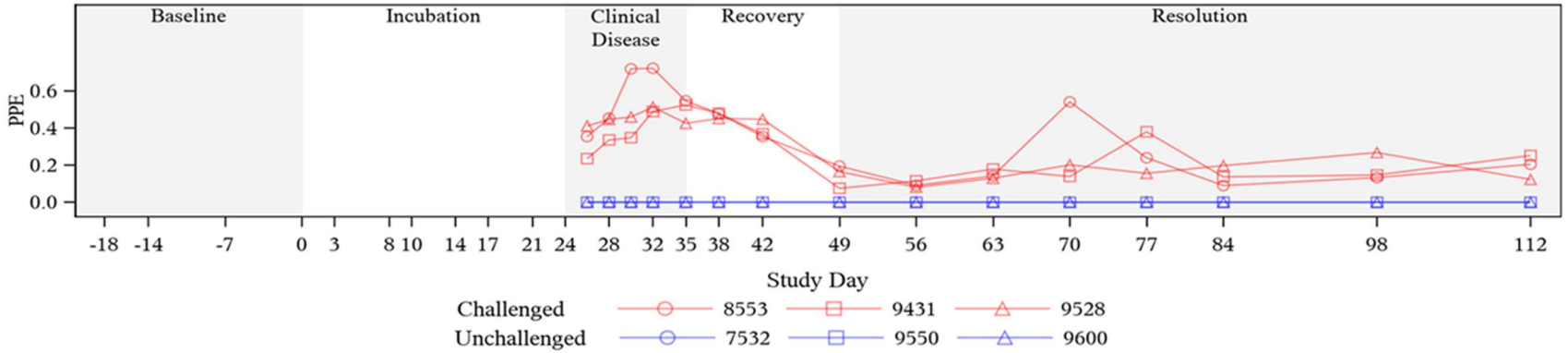

Figure 7. Progression of percent parasitized erythrocytes (PPE). Progression of PPE (%) among *A. marginale*-challenged and unchallenged bulls.

Impact of clinical anaplasmosis on bull breeding soundness examination metrics

Overview

Breeding soundness examination results are presented overlaid with different periods of clinical anaplasmosis progression: baseline (time prior to *A. marginale* inoculation), incubation period (period of time prior to display of clinical signs), clinical disease (period of time when clinical signs – fever and anemia -- are exhibited), recovery (period of time when clinical signs are returning towards baseline values), and resolution (period of time after anemia and fever are fully resolved with values similar to baseline values).

The occurrence of clinical anaplasmosis coincided with loss of breeding soundness for a variable but extended time period. All *Ana* bulls fell below satisfactory breeding potential standards beginning 28 days post-inoculation with *A. marginale*, coinciding with peak bacteremia. Bull 9431 never returned to satisfactory breeding potential within the time span of the study (unsatisfactory for more than 72 days), bull 8553 returned to satisfactory breeding potential 72 days after initial failure, and bull 9528 returned to a satisfactory breeding potential 28 days after initial failure. The CON bulls 7532 and 9600 maintained satisfactory breeding soundness throughout the study, while one bull in the CON group (9550) had unsatisfactory BSE results 67 days after study start and never returned to passing. This bull had changes in testicular texture and size consistent with insult related to extreme cold temperatures experienced during the study period. This bull remained negative for *A. marginale* throughout the study.

Physical Examination

None of the *Ana* nor the CON bulls developed abnormalities related to feet, legs, or eyes. None of the *Ana* nor the CON bulls developed any internal genitalia abnormalities at any time point via examination by rectal palpation. All *Ana* and CON bulls started with and maintained their ability to extend their penis, acquire an erection, and ejaculate via electroejaculation. Although not specifically measured, additional physical response changes were observed in some *Ana* bulls during clinical disease including, prolonged reaction time (time to produce ejaculate) and poor erection response. Two bulls in the *Ana* group (9431, 9528) developed weak erection response (did not achieve full engorgement, remained slightly flaccid at the time of ejaculation) during peak bacteremia (24-days post-inoculation) but returned to satisfactory response within 21 days of peak bacteremia (45-days post-inoculation).

All *Ana* bulls had decreased body condition from baseline (Figure 8, Table 5) with transverse processes and ribs becoming more palpable. The most significant decrease in body condition from baseline was by 3 scores in two bulls (9431 and 9528). Notable reductions in body condition score began 21-days post inoculation. Two bulls (9528 and 8553) returned to baseline body condition 56 days after inoculation; one bull (9431) never returned to baseline but regained some condition before the end of the study. All CON bulls maintained a BSC between 5 and 7 throughout the study.

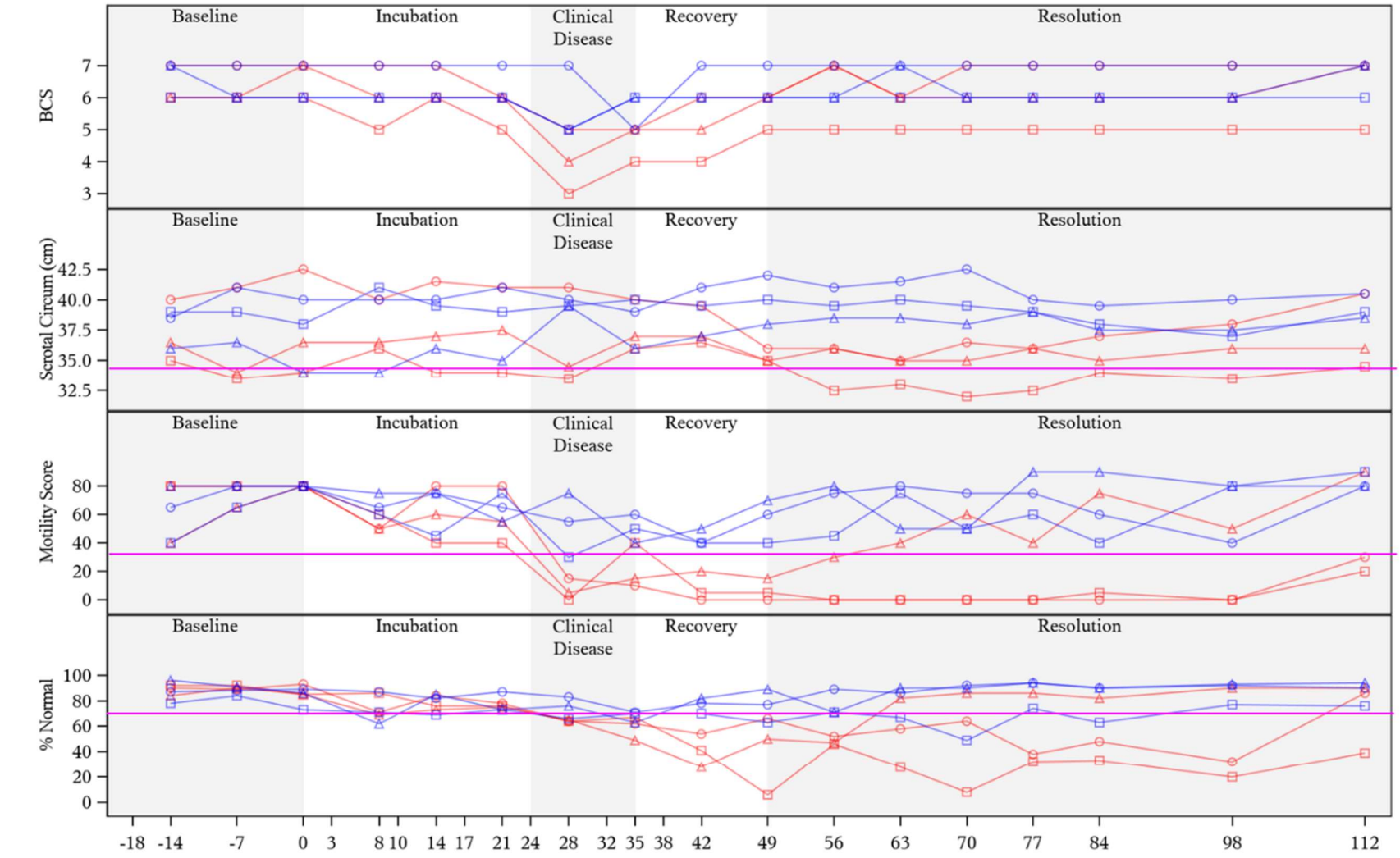

Figure legend:

—: Cutoff for least satisfactory breeding requirements

Figure 8. Changes in bull breeding soundness examination parameters over a course of clinical anaplasmosis. Top: Body Condition Score (BCS) using a 9-point scale. Middle top: Scrotal circumference (cm). Middle bottom: Percentage of progressively motile spermatozoa. Bottom: Percentage of morphologically normal spermatozoa. Figure background shading represents phases of clinical anaplasmosis with Day 0 representing day of *A. marginale* inoculation in challenged bulls.

Table 5. Summary statistics for bull body condition scores (BCS) and scrotal circumference throughout a course of clinical anaplasmosis. Mean, minimum and maximum BCS and scrotal circumference (cm) among *A. marginale*-challenged (*Ana*) and unchallenged bulls (CON). [1]BSC determined using a 9-point scale

Phase	Study Day	BCS						Scrotal circumference (cm)					
		Ana			CON			*Ana*			CON		
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Baseline	-14	6.3	6	7	6.7	6	7	37.2	35.0	40.0	37.8	36.0	39.0
	-7	6.3	6	7	6.3	6	7	36.2	33.5	41.0	38.8	36.5	41.0
	0	6.7	6	7	6.3	6	7	37.7	34.0	42.5	37.3	34.0	40.0
Incubation	8	6.0	5	7	6.3	6	7	37.5	36.0	40.0	38.3	34.0	41.0
	14	6.3	6	7	6.3	6	7	37.5	34.0	41.5	38.5	36.0	40.0
	21	5.7	5	6	6.3	6	7	37.5	34.0	41.0	38.3	35.0	41.0
Clinical disease	28	4.0	3	5	5.7	5	7	36.3	33.5	41.0	39.7	39.5	40.0

Recovery	**35**	4.7	4	5	5.7	5	6	37.7	36.0	40.0	38.3	36.0	40.0
	42	5.0	4	6	6.3	6	7	37.7	36.5	39.5	39.2	37.0	41.0
Resolution	**49**	5.7	5	6	6.3	6	7	35.3	35.0	36.0	40.0	38.0	42.0
	56	6.3	5	7	6.3	6	7	34.8	32.5	36.0	39.7	38.5	41.0
	63	5.7	5	6	6.7	6	7	34.3	33.0	35.0	40.0	38.5	41.5
	70	6.0	5	7	6.3	6	7	34.5	32.0	36.5	40.0	38.0	42.5
	77	6.0	5	7	6.3	6	7	34.8	32.5	36.0	39.3	39.0	40.0
	84	6.0	5	7	6.3	6	7	35.3	34.0	37.0	38.3	37.5	39.5
	98	6.0	5	7	6.3	6	7	35.8	33.5	38.0	38.2	37.0	40.0
	112	6.3	5	7	6.7	6	7	37.0	34.5	40.5	39.3	38.5	40.5

Scrotal Circumference and Testicular Texture

Each *Ana* bull had decreases in scrotal circumference from baseline (Table 6, Table 7). Peak reductions in scrotal circumference were 7% on average in *Ana* bulls compared to baseline values (Table 6). Greatest percent reductions in individual *Ana* bull scrotal circumference during the study compared to baseline values was 15%, 6.3%, and 4.7% in 8553, 9431, and 9528, respectively. Compared to baseline, average decreases in scrotal circumference in the *Ana* bulls were not significantly different during clinical disease (Day 28, 2% decrease, $P = 0.487$), but were significant after resolution of clinical disease on Day 70 (7% decrease, $P = 0.043$) (Table 6). Compared to the changes from baseline of time-matched CON bulls, average changes in scrotal circumference were not significant on Day 28 (6% difference, $P = 0.134$), but were significant on Day 70 (12% difference, $P = 0.02$) and neared significance on Day 63 (12% difference, $P = 0.052$). None of the *Ana* bulls returned to baseline scrotal circumference by the end of the study despite meeting minimum satisfactory standards by the end of the study. One *Ana* bull (9431) had consistently soft testicles on palpation starting 14 days after inoculation which remained abnormal for approximately 63 days through the end of the study.

