PUBERTAL CHANGES IN THE EXPRESSION OF FERTILITY ASSOCIATED ANTIGEN IN *BOS INDICUS* AND *BOS TAURUS* BULLS

A Thesis

by

AARON M. NOVOSAD

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

December 2005

Major Subject: Physiology of Reproduction

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Approved by:

Co-Chairs of Committee,	David Forrest
	L. R. Sprott
Committee Members,	Kerry Barling
	Jim Sanders
Head of Department,	Gary Acuff

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ABSTRACT

Pubertal Changes in the Expression of Fertility Associated Antigen in *Bos Indicus* and *Bos Taurus* Bulls. (December 2005)

Aaron M. Novosad, B.S., Texas A&M University

Co-Chairs of the Advisory Committee: Dr. David Forrest
Dr. L. R. Sprott

Fertility Associated Antigen (FAA) produced by the accessory sex glands and contained within the seminal fluid binds heparin and facilitates capacitation in ejaculated sperm, resulting in improved fertility in bulls capable of producing the protein. In this study, a total of 206 bulls derived from three populations were evaluated for the presence or absence of FAA through utilization of the Repro Test at three semen collections over a 60-d period. Across all collections, the percentage of FAA Negative bulls ranged from 13.64 to 36.11%. Within the three populations, 32, 33, and 67 bulls were observed at three different collections, of which 3.03, 9.09 and 4.48% were FAA Negative at all three collections, respectively. Furthermore, 27.27, 33.33, and 20.90% of bulls were observed to have variations within their FAA status after providing an initial FAA Positive result, respectively. Bull age, sperm concentration, progressive forward motility, percent normal sperm, ejaculate volume, and scrotal circumference were determined to be significantly different between FAA Negative and FAA Positive bulls in at least one collection. However, no consistent trend was observed across populations, or collections within a population, with regard to a relationship between these variables and FAA. Furthermore, of fourteen bulls that produced an ejaculate in which no sperm

was detected, 78.57% (n=11) were FAA Positive despite the lack of sperm within the ejaculate. No single variable commonly measured to determine bull fertility was consistent in predicting the FAA status of bulls. The ability to produce FAA precedes puberty and the Repro Test can be used to identify FAA in prepuberal bulls. However, a large percentage of bulls, both prepuberal and peripuberal, are capable of displaying variation in their FAA status (as determined by the Repro Test) over time.

This work is dedicated to my wife:

Jennifer Lee Novosad, your sacrifice, steadfast support, patience and understanding often go unmentioned, but are never unnoticed. You are my drive, for without you none of this would have been possible. I love you and can never thank you enough for what you have done for me. For your love, I am eternally grateful.

Jenn, thanks for everything!

Your Husband,

Aaron M. Novosad

and to my parents:

Mike and Jan Novosad, who helped me believe I can do anything if I put my mind to it. Thanks Mom and Dad for all your love and support!

Your son,

Aaron M. Novosad

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I gratefully acknowledge Repro Tec Inc. (Tucson, AZ) and Midland Bioproducts (Boone, IA) for donating the Repro Test Lateral-Flow Cassettes utilized in this experiment.

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INTRODUCTION

Improving herd fertility is the most efficient way to increase production within any given beef or dairy operation. Increasing pregnancy rates improves the herd's percent calf crop weaned and provides more total pounds of calf weaned per cow exposed in herds of high fertility compared to herds of low fertility. In fact, each 1% increase in percent calf crop weaned returns the same economic benefit as improving weaning weight by 2.36 kg (Sprott et al., 1998). While the sire contributes half of the genetic make up of the resulting calf crop, he more importantly performs a vital role in initiating each pregnancy that occurs within the herd. For single sire operations, it is imperative that the chosen herd sire be highly fertile. While a yearly breeding soundness exam (BSE) can diagnose common causes of infertility, it cannot explain the difference in fertility between two bulls with similar exam results. However, the difference can be at least partially explained by variations in the molecular composition of the ejaculate, particularly with regard to several proteins secreted by the accessory sex glands.

Heparin-binding proteins (HBP), also referred to as bovine seminal plasma proteins (BSP), which are produced by the Cowper's gland, prostate, and seminal vesicles, are mixed with sperm during ejaculation and aid in increasing fertility in bulls which express the genotype to produce these proteins (Bellin et al. 1994, 1996, 1998). Repro Tec Inc. (Tucson, AZ) has developed the Repro Test, a chute-side lateral-flow

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

cassette to detect the presence of a specific category of HBP known as fertility associated antigen (FAA) in an ejaculate. For a minimal cost, a beef or dairy producer can test a potential herd sire for the presence of FAA at the same time the initial BSE is performed. The results of this fast and low-cost analysis would equip the producer with additional information regarding a bull's fertility potential above that which can be determined through visual observation of the neat sample obtained during a BSE. Ultimately, this information will allow the producer to make a more informed decision as to the fertility potential of a herd sire. While research has been conducted to determine the relative accuracy of this chute side test, ontogeny of FAA production in the peripuberal bull has not been characterized.

The objectives of this project were to determine the relationships among age and/or pubertal changes in scrotal circumference, sperm cell concentration, sperm cell motility and sperm cell morphology on the initial appearance of FAA in ejaculates of prepuberal and peripuberal beef bulls. We tested the hypothesis that a relationship exists between FAA and fertility factors influenced by puberty, whereby prepuberal bulls would be unable to produce detectable levels of FAA as determined by the Repro Test.

LITERATURE REVIEW

Capacitation and the Acrosome Reaction

To successfully bind and fertilize the oocyte, sperm must undergo a process know as capacitation and the acrosome reaction (AR). The presence of several intrinsic molecules within the female reproductive tract aids sperm in achieving capacitation and allows for a more efficient rate of AR. Glycosaminoglycans (GAG), such as heparin, and high-density lipoprotein (HDL) originate in the female reproductive tract and react with components of the sperm membrane to act as a catalyst for capacitation within the female reproductive tract (Marks and Ax, 1985, Therien et al., 1998). Although the exact mechanism of capactiation is not fully understood, the general roles and interactions among GAG, HDL, bovine seminal binding proteins (BSP), and FAA, are well documented.

Cholesterol and Phospholipid Efflux from the Sperm Membrane

As cited in Therien et al. (1998), capacitiation of bovine sperm is the result of many concurrent events, one of which involves an efflux of cholesterol from the sperm membrane. While HDL can successfully stimulate cholesterol efflux in epididymal sperm, the addition of BSP has a synergistic effect on the rate of cholesterol efflux (Therien et al., 1998, 2001). In fact, epididymal sperm exposed to BSP during an 8-h incubation period had a greater rate of cholesterol efflux: 42% in sperm incubated in media containing 2% seminal plasma compared to 11.5% in the control (Therien et al., 1998). Furthermore, incubation with a single purified version of BSP-A1/-A2, BSP-3, or BSP-30 kDa resulted in a 31-35% efflux of cholesterol from the sperm membrane.

The highest rate of efflux occurred within the first 2 h of the experiment, with the majority of the efflux occurring within the first 4 h (Therien et al., 1998). Closer observation investigating the rate of cholesterol efflux during the first 90 min after the addition of BSP found that the rate of cholesterol efflux was greatest within the first 20 min (Moreau et al., 1999).

An efflux of phospholipids from the sperm membrane has also been shown to be an integral step during capacitation. BSP-30-kDa, BSP-A1/A2, and BSP-3 at concentrations above 40 μg/ml significantly increased the amount of phospholipid efflux from the sperm membrane in a dose-dependent manner (Therien et al., 1999). BSP-30-kDa (120 μg/ml) improved the level of phospholipid efflux from 11.5% in the control to 29.5% when added to epididymal sperm (Therien et al., 1999). This was similar to results achieved with the addition of BSP-A1/A2 (120 μg/ml) in which the amount of phospholipid lost from the sperm membrane was 28.1% (Therien et al., 1999).

Earlier research documented the importance of BSP during capacitation, through their ability to remove cholesterol and phospholipids from the sperm membrane. However, the mechanism of action by which BSP achieves these results is still unclear. Moreau et al. (1999) cited earlier research in which binding between BSP and immobilized cholesterol did not occur. This led researchers to hypothesize that BSP was acting indirectly to yield changes in the structure of the sperm membrane during capacitation. Moreau et al. (1999) demonstrated that BSP-A1/-A2, when incubated with choline prior to being added to human fibroblasts, failed to produce a significant amount of phospholipid efflux compared to the control. Furthermore, cholesterol efflux was

reduced from 8.6% to 2.91% when free choline was added to BSP-A1/-A2 prior to incubation with human fibroblasts (Moreau et al., 1999).

Manjunath and Therien (2002) suggested that capactition of bovine sperm begins at ejaculation as sperm are mixed with seminal plasma derived from the accessory sex glands. BSP bind to choline phospholipids on the sperm membrane and facilitate phospholipid efflux followed by an efflux of cholesterol. This process occurs at a molecular ratio of 1.5-2:1 cholesterol to phospholipid (Moreau et al., 1999) and results in an efflux of cholesterol ranging from 7-15% during the 15-30 min time frame in which sperm are in contact with seminal fluid after ejaculation (Manjunath and Therien, 2002). As sperm progress through the female reproductive tract, they depart from the seminal plasma. BSP which remain bound to the sperm membrane during this progression are removed when contact with HDL occurs (Therien et al., 2001). HDL removes the remaining BSP which causes further efflux of phospholipids and cholesterol, completing the process of capacitation (Manjunath and Therien, 2002).

Ultimately, improved capactitation of ejaculated sperm allows for a greater number of cells to achieve the AR and become capable of fertilizing an oocyte. BSP derived from the seminal plasma stimulate phospholipid and cholesterol efflux from the sperm membrane, which are important steps during the process of capacitation. Therien et al. (1998, 1999) reported induced AR rates ranging from 11.2 to 15.1% in control samples. Researchers improved the rate of AR to a range of 30.5 to 38.5% with the addition of any one of three purified BSP. Even without the use of an AR induction agent, the addition of BSP-30-kDa during the incubation period also allowed epididymal

sperm to achieve an 11.5 percentage point advantage over the control with regard to the number of cells which underwent the AR (Therien et al., 1999).

Heparin and Heparin Binding Proteins

Parrish et al. (1988) demonstrated that incubation of ejaculated sperm with heparin for 4 h significantly improves oocyte penetration ($p<.001$) during *in vitro* fertilization (IVF). Sperm incubated in heparin for 4 h prior to the addition of 100 µg/ml of lysophosphatidylcholine (LC) underwent the AR at a rate of 60 percentage points greater than sperm not incubated in heparin. Lane et al. (1999) observed similar results as LC-induced AR rate improved to 38.4% in sperm which underwent the AR when incubated in media containing 12 µg/ml of heparin, compared to 12.9% in sperm not incubated with heparin. A dose response was also observed with regard to the heparin concentrations during the 4 h incubation period. The number of cells that underwent the AR improved until the incubation concentration of heparin reached 5 µg/ml and remained constant at concentrations up to 10 µg/ml (Parrish et al., 1988). Incubation with heparin also improved sperm motility and number of acrosome-reacted sperm when a more concentrated dose of LC, (200 µg/ml compared to 100 µg/ml), was administered (Parrish et al., 1988).

Heparin facilitates capacitation in bovine sperm though an indirect mechanism by utilizing HBP derived from seminal fluid (Lane et al., 1999). The addition of polycolonal antibodies against HBP to sperm prior to incubation with heparin decreased the rate of AR in tangent with the concentration of antibody in each sample (Lane et al., 1999). In fact, samples subjected to antibody concentrations of 40 µg/ml had similar AR

rates as sperm incubated without heparin. Since binding of several different antibodies to their respective HBP reduced the rate of capacitation to a level similar to that of sperm incubated in the absence of heparin, this study suggests that heparin binds to proteins within the seminal plasma rather than directly to sperm membrane. Still, low rates of LC-induced AR are observed in sperm incubated without heparin (Parrish et al., 1988; Lane et al., 1999), as well as in sperm subjected to polycolonal antibodies against HBP (Lane et al., 1999). It is important to note that capacitation and the subsequent AR of ejaculated bovine sperm can be achieved without the presence of heparin; however the efficiency is greatly reduced.

