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Relationship of Polymorphisms in The FSH Beta Subunit Gene With Reproduction in Bos Taurus and Bos Indicus Cattle

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RELATIONSHIP OF POLYMORPHISMS IN THE FSH BETA SUBUNIT GENE WITH REPRODUCTION IN *BOS TAURUS* AND *BOS INDICUS* CATTLE

A thesis submitted in partial fulfillment
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Abstract

Relationship of polymorphisms in the FSH beta subunit gene with reproduction in *Bos taurus* and *Bos indicus* cattle

Two experiments were conducted to characterize polymorphisms (SNP) in the bovine FSHβ gene promoter region to examine breed differences in SNP, and to determine effects of SNP on reproduction in beef cattle. Experiment 1 - DNA samples were collected from 5 Angus (ANG), 13 Balancer (BAL), & 16 Brahman influenced (BI) bulls. Polymorphisms were identified by sequencing of 3 sequential PCR products from the promoter region revealed 17 SNP & 4 insertion/deletions (INDEL). Semen samples were collected and sperm quality variables determined viacomputer assisted sperm analysis (CASA) or stained smears. The MIXED procedure for ANG & BAL indicated interactions of week and SNP 485for LIN, AREA, & MINAB ($P \leq 0.05$), effects of SNP 169 & 170 on MINAB & TOTAB ($P \leq 0.07$), SNP 485 on MOT, PROG, RAP, VAP, VSL, VCL, ALH, BCF, AREA, & LIVE ($P \leq 0.05$),and SNP 1130 on VCL, ALH, LIN ($P \leq 0.05$), & STR ($P = 0.06$). For BI bulls effects of SNP 171, 225, 353, 410, 411, 412, & INDEL 413-414 on MINAB & TOTAB ($P \leq 0.09$), SNP 783 on MINAB & TOTAB ($P \leq 0.03$), BCF & MAJAB ($P \leq 0.09$), SNP 1130 on VCL ($P = 0.05$), VAP, & VSL ($P \leq 0.09$), and SNP 1702 on MINAB & TOTAB ($P \leq 0.04$). Experiment 2 - DNA samples were collected from early & late pubertal Brahman heifers and SNPwere identified by sequencing 2 upstream segments of FSHβ promoter region. Chi square revealed effects upon pubertyfor SNP 783 ($P = 0.02$) and 171 ($P = 0.08$), breed differences in frequency of occurrence for10 SNP (171, 225, 321, 353, 410, 411, 412, 783, 887, 1702), and 2 INDEL (411-412 INDEL & 413-414 INDEL). Results indicate breed differences in frequency of occurrence of SNP in FSHβ promoter region,

and that SNP may be useful as markers related to semen quality in bulls and puberty in Brahman heifers.

This thesis is approved for recommendation
to the Graduate Council.

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Dedication

This work is dedicated to my husband, Nathanael Paul Davis. I love you more than I could ever say. Thank you for all that you have done, and been willing to sacrifice, in order to support me in following my dreams. SHMILY!

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Chapter 1: Literature Review

FSH Structure and Function

Follicle Stimulating Hormone (FSH) is a glycoprotein secreted by the anterior pituitary gland primarily in response to gonadotropin-releasing hormone (GnRH) and plays a role in the growth, development, and function of the ovaries and testes. Follicle Stimulating Hormone is a heterodimer consisting of a non-covalently linked alpha (α) and beta (β) subunit. The α subunit of FSH is identical to that of luteinizing hormone (LH), thyroid stimulating hormone (TSH), and chorionic gonadotropin (CG), whereas the β subunit is responsible for the specific biological actions of FSH and its interaction with the FSH-receptor (Bernard, et al., 2010).

The sequence of the bovine FSHβ subunit gene has been reported by Kim, et al., (1988). The 6,610 bp gene is located on chromosome 15 and consists of a 1,787 bp promoter region located in the 5' region upstream of the transcription start site, followed by three exons and two introns. The ATG translation start site occurs at bp 2579 within exon 2, stops at bp 4536 within exon 3, and encodes a 119 amino acid precursor peptide that is cleaved to produce a 110 amino acid mature peptide.

Factors that influence the biological activity or the pattern of secretion of FSH can alter the development and function of the ovaries and testes and thereby affect fertility (Bernard, et al., 2010). In females FSH is responsible for follicular development by binding to receptors on granulosa cells leading to the conversion of testosterone to estradiol. Estradiol functions in expression of sexual behavior, regulation of GnRH, uterine secretions, and aids in transport of sperm through the female reproductive tract (Senger, et al., 2003). Mutations within the FSHβ gene and its promoter in females have been associated with primary and secondary amenorrhea

(absence of a menstrual period in women) and arrested follicular development (Grigorova, et al., 2010).

In males, FSH promotes spermatogenesis, the process of sperm development, in the seminiferous tubules primarily via actions on Sertoli cells (Sairam, et al., 2001). For this reason, polymorphisms within the FSHβ gene and its promoter are of particular interest to male fertility.

The proliferation of Sertoli cells is activated by FSH during fetal and neonatal development as well as during the pubertal phase (Grigorova, et al., 2010). Sertoli cells are the only somatic cells found within the seminiferous tubules and serve as nurse cells for the developing male germ cells (Amann, et al., 1983). Sertoli cells are large structures with overflowing cytoplasmic envelopes extending to the lumen of the seminiferous tubules, providing the only communication link across the blood-testes barrier, which functions to prevent haploid sperm from causing an immune reaction (Amann, et al., 1983).

Spermatogenesis is the developmental process in which spermatogonia are transformed into spermatozoa. FSH plays a critical role in the hormonal control of spermatogenesis by binding to FSH-receptors on Sertoli cells and stimulating primary spermatocytes to undergo the first meiotic division to form secondary spermatocytes (Senger, et al., 2003). Since each Sertoli cell can only support a limited number of developing male germ cells at a time, it is important that we understand how polymorphisms of the FSHβ subunit gene can affect male fertility (Sairam, et al., 2001)

The synthesis and release of FSH is controlled by a number of factors including the pulsatile release of gonadotropin releasing hormone (GnRH), the gonadal steroids, estrogen (E_2), progesterone (P_4), testosterone (T), as well as activin, inhibin, and follistatin (FS; Burger, et al.,

2004). The rate limiting step in FSH production is the synthesis of the FSH beta subunit which is controlled by the FSH β-subunit gene (Dai, et al., 2009).

Hypothalamic-Pituitary-Gonadotropic Axis

The expression of gonadotropins is a complex system controlled by endocrine and paracrine interactions between the hypothalamus, pituitary, and gonads (Burger, et al., 2004). The hypothalamus is located at the base of the brain and is connected to the pituitary gland via the pituitary stalk. The pituitary gland is divided into two regions based on the sources from which they derive during embryonic development.

The posterior pituitary is derived from an outgrowth of neural tissue from the hypothalamus while the anterior pituitary develops from the upward growth of embryonic pharyngeal epithelium (Guyton, et al., 2006). The anterior pituitary consists of five cell types which secrete six hormones controlling important functions within the body. Approximately 30 - 40% of the cells of the anterior pituitary are somatotropes secreting growth hormone while another 20% are corticotropes which secrete adrenocorticotropin (ACTH). The other cell types are the thyrotropes, which produce TSH, lactotropes, responsible for producing prolactin (PRL), and the gonadotropes which secrete LH and FSH (Guyton, et al., 2006). Therefore this review will focus on factors affecting the expression of FSH.

GNRH Regulation of FSH

Expression of FSH occurs in response to the hypothalamic release of gonadotropin releasing hormone (GnRH) and positive/negative feedback regulation upon GnRH from steroid and peptide hormones (Burger, et al., 2004). The hypothalamus consists of clusters of nerve

cells, known as hypothalamic nuclei. The hypothalamic nuclei found to influence reproduction in females, by releasing GnRH, are referred to as the surge and tonic center. The tonic center of the hypothalamus is responsible for releasing small pulses of GnRH over a sustained period of time favoring the small pulsatile release of FSH and LH, and is regulated by negative feedback mechanisms of the steroid hormones estrogen and progesterone. The surge center of the hypothalamus is responsible for the high amplitude, high frequency GnRH surge resulting in a rapid increase in LH and FSH prior to ovulation (Maeda, et al., 2010).

In females FSH binds to receptors on ovarian granulosa cells, stimulating follicular growth development and the conversion of testosterone to estradiol via the aromatase enzyme (Senger, et al., 2003). Studies involving ovariectomized rats revealed rapid increases in serum FSH concentrations, FSHβ mRNA, and FSHβ primary transcript (PT) due to elevated transcription rates resulting from the loss of E_2 negative feedback regulation upon the hypothalamus. Primary transcripts are new RNA molecules that contain exon and intron sequences, and are therefore closely associated to gene transcription and the formation of mRNA. Although administration of a GnRH agonist or E_2 resulted in decreased production of LHβ PT and mRNA, a milder effect was observed for FSHβ PT and mRNA indicating the importance of inhibin, activin, or FS regulation of FSH expression (Burger, et al., 2004).

GnRH induced regulation of FSH is different in males as opposed to females. In males, lack of development of the hypothalamic surge center results in small frequent pulses of GnRH stimulating the pulsatile release of LH and FSH (Senger, et al., 2003). LH acts upon the Leydig cells of the testes to produce testosterone while FSH binds to receptors on the Sertoli cells of the seminiferous tubules promoting the process of spermatogenesis and the conversion of testosterone to dihydrotestosterone (DHT) and E_2 (Senger, et al., 2003). Increased GnRH

secretion following castration in male rats resulted in increased LHβ and FSHβ mRNA but not FSHβ PT (Burger, et al., 2004). However Huang, et al., (2001) reported increased FSHβ transcription via increased promoter activity in ovine FSHβ-luciferase reporter gene transgenic mice.

Steroid Hormone Regulation of FSH

By binding to receptors on granulosa cells, FSH facilitates the conversion of testosterone to estradiol resulting in the expression of sexual behavior, regulation of GnRH, production of uterine secretions, and aids in transport of sperm through the female reproductive tract (Senger, et al., 2003). Studies in rodents have shown that ovariectomy resulted in increased circulatory LH and FSH concentrations. Studies involving knock out models have revealed that the negative feedback regulation of E_2 upon the gonadotropes occurs through the E_2 receptor. However, it appears that E_2 only partially regulates suppression of FSH as E_2 administration following ovariectomy resulted in decreased, but not total, suppression of FSH (Thackray, et al., 2010). Thackray, et al.,(2010) proposed that regulation of FSH by E_2may occur indirectly through actions upon GnRH or activin since expression of FSHβ mRNA was not altered by E_2 in GnRH antagonized ovariectomized rats or by insertion of a murine FSHβ promoter in the LβT2 cell line (Thackray, et al., 2006). There is also evidence that E_2 increased pituitary induced secretion of FS primary transcript and mRNA in female rodents and decreased the production of the activin βB subunit mRNA in pituitary cells of sheep (Burger, et al., 2007).