Table 6. Changes in scrotal circumference during a course of clinical anaplasmosis. Mean percent (%) scrotal circumference (cm) change in *A. marginale*-infected (*Ana*) bulls compared to baseline and unchallenged control (CON) bulls over time.

	Compared to unchallenged

Phase	**Day**	**Treatment**	**Mean % change**	**P-value for testing change**	**Diff in mean % change**	**P-value for testing diff. ≠0**
Incubation	8	*Ana*	2%	0.58	1%	0.829
		CON	1%	0.797	.	.
	14	*Ana*	1%	0.228	0%	0.985
		CON	1%	0.236	.	.
	21	*Ana*	1%	0.423	1%	0.796
		CON	1%	0.643	.	.
Clinical disease	28	*Ana*	-2%	0.487	-6%	0.134
		CON	5%	0.133	.	.
Recovery	35	*Ana*	2%	0.378	1%	0.715
		CON	1%	0.685	.	.
	42	*Ana*	2%	0.403	-1%	0.792
		CON	3%	0.253	.	.
Resolution	49	*Ana*	-4%	0.285	-9%	0.112
		CON	5%	0.176	.	.
	56	*Ana*	-5%	0.15	-10%	0.084
		CON	5%	0.219	.	.
	63	*Ana*	-7%	0.097	-12%	0.052
		CON	5%	0.162	.	.
	70	*Ana*	-7%	0.043	-12%	0.02
		CON	5%	0.076	.	.
	77	*Ana*	-5%	0.193	-9%	0.138
		CON	4%	0.35	.	.
	84	*Ana*	-4%	0.196	-5%	0.244
		CON	1%	0.722	.	.
	98	*Ana*	-3%	0.344	-3%	0.415
		CON	1%	0.841	.	.
	112	*Ana*	0%	0.96	-4%	0.235

		CON	4%	0.113	.	.

Throughout the study among CON bulls, the mean change in scrotal circumference varied 1% to 5% above baseline. Two CON bulls (7532, 9600) had overall scrotal growth from baseline by the end of the study, which was expected as these were younger and still maturing bulls. One CON bull (9550) had an overall 4 cm decrease in scrotal circumference during the study, with consistent decreases most notably in the left testicle starting at Day 77. Despite abnormal testicular palpation, the overall scrotal circumference for this bull met minimum satisfactory standards during the study.

Sperm Morphology

Loss of morphologically normal sperm was observed in all *Ana* bulls (Figure 8, Table 7). All *Ana* bulls failed to meet minimum sperm morphology standards beginning at peak clinical anaplasmosis and had a progressively increasing percentage of sperm morphology abnormalities during the anaplasmosis recovery period (Figure 8). *Ana* bulls 9528, 8553, and 9431, did not meet minimum sperm morphology requirements for 28-, 70- and at least 84-days, respectively. Bull 9431 never regained satisfactory normal sperm morphology results prior to study completion. The lowest percentage of morphologically normal sperm for bulls 9528, 8553, and 9431 was 28% (42-days post-inoculation), 32% (98-days post-inoculation), and 6% (49-days post-inoculation), respectively, all occurring during clinical anaplasmosis recovery or after resolution. Compared to time-matched CON bulls, the percentage of morphologically normal sperm differed significantly from *Ana* bulls on Days 28, 42, and 56 (P = 0.049, 0.013,

0.026, respectively; Table 7). Morphologically normal sperm (Figure 9L) percentages for *Ana* bulls ranged from 86.3% - 90.7% at baseline; 6% - 32% during peak clinical anaplasmosis; and 39% - 90% after resolution of clinical anaplasmosis. The overall mean percentage of morphologically normal sperm from peak clinical disease through the remainder of the study was 53% for *Ana* bulls.

Table 7. Summary statistics for normal morphology sperm. Mean, minimum, and maximum percent of sperm with normal morphology among *A. marginale*-challenged (*Ana*) and unchallenged (CON) bulls.

Phase	Study Day	% of sperm with normal morphology					
		Ana			CON		
		Mean	Min	Max	Mean	Min	Max
Baseline	-14	89%	84%	92%	87%	78%	96%
	-7	90%	89%	92%	88%	84%	91%
	0	88%	85%	93%	83%	73%	89%
Incubation	8	75%	69%	86%	73%	62%	87%
	14	78%	73%	84%	79%	69%	85%
	21	76%	75%	78%	78%	73%	87%
Clinical disease	28	64%	64%	65%	75%	66%	83%
Recovery	35	59%	49%	67%	68%	63%	71%
	42	41%	28%	54%	77%	70%	82%
Resolution	49	41%	6%	66%	76%	63%	89%
	56	48%	46%	52%	77%	71%	89%
	63	56%	28%	82%	81%	67%	90%
	70	53%	8%	86%	77%	49%	92%
	77	52%	32%	86%	87%	74%	94%

	84	54%	33%	82%	81%	63%	90%
	98	47%	20%	90%	87%	77%	93%
	112	72%	39%	90%	87%	76%	94%

Table 8. Changes in the percent of morphologically normal sperm during a course of clinical anaplasmosis. Mean percent (%) of normal morphology sperm change in *A. marginale*-infected (*Ana*) bulls compared to baseline and unchallenged control (CON) bulls over time.

				Compared to unchallenged	
Phase	**Study day**	**Treatment**	**Mean w/ baseline adjusted**	**Diff. in mean w/ baseline adjusted**	**P-value for testing diff ≠0**
Incubation	8	*Ana*	75%	2%	0.881
		CON	73%	.	.
	14	*Ana*	76%	-5%	0.129
		CON	81%	.	.
	21	*Ana*	76%	-3%	0.682
		CON	78%	.	.
Clinical disease	28	*Ana*	63%	-13%	0.049
		CON	76%	.	.
Recovery	35	*Ana*	59%	-9%	0.318
		CON	68%	.	.
	42	*Ana*	39%	-40%	0.013
		CON	79%	.	.
Resolution	49	*Ana*	38%	-41%	0.169
		CON	79%	.	.
	56	*Ana*	48%	-30%	0.026
		CON	78%	.	.
	63	*Ana*	55%	-27%	0.279

		CON	82%	.	.
	70	*Ana*	49%	-31%	0.402
		CON	80%	.	.
	77	*Ana*	52%	-36%	0.21
		CON	88%	.	.
	84	*Ana*	53%	-30%	0.24
		CON	83%	.	.
	98	*Ana*	48%	-39%	0.253
		CON	87%	.	.
	112	*Ana*	70%	-17%	0.466
		CON	88%	.	.

Collectively, unsatisfactory percentages of sperm head, midpiece, and tail abnormalities were observed in *Ana* bulls (Figure 9, Table 9, Figure 10). The most common sperm morphology abnormalities were head abnormalities for bulls 8553 and 9431 and midpiece abnormalities for bull 9528, although head, midpiece, and tail abnormalities were observed in all *Ana* bulls.

Among *Ana* bulls, the most common types of head abnormalities were nuclear vacuolation, pyriform heads, and free abnormal heads (Figure 9A-C). The most common types of midpiece abnormalities were distal midpiece reflections (DMRs), and proximal droplets (Figure 9D-F). The most common types of tail abnormalities were coiled tails and, to a lesser extent, broken principal pieces (Figure 9F-H). Other abnormal cell types observed in the semen of *Ana* bulls included immature sperm cells (i.e. spermatocytes, medusa cells) (Figure 9I, 9J). Erythrocytes were never observed in Wright-Giemsa stained semen samples.

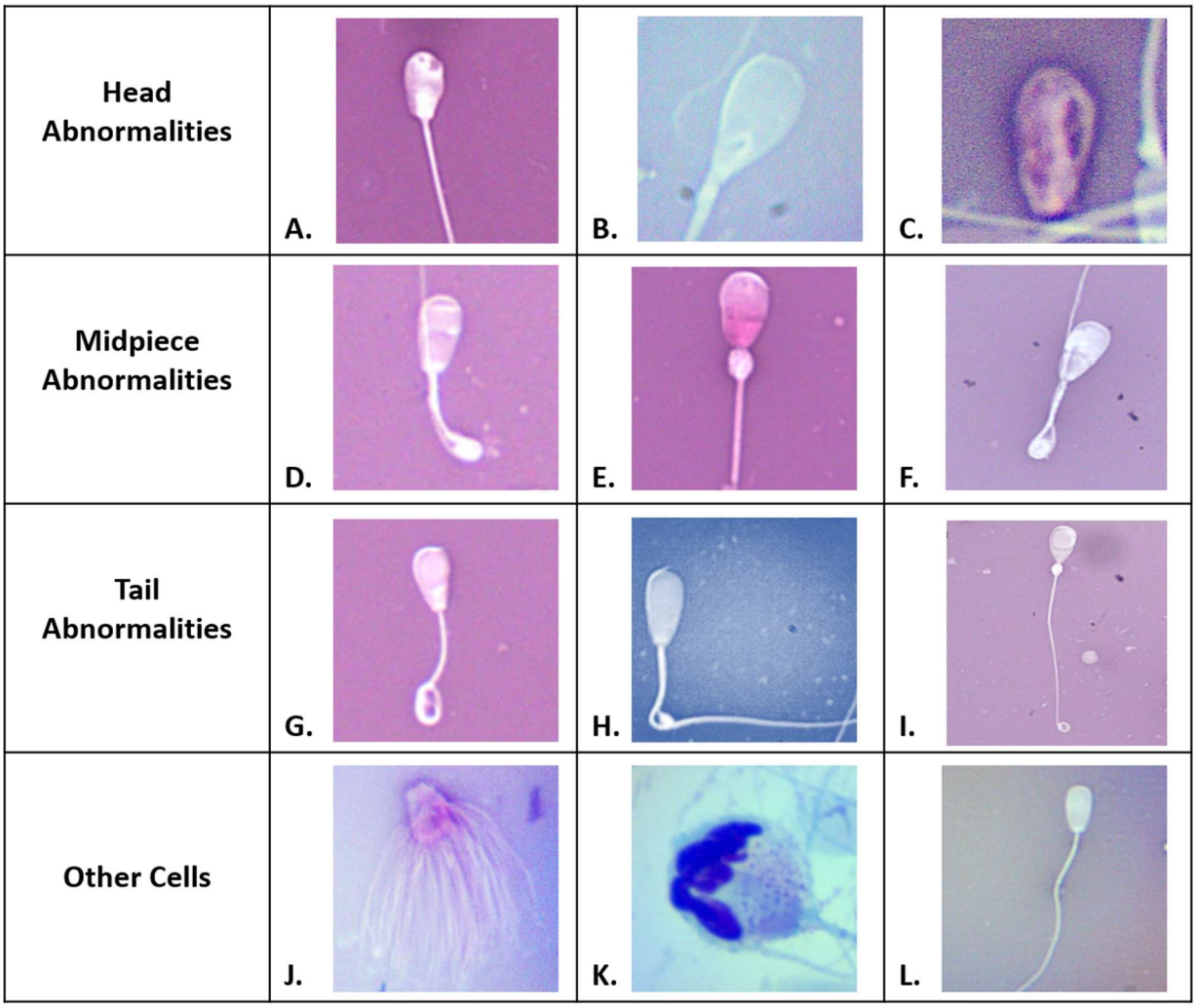

Figure 9. Abnormal sperm morphologies and other cells in semen samples collected from bulls experimentally-infected with *A. marginale*. Head Abnormalities: A. Nuclear Vacuolation; B. Pyriform Head; C. Abnormal Free Head; Midpiece Abnormalities: D. Distal Midpiece reflection (DMR); (E) proximal droplet; (F) DMR; Tail Abnormalities: (G) coiled tail; (H) bent principal piece; (I) distal coil with proximal droplet (midpiece); Other Cells: (J) medusa cell; (K) spermatocyte (immature sperm cell); (L) normal, mature sperm cell

Table 9. Summary statistics for observed abnormal sperm morphology categories. Mean, minimum, and maximum of abnormal morphology sperm categories among *A. marginale*-challenged (*Ana*) and unchallenged (CON) bulls.

