FERTILITY ASSOCIATED ANTIGEN IN PERIPUBERTAL BEEF

BULLS AS AN INDICATOR OF POTENTIAL FERTILITY

A Thesis

by

JOANNA LYNN GALLINO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Physiology of Reproduction

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Approved by:

Co-Chairs of Committee,	Lesly R. Sprott
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ABSTRACT

Fertility Associated Antigen in Peripubertal Beef Bulls as an

Indicator of Potential Fertility. (August 2005)

Joanna Lynn Gallino, B.S., California State Polytechnic University

Co-Chairs of Advisory Committee: Dr. L. R. Sprott
                                                          Dr. D. W. Forrest

Ejaculates from peripubertal Angus (n=106), Brahman (n=156) and Brangus (n=212) bulls were evaluated for the presence of a 31 kDa protein known as fertility associated antigen (FAA) using the ReproTest for Bulls (ReproTech, Tucson, AZ). This study was designed to test the repeatability of FAA detection using the chute-side cassette and to quantify the relationship of age, scrotal circumference, sperm motility, ejaculate volume and sperm concentration with the presence of FAA in ejaculates from peripubertal bulls. A total of 776 ejaculates were collected, and 77% (n=598) were classified as FAA^+. Three ejaculates were obtained from 133 bulls (Angus, n=33; Brahman, n=100) on d 0, 30 and 60. Brahman bulls were older (p<0.0001) at puberty than Angus bulls. Ejaculate volume was the only trait that differed between bulls that were classified as FAA^+ and FAA^- at first collection. Mean ejaculate volume was greater (p<0.0001) for FAA^- than for FAA^+ ejaculates. Serial ejaculates were profiled according to one of four FAA classification patterns (0 = three FAA^- ejaculates; 1 = three FAA^+ ejaculates; 2 = first ejaculate FAA^-; and 3 = first ejaculate FAA^+). A majority of the bulls were FAA^+ on all three ejaculates (57%) or at least on the first

ejaculate (25%). Seven bulls (5%) were consistently FAA$^-$, while the remaining bulls (12%) were FAA$^-$ on the first ejaculate. There were no differences in scrotal circumference or sperm concentration among FAA profiles. FAA profile-3 bulls were older (p<0.05) than FAA profile-1 bulls at 2nd collection. Sperm motility was greater (p<0.05) for FAA profile-1 than for FAA profile-3 bulls at 1st collection. Ejaculate volume was greater (p<0.05) in FAA profile-0 Angus bulls than for FAA profile-1 at 1st collection and in FAA profile-0 Brahman bulls than FAA profile-2 at 2nd collection. These data indicate that FAA classification was not affected by sperm motility, sperm concentration or scrotal circumference in peripubertal bulls. Repeatability of FAA classification was higher for bulls that were FAA$^+$ at first ejaculation. Thus, peripubertal FAA$^-$ bulls should be re-evaluated to increase FAA-classification accuracy for identification of higher fertility as well as lower fertility animals prior to breeding.

DEDICATION

This is dedicated to my family, who has been my foundation in all of my endeavors. To my parents, for giving me wings to soar, and to my siblings for keeping me grounded. Without the strong family network that we have, I would not have been able to reach for the stars. Thank you, Mom and Dad, for the support, the guidance, and the not-so-gentle reminders that I can't stay a student forever. Thank you for the financial support to make these dreams of mine come true. It is said that life is the journey, not the destination. It has been an incredible journey thus far. Thank you for accepting my application to the "Donald and Eileen Gallino Educational Enhancement Scholarship Fund" in the summer of 2004 in support of my study abroad trip to South Africa. That was an absolutely incredible experience that I will take with me the rest of my life.

Thank you to my sister Jan, for your support, encouragement, advice, and ruthless editing. Thank you for the time spent on the phone listening to me whining and complaining, and still calling me back the next weekend. I feel truly blessed to have you as my sister.

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INTRODUCTION

Efficient reproductive management of cattle requires the identification and selection of dams and sires that exhibit high fertility potential in order to consistently produce offspring at 12-month intervals and successfully maintain a profitable business. Incorporation of sub-fertile bulls into herds as sires can decrease pregnancy rate, calf crop and income. Much effort has been placed on improving fertility and conception rate in cows by selecting cows that initiate estrous cyclicity within 45-60 days following parturition, thereby decreasing the post-partum interval and allowing the cow to produce more calves during her lifetime. Cows that fail to conceive within 60-85 days of parturition (the time needed to maintain a 12-month calving interval) may be culled from the herd if it is thought that the cows did not rebreed because of perceived fertility problems. However, the inability to rebreed may be due to use of a sub-fertile bull rather than sub-fertility of the cow. Accurate diagnosis of infertile or sub-fertile bulls before the start of the breeding season is of paramount importance for maintaining proper management guidelines.

Fertility and breeding soundness are important characteristics in bulls that are selected as sires. Potential fertility of mature bulls is a characteristic that has been evaluated based upon an array of physical and behavioral traits. Some traits, such as scrotal circumference, have subsequently been used to predict fertility of offspring as

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This thesis follows the style of the Journal of Animal Science.

well. However, the criteria that are routinely used to evaluate the fertility of sires only provide an estimate of potential fertility, and are not a direct indicator of fertility. Breeding soundness evaluations (BSE) are used to evaluate sperm quality and the physical conformation of the bull, while libido (desire to mate) and serving capacity (number of matings achieved during a specific time period) can also be tested in order to estimate the number of females a bull can be expected to service during the breeding season (Chenoweth, 1997). Previous studies have indicated positive correlations among ejaculate characteristics, mating ability, and desire to mate with the pregnancy rate achieved in the herd (Bellin et al., 1994, 1996, 1998). However, some bulls may exhibit excellent semen characteristics, achieve a satisfactory score on their BSE and exhibit acceptable libido/serving capacity while still not achieving high pregnancy rates.

Recent studies of bull semen characteristics have indicated that other components of an ejaculate might play a role in increasing fertility potential. Several compounds, such as heparin (Handrow et al., 1984; Parrish et al., 1988), have been shown to enhance the ability of sperm to undergo capacitation and the acrosome reaction *in vitro*. Proteins found in ejaculated semen samples have high affinity for binding to heparin-like compounds, such as glycosaminoglycans (GAG), which are found in the female reproductive tract. Following natural service or artificial insemination, seminal proteins bind to the female GAG and enhance the fertilization ability of sperm by facilitating penetration of the zona pellucida and fertilization of the oocyte. Bellin et al. (1998) identified a specific member of the heparin-binding protein family in bovine semen which was termed fertility-associated antigen (FAA).

The ability to detect FAA in semen samples allows for identification of sires possessing an increased potential for greater fertility. Testing of these sires for the presence of FAA can benefit producers by offering them another tool to be used for evaluation in conjunction with a BSE. Bulls that test positive for FAA during regular exams have been shown to exhibit increased fertility by siring more calves earlier in the breeding season than bulls that tested negative for FAA (Bellin et al., 1998). This is especially important since earlier conceiving cows, thus earlier calving cows, produce the oldest and heaviest calves in the herd. Such calves will generate more income compared to their younger, lighter herdmates.

Initial studies tested semen of sexually mature bulls for the presence of heparin binding proteins (HBP), and techniques were subsequently developed to specifically detect the presence of FAA (Bellin et al., 1994, 1998).   The ontogeny of FAA production by the accessory sex glands has not been investigated. Although it has been shown that FAA production in rats is testosterone dependent (Nass et al., 1990), it is not known whether testosterone concentrations must reach the threshold levels required for sperm production to begin before production of FAA may commence. The timing of the initiation of FAA synthesis and secretion into the seminal plasma during post-natal development remains to be characterized. Testing of young pre- or peri-pubertal bulls for the presence of FAA will allow producers the opportunity to select potential sires at an earlier age.

Detection of FAA has been made more efficient by the development of a "chute-side" test cassette, the ReproTest for Bulls[®] (ReproTech, Tucson, AZ) that can analyze

neat semen samples for the presence of FAA. McCauley et al. (2004) evaluated over 900 bulls for the presence of FAA using the chute-side test cassette and determined that analysis for the presence or absence of FAA could be accurately assessed within 5-10 minutes. Semen samples collected during annual BSE exams are currently evaluated for concentration, motility and morphology. Simultaneous evaluation for the presence of FAA using the chute-side test cassette is another tool that can be used to evaluate the potential fertility of potential sires.

OBJECTIVES

This study was designed to test whether the ReproTest® cassette can distinguish between FAA-positive and FAA-negative peripubertal beef bulls in which spermatozoa may or may not be present in the ejaculate. Up to three ejaculates were obtained at 30-d intervals during the peripubertal period from beef bulls (n=474) of three breed types. The objectives of this study were to: (1) assess the repeatability of FAA classification among the three ejaculates from each bull, and (2) quantify the relationship within and across breed type of age, sperm concentration, sperm motility, ejaculate volume and scrotal circumference with FAA classification.

LITERATURE REVIEW

Evaluation of Fertility

Evaluation of potential sires is vital to increase the probability of reproductive success during the breeding season. Selection of sub-fertile or infertile animals can lead to decreased production efficiency. Positive correlations exist between sires with a larger scrotal circumference and the reproductive traits of their offspring (Smith et al., 1989), however recent studies have indicated that selection of yearling bulls based solely on physical traits may not be as effective as once thought (Martinez-Velazquez et al., 2003). Increased knowledge of the interactions among measurable reproductive traits in young bulls is necessary in order to enhance selection accuracy for potential fertility.

Puberty

Puberty is the process of acquiring the ability to reproduce. The attainment of puberty is typically marked by the development of the secondary sex characteristics in response to increased androgen production, namely testosterone, as well as the onset of spermatogenesis (Thibier, 1975; Lunstra et al., 1978; Amann and Walker, 1983). Puberty has been defined in several ways: attainment of a minimum scrotal circumference or the presence of a minimum sperm concentration in an ejaculate. A minimum of 50 X 10^6 spermatozoa in an ejaculate, with >10% being progressively motile was used to define achievement of puberty in young bulls by Wolf et al. (1965). A report by Lunstra et al. (1978) indicated that over one half of the Angus, Hereford and reciprocally crossed bulls in their study with a scrotal circumference of 27.9±0.2 cm had achieved puberty, while Aravindakshan et al. (1999) also reported average scrotal

circumference measurements of 28.0±0.2 cm in bulls achieving puberty. Sexual maturity has also been defined as two consecutive ejaculates containing $\geq 500 \times 10^6$ sperm/ejaculate with one ejaculate exhibiting >50% motility or 4 consecutive ejaculates with $\geq 500 \times 10^6$ spermatozoa (Tatman et al., 2004). The ease in obtaining scrotal circumference measurements compared to semen collection and evaluation aids in the prediction of potential fertility in young, untrained animals. A delay in the attainment of puberty precludes the ability to evaluate potential fertility in yearling bulls by conventional methods.