One of the functions of E_2 is to stimulate the expression of the progesterone receptor. Progesterone (P_4) has been shown to act upon gonadotropin synthesis through direct regulation of the anterior pituitary and indirect regulation of GnRH production; however, there does appear

to be differences among species. Administration of P_4 and E_2 resulted in increased FSHβ mRNA in rats while P_4 was found to suppress FSHβ mRNA in sheep (Burger, et al., 2004).

Studies involving the overexpression of progesterone receptor in the immortalized LβT2 cell line upon LH revealed a region on the LHβ promoter essential for suppression of LH,as deletion in this region abolished progesterone suppression (Thackray, et al., 2010). However, studies involving the P_4 induced expression of FSHβ revealed increased levels of FSHβ mRNA in rats treated with E_2andP_4(Attardi, et al., 1990) and that P_4antagonist, such as the antiprogestins RU486 and ZK98299, blocked FSH production and expression of FSH mRNA during pre-ovulatory and secondary FSH surges (Ringstrom, et al., 1997). Progesterone was found to induce the FSHβ promoter in the LβT2 cells as a reporter gene containing the FSHβ promoter from rats and sheep was shown to be responsive to progesterone treatment of rat and ovine pituitary cultures; as opposed to the LHβ, direct binding and transactivation of the progesterone reporter was required for the progesterone activation of the murine FSHβ promoter (Thackray, et al., 2006). Based on these observations Thackray, et al. (2010), concluded that progesterone acts in a direct manner to regulate FSHβ expression instead of altering the activin/inhibin/follistatin feedback regulation.

Along with estrogens and progestins, testosterone appears to stimulate expression of FSHβ via the pituitary gonadotropes (Spady, et al., 2004). Spady, et al., (2004) suggested that androgen indirectly stimulates FSHβ production by modification of the pituitaries activin/FS pathway based on a two fold increase in FSHβ mRNA observed in GnRH antagonist treated rodents following administration of T and in pituitary cell cultures from male and female rats. Through the use of the LβT2 cell line, Spady, et al.,(2004) showed that androgen stimulates FSHβ expression in a dose dependent manner and that mutation of either of the two known

androgen response elements proximal to the FSHβ gene promoter eliminates androgen stimulation. Research conducted by Thackray, et al (2006) revealed that hormone response elements exist within the first 500 bp of the FSHβ promoter for not only T, but progestins and glucocorticoids as well. The receptors for androgens, progestins, and glucocorticoids are all similar in that they are members of the class I family of steroid receptors binding directly to the DNA at six hormone response elements containing the TGTTCT half site (Thackray, et al., 2006). Receptor binding of androgens, progestins, and glucocorticoids at the -381 site of the FSHβ promoter has been found to stimulate FSHβ gene expression. Although the -230 and -273 site is necessary for the expression of FSHβ by androgens and progestins, it is not essential for glucocorticoid induced expression of the FSHβ gene (Thackray, et al., 2006).

Activin, Inhibin, and Follistatin Regulation of FSH

Activin and inhibin, which are glycoproteins consisting of an alpha and beta subunit, belong to the transforming growth factor beta (TGFβ) superfamily which also includes bone morphogenetic proteins (BMP), growth differentiation factors (GDF), and anti-müllerian hormone (AMH; Bilezikjian, et al., 2011). Activin is a dimer of two beta subunits represented in four different isoforms ($\beta_A - \beta_D$), but only the β_A and β_B appear to regulate FSH expression. Activin stimulates FSHβ expression on the transcriptional level by increasing the amount of FSHβ mRNA (Burger, et al., 2004). Signaling for members of the TGFβ family occurs through two types of transmembrane receptor serine/threonine kinases: type I receptors (ActRIB/ALK4) and type II receptors (ActRII and ActRIIB; Bilezikjian, et al., 2011). The process of activin binding to its type II receptor results in the formation of a heteromeric complex on the cell surface due to paring with the type I receptor. The type I receptor then phosphorylates a class of

intracellular signaling proteins known as mothers against decapentaplegic (Smad; Burger, et al., 2004).

Eight Smads are responsible for the intracellular signaling from the receptor to the nucleus. Smads2 and 3 appear to be required for activin signaling as they bind to the type I receptor and partner with Smad4, which in turn binds to DNA thereby altering gene activity. However Smad7 is an inhibitory Smad which prevents the phosphorylation of Smad2 and 3 by binding to the type I receptor (Burger, et al., 2004).

It is important to limit the actions of activin upon the body as overexpression of FHS has been related to infertility in gain-of-function transgenic mice (Kumar, et al., 1999). In addition to Smad7, inhibin effectively regulates FSHβ expression by preventing activin from binding to its type II receptors inhibiting the phosphorylation of the type I receptor (Burger, et al., 2004). Inhibin is a heterodimer of an activin subunit and a unique alpha subunit, which is produced by the granulosa cells of the ovary upon selection of a dominant follicle to suppress FSH secretion in females. In males, inhibin is produced by the Sertoli cells of the seminiferous tubules in response to androgen stimulation (Anderson, et al., 2000 and Luisi, et al., 2005). Although the mechanism by which inhibin suppress FSHβ transcription remain unclear, inhibin appears to bind with the type II receptors without stimulating the formation of the complex between the type I and II receptors, suggesting that inhibin functions as an activin antagonist via the activin receptor (Burger, et al., 2004).

Another inhibitor of activin induced expression of FSH is follistatin. Follistatin is a glycoprotein produced by pituitary folliculostellate cells and functions in local control of FSHβ and LHβ gene expression (Blount, et al., 2009). Follistatin functions as a ligand antagonist blocking activin stimulation of FSHβ gene expression and is also produced by granulosa cells of

dominant follicles where it was first isolated (Ueno, et al., 1987). Follistatin prevents the binding of activin to its type I receptor by binding activin directly in a 2:1 ratio neutralizing the activin receptor binding site (Thompson, et al., 2005). Because of its high affinity for activin, the binding of follistatin to activin is considered permanent as the dissociation rate of follistatin from activin is relatively low. Studies involving rat pituitary cultures have shown that follistatin production was increased by activin, GnRH, and adenylatecyclase-activating polypeptide, but decreased by testosterone and follistatin itself. However, studies in primates have shown that follistatin production is increased by testosterone and unaffected by GnRH stimulation (Burger, et al., 2004).

Puberty

FSH levels decline following fetal and neonatal development for both sexes until puberty. Puberty has been defined as a period of time leading to the cascade of morphological, physiological, and behavioral events leading to increased gonadal activity (Krishnamurthy, et al., 2001). Simply puberty is the point at which an animal is capable of reproducing while sexual maturity is the point at which an animal reaches its full reproductive potential. Age at which an animal reaches puberty is influenced by body weight, breed, genetic, nutritional, environmental, social, and hormonal factors (Abeygunawardena and Dematawewa, 2004; Cammack, et al., 2009). As FSH functions in follicular growth and recruitment in females and promoting the process of spermatogenesis in males (Ulloa-Aguirre and Timossi, 1998), it is possible that differences in age at puberty could be dueat least in part to mutations in the FSHβ gene promoter region.

Heritability of female reproductive traits is low; however, studies have demonstrated that age at puberty in heifers is correlated to scrotal circumference in bulls, a highly heritable trait (Brinks, 2010). Smith, et al. (1989) showed that Hereford, Angus, and Red Angus bulls with larger scrotal circumferences have the ability to sire offspring with larger testicles, improved milking ability, and daughters that reached puberty at an earlier age. A similar result was observed by Vargas, et al. (1998) between increased scrotal circumferences in Brahman bulls and earlier ages at puberty in Brahman heifers. Increased testicular size results in greater capacity for sperm production due to increased Sertoli cell numbers. Bull calves undergoing FSH treatment from 4 to 8 weeks of age displayed increased testicular growth, increased Sertoli cell proliferation, and attainment of puberty at an earlier age compared with those treated with LH and control calves (Bagu, et al., 2004). However, great diversity exists for the age of puberty between *Bos taurus* and *Bos indicus* cattle, as well as, within species (Freetly, et al., 2011).

The incorporation of Brahman cattle have proven advantageous to cattle producers due to their increased tolerance to high temperatures and humidity, parasite resistance, and their ability to more effectively utilize poor quality forages. However a drawback associated with Brahman cattle is a lower reproductive rate than*Bos taurus* cattle (Randel, 2000). It has been estimated that *Bos taurus* heifers reach puberty at earlier ages than *Bos indicus* heifers (Randel, 2011). Freetly, et al. (2011) conducted a study to determine the relationship of different growth curves in heifers to mature size and puberty. Crossbred cows sired by three breeds of heat tolerant bulls (Brahman, Boran, and Tuli) as well as Angus, Hereford, and Belgian Blue bulls were used in this study. Hereford and Angus sired cows were found to have the heaviest mature body weights. Angus sired cows matured faster than cows sired by Hereford, Brahman, Boran, orTuli bulls, but did not differ from Belgian Blue sired cows. Brahman sired cows took the longest to mature (58

weeks versus 49 weeks for Angus and Hereford sired cows, respectfully) and reached a greater percentage of their mature body weight at puberty than Hereford, Tuli, and Belgian Blue sired cows. Within species, the proportion of mature body weight at puberty ranged from 56 – 58% for cow sired from *Bos taurus* bulls and 60% for cows sired by *Bos indicus* bulls (Freetly, et al., 2011).

Although reducing the amount of time it takes for an animal to reach puberty has tremendous potential to increase profitability of producers, little research had been done to determine if there is a link between age of puberty and mutations of the FSHβ gene. Several case studies in men and women have reported mutations of the FSHβ gene affecting development of secondary sex characteristics and infertility. In a study by Krishnamurthy, et al. (2001), on testicular function and spermatogenesis of FSHβ receptor knockout (FORKO) mice, their data showed that compared to wild-type heterozygous males, 7 day old FORKO male mice displayed severely reduced testicular weight and seminiferous tubule shrinkage which continued into adulthood for male FORKO. By day 21 of the study, wild-type heterozygous males displayed plentiful round spermatids in the seminiferous tubules as opposed to FORKO mice which displayed delayed puberty. At day 35, no elongated spermatids were observed in the tubules of FORKO mice. By day 49 continuing into adulthood, only abnormal sperm was detected. Delayed testicular development and impaired spermatogenesis were attributed to loss of signal between FSH and its receptor (Krishnamurthy, et al., 2001).

Previous Research

Females. Polymorphisms of the FSHβ subunit gene were found to positively affect the litter size and the total number of pigs born alive to multiparous Beijing Black Pig sows

(Luoreng, et al., 2007) with similar results being observed by Humpolicek, et al., (2006) in the Czech Large White sows. Studies with female Japanese Flounder revealed a polymorphism in exon 3 associated with increased serum testosterone concentrations and an increased gonadosomaticindex, defined as the gonad mass represented as a portion of the total body mass (Feng, et al., 2010).