Phase	Study Day	% head abnormalities						% midpiece abnormalities						% tail abnormalities					
		Ana			CON			*Ana*			CON			*Ana*			CON		
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Baseline	-14	5%	3%	6%	7%	1%	15%	4%	2%	5%	3%	1%	5%	3%	1%	6%	3%	2%	5%
	-7	3%	0%	5%	8%	7%	10%	2%	1%	3%	2%	0%	4%	5%	3%	8%	2%	0%	3%
	0	7%	1%	10%	6%	2%	11%	3%	1%	5%	5%	2%	9%	3%	1%	4%	6%	5%	7%
Incubation	8	3%	1%	7%	3%	3%	4%	14%	2%	23%	17%	5%	24%	7%	5%	12%	6%	4%	10%
	14	8%	5%	12%	10%	3%	18%	11%	10%	12%	8%	6%	9%	3%	1%	8%	4%	3%	4%
	21	7%	4%	10%	12%	8%	17%	11%	9%	12%	7%	5%	9%	6%	5%	7%	3%	0%	8%
Clinical disease	28	15%	7%	23%	13%	4%	24%	16%	9%	25%	10%	9%	10%	5%	4%	7%	2%	0%	4%
Recovery	35	14%	4%	21%	20%	8%	28%	20%	10%	40%	9%	3%	14%	6%	4%	7%	4%	1%	7%
	42	22%	12%	32%	12%	3%	25%	24%	12%	38%	9%	5%	12%	13%	4%	22%	2%	0%	3%

Resolution	**49**	35%	16%	52%	10%	1%	24%	10%	6%	14%	10%	7%	15%	15%	4%	36%	4%	3%	4%
	56	33%	14%	44%	14%	7%	20%	13%	6%	26%	7%	4%	12%	6%	0%	13%	2%	0%	3%
	63	31%	8%	66%	13%	5%	26%	6%	0%	14%	2%	0%	4%	7%	6%	8%	4%	2%	6%
	70	39%	8%	84%	16%	4%	40%	6%	3%	10%	5%	2%	9%	2%	2%	3%	1%	1%	2%
	77	32%	7%	54%	10%	4%	22%	9%	3%	15%	2%	1%	4%	6%	4%	11%	0%	0%	1%
	84	25%	5%	36%	11%	3%	24%	15%	6%	23%	4%	2%	9%	6%	0%	10%	4%	2%	5%
	98	33%	4%	50%	9%	3%	19%	13%	2%	20%	3%	3%	3%	7%	4%	10%	1%	0%	2%
	112	13%	2%	33%	9%	3%	19%	10%	4%	20%	3%	2%	5%	5%	3%	8%	1%	0%	1%

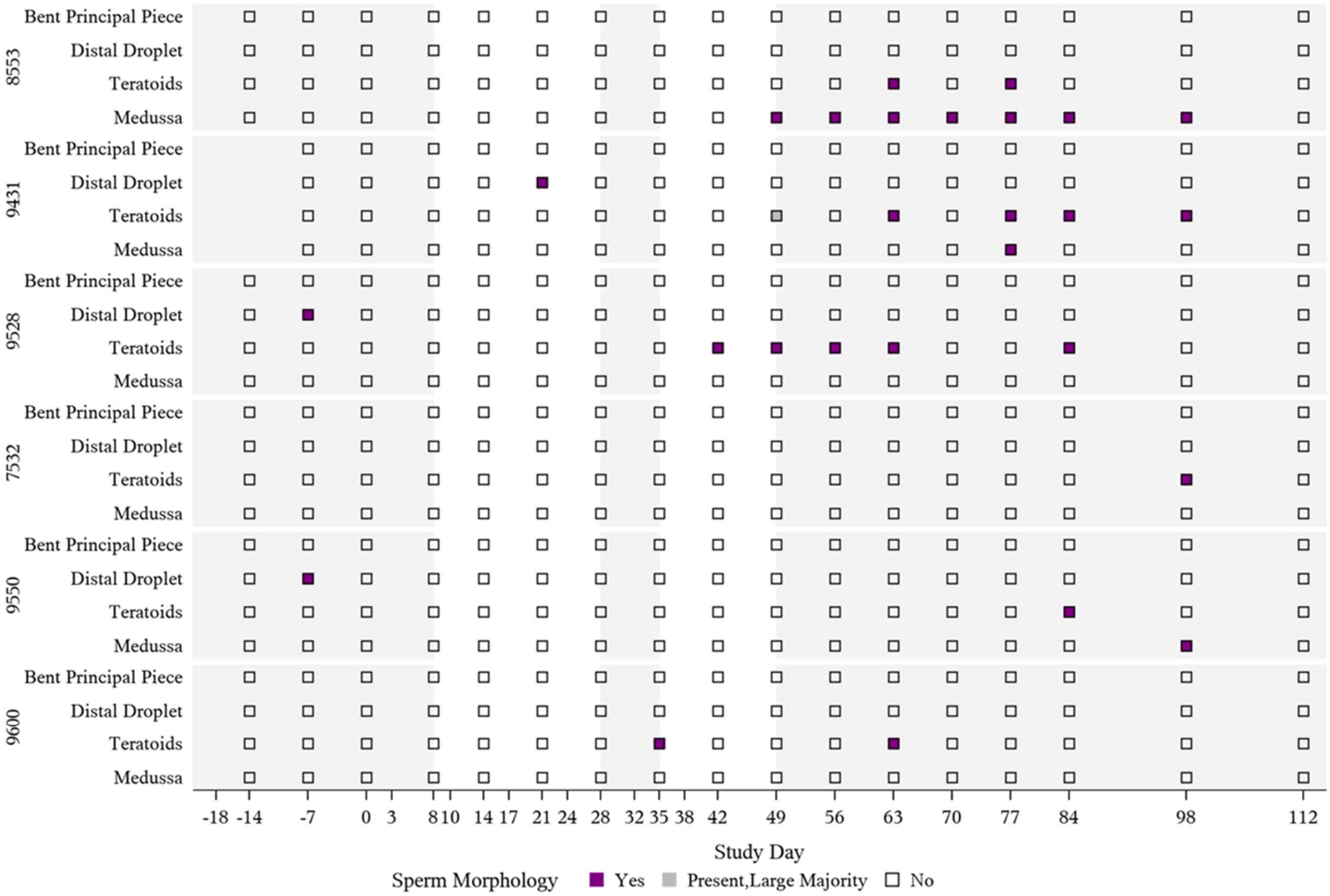
8553
9431
9528
7532
9550
9600
Bent Principal Piece
Distal Droplet
Teratoids
Medussa
-18 -14 -7 0 3 8 10 14 17 21 24 28 32 35 38 42 49 56 63 70 77 84 98 112
Study Day
Sperm Morphology
Yes
Present,Large Majority
No

Figure 10. Occurrence of specific sperm morphology abnormalities observed in individual *A. marginale*-challenged and unchallenged bulls. Purple shading indicates the abnormality was present; no shading indicates the abnormality was not present; and gray shading indicates the abnormality was present in abundance. Bulls 8553, 9431, and 9528 were challenged with *A. marginale* and bulls 7532, 9550, 9600 served as unchallenged controls.

For CON bulls 7532 and 9600, the percentage of morphologically normal sperm remained above 70% throughout the study (Figure 8). One bull in the CON group (9550) had sporadic sperm morphology scores below minimum requirements for passing during the study, occurring on study Day 14, 28, 49, 63, 70, and 84 (Figure 8). Collectively, morphologically normal sperm percentages for CON bulls ranged from 49%-96% throughout the study with an average of 79%.

Sperm Motility

Loss of progressive sperm motility was observed in all *Ana* bulls, with sperm motility below minimum satisfactory standards first noted during peak bacteremia and clinical disease (Figure 8, Figure 11, Table 10, Table 11). *Ana* bulls 9528 and 8553 fell below the minimum satisfactory sperm motility standards beginning 35 days post-inoculation and consistently remained below these standards for 28- and 70-days, respectively (Figure 8, Figure 11). *Ana* bull 9431 fell below the minimum satisfactory sperm motility standards beginning 28-days post-inoculation, rebounded to meeting minimum standards at Day 35, and then consistently fell below minimum satisfactory standards from Day 42 until the end of the study (at least 70 days) (Figure 8). The percentage of progressively motile sperm for the *Ana* group bulls ranged from 62% - 80% at baseline; 0% - 5% during clinical anaplasmosis; and 20% - 90% on the last day of the study (Figure 6). Overall mean percentage of progressively motile sperm from Day 28 (peak clinical disease) to the study end for the *Ana* group was 17%. Compared to time-matched CON bulls, mean progressive sperm motility was significantly lower in *Ana* bulls beginning at peak

clinical disease on Day 28 (49% difference, *P* = 0.027), and for 35 days after that (Table 10, Table 11).

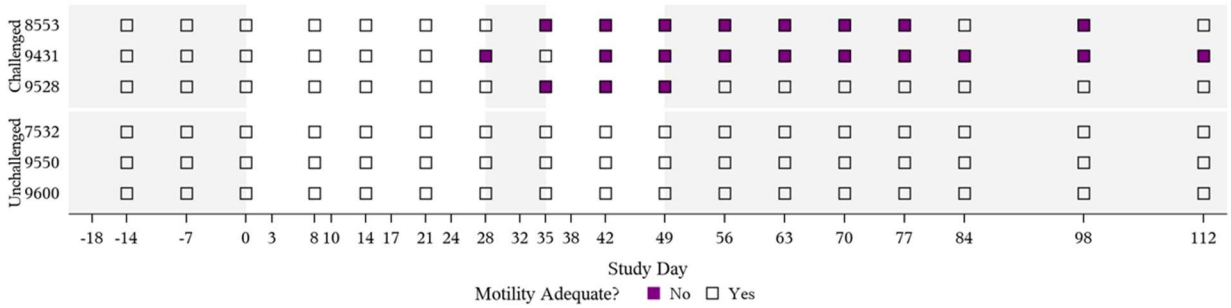

Figure 11. Sperm motility outcomes among uninfected and infected bulls throughout the study. Semen samples from bulls experimentally infected with *A. marginale* (8553, 9431, 9528) were assessed for satisfactory (≥ 30% progressively motile sperm) sperm motility compared to time-matched uninfected control bulls (7532, 9550, 9600). Figure background shading represents disease course of clinical anaplasmosis with Day 0 representing day of *A. marginale* inoculation in challenged bulls.