Breedtype Differences. Animals of different breedtypes progress through puberty differently. *Bos indicus* breeds such as Brahman achieve puberty at an older age compared to their *Bos taurus* counterparts. In the study by Tatman et al. (2004) spring- and fall- born Brahman bulls achieved puberty between 14-16 months of age, with spring-born bulls then reaching sexual maturity approximately two months earlier than the fall-born bulls. Conversely, several *Bos taurus* breeds, including, Angus, achieve puberty at an average age of 10-11 months (Lunstra et al., 1978).

Breeding Soundness Evaluations

History and Components. Prediction of fertility in cattle has been the ultimate goal of many exams and evaluations. Examinations of physical and seminal characteristics have long been used in an attempt to discern animals with increased fertility from animals that are sub-fertile. The use of breeding soundness evaluations (BSE) on potential herd sires allows producers to cull those animals that are not performing up to specific standards. Standardization of the process for evaluating the

breeding soundness of bulls, as introduced by the Colorado State University Animal Reproduction Section (Carroll et al., 1963) and further developed by the Society for Theriogenology, has been an ongoing process. Bulls undergo a physical examination as well as a semen evaluation. Chenoweth et al. (1992) recommended minimum required standards for each category tested (scrotal circumference, sperm motility and sperm morphology) rather than the cumulative scoring system that was previously used. The cumulative system allowed a satisfactory rating of a bull that was sub-standard in one or more categories if exceptional marks were achieved in other categories

Physical Examination. A BSE consists of a physical examination to evaluate a young bull's structural conformation, including body condition and correctness of the mouth, as well as an examination the penis, scrotum and a palpation per rectum of the accessory sex glands. For bulls up to 15 months of age, a classification of satisfactory requires that the minimum scrotal circumference should be 30 cm (Chenoweth et al., 1992). Bulls with incorrect structural conformation or problems with their feet could possibly have difficulty mounting the females, while bulls with deformed or broken jaws would not be able to eat properly, leading to an overall decline in body condition and health. Animals in poor body condition do not usually have the energy stores that are required during intensive breeding seasons. Conversely, over-conditioned bulls may also exhibit difficulty in successful breeding due to increased fat deposition around the testicles and spermatic cord, leading to decreased testicular function due to inhibition of proper thermoregulation processes (Barth et al., 1995). Testing and examination of bulls several months prior to breeding allows producers time to treat or correct any maladies

that may be affecting the bull, such as illnesses or infections that require drug treatments or altering an animal's diet, thereby restoring the animal to the proper body condition.

Semen Evaluation. Semen samples are analyzed according to sperm motility, normality, classification of abnormalities and the presence of other types of cells such as white or red blood cells or somatic cells. To be classified as a satisfactory potential breeder, sperm motility must be at least 30% with at least 70% of sperm morphologically normal (Chenoweth et al., 1992). Concentration of sperm is not used in the strict measure of a BSE, however, the concentration of sperm must be known in order to correctly process the semen for artificial insemination purposes. Color of the ejaculate should be a creamy, milky white, which is indicative of a high sperm concentration, whereas a clear, watery color indicates a very low concentration and a yellowish color may indicate urine contamination of the sample.

BSE Classification Results. Following completion of a BSE, animals will be classified as a satisfactory potential breeder, unsatisfactory potential breeder, or classification deferred which indicates that the animal should be retested at a later date (Chenoweth et al., 1992). Each bull must meet or exceed the minimum requirement in all categories to be classified as satisfactory. Failure to meet one or more requirements would result in the animal being deferred for retesting at a later date. Should the animal fail to meet the minimum standards at that time, he would be classified as an unsatisfactory potential breeder. Annual BSE exams are highly recommended for every bull that is to be used as a sire during the breeding season.

Libido and Serving Capacity. Although not commonly tested due to time and labor constraints, libido and serving capacity are also important characteristics to consider. Libido is defined as the desire to mate. Bulls with little or no desire to mate with females will not produce many, if any, offspring. Lack of libido in bulls may be caused by a number of factors, including poor nutrition, back problems, abnormalities of the feet and legs or abnormalities of the penis and prepuce (Chenoweth, 1983). Serving capacity of bulls is also an important characteristic (Osborne et al., 1971; Blockey, 1981). Bulls must be able to achieve intromission in order to successfully breed females while in estrus. Bulls that are not able to complete intromission, despite their willingness to breed, will also not produce many calves. To ensure that all cows in a breeding herd are successfully serviced and bred, it becomes important to also test libido and serving capacity in order to fully estimate potential fertility.

Fertilization of the Ovum

Sperm Transport. During ejaculation, spermatozoa leave the epididymis and are transported through the vas deferens and the urethra, where they are bathed in seminal plasma fluids. These fluids are produced by the accessory sex glands, which include the seminal vesicles, prostate, bulbourethral gland and ampulla. Secretions in the fluids from these glands serve many functions, including cleansing the urethra of residual urine prior to ejaculation, providing a source of energy for the spermatozoa and initiating the maturation process required for fertilization (Harper, 1994). There are many proteins produced by the accessory sex glands that can be present in an ejaculate. Some of these proteins have been shown to have an apparent effect on the ability of the spermatozoa to

fertilize the ovum as well as influence the overall fertility in several species, including the bull.

One family of proteins has been classified as heparin-binding proteins (HBP). These proteins are produced mainly by the seminal vesicles and prostate gland (Nass et al., 1990; McCauley et al., 1999) and have been found to positively affect fertility. These proteins can bind to the sperm membrane (Bellin et al., 1996, 1998), apparently during ejaculation. Heparin-binding proteins have an increased affinity for binding compounds similar to heparin, such as glycosaminoglycans (GAG), which are produced throughout the female reproductive tract (Lee and Ax, 1984; Ax and Lenz, 1987). Once bound to the sperm membrane, the HBP provide an increased number of binding sites for heparin (Miller et al., 1990) and therefore the GAG found in the female reproductive tract, thereby facilitating capacitation and acrosome reaction.

Capacitation. Spermatozoa must undergo physiological changes, capacitation and acrosome reaction, before becoming reproductively competent. Although spermatozoa have been found in the oviduct within minutes of insemination, they are not capable of fertilization at that time (Harper, 1994) because they have not had the required capacitation time. These physiological changes begin when HBP in seminal fluid bind to the spermatozoa and conclude with the acrosome reaction and penetration of the zona pellucida. The precise mechanisms of capacitation are still poorly understood. However, it is accepted that capacitation is the process by which the plasma membrane of the spermatozoa is altered by the addition of certain seminal plasma proteins during ejaculation and then the removal of other proteins by fluids produced in

the female tract during transport. Spermatozoa must undergo capacitation before initiation of the acrosome reaction can occur. Capacitation may take several hours as spermatozoa are transported through the cervix, uterus and oviduct. Capacitation of a spermatozoa is not a terminal change; rather, it is a reversible process, provided the spermatozoa are reintroduced to seminal plasma; essentially undergoing de-capacitation. Spermatozoa then require additional capacitation time to regain fertilization capacity.

Acrosome Reaction. Following capacitation, spermatozoa must undergo the acrosome reaction in order to achieve full fertilization competence. In a spermatozoon, the acrosome is a membrane-bound organelle, lying just under the plasma membrane, containing proteolytic enzymes required for penetration of the zona pellucida and fertilization (Yanagimachi, 1994). The acrosome covers up to one-half of the nucleus and is comprised of an inner- and outer- acrosomal membrane (Yanagimachi, 1994). The acrosome reaction occurs when the outer acrosomal membrane fuses with the plasma membrane, creating vesicles that allow the release of acrosomal contents. One component is the enzymatic compound proacrosin, the inactive precursor of acrosin. Acrosin has been shown to aid in the binding of the spermatozoa to the zona pellucida. It was determined by Wincek et al. (1979) that a GAG found in porcine uterine flushes had the ability to induce the conversion of the enzymatically-inactive proacrosin precursor to acrosin, the active form, following the acrosome reaction. Parrish et al., (1980) found that GAG derived from different species were able to induce the conversion of porcine proacrosin to acrosin, indicating that the transformation is not species- or tissue-specific. GAG from several tissues in the female reproductive tract

may be involved in the process of capacitation and the acrosome reaction during sperm transport in the female to the site of fertilization.

In vitro studies have indicated that the addition of heparin (Handrow et al., 1984; Parrish et al., 1988) or heparin-like compounds such as chondroitin sulfate (Lenz et al., 1982; Ax et al., 1985) to semen samples facilitates capacitation, and subsequently the acrosome reaction. Although heparin is not found in the female reproductive tract, GAG similar in structure to heparin are present in bovine follicular, oviductal and cervical fluid and these compounds may facilitate sperm capacitation (Lenz et al., 1982; Lee and Ax, 1984; McNutt and Killian, 1991) and the acrosome reaction (Lenz et al., 1982, 1983) in the female following natural and artificial insemination. Therien et al. (2005) recently reported on the potency of follicular GAG, concluding that, while there were strong interactions between the follicular GAG and seminal proteins, heparan sulfate had a greater effect on capacitation compared to chondroitin sulfate B. *In vitro* studies by Parrish et al. (1988) and Miller et al. (1990) have shown that epididymal sperm must first be exposed to seminal plasma prior to incubation with heparin in order to undergo zonae pellucidae-induced acrosome reaction.

Heparin Binding Proteins

HBP-5. Heparin binding proteins are produced by the accessory sex glands, primarily the seminal vesicles (Nass et al., 1990), but also by the prostate and bulbourethral glands (McCauley et al., 1999) and have been shown to be testosterone-dependent in rats (Nass et al., 1990). HBP have variable binding affinities for heparin or heparin-like compounds (Handrow et al., 1984; Chandonnet et al., 1990; Bellin et al.,

1994). Marks and Ax (1985) noted that bulls of known increased fertility produced sperm with an increased affinity for heparin. Bellin et al. (1994) found that ejaculates from bulls with detectable concentrations of sperm-associated HBP-B5 (the HBP complex with the greatest affinity for heparin) achieved a pregnancy rate that was 17 percentage points higher than bulls with undetectable concentrations of HBP-B5 or HBP-B5 detectable only in seminal fluid. HBP-B5 was comprised of three proteins of different sizes (21 kDa, 24 kDA and 30 kDa) and were recognized by a monoclonal antibody (M1) developed by Bellin et al. (1996). McCauley et al. (1996) using M1, showed that HBP were localized to specific regions of the sperm membrane of ejaculated sperm but not epididymal sperm. The three proteins comprising HBP-B5 that were recognized by M1 were the proteins expressed by the bulls with increased fertility (Bellin et al., 1996).