Matthews, et al. (1993) reported the first mutation of the FSHβ gene found to occur in a female. The patient, a 27 year old Italian woman, displayed primary amenorrhea, infertility, and lacked breast development. DNA sequencing revealed the patient to have inherited a 2 bp deletion (TG) in codon 61 resulting in a frame shift in transcription, altering the amino acids 61 – 86 before leading to a premature stop codon (Val61X). The last 24 amino acids of the protein were found to be missing, leading to impaired dimerization of the FSH α and β subunits required to form bioactive FSH (Matthews, et al., 1993). A similar observation was made by Layman, et al., (1997) who reported a compound heterozygous mutation of the FSHβ gene, in the coding region of exon 3, in a 15 year old girl that resulted in delayed puberty. The patient displayed poor breast development, amenorrhea, undetectable serum FSH concentrations, and increased LH concentrations. DNA sequencing revealed the patient to have inherited the same 2 bp deletion described by Matthews (et al., 1993) from her mother and a missense mutation in codon 51 from her father that changed a cysteine to a glycine (Cys51Gly). Both mutations impaired dimerization of the FSH α and β subunits resulting in failure to activate the FSH receptor (Layman, et al., 1997).

The first FSHβ mutation found to affect siblings was reported by Layman, et al., (2002)in Brazil. The female sibling (32 years old) exhibited poor breast development, primary amenorrhea, and infertility, while her brother (30 years old) exhibited reduced testicular size and

azoospermia. Both patients were found to be homozygous for a C to A nonsense substitution in codon 76, resulting in the change of a tyrosine to a premature stop codon (Tyr76X). Although the two patients in this case displayed at least partial pubertal development compared to those previously reported, patients suffering the Val61X, Cys51Gly, and Tyr76X mutations all exhibited undetectable FSH concentrations (Layman, et al., 2002).

Berger et al., (2005) also reported a case study involving a loss-of-function mutation of the FSHβ subunit gene in a 16 year old Brazilian female. The patient displayed delayed puberty, primary amenorrhea, and partial breast development. DNA sequencing of the patient revealed the same C to A substitution in codon 76 of exon 3 reported by Layman, et al., (2002). The patient's mother was found to heterozygous for the same mutation.

Males. A case study of an eighteen year old male seeking medical attention for delayed puberty was investigated by Phillip, et al., (1998). Although the patient reported having normal erections and ejaculations, he exhibited small soft testicles, abnormal development of secondary sex characteristics, and white ejaculates with no sperm. Radioimmunoassay revealed low serum FSH, total and free testosterone concentrations, and increased serum luteinizing hormone (LH) concentrations. Two asymptomatic male family members, the patient's younger brother and father, were also examined. These individuals exhibited normal FSH concentrations, normal development of secondary sex characteristics, and were considered to be completely fertile. DNA sequencing of the coding region of the FSHβ subunit gene revealed a homozygous deletion of two bp (TG) in codon 61 causing a frame shift in transcription resulting in a premature stop codon (Val61X; Phillip, et al., 1998).

Although the homozygous deletion identified in this study may explain the decreased serum FSH concentration, the basis for the relationship between polymorphisms and decreased testosterone and increased LH concentrations is not clear. Secretion of GnRH by the hypothalamus results in the pulsatile release of LH and FSH from the anterior pituitary (Maeda, et al., 2010). While FSH acts upon the Sertoli cells, LH binds to receptors upon the Leydig cells of the testes stimulating the production of testosterone (Nalbant, et al., 1998). Therefore the low testosterone concentration observed in this study is curious. The authors suggested the observed low testosterone concentrations may have been due to the absence of an unidentified substance, normally produced when FSH binds to Sertoli cells, which stimulates Leydig cells to produce testosterone (Phillip, et al., 1998).

A study was conducted comparing the frequency of occurrence of polymorphism of the FSHβ gene and its promoter on two Caucasian populations, Danes and Finns. Two hundred DNA samples were collected from healthy randomly selected individuals from the two populations (Finland n = 101, Denmark n = 99). Polymerase chain reaction (PCR) was used to amplify DNA of 50 individuals from each population for the coding region of the gene while only 15 Finnish and 10 Danish samples were used to investigate the frequency of occurrence of polymorphisms in a 430 bp segment upstream of exon 1. A known polymorphism occurring in exon 3, Y76 TAT→TAC which may be associated to polycystic ovarian syndrome in obese women, was isolated using PCR amplification and digestion via a restriction enzyme (Lamminen, et al., 2005).

When compared to the published FSHβ sequence, two previously unreported and one previously reported polymorphism were detected. All SNP identified within the coding region of exon 3 were found to be silent, resulting in no change in the amino acid structure of the protein.

The frequency of occurrence of the novel SNP were different between the two populations with SNP K58 AAG→AAA only occurring in 16 percent of the Danish population and SNP K104 AAG→AAA in 8 percent of the Finnish population. Although samples used in this study were limited and represent only healthy individuals, data suggests population differences in the frequency of occurrence of polymorphisms even though the authors determined the gene to be highly conserved (Lamminen, et al., 2005).

The first SNP of the FSHβ gene promoter region shown to affect serum FSH concentrations in men was discovered by Grigorova, et al. (2008). This study was conducted over a two year period on 554 voluntary normal healthy men (ages 19.2 ± 1.7 years) from the Centre of Andrology, Tartu University Hospital, Estonia. After the subjects fasted overnight, a single blood sample was collected from 8:00 – 10:00 am for analysis of serum FSH, LH, and sex hormone binding globulin (SHBG), testosterone, and estradiol (E_2) concentrations. Semen samples were collected in a collection tube via masturbation and semen volume, sperm motility, concentration, and sperm count determined. Patients were subject to a physical examination in order to determine the total testes volume.

Variations in DNA sequences were investigated by PCR and restriction enzyme digestion. A G211T SNP was identified upstream of the mRNA transcription start site. Four hundred and twenty three individuals were found to be homozygous for the major allele (GG, 76.4%), 125 heterozygotes (GT, 22.6%), and 6 were homozygous for the minor allele (TT, 1.1%; Grigorova, et al., 2008).Linear regression analysis revealed SNP G211T to be related to serum FSH concentrations and the free testosterone index (FTI). The GG genotype had higher concentrations of FSH and FTI over heterozygotes which were higher than TT homozygous individuals. The difference between the lower FTI in the heterozygous males compared to the

GG homozygous males was attributed to the lower overall testosterone concentrations while the difference between the GG and TT homozygous males was due to increased concentrations of SHBG. Data also suggested that SNP G211T may be related to the decreased combined testes volume, increased E_2 concentrations, and increased semen volume of TT homozygous males (Grigorova, et al., 2008).

A subsequent study was conducted by Grigorovia, et al., (2010) to discover if genetically reduced FSH concentrations affected male reproduction and to replicate the association between the SNP G211T and serum FSH concentration among infertile males. The authors hypothesized that since FSH is essential for normal spermatogenesis, abnormalities in the actions of FSH during post fetal development could result in decreased FSH concentrations affecting male reproduction. The study was performed on a group of 1029 infertile men with low sperm counts from couples who had tried and failed to conceive a child for at least one year. Of this group of men, 750 were classified with idiopathic infertility, while 279 were classified with causal factors affecting male fertility such as obstructions, cryptorchidism, chromosomal abnormalities, hypogonadotrophichypogonadism, etc. The control group used in this experiment was the 554 normal healthy men described from the previous study (Grigorova, et al., 2010).

A blood sample was collected between 8:00 to 10:00 am following an overnight fast or light morning meal and DNA extracted. Serum concentrations of FSH, LH, testosterone, E_2, and inhibin B were determined. Semen samples were collected via masturbation in a sterile collection tube. Semen volume, sperm motility, and concentration were calculated, stained smears were prepared to determine morphology, and a physical examination performed to determine the combined testes volume. Genotyping of alternative alleles was performed using PCR and restriction enzyme technique reported byGrigorova, et al., (2010).

Genotyping of the 1029 infertile men in this study revealed 746 GG homozygotes, (72.5%), 258 heterozygotes (25.1%), and 25 TT homozygous males (2.4%). When compared to the control group, differences were observed for both the allele and genotype frequencies between the infertile and healthy men. Compared to normal health males, infertile males were found to have increased frequencies of T-alleles and increased frequencies of genotype GT and TT over healthy males. TT homozygous males exhibited approximately a 50% decrease in serum FSH concentrations which were correlated to decreasing FSH:LH ratios. Smaller combined testes weights and decreased sperm motility were also associated with TT homozygous males. Researchers suggest that infertility observed in T allele carriers, may be due to reduced FSH concentrations during fetal and neonatal development or during the pubertal phase (Grigorova, et al., 2010).

Dai, et al. (2009) conducted a study in bulls evaluating the effects of SNPs of the FSHβ gene on semen quality and fertility of 56 normal bulls in both fresh and frozen semen. Thirty five Simmental, 13 Charolais, and 8 Limousin bulls (avg age was 37.5 ± 1.3 months) from the Institute of High-quality Cattle, Changchun, Julin, Northeast China were used in the study. Semen was collected via artificial vagina every 3 to 4 days for 3 collections during May. Following collection, ejaculates were evaluated for fresh sperm quality variables then prepared for cryopreservation. Following cryopreservation for 4 to 5 days, two straws were randomly selected from each ejaculate, thawed, and examined for frozen semen quality variables. Non-returned rates were determined for the analyzed batches from 14,416 inseminations of cows with a post-partum anestrus period of at least 60 days.

DNA was isolated from blood samples containing acid citrate dextrose. Six sets of primers were used to amplify bases in the 5' upstream regulatory region, exon 1, exon 2, and

exon 3. Single strand conformation polymorphism (SSCP) was used for identify polymorphisms based of differing migration patterns observed in the SSCP gels. Following purification, PCR products were cloned into Escherichia coli component cells and transformed with the recombinant plasmid. Plasmid DNA was then extracted from cultures, incubated overnight, and positive inserts confirmed by PCR and digested using restriction enzymes. DNA sequencing was performed for at least 4 positive clones of selected amplifications or from two PCR amplifications from the same bulls. FSH concentrations were determined from blood samples free of anticoagulant via radioimmunoassay (Dai, et al., 2009).

Six SNP (G782A, T170C, C224G, C352A, G409T, and G500T) and one insertion (409_500insTAAC) were identified in the 5' upstream regulatory region of the gene. Web-based software TFSEARCH (1.3) allowed Dai, et al, (2009) to confirm that the polymorphisms observed in the 5' upstream regulatory region of the gene altered potential transcription factor binding sites. Radioimmunoassay revealed that bulls with mutations in the promoter region of the FSHβ gene tended to have decreased serum FSH concentration over the control group.