Table 10. Changes in the percent of progressively motile sperm during a course of clinical anaplasmosis. Mean percent (%) of progressively motile sperm change in *A. marginale*-infected (*Ana*) bulls compared to baseline and unchallenged control (CON) bulls over time.

				Compared to unchallenged	
Phase	**Study day**	**Treatment**	**Mean w/ baseline adjusted**	**Diff in mean w/ baseline adjusted**	**P-value for testing diff. ≠0**
Incubation	8	*Ana*	53%	-14%	0.053

		CON	67%	.	.
	14	*Ana*	59%	-6%	0.721
		CON	66%	.	.
	21	*Ana*	59%	-6%	0.706
		CON	65%	.	.
Clinical disease	28	*Ana*	6%	-49%	0.027
		CON	54%	.	.
Recovery	35	*Ana*	22%	-29%	0.107
		CON	50%	.	.
	42	*Ana*	9%	-34%	0.018
		CON	43%	.	.
Resolution	49	*Ana*	6%	-51%	0.019
		CON	57%	.	.
	56	*Ana*	10%	-57%	0.046
		CON	67%	.	.
	63	*Ana*	15%	-52%	0.015
		CON	67%	.	.
	70	*Ana*	21%	-36%	0.168
		CON	57%	.	.
	77	*Ana*	14%	-61%	0.042
		CON	75%	.	.
	84	*Ana*	28%	-35%	0.344
		CON	62%	.	.
	98	*Ana*	18%	-47%	0.079
		CON	65%	.	.
	112	*Ana*	49%	-33%	0.107
		CON	81%	.	.

Table 11. Sperm progressive motility results. Number of *A. marginale*-challenged (*Ana*) and unchallenged (CON) bulls with a satisfactory percentage (>30%) of sperm with progressive motility; and summary statistics (mean, minimum, maximum) of sperm with progressive motility.

Phase	Study Day	# of bulls with satisfactory progressive sperm motility			% of sperm with progressive motility					
		Ana		CON	*Ana*			CON		
		Yes	No	Yes	Mean	Min	Max	Mean	Min	Max
Baseline	-14	3	.	3	67%	40%	80%	62%	40%	80%
	-7	3	.	3	75%	65%	80%	75%	65%	80%
	0	3	.	3	80%	80%	80%	80%	80%	80%
Incubation	8	3	.	3	53%	50%	60%	67%	60%	75%
	14	3	.	3	60%	40%	80%	65%	45%	75%
	21	3	.	3	58%	40%	80%	65%	55%	75%
Clinical disease	28	2	1	3	7%	0%	15%	53%	30%	75%
Recovery	35	1	2	3	22%	10%	40%	50%	40%	60%
	42	.	3	3	8%	0%	20%	43%	40%	50%
Resolution	49	.	3	3	7%	0%	15%	57%	40%	70%
	56	1	2	3	10%	0%	30%	67%	45%	80%
	63	1	2	3	13%	0%	40%	68%	50%	80%
	70	1	2	3	20%	0%	60%	58%	50%	75%

	77	2	1	3	13%	0%	40%	75%	60%	90%
	84	2	1	3	27%	0%	75%	63%	40%	90%
	98	1	2	3	17%	0%	50%	67%	40%	80%
	112	2	1	3	47%	20%	90%	83%	80%	90%

All CON bulls remained above the 30% progressive motility minimum satisfactory standard throughout the study. Progressive sperm motility among CON bulls ranged from 30%-90%. Overall, the mean percentage of progressively motile sperm for the CON group from Day 28 to study end was 62%, nearly four times the mean of the time-matched *Ana* group.

Discussion

In this pilot study, we demonstrate that clinical anaplasmosis negatively impacts beef bull breeding potential in animals previously determined of satisfactory breeding potential. Further, loss of satisfactory breeding potential occurred for an extended time period in bulls newly infected with *A. marginale*, including past resolution of clinical anaplasmosis. All experimentally-infected *Ana* bulls developed classic clinical signs of acute anaplasmosis, including anemia, fever, and loss of body condition. Upon peak *A. marginale* infection and accompanying clinical disease, bulls exhibited reductions in percentage of morphologically normal sperm, reduced progressive sperm motility, decreased scrotal circumference, and loss of body condition. Loss of satisfactory breeding potential status varied in duration among *Ana* bulls, but an ultimate return to satisfactory breeding potential was observed in 66.7% (2/3) of the *Ana* bulls.

Efficient spermatogenesis requires healthy testicular tissue, hormone balance, overall bull health, and appropriate environmental conditions [53,60,72]. The process of spermatogenesis from immature cell to the mature sperm cell in the bull typically takes 61 days [53]. Insults to the spermatogenic cycle can affect breeding potential by limiting production of morphologically

normal and progressively motile, mature sperm. Location of insult and stage of sperm cell development at the time of insult often determine when these defects are observed in the ejaculate [89]. Environmental and external insults are typically observed more immediately in the ejaculate, while systemic insults may take weeks to be observed in the ejaculate [49]. For example, changes in ambient temperature typically affect sperm in the distal-most aspect of the spermatogenic cycle and morphologic sperm abnormalities associated with this type of insult typically resolve within days as affected sperm are naturally expelled [72]. In contrast, systemic internal insults such as oxidative stress and impaired scrotal thermoregulation from anemia and fever [108-110] can cause direct damage to testicular tissue, requiring a full sperm maturation cycle after resolution of the insult to achieve a successful load if no permanent damage occurs to the testicular parenchyma. Effects from these systemic, internal insults are seen in the ejaculate much later, up to weeks after the insult.

Anaplasmosis causes a hemolytic anemia through removal of infected red blood cells by the reticuloendothelial system [12]. Anemic animals can experience reproductive tissue hypoxia leading to oxidative stress which can interfere with spermatogenesis, hormonal influences, and directly on the mature sperm cell, overall reducing fertility [46,59,96,97,111]. Concurrent with PCV nadirs, our *Ana* bulls developed significant reductions in progressive sperm motility that continued beyond resolution of anemia (Figure 6). Although morphologic sperm abnormalities increased to unsatisfactory levels concurrently with PCV nadirs, further reductions in morphologically normal sperm counts were observed for weeks after PCV nadirs (Figure 6). These findings are consistent with previous studies evaluating BSE-related outcomes in bulls undergoing disease leading to anemia [36,59,112,113]. However, one study assessing the effects of

Theileria orientalis, another tick-borne pathogen that causes anemia and fever, in Friesian bulls in New Zealand, found no effect on sperm quality parameters despite reductions in libido [114]. Possible explanations for this discrepancy include a less severe PCV nadirs (mean PCV nadir 25%) and more mild systemic infection (no fever, no overt clinical signs) compared to what was observed among *Ana* bulls (mean PCV nadir 15%, fever, overt clinical signs, Table 2) in our study.

As with classic clinical anaplasmosis, all *Ana* bulls developed acute fever (Figure 6). Increased metabolic rate and oxygen consumption during fever impair sperm cells and their maturation due to hypoxia in the testicular tissue [35,36,81,86,115]. In their review, Kastelic et al [69], described several studies demonstrating that scrotal insulation and increased testicular temperature alter normal sperm morphology, motility, and concentration of semen samples beyond the time of insulation. In our study, even when bulls recovered from clinical anaplasmosis and appeared healthy, semen quality continued to be impaired for months after peak infection. A study by Wildeus and Entwistle documented how various morphologic sperm abnormalities occurred at different time points subsequent to the insult in *Bos indicus* x *Bos Taurus* bulls with insulated scrota and elevated testicular temperature [116]. In that study, head abnormalities began 6 days after elevated scrotal temperature and lasted 14 days after insulation. Tail and midpiece abnormalities emerged 12 and 17 days after insult, respectively, and remained present for at least 23 days. In our study, sperm morphology fell below minimum satisfactory standards in the *Ana* bulls starting at peak clinical disease when they experienced the highest body temperatures. Head, midpiece, and tail abnormalities peaked 42-, 14-, and 21-days post peak fever, respectively (Table 9). All *Ana* bulls had morphologically normal sperm

percentages below minimum satisfactory standards for 35 to at least 84 days post-fever. Despite experimental differences (e.g. cause of insult) between the Wildeus study and ours, recovery to satisfactory breeding potential is in agreement with the spermatogenic cycle after resolution of the insult [49].

Most bulls of satisfactory breeding potential will have some proportion of morphologically abnormal sperm, though this value should always be less than 30% of the minimum 100 sperm cells examined. These expected abnormalities can be due to post-ejaculate sample handling or cold shock of the sample. Ejaculate with >30% morphologically abnormal sperm likely indicates a change in bull health status and a reduced likelihood of reproductive success (e.g. decreased fertilization efficiency, conception rate, embryonic development) [81,117]. The specific morphologic abnormality can assist in deducing the location and potential cause of the insult [49,118]. In this study, the primary insult in the *Ana* bulls was fever and anemia from acute anaplasmosis, that likely affected spermatogenesis at both the cellular and tissue level. Regulation of testicular temperature within the scrotum is incredibly responsive to temperature changes. Anything that interferes with the thermoregulatory capacity within the scrotum— including increases in deep body temperature like fever—can cause damage to spermatogenic tissue and epithelium and lead to morphologically abnormal sperm [54]. The anatomy and physiology of sperm cells make them particularly vulnerable to the effects of oxidative stress caused by anemia and fever, which can further lead to morphologic abnormalities [67,68]. Collectively, the morphologic sperm abnormalities commonly observed in the *Ana* bulls during this study reflect damage to both testicular tissue and sperm cells. The presence of specific abnormalities indicate multiple phases of the spermatogenic cycle were

affected, which would be consistent with anaplasmosis causing a systemic disease. The observed abnormalities could reasonably be explained by impaired scrotal thermoregulation and oxidative stress from fever, anemia, and systemic illness caused by acute anaplasmosis. While histopathology is required to provide evidence of direct testicular damage, the presence and persistence of abnormal sperm cells in *Ana* bulls further suggests that clinical anaplasmosis results in testicular damage. In a production setting, the implication of these morphologic sperm abnormalities points to decreased likelihood of fertilization in the female [49,109,117,119,120].