In order for heparin to initiate capacitation of sperm, HBP must be present within the ejaculate. Cauda epididymal sperm from bulls of varying fertility (determined by previous non-return rates) achieved greater penetration rates of zona-free oocytes when incubated in seminal plasma derived from high fertility bulls, compared to incubation in seminal plasma of lower fertility bulls (Henault et al., 1995). Cauda epididymal sperm from low fertility bulls, when incubated in their respective accessory fluid achieved penetration rates of 34.7%. However, penetration rates improved to 65.3% when sperm from low fertility bulls were incubated with accessory fluid from a high fertility sire. Furthermore, a reduction of 33.2 percentage points occurred in zona-free oocyte penetration when cauda epididymal sperm from high fertility sires was incubated in accessory fluid from low fertility sires instead of the bulls' own seminal fluid. Despite these significant results, a large amount of variation was observed among bulls. In fact, three bulls of low fertility achieved greater penetration rates when incubated in their own

seminal plasma rather than seminal plasma from higher fertility bulls. Moreover, in several trials in which sperm mixed with seminal plasma from lower fertility sires had significantly greater penetration rates, the seminal plasma was derived from one of the three aforementioned bulls, indicating factors other than the seminal plasma were resulting in reduced fertility.

In general, higher fertility bulls produce an ejaculate containing HBP, causing sperm cells to have a much greater affinity for heparin (Parrish et al., 1988; Lane et al., 1999). As a result, these bulls generate sperm that have an improved response to the chemical factors that stimulate capacitation and the AR (Marks and Ax, 1985). Ultimately, this leads to greater oocyte penetration (Henault et al., 1995) and corresponds to the high fertility bull's ability to generate greater pregnancy rates.

Fertility Associated Antigen

Heparin-binding protein B-5 (HBP-B5) is a protein complex, which contains the 30-kDa FAA molecule and four other heparin-binding proteins of 66, 49, 24, and 21.5 kDa (Miller et al., 1990). Bellin et al. (1994) reported the effects of HBP-B5 on the fertility of 300 bulls, representing the following breed types: Red Angus, Gelbvieh, Santa Gertrudis, and Gelbvieh x Santa Gertrudis. The presence or absence of HBP-B5 on the sperm membrane and/or in the accessory fluids was determined using heparin-affinity high-performance liquid chromatography of pelleted sperm and purified seminal fluid from each bull. Among all bulls in this study, 37, 20, 16, and 27% of bulls had HBP-B5 on the sperm membrane but not in the seminal plasma, on the sperm membrane and in the seminal plasma, only in the seminal plasma, or no detectable HBP-B5,

respectively. A significantly higher percentage of Gelbvieh x Santa Gertrudis bulls had no detectable HBP-B5 in the ejaculate, while a significantly higher percentage of Gelbvieh bulls had HBP-B5 detectable in both the seminal plasma and the sperm membrane.

A subsequent study revealed that, within a bull battery of 63 Santa Gertrudis sires, 40% of the population failed to produce HBP-B5 as detected through ELISA testing utilizing a monoclonal antibody specific for HBP-B5 (Bellin et al., 1996). Nineteen percent of the bulls had detectable levels of HBP-B5 on the sperm membrane but not in the seminal fluid, while 16% of the bulls had HBP-B5 concentrations in the seminal fluid but not on the membrane. The remaining 25% of the bulls had HBP-B5 in both the seminal fluid and on the sperm membrane.

While BSP and HBP play intrinsic roles in the process of capacitation, they comprise a relatively small volume of the total ejaculate, especially when compared to other components. In fact, the presence of all BSP accounts for only 31.4-46.7 mg/ml within the 73.5-93 mg/ml protein fraction contained within the raw ejaculate (Nauc and Majunath, 2000). Work by McCauley et al. (1999) recorded HBP concentrations of 20 mg/ml in purified samples of seminal fluid derived from a vasectomized bull. In respect to FAA, average concentrations were determined to be 0.4% or 0.08 mg/ml within the 20 mg/ml HPB fraction (McCauley et al., 1999). Variation in BSP-30-kDa concentration was observed not only between bulls, but also within various ejaculates of a single bull (Nauc and Majunath, 2000). Comparison of five bulls displayed a range in average BSP-30-kDa concentration from 2.04 to 6.07 mg/ml of ejaculate (Nauc and Majunath,

2000). Standard deviations in respect to BSP-30-kDa concentration across five ejaculates for each bull ranged from 0.19 to 0.57 (Nauc and Majunath, 2000).

Nauc and Majunath (2000) reported that, after pelletization and triple washing of ejaculated sperm, only 5% of the protein bound to the sperm membrane was attributed to BSP. Across all samples, the average concentration of BSP-30-kDa bound to the sperm membrane was 0.833 % of the total protein, or at a ratio of 1:5 BSP-30-kDa to BSP. The ratio of BSP-30-kDa to total BSP bound to the cell membrane was much improved from the average ratio of 1:11 observed in the seminal fluid of the same ejaculates.

Bellin et al. (1996) also analyzed the population dynamics with respect to the profile of three particular proteins that comprise the HBP-B5 complex. In 49 three-year-old Santa Gertrudis bulls, 67.3% of the bulls produced FAA as well as the 21.5 and 24 kDa molecules. Only 4.1% of the bulls produced only the FAA molecule, while 12.2% of the bulls produced the 21.5 kDa molecule and FAA without the presence of the 24 kDa molecule. The remaining 16.3% of the population failed to produce FAA, the 21.5 kDa molecule, or the 24 kDa molecule, either alone or in combination with one another. An additional group of 30 two-year-old bulls were also evaluated. Two-thirds of these bulls were FAA Positive and produced the 21.5 and 24 kDa molecules, while the remaining population did not produce these three molecules.

Later work focusing primarily on FAA rather than the HBP-B5 complex revealed that 88% of 2,191 bulls from various breeds were indeed FAA Positive (Bellen et al., 1998). Across all breeds, 50% of the bulls produced the 21.5 and 24 kDa molecules in conjunction with FAA, while 38% of the bulls produced only FAA and the 21.5 kDa

molecule. Interestingly, 3% of Santa Gertrudis bulls and 2% of Santa Cruz bulls were the only animals to produce FAA without the 21.5 or 24 kDa HBP molecules, which are commonly associated with HBP-B5. Due to their small population and unique HBP profile, these bulls were excluded from the across breed percentages.

Effects on Fertility

A subset of the previously mentioned bulls from Bellin et al. (1994) were utilized in multiple-sire pastures for a 60-d breeding season, where each pasture contained sires with the same HBP-B5 profile stocked at a 1:25 bull to cow ratio. Rectal palpation 60 d later revealed that a higher percentage of cows conceived during the breeding season when pastured with bulls which produced ejaculates containing HBP-B5 only on the sperm membrane. Bulls which had HBP-B5 in the seminal fluid, but absent on the sperm membrane, achieved pregnancy rates similar to those of bulls which failed to produce HBP-B5. These results were confirmed by Bellin et al. (1996) when HBP-B5-positive sires, after the end of a 60-d breeding season, achieved pregnancy rates that were 11 percentage points higher than bulls which lacked HBP-B5 on the sperm membrane. Once again, there was no significant difference in fertility with regard to the presence or absence of HBP-B5 in the seminal fluid when the protein complex was absent on the sperm membrane. Considering the large number of animals devoted to these studies, it seems that the location of HBP-B5 within the ejaculate plays an important role in a sire's fertility.

Still, the location of HBP-B5 within the ejaculate is not the only factor responsible for differences in fertility of FAA Positive bulls. The particular combination

of proteins, which account for a bull's HBP-B5 profile, also seems to have an important role in fertility. Bellin et al. (1996) reported that bulls that produced at least one of three components of HBP-B5 achieved an average pregnancy rate of 81.3%. The pregnancy rates for the aforementioned bulls ranged from 74.4 to 89.9%. Bulls in this study which failed to produce FAA and the 24 and 21.5 kDa molecules achieved significantly lower pregnancy rates of 61.3%.

Bellin et al. (1998) determined group pregnancy rates from multiple-sire pastures comprised of bulls with identical HBP-B5 composition profiles. Pastures stocked with bulls, which produced all three heparin-binding proteins, had pregnancy rates of 86% which is consistent with the results of Bellin et al. (1996). Sires that produced the 21.5 kDa HBP and FAA without HBP 24 were able to achieve a further increase in pregnancy rates of 92%. No significant difference in fertility was observed in FAA Negative bulls regardless of their profile for other heparin binding proteins. Furthermore, FAA Negative bulls in this study achieved an average pregnancy rate of 78.9%, which was nine percentage points lower than FAA Positive bulls regardless of their HBP profiles. In summary, FAA Positive sires had pregnancy rates of 82.6 to 97.4% with an average pregnancy rate of 87.5% (Bellin et al., 1998). FAA Negative sires, on the other hand, achieved pregnancy rates of 78.3 to 79.2% with an average of 78.9%.

High serving capacity may overcome the reduction in fertility observed in FAA Negative bulls when these animals are compared to low serving capacity, FAA Positive bulls. FAA Negative bulls with high serving capacities were able to achieve a greater average pregnancy rate of 78% while low serving capacity, FAA Positive sires achieved

only 69% (Bellin et al., 1998). This phenomenon can probably be attributed to the high serving capacity bull's increase in mounting activity and subsequent ejaculations allowing a greater potential for pregnancy simply due to the number of services as compared to lower serving capacity bulls. Unfortunately, due to the difficulty in determining serving capacity, many cattlemen fail to utilize serving capacity tests when evaluating potential herd sires. More importantly, high serving capacity, FAA Positive bulls are more efficient in impregnating cows when compared to high serving capacity, FAA Negative bulls, and as a result will impregnate more cows earlier in breeding season. Bellin et al. (1998) reported that FAA Positive bulls impregnated 69% of the cowherd during the first 40 d of the breeding season compared to FAA Negative bulls, which impregnated only 58%. Cows that conceive earlier in the breeding season will wean more total pounds of calf simply due to the age advantage of their offspring. This advantage in net weaning weight will account for more total pounds of marketable beef product and ultimately more net income for the operation.

High fertility in FAA Positive sires has also been demonstrated using artificial insemination. Sprott et al. (2000) compared the first service conception rates of females inseminated with FAA Positive semen as compared to FAA Negative semen. Heifers and mature cows from three herds were subjected to estrous synchronization protocols and randomly inseminated with semen from either FAA Positive or FAA Negative bulls. Subsequent palpation revealed that first service conception rates were 7.2 to 9.1 percentage points higher in females inseminated with semen from FAA Positive bulls as compared to FAA Negative bulls.

The impact of BSP on pregnancy rates may be somewhat reduced during artificial insemination, as frozen-thawed semen has been shown to bind less HBP to the sperm membrane, compared to freshly ejaculated sperm. (Nauc and Majunath, 2000). In fact, the concentrations of HBP bound to the sperm membrane were 70-80% lower in frozen-thawed samples compared to their respective pre-frozen results(Nauc and Majunath, 2000). Sperm membrane bound BSP concentrations declined by an average of 74% during the cryopreservation process (Nauc and Majunath, 2000).

Explanation for this result lies within the extender utilized during the cryopreservation process. Sperm equilibrated in extender containing hen's egg yolk bound 50% less BSP than sperm preserved in extenders which did not contain hen's egg yolk (Bergeron et al., 2004). Findings from this study suggest that the low-density lipoprotein fraction contained in hen's egg yolk, binds to BSP-30-kDa and other BSP, which reduces the ability of sperm to undergo phospholipid and cholesterol efflux. Nonetheless, the added cryopreservation benefits of hen's egg yolk outweigh the disadvantages of utilizing it as an extender during artificial insemination.