Seven SNP were also observed in the coding region of exon 3 (T4338C, C4341T, G4350A, C4452T, A4453C, C4461T, A4489C). The polymorphism A4453C resulted in a change in the amino acid structure of the protein, Ser103Arg, while the other SNP were silent. Bioinformatics analysis indicated linkage among SNP and that bulls with this genotype exhibited decreased sperm concentrations, increased percentage of abnormal sperm, and decreased percent acrosome integrity in fresh semen. In frozen semen, SNP resulted in an increased number of abnormal sperm, decreased percent acrosome integrity, and decreased sperm motility. Lower non-return rates (NRR) were observed for bulls with this genotype as well. The authors

suggested that mutations within the regulatory region of the gene altered transcription factor binding sites possibly resulting in alterations in serum FSH concentrations (Dai, et al., 2009).

Summary

Data on polymorphisms suggest that polymorphisms of the FSHβ gene and its promoter are related to: decreased serum FSH concentrations, decreased sperm motility, decreased acrosome integrity, increased number of abnormal sperm, and delayed puberty. Transcriptional regulation is an important component in control of gene expression. Initiation of transcription is the first rate limiting step in gene expression and is regulated by interactions of transcription factors with transcription factor binding sites in the promoter region (Dai, et al., 2009). Since transcription controls the synthesis of FSH, mutations of the regulatory region of a gene could result in either altered transcription factor binding sites or transcription initiation sites resulting in altered gene expression (Dai, et al., 2009). Effects of FSH on Sertoli cell proliferation and development of spermatids can potentially alter male fertility and previous research suggests that polymorphism of the FSHβ subunit gene have been associated with decreased FSH, delayed puberty, and male fertility. Therefore, the objectives of this study was to characterize polymorphisms (SNP) in the promoter region of the bovine FSHβ gene, examine breed differences in SNP, and determine the relationship of SNPin the FSHβ gene promoter region with measures of semen quality in bulls and early and late puberty in Brahman heifers.

Chapter 2: Relationship of Polymorphisms in the FSH Beta Subunit Gene with Reproduction in Bos Taurus and Bos Indicus Cattle

Abstract

Two experiments were conducted to characterize polymorphisms (SNP) in the bovine FSHβ gene promoter region to examine breed differences in SNP, and to determine effects of SNP on reproduction in beef cattle. Experiment 1 - DNA samples were collected from 5 Angus (ANG), 13 Balancer (BAL), & 16 Brahman influenced (BI) bulls. Polymorphisms were identified by sequencing of 3 sequential PCR products from the promoter region revealed 17 SNP & 4 insertion/deletions (INDEL). Semen samples were collected and sperm quality variables determined viacomputer assisted sperm analysis (CASA) or stained smears. The MIXED procedure for ANG & BAL indicated interactions of week and SNP 485for LIN, AREA, & MINAB ($P \leq 0.05$), effects of SNP 169 & 170 on MINAB & TOTAB ($P \leq 0.07$), SNP 485 on MOT, PROG, RAP, VAP, VSL, VCL, ALH, BCF, AREA, & LIVE ($P \leq 0.05$),and SNP 1130 on VCL, ALH, LIN ($P \leq 0.05$), & STR ($P = 0.06$). For BI bulls effects of SNP 171, 225, 353, 410, 411, 412, & INDEL 413-414 on MINAB & TOTAB ($P \leq 0.09$), SNP 783 on MINAB & TOTAB ($P \leq 0.03$), BCF & MAJAB ($P \leq 0.09$), SNP 1130 on VCL ($P = 0.05$), VAP, & VSL ($P \leq 0.09$), and SNP 1702 on MINAB & TOTAB ($P \leq 0.04$). Experiment 2 - DNA samples were collected from early & late pubertal Brahman heifers and SNP were identified by sequencing 2 upstream segments of FSHβ promoter region. Chi square revealed effects upon puberty for SNP 783 ($P = 0.02$) and 171 ($P = 0.08$), breed differences in frequency of occurrence for 10 SNP (171, 225, 321, 353, 410, 411, 412, 783, 887, 1702), and 2 INDEL (411-412 INDEL & 413-414 INDEL). Results indicate breed differences in frequency of occurrence of SNP in FSHβ promoter region,

and that SNP may be useful as markers related to semen quality in bulls and puberty in Brahman heifers.

Introduction

Follicle stimulating hormone (FSH) is an important reproductive hormone functioning in follicular maturation in females, spermatogenesis in males, and steroidgenesis (Bernard, et al., 2010).Follicle stimulating hormone belongs to thesame glycoprotein family asluteinizing hormone (LH), thyroid stimulating hormone (TSH), and chorionic gonadotropin (CG). Like other glycoproteins from this gene family, FSH is a heterodimer containing an alpha (FSHα) and beta (FSHβ) subunit. While the alpha subunit is the same for FSH, LH, TSH, and CG, the beta subunit is unique and is responsible for the protein's specific biological actions (Burger, et al., 2004).

Sequencing of the bovine FSHβ subunit gene was successfully completed by Kim, et al., (1988). The gene was found be approximately 6,610 kb consisting of 3 exons separated by two introns, with a 1787 bp promoter region located upstream from the transcription initiation site (Kim, et al., 1988). Regulation of FSHβ synthesis and secretion occurs through control of the hypothalamic-pituitary-gonadal axis. Although the most prominent regulator of FSH is the pulsatile release of gonadotropin releasing hormone (GnRH), additional controls include steroid hormones, activin, inhibin, and follistatin (Burger, et al., 2004).

Transcriptionof the FSHβ subunit is the rate limiting step in production of the mature hormone, as it is the amount of FSHβ subunit created that determines the quantity of mature protein produced (Bernard, et al., 2010). Mutations occurring in the FSHβ subunit genes 5'

regulatory region have the potential to alter transcription factor bind sites or transcription initiation sites leading to altered gene expression (Dai, et al., 2009).

Follicle stimulating hormone is integral in the process of spermatogenesis by binding to receptors on Sertoli cell membranes stimulating the meiotic division of primary spermatocytes to secondary spermatocytes (Senger, et al., 2003). Fetal and neonatal expression of FSH is also essential for normal testicular development and expression of secondary sex characteristics (Sharpe, et al., 2003).An increase in Sertoli cell proliferation occurs during fetal/neonatal development and the pre-pubertal phase primarily due to FSH stimulation (Sharpe, et al., 2003). As testicular size and daily sperm production is dependent upon the number of mature Sertoli cells, it is reasonable to determine that factors affecting fetal and neonatal Sertoli cell development will affect adult testicular function (Sharpe, et al., 2003). Exogenous FSH administration every two days to bull calves at four to eight weeks of age accelerated onset of puberty and resulted in greater Sertoli cell proliferation, increased number of elongated spermatids and spermatocytes per seminiferous tubule by the time the calves were 56 weeks old (Bagu, et al., 2004).

Several case studies have reported mutations of the FSHβ subunit gene resulted in delayed puberty, with primary amenorrhea and arrested follicular development in women, and azoospermia in men (Huhtaniemi, 2000). The first study indicating polymorphisms in the FSHβ subunit gene to affect serum FSH concentrations in men was conducted by Grigorova, et al., (2008) on a cohort of normal healthy men. Results indicated that compared to men homozygous for the primary allele, heterozygotes and men homozygous for the minor allele had 15.7% and 40% decreased serum FSH concentrations, respectfully. The association of the minor allele to reduced serum FSH concentrations was further confirmed by increased frequency of occurrence

among infertile men (Grigorova, et al., 2010). Dai, et al., (2009) showed that polymorphisms occurring in the regulatory and coding region of the FSHβ gene in bulls resulted in decreased serum FSH concentration along with decreased sperm concentrations, increased percent abnormal sperm, and decreased percent acrosome integrity in fresh semen which became even more pronounced in frozen semen.

The objective of this study was to characterize polymorphisms (SNP) in the promoter region of the bovine FSHβ gene, examine breed differences in SNP, and to determine the relationship of polymorphisms of the FSHβ gene with measures of semen quality in bulls, as well as, and early and late maturity in Brahman heifers.

Materials and Methods

All animal procedures used in this study were approved by the University of Arkansas Animal Care and Use Committee (Protocol # 11001) or the Committee of Animal Welfare, USDA-ARS Dale Bumpers Small Farms Research Center in Booneville, AR.

Experiment 1

Animal management and semen collection.

Angus and Balancer bulls. Five Angus and 13 Balancer bulls, located at the University of Arkansas Beef Research Unit located near Savoy, AR were used. Bulls averaged 5.94 ± 1.47 years of age. Animals were managed in dry lots in two separate groups to reduce stress associated with dominance and were fed a 0.45 kg high concentrate ration three times per week with ad libitum access to grass hay. Semen samples were collected via electro-ejaculation prior to the start of the trial to ensure that sperm variables exceeded minimal standards required to pass

a breeding soundness evaluation (BSE). Thereafter, semen samples were collected weekly for a period of nine weeks from July through September 2010 via electro-ejaculation.

Brahman-influenced bulls. Brahman-influenced bulls (n = 16), at a mean age of 15.13 ± 0.34 months at the start of the trial, were located at the USDA-ARS Dale Bumpers Small Farms Research Center in Booneville, AR. Prior to the start of semen collections, bulls were kept on common Bermudagrass pastures overseeded with Elbon rye. Semen samples were collected monthly from June through August 2007 via electro-ejaculation.

Semen analysis. Within five minutes post collection, ejaculates were analyzed by computer assisted sperm analysis (CASA; Hamilton Thorne Biosciences; Beverly, MA) to evaluate percent motile, progressive, and rapid sperm. Each sample was diluted with Dulbecco's PBS to achieve a concentration of ~ 25 $x10^6$ sperm/ml then loaded onto a 2X-CEL (Hamilton Thorne Biosciences, Beverly, MA) slide. Eight to ten areas along the length of the slide were scanned while capturing thirty video frames per viewing area to create a composite of the sperm motility variables. A minimum of 400 sperm cells were counted per slide to achieve an accurate representation of each semen sample. Percent live/dead sperm and sperm morphology was determined by applying nigrosin-eosin live dead stain to a microscope slide containing a diluted sperm sample. Sperm morphology measurements included the percent major (MAJAB), minor (MINAB), & total abnormalities (TOTAB). Sperm quality variables (Appendix A) measured via CASA were as follows: the percent motile (MOT), progressive (PROG), rapid (RAP), & live (LIVE) sperm; path velocity (VAP), progressive velocity (VSL), & track speed (VCL); lateral

amplitude (ALH); beat frequency (BCF); percent straightness (STR) & linearity (LIN); minor:major axes of all sperm heads (ELONG); and average size of sperm heads (AREA).