Presence of other abnormal cell types were also observed in *Ana* bull ejaculates. Spermatocytes (Figure 9J), or immature sperm cells, were observed in *Ana* bulls 8553 and 9431 starting 21 days after inoculation prior to clinical disease, peaked at 6 weeks after inoculation, and continued for several weeks after resolution of clinical disease. The presence of high spermatocyte numbers can indicate testicular degeneration, regeneration, or immaturity based on history and other cells present in the spermiogram. In this study, the high number of spermatocytes was likely associated with a regenerative response after transient damage to testicular tissue, with a full spermatogenic cycle required for bulls to achieve mature sperm cells in the ejaculate [48]. Medusa cells (Figure 9K) were observed in *Ana* bulls 8553 and 9431 for 6 consecutive weeks and 1 week, respectively. Medusa cells can be an indicator of significant testicular or epididymal pathology when present in increased numbers [121]. Spermatocytes and medusa cells were not observed for *Ana* bull 9528; however, teratoids were uniquely observed in this bull for 5 weeks. Although presence of specific abnormal cell types varied in *Ana* bull ejaculate, the presence of spermatocytes, medusa cells, and/or teratoids collectively suggests impaired spermatogenesis and damage to the seminiferous epithelium, a precursor or indicator

of testicular degeneration [48,72,81]. No white blood cells were observed in spermiograms of *Ana* or CON bulls which ruled out the possibility of epididymitis or orchitis influencing BSE outcomes, making altered testicular thermoregulation from systemic disease the main differential for the outcomes in the *Ana* bulls. However, it should be mentioned that there is conflicting evidence whether the presence of white blood cells in the ejaculate affect semen quality, particularly sperm morphology[122,123]. Additionally, no red blood cells were observed in semen samples, nor was *A. marginale* organism visually detected in any stained semen preparations. Interestingly, *A. marginale* was inconsistently detected via qPCR in several *Ana* bull semen samples (semen was tested by qPCR out of curiosity). As *A. marginale* resides in red blood cells, these findings are curious, and may simply be due to sample contamination; however, contamination of semen samples with frank blood was never observed. Further research is required to identify whether PCR-positive semen from infected bulls poses a transmission risk.

Annual breeding soundness examination, including assessment of physical exam parameters, sperm morphology, and progressive sperm motility is one of the most cost-effective measures to confirm satisfactory bull breeding potential [89,90,124]. When a bull does not pass any one or more of the four parts of a BSE, it's given an "unsatisfactory" or "deferred" breeding status. The latter is typically given when the BSE changes appear to be transient or resolving particularly around extreme weather conditions, or with younger animals, lameness expected to resolve, lack of appropriate nutrition, or co-morbidities not directly to the reproductive tract. Current recommendations for bulls not passing a BSE with a "deferred" status typically include waiting 60 days before re-examination (based on the normal

spermatogenic cycle) [49,118], although sperm-related changes due to ambient temperatures typically resolve in a matter of days [72]. A bull is assigned an "unsatisfactory" breeding status when the BSE changes are associated with more permanent abnormalities, such as changes to testicular size or texture, upper limb lameness or other chronic disease, or when multiple deferred BSEs have already occurred and ongoing abnormal sperm morphology is present such as high numbers of proximal droplets particularly in older bulls. Two of the *Ana* bulls in this study (8553, 9431) did not return to a satisfactory breeding status for at least 63 days after resolution of clinical anaplasmosis, longer than the typical 61-day spermatogenesis cycle and recommended re-testing period for unsatisfactory bulls. Importantly, bulls that survive clinical anaplasmosis can return to satisfactory breeding potential (e.g. 8553, 9528), however, they may require a longer period before re-testing, such as bull 8553 that required 72 days to return to satisfactory breeding potential. Bull 9431, that failed to recover satisfactory breeding potential within the study period (72 days past initial failure), may never pass if the testicular tissue was permanently damaged during clinical disease. Our pilot study demonstrates that bulls recovered from clinical anaplasmosis can have extended transient or possibly lasting testicular damage leading to delays in production of normal mature sperm. Future studies incorporating testicular biopsies could address how clinical anaplasmosis damages testicular tissue and to what degree this damage is reversible.

Our findings are similar to an earlier study examining the impact of anaplasmosis on bull breeding soundness by Swift et al. in 1979 [36]. In that study, experimentally-infected bulls developed anemia (mean nadir PCV of 21%) and reproductive changes that included unsatisfactory sperm motility and morphology, testicular degeneration, and reduced libido.

Two of the four *A. marginale* inoculated bulls in that study demonstrated improvements in sperm morphology after clinical disease resolved, similar to the outcome for the bulls in our study. Swift et al. further observed reduced libido response to cycling cows by bulls during peak clinical disease. Bull behavior around cycling females was not investigated in our study. Despite a difference of 40 years, differences in equipment and reagents, and likely different *A. marginale* strains, our study and the Swift study had remarkably similar outcomes. Despite a small sample size, our findings agree with those of Swift et al. on how anemia and fever can reduce bull breeding potential.

Building on the results of this pilot study, future studies are needed to mechanistically address how individual hallmark clinical signs of anaplasmosis (e.g. anemia and fever) specifically affect semen quality and reproductive success. Additional variables that could further affect clinical anaplasmosis outcomes and contribute to bull breeding soundness include bull signalment, nutrition status, and co-morbidities. Incorporation of testicular histopathology, ultrasonography, and thermography would enable characterization of anaplasmosis-associated testicular degeneration and injury as prognostication for return to breeding soundness. This is especially important for veterinarians offering recommendations to producers to consider a longer bull retesting window. If the damage caused to the testicle is reversible, retaining bulls longer for retesting is more economic than culling valuable bulls capable of producing viable sperm. However, if testicular damage from disease is irreversible and the bull is retained in the herd for the sake of a longer retesting window, the cost the producer incurs waiting to cull an infertile bull is counted as a loss. Additionally, detection of *A. marginale* DNA in semen samples also warrants effort into whether semen directly, or breeding

activities that can cause reproductive mucosal damage, contribute to disease transmission. Finally, whether bulls with chronic anaplasmosis exhibit lower satisfactory semen quality parameters would be useful to assess as most cattle that survive clinical anaplasmosis develop chronic anaplasmosis.

Conclusion

Pervasive and expanding infectious diseases of cattle such as bovine anaplasmosis hampers the potential for profitable beef production. Under the conditions of our pilot study, we demonstrate that clinical anaplasmosis causes reduced breeding potential through reduced semen quality, scrotal circumference, and body condition in bulls with previous satisfactory breeding potential. The most striking reductions were in sperm motility and morphology. Although clinical anaplasmosis was associated with reductions in several breeding soundness evaluation metrics, this loss was temporary and a return to satisfactory breeding potential was observed for two of three *A. marginale*-infected bulls. Anaplasmosis should be considered as a differential-diagnosis for bulls with unsatisfactory BSE results or poor reproductive performance in anaplasmosis-endemic areas, especially if there is evidence of recent seroconversion. Veterinarians performing BSEs should consider a longer retesting window of 90 days or longer for deferred bulls, and anaplasmosis testing for breeding bulls in anaplasmosis-endemic areas. Producers should take caution when introducing anaplasmosis-naïve, reproductively sound bulls into an anaplasmosis-endemic herd. Producers living in endemic areas purchasing new bulls should employ diagnostic testing to determine infection status, whether by qPCR or cELISA. Naive bulls in endemic areas are considered at highest risk for the

development of clinical disease especially during peak vector season. Considerations for valuable bulls in these areas should include minimizing vector exposure, controlled turn-out and perhaps collection and artificial insemination during highest peak rather than pasture turn-out. It should be stressed that in any case, but especially in endemic regions, producers should not re-use needles due to the risk of blood from infected animals causing acute disease in unexposed animals. Producers should also avoid fall calving to avoid the stress of breeding season and calving during peak vector season, and should consider confine feeding CTC during peak vector season as a means to control active anaplasmosis per the label guidelines. The results of our study demonstrate the detrimental effects of clinical anaplasmosis on bull breeding potential, thus it is imperative that cow-calf herd management, testing, and treatment protocols for *A. marginale* consider risks and impacts of the disease in both females and bulls.

Chapter 3 – Cross-sectional study of Kansas beef bulls to examine associations between chronic bovine anaplasmosis and breeding soundness

Introduction:

Anaplasma marginale is the tick-borne bacterial agent of bovine anaplasmosis, an endemic disease of cattle in the United States (U.S.) and, more broadly, throughout cattle production regions of the world. In particular, anaplasmosis is endemic in the midwestern state of Kansas. In a recent statewide survey of cows in Kansas cow-calf herds by Spare, et al. 2020., 52.5% (486/925) of cow herds were found seropositive for *A. marginale*[101]. While *A. marginale* infection is common among Kansas cattle, most infected animals appear clinically healthy, existing as chronically-infected asymptomatic carriers[4]. Hallmarks of clinical anaplasmosis include anemia and fever leading to pallor, lethargy, and anorexia[3,10] which may easily go unrecognized in cattle on pasture. In some cases, clinical disease may progress to death without the producer appreciating signs of clinical anaplasmosis[125]. Cattle that recover from acute clinical disease become chronically-infected transmission reservoirs[5]. These carriers experience low-level, waxing and waning bacteremia, managed in part by a competent immune system that prevents clinically-relevant high bacteremia levels[126]. Recrudescent clinical disease can manifest from immunosuppression due to co-morbidities such as pneumonia, or extreme stress, such as transport[15]. Less appreciated, is the impact of subclinical anaplasmosis on cattle

production, which may occur during periods of transient immunosuppression such as during temporary environmental stress, breeding season, or herd-level changes.

Studies involving bovine anaplasmosis prevalence primarily focus on cows because they are the dominant sex retained as replacements in cow-calf herds. Data on bovine anaplasmosis among breeding bulls is limited despite the fact that bulls are often retained for multiple years of service. From our previous work and others, clinical anaplasmosis may render beef bulls of unsatisfactory breeding potential for 10 weeks or longer[35,36,127]. Whether clinical anaplasmosis can cause long-term reproductive damage or whether lower bull breeding performance and chronic anaplasmosis are associated is unknown.

In a 2008 APHIS bull management survey, 92.6% of U.S. cow-calf operations used only natural service by bulls to breed females[128]. An updated APHIS national beef cow-calf management survey in 2017 identified that females bred by natural service was 90.7%[129]. Another survey report of Kansas, Oklahoma, and Texas ranches from the USDA Beef Checkoff in 2015 reported an average cow/bull ratio of 18.2, reaching numbers as high as 40 cows/bull in Kansas[130]. According to 2022 USDA survey data, there were 85,000 head of bulls over 500 pounds[131] in Kansas (ranked 6th in U.S. beef production) to service over 20,000 cow-calf herds (average Kansas cow-calf herd size 20 to 49 cows)[132].

Bulls that are intended for breeding in cow-calf operations can be sourced from within the herd or purchased from seedstock producers, the latter of which may come from outside states. According to the annual Seedstock 100 report from BEEF magazine, 56,787 bulls were sold in 2022 from the top 100 seedstock operations[41], with the highest selling operation from

South Dakota, and the second highest selling operation from Kansas, states with a low and high anaplasmosis prevalence, respectively. Movement of bulls into or out of endemic areas may facilitate *A. marginale* transmission and risk of anaplasmosis. For example, asymptomatic *A. marginale* carrier bulls pose an infection transmission risk when introduced into naïve herds. Conversely, naïve bulls moving into anaplasmosis endemic herds are at risk of acquiring *A. marginale* infection through biologic or mechanical transmission events and developing clinical anaplasmosis. Anaplasmosis-naïve cattle (including bulls) 2-years-old or older are at increased risk for poor clinical disease outcomes[2,5,11]. While any animal loss is economically devastating to a cow-calf producer, the loss of a valuable breeding bull is even more so, as bulls represent a significant input cost[133]. Secondly, production loss from sub- or infertile bulls due to infectious diseases such as anaplasmosis could also be economically devasting as cow-calf operation success is based on breeding success towards producing a marketable calf crop, and breeding efficiency is critical to profitability and sustainability.