Genetics and Breed Effects

With such a large proportion of the population producing one or more of the proteins that comprise the HBP-B5 complex, the question arises as to the gene frequency of the FAA allele. Research has estimated the average gene frequency, across several breeds, for the dominant FAA Positive allele to be 0.6 (Dawson et al., 2002). This suggests that 36% of the population is homozygous dominant for the gene that codes for the production of FAA. Only 16% of the population would be homozygous recessive for

the FAA allele and would consequently be FAA Negative. The remaining 48% of the population would be expected to be heterozygous for the FAA allele. The genetic breeding value of the heterozygote, with regard to fertility effects directly linked to FAA, is not entirely known. The female's role in the heredity of FAA is also not well understood. Ultimately, across many of the popular U.S. beef breeds, the percentage of FAA Positive bulls tends to be similar (Bellin et al., 1998). However, there is great variation among ranches which appears to be related to their historical rate of selection pressure for fertility (Bellin et al., 1998).

Development of a "Chute-Side" FAA Analysis

Midland Bioproducts (Boone, IA) recently manufactured a chute-side ELISA test for FAA in order to avoid the cumbersome and expensive laboratory analysis conducted in the previously mentioned studies. The device, known as the Repro Test Lateral-Flow Cassette, is marketed by Repro Tec, Inc. (Tucson, AZ). Repro Test yields cost-effective and timely analysis regarding the FAA status of a bull's ejaculate. More importantly, this analysis can be conducted at the time of a routine BSE or any time semen is collected, and results are available within 1-h. The Repro Test has sensitivity for FAA at 20 ng/mL of ejaculate and utilizes an anti-FAA antibody that will migrate, by means of capillary action, across a membrane that contains one band of immobilized antibody and a separate band of a control reagent (McCauley et al., 2004). Ultimately, this migration will yield a two-band coloimetric result for FAA Positive samples or a singe band reaction for FAA Negative samples (McCauley et al., 2004).

Validation of this ELISA test was conducted by testing the ejaculates of 914 bulls of various breeds from 18 ranches. Across all samples, 26% of the samples were deemed FAA Negative through the use of the Repro Test. Although this result is larger than the 15% average occurrence of FAA Negative bulls in approximately 6,000 samples documented in previous research which utilized Western blots to determine FAA status, the convenience of the chute-side analysis is appealing (McCauley et al., 2004). The variation in these results may be attributed to differences in sensitivity between the two types of analysis.

MATERIALS AND METHODS

Subject Animals

Three cooperative ranches provided a combined total of 206 bulls for three semen collections over a 60-d period and subsequent analysis for the presence of FAA in each ejaculate. Ranches A, B, and C provided 48 Angus bulls, 47 Brahman bulls, and 111 Brahman bulls, respectively. Of the total population, 32, 33, and 67 bulls from Ranches A, B, and C were observed at all three collections at each ranch. The average age of the bulls from each ranch at the date of the first collection was 305, 582, and 562 d, respectively. The total duration of the serial collection was approximately 60 d with collection intervals of approximately 30 d.

Semen Collection, On-Site Evaluation and Measurement

At the ranch, each bull was restrained in a squeeze chute and scrotal circumference was measured in centimeters. Then, each animal was electroejaculated by means of an Electrojac III rectal probe with the sperm-rich fraction of the resulting ejaculate collected in a 14-mL plastic conical tube with lid. The clear seminal fluid preceding the sperm-rich fraction was not collected. Ejaculate volume was observed and recorded. A motility slide was prepared to assess progressively motile sperm by placing a drop of semen on a 3 x 1-inch glass microscope slide followed by the application of a cover slip. After viewing an average of five fields under 10x magnification, the average percentage of progressively motile sperm cells were assessed and recorded. Next, one drop each of semen, Eosin, and finally Nigrosen was applied to a 1x 3-inch glass microscope slide. A slide was prepared to assess sperm morphology by smearing the

mixture across the slide with the edge of another glass slide. Once prepared, the morphology slide was stored for interpretation and scoring at a later date. Sperm concentration was measured by suspending 47 µl of the collected semen in 3.4 mL of formalin within a plastic cuvette, which was capped and placed in a Densimeter Sperm Counter to obtain a sperm concentration for the sample. Finally, a one-mL aliquot of semen was taken from the plastic 14-mL conical tube and placed into a plastic 1-milliliter flip top centrifuge tube. Caps were applied to seal both containers. The 14-mL conical tube and the 1-mL centrifuge tube, which were labeled with the collection code and bull identification number, were frozen on dry ice at chute side and stored in a –80 centigrade freezer upon returning to the lab that afternoon. Morphology evaluations of the prepared slides, as well as testing for FAA utilizing the Repro Test, were conducted in the laboratory due to time constraints in the field.

Morphology Evaluation

With the aid of a laboratory counter and following specifications and guidelines for the Major/Minor Classification System as outlined in Barth and Oko (1989), sperm morphology scores were determined by counting 200 sperm cells under 40x magnification from each of the previously prepared morphology slides. If less than 200 sperm cells were found on any particular slide, every cell on the slide was analyzed and resulting morphology score was given.

Repro Test Analysis

Once all semen collections had been completed, the frozen 1-mL centrifuge tubes were packed on dry ice and shipped to the laboratory of Dr. Roy Ax at the University of

Arizona-Tucson, in order to conduct FAA analysis utilizing the Repro Test Lateral-Flow Cassette. Upon processing, each centrifuge tube was thawed in an ice water bath. Next, 200 µl of the thawed semen sample was mixed with 200 µl of 1M NaCl buffer in a centrifuge tube. Then, a 150-µl aliquot of the diluted semen were placed in the sample well of the Repro Test cassette. Once the control line appeared in the observation window to insure the test was valid, the test line was read. Each negative result was recorded after approximately a 1-hr development period.

Statistical Analysis

Data generated from this experiment were analyzed using SAS (8.1, SAS Institute, Cary, NC). Following guidelines and examples from SAS Institute Inc. (1999), bull age, scrotal circumference, ejaculate volume, sperm cell concentration, percentage of progressively motile sperm, and percentage of normal sperm were regressed against FAA status (0-FAA positive; 1-FAA Negative) utilizing the PROC LOGISTIC, SELECTION=STEPWISE procedure. The DESCENDING command was added to the PROC LOGISTIC statement to regress the likelihood of a FAA Positive result. Parameters for entry into and from the final model were set at $P<0.30$ and $P<0.35$, respectively. The RSQUARE, and LACKFIT commands were utilized to generate R-Square, Max-rescaled R-Square, and Hosmer and Lemeshow Goodness-of-Fit test statistics. Data were analyzed on a collection by collection basis. Bulls that were FAA Negative at the first collection but progressed to and remained FAA Positive at collection two and/or three were deemed “transition bulls.” Transition bulls were grouped on a Ranch by Ranch basis and data from the three collections were combined

for analysis. The percentages of bulls that were FAA Positive, FAA Negative, and had variable FAA results were recorded. Puberty was defined as the point when an ejaculate contained 50 million sperm cells/ejaculate.

RESULTS

Ranch A

Observations at Ranch A, located in central Texas, consisted of serial semen collections from a population of 48 yearling Angus bulls. Within this population, 32 bulls were collected three times and 16 bulls were only collected twice. Within the bulls collected twice, two animals were observed at collections one and two, two animals at collections one and three, and 12 animals at collections two and three.

Ranch A, Collection One

The average and range in age, scrotal circumference, ejaculate volume, and sperm concentration for bulls evaluated at Ranch A, collection one were: 304 d (266-352 d), 31.56 cm (27.5-36.5 cm), 4.19 mL (2-11 mL), and 137 million cells/mL (0-499 million cells/mL), respectively. For bulls that produced an ejaculate which contained detectable amounts of sperm, the average and range of percent motile sperm and percent normal sperm were 33.75% (10-80%) and 78.57% (58-97%), respectively.

Ultimately, ReproTest analysis of 36 samples collected during collection one revealed that 63.89% (n=23) were FAA Positive (Figure 1). Two of three bulls that failed to produce an ejaculate containing a detectable amount of sperm were FAA Positive at this collection. These three bulls were the only bulls determined to be perpuberal at this collection. The final model determined that the volume of ejaculate ($P=0.09$) and sperm cell concentration ($P<0.04$) were associated with a bull's FAA status within this collection (Table 1). Bull age ($P<0.05$) also affected the expression of FAA (Table 1). Estimates for the age, volume and concentration suggest that, within this

population, younger bulls with lower total volume of ejaculate but with greater concentrations of sperm per mL of ejaculate were more likely to produce a FAA Positive sample (Table 2). While bull age and sperm cell concentration affected the likelihood of a FAA Positive sample, each 1-ml decrease in ejaculate volume increased the odds a bull would produce an ejaculate containing FAA by 41% (Table 3). Furthermore, the final model generated a Max-rescaled R-Square of 0.4388 (Table 1), with no evidence of lack of fit (P=0.67) as determined by the Hosmer and Lemeshow Goodness-of-Fit Test (GFT) statistic. Within this data set, the final model was able to correctly identify a bull's FAA Status 85.4% of the time (Table 4).

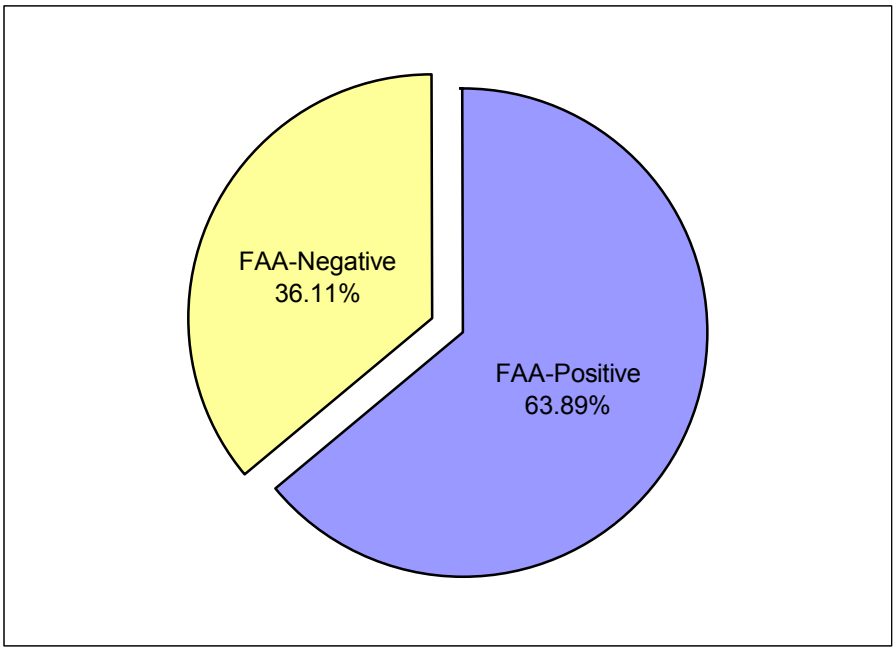

Figure 1. FAA status of bulls from Ranch A at collection one.

Table 1. Summary of stepwise logistic regression and respective R-Square and Max-rescaled R-Square values for Ranch A, collection one

Step	Variable	DF	Score Chi-Square	Pr > ChiSquare	R-Square	Max-rescaled R-Square
1	Ejaculate Volume	1	2.8743	0.090	0.0861	0.1173
2	Sperm Concentration	1	4.4429	0.035	0.2216	0.3021
3	Age	1	3.9303	0.047	0.3219	0.4388

Table 2. Analysis of maximum likelihood estimates for Ranch A, collection one

Parameter	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSquare
Intercept	1	14.4537	7.4200	3.7945	0.051
Age	1	-0.0455	0.0247	3.3885	0.066
Ejaculate Volume	1	-0.5268	0.2524	4.3563	0.037
Sperm Concentration	1	0.0195	0.0085	5.2817	0.022

Table 3. Odds ratio estimates from Ranch A, collection one

Effect	Point Estimate	95% Wald Confidence Limits	
		Lower	Upper
Age	0.955	0.910	1.003
Ejaculate Volume	0.590	0.360	0.968
Sperm Concentration	1.020	1.003	1.037

Table 4. Association of predicted probabilities and observed responses from Ranch A, collection one

	Percent
Concordant	85.4
Discordant	14.2
Tied	00.4

Ranch A, Collection Two

At collection two, analysis of 46 bulls found 78.26% (n=36) bulls to be FAA Positive (Figure 2). Of 13 bulls which were FAA Negative at collection one, 76.92% (n=10) were positive at collection two. Interestingly, five (23.81%) of 21 bulls, which were FAA Positive at collection one had become FAA Negative at collection two. One

of three bulls that failed to produce an ejaculate containing a detectable amount of sperm was FAA Positive at this collection. These three bulls were the only bulls observed as being prepuberal at this collection.