Fertility Associated Antigen. In the study by Bellin et al. (1996), bulls grouped according to the differential expression of the three M1-recognized proteins on sperm membranes exhibited differences in fertility, with those bulls expressing the 30 kDa protein achieving the highest pregnancy rate. Bellin et al. (1998) termed the 30 kDa protein Fertility Associated Antigen (FAA). Bulls expressing sperm-associated FAA (FAA-positive) had greater fertility than those bulls that were FAA-negative and, coupled with increased serving capacity (libido), sired more calves earlier in the breeding season (Bellin et al., 1998). In a study by Sprott et al. (2000), it was noted that artificial insemination (AI) of mature cows and replacement heifers resulted in higher

pregnancy rates when using semen from FAA-positive bulls compared to semen from FAA-negative bulls, regardless of AI service at spontaneous or synchronized estrus.

Purification and characterization of FAA by McCauley et al. (1999) yielded an amino acid (AA) sequence similar to that of human DNase I-like protein. This AA sequence was then used to generate antibodies that were used to develop technology to detect FAA in neat semen at chute-side during a BSE (McCauley et al., 2004). According to McCauley et al. (2004), only semen from older, sexually mature bulls was used for validation of the lateral-flow, chute-side test cassette for FAA. There is little information regarding the evaluation of potential fertility of young pubertal bulls, and no information on the age at which the presence of FAA can be initially detected in young bulls. The ability to evaluate the potential fertility of pre- or peri-pubertal bulls would be of great benefit to producers who regularly evaluate the fertility potential of herd replacement animals.

Other Fertility Related Proteins

McCauley et al., (2001) reported the identification of a 24 kDa HBP that was recognized by the same monoclonal antibody (M1) that had previously recognized the HBP-B5 group that included the 31 kDa HBP protein, FAA. Characterization of the 24 kDa HBP-B5 member indicated a strong similarity to the 24 kDa tissue inhibitor of metalloproteinases-2 (TIMP-2), a factor previously reported to be involved with the remodeling of the extracellular matrix. TIMP-2 was subsequently found to be produced by the bulbourethral gland, prostate and seminal vesicles. In the study by Bellin et al.

(1996), bulls lacking the 24 kDa-fraction of HBP-B5 exhibited reduced fertility compared to those animals expressing all three HBP-B5 components or FAA alone.

Two other proteins have also been identified in the seminal plasma of Holstein bulls of known high fertility. These proteins include the 26 kDa lipocalin-type prostaglandin D synthase (PGD synthase), produced by the epididymis (Gerena et al., 1998, 2000) and the ubiquitous 55 kDa osteopontin (OPN) produced by the seminal vesicles and ampulla as well as many other tissues (Cancel et al., 1997, 1999). Gerena et al. (1998) hypothesized that PGD synthase functions as a carrier for blood-derived substances across the blood-testis barrier, since many in the lipocalin family serve as transporters. A role for OPN in fertility has yet to be elucidated. However, since Cancel et al. (1999) were unable to localize OPN on epididymal, ampullar or ejaculated sperm, it was hypothesized that OPN plays an indirect role in increasing fertility by actions in the male reproductive tract, rather than directly affecting the spermatozoa.

MATERIALS AND METHODS

Animals

Purebred, peri-pubertal Angus (n= 106), Brahman (n= 156) and Brangus (n= 212) bulls were used in this trial. Bulls were maintained on the premises of their respective owners during data collection. Ranches one, two, and three each housed one breedtype while ranch four housed two breedtypes of animals (ranch #1 – Angus; ranch #2 – Brahman; ranch #3 – Brahman; ranch #4 – Angus, Brangus). Although exact nutritional strategies were not examined, bulls on ranches two, three and four were on full-feed growing rations while bulls housed on ranch one were on forage (late summer coastal Bermuda followed by oat grazing in November and December).

Angus bulls from ranch one weighed approximately 330 kg with an average age of 9.9 months at first collection, while Angus bulls from ranch four weighed approximately 435 kg with an average age of 11.8 months. Weights on Brahman bulls from ranches two and three were not available, however the Brahman bulls had an average age of 18.9 (ranch two) and 18.2 (ranch three) months at first collection. Brangus bulls from ranch four averaged 11.4 months of age. Animals housed at ranches one, two, and three were examined at 28-30 d intervals on approximately days 0, 30 and 60, with the first collection date at each ranch termed day 0. Some animals were not present for all three examinations. Several bulls from ranches one, two and three were examined only one or two times during the three collection days. Therefore, analysis of all animals includes those present at any of the three collection times, while animals present for all three collections were analyzed separately. Animals on ranch four were

examined once with one semen sample collected. Semen samples were collected and immediately evaluated for volume, sperm concentration and sperm motility with results immediately recorded. Scrotal measurements were taken for every bull present for evaluation, even if an ejaculate could not be collected.

Ejaculates collected at each ranch were coded with a number unique for that collection day so that ranches one, two, and three each had three separate collection codes, while ranch four had one collection code. Not all bulls evaluated were able to produce an ejaculate, therefore, although only breed, age and scrotal measurements were recorded for those animals; ejaculate characteristics were not.

Scrotal Circumference

Scrotal circumference measurements were taken prior to electroejaculation using a scrotal circumference measuring tape at the widest point of the scrotum, in part to determine peripubertal status.

Semen Collection and Processing

Semen Collection. Semen samples were collected via electroejaculation using a rectal probe (Electrojac III, Ideal Instruments, Chicago, IL). Ejaculates were collected in 15-ml conical tubes via a plastic semen collection-cone funnel.

Sperm Concentration. Sperm concentration was evaluated using a Densimeter (Animal Reproduction Systems, Chino, CA) with pre-programmed software for evaluating bull semen. Sperm concentration of each ejaculate was analyzed using a ratio of 79:1 F10 formalin (3.42mL) to semen (43.2 μL).

Sperm Motility. The percentage of motile sperm of each ejaculate was estimated using microscopic evaluation. Microscope slides were warmed prior to motility evaluation.

Semen Aliquots. Aliquots of semen (1 mL) were frozen separately in 1.5-mL micro-centrifuge tubes for FAA analysis at a later date. Collection and aliquot tubes were then frozen on dry ice for transportation back to the laboratory (Texas A&M University, College Station, TX) where all samples were then transferred to storage at -80ºC.

FAA Analysis Using the FAA Chute-Side Cassette

FAA analysis was conducted using the ReproTest for Bulls (ReproTech, Tucson, AZ) chute-side lateral-flow diagnostic cassette (McCauley et al., 2004). Analysis was performed on 782 ejaculates from 474 peripubertal Angus, Brahman and Brangus beef bulls. Semen aliquots frozen in 1.5ml micro-centrifuge tubes were shipped on dry ice to the laboratory of Dr. Roy Ax at the University of Arizona, Tucson for FAA analysis. Aliquot groups were thawed according to their unique collection code number so that samples from the same ranch, and therefore the same bulls, were not analyzed at the same time. Once thawed, micro-centrifuge tubes were lightly vortexed to re-suspend any sperm cells that may have settled.

For FAA analysis, equal volumes of semen (200 µL) and buffer (50mM TRIS, pH 7.5, 0.19 azide, 1.0 M NaCl, 5 mM EDTA [Midland Bioproducts, Boone, IA]) were mixed in a micro-centrifuge tube. An aliquot (150 µL) of the semen-buffer mixture was then placed in the test well of the ReproTest for Bulls® and exposed to gold-labeled

anti-FAA antibody previously adhered to the membrane. FAA present in the sample adhered to gold-labeled recombinant anti-FAA and migrated laterally across the nitrocellulose membrane. The migrating gold-labeled antibody conjugate binding to immobilized antibody, located at a test strip section of the membrane, resulted in a positive visual confirmation. Samples positively-reacting with the test strip within 5 min were noted as positive. The remaining samples were allowed up to 30 min to react positively with the test strip before being recorded as negative. Migrating, unconjugated gold-labeled antibody, bound to a control protein adhered to the membrane at the control strip (downstream from the test strip) to verify proper performance of the cassette kit, regardless of the presence FAA.

FAA-positive results were recorded as soon as a colormetric change at the test-strip on the nitrocellulose membrane was apparent. Results with an increased intensity color change at the test strip were rated as slightly positive, moderately positive and highly positive, based on the intensity of the color change at the test strip.

Following FAA analysis, semen samples were sent on dry ice to the laboratory at Texas A&M University, College Station, Texas.

Sperm Concentration Validation of Densimeter Readings

Validation of sperm concentrations as measured by the Densimeter were conducted using single-use, 10-ul Unopettes (Becton-Dickson and Co., Franklin Lakes, NJ) on a hemocytometer microscope slide following the protocol previously described by Sorensen (1979). A subsample of all ejaculates was selected to represent all ranches and a cross section of all sperm concentration ranges previously measured. Ejaculate

samples were grouped according to the following sperm concentration ranges: 0-99, 100-299; 300-499, 500-749, 750-999, 1000-1249, 1250-1499 and >1500 million sperm cells per mL. The samples were allocated to a concentration level based according to the sperm concentration measurements determined by the original Densimeter readings.

An aliquot of semen was taken with a 10μ capillary tube and mixed with 1.99 mL of a pre-mixed diluent in a reservoir container. Following thorough mixing, the capillary tube was then used to place a drop of the semen/diluent mixture on a Neubauer Brightline Hemacytometer (Hausser Scientific, Horsham, PA). Sperm cells were viewed at 10X using a Nikon Alphaphot 2 microscope (Japan). Counts were taken by counting sperm cells in 5 of 25 squares on the hemacytometer grid. Four separate counts were taken for each sample and averaged together for comparison against Densimeter readings.

Statistical Analysis

Data analysis was performed according to ranch, breed, total number of animals examined and animals that were present for all examination and had three ejaculates collected. The FAA data were analyzed using SAS Analyst PROC GLM (SAS 8.1, SAS Institute Inc., Cary, NC) against breed, age in months, scrotal circumference, sperm concentration, sperm motility, ejaculate volume, FAA profile, and all appropriate interactions. Correlation of all variables was conducted using the SAS Analyst correlations program. Sperm concentration validation was conducted using SAS paired t-tests to compare the means of each sperm concentration level measured by the Densimeter and hemacytometer.