The breeding soundness examination (BSE) is a veterinary tool for systematic assessment of bull breeding potential (satisfactory, unsatisfactory, or deferred). Components of the BSE include general physical examination, internal and external genitalia including scrotal circumference, and semen quality as assessed by progressive motility and sperm morphology[49]. Variables that may influence overall BSE satisfactory rates include geographic location and climate, veterinary examiner subjectivity, diet changes, herd environment and management, production season (e.g. breeding), and concurrent infectious diseases[133,134]. In general, 1 in 5 bulls in any population are expected to have an unsatisfactory BSE outcome[135-137]. In a study by Higdon et al., BSE outcomes were evaluated in 2,898 bulls in Canada and 93.1% demonstrated

satisfactory breeding potential[138]. In another study of 3,648 U.S. bulls from Tennessee and South Carolina, 76.2% bulls demonstrated satisfactory breeding potential[139]. While the bulls evaluated in these studies were yearlings and therefore not reproductively mature, the majority of unsatisfactory outcomes in both studies were related to semen quality (sperm morphology and/or motility), followed by inadequate scrotal circumference and testicular abnormalities. Similarly, Carson et. al investigated BSE outcome trends at a single institution in bulls ages <15 months to >7 years over the course of 20 years, and abnormal sperm morphology composed by far the majority of unsatisfactory outcomes at any time point[140]. Important considerations for bulls in *A. marginale* endemic areas include exposure of naïve bulls to *A. marginale*, and recrudescent clinical or transient subclinical anaplasmosis in chronically-infected bulls. Bulls recovering from clinical anaplasmosis can experience prolonged effects from anemia and fever that could lead to lasting, degenerative impacts on their reproductive system, namely, the testicles[72,112,141]. Little to no information is available on the lasting effects of clinical anaplasmosis on bull reproductive performance, if chronic *A. marginale* infection reduces bull reproductive performance, or if carrier animals trend lower in satisfactory BSE evaluation metrics.

Considering the essential role of bulls in U.S. cow-calf operations, the widespread anaplasmosis prevalence among most cow-calf production states, and the impact of clinical anaplasmosis on bull breeding soundness, the main objectives of this study were to: i) evaluate the prevalence of chronic anaplasmosis among bulls in eastern Kansas, and ii) investigate whether bulls with chronic anaplasmosis were more likely to have unsatisfactory BSE outcomes. To achieve these study objectives, *A. marginale* infection status and BSE outcomes were

determined for 535 healthy Kansas bulls, and associations between *A. marginale* status, BSE outcomes, and animal signalment (e.g. age and breed) were investigated.

Materials and Methods:

Study Design

All animal studies were carried out under an approved Institutional Animal Care and Use protocol (IACUC 4476) and an approved Institutional Biosafety Committee protocol (IBC 1495) on file at the University Research Compliance Office at Kansas State University, Manhattan, KS. Bull owners signed study consent forms for sample and data collection. Owner and animal information was de-identified to ensure confidentiality. All methods were carried out in accordance with relevant guidelines and regulations in compliance with ARRIVE guidelines[142].

A total of 583 producer-owned beef bulls of various breeds from eastern Kansas were sampled and evaluated for study enrollment. Requirements for bull enrollment included: otherwise healthy with the exception of lameness on initial physical examination; ≥15-months of age; residing in the state of Kansas; and having a complete BSE as described by the Society for Theriogenology (SFT)[49]. Based on these inclusion criteria, one bull was excluded because it was <15-months old, 46 bulls were excluded because they did not reside in Kansas; and one bull was excluded because it had insufficient BSE data, leaving 535 bulls that met study inclusion criteria for analysis. Enrolled bulls had BSEs performed in-clinic or on producer premises by local, practicing veterinarians in the state of Kansas from December 2020 – May 2021. Breeding soundness examination forms from the SFT were provided to participating veterinarians.

Participating veterinarians (n=8) were requested to complete the entire SFT BSE form according to SFT guidelines. In cases where incomplete BSE forms were returned, data from only completed sections were used. Bull signalment and county of origin were also recorded. Temperature humidity index (THI) was calculated for each sampled set of bulls based on the humidity (%) and highest temperature for the county on that day (°C) using previously described calculations[78].

Blood samples were collected from all bulls at the time of BSE by the veterinarian via coccygeal or jugular vein. The blood was divided into evacuated tubes containing EDTA (1.8 mg/mL whole blood) or no anticoagulant and shipped on ice back to Kansas State University for sample processing within one week of collection. Packed cell volume was determined for each animal from EDTA blood samples by centrifuging blood in microhematocrit capillary tubes for 5 min and evaluating PCV using a manual hematocrit reader. A bull was considered anemic if their PCV was <22%, or hemo-concentrated if their PCV was >33% [105]. Blood samples collected into tubes without anticoagulant were centrifuged at 3,000 rpm for 10 min at 20°C to separate and collect serum. Serum samples were aliquoted and stored at -80°C prior to testing.

Breeding soundness examination (BSE)

All BSEs were performed by participating veterinarians using the Society for Theriogenology Manual for Breeding Soundness Examination of Bulls, 2nd Edition guidelines[49]. Semen samples were collected via electroejaculation. As part of a routine BSE, the following were evaluated unless otherwise noted by the veterinarian and are described in detail below: general physical health including external and internal genitalia, scrotal circumference

measurement, electroejaculation response, sperm morphology, and sperm progressive motility. All examination findings were reported on a SFT BSE form provided to the participating veterinarian[49].

General physical examination included an overall health assessment with specific evaluation of foot and leg soundness, vision, external genitalia, and internal genitalia. Body condition score (BCS) assessment was considered part of the general physical exam, and was assigned based on a 9-point scoring system[107]. External genitalia assessment included palpation of penile sheath and prepuce, penis, scrotum and testes. Scrotal circumference was determined using a scrotal measuring tape (cm) around the widest girth of the scrotum. Internal genitalia assessment was performed by rectal palpation of seminal vesicles, ampullae, prostate, and inguinal rings. Any observed abnormalities were recorded on the BSE form.

Sperm morphology was evaluated from a minimum standard of 100 sperm cells via microscopy using semen stain under 1,000X magnification in oil. Individual sperm cells were categorized into “normal”, “head abnormality”, “midpiece abnormality”, or “tail abnormality,” with a minimum of 70% morphologically normal sperm cells required for passing[49]. Presence or absence of other abnormal cells in the ejaculate, such as white cells (i.e. neutrophils, macrophages) or spermatocytes were recorded. No spermatocytes and no more than one white blood cell per high-powered field were required for satisfactory breeding potential.

Sperm motility was assessed chute-side using a compound microscope and scored on either a satisfactory/unsatisfactory basis, or on a percentage basis, with a minimum satisfactory requirement of 30% of cells moving in a forward linear motion per high powered field.

Collectively, bulls were considered of satisfactory breeding potential if they had: i) no abnormalities on general physical examination; ii) no abnormalities on assessment of genitalia; iii) appropriate scrotal circumference based on age; iv) ≥70% sperm cells of normal morphology; and, v) adequate or ≥30% progressive sperm motility. Bulls were considered of unsatisfactory breeding potential if they did not meet minimum requirements in any of the above categories.

DNA extraction and quantitative PCR (qPCR) for detection of *A. marginale* infection.

DNA extraction from blood samples and quantitative PCR to evaluate *A. marginale* infection was performed as previously described[127]. Briefly, total genomic DNA (gDNA) was extracted from 100 µL of whole blood collected into EDTA tubes according to the extraction kit manufacturer instructions, eluted in 35 µL of DNA Elution Buffer, and stored at -20°C. A quantitative, real-time PCR (qPCR) assay targeting a portion of the single-copy *A. marginale* Msp5 gene[29] was used to quantify *A. marginale* infection levels. PCR mastermix preparations were set up in 20 µL reaction volumes, each containing 0.2 µM of each primer (Am msp5 F: ATA CCT GCC TTT CCC ATT GAT GAG GTA CAT and Am msp5R AGG CGA AGA AGC AGA CAT AAA GAG CGT), 10 µL of an intercalating dye mastermix, and 2 µL gDNA. Real-time PCR reaction cycling parameters were as follows: one cycle of 98°C for 2 min; followed by 40 cycles at 98°C for 5 sec, 60°C for 5 sec and 74°C for 15 sec; and, a final melt curve cycle of 65-95°C with increasing 0.5°C temperature steps at 10 sec/step. Real-time qPCR data was visualized and analyzed using instrument software.

Competitive ELISA

Serum samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA) for *A. marginale* serological screening using a commercial cELISA that detects host antibody produced against *A. marginale* Msp5. Animals with a percent inhibition score ≥30% were considered seropositive for *A. marginale*.

Statistical analysis

For the purpose of statistical modeling, age (yr) was categorized into three groups: [1.25, 4],(4, 6], and (6, 11]; THI was categorized into four quartiles: [low, 55.74], (55.74, 60.89], (60.89, 64.15], and (64.15, high]. For statistical modeling with breed being an explanatory variable, 16 animals were excluded from the analysis because their breed was either not recorded or infrequent (<5 animals per breed).

Binary response variables were analyzed using the logic linear model. Model goodness of fit was verified based on the ratio of deviance to degrees of freedom. For *A. marginale* prevalence, explanatory variables included age group, PCV and breed. For overall BSE outcome and motility status, explanatory variables included age group, THI group and *A. marginale* PCR status.

Scrotal circumference was analyzed using the linear model with predictors being age group, THI group and *A. marginale* PCR status. Model assumption was verified by the residual plot.

Numeric outcomes for sperm morphology, including normality, head abnormality, midpiece abnormality and tail abnormality, did not satisfy the assumption of linear models.

Instead, these were analyzed by the non-parametric method that utilized data values as scores to construct test. Two explanatory variables were considered: age group and *A. marginale* PCR status. The effect of one explanatory variable was evaluated while controlling (i.e. stratifying on) the other.

Kappa statistics was used to measure the agreement between *A. marginale* infection status detected by PCR and cELISA. Significance was defined *a priori* as $p \leq 0.05$.

Statistical analyses were executed via Statistical Analysis Software (SAS version 9.4; Cary, NC) LOGISTIC, GLIMMIX, NPAR1WAY and FREQ procedures.

Results:

Description of study bulls

A total of 535 convenience-sampled bulls that met inclusion criteria were analyzed for this study. Eighteen beef breeds, including crossbred bulls, were represented, of which Aberdeen Angus (35.5%), Simmental cross (29.7%), Gelbvieh cross (8.6%), Hereford (7.9%), and Red Angus (6.7%) were most common. Bulls originated from 14 Kansas counties; however, the majority of bulls were from Cloud (50.3%), Clay (15.0%), and Republic (12.9%). Collectively, study bulls were owned by 85 producers, with an individual participating producer owning 1 to 28 bulls (median 5 bulls). Bull ages ranged from 1.5 – 11.0 years (median = 4.0, mean = 4.0). Body condition score ranged from 3 to 8 (median = 6, mean = 5.7). Packed cell volume ranged from 12.0% to 50.0% (median = 34.0%, mean = 34.0%). Scrotal circumference ranged from 32.0 – 51.0 cm (median = 40.3 cm, mean = 40.5 cm), sperm progressive motility scores ranged from

0% to 100% (median = 90%, mean = 84%) and normal morphology scores from 0% to 100% (median = 90%, mean = 86%). Temperature humidity index ranged from 43.4 to 71.5 (median = 60.9, mean = 60.6). A total of eight veterinarians performed BSEs on bulls in this study, although three veterinarians performed BSEs on three or fewer bulls. The other five veterinarians performed BSEs on 57 or more bulls for this study. Veterinarians that performed ≥57 BSEs had bull pass rates between 69%-93%.