Logistic regression revealed that sperm motility was the only significant variable ($P<0.02$) with regard to the expression of FAA (Table 5). Considering this selection criteria for entry into the model, age ($P<0.25$), ejaculate volume ($P<0.21$), and sperm morphology ($P<0.14$) were included in the model (Table 5). GFT results confirm the fit of the model ($P=0.6055$). At collection two, bulls that were FAA Positive had significantly lower sperm motility compared to FAA Negative bulls (Table 6). Despite its significance, for each one percent decrease in sperm motility, the likelihood of a bull producing an FAA Positive sample only improved by 7% (Table 7). Addition of ejaculate volume and sperm morphology into the final model improved the Max-rescaled R-Square from 0.2895 with sperm motility as the only variable to 0.4431, which was able to identify the correct FAA status of bulls from this collection 83.8% of the time (Table 8).

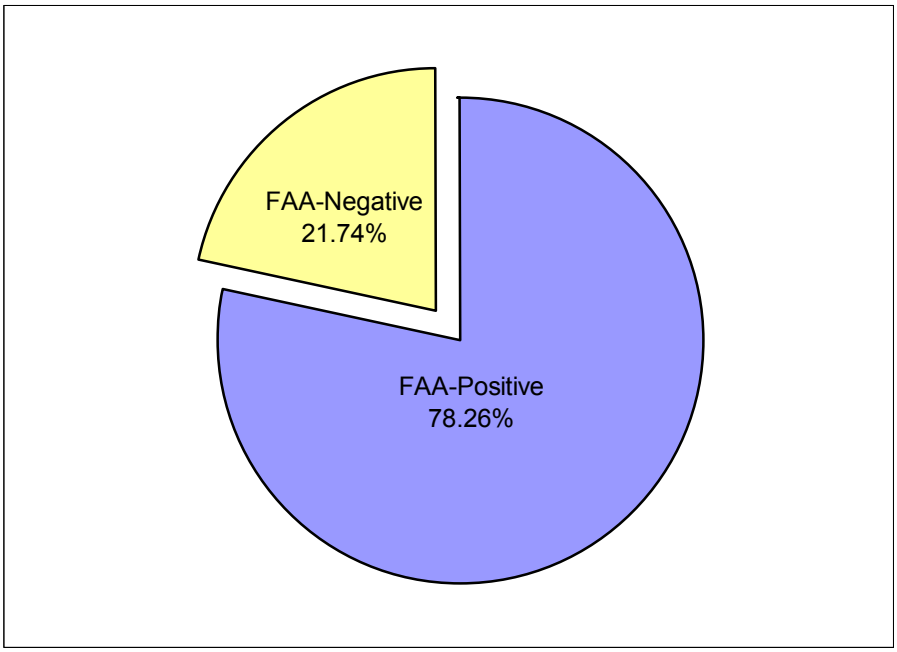

Figure 2. FAA status of bulls from Ranch A at collection two.

Table 5. Summary of stepwise logistic regression and respective R-Square and Max-rescaled R-Square values for Ranch A, collection two

Step	Variable	DF	Score Chi-Square	Pr > ChiSquare	R-Square	Max-rescaled R-Square
1	Sperm Motility	1	6.3978	0.011	0.1802	0.2895
2	Sperm Morphology	1	2.2595	0.133	0.2223	0.3572
3	Ejaculate Volume	1	1.5793	0.209	0.2513	0.4037
4	Age	1	1.3485	0.246	0.2758	0.4431

Table 6. Analysis of maximum likelihood estimates for Ranch A, collection two

Parameter	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSquare
Intercept	1	7.1095	8.5601	0.6898	0.406
Age	1	-0.0234	0.0208	1.2702	0.260
Ejaculate Volume	1	-0.2981	0.2374	1.5763	0.210
Sperm Motility	1	-0.0731	0.0371	3.8880	0.049
Sperm Morphology	1	0.0975	0.0770	1.6068	0.201

Table 7. Odds ratio estimates from Ranch A, collection two

		95% Wald Confidence Limits	
Effect	Point Estimate	Lower	Upper
Age	0.977	0.938	1.017
Ejaculate Volume	0.742	0.466	1.182
Sperm Motility	0.930	0.864	1.000
Sperm Morphology	1.102	0.948	1.282

Table 8. Association of predicted probabilities and observed responses for Ranch A, collection two

	Percent
Concordant	83.8
Discordant	16.2
Tied	00.0

Ranch A, Collection Three

At collection three, 46 bulls, all of which were observed at collection two with the exception of two bulls observed only at collection one, produced 84.78% (n=39) FAA Positive results (Figure 3). Three bulls (FAA Negative at collections one and two) were FAA Positive at collection three. Nine of the 10 bulls, which progressed to FAA positive at collection two, were observed at collection three. Within these bulls, all but two remained FAA Positive during collection three. Two bulls, which were observed first at collection two and were FAA Negative, had become FAA Positive at collection three. Furthermore, one of two bulls, which was FAA Positive at collection one but not viewed at collection two, became FAA Negative at collection three. Two bulls that failed to produce an ejaculate containing a detectable amount of sperm were FAA Positive at this collection. The two aforementioned bulls were the only prepuberal bulls at this collection.

Statistical analysis of data collected during the third evaluation determined that no single variable, nor combination of variables, was significant at predicting a bull's FAA status. Furthermore, based on measurements taken at this collection, 32 bulls were capable of passing a BSE. Of these bulls, seven were FAA Negative. Furthermore, of 11 bulls which would have been deferred, three were FAA Negative. Statistical analysis (Fisher's Exact Test) of the distribution of FAA Negative samples indicated no relationship ($P>0.05$) between the presence of absence of FAA within the ejaculate and a bull's ability to pass a BSE at that collection.

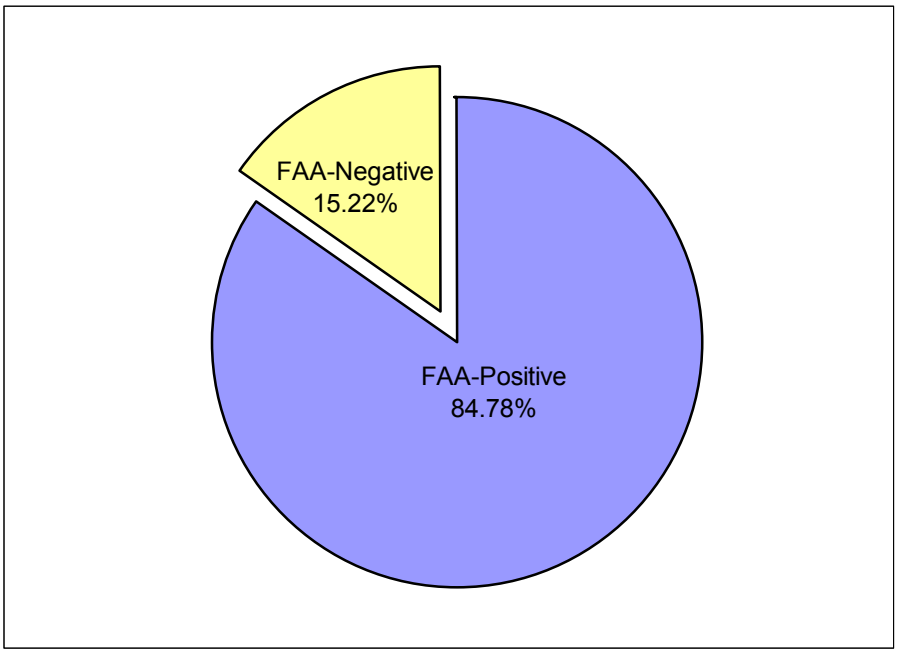

Figure 3. FAA status of bulls from Ranch A at collection three.

Summary of All Collections at Ranch A

Across all collections the percentage of FAA Positive bulls improved from 63.89% at collection one to 84.78% by collection three. Of 32 bulls, which were

observed at all three collections, 12 bulls were FAA Negative at collection one. Of these 12 bulls, 75% (n=9) provided at least one FAA Positive sample at collections two and/or three. Furthermore, nine bulls (27.27%) had variation in their FAA status, moving from FAA Positive to FAA Negative, after their first FAA Positive result (Figure 4). Of these nine bulls, five were FAA Negative at collection two after being FAA Positive at Collection one. At collection three, four of the five bulls provided a FAA Positive sample. Ten bulls that were FAA Negative at collection one progressed to FAA Positive by collection two. However, 20% (n=2) of these bulls failed to remain FAA Positive at collection three. Regardless of this variation, across all collections, 96.97% (n=31) of these 32 bulls provided at least one ejaculate that was FAA Positive (Figure 4).

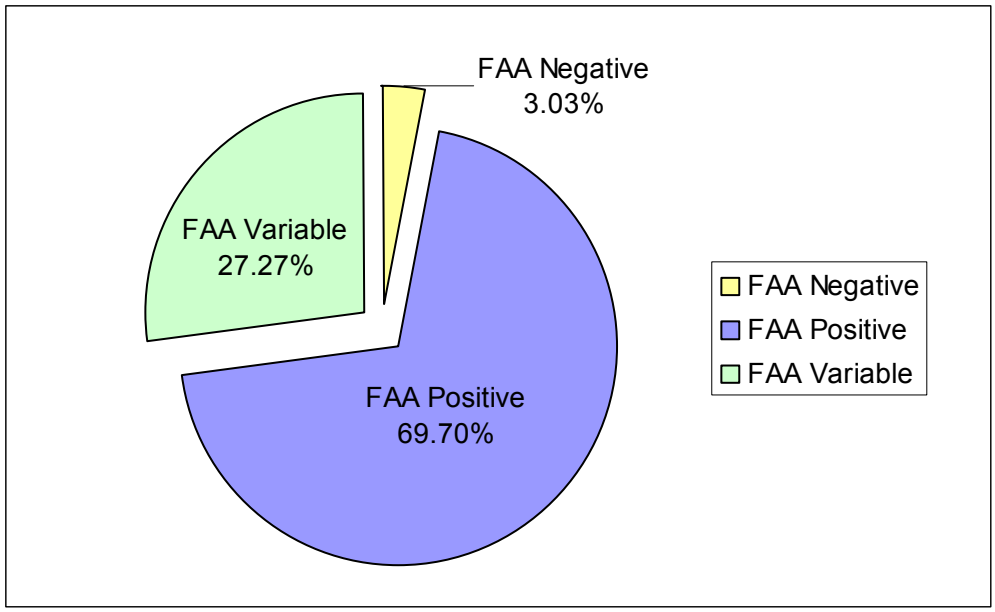

Figure 4. FAA status of 32 bulls from Ranch A observed at all three collections.

Bulls That Transitioned from FAA Negative to FAA Positive at Ranch A

After evaluating data from 32 bulls which were observed at all three collections, nine bulls were identified as being transition bulls, or bulls that were FAA Negative at collection one but progressed to FAA Positive and remained FAA Positive at collection two and/or three. Analysis of the entire data set collected from these nine bulls found that age (P=0.001) and sperm morphology (P=0.003) were significant in predicting the presence of FAA (Table 9). The point estimates for these variables suggest a positive relationship between FAA and sperm morphology, as each one unit increase in percent normal sperm improved the odds ratio of an FAA Positive sample by 31.6% (Table 10). FAA Positive samples were also more frequently observed in older bulls compared to their younger contemporaries (Tables 10, 11). In fact, for each additional day of age, the likelihood a bull would produce FAA increased by 11.5% (Table 11). The final model generated a Max-rescaled R-Square of 0.8276, with a satisfactory GFT (P=0.66) and predicted the FAA status of the bulls within this subset of bulls in 96% of the time (Tables 9, 12).