RESULTS

Animals Tested

Across three breeds and four ranches, 776 ejaculates were analyzed for FAA, resulting in 77% (n=598) classified as FAA-positive. An ejaculate was collected on three dates (at approximately 30-d intervals) from peripubertal Angus (n=48) and Brahman (n=154) bulls that were located on ranch one, two or three, resulting in 514 ejaculates analyzed for FAA with 80% of the ejaculates (n=409) classified as FAA-positive. A single ejaculate was collected from peripubertal Angus (n=56) and Brangus bulls (n=206) located on ranch four. A total of 262 ejaculates collected from ranch four were analyzed for FAA. Among Angus bulls, 73% (n=41) were FAA-positive and 78% (n=148) of Brangus bulls were FAA-positive.

Peripubertal Status

A total of 450 bulls were present at first collection for scrotal circumference measurements. Three bulls (Angus, n=1; Brangus, n=2) had a scrotal circumference less than 28 cm at first collection. Among all scrotal circumference measurements, 12% of bulls (n=53: Angus, n=15; Brahman, n=19; Brangus, n=19) had a scrotal circumference less than or equal to 30 cm.

Breed Comparisons

Least square means (LSM) for age, scrotal circumference and ejaculate characteristics for Angus, Brangus and Brahman bulls at collection of the first ejaculate are presented in Table 1. At first collection (d 0), Brahman bulls were older (p<0.0001) than Angus and Brangus bulls. Scrotal circumference was smaller in Brahman bulls

Table 1. LSMean age, scrotal circumference, sperm concentration, motile sperm and ejaculate volume by breed at collection of the first ejaculate

	Characteristic				
Breed	Age (mo)	Scrotal circumference (cm)	Concentration ($\times 10^6$ sperm/mL)	Motile sperm (%)	Ejaculate volume (mL)
Angus	11.1^a	34.9^e	263^g	56.3^c	5.8^a
Brahman	18.3^b	33.8^f	325^g	56.0^c	5.7^a
Brangus	11.4^a	34.5^e	274^g	49.9^d	4.6^b

LSMeans without a common superscript differ (^{a,b} $p<0.0001$, ^{c,d} $p<0.005$, ^{e,f} $p<0.05$, and ^{g} $p>0.1$)

than Angus (p<0.01) or Brangus (p<0.05) bulls, however, there were no differences in sperm concentration. Sperm motility and ejaculate volume were lower in Brangus bulls (p<0.005; p<0.0001 respectively). However, when the comparison was made between animals classified as FAA-positive or FAA-negative, there were no differences between the two groups for age, scrotal circumference, sperm concentration or sperm motility. However, LSM for ejaculate volume was higher (p<0.0001) in FAA-negative ejaculates than for FAA-positive ejaculates (Table 2).

LSM for age, scrotal circumference and ejaculate characteristics for Angus and Brahman bulls at last collection (d 60) are presented in Table 3. Brahman bulls were older than Angus bulls (p<0.0001), had larger scrotal circumferences (p<0.005) and produced ejaculates with greater volume (p<0.0005). There was a tendency for sperm concentration to be greater in Brahman bulls (p=0.08) and there was no difference in motility between the two breeds. When comparisons were made between animals classified as FAA-positive or FAA-negative at last collection (Table 4), bulls classified as FAA-negative were older (p<0.0005) than bulls classified as FAA-positive. There was a tendency for ejaculate volume to be higher (p=0.06) in FAA-negative bulls than FAA-positive bulls. There were no differences between FAA classification for scrotal circumference, sperm concentration or sperm motility (Table 4).

Breeds

Angus Bulls. Angus bulls (n=48) from ranch one, ranging in age from 8-11 mo (mean = 9.9 mo) at first collection, were evaluated three times, with a total of 128 ejaculates analyzed. Ejaculates were not collected from all bulls during the first

Table 2. LSMean age, scrotal circumference, sperm concentration, motile sperm and ejaculate volume by FAA classification at first collection

Variable	FAA-positive	FAA-negative	p-Value
Age (mo)	12.7	12.7	0.8769
Scrotal circumference (cm)	34.2	34.1	0.6759
Concentration ($x10^6$ sperm/mL)	236	221	0.6375
Ejaculate volume (mL)	3.5	9.3	0.0001
Sperm motility (%)	51.3	49.8	0.4518

Table 3. LSMean age, scrotal circumference, sperm concentration, motile sperm and ejaculate volume by breed at collection of the last ejaculate

	Characteristics				
Breed	Age (mo)	Scrotal circumference (cm)	Concentration ($x10^6$ sperm/mL)	Motile sperm (%)	Ejaculate volume (mL)
Angus	11.8[a]	32.8[e]	355[g]	56.6[i]	4.3[c]
Brahman	20.2[b]	34.3[f]	466[h]	60.4[i]	5.6[d]

LSMeans without a common superscript within a column differ ([a,b] p<0.0001, [c,d] p<0.0005, [e,f] p<0.005, and [g,h] p=0.08, [i] p>0.1)

Table 4. LSMean age, scrotal circumference, sperm concentration, motile sperm and ejaculate volume by FAA classification at last collection

Variable	FAA-positive	FAA-negative	p-Value
Age (mo)	15.6	16.4	0.0005
Scrotal circumference (cm)	33.7	33.4	0.5255
Concentration ($x10^6$ sperm/mL)	388	432	0.5285
Sperm motility (%)	58.3	58.7	0.9022
Ejaculate volume (mL)	4.6	5.3	0.0620

collection, resulting in three ejaculates collected from 32 bulls while two ejaculates were collected from 16 bulls and analyzed for FAA. All but one of the 48 Angus bulls located at ranch one had at least one FAA-positive ejaculate, while 77% (n=38) had at least two FAA-positive ejaculates. Among the 32 bulls collected three times, 40% (n=13) were FAA-positive for all three ejaculates, while another 40% (n=13) of the bulls were FAA-positive for two ejaculates. Twenty-two percent (n=7) of the bulls were FAA-negative for the first collection and then were FAA-positive for the second and/or third collection. Conversely, 18.75% (n=6) of the bulls were FAA-positive for the first collection, and then were FAA-negative for the second and/or third collection. One bull was FAA-negative for all three ejaculates. Figure 1 illustrates the mean age of Angus bulls classified as FAA-positive or FAA-negative. There were no differences in age between bulls classified as FAA-positive or FAA-negative within each collection. Figure 2 depicts the percentage of FAA-positive Angus bulls at ranch one by age at time of ejaculate collection for bulls that had ejaculates collected three times vs. bulls with ejaculates collected less than three times.

Positive and negative correlations existed between FAA and several characteristics measured at ranch one. There was a tendency for ejaculates with higher sperm concentration to be FAA-positive for the first collection (r=0.281; p<0.1). However, this trend disappeared during subsequent collections. At the second collection, ejaculate volume and sperm motility were negatively correlated to FAA presence (r=-0.320, p<0.04; r=-0.370, p<0.02; respectively). There were no significant correlations between FAA and any of the characteristics measured for the third collection. LSM for
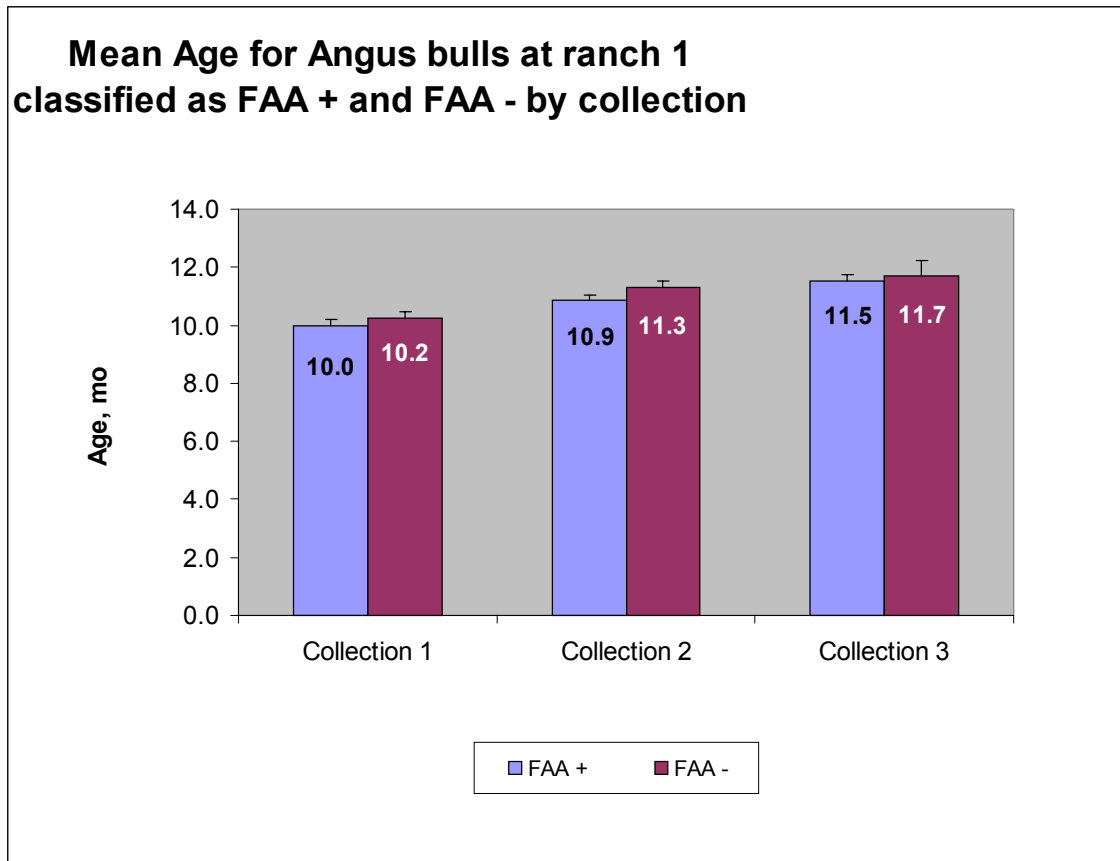
Figure 1. Mean age of peripubertal Angus bulls at ranch 1 classified as FAA-positive and FAA-negative for each collection.
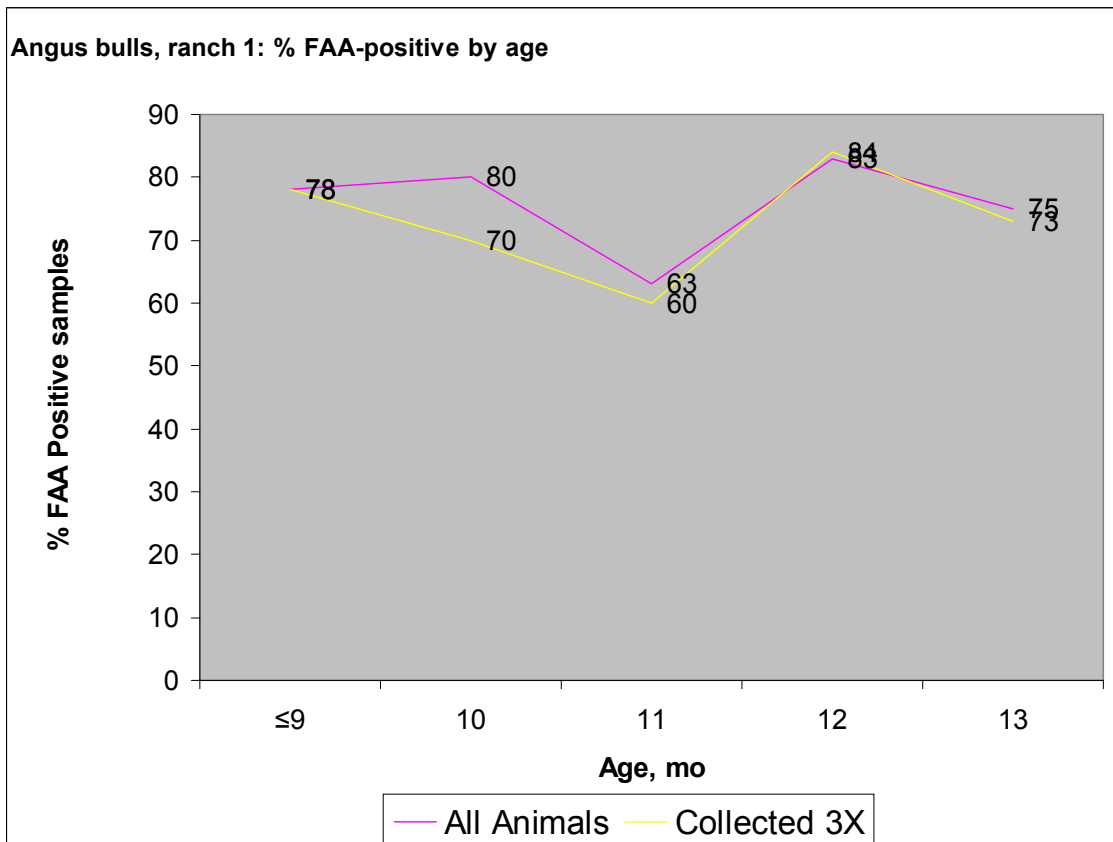
Figure 2 Percentage of peripubertal Angus bulls at ranch one that were classified as FAA-positive based upon either three or fewer than three ejaculates by age.