Probability of *A. marginale* qPCR status based on cELISA status, age category, breed category, PCV, and body condition score

Among study bulls, 45.8% (245/535) were qPCR-positive for *A. marginale* infection and 52.7% (282/535) were seropositive for *A. marginale*. No study bulls displayed signs of clinical anaplasmosis; therefore, any qPCR-positive bull was considered to be a chronically-infected carrier. The test agreement for both common diagnostic tests was near perfect with a kappa value 0.9213 (SE ±0.0168). Overall, *A. marginale*-carrier status was significantly associated with age group ($p < 0.001$) and bull breed ($p = 0.022$), but not PCV ($p = 0.468$) (Figure 12). The average age of *A. marginale* carrier bulls was 4.5 years (range 1.5 – 10 years, median = 4.0). Bulls less than 4 years of age were significantly less likely to be infected than older bulls ($p = <0.001$) (Figure 12A). The highest *A. marginale* infection prevalence rate among represented breeds was for Simmental (79% ±8.6%) followed by Aberdeen angus (56% ±3.9%), Hereford (53% ±8.4%), Gelbvieh x Angus (52% ±8.0%) and Simmental x Angus (47% ±4.4%) (Figure 12B). The prevalence of *A. marginale* infection was lowest for Charolais bulls (27% ±9.4%). Of the *A. marginale* carrier bulls, 53% ±4.4% (136/245) had an abnormal PCV (<22% or >33%) and 2.4%

(6/245) had an abnormal BCS (<4 or >7). Comparatively, of the uninfected bulls, 56.5% (164/290) had an abnormal PCV; 0.7% (2/290) had an abnormal BCS; and average age was 3.6 years (Range 1.0 – 11 years, median 3.0).

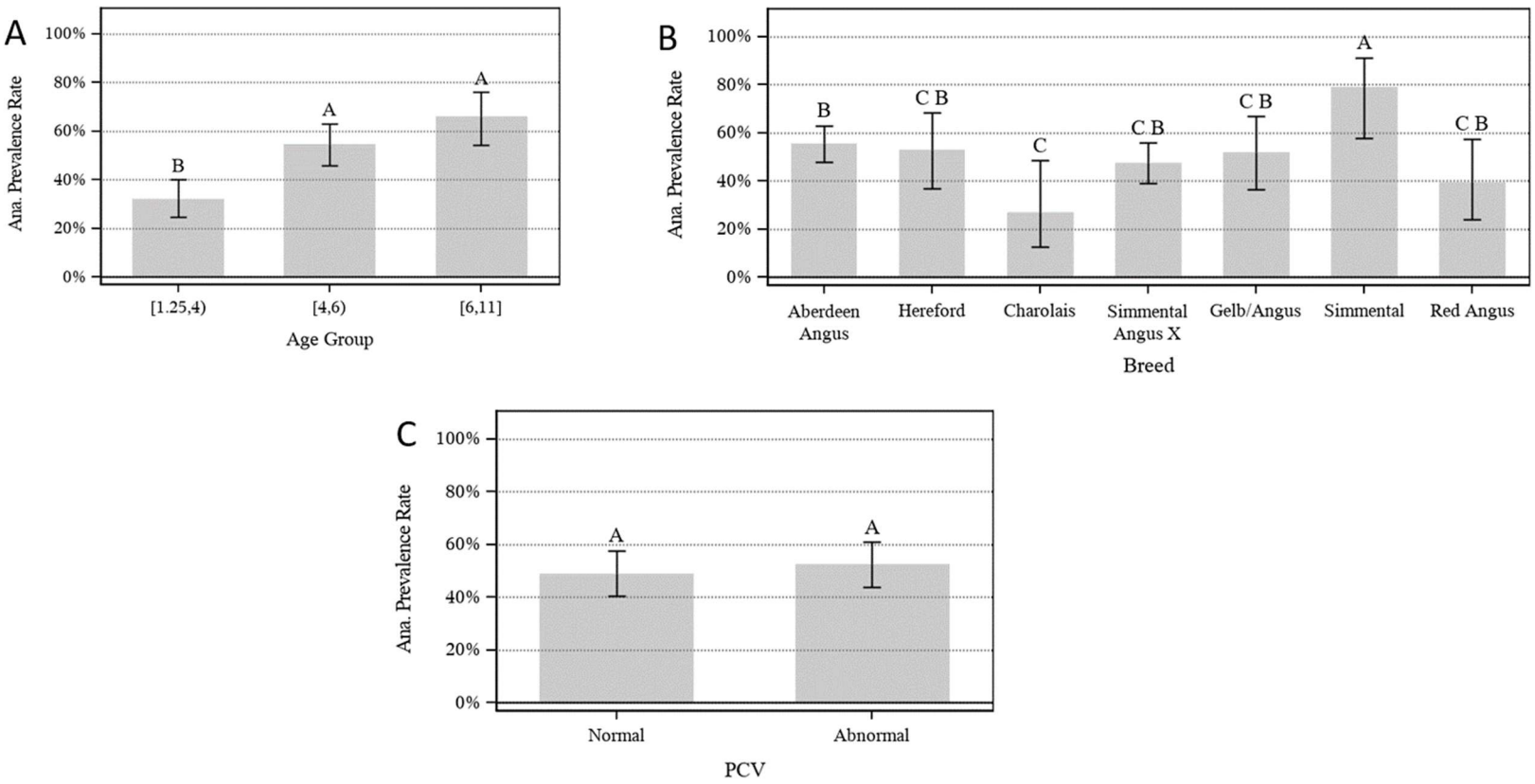

Figure 12. *Anaplasma marginale* (Ana) carrier prevalence among eastern Kansas bulls based on PCR status related to measured variables. Panel A: *A. marginale* prevalence versus bull age in years; B. *A. marginale* prevalence versus bull breed; C. *A. marginale*

prevalence versus bull packed cell volume (Normal 22-33% PCV); D. *A. marginale* prevalence versus bull anaplasmosis vaccine status. Plotted values with different letters are significantly different (p <0.05).

Probability of satisfactory breeding potential based on *A. marginale* qPCR status, age category, breed category, and body condition score

Of surveyed bulls, 12.5% (67/535) had an unsatisfactory BSE outcome. The majority of unsatisfactory outcomes (77.6%, 52/67) were due to poor semen quality, specifically unsatisfactory progressive motility and/or sperm morphology. Five study bulls produced ejaculate with poor sperm concentration and motility, limiting the veterinary observer from assessing sperm morphology. Another five bulls (7.5%) had an unsatisfactory BSE outcome due to physical examination abnormalities alone (e.g. lameness). Several bulls (20/67) did not pass their BSE based on a combination of poor semen quality, external genitalia abnormalities, and/or physical examination abnormalities.

Overall, neither *A. marginale* qPCR status ($p = 0.324$), THI ($p = 0.677$), nor age group ($p = 0.672$) were significantly associated with an overall unsatisfactory BSE outcome among this sample set (Figure 13). Of *A. marginale* qPCR-positive bulls, the overall unsatisfactory BSE outcome rate was slightly higher in carrier bulls (15.0% ±2.4% versus 12.0% ±2.2%) than in uninfected bulls, but this difference was not significant (Figure 13A). The percentage of bulls with an unsatisfactory BSE outcome tended to increase with age, as bulls >6-years-old had the highest unsatisfactory BSE rate (16.0% ±3.8%) of the three age groups (Figure 13B). No obvious trends were appreciated with THI quartile, though all bulls were tested below reported the threshold (study THI maximum = 71.5) for thermal comfort (THI <72)[78].

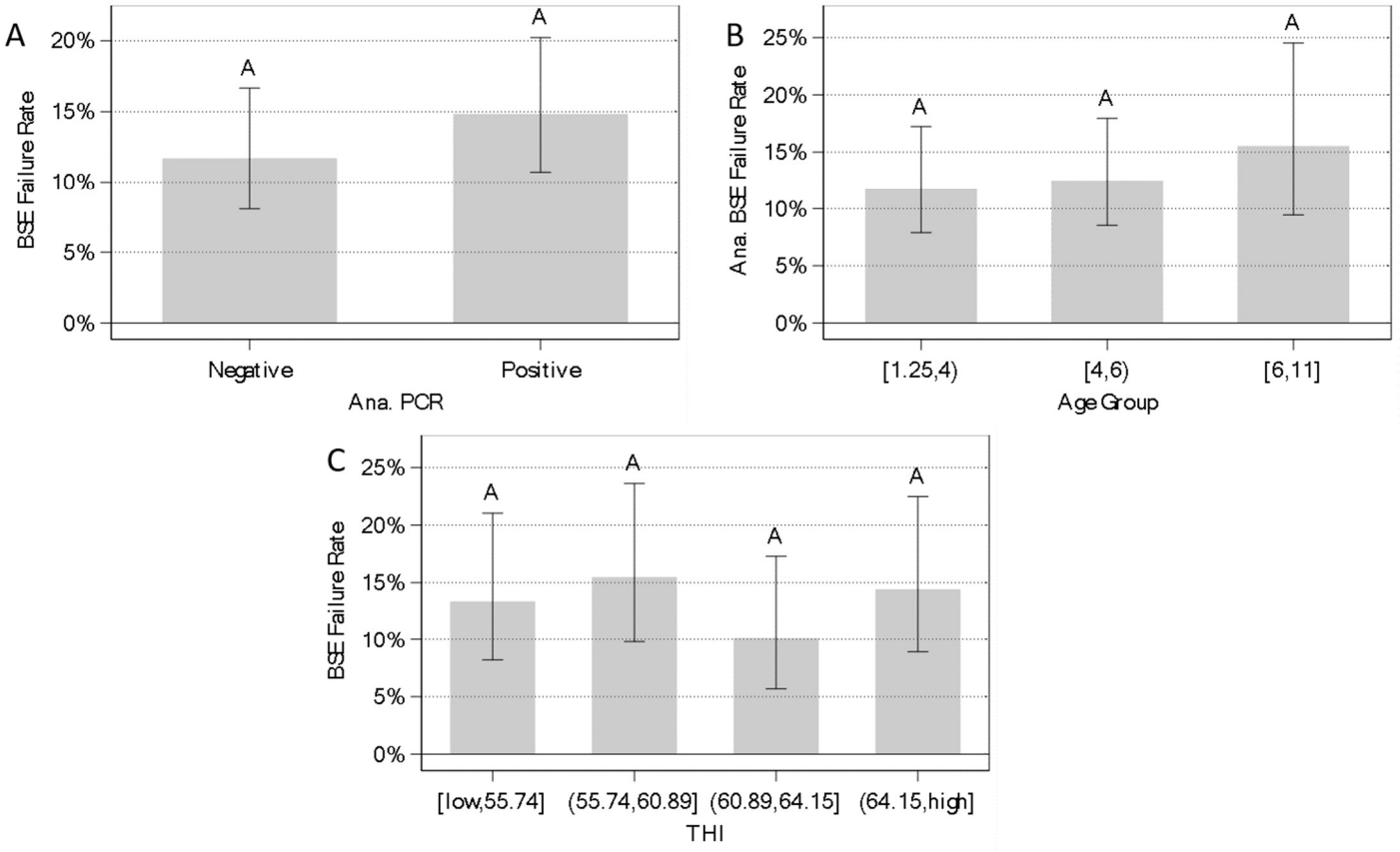

Figure 13. Breeding soundness examination (BSE) outcomes related to measured variables. Panel A: BSE failure rate versus bull *A. marginale* (Ana) PCR status; B. BSE failure rate versus anaplasmosis vaccine status; C. BSE failure rate versus temperature humidity index (THI); D. BSE failure rate versus bull age in years. Plotted values with different letters are significantly different (p <0.05).