Blood collection and DNA extraction. Blood samples were collected from the median caudal vein into 8 ml EDTA vacuum tubes, placed on ice, and transported to the laboratory. Following centrifugation at 1,200 x g for 20 minutes at room temperature, buffy coats were removed and stored at -20°C until genomic DNA was extracted. A Puregene DNA purification kit (cat. no. 158445, Quiagen, Valencia, CA) was used for extracting DNA from whole blood. The manufacturer's protocol was used as described for DNA extraction except that 100 µl of buffy coat was used rather than 300 µl of whole blood (Appendix B).

Primer design and Polymerase Chain Reaction. Three successive regions of the promoter of the FSHβ subunit gene were amplified via PCR in order to determine the DNA sequence of the majority of the promoter region of the gene. Primers were designed by Primer3 version 0.4.0 (Rozen&Skaletsky, 2000; Whitehead Institute for Biomedical Research, Cambridge, MA) and purchased from Invitrogen (Invitrogen, Eugene, OR; Table 1).

Amplification was achieved at a final reaction volume of 50 µl. Reagents were thawed and stored on ice prior to and during use. Samples were prepared by adding 34.4 µl PCR grade H_2O, 5 µl of 10xNH_4 PCR reaction buffer, 2 µl 50 mM $MgCl_2$, 2.6 µl 4 mMdNTP mix, 1 µl Biolase Red DNA Polymerase (Bioline USA Inc., Taunton, MA), and 2 µl 25 µM forward and reverse primers (Invitrogen, Eugene, OR) to a 1.5 µl microcentrifuge tube and mixed by inversion. Polymerase chain reaction tubes were labeled and 49 µl of PCR mix was added to

each tube followed by 1 µl DNA template(~ 20 ng/µl), mixed by inversion, and briefly spun to remove droplets from the lid.

Polymerase chain reaction was performed using a Veriti 96 well thermal cycler (Applied Biosystems, Life Technologies, Foster City, CA) and programmed as follows: stage 1 - DNA denaturing process performed in one cycle at 95˚C for 5 minutes; stage 2 - performed in 30 alternating cycles of denaturing (95˚C – 1 ½ minutes), annealing (56˚C – 1 minute), and extension (72˚C - 1 ½ minutes); with stage 3 - final extension occurring at 72˚C for 7 minutes; and held indefinitely at 4˚C upon completion. Following PCR, the appearance of a single band of appropriate size was confirmed by electrophoresis of PCR products on a 6 1/2 cm x 10 cm 1% agarose (cat. no. BIO-41026, Bioline USA Inc., Taunton, MA) gel in 1x TBE buffer at 85 volts for 35 minutes. Hyperladder I (BIO-33053, Bioline USA Inc., Taunton, MA) was included in each gel as a reference standard, and bands were visualized on a UVP Laboratory Products EpiChemi II Darkroom imaging system. Two PCR reactions for each amplicon were done simultaneously and combined to yield adequate DNA for subsequent purification and sequencing.

PCR product purification, quantification, and sequencing. Following PCR, duplicate products were combined and then purified using a Qiagen: QIAquick PCR Purification Kit (cat. no. 28104, Qiagen, Valencia, CA; Appendix C) according to manufacturer's instructions and quantified on a Qubit® 2.0 Fluorometer (Invitrogen, Eugene, OR). Purified PCR products were prepared for sequencing as follows. Three point four µl of 1µM forward primer was pipetted into labeled PCR tubes followed by 8.6 µl of PRC grade H_2O and 1 µl of purified PCR product (~ 20 ng/µl). If product concentrations were below 15 ng/µl, the amount of purified product was

increased and the amount of PCR grade H_2O was decreased to give a final volume of 13 µl. Process was repeated for the reverse primer and samples submitted to the University of Arkansas DNA Resource Center for sequencing using a ABI 3100 DNA Sequencer (Applied Biosystems; Foster City, CA).

Identification of polymorphisms. Polymorphisms were identified by comparing amplicons to the published sequence of the bovine FSHβ subunit gene (GenBank: M83753.1, GI:163063, NCBI) using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and electropherograms were individually examined via Finch TV (Geospiza, Inc., Seattle, WA).

Statistical analysis. Chi square was used to examine breed differences in the frequency of occurrence of SNP identified in the 5 Angus, 13 Balancer, and 16 Brahman influenced bulls by Chi square using JMP® Statistical Discovery Software (SAS Inst. Inc., Cary, NC). The PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) was used to determine: effects of week and SNP upon semen quality for the Angus & Balancer bulls, and the effects of month and SNP upon semen quality for the Brahman influenced bulls.

Experiment 2

Animal management and classification. DNA samples from registered Brahman heifers (n = 35) from the Texas AgriLife Research and Extension Center, Texas A&M University System, Overton, TX were used in this study. Heifers were kept on pastures of coastal Bermudagrassoverseeded with ryegrass. Costal hay, grain, and soybean meal supplements were provided as needed. At 12 months of age, heifers were placed with a bull of

proven fertility and remained with a fertile bull continually until they conceived. Heifers were classified as early or late puberty based on the age at first calving. Age at conception was determined by subtracting 292 days from their age at first calving. Heifers calving at 24 to 26 months of age were classified as early pubertal (n = 18) while those calving at 36 months of age were classified as late pubertal (n = 17).

Blood collection and DNA extraction. Blood was collected by venipuncture into vacuum tubes containing EDTA, frozen, and shipped to the University of Arkansas, Department of Animal Science in Fayetteville, AR. Genomic DNA was extracted according to manufacturer's instructions using the QiagenQIAamp DNA Mini Kit (cat. no. 51304, Qiagen, Valencia, CA; Appendix D). Due to difficulty in the PCR procedure with these samples, DNA samples were further purified to ensure PCR amplification as described. Isopropanol (300 µl) and 3M Na Acetate (20 µl) were added to a 1.5 ml microcentrifuge tube containing 200 µlDNA solution with approximately 2.95 to 18.1 ng/µl DNA and mixed by inversion. Samples were centrifuged at 16,000 x g for 15 minutes. The isopropanol was carefully decanted and the DNA pellet rinsed by adding 200 µl 80% ethanol and mixing by inversion. Samples were again centrifuged at 16,000 x g for 15 minutes, the ethanol decanted, and allowed to air dry for 15 minutes. DNA pellet was resuspended in 50 µl PCR grade H_2O and stored at -20°C.

Primer design and Polymerase Chain Reaction. Two regions of DNA covering a 1215 bp section of the upstream FSHβ gene promoter region were amplified via PCR in order to determine the DNA sequence. Primers used (primer set 1 and 2, Table 1) were identical to those used in Experiment 1. Due to the low DNA concentrations obtained, PCR amplification was

performed in duplicate for each sample and the appearance of a single band of the correct size confirmed by electrophoresis as described in Experiment 1.

PCR product purification, quantification, sequencing, and identification of polymorphisms. Polymerase chain reaction products were purified using a Qiagen: QIAquick PCR Purification Kit (cat. no. 28104, Qiagen, Valencia, CA; Appendix C) according to manufacturer's instructions and quantified on a Qubit® 2.0 Fluorometer (Invitrogen, Eugene, OR). Purified PCR products were prepared for sequencing as described in experiment 1 and submitted to the University of Arkansas DNA Resource Center for sequencing. Polymorphisms were identified by comparing amplicons to the published sequence of the bovine FSHβ subunit gene (GenBank: M83753.1 GI:163063, NCBI) using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and electropherograms were individually examined via Finch TV (Geospiza, Inc., Seattle, WA).

Statistical analysis. Breed differences in the frequency of occurrence of SNP were identified in the 5 Angus, 13 Balancer, 16 Brahman influenced bulls, and 35 Brahman heifers and effects of SNP upon maturity in early and late pubertal Brahman heifers using Chi Square in the PROC FREQ procedure of SAS (SAS Inst. Inc., Cary, NC).

Results

This study was conducted to identify polymorphisms (SNP) in the promoter region of the bovine FSHβ gene and to determine the effects of polymorphisms of the FSHβ subunit gene

upon measures of semen quality in bulls and sexual maturity in early and late pubertal Brahman heifers.

Experiment 1

Genomic DNA samples were collected from 5 Angus (ANG), 13 Balancer (BAL), & 16 Brahman influenced (BI) bulls. Polymorphisms were identified by sequencing 3 sequential PCR products from the FSHβ promoter region. Seventeen SNP (A169G, G170T, T171C, C225G, C353A, G410T, G411T, T412A, G485A, A643G, G783A, A887G, C1130G, C1369T, C1376T, A1494T, C1702T) and 4 insertion/deletions (INDEL: CC413-414CAC, TC1063-1064TCC, TG1256-1257TCG, GA1703-1704GCA) were identified by comparing amplicons to the bovine FSHβ published sequence (GenBank: M83753.1 GI:163063; NCBI).

Effects of SNP upon semen quality. Semen samples were collected monthly from BI bulls and weekly from the ANG and BAL bulls. Within five minutes of collections, semen samples were analyzed via computer assisted sperm analysis and stained smears were prepared to determine semen quality variables. The MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) was used to determine the following: effects of week and SNP upon semen quality for the ANG & BAL bulls, and the effects of month and SNP upon semen quality for the BI bulls.

Angus and Balancer bulls. Four SNP appeared to affect semen quality for ANG and BAL bulls. Results indicate that SNP G485A affected the most semen quality variables (Table2). Animals homozygous for the major allele (GG) had a decreased percentage of motile (MOT), progressive (PROG), rapid (RAP), and live (LIVE) sperm compared to heterozygous

bulls ($P \leq 0.01$). Homozygous GG bulls also had a decreased path velocity (VAP), progressive velocity (VSL), track speed (VCL), and lateral amplitude (ALH), and an increased average size of sperm heads (AREA) and beat frequency (BCF) compared to heterozygous bulls ($P \leq 0.05$).

Bulls homozygous for the major allele occurring at SNP C1130G (Table3) had decreased track speed (VCL) and lateral amplitude (ALH), and an increased percent linearity (LIN) compared to CG heterozygous bulls ($P \leq 0.05$). Sperm from CC homozygous bulls also had a strong tendency to move in a straighter pattern compared to heterozygous bulls (STR, $P = 0.06$).

Fewer total abnormalities (TOTAB) tended to occur in bulls homozygous for the major allele at SNP A169G (Table 4) and SNP G170T (Table 5) than for bulls homozygous for the minor allele ($P = 0.06$). These bulls also tended to have fewer minor abnormalities (MINAB; $P = 0.07$).

Interactions between week and genotype for SNP G485A were observed for the following semen quality measurements: LIN, AREA, and MINAB ($P \leq 0.05$). Genotype differences were observed for SNP G485A on percent linearity for weeks 1 and 5 of semen collections (Figure 2). Linearity is a measure of sperm motility depicting the ration of the progressive velocity (VSL) to the track speed, commonly referred to as the total distance traveled by the sperm (VCL). Bulls homozygous for the primary allele (GG) had an increased percent linearity (LIN) compared to heterozygous bulls (55.60 versus 47.08%, respectfully) in week 1 ($P = 0.02$) which became drastically reduced by week 5 ($P < 0.01$).