age, scrotal circumference, and ejaculate volume were not different between animals classified as FAA-positive or FAA-negative across collections.

Angus bulls from ranch four (n=56), ranging in age from 10-12 mo (mean = 11.8 mo) were evaluated once with FAA analysis performed on 56 ejaculates. Of the 56 ejaculates collected, 73% (n=41) were positive. As with the Angus bulls from ranch one, a negative correlation existed between presence of FAA and ejaculate volume (r=-0.319; p<0.0001). Table 5 depicts the mean (±SEM) age, scrotal circumference, sperm concentration, motility and ejaculate volume at the first collection of all Angus bulls (ranches 1 and 4) vs. the three collections for Angus bulls from ranch 1.

Analysis of the first or only collection from all Angus bulls from both ranches revealed no significant correlations between FAA and any of the characteristics measured. However, as would be expected, for all Angus bulls there were positive correlations between age and scrotal circumference (p<0.0001), sperm concentration (p<0.0001) and sperm motility (p<0.0001), and between scrotal circumference and sperm concentration (p<0.0001), sperm motility (p<0.0003) and semen volume (p=0.0001). Figure 3 illustrates the percentage of all Angus bulls that were classified as FAA-positive grouped by age at time of ejaculate collection.

Brahman Bulls. Brahman bulls from ranches two (n=45) and three (n=109), ranged in age from 16-21 mo (mean=18.5 mo) and 16-21 (mean=18.2 mo) at first collection, respectively. Three ejaculates (n=102), two ejaculates (n=35) or one ejaculate (n=19) from each Brahman bull were evaluated for semen traits and FAA classification. Figure 4 illustrates the mean age of Brahman bulls that were classified as

Table 5. Mean (±SEM) age, scrotal circumference, sperm concentration, sperm motility and ejaculate volume by ejaculate number for Angus bulls on ranches one and four and for ejaculate one for all Angus bulls

|		Age (mo)		Scrotal circumference (cm)		Concentration (x10^6 sperm/mL)		Sperm motility (%)		Ejaculate volume (mL)	
FAA Status		+	−	+	−	+	−	+	−	+	−
All Angus: Collection 1	Ejaculate 1	11.2± 0.1	11.0±0.2	35.0±0.4	34.5±0.5	275±30	244±38	58.4±2.2	52.3±3.4	5.2±0.3	6.2±0.5
Angus bulls: Ranch 4	Ejaculate 1	11.8±0.1	11.7±0.2	36.7±0.5	36.4±0.6	319±42	347±60	62.3±2.0	58.2±2.8	5.6±0.4	6.5±0.7
Angus bulls: Ranch 1	Ejaculate 1	10.0±0.2	10.2±0.3	31.9±0.4	32.6±0.4	198±26	134±18	51.2±4.9	45.4±6.1	4.6±0.4	5.9±0.8
	Ejaculate 2	10.9±0.2	11.3±0.2	33.4±0.4	34.2±1.2	229±39	273±58	45.7±4.3	68.8±3.4	4.3±0.3	5.4±0.9
	Ejaculate 3	11.5±0.2	11.7±0.5	33.0±0.4	32.7±0.5	334±42	370±95	56.2±3.5	57.9±7.0	4.0±0.3	4.6±0.4
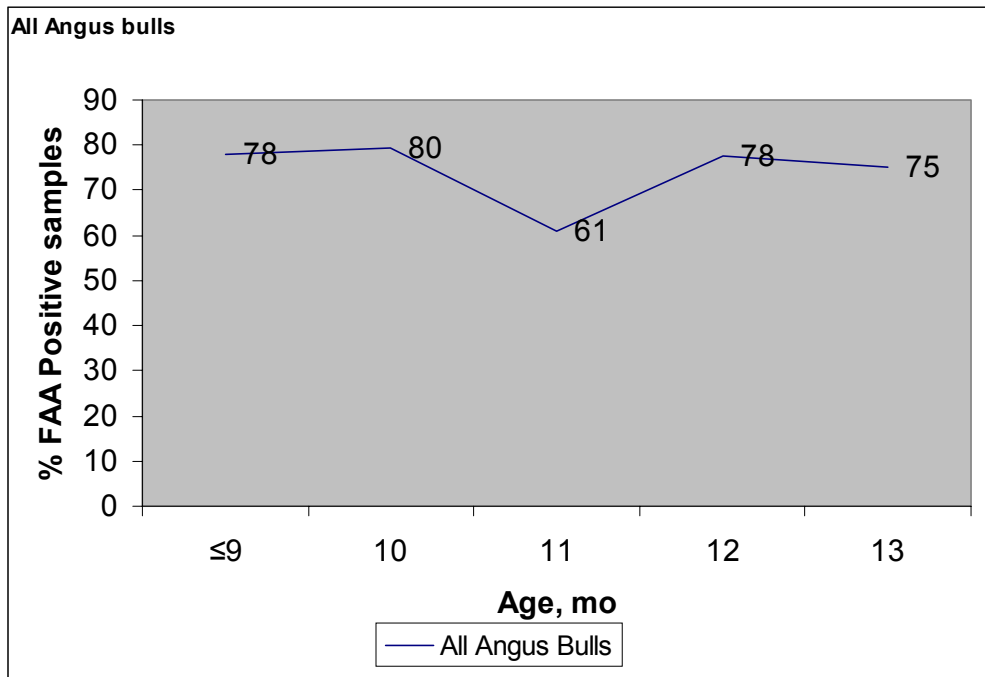
Figure 3. Percentage of all Angus bulls (ranches one and four) that were classified FAA-positive by age at ejaculation.
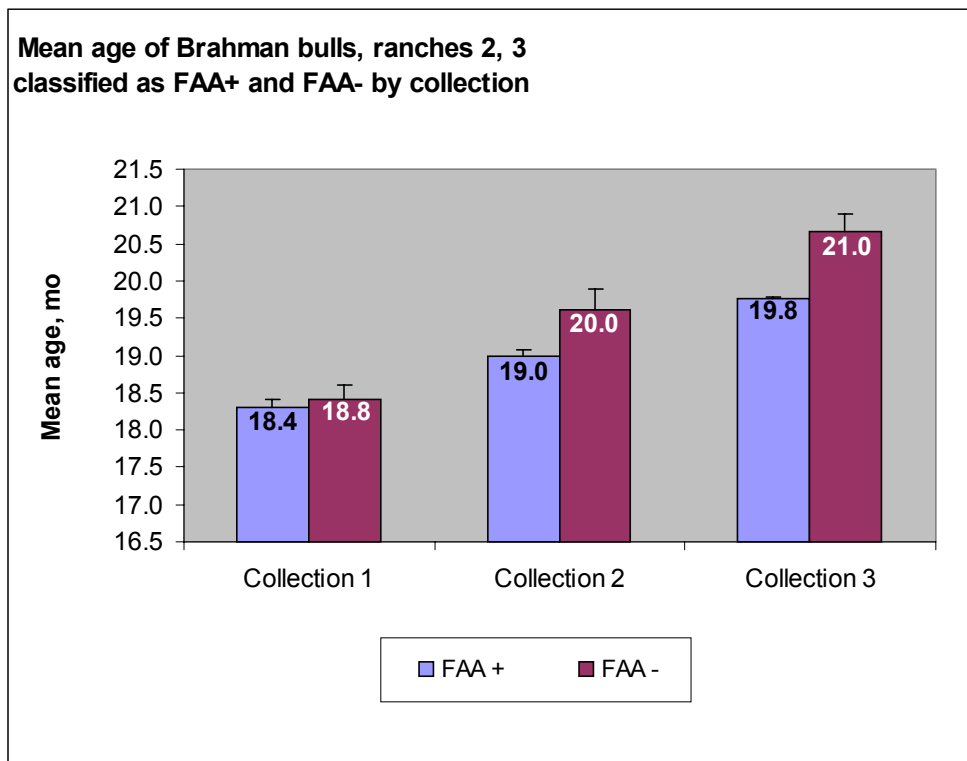
Figure 4. Mean age of peripubertal Brahman bulls from ranches 2 & 3 classified as FAA-positive and FAA-negative for each collection.