Individual BSE components investigated included sperm progressive motility, scrotal circumference, and sperm morphology. When investigating unsatisfactory sperm progressive motility, neither *A. marginale* qPCR status (p = 0.156), age category (p = 0.147), nor THI (p = 0.832) were significantly associated with unsatisfactory sperm progressive motility. Although not statistically significant, *A. marginale* qPCR-positive bulls had greater unsatisfactory motility scores (6.3% ± 1.6%) than negative bulls (3.5% ± 1.2%). Similarly, bulls ≥6-years-old had a greater incidence of unsatisfactory motility scores (8.1% ± 2.8%) than younger age cohorts ([1.25,4): 3.7% ± 1.3% and [4, 6): 3.4% ± 1.3%), though this was not significant between age groups. No notable motility trends were appreciated in the bulls tested at study THIs. When investigating scrotal circumference, neither THI group (p = 0.211) nor *A. marginale* qPCR status (p = 0.149) were significantly associated with scrotal circumference, but age category was significantly associated with scrotal circumference (p = <0.001). Bulls had significantly larger scrotal circumference with each increase in age group ([1.25, 4): 39.12 cm ± 0.20 cm; [4, 6): 40.86 ± 0.20 cm; and [6, 11): 42.35 cm ± 0.30 cm) (p <0.001).

When investigating sperm morphology, the percentage of normal morphology sperm was not significantly associated with bull *A. marginale* qPCR status (p = 0.479) or age category (p = 0.491). Sperm midpiece abnormalities were the most frequently described abnormalities by veterinarians in this study (mean = 22.9% in failing bulls).

Discussion:

Findings from this study support that *A. marginale* is endemic in eastern Kansas, including among bulls in eastern Kansas. In the context of this sample set, chronic *A. marginale*

infection was not significantly associated with unsatisfactory BSE outcomes. This study also briefly describes important variables that could influence study outcomes, such as timing of BSE and variation among technique and precision among veterinarians performing BSEs in a clinical practice setting.

Based on this sample population of bulls, the prevalence of chronic *A. marginale* infection among bulls in eastern Kansas is high, nearing 50%. Other studies have demonstrated 4.4%-29.0% *A. marginale* infection prevalence in cattle in states throughout the U.S.[37-39], and 52.5% herd-level seroprevalence in Kansas cattle[101]. Other studies have also demonstrated deleterious health effects of chronic *A. marginale* infection, including reduced milk production in dairy cattle, reduced weight gain, and transplacental infection of offspring, leading to economic losses for the producer[34,143,144]. However, this is the first study to describe prevalence of *A. marginale* in Kansas bulls and investigate associations between chronic *A. marginale* infection and breeding soundness parameters. In this study, unsatisfactory BSE outcomes were low at 12.5% among the sampled eastern Kansas bulls, considerably lower than the expected 20% among a tested population of bulls[135-137]. The percentage of bulls with unsatisfactory BSE outcomes was slightly, but not significantly, higher among bulls with chronic *A. marginale* infection (15.0% ± 2.4% vs 12% ±2.2%). While a reasonable explanation for this could be that carrier bulls are minimally affected by long-term *A. marginale*-infection, personal biases or variability by the veterinarians performing the BSEs may have unintentionally influenced results[50,145]. Additionally, the sampling strategy for this study may have inadvertently selected for bulls that receive more regular veterinary oversight considering that the participating

producers pursue routine BSE screening and may prioritize more routine veterinary preventive care.

The lower prevalence rate of *A. marginale* carriers among younger animals in this study was expected as *A. marginale* infection incidence and age are positively correlated[6]. The longer cattle are retained (the longer they live), the more opportunities for transmission and disease exposure, though cattle first exposed to *A. marginale* after >2 years of age are more likely to suffer from severe, acute disease[6]. Pairing this information with the data demonstrating slightly higher unsatisfactory BSE outcomes in PCR-positive animals, it is important to note that regardless of *A. marginale* status, bulls may begin to develop abnormalities on the BSE starting at 5 years of age, which has been attributed to a more pendulous scrotum and greater opportunities for multiple testicular insults over time[95,146]. Finally, there were some trends in *A. marginale* infection status among varying breeds, with Charolais bulls demonstrating a considerably lower prevalence rate of chronic *A. marginale* infection compared to breeds such as Simmental. Whether there are breeds of *Bos taurus* cattle more resistant to infection with *A. marginale* warrants further investigation.

Several studies have outlined factors contributing to reduced breeding soundness in beef bulls[48,95]. Previous studies have addressed the impact of hemolytic diseases on bull BSE outcome[35,59,112-114,127], though these investigate clinical disease rather than the carrier state or recovery period on breeding soundness. Carrier status was not significantly associated with unsatisfactory BSE outcomes in this study. Although timing of initial clinical disease could not be

estimated in this study, producers living in endemic areas should have confidence that *A. marginale* carrier bulls can demonstrate satisfactory breeding soundness.

Anemia and fever, like that during clinical anaplasmosis, especially when prolonged, can lead to testicular degeneration and damage of sperm DNA as a result of oxidative stress[35,36,114]. Testicular degeneration can affect spermatogenesis to the degree that no viable sperm are produced, either transiently or permanently. For the entire study population, sperm motility and morphology abnormalities were low, with less than 10% of all study bulls (52/535, 9.7%) having unsatisfactory BSE outcomes related to semen quality. However, of the bulls that did not pass, semen quality alone or in conjunction with other parameters below minimum requirements was the number one reason for BSE failure (52/67, 77.6%). It is important to identify specific abnormalities of the sperm head, midpiece and tail to differentiate between compensable and non-compensable defects, which provide a more defined picture of fertility potential. Compensable sperm defects (e.g. knobbed acrosomes, bent tails) are those that can be overcome by increasing the volume of ejaculate[147]. Non-compensable defects (e.g. nuclear vacuoles, pyriform heads) are those which can be overcome by increasing the dose of morphologically normal, progressively motile sperm cells in the ejaculate. The degree of these defects for bulls in this study was not specifically captured and thus could not be analyzed. A limitation of this study was that most participating veterinarians divided sperm morphology by head, midpiece, and tail alone—the minimum requirements of a veterinarian performing a BSE. Beyond these three basic categories, it is strongly advised to further discriminate the defects in each category to identify sperm abnormalities that could influence future fertility. Although the cause for the poor-quality semen in these samples was not determined, previous clinical

anaplasmosis should be considered as a differential in those bulls, as bulls recently recovering from clinical anaplasmosis may have prolonged unsatisfactory semen characteristics[127]. It should also be emphasized that while carrier bulls can produce ejaculate with normal sperm morphology and motility during breeding soundness examination, the stress of the breeding season may lead to recrudescence of clinical or transient subclinical disease that may alter semen quality, particularly when new escape variants are emerging in animals with chronic anaplasmosis (chronic anaplasmosis is characterized by a cyclical bacteremia, where chronic infection is maintained through a continuous cycle of new immune escape variants emerging every four to six weeks).

Limitations for interpreting effects of scrotal circumference on study outcomes include age and breed effect on scrotal circumference. Multiple ages of bulls were analyzed in this study, and scrotal circumference is expected to increase with age. Puberty is expected to continue in bulls up to four years of age[49]. Older bulls would be expected to have the largest circumference due to maturity. Finally, while the BSE seeks to improve uniformity among veterinarians during assessment of bull breeding soundness, variations exist among conditions in which the BSE is performed and veterinary technique and experience. Assessment of variability among veterinarians that performed BSEs was not compared as each enrolled bull was evaluated by a single veterinarian. Important considerations when comparing BSE outcomes across veterinarians would include technique, experience, personal bias, and skill of the performing veterinarian. Studies are limited in regard to veterinary BSE variability[50,82,145], though this can be challenging to measure due to individual bulls being assessed once every several months at most and due to the variations described above. The high number of

identified midpiece abnormalities in this study could be attributed to true deficits of the spermatocyte; changes after sample handling; quality of the microscope and objective used; veterinarian comfort in identifying midpiece abnormalities; and decreased veterinarian experience in identifying more subtle abnormalities in the sperm head. One potential pathway to have overcome these discrepancies would have been to create a protocol for participating veterinarians to submit prepared morphology slides to the primary veterinary investigator or a boarded veterinary theriogenologist for consistency in evaluation of sperm morphology.

In conclusion, chronic *A. marginale* infection was not significantly associated with an unsatisfactory BSE outcome in this population of bulls with a nearly 50% *A. marginale* infection prevalence. Investigating prevalence of *A. marginale* in other bull populations and subtle impacts of chronic *A. marginale* infection during different times of the production year on BSE outcomes warrants further investigation. The impact of endemic infectious diseases of cattle such as bovine anaplasmosis is important to study in both cows and bulls to minimize disease impact on cattle production and operation profitability.

Chapter 4 – Conclusions

This research highlights many key features of anaplasmosis in beef bulls, including how this disease impacts bull fertility. Key findings from this program of work include: i) overall finding that bull fertility is negatively impacted by acute anaplasmosis; ii) the probable insult to spermatogenic tissue that occurs during acute anaplasmosis may warrant a recovery window longer than 60 days; iii) recovered carrier bulls undergoing routine BSEs by general practitioners have similar outcomes to uninfected bulls in the investigated population; iv) prevalence of anaplasmosis in Eastern Kansas bulls nears 50%; and v) discrimination variability between general practitioner veterinarians when performing BSEs likely influence BSE outcomes despite a standardized system of examination.

The project investigating acute clinical anaplasmosis in beef bulls paves the way for many future projects. In addition to the need for a larger clinical trial, future studies would benefit from incorporating testicular tissue biopsy to characterize the extent and potential for tissue insult recovery after resolution of clinical anaplasmosis. While testicular biopsy is certainly useful in the research setting or in bulls that have concerningly abnormal spermiograms, it should be noted that this diagnostic approach is not a routine aspect of the BSE because there is some risk for testicular fibrosis, calcification, and/or inflammation at the biopsy site[148]. Other methods for evaluating the effects of anaplasmosis on testicular tissue during peak clinical disease include the use of imaging such as ultrasonography and thermography. Limitations to this project included the small sample size and lack of histopathology of testicular tissue.

The project investigating differences between apparently healthy *A. marginale* carrier bulls and uninfected bulls undergoing routine BSE creates a standard template for similar investigations of *A. marginale* prevalence throughout the U.S. There are several prevalence studies in female cattle throughout the U.S. especially in endemic southern regions[34,37,38,101,149]. Bulls undergoing routine BSEs in these regions could be enrolled in similar prevalence studies. The changing climate in the U.S. due to global warming and animal movement will continue to warrant anaplasmosis prevalence studies, including in areas not historically considered to be endemic for anaplasmosis. Additionally, outcomes from this project highlight the need to investigate agreement among veterinary observers in regard to the bull BSE. Data from a controlled study investigating veterinary observer agreement of the BSE could identify potential pitfalls in the training of the veterinary student or general practitioner in performing an accurate and effective BSE, similar to that investigated by Johnson in 1996[82]. Similar studies could also investigate prevalence among bulls in a particular region or population with a known or controlled anaplasmosis vaccine history, as well as bulls with a known timeline post recovery from clinical disease. Limitations to this work include the narrow Eastern Kansas demographic of the bull population investigated, gaps in known history of bulls sampled, and variability among veterinary observers and equipment used to perform examination.

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