Table 9. Summary of stepwise logistic regression and respective R-Square and Max-rescaled R-Square values of nine transition bulls from Ranch A

Step	Variable	DF	Score Chi-Square	Pr > Chi-Square	R-Square	Max-rescaled R-Square
1	Age	1	12.2285	0.001	0.4304	0.5807
2	Sperm Morphology	1	8.9988	0.003	0.6135	0.8276

Table 10. Analysis of maximum likelihood estimates of nine transition bulls from Ranch A

Parameter	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSquare
Intercept	1	-60.2795	23.9455	6.3371	0.012
Age	1	0.1087	0.0468	5.3957	0.020
Sperm Morphology	1	0.2747	0.1230	4.9865	0.026

Table 11. Odds ratio estimates of nine transition bulls from Ranch A

		95% Wald Confidence Limits	
Effect	Point Estimate	Lower	Upper
Age	1.115	1.017	1.222
Sperm Morphology	1.316	1.034	1.675

Table 12. Association of predicted probabilities and observed responses of nine transition bulls from Ranch A

	Percent
Concordant	96.0
Discordant	04.0
Tied	00.0

Ranch B

Ranch B, located near the Texas gulf coast provided 47 Brahman bulls for semen collection and FAA analysis. Of this population, 33 bulls were evaluated three times, nine bulls were observed twice, and five bulls were collected only once. Of the bulls collected twice, five bulls were evaluated at collections one and two, two bulls were evaluated at collection one and three, and two bulls were evaluated at collections two and three. Of bulls observed only once, four were evaluated at collection one and one bull was evaluated at collection three.

Ranch B, Collection One

The average and range in age, scrotal circumference, ejaculate volume, and sperm concentration for bulls at Ranch B, collection one were: 582 d (510-886 d), 35.83 cm (30-43 cm), 4.44 mL (1-11 mL), and 313 million cells/mL (0-1,385 million cells/mL), respectively. For bulls that produced an ejaculate which contained detectable amounts of sperm, the average and range of percent motile sperm and percent normal sperm were 48.78% (10-90%) and 69.87% (47-87%), respectively.

At collection one, 44 bulls were evaluated and 86.36% (n=38) were deemed FAA Positive through ReproTest analysis (Figure 5). Three bulls that failed to produce an ejaculate containing a detectable amount of sperm were FAA Positive at this collection. In addition, these were the only bulls that were determined to be prepuberal at this collection. Further statistical analysis utilizing Stepwise Logistic Regression of data collected from Ranch B at collection one revealed that no variable, or combination of variables, significantly explained a bull's FAA status. Due to the conditions of the model statement, age ($P<0.13$) was included as the only variable in the final model, with an estimate of –0.0080, suggesting that younger bulls perhaps have a slight tendency to more frequently express FAA (Tables 13, 14, 15). As expected by the lack of significant explanatory variables within the data set, the model was only able to explain 8.49% of the variation in FAA status of bulls observed at this collection (Table 13). The final model was only able to correctly predict the FAA status of 55.4% of the bulls within this population (Table 16).

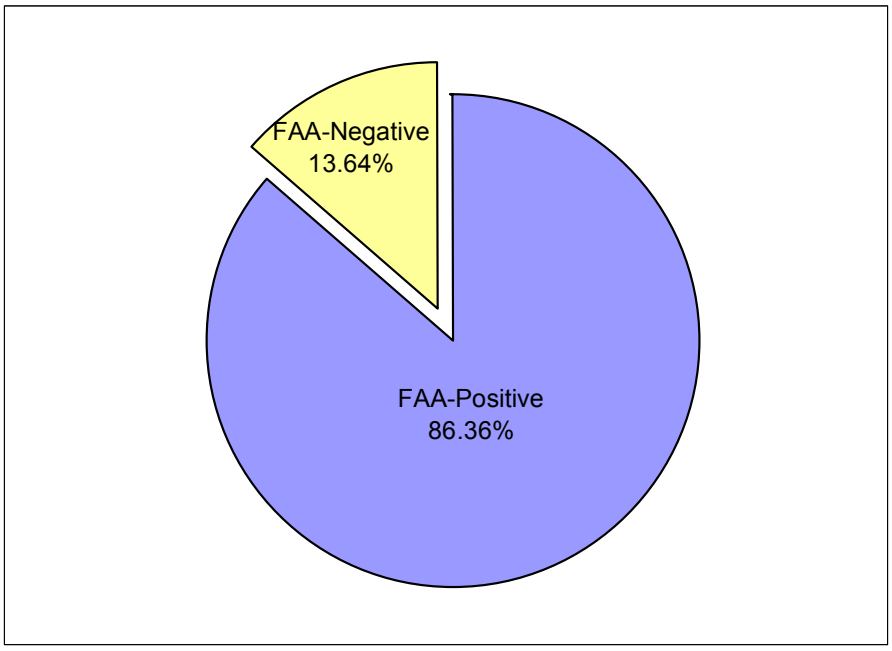

Figure 5. FAA status of bulls from Ranch B at collection one.

Table 13. Summary of stepwise logistic regression and respective R-Square and Max-rescaled R-Square values from Ranch B, collection one

Step	Variable	DF	Score Chi-Square	Pr > Chi-Square	R-Square	Max-rescaled R-Square
1	Age	1	2.3201	0.128	0.0499	0.0849

Table 14. Analysis of maximum likelihood estimates from Ranch B, collection one

Parameter	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSquare
Intercept	1	6.4586	3.6462	3.1375	0.077
Age	1	-0.0080	0.0059	1.8132	0.178

Table 15. Odds ratio estimates from Ranch B, collection one

Effect	Point Estimate	95% Wald Confidence Limits	
		Lower	Upper
Age	0.992	0.981	1.004

Table 16. Association of predicted probabilities and observed responses from Ranch B, collection one

	Percent
Concordant	55.4
Discordant	43.5
Tied	01.1

Ranch B, Collection Two

Collection two was conducted on 40 bulls, composed of 38 bulls observed at collection one and two additional bulls. At this testing, only 67.5% (n=27) produced an ejaculate that contained FAA as determined by the ReproTest (Figure 6). Within six bulls, which were FAA Negative at collection one, only one animal progressed to FAA Positive at collection two. However, 21.86% (n=7) of the FAA Positive bulls from collection one that were observed at collection two (n=32) produced a FAA Negative ejaculate at collection two. Two bulls that failed to produce an ejaculate containing a detectable amount of sperm were FAA Positive at this collection. Additionally, these two bulls were the only prepuberal bulls evaluated at collection two.

Bull age ($P<0.03$) was a significant indicator of FAA status, with younger bulls being more likely to be FAA Positive as indicated by the estimate of -0.0156 (Tables 17, 18). Ejaculate volume ($P<0.03$) was also observed as a significant explanatory variable, as each 1-mL decrease in ejaculate volume increased the likelihood of an FAA Positive sample by 35% (Tables 17, 18, 19). The Max-rescaled R-Square value for the final model was 0.3453 and was able to correctly identify the FAA status in 79.2% of the bulls observed at this collection (Tables 17, 20). Although the Max-rescaled R-Square for the final model was low, results of the GFT ($P=0.29$) indicates no lack of fit. In

summary, within this collection, FAA Positive bulls were significantly younger and had a significantly lower ejaculate volume compared to FAA Negative bulls (Tables 17, 18, 19).

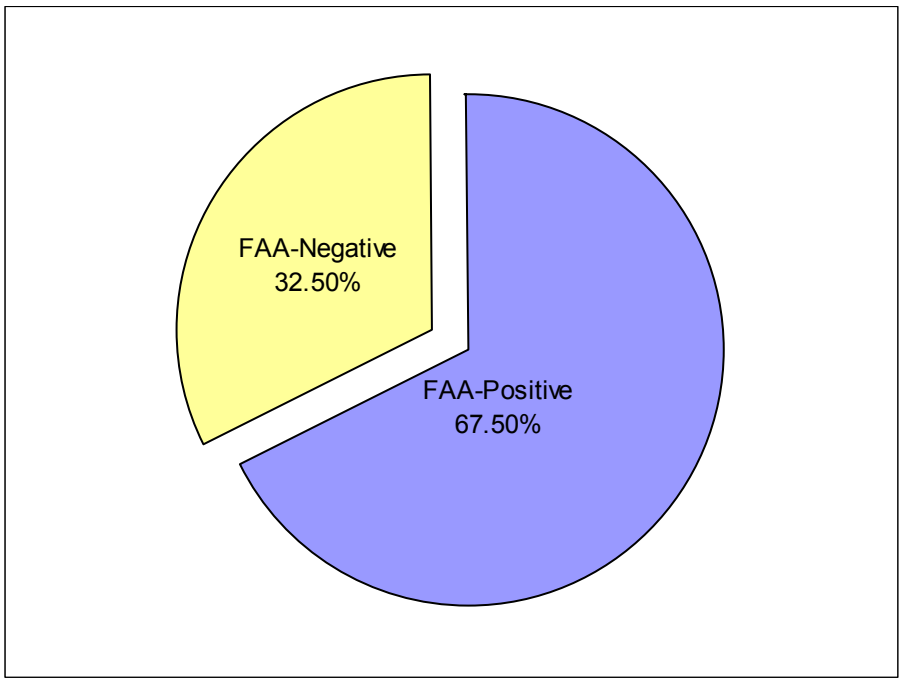

Figure 6. FAA status of bulls from Ranch B at collection two.

Table 17. Summary of stepwise logistic regression and respective R-Square and Max-rescaled R-Square values from Ranch B, collection two

Step	Variable	DF	Score chi-square	Pr > chi-square	R-Square	Max-rescaled R-Square
1	Age	1	5.0275	0.025	0.1377	0.1895
2	Ejaculate Volume	1	5.0324	0.025	0.2509	0.3453

Table 18. Analysis of maximum likelihood estimates from Ranch B, collection two

Parameter	DF	Estimate	Standard Error	chi-square	Pr > chi-square
Intercept	1	12.2711	5.3618	5.2377	0.022
Age	1	-0.0156	0.0082	3.6226	0.057
Ejaculate Volume	1	-0.4306	0.2078	4.2927	0.038

Table 19. Odds ratio estimates from Ranch B, collection two

		95% Wald Confidence Limits	
Effect	Point Estimate	Lower	Upper
Age	0.985	0.969	1.000
Ejaculate Volume	0.650	0.433	0.977

Table 20. Association of predicted probabilities and observed responses from Ranch B, collection two

	Percent
Concordant	79.2
Discordant	20.8
Tied	00.0

Ranch B, Collection Three

Collection three resulted in an improvement in the percentage of FAA Positive bulls, with 76.32% (n=29) of 38 bulls providing a positive result (Figure 7). The single bull that was FAA Negative at collection one but was FAA Positive at collection two remained FAA positive. Only three of the six bulls deemed FAA Negative at both collections one and two were observed at collection three. All three bulls were FAA negative at collection three as well. Of the seven bulls that transitioned from FAA Positive at collection one to FAA Negative at collection two, 71.42% (n=5) regained their FAA Positive status at collection three. One bull, observed only at collections two and three, shifted from FAA Negative at collection two to FAA Positive at collection three. One bull failed to produce an ejaculate containing a detectable amount of sperm and was FAA Positive at this collection. This bull was also the only prepuberal bull evaluated at this collection. Due to cold ambient temperatures, accurate scrotal circumference measures could not be obtained at this collection.