FAA-positive or FAA-negative for each collection. Figure 5 depicts the percentage of FAA-positive Brahman bulls by age at time of semen collection for bulls tested three times vs all bulls tested. A total of 386 ejaculates resulted in 81% (n=311) as FAA-positive. Of the 100 animals evaluated three times, 62% (n=62) had three FAA-positive ejaculates, 32% (n=32) had one or two FAA-positive ejaculates and 6% (n=6) were FAA-negative for all three ejaculates.

The LSM of scrotal circumference, sperm concentration, or sperm motility did not differ for ejaculates classified as FAA-possitive or FAA-negative. As with the Angus bulls, there was a tendency for ejaculates with higher volumes to be FAA-negative for tests one, two and three (p<0.5, p=0.07, and p=0.09, respectively). As expected, scrotal circumference was larger for older bulls than younger bulls at semen collections one and two (p<0.02; p<0.05 respectively). However, this difference decreased for test three (p=0.06). Bulls with larger scrotal circumferences also produced ejaculates with higher sperm concentrations than those with smaller scrotal circumferences (p<0.002, p<0.002, p<0.05 for collections one, two, and three, respectively). Table 6 depicts the means (±SEM) for age, scrotal circumference, sperm concentration, motility and ejaculate volume for Brahman bulls from ranches two and three for all three collections.

FAA Profile

A total of 133 Angus and Brahman bulls were evaluated three times. Animals were grouped according their FAA profile for all three ejaculates. FAA profiles were defined as: FAA profile-0: all three ejaculates were negative (5%; n=7); FAA profile-1:
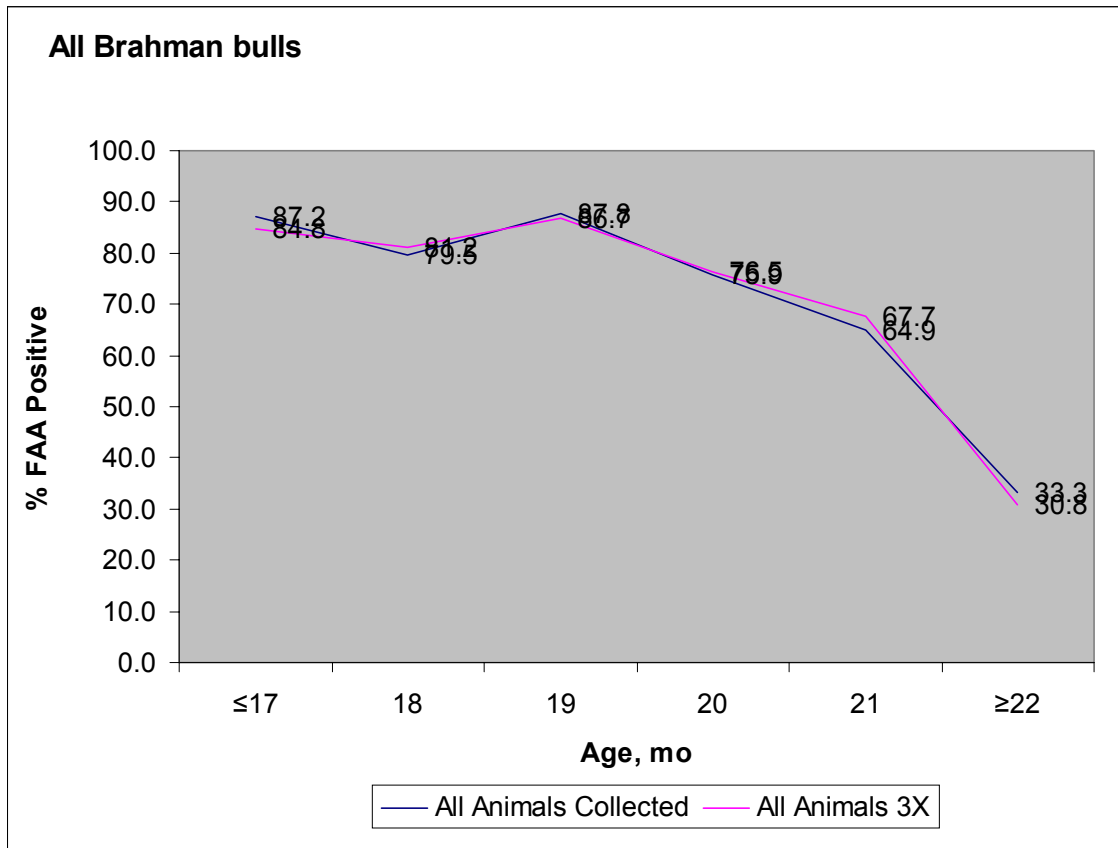
Figure 5. Percentage of FAA positive scores for all Brahman bulls vs those bulls tested three times relative to the age of the bull at time of test.

Table 6. Mean (±SEM) for age, scrotal circumference, sperm concentration, sperm motility and ejaculate volume for all Brahman bulls with three collections

	Age (mo)		Scrotal circumference (cm)		Concentration $(x10^6$ sperm/mL)		Sperm motility (%)		Ejaculate volume (mL)	
FAA Status	+	−	+	−	+	−	+	−	+	−
All Brahman bulls tested										
Ejaculate 1	18.4± 0.2	18.8±0.5	34.0±0.3	33.3±0.7	339±32	312±51	57.6±2.1	51.9±5.1	5.0±0.2	5.9±0.5
Ejaculate 2	19.0±0.1	20.0±0.5	33.7±0.3	34.0±0.7	364±31	377±63	58.9±2.1	57.5±3.7	4.7±0.2	5.4±0.4
Ejaculate 3	19.8±0.1	21.0±0.4	34.5±0.3	34.1±0.7	449±44	491±64	60.5±1.7	60.7±3.6	5.2±0.2	6.1±0.4

all three ejaculates were positive (57%; n=76); FAA profile-2: first ejaculate was negative (12%; n=16); FAA profile-3: first ejaculate was positive (25%; n=34). Table 7 depicts LSM for age, scrotal circumference, sperm concentration, sperm motility and ejaculate volume at first collection for animals classified according to FAA profile. Table 8 depicts LSM for age, scrotal circumference, sperm concentration, sperm motility and ejaculate volume between Angus and Brahman bulls within each collection for all animals collected three times.

Least square mean age of animals did not differ among FAA profiles at the first collection; however, for collection two and three, bulls classified as FAA profile-3 were older than bulls classified as FAA profile-1 ($p < 0.05$; $p = 0.08$, respectively). Between breeds, Brahman bulls were older than Angus bulls across all FAA profiles ($p < 0.0001$). Within breeds, there were no differences between FAA profiles for Angus bulls. However, Brahman bulls classified as FAA profile-3 were older than Brahman bulls classified as FAA profile-1 at first collection ($p < 0.05$), second collection ($p < 0.01$) and third collection ($p < 0.01$).

Least square mean scrotal circumference of all Brahman bulls tended to be larger ($p = 0.07$) than LSM scrotal circumference for all Angus bulls at first collection, however, that difference disappeared for collection two and three. When bulls were classified only according to FAA profile, scrotal circumference was not different across the four FAA profiles at all three collections. However, between breeds, at first collection, scrotal circumference of Brahman bulls classified as FAA profile-1 and FAA profile-3 was larger than Angus bulls classified as FAA profile-1 and FAA profile-3 ($p < 0.001$;

Table 7. LSMean at first collection for age, scrotal circumference, sperm concentration, sperm motility and ejaculate volume for FAA profile classifications

	FAA Profile				
Variable	0	1	2	3	P - Value
Age (mo)	14.2	14.0	14.1	14.3	0.4995
Scrotal circumference (cm)	32.8	33.1	32.5	33.0	0.9202
Concentration ($x10^6$ sperm/mL)	234	280	184	245	0.7371
Sperm motility (%)	55.8	60.1	51.1	49.9	0.1246
Ejaculate volume (mL)	7.3[a]	4.7[b]	6.0[a]	4.7[b]	0.0462

Within a row, means with uncommon superscript ([a,b]) are different (p<0.05)

Table 8. LSMean for age, scrotal circumference, sperm concentration, sperm motility and ejaculate volume between breeds and among collections 1, 2 and 3 for bulls collected three times

	Collection					
	1		2		3	
Variable	Angus	Brahman	Angus	Brahman	Angus	Brahman
Age (mo)	10.3	18.8	11.5	19.6	12.3	20.5
Scrotal circumference (cm)	32.1	34.2	34.9	35.1	33.1	34.1
Concentration ($x10^6$ sperm/mL)	158	319	297	384	362	429
Sperm motility (%)	46.0	56.5	55.2	61.3	59.1	60.8
Ejaculate volume (mL)	4.5	10.0	7.9	5.0	4.8	5.7

p<0.05, respectively). At the third collection, scrotal circumference of Brahman bulls classified as FAA profile-1 was larger ($p < 0.05$) than Angus bulls classified as FAA profile-1. There were no differences between the breeds across FAA profiles for the second collection. Scrotal circumference differed within the Brahman breed across FAA profile at collection two, but not for collection one or three. Scrotal circumference at collection two of Brahman bulls classified as FAA profile-0 was larger than Brahman bulls with FAA profiles -1, -2 or -3 ($p < 0.05$; $p < 0.05$; $p < 0.05$, respectively). There were no differences in scrotal circumference within Angus bulls across FAA profiles.

Ejaculate volume was not different between breeds at any collection. However, at first collection, when ejaculate volumes were classified according to FAA profile, volume was greater in FAA profile-0 ejaculates than FAA profile-1 ($p < 0.05$) and FAA profile-3 ($p < 0.05$) ejaculates. FAA profile-2 ejaculates were larger ($p < 0.05$) than FAA profile-1 ejaculates (Table 7). At second collection, FAA profile-0 ejaculate volume was still greater than FAA profile-1 ($p < 0.01$), FAA profile-3 ($p < 0.01$) and FAA profile-2 ($p < 0.05$) ejaculate volume. However, these differences were not apparent at the third collection.