Differences were observed between genotypes on weeks 1 and 4 for sperm head area (AREA, Figure 3). Sperm head area gradually increased for heterozygous bulls from week 1 through 7 of the study before beginning to decrease on weeks 8 through 9. Measurements of sperm head area were more variable for GG bulls however. On week 1 GG bulls had an

increased sperm head area (4.96 μm^2, P< 0.01) which became drastically reduced (4.52 μm^2) in week 2 before beginning an upward trend in weeks 3 and 4. By week 4 sperm head area peaked (4.96 μm^2, P = 0.03) for GG bulls before declining in weeks 5 and 6 and increasing again in weeks 7 through 9.

Interactions between week 4 and genotype were also observed for the percent minor abnormalities (MINAB; Figure 4). GG bulls had decreased percent minor abnormalities on week 1 (1.02%) which increased to 2.9% by week 4 (P = 0.01) before dropping to 0.67% in week 8. Although heterozygous bulls also exhibited an increased percentage of abnormal sperm from week 1 to week 4 (1.21 to 1.59%), the change was not as drastic as that observed in the GG bulls.

Brahman influenced bulls. Bulls homozygous for the major allele at SNP G783A (Table6) had a decreased percentage of minor (MINAB, P = 0.03) and total abnormalities (TOTAB, P = 0.03) compared to heterozygous animals. GG homozygous bulls also tended to have fewer sperm with major abnormalities (MAJAB, P = 0.09) and an increased beat frequency (BCF, P = 0.07) over heterozygous animals.

Three genotypes were observed at SNP C1702T (Table7). Heterozygous CT bulls had an increased number of minor (MINAB, P = 0.04) and total abnormalities (TOTAB, P = 0.03) compared to bulls homozygous for the major allele, but only tended to differ from bulls homozygous for the minor allele (TT). TT bulls tended to have a decreased percent total abnormalities (TOTAB, P = 0.06) compared to heterozygous bulls. CC homozygous bulls tended to have an increased beat frequency (BCF, P = 0.07) and fewer major abnormalities (P = 0.09) compared to heterozygous animals.

The SNP C1130G had a strong tendency to affect track speed (VCL; Table8) as heterozygous bulls exhibited increased track speed compared to CC homozygous bulls (VCL; P = 0.06); as well as, increased path velocity (VAP; P = 0.07) and progressive velocity (VSL; P ≤ 0.09).

Bulls homozygous for the major allele at T171C (Table 9), C225G (Table 10), C353A (Table 11), G410T (Table 12), G411T (Table 13), T412A (Table 14), and bulls without the insertion at INDEL CC413-414CAC (Table 15) tended to have fewer minor and total abnormalities (MINAB, TOTAB; P ≤ 0.08) over heterozygotes and bulls with insertions. No affect was observed upon the sperm quality measurements tested for the following SNP for the Brahman influenced animals: G485A, C1369T, A1494T, and INDEL GA1703-1704GCA.

Experiment 2

Genomic DNA samples were collected from early and late pubertal purebred Brahman heifers. Polymorphisms were identified by sequencing two PCR products from a 1215 bp section of the upstream FSHβ promoter region. Twelve SNP (T171C, C225G, G321A, C353A, G410T, G411T, T412A, C620T, T622A, C623T, G783A, A887G) and 3 insertion/deletions (INDEL, GT411-412GAACTT, CC413-414CAC, TC1063-1064TCC) were identified by comparing amplicons to the most recent bovine FSHβ published sequence (GenBank: M83753.1 GI:163063, NCBI).

Relationship of SNP with puberty. Effects of SNP in the FSHβ promoter region upon puberty were determined for 35 early and late pubertal BRAH heifers using Chi square.Although 9 SNP and 3 INDEL were identified in the BRAH heifers, few were found to affect puberty.The

G783A SNP was a guanine to adenine substitution occurring in early and late pubertal heifers (Table 16). One hundred percent of the heifers heterozygous for SNP G783A displayed early puberty (P = 0.02). Although the GG genotype was not observed in any of the early or late pubertal heifers in this study, 100% of the late and 72% of the early pubertal heifers were homozygous for the minor allele (AA).The only other SNP appearing to affect puberty was a thiamine to cytosinesubstitution occurring at bp 171 (SNP T171C, Table 17). While none of the early pubertal heifers were homozygous for the primary allele, 22.22% were heterozygous and 77.78% were homozygous for the minor allele. Unlike late pubertal heifers of which 23.53% were homozygous for the primary allele, 11.76% were heterozygous, and 64.71% homozygous for the minor allele (P = 0.08).

Breed differences in the frequency of occurrence of SNP. Chi square was used to determine breed differences in the frequency of occurrence of SNP identified in an upstream section of the FSHβ promoter region (starting at bp 1324 and proceeding upstream to bp 109) in early and late pubertal Brahman heifers, and the corresponding amplified regions in the Angus, Balancer, and Brahman influenced bulls from Experiment 1. In this 1215 bp region a greater number of SNP and INDEL were observed in the Brahman heifers (9 SNP and 3 INDEL) and Brahman influenced bulls (11 SNP and 3 INDEL) than in the Angus (4 SNP and 0 INDEL) and Balancer (6 SNP and 1 INDEL) bulls. Of the SNP identified, Chi square indicated breed differences in the frequency of occurrence for 9 SNP (A169G, G170T, T171C, C225G, C353A, G410T, G411T, T412A, and G783A) and 2 INDEL (GT411-412GAACTT and CC413-414CAC).

The A169G and G170T SNP occurred in 80% of the Angus bulls, 30.77% of the Balancer, and did not occur in any of the Brahman influenced bulls nor the Brahman heifers ($P < 0.01$, Table 18). Eight SNP were found to be unique to the Brahman influenced bulls and 7 to the Brahman heifers as they did not occur in any of the Angus and Balancer bulls (Table 18). Over forty percent of Brahman influenced bulls (43.75%) were found to be heterozygous for SNP T171C, C225G, C353A, G410T, G411T, and T412A, while the percent Brahman heifers heterozygous and homozygous for the minor allele were as follows: SNP T171C = 17.14 and 71.43%, SNP C225G and G411T = 31.43 and 60%, SNP C353A = 22.86 and 71.43%, SNP G410T = 34.29 and 57.14% ($P < 0.01$), and SNP T412A = 31.43 and 0% ($P = 0.02$). The SNP G783A also did not occur in any of the Angus and Balancer bulls, but occurred in Brahman influenced bulls (31.25% heterozygous) and Brahman heifers (14.29% heterozygous and 85.71% homozygous for the minor allele; $P < 0.01$). Additional sequencing of a section of the FSHβ promoter region from bp 1276 – 1897 (5' to 3') revealed breed differences in the frequency of occurrence of one additional SNP (C1702T) among Angus, Balancer, and Brahman influenced bulls. One hundred percent of the Angus and Balancer bulls and 56.25 % of Brahman influenced bulls were homozygous for the major allele; while 37.5% of Brahman influenced bulls were heterozygous and 6.25% were homozygous for the minor allele ($P = 0.01$, Table 18).

The insertion GT411-412GAACTT was unique to the Brahman heifers (Table19). The majority of heifers homozygous for the minor allele at SNP G410T and G411T were found to have a homozygous insertion of 4 base pairs (AACT) between bp 411 and 412 when compared to the published sequence ($P < 0.01$,Figure 1). Although Brahman heifers and Brahman influenced bulls heterozygous at SNP G410T and G411T did not have INDEL GT411-412GAACTT, they were heterozygous at SNP T412A as well as having an insertion of an

adenine between two cytosine bases at bp 413-414 (INDEL CC413-414CAC). The INDEL CC413-414CAC was found in 31.43% of Brahman heifers and 43.75% of Brahman influenced bulls, but did not occur in Angus or Balancer bulls (P = 0.02,Table 20).

Discussion

Effects of SNP upon semen quality

In males, FSH functions in the hormonal control of spermatogenesis by binding to receptors on Sertoli cells stimulating the meiotic division of primary spermatocytes to secondary spermatocytes (Senger, et al., 2003).Sertoli cells are large structures with overflowing cytoplasmic envelopes extending to the lumen of the seminiferous tubules providing the only communication link across the blood-testes barrier, preventing haploid sperm from causing an immune reaction (Amann, et al., 1983 andSenger, et al., 2003). Therefore it is important to understand how polymorphisms occurring in the promoter region of the FSHβ subunit gene can affect male reproduction.

Seventeen SNP (A169G, G170T, T171C, C225G, C353A, G410T, G411T, T412A, G485A, A643G, G783A, A887G, C1130G, C1369T, C1376T, A1494T, C1702T) and 4 insertion/deletions (INDEL; CC413-414CAC, TC1063-1064TCC, TG1256-1257TCG, GA1703-1704GCA) were identified in Experiment 1. Of those, 4 SNP (T171C, C225G, C353A, and G410T) appear to coincide with finding presented by Dai, et al. (2009) in Simmental, Charolais, and Limousin bulls. Dai, et al., (2009) reported five SNP and one INDEL to occur in the 5' upstream regulatory region of the FSHβ gene at bp 170(T > C), 224 (C > G), 352 (C > A), 409 (G > T), 500 (G > T), and 409_500 (TAAC insertion). Comparison of SNP identified by Dai, et al., (2009) to the most recent FSHβ published sequence (GenBank: M83753.1 GI:163063,

NCBI) revealed the following SNP to coincide with SNP identified in this study: 170(T > C) with T171C, 224 (C > G) with C225G, 352 (C > A) with C353A, and 409 (G > T) coincided with G410T.

Angus and Balancer bulls heterozygous for the SNP identified in this study were associated with an increased percentage of live, motile, rapid, and progressively motile sperm. These bulls also exhibited increased path velocity, progressive velocity, track speed, and lateral amplitude, but a reduced sperm head area, beat frequency, and percent linearity and straightness. Heterozygous Brahman influenced bulls were also associated with increased track speed, path, and progressive velocity. Although most of the sperm quality measures evaluated appeared to be positively influenced by polymorphisms, Angus, Balancer, and Brahman influenced bulls heterozygous for the SNP identified appear to have an increased percentage of minor and total abnormalities when compared to bulls homozygous for the major allele.