Statistical analysis of data derived from collection three found age ($P<0.01$) to be highly significant, and sperm concentration ($P=0.02$) to be significant with regard to FAA expression (Table 21). Sperm morphology ($P<0.07$) tended to affect a bull's FAA status as well (Table 21). FAA Positive bulls were younger than FAA Negative bulls, and for each daily increase in age, were 3.3% less likely to produce an ejaculate that contained FAA (Tables 22, 23). Furthermore FAA Positive bulls at this collection had a significantly less concentrated ejaculate with respect to sperm concentration ($P=0.02$), and tended to have a higher percentage of normal sperm than FAA Negative bulls (Tables 21, 22, 23). Although the volume of ejaculate was not significant, it was included in the final model and improved the Max-rescaled R-Square from 0.5192 to 0.5847 (Table 21). Nonetheless, the final model was able to successfully categorize 93.8% of the bulls in this study by their respective FAA-Profile given the four previously mentioned explanatory variables (Table 24).

Measurements of semen quality taken at this collection, coupled with scrotal circumference measures recorded at collection two, reviled that 28 bulls were capable of passing a BSE. Of these bulls, seven were FAA negative. Additionally, 10 bulls would have been deferred based on our measurements at this collection. Two bulls that would have been deferred were FAA Negative. Statistical analysis of this distribution by way of Fischer's Exact Test reviled no difference ($P>0.05$) between FAA status and a bull's ability to pass a BSE.

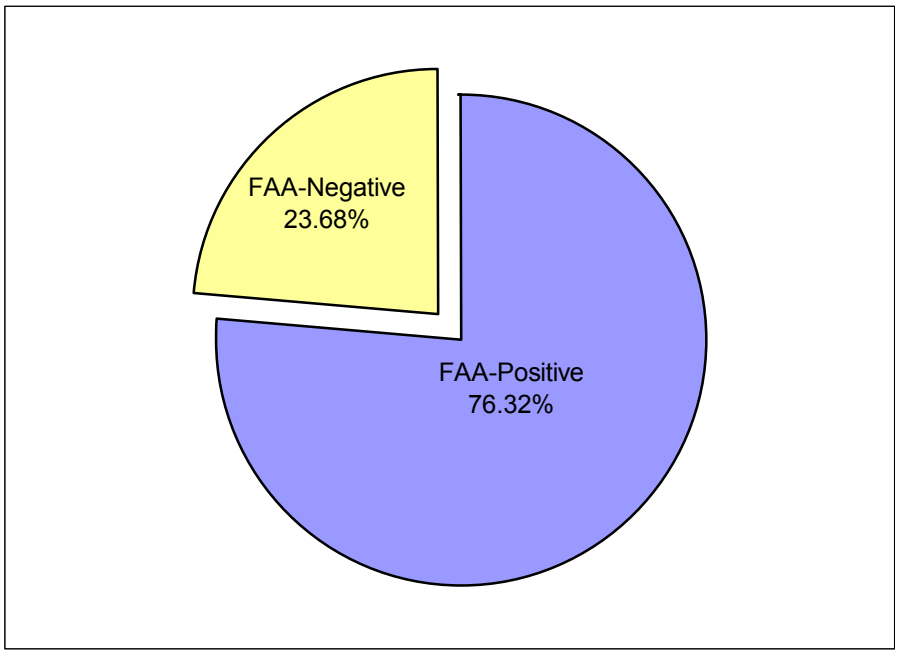

Figure 7. FAA status of bulls from Ranch B at collection three.

Table 21. Summary of stepwise logistic regression and respective R-Square and Max-rescaled R-Square from Ranch B, collection three

Step	Variable	DF	Score chi-square	Pr > chi-square	R-Square	Max-rescaled R-Square
1	Age	1	7.7010	0.006	0.1952	0.2891
2	Sperm Concentration	1	5.4161	0.020	0.3020	0.4473
3	Sperm Morphology	1	3.3686	0.067	0.3506	0.5192
4	Ejaculate Volume	1	2.5430	0.111	0.3948	0.5847

Table 22. Analysis of maximum likelihood estimates from Ranch B, collection three

Parameter	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSquare
Intercept	1	18.9517	9.0772	4.3590	0.037
Age	1	-0.0333	0.0158	4.4559	0.035
Ejaculate Volume	1	-0.4670	0.3175	2.1634	0.141
Sperm Concentration	1	-0.0042	0.0018	5.3457	0.021
Sperm Morphology	1	0.1065	0.0530	4.0419	0.044

Table 23. Odds ratio estimates from Ranch B, collection three

		95% Wald Confidence Limits	
Effect	Point Estimate	Lower	Upper
Age	0.967	0.938	0.998
Ejaculate Volume	0.627	0.336	1.168
Sperm Concentration	0.996	0.992	0.999
Sperm Morphology	1.112	1.003	1.234

Table 24. Association of predicted probabilities and observed responses from Ranch B, collection three

	Percent
Concordant	93.8
Discordant	06.2
Tied	00.0

Summary of All Collections at Ranch B

For Ranch B, the percentage of FAA Positive samples during three collections ranged from 67.5-86.36%. Out of 46 bulls which were observed two or more times over the course of the study, 26.08% (n=12) had variations with regard to their FAA status. Furthermore, a subset of the population presented by Ranch B, comprised of 33 bulls observed at all three collections, found 33.33% (n=11) of bulls to have shifts within their FAA status over the course of the study. Of these 33 bulls, one bull, which was FAA negative at collection one, progressed to FAA Positive at collections two and three. Ten bulls (30.30%) within this subset transitioned to FAA Negative after producing a FAA Positive result at one or more collections. Three (9.09%) of the ten previously mentioned bulls provided FAA Positive ejaculates at collections one and two, yet produced an FAA Negative sample at collection three. The remaining seven bulls of this group of ten were FAA Positive at collection one but were FAA Negative at collection two. Of these

seven bulls, five regained their FAA Positive status at collection three, while two did not. Nonetheless, of 33 bulls observed at all three collections, 90.9% (n=30) produced at least one FAA Positive result over the course of this study.

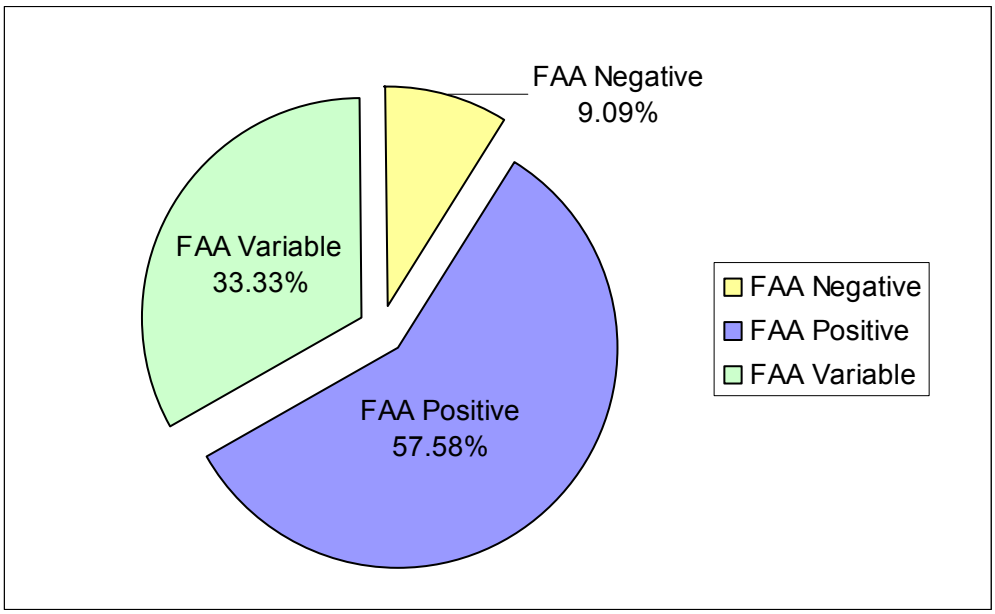

Figure 8. FAA status of 33 bulls from Ranch B observed at all three collections.

Bulls That Transitioned from FAA Negative to FAA Positive at Ranch B

Across all collections from Ranch B, only two bulls observed at all three collections progressed from FAA Negative to FAA Positive and remained FAA Positive for the remainder of the study. Due to the small sample size, a valid statistical analysis could not be generated.

Ranch C

Ranch C, located near the Texas gulf cost, provided 111 Brahman bulls for semen collection and FAA analysis. From this population of 111 Brahman bulls, 272 semen samples were obtained. Within the population, 67 animals were collected three

times, 27 animals were collected twice, and 17 animals were collected once. Of the bulls collected twice, 12 were viewed at collection one and two, 12 were viewed at collection two and three, and three were viewed at collection one and three. Within sires for which only one ejaculate was obtained, 14 bulls were tested at collection one, two at collection two, and one at collection three.

Ranch C, Collection One

The average and range in age, scrotal circumference, ejaculate volume, and sperm concentration for bulls evaluated at Ranch C, collection one were: 563 d (506-633 d), 32.97 cm (28-41.5 cm), 5.54 mL (1-13.5 mL), and 346 million cells/mL (49-1,688 million cells/mL), respectively. For bulls that produced an ejaculate which contained detectable amounts of sperm, the average and range of percent motile sperm and percent normal sperm were 59.53% (10-75%) and 77.77% (3-95%), respectively.

At collection one, a group of Brahman bulls (n=96) was collected in which 79.17% (n=76) were FAA Positive (Figure 9). Furthermore, every bull that was observed at this collection was pubertal. Stepwise Logistic Regression revealed a significant difference in ejaculate volume ($P<0.02$) between bulls which were FAA Positive compared to bulls which were FAA Negative (Table 25). FAA Positive bulls had a lower ejaculate volume compared to FAA Negative bulls, as indicated by the estimate given in Table 26. Each 1-mL decrease in ejaculate volume improved the odds ratio that the sample would be FAA Positive by 26% (Table 27). The final model Max rescaled R-Square was low at 0.1086, however GFT indicated no lack of fit ($P<0.16$) and

the model was able to correctly identify the FAA status of 62.4% of the bulls within this study (Table 28).

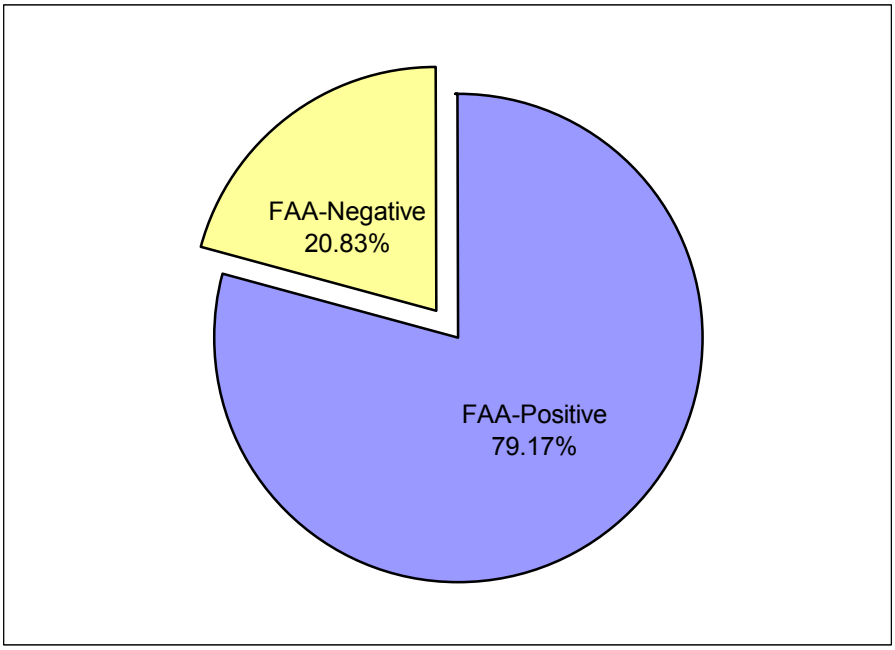

Figure 9. FAA status of bulls from Ranch C at collection one.