When ejaculate volume was classified according to FAA profile within and between breeds, there were no differences between breeds across FAA profiles for collections one, two or three. However, within breeds, differences in ejaculate volume between FAA profiles existed. At collection one among Angus bulls, ejaculate volume for bulls classified as FAA profile-0 was greater than the ejaculate volume for bulls classified as FAA profile-1 ($p < 0.05$) or FAA profile-3 ($p < 0.05$) and ejaculate volume for

bulls classified as FAA profile-2 was greater (p<0.05) than for bulls classified as FAA profile-1. At collection two among Angus bulls, ejaculate volume for bulls classified as FAA profile-0 was still greater than ejaculate volume for bulls classified as FAA profile-1 (p<0.05) or FAA profile-3 (p=0.05). At collection three, there were no differences in ejaculate volume among Angus bulls classified according to FAA profile. Among Brahman bulls, there were no differences at collection one or three for ejaculate volume when classified according to FAA profile. At collection two, ejaculate volume among Brahman bulls classified as FAA profile-0 was greater than ejaculate volume for Brahman bulls classified as FAA profile-1 (p<0.01) and FAA profile-3 (p<0.01).

Sperm concentration was not different among animals classified according to FAA profile. However, at first collection, there was a tendency for sperm concentration of Brahman bulls to be greater in bulls classified as FAA profile-1 (p=0.07) and FAA profile-3 (p=0.08) than Angus bulls of the same FAA profile classification. This trend disappeared at subsequent collections. There were no differences between FAA profiles within each breed.

Least square mean sperm motility was not different between all Brahman and Angus bulls for collections one, two or three. However, within FAA profile-3, there were differences between the breeds. When grouped according to FAA profile, sperm motility for FAA profile-3 classified Brahman bulls was greater at first (p<0.01) and second (p<0.05) collection than sperm motility for FAA profile-3 classified Angus bulls. When all bulls were classified only according to FAA profile, at collection one, sperm motility in all bulls classified as FAA profile-1 was greater (p<0.05) than in bulls

classified as FAA profile-3 and tended to be greater (p=0.09) than in bulls classified as FAA profile-2. There were no differences in sperm motility between bulls classified only according to FAA profile at the second or third collection. Within breeds, however, sperm motility varied across FAA profile classifications. At first collection, motility in Angus bulls classified as FAA profile-1 was greater (p<0.05) than in Angus bulls classified as FAA profile-3. There were no other differences among FAA profiles within the Angus breed at the second or third collection. At first collection among Brahman bulls, sperm motility was greater in bulls classified as FAA profile-1 (p<0.01) or as FAA profile-3 (p<0.05) than in bulls classified as FAA profile-0. At second collection, sperm motility among Brahman bulls classified as FAA profile-1 (p<0.05) and FAA profile-3 (p<0.01) was greater than bulls classified as FAA profile-0. There were no other differences among FAA profiles within the Brahman breed at the third collection.

As expected, significant correlations existed between age and scrotal circumference (r=0.366, p<0.0001), sperm concentration (r=0.280, p<0.001), and sperm motility (r=0.301, p<0.005) at first collection. Scrotal circumference was highly correlated to sperm concentration at all three collections, (r=0.282, p<0.001; r=0.170, p<0.05; r=0.256, p<0.01, respectively).

Densimeter Validation

Mean sperm concentration as determined by the hemacytometer was lower than the mean sperm concentration as determined by the Densimeter technique (Table 9). Sperm concentration as determined by a hemacytometer was more variable with a

Table 9. Mean (±SEM) sperm concentration as determined by either the Densimeter or the hemacytometer technique within each sperm concentration category

Sperm concentration category[a] ($x10^6$ sperm/mL)	Techniques used to determine sperm concentration		t-test value
	Densimeter	Hemacytometer	
	Mean ± SEM ($x10^6$ sperm/mL)	Mean ± SEM ($x10^6$ sperm/mL)	
0 – 99	64.6 ± 3.7	40.0 ± 11.8	0.0460
100 – 299	188.1 ± 8.5	138.3 ± 10.6	0.0001
300 – 499	386.7 ± 9.5	295.3 ± 24.1	0.0002
500 – 749	627. 1 ± 19.2	445.7 ± 33.6	0.0003
750 – 999	846.0 ± 25.7	660.9 ± 30.0	0.0010
1000 – 1249	1083.0 ± 17.8	796.5 ± 39.8	0.0001
1250 – 1499	1335.2 ± 24.7	1013.3 ± 100.9	0.0342
> 1500	1734. 7 ± 65.8	1178.3 ± 136.66	0.0026

[a] Determined by Densimeter technique

consistently larger standard error than for the Densimeter technique.   Across all categories, average sperm concentration was 25% higher when determined by the Densimeter technique (713.2 x 10^6 sperm/mL) than when determined by the hemacytometer technique (571.0 x 10^6 sperm/mL).

DISCUSSION

In the present study, 776 ejaculates were collected from 464 peripubertal beef bulls of two breedtypes over a 60-d period. This experiment was conducted to ascertain whether significant relationships exist for breedtype, age, scrotal circumference, sperm concentration and sperm motility (characteristics evaluated during annual breeding soundness evaluation (BSE) exams) of peripubertal beef bulls with the presence of fertility associated antigen (FAA) in the ejaculate.

Angus and Brahman bulls (n=133) were used to assess the repeatability of FAA classification among serial ejaculate collections. Analysis of FAA was conducted using the ReproTest® for Bulls (ReproTech, Tucson, AZ) chute-side diagnostic cassette (McCauley et al. 2004). Seventy-seven percent of the 776 (n=598) ejaculates collected from peripubertal Angus, Brahman and Brangus beef bulls were classified as FAA-positive. These results are in line with those reported by McCauley et al. (2004).

Previous studies of fertility in bulls have used the presence or absence of FAA (or HBP) in an ejaculate as the marker by which to compare fertility rates of animals. The presence of these proteins has been shown to be an indicator of increased fertility in natural breeding operations (Bellin et al., 1994, 1996, 1998) as well as artificial insemination (Sprott et al., 2000). Bellin et al. (1994) reported a 17 percentage point increase in fertility in bulls expressing a particular HBP-B5 profile that included the 31 kDa protein (FAA) and Sprott et al. (2000) showed increased conception rates in cows bred via artificial insemination (AI) to FAA-positive bulls compared to FAA-negative bulls, regardless of whether AI service was at spontaneous or synchronized estrus.

However, the bulls used in these previous studies testing the effect of FAA on fertility were sexually mature, reproductively competent bulls. There are no results reported on the presence of FAA in young, peripubertal bulls or the use of FAA in selection of young bulls as replacement sires.

Selection of young animals with the potential of increased fertility is one way for producers to increase their overall production efficiency. Historically, evaluation of fertility of a bull required that he begin producing offspring that could then be evaluated as sires themselves. In recent decades, however, the development and refinement of the BSE has greatly aided in evaluating potential fertility of bulls. By itself, though, a BSE does not completely assess all factors related to prediction of potential fertility. The discovery that certain proteins in semen, such as FAA, have an affect on the fertility of the animal has necessitated the development of tests designed to detect these proteins.

Previous studies used Western blot technology to detect the presence of these proteins associated with the sperm membrane as well as seminal plasma (Bellin et al. 1994, 1996; Sprott et al., 2000). While results from Western blots are very accurate, they are expensive and time consuming. The ability to detect FAA using neat semen samples would make testing for FAA in young or mature bulls quicker and more cost effective for the producer. One such new technology is the chute-side cassette test which allows producers to evaluate ejaculates collected during a BSE for the presence of FAA. Other proteins in semen recently identified also appear to be related to fertility.

It was the purpose of this study to evaluate two different breedtypes of peripubertal beef bulls, *Bos Indicus* and *Bos taurus*, over a period of 60 days including

monitoring changes of several characteristics, such as age, scrotal circumference, sperm concentration, sperm motility and ejaculate volume and relating those results to the presence of FAA in the ejaculate. As a part of our analysis, we quantified relationships between BSE exam results, or the changes in BSE results over time, to the presence of FAA or a change in the FAA profile of a bull over time.

In this study, serial ejaculates were collected from peripubertal bulls of two breedtypes (Angus and Brahman), targeting the relationships between the presence of FAA and semen characteristics of ejaculates collected during an annual BSE exam. This experiment was conducted to test three main hypotheses:

1) The relationship of FAA to the age, breedtype and pubertal status of the animal at time of ejaculate collection.

2) The relationship of FAA to scrotal circumference, sperm concentration, sperm motility and ejaculate volume.

3) The repeatability of the chute-side cassette when testing serial ejaculate collections from peripubertal bulls.

In general, comparisons of the two breedtypes of peripubertal animals indicated few differences between Brahman and Angus bulls. There were differences in age between Brahman and Angus bulls as Brahman bulls achieve puberty at an older age than Angus bulls. When scrotal circumference is used to determine pubertal status, Angus bulls achieve puberty at an earlier age (9 to 10 mo; Lunstra et al., 1978), than Brahman bulls (16 to 17 mo; Fields et al., 1982; Morris et al., 1989).

Puberty has been defined using minimum scrotal circumference measurements (Lunstra et al., 1978; Aravindakshan et al., 1999) and minimum sperm concentrations (Wolf et al., 1965; Lunstra and Echternkamp, 1982). According to Chenoweth et al. (1992), the minimum required scrotal circumference measurement for bulls up to 15 mo of age is 30 cm and for bulls 15 to 18 mo of age, 31 cm. For this study, bulls were considered to have achieved puberty with a scrotal circumference measurement greater than or equal to 28 cm.

All but three of the bulls in this study had a scrotal circumference measurement of $\geq$ 28 cm. Animals with scrotal circumference measurements less than 28 cm were considered to be pre-pubertal animals, while the rest were considered to be in the peripubertal stage. In our study, 12% (n=53) of bulls tested had a scrotal circumference less than 30 cm, and 20% (n=88) were less than 31 cm at first collection.

In the past, minimum sperm concentrations have been used to define puberty (50 X 10^6 sperm cell per mL ejaculate) (Wolf et al., 1965; Lunstra et al., 1982) and sexual maturity (two consecutive ejaculates containing $\geq$500 x 10^6 sperm/ejaculate with one ejaculate exhibiting >50% motility; (Tatman et al., 2004). Our results indicate that although most of the bulls had a scrotal circumference that was greater than the minimum requirement, sperm concentration was not high enough in the Angus, Brahman or Brangus bulls to indicate the traditionally accepted level of sexual maturity. Because they did not meet standards indicating sexual maturity, the scrotal circumference of the bulls used in this study indicated that the bulls used were within the parameters of the peripubertal stage.