Similar effects of polymorphisms in the FSHβ promoter region upon semen quality were observed by Dai, et al, (2009) in fresh and frozen semen. Bulls exhibiting mutations in the regulatory region of the FSHβ gene were also found to have mutations in exon 3. Bulls with this genotype expressed decreased serum FSH concentrations, decreased sperm concentration in fresh semen, decreased percentage of acrosome integrity in fresh and frozen semen,and increased percentage of abnormal sperm in fresh semen, which became more pronounced in frozen semenand decreased sperm motility in frozen semen. A transcription factor binding site database allowed Dai, et al., (2009) to conclude that mutations occurring in the regulatory region of the gene altered transcription factor binding sites resulting in altered gene transcription.

A study investigating the occurrence of polymorphisms in the promoter region of the FSHβ subunit gene in infertile men suggested that SNP occurring in the promoter region were

associated with infertility and reduced serum FSH concentration compared to control men (Grigorova, et al., 2010). Case studies involving nonsense mutations in men reported delayed puberty, reduced testicular size, and azoospermia. Phillip, et al., (1998) reported an FSHβ mutation occurring in an 18 year old Israeli male due to a homozygous deletion of two bp occurring in codon 61 of exon 3 (Val61X) while Layman, et al., (2002) reported a 30 year old Brazilian man with a homozygous C to A substitution in codon 76 (Tyr76X). Both mutations caused in a change in the amino acid structure of the protein resulting in a premature stop codon.

In this study interaction between genotype and week were observed for SNP G485A for the percent linearity, sperm head area, and the percent minor abnormalities. The complete process of spermatogenesis takes 61 days in bulls (Senger, et al., 2003). Factors such as method of semen collection, environment, genetics, and breedhave the potential to affect semen quality measurement (Foote, 1978). Therefore changes in anyof these factors 61 days prior to weeks 1, 4, and 5 could explain the genotype by week interactions observed for SNP G485A.

Semen was collected via electroejaculation twice weekly for 9 weeks from July through September for Angus and Balancer bulls. Although artificial vagina is the preferred method of collection for bulls due to higher quality ejaculates than those observed with electroejaculation, electroejaculation is often the easiest method of collection for bulls that are difficult to handle or that have not been trained to mount a dummy or teaser animal (Foote, 1978). Frequency of collection also has the potential to alter semen quality characteristics since it affects the sperm reservoirs of the epididymis influencing the number of spermatozoa available for ejaculation (Foote, 1978).

An environmental factor possibly contributing to the week by genotype interactions observed for SNP G485A is temperature. According to past weather reports for Springdale, AR

from the United States Government's National Oceanic and Atmospheric Administration (WeatherSource; http://weathersource.com) 61 days prior to beginning of the trial, the temperature highs ranged from 64.4 to 73.4°F with the exception of one day in which the high was 82.4°F. Temperature highs 61 days prior to week 4 and 5 ranged from 82.4 to 89.6°F. Although temperature highs in the upper 80° are typically not considered as extreme temperatures, it is possible that heat stress could have been a contributing factor leading to interaction between week and genotype for SNP G485A. Other factors potentially affecting semen quality could have been animal health, periods of prolonged stress, and nutritional status of the animal (Foote, 1978).

Relationship between SNP and puberty

*Bos indicus*heifers typically have reduced reproductive performance and increased age at puberty than *Bos taurus* heifers (Randel, 2011). Thus the identification of polymorphisms that can serve as genetic markers for improved reproductive performance is important to improving *Bos indicus* cattle breeds. Although 9 SNP and 3 INDEL were identified in the 5' upstream regulatory region of the FSHβ gene in BRAH heifers, only two SNP (G783A and T171C) appeared to affect puberty. One hundred percent of the heifers heterozygous at SNP G783A displayed early puberty while all late pubertal heifers were homozygous for the minor allele. For SNP T171C all early pubertal heifers were C allele carriers (22.22% TC and 77.78 % CC) where as 23.53% of the late pubertal heifers were homozygous for the major allele and 76.47% were C allele carriers (23.53% TT, 11.76% TC, and 64.71% CC).

Polymorphisms of the FSHβ gene in women have been associated with delayed puberty and infertility (Matthews, et al., 1993,Layman, et al., 1997, and Layman, et al., 2002).

Matthews, et al. (1993) reported a nonsense deletion of two bp (TG) in codon 61 of exon 3 of the FSHβ gene in a 27 year old Italian woman. The patient displayed primary amenorrhea, infertility, and lacked breast development. A similar observation was made by Layman, et al., (1997) in a 15 year old girl with delayed puberty. The patient displayed poor breast development, amenorrhea, and undetectable serum FSH concentrations. Gene sequencing revealed the patient to have a compound heterozygous mutation of the FSHβ gene in the coding region of exon 3. The patient inherited the same two bp deletion described by Matthews, et al., (1993) from her mother and a missense mutation in codon 51 from her father changing a cysteine to a glycine (Cys51Gly). Both mutations impaired dimerization of the FSH α and β subunits resulting in failure to activate the FSH receptor (Layman, et al., 1997). Berger et al., (2005) also reported a case study involving a loss-of-function mutation of the FSHβ subunit gene in a 16 year old Brazilian female. The patient displayed delayed puberty, primary amenorrhea, and partial breast development. Analysis of the patients FSHβ gene DNA sequence revealed a C to A substitution in codon 76 of exon 3 changing the amino acid structure from atyrosine to a premature stop codon (Tyr76X).

Although FSHβ knockout male mice were considered fertile even though they displayed impaired spermatogenesis with reduced testicular size, female FSHβ knockout mice were infertile displaying small thin ovaries and uteri, lacking corpus luteum development, and blocked follicular maturation prior to antral follicle formation (Kumar, et al., 1997). An additional study investigating testicular function and spermatogenesis in FSHβ receptor knockout (FORKO) mice indicated that when compared to control mice, FORKO displayed delayed puberty and testicular development as well as impaired spermatogenesis due to loss of signal between FSH and its receptor (Krishnamurthy, et al., 2001).

Breed differences in the frequency of occurrence of SNP

Breed differences in the frequency of occurrence of SNP in the promoter region of the FSHβ gene were found in the majority of SNP identified in animals from this study. Comparison of amplicons in a 1215 bp region from bp 109 – 1324 (5' to 3') revealed breed differences for 9 SNP and 2 INDEL. A greater number of SNP were observed in the Brahman heifers and Brahman influenced bulls compared to Angus and Balancer bulls.

The Brahman influenced bulls used in this study were Angus sired calves with approximately 1/8 to 3/16 Brahman influence, raising the question as to whether or not it was the Brahman influence in these crossbred bulls attributing to the increased frequency of occurrence of SNP. The majority of *Bos indicus* cattle found in the United States today are Brahman. The Brahman breed was developed in the Gulf Coast region of the United States by crossbreeding native U.S. cattle of *Bos taurus* origin to various breeds of *Bos indicus* cattle from India and later Brazil (Randel, 2011; and Sanders, 1980). It is possible that the greater number of SNP identified in this study in the Brahman and Brahman influenced cattle was due to genetic diversity among the native cattle and *Bos indicus* cattle used to develop the Brahman breed.

At the time of this study, no research has been found reporting breed related differences in the frequency of occurrence of polymorphisms in the FSHβ gene. A genome-wide association study was performed for the age at first corpus luteum (AGECL) and postpartum anestrous interval (PPAI) using the bovine SNP50 chip in Brahman and Tropical Composite (approximately ½ Brahman and ½ non-tropically adapted *Bos taurus* breeds consisting of a mixture of Red Angus, Hereford, Shorthorn, Red Poll and Charolais) breeds of cattle (Hawken, et al., 2012). One hundred sixty nine SNP were found to be associated with AGECL in Brahman

cattle compared to 84 SNP for Tropical Composite cattle. Polymorphisms related to AGECL were primarily located on chromosome 14 (41%, respectfully) and chromosome 5 (9% respectfully) compared to Tropical Composite cattle which displayed a more even distribution of SNP throughout the genome. Sixty six SNP were related to PPAI in Brahman (14% - chromosome 3, 17% - chromosome 14, and 9% - chromosome 21, respectfully) versus 113 SNP in Tropical Composite cattle (19% - chromosome 5, and 17% - chromosome16, respectfully). Although a large number of SNP were identified to affect AGECL and PPAI, comparison of SNP between breeds only indicated one SNP to affect AGECL between both breeds of cattle indicatingbreed difference in the distribution of SNP (Hawken, et al., 2012). A similar study was conducted to determine association of SNP for genomic prediction in Holstein, Jersey, and Brown Swiss bulls (Wiggans, et al., 2009). Data suggested that although thousands of SNP observed in Holsteins were polymorphic, the same SNP were monomorphic in Jersey and Brown Swiss cattle indicating breed specific polymorphisms.

Conclusion

Although the number of animals used in this study was limited, data suggests breed differences in the frequency of occurrence of SNP in the FSHβ gene promoter region, and that SNP may be useful as markers related to semen quality in bulls and puberty in Brahman heifers. Further research is needed to verify these results, and to determine if the polymorphisms identified herein resulted in altered transcription factor binding or transcription initiation sites therefore potentially altering gene expression.

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Tables

Table 1: Primers Used in PCR to Amplify the FSHβ-Gene Promoter Region

Experiment 1

Set	Length (bp)	Amplified Region	Forward Primer (5’ – 3’)	Reverse Primer (5’ – 3’)
1	477	586 - 109	ACACACCAGTCAGGATTTTGCTCC	TCTGGGAGTCATTGACATAAGC
2	811	1324 - 513	CCAAACCCAGTTTCAGCATT	TCTGCTGATTCATTTCTCTAGCTC
3	621	1897 - 1276	GGGGGTAAGCAAGGACATTT	CCTTGTCTGGGGAAAGCTGTAG

Experiment 2

Set	Length (bp)	Amplified Region	Forward Primer (5’ – 3’)	Reverse Primer (5’ – 3’)
1	477	586 - 109	ACACACCAGTCAGGATTTTGCTCC	TCTGGGAGTCATTGACATAAGC
2	811	1324 - 513	CCAAACCCAGTTTCAGCATT	TCTGCTGATTCATTTCTCTAGCTC

Table 2: Effect of SNP G485A on mean[a] semen quality measurements for Angus and Balancer bulls

	SNP G485A			
Variable	GG	GA	SE	P Value
MOT	46.32	64.66	4.65	< 0.01
PROG	33.16	44.99	3.64	< 0.01
RAP	43.89	60.95	4.77	< 0.01
VAP	120.48	133.72	5.23	0.02
VSL	94.73	105.38	4.28	0.02
VCL	210.83	231.91	9.00	0.03
ALH	7.91	8.76	0.34	0.02
BCF	23.59	21.44	1.04	0.05
STR	78.71	78.76	1.15	0.97
LIN	48.33	48.32	1.26	1.00
ELONG	43.97	44.79	0.78	0.31
AREA	4.73	4.58	0.07	0.04
LIVE	66.40	76.22	3.08	< 0.01
TOTAB	2.63	2.69	0.46	0.91
MAJAB	1.11	1.36	0.28	0.38
MINAB	1.53	1.34	0.31	0.54