Table 25. Summary of stepwise logistic regression and respective R-Square and Max-rescaled R-Square from Ranch C, collection one

Step	Variable	DF	Score Chi-Square	Pr > Chi-Square	R-Square	Max-rescaled R-Square
1	Ejaculate Volume	1	5.9924	0.014	0.0670	0.1086

Table 26. Analysis of maximum likelihood estimates from Ranch C, collection one

Parameter	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSquare
Intercept	1	3.2762	0.8790	13.8920	0.001
Ejaculate Volume	1	-0.3010	0.1312	5.2646	0.022

Table 27. Odds ratio estimates from Ranch C, collection one

		95% Wald Confidence Limits	
Effect	Point Estimate	Lower	Upper
Ejaculate Volume	0.740	0.572	0.957

Table 28. Association of predicted probabilities and observed responses from Ranch C, collection one

	Percent
Concordant	62.4
Discordant	31.2
Tied	06.4

Ranch C, Collection Two

Collection two involved sampling a population of Brahman bulls (n=93) comprised of 67 bulls from collection one, and 26 additional bulls that were not observed at collection one. In addition, every bull that was observed at this collection was pubertal. FAA results from this population revealed that 88.17% (n=82) were indeed FAA positive (Figure 10). Interestingly, three bulls identified as FAA Positive during the first collection provided FAA Negative samples at collection two. Conversely, within 16 bulls which were FAA Negative at collection one, 62.5% (n=10) progressed to FAA Positive at collection two.

Data recorded from collection two revealed that FAA Positive bulls at this collection had larger scrotal circumference ($P<0.01$) and improved sperm motility ($P<0.02$) (Tables 29, 30). In fact, for each 1-cm increase in scrotal circumference, bulls were 24.8% more likely to be FAA Positive (Table 31). Furthermore, for each one-percent decrease in percent normal sperm, bulls within this population were 14% more likely to produce a FAA Positive ejaculate (Table 31). No lack of fit for the final model

was observed by GFT (P=0.7376) despite the again relatively low, (0.2846), Max-rescaled R-Square (Table 29). Within this data set, the model was able to correctly predict a bull's FAA status 80.8% of the time, as indicated in Table 32.

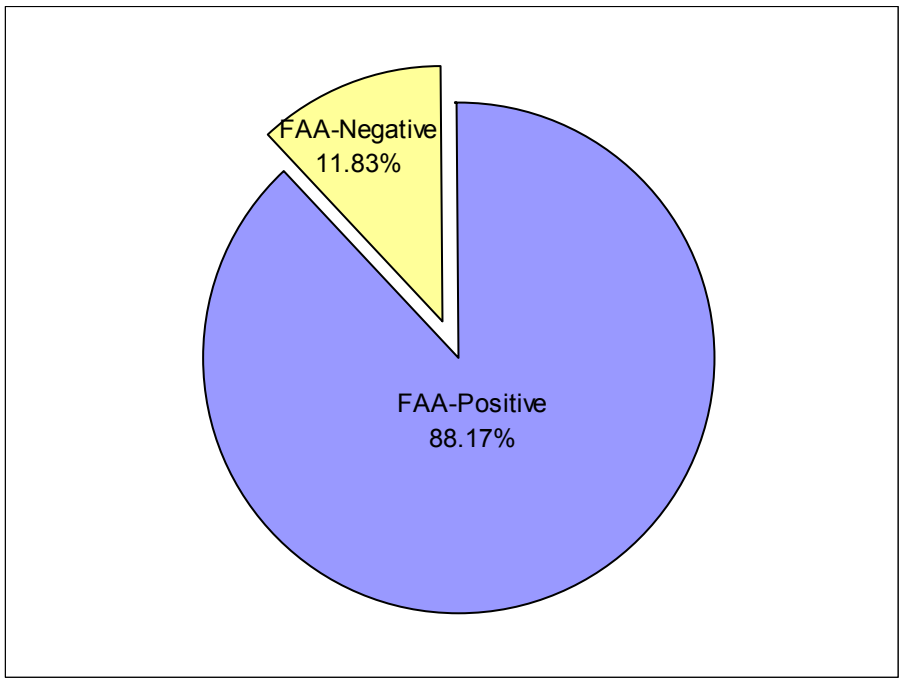

Figure 10. FAA status of bulls from Ranch C at collection two.

Table 29. Summary of stepwise logistic regression and respective R-Square and Max-rescaled R-Square from Ranch C, collection two

Step	Variable	DF	Score Chi-Square	Pr > Chi-Square	R-Square	Max-rescaled R-Square
1	Scrotal Circumference	1	8.0975	0.004	0.0593	0.1264
2	Sperm Morphology	1	1.1416	0.285	0.0789	0.1680
3	Sperm Motility	1	5.6291	0.018	0.1336	0.2846

Table 30. Analysis of maximum likelihood estimates from Ranch C, collection two

Parameter	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSquare
Intercept	1	3.3217	4.2809	0.6021	0.438
Scrotal Circumference	1	0.2213	0.0976	5.1357	0.023
Sperm Motility	1	0.0713	0.0345	4.2731	0.039
Sperm Morphology	1	0.1513	0.0798	3.6004	0.058

Table 31. Odds ratio estimates from Ranch C, collection two

Effect	Point Estimate	95% Wald Confidence Limits	
		Lower	Upper
Scrotal Circumference	1.248	1.030	1.511
Sperm Motility	1.074	1.004	1.149
Sperm Morphology	0.860	0.735	1.005

Table 32. Association of predicted probabilities and observed responses from Ranch C, collection two

	Percent
Concordant	80.8
Discordant	18.7
Tied	00.5

Ranch C, Collection Three

At collection three, 83 samples were taken, with 67 samples coming from bulls seen at collections one and two, 12 samples coming from bulls collected first at collection two, and one sample derived from a bull viewed only at collection three. Every bull that was observed at this collection was determined to be pubertal. Across all samples obtained during collection three, 78.31% (n=65) were FAA Positive (Figure 11). Of the bulls which were FAA Negative at collections one and two (n=6), three

progressed to FAA Positive at collection three. However, 62.5% (n=5) of eight bulls which were FAA Negative at collection one and FAA Positive at collection two were FAA Negative at collection three. Furthermore, of the three bulls that were FAA Positive at collection one, and FAA Negative at collection two, two had regained their FAA Positive status at collection three. Six bulls that were FAA Positive at collections one and two had become FAA Negative at collection three.

Bull age (P=0.02) was the only significant variable with respect to explaining the difference in FAA status among bulls observed at this collection (Table 33). At this collection, FAA Positive bulls tended to be older. In fact, for each additional day of age, the likelihood a bull would produce FAA improved by 2.6% (Tables 34, 35). Ejaculate volume, which was not significant, was included in the final model that had a Max-rescaled R-Square of 0.1556 and was able to correctly identify the FAA status of bulls observed at this collection 73.3% of the time, based on these two explanatory variables (Tables 33, 36).

Of the 83 bulls observed at this collection, 73 would have passed a BSE at this collection. Fifteen of these bulls were FAA Negative at this collection. Within the 10 bulls that would have been deferred, three were FAA Negative. Statistical analysis by Fisher's Exact Test reveled no difference (P>0.05) between bulls ability to pass a BSE based on the presence or absence of FAA within the ejaculate at the time of evaluation.

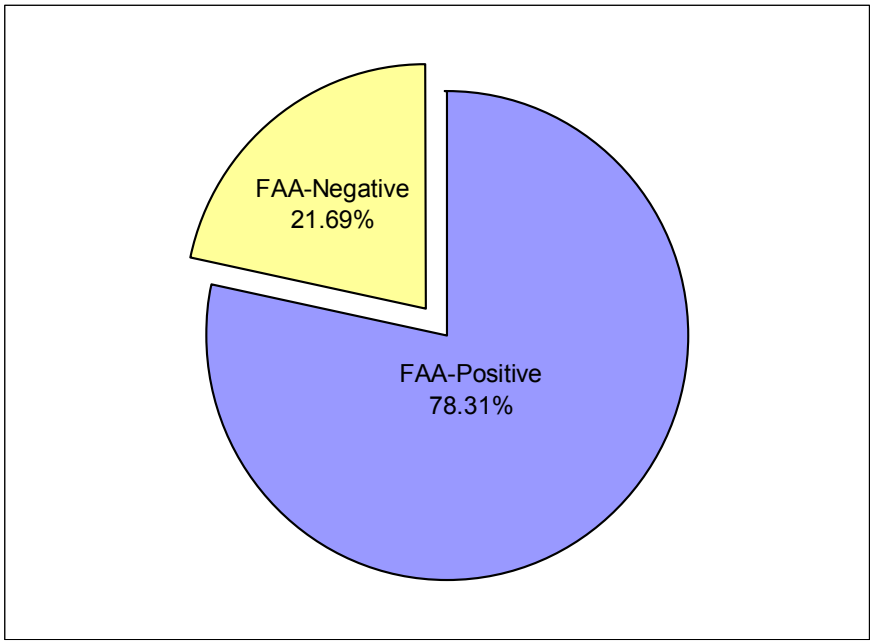

Figure 11. FAA status of bulls from Ranch C at collection three.

Table 33. Summary of stepwise logistic regression and respective R-Square and Max-rescaled R-Square from Ranch C, collection three

Step	Variable	DF	Score Chi-Square	Pr > Chi-Square	R-Square	Max-rescaled R-Square
1	Age	1	5.7380	0.017	0.0697	0.1073
2	Ejaculate Volume	1	2.8374	0.092	0.1011	0.1556

Table 34. Analysis of maximum likelihood estimates from Ranch C, collection three

Parameter	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSquare
Intercept	1	18.8526	7.2921	6.6839	0.010
Age	1	-0.0264	0.0115	5.2468	0.022
Volume	1	-0.2120	0.1305	2.6378	0.104

Table 35. Odds ratio estimates from Ranch C, collection three

		95% Wald Confidence Limits	
Effect	Point Estimate	Lower	Upper
Age	0.974	0.952	0.996
Volume	0.809	0.626	1.045

Table 36. Association of predicted probabilities and observed responses from Ranch C, collection three

	Percent
Concordant	73.3
Discordant	25.8
Tied	00.9

Summary of All Collections at Ranch C

Across all collections, the number of FAA positive bulls ranged from 78.31-88.17%. Of the 111 bulls tested, only 67 animals were observed at all three collections. Among bulls observed at all three collections, 4.48% (n=3) were FAA Negative at every collection (Figure 12). Furthermore, 20.9% (n=14) had variation in their FAA status after recording their first FAA Positive result at either collection one or collection two (Figure 12). However, within a subset of 67 bulls, 95.52% (n=65) had at least one positive FAA result (Figure 12). Moreover of the 20.8% (n=14) that began the study as FAA Negative at collection one, 64.29% (n=9) progressed to a FAA Positive status at one or more collections. However, of these nine bulls, five produced a FAA Negative ejaculate at collection three after providing a FAA Positive sample at collection two.

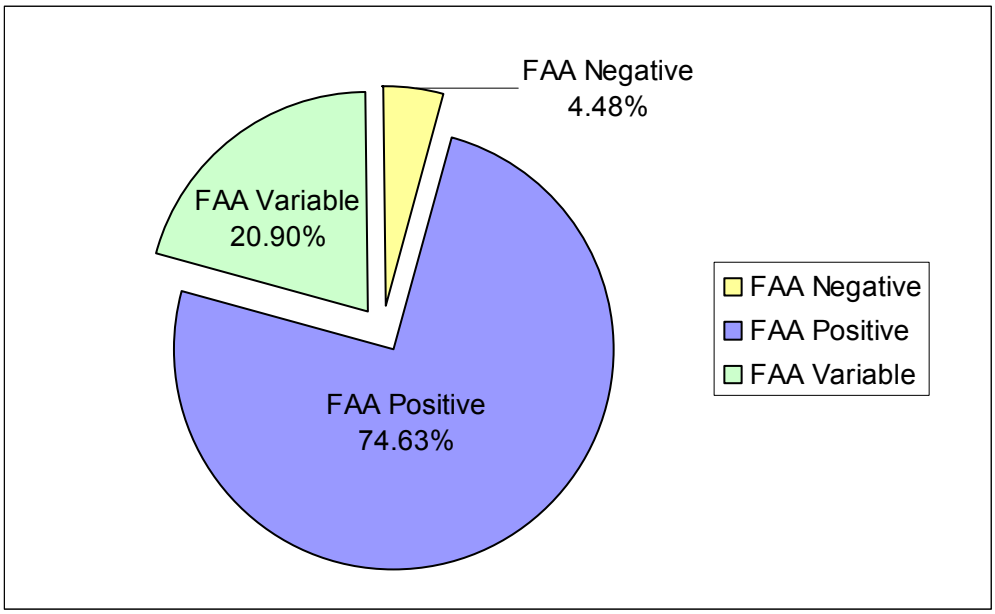

Figure 12. FAA status of 67 bulls from Ranch C observed at all three collections.