When the measurements from all animals are analyzed, scrotal circumferences among all Angus bulls at first collection (d 0) were larger than Brahman or Brangus bulls; the Angus bulls from ranch 4 were older than the Angus bulls from ranch 1 by two months. However, when only the animals grouped according to FAA profile with three collections (Angus and Brahman) are compared, Brahman bulls had a larger mean scrotal circumference than Angus bulls at first collection, but not at the second or third.

Sperm concentration across the three breeds was not different, indicating that testicular size differences at the time of the first collection did not significantly impact spermatogenesis. Sperm motility was lower in Brangus bulls than Angus or Brahman bulls.

There were no breedtype-associated differences in FAA presence seen in this study. When all Angus, Brahman and Brangus bulls with ejaculates collected during the first collection were compared by FAA classification, no evident pattern exists between classification as FAA-positive or FAA-negative and the other parameters investigated, including age, scrotal circumference, sperm concentration or sperm motility. The absence of a significant correlation indicates that FAA presence in an ejaculate during the peripubertal period is not dependent on age or scrotal circumference.

 It was anticipated that FAA would not be present in some ejaculates of the peripubertal bulls tested, although the physiological reasons behind its absence are not yet understood. Because testosterone plays such an intricate role in sexual maturation and spermatogenesis, it is reasonable to conclude that it may play a role in production and secretion of FAA by the male reproductive tract as well. If the presence of FAA

was dependent upon the animal reaching threshold levels of testosterone or sperm concentration, it would be reasonable to expect that ejaculates classified as FAA-negative could change to FAA-positive as age, scrotal circumference and sperm concentration increased.

Several ejaculates were collected from Angus and Brahman bulls that, upon immediate microscopic evaluation, did not contain any sperm. However, all of these ejaculates, when tested, were FAA-positive. This indicates that sperm production is not necessarily required for FAA synthesis and suggests that the cassette can detect FAA in seminal plasma that is apparently devoid of sperm. Testing for FAA may not be predicated on having a minimum sperm concentration in the ejaculate, meaning, if sperm presence is not necessary, then motile spermatozoa need not be present for an ejaculate to be tested.

The presence of FAA in ejaculates, regardless of the presence of spermatozoa, would allow for the testing of FAA in pre- or peri- pubertal beef bulls as soon as an ejaculate could be collected, allowing for earlier detection of enhanced fertility potential. FAA presence could be used as a tool to aid in selection of certain high-fertility-potential animals to undergo further performance testing at a later age.

Ejaculate volume at the second collection was negatively correlated to the presence of FAA with greater volume ejaculates being predominantly FAA-negative. It is not clear why bulls with higher ejaculate volumes were more likely to be classified as FAA-negative than bulls with smaller ejaculate volumes. This negative relationship could be due to the increased dilution of FAA by fluid from a particular accessory sex

gland to a point below the sensitivity of the cassette. Ejaculates with larger volumes, and consequently, a potentially more dilute FAA concentration, may be outside the ability of the gold-labeled antibody on the test cassette to adhere to FAA in the sample, resulting in a false-negative result.

Only 12% of bulls tested had an "FAA -negative to -positive" FAA profile, meaning that they initially tested negative, then later tested positive. Another 25% of bulls had an "FAA -positive to -negative" FAA profile, meaning they tested positive for FAA then later tested negative. Four of the "FAA -positive to -negative" FAA profiled bulls had two subsequent FAA-negative tests. More investigation into the physiology behind the possible transient nature of FAA secretion is warranted, however, these data indicate the possibility that the first test may have been a "false-positive".

The incidence of FAA-negative bulls (23% of 782 samples tested) using the test cassette in the current study is similar to results reported by McCauley et al. (2004). Validation trials conducted by McCauley et al. (2004), using the FAA chute-side test cassette, resulted in 26% of 914 bulls testing FAA-negative using the cassette, compared to 15% testing FAA-negative with Western blots. These differences indicate, however, that there is the possibility for a bull to be falsely classified as FAA-negative when using the chute-side cassette.

While some tests may produce false results, our findings also indicate that the test should be repeated at a later date, as it is possible for bulls that initially tested negative to test positive at a later date. It is important to remember that a bull may not achieve a satisfactory rating on all components of a BSE exam every time he is tested,

which is why classification of breeding potential is deferred in some animals for a short time period until retesting can be conducted.

Because the physiological processes governing production and secretion of FAA are not yet fully understood, it is plausible to assume that FAA prevalence in the ejaculate may be impacted by the semen collection process itself. While ejaculates are produced using electroejaculation, complete sexual arousal and erection do not always result, making it difficult to ascertain whether the results are indicative of a physiological fault in the animal or resulting from less-than-ideal collection procedures. Previous studies provide evidence that volume of an ejaculate collected during a BSE is affected by the process of electroejaculation (Austin et al., 1961). Austin et al. (1961) showed that bulls undergoing electroejaculation produced higher ejaculate volumes with lower sperm concentrations compared to ejaculates collected using an artificial vagina. It was also noted in the study by Austin et al. (1961) as well as Seidel and Foote (1969) that differences in the concentration of secretions from the accessory sex glands, including the seminal vesicles, occur when the animal is subjected to electroejaculation rather than ejaculate collection via artificial vagina. Further research into the effects of electroejaculation on individual sex glands and their secretions might offer sources of insight into variability in volume of ejaculate produced and the apparent differences between FAA-positive and FAA-negative samples.

Little is known about regulation of FAA synthesis; however Nass et al. (1990) reported that HBP production was testosterone-dependent in rats. When testosterone replacement therapies were used in castrated rats, HBP content of the accessory sex

glands increased to the levels of sham-operated controls. While FAA production is testosterone-dependent, testosterone levels apparently do not need to attain a level that is sufficient to initiate sperm production before FAA synthesis is upregulated. It is also not known what affect other factors, such as nutrition, ambient temperature as affected by season of the year, or sexual arousal have on FAA production. These "stressors" could therefore influence the results of the FAA chute-side test cassette. If FAA synthesis is not constant, but rather pulsatile or episodic, then it is possible that ejaculates with lower or higher concentrations of FAA could be randomly collected at any time.

It is not known where, or if, FAA is expressed in the cow, or even if FAA would have such an effect on female fertility as it does in the male. Currently, FAA testing in bulls means that only half of the bovine population can be selected for FAA. Future technological developments may allow for the testing of cows for the expression of the FAA gene as well as testing animals for the presence of one or two alleles. Some offspring should receive two copies of the FAA allele (homozygous), while others may receive only one (heterozygous). It is not currently possible to test the DNA for FAA homozygosity or heterozygosity.

Proteins, such as FAA, are products of particular sequences that are coded for by an animal's DNA. Since FAA is coded for by a particular gene or genes, there is the possibility that slight mutations (single nucleotide polymorphisms [SNP]) in the code sequence could occur, thereby rendering the aberrant FAA protein unrecognizable by the immobilized gold-labeled antibody on the nitrocellulose membrane of the test cassette. This is another possible reason for the FAA-negative results seen in some of the animals.

Variation in the colormetric signal of the test cassette was seen between and within animals. The weaker test cassette results may be the result of a decreased mRNA message from heterozygous animals, while stronger signals could be from homozygous animals.

FAA detection tests should be used in conjunction with, but not in place of, all aspects of the standard BSE evaluation of peripubertal beef bulls. Animals that are FAA-positive but are deficient in other areas such as small scrotal circumferences, low sperm concentration and motility or increased abnormal sperm morphology should be evaluated with a second BSE exam at a later date. Failure of at least two consecutive BSE exams should result in the animal being culled as a potential breeder, even if that animal is FAA-positive.

The evaluations conducted during a typical BSE evaluation, while somewhat subjective, are macroscopic evaluations of the ejaculate itself, not an indicator of the physical composition of the ejaculate or its ability to fertilize oocytes. Bulls may produce ejaculates with high sperm concentration, morphology and motility and yet, are still unable to produce offspring. The presence of FAA in an ejaculate is a physiological indicator of an increased potential for greater fertilization rates. Since semen characteristics are commonly measured during annual BSE exams, the discovery of significant relationships between these characteristics and the presence of FAA in the ejaculates in peripubertal animals would aid in the prediction of potential fertility of young animals.

Our results indicate a consistently higher sperm concentration as determined by the Densimeter as compared to measurements via hemacytometer. This indicates that sperm concentration may be slightly over estimated when using the Densimeter. Ejaculates may contain other foreign bodies such as somatic cells, lymphocytes or red blood cells. The Densimeter can not distinguish between sperm cells and these other contaminants that may be present in an ejaculate. If an ejaculate is not obtained using an artificial vagina, but through electroejaculation, it is possible for hair or dirt from the prepuce area to contaminate the sample during collection, and therefore alter the Densimeter results. These contaminants could contribute to an overestimation of sperm concentration via the Densimeter. However, use of a hemacytometer will aid detection of the presence of foreign bodies such as the lymphocytes or red blood cells, which can indicate a health problem that can then be identified and treated.

CONCLUSIONS

The results of this study indicate that there is much variability between the relationship of FAA-positive ejaculates and the BSE exam characteristics measured, such as age, scrotal circumference, sperm concentration, motility and morphology. The inverse relationship of ejaculate volume with the presence of FAA is an aspect that is deserving of future investigation. It is unknown whether the increased seminal fluid diluted the FAA protein concentration beyond that which the test cassette could detect. Not all high volume ejaculates were classified as FAA-negative, though, so care must be taken when assessing an ejaculate. These results indicate that there is not one specific measurable characteristic that is definitively tied to FAA production. It is also evident from these results that the presence of FAA in an ejaculate did not differ among the three breedtypes in the current study.

All bulls that are to be used as herd sires should have a BSE exam annually prior to every breeding season so that any problems that may arise can be corrected. The availability of a quick, easy-to-use, chute-side diagnostic test cassette that will positively identify the presence of FAA in an ejaculate collected during the annual BSE exam gives producers an added tool to use, in addition to the BSE, for evaluation of potential herd sires, whether for cow/calf operations or for young purebred bulls undergoing performance testing. However, bulls should be evaluated on physical conformation, ejaculate characteristics, libido, and serving capacity, as well as FAA,

Just as an animal may not pass a portion of the BSE exam and be classified deferred to be retested again at a later date, so should young bulls that test FAA-negative

during their first exam be classified deferred for retesting at a later date, especially if the ejaculate volume is increased. If an animal is classified as FAA-negative for the first test, he should be classified as deferred and tested again at a later date to be confident in the FAA-negative classification. Young animals should be evaluated on all aspects of a BSE before the decision to cull should be made. The absence of FAA does not make an animal infertile while the presence of FAA does not ensure increased future fertility rates. The presence of FAA should be used in junction with a BSE for evaluation and selection of superior breeding animals.

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