Bulls That Transitioned from FAA Negative to FAA Positive at Ranch C

Six bulls within the population of 111 animals observed at this ranch were identified as transition bulls. Statistical analysis revealed that no explanatory variable was significant in predicting the FAA status of an ejaculate produced by these six transition bulls. Furthermore, no variable met the 0.3 level of significance for entry into the model, therefore a model was not generated.

DISCUSSION

Percentage calf crop weaned is potentially the most influential factor concerning economic success and profitability in any beef or dairy operation. While embryonic and fetal death loss, dystocia and death loss postpartum impact overall profitability, no single factor affects this equation more than the number of females that conceive during the breeding season. In single sire herds, the chosen herd sire must be of the highest level of fertility to ensure that all fertile females within the herd conceive during the breeding season. Therefore, factors affecting bull fertility should be of the utmost importance in any cattle operation.

Ejaculated sperm must undergo the process of capacitation and the acrosome reaction within the female reproductive tract in order to successfully fertilize an oocyte. Molecules within the reproductive tract of the female such as GAG, Heparin, and HDL bind with proteins in the ejaculate which were secreted by the accessory sex glands during ejaculation (Marks and Ax, 1985, Therien et al., 1998). At ejaculation, BSP bind to choline phopholipids on the sperm membrane and stimulate phospholipid efflux from the sperm membrane during the first 15-30 minutes post-ejaculation (Manjunath and Therien, 2002). An efflux of cholesterol soon follows the phospholipid efflux and further destabilizes the sperm membrane (Therien et al., 1998, 2001, Moreau et al., 1999). This destabilization of the sperm membrane is know as capacitation and prepares the sperm for the acrosome reaction.

While BSP play an important role in the cholesterol and phospholipid efflux from the sperm membrane, FAA with its much greater affinity for heparin also

stimulates the AR. After ejaculation, HBP, including FAA, binds with heparin, and, through an indirect mechanism which is not well understood, enables heparin to stimulate greater rates of capacitation in ejaculated sperm, which results in improved oocyte penetration and corresponds to higher fertility (Henault et al., 1995). Ultimately, several proteins within the ejaculate influence the rate of capacition in ejaculated sperm through phospholipid and cholesterol efflux as well as interactions with heparin.

Bellin et al. (1994) reported that within two populations of bulls from the same ranch, FAA Negative bulls composed approximately 27% and 40% of the entire population, suggesting variation among herds and/or breed types, even on the same ranch. Western blot analysis of approximately 6,000 samples derived from many different breed types and locations suggest a slightly lower rate, with an average of 15% FAA Negative (McCauley et al., 2004). ReproTest analysis of 914 samples from several different populations revealed that an average of 26% of bulls were FAA Negative (McCauley et al., 2004). If the estimates of the gene frequency that code for the production of FAA are correct, one would expect that across the entire population, 16% of the bulls would be FAA negative due to genotype (Dawson et al., 2002). This estimate is complimentary to results observed with Western blots. The larger percentage of FAA Negative results observed with ReproTest analysis may be due to either population dynamics of the test population, or discrete differences in sensitivity between the Western Blot and the ReproTest.

At Ranch A, 36.11, 21.74 and 15.22% of the sample population were FAA Negative at collections one, two, and three, respectively. At Ranch B, 13.64, 32.5, and

23.68% of bulls were FAA Negative at collections one, two, and three, respectively. Ranch C had 20.83, 11.83, and 21.68% of samples return FAA Negative results for collections one, two and three, respectively. While results on a collection by collection basis may seem similar to those reported in Bellin et al. (1994, 1996) and McCauley et al. (2004), a look at bulls that were FAA Negative at all three collections paints a much different picture. In fact, of bulls observed at all three collections at Ranch A, B, and C, the percentage of FAA Negative bulls (bulls that were FAA Negative at all three collections) was 3.03, 9.09 and 4.48%, respectively. More intriguing was the number of bulls that displayed variation within their expression of FAA. These bulls, which were observed at all three collections, provided one or more FAA Negative samples after producing an ejaculate at collection one or two that contained FAA. Within the sample populations from each ranch, 27.27, 33.33, and 20.90% of bulls displayed variation within their FAA status at Ranch A, B, and C, respectively. Considering that one-fifth to two-third of the entire population displayed variation in their FAA status during our study, it becomes quite clear that FAA classification based on a single test result may not be adequate in identifying the true FAA status of a considerable portion of the bulls within the population. Furthermore, the fertility of bulls that show variation in their FAA status has not been evaluated.

The first objective of this study was to determine if a relationship exists between the expression of FAA and common variables evaluated during an routine BSE. These variables included age, scrotal circumference, ejaculate volume, sperm concentration, sperm motility, and percent normal sperm. Statistical analysis of data collected from

Ranch A revealed that bull age ($P<0.05$) and sperm concentration ($P<0.05$) were significant variables in determining the FAA status of bulls at collection one. However, at collection two, sperm motility ($P<0.05$) was the only significant variable, with age included in the final model, but not significant. No significant explanatory variables were identified during the third collection at Ranch A. Point estimates for age and sperm concentration suggest that at collection one, FAA Positive bulls tended to be younger and had a more concentrated ejaculate. At collection two, age and sperm concentration differences between FAA Positive and FAA Negative bulls relaxed to a level of insignificance, yet impaired sperm motility was observed in FAA Positive bulls compared to FAA Negative bulls. The reason for the significant improvement in sperm motility of FAA Negative bulls, as observed at collection two is still unclear. Results from collection one perhaps display the genetic effects concerning variations in the onset of puberty within a population. Despite their younger age, only three bulls were prepuberal. Given the significantly greater number of sperm cells observed within the ejaculate, FAA Positive bulls within this population had likely reached puberty at an earlier age compared to their FAA Negative contemporaries. Differences in SC were not observed, suggesting similar testicular development amongst the two groups, and that SC may not be a good measure of puberty as defined by our definition. Therefore, some factor resulting in a suppressed rate of spermatogenesis and a delayed response to the effects of puberty within these FAA Negative bulls, could potentially explain the delayed expression of FAA in bulls which go on to produce FAA in later collections. This assumption could not be confirmed as statistical evaluation of the transition bulls

from Ranch A revealed positive relationships between FAA and age and FAA and sperm morphology, both of which were highly significant. A relationship between FAA and sperm concentration was not observed in these transition bulls, suggesting that puberty may in fact not be related to the expression of FAA. However, sperm morphology improved ($P<0.01$) as these bulls transitioned to FAA positive, suggesting an improved normality of spermatogenesis, which correlates favorably to the conditions observed in collection one of the entire population at Ranch A.

Ranch B followed the age-associated trend with respect to FAA as observed at Ranch A, despite the distinct differences in biological type concerning the two different populations of bulls. Bull age was significant at collection two, and highly significant at collection three with regard to the expression of FAA. Ejaculate volume was significantly less ($P<0.05$) in bulls which were FAA Positive at collection two, but insignificant at collections one and three. At collection three, FAA Positive bulls also had a lower concentration of sperm compared to FAA Negative bulls.

At Ranch C, SC was highly significant at collection two, with FAA Positive bulls having a larger SC. Ejaculate volume was less ($P<0.05$) in FAA Positive bulls at collection one, which was similar to the results for collection two at Ranch B. Sperm motility was also significantly higher in FAA Positive bulls at collection two.

Ejaculate volume, while only significant in two of nine collections, was included in the final model in six of eight collections for which a final model was generated. In all six models, the point estimate generated for this variable was negative, suggesting FAA Negative bulls produce a larger volume of ejaculate compared to FAA Positive

bulls. Sperm concentration of FAA Positive bulls was similar to or less than FAA Negative bulls in all but one collection. Considering these observations, the increased volume of ejaculate may be a compensatory mechanism by the FAA Negative bull to combat a lower rate of AR. By increasing the volume of ejaculate, the FAA Negative bull is able to improve the number of AR reacted sperm, despite the impaired rate of AR, simply due to an increase in the total number of ejaculated sperm. However, even if this mechanism exists, work cited earlier in this paper still leaves little doubt that FAA Positive bulls will often times be more fertile than FAA Negative bulls of similar serving capacity (Bellin et al., 1994, 1996, 1998).

It is important to note that oftentimes SC is utilized as an indicator of puberty in bulls. If an interaction between puberty and FAA does exist, one would expect to observe a larger SC in FAA Positive bulls compared to FAA Negative bulls. Increased SC is directly due to up regulation of the androgenic axis of the endocrine system as a result of the onset of puberty. While this would suggest a positive relationship between puberty and the expression of FAA, this phenomenon was not evident at any collection at Ranch A or B in which FAA Positive bulls tended to be younger.

For all bulls in which no sperm were detectable in the ejaculate (n=14), 78.57% (n=11) produced a FAA Positive ejaculate, despite the lack of sperm in the sample. These bulls by definition were prepuberal. Moreover, the distribution of FAA Positive samples compared to FAA Negative samples at the third collection from all three ranches was not different ($P>0.05$), suggesting that any outward measure relating to the BSE did not effect the percentage of FAA Positive bulls. Therefore, it would appear that

the ability to express FAA relies on some factor not affiliated with sperm concentration and/or puberty.

SUMMARY AND CONCLUSIONS

The results presented in this thesis support the following conclusions:

1. No single variable was consistent in explaining the presence or absence of FAA within the ejaculate,
2. FAA was present in detectable amounts in the ejaculate of prepuberal bulls, including bulls that had no sperm within the ejaculate,
3. Across multiple collections, the number of bulls that were consistently classified as FAA Negative is more conservative than previous estimates, and
4. Despite their ability to produce FAA, a significant fraction of bulls fail to produce detectable amounts of FAA, as determined by the Repro Test, at every ejaculation, and

Future research on FAA is merited due to the conflicting results of this study compared to previous research. This study is one of the first to evaluate a population of bulls across multiple collections. Considering the large number of bulls observed within this study which had variation in their FAA status, areas of further research should include:

1. An evaluation of the fertility potential of bulls with variable FAA results, and
2. Determination of whether or not FAA variability diminishes as a bull matures.

Results generated from this experiment suggest that the ability to produce FAA can preceed the onset of puberty and has no consistent relationship with factors commonly

evaluated during the BSE. Therefore, under the conditions of this experiment, we reject our hypotheses that:

1. Consistent and repeatable relationships exists among age and/or pubertal changes in scrotal circumference, sperm cell concentration, sperm cell motility and sperm cell morphology on the initial appearance of FAA, and
2. Prepuberal beef bulls are unable to express FAA.

IMPLICATIONS

The presence of Fertility Associated Antigen within the ejaculate stimulates capacitation and results in an improvement in the rate of the acrosome reaction in ejaculated sperm, resulting in improved fertility in bulls capable of producing the protein. While other proteins secreted in the accessory fluid also impact the rate of capacitation and the AR, FAA has been shown to improve fertility in bulls that are capable of producing this protein. It is possible to identify FAA Positive bulls before puberty while utilizing the Repro Test. However, prepuberal and peripuberal bulls evaluated with the Repro Test and classified as FAA Negative based upon the results of a single evaluation should be subjected to at least one additional evaluation (a minimum of 30 d between evaluations) to provide an acceptable level of accuracy of the FAA Negative classification.

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