[a] Least square means

Table 3: Effect of SNP C1130G on mean[a] semen quality measurements for Angus and Balancer bulls

	SNP C1130G			
Variable	CC	CG	SE	P Value
MOT	58.79	66.83	6.31	0.22
PROG	41.55	43.83	4.90	0.65
RAP	55.43	63.33	6.37	0.23
VAP	128.66	140.03	6.84	0.12
VSL	101.94	106.47	5.73	0.44
VCL	222.30	251.35	11.66	0.02
ALH	8.37	9.59	0.45	0.02
BCF	22.07	22.11	1.41	0.98
STR	79.16	76.06	1.51	0.06
LIN	48.81	45.17	1.68	0.05
ELONG	44.44	45.56	0.99	0.28
AREA	4.61	4.68	0.09	0.46
LIVE	73.53	75.56	4.29	0.64
TOTAB	2.75	2.32	0.59	0.48
MAJAB	1.32	1.02	0.36	0.41
MINAB	1.43	1.32	0.42	0.79

[a] Least square means

Table 4: Effect of SNP A169G on mean[a] semen quality measurements for Angus and Balancer bulls

	SNP A169G			
Variable	AA	GG	SE	P Value
MOT	59.48	60.16	4.30	0.88
PROG	40.98	42.77	3.33	0.60
RAP	56.00	56.89	4.35	0.84
VAP	129.73	130.19	4.71	0.92
VSL	101.53	103.42	3.88	0.63
VCL	227.49	223.73	8.16	0.65
ALH	8.56	8.47	0.32	0.77
BCF	21.69	22.58	0.93	0.35
STR	78.45	79.18	1.01	0.48
LIN	47.67	49.18	1.12	0.19
ELONG	44.08	45.13	0.66	0.13
AREA	4.60	4.65	0.06	0.36
LIVE	74.11	73.48	2.79	0.82
TOTAB	2.31	3.09	0.38	0.06
MAJAB	1.15	1.42	0.24	0.28
MINAB	1.16	1.68	0.27	0.07

[a] Least square means

Table 5: Effect of SNP G170T on mean[a] semen quality measurements for Angus and Balancer bulls

	SNP G170T			
Variable	GG	TT	SE	P Value
MOT	59.48	60.16	4.30	0.88
PROG	41.00	42.77	3.31	0.60
RAP	56.00	56.89	4.35	0.84
VAP	129.73	130.19	4.71	0.92
VSL	101.53	103.42	3.88	0.63
VCL	227.49	223.73	8.16	0.65
ALH	8.56	8.47	0.32	0.77
BCF	21.69	22.58	0.93	0.35
STR	78.45	79.18	1.01	0.48
LIN	47.67	49.18	1.12	0.19
ELONG	44.08	45.13	0.66	0.13
AREA	4.60	4.65	0.06	0.36
LIVE	74.11	73.48	2.79	0.82
TOTAB	2.31	3.09	0.38	0.06
MAJAB	1.15	1.42	0.24	0.28
MINAB	1.16	1.68	0.27	0.07

[a] Least square means

Table 6: Effect of SNP G783A on mean[a] semen quality measurements for Brahman influenced bulls

	SNP G783A			
Variable	GG	GA	SE	P Value
MOT	65.33	55.80	8.28	0.27
PROG	49.82	42.33	7.67	0.35
RAP	58.39	49.60	8.45	0.32
VAP	114.07	109.69	9.52	0.65
VSL	97.64	94.18	8.88	0.70
VCL	187.25	177.75	14.44	0.52
ALH	7.19	6.63	0.48	0.27
BCF	28.33	25.02	1.68	0.07
STR	84.15	78.80	3.83	0.18
LIN	54.03	51.73	3.27	0.49
ELONG	46.36	43.07	2.15	0.15
AREA	4.83	4.55	0.23	0.24
LIVE	77.48	73.00	4.97	0.38
MAJAB	10.09	14.80	2.57	0.09
MINAB	13.24	23.33	4.22	0.03
TOTAB	23.33	38.13	5.94	0.03

[a] Least square means

Table 7: Effect of SNP C1702T on least squares means of semen quality measurements for Brahman influenced bulls

	SNP C1702T				
Variable	CC	CT	TT	SE	P Value
MOT	65.11	55.50	78.67	16.80	0.31
PROG	49.30	42.00	64.00	15.71	0.35
RAP	57.81	49.72	71.67	17.27	0.40
VAP	113.68	111.49	111.07	19.89	0.97
VSL	97.24	95.33	97.87	18.65	0.98
VCL	186.94	182.23	172.77	29.52	0.86
ALH	7.24	6.81	6.27	0.97	0.48
BCF	28.41	25.09	30.47	3.44	0.12
STR	83.74	79.78	87.33	8.00	0.49
LIN	53.33	52.50	58.00	6.65	0.72
ELONG	46.00	43.89	48.00	4.57	0.53
AREA	4.86	4.58	4.73	0.49	0.53
LIVE	76.70	73.56	85.67	10.10	0.49
MAJAB	10.07[b]	14.78[a]	5.67[b]	5.26	0.09
MINAB	13.11[b]	22.89[a]	7.00[b]	8.70	0.09
TOTAB	23.19[d]	37.67[c]	12.67[d]	12.09	0.06

[ab]Least squares means tended to differ. P = 0.09

[cd] P = 0.06

Table 8: Effect of SNP C1130G on mean[a] semen quality measurements for Brahman influenced bulls

	SNP C1130G			
Variable	CC	CG	SE	P Value
MOT	59.98	79.33	16.17	0.25
PROG	44.98	66.00	15.04	0.19
RAP	52.98	77.00	16.44	0.17
VAP	110.30	147.87	19.05	0.07
VSL	94.32	126.60	17.88	0.09
VCL	180.98	242.07	28.78	0.05
ALH	6.97	8.47	0.96	0.14
BCF	26.95	29.00	3.58	0.58
STR	81.93	85.33	8.22	0.69
LIN	52.88	54.67	6.75	0.80
ELONG	45.12	45.67	4.56	0.91
AREA	4.74	4.83	0.48	0.85
LIVE	74.81	84.33	9.73	0.35
MAJAB	12.21	8.33	5.19	0.47
MINAB	17.52	10.00	8.83	0.41
TOTAB	29.74	18.33	12.31	0.37

[a] Least square means

Table9: Effect of SNP T171C on mean[a] semen quality measurements for Brahman influenced bulls

	SNP T171C			
Variable	TT	TC	SE	P Value
MOT	65.11	58.81	7.77	0.43
PROG	49.30	45.14	7.25	0.58
RAP	57.81	52.86	7.95	0.54
VAP	113.68	111.43	9.02	0.81
VSL	97.24	95.69	8.47	0.86
VCL	186.94	180.88	13.47	0.66
ALH	7.24	6.73	0.44	0.28
BCF	28.41	25.86	1.61	0.14
STR	83.74	80.86	3.70	0.45
LIN	53.33	53.29	3.06	0.99
ELONG	46.00	44.48	2.08	0.48
AREA	4.86	4.60	0.22	0.27
LIVE	76.70	75.29	4.63	0.76
MAJAB	10.07	13.48	2.45	0.19
MINAB	13.11	20.62	4.08	0.09
TOTAB	23.19	34.10	5.73	0.08

[a] Least square means

Table10: Effect of SNP C225G on mean[a] semen quality measurements for Brahman influenced bulls

	SNP C225G			
Variable	CC	CG	SE	P Value
MOT	65.11	58.81	7.77	0.43
PROG	49.30	45.14	7.25	0.58
RAP	57.81	52.86	7.95	0.54
VAP	113.68	111.43	9.02	0.81
VSL	97.24	95.69	8.47	0.86
VCL	186.94	180.88	13.47	0.66
ALH	7.24	6.73	0.44	0.28
BCF	28.41	25.86	1.61	0.14
STR	83.74	80.86	3.70	0.45
LIN	53.33	53.29	3.06	0.99
ELONG	46.00	44.48	2.08	0.48
AREA	4.86	4.60	0.22	0.27
LIVE	76.70	75.29	4.63	0.76
MAJAB	10.07	13.48	2.45	0.19
MINAB	13.11	20.62	4.08	0.09
TOTAB	23.19	34.10	5.73	0.08

[a] Least square means

Table11: Effect of SNP C353A on mean[a] semen quality measurements for Brahman influenced bulls

	SNP C353A			
Variable	CC	CA	SE	P Value
MOT	65.11	58.81	7.77	0.43
PROG	49.30	45.14	7.25	0.58
RAP	57.81	52.86	7.95	0.54
VAP	113.68	111.43	9.02	0.81
VSL	97.24	95.69	8.47	0.86
VCL	186.94	180.88	13.47	0.66
ALH	7.24	6.73	0.44	0.28
BCF	28.41	25.86	1.61	0.14
STR	83.74	80.86	3.70	0.45
LIN	53.33	53.29	3.06	0.99
ELONG	46.00	44.48	2.08	0.48
AREA	4.86	4.60	0.22	0.27
LIVE	76.70	75.29	4.63	0.76
MAJAB	10.07	13.48	2.45	0.19
MINAB	13.11	20.62	4.08	0.09
TOTAB	23.19	34.10	5.73	0.08

[a] Least square means

Table 12: Effect of SNP G410T on mean[a] semen quality measurements for Brahman influenced bulls

	SNP G410T			
Variable	GG	GT	SE	P Value
MOT	65.11	58.81	7.77	0.43
PROG	49.30	45.14	7.25	0.58
RAP	57.81	52.86	7.95	0.54
VAP	113.68	111.43	9.02	0.81
VSL	97.24	95.69	8.47	0.86
VCL	186.94	180.88	13.47	0.66
ALH	7.24	6.73	0.44	0.28
BCF	28.41	25.86	1.61	0.14
STR	83.74	80.86	3.70	0.45
LIN	53.33	53.29	3.06	0.99
ELONG	46.00	44.48	2.08	0.48
AREA	4.86	4.60	0.22	0.27
LIVE	76.70	75.29	4.63	0.76
MAJAB	10.07	13.48	2.45	0.19
MINAB	13.11	20.62	4.08	0.09
TOTAB	23.19	34.10	5.73	0.08

[a] Least square means

Table 13: Effect of SNP G411T on mean[a] semen quality measurements for Brahman influenced bulls

	SNP G411T			
Variable	GG	GT	SE	P Value
MOT	65.11	58.81	7.77	0.43
PROG	49.30	45.14	7.25	0.58
RAP	57.81	52.86	7.95	0.54
VAP	113.68	111.43	9.02	0.81
VSL	97.24	95.69	8.47	0.86
VCL	186.94	180.88	13.47	0.66
ALH	7.24	6.73	0.44	0.28
BCF	28.41	25.86	1.61	0.14
STR	83.74	80.86	3.70	0.45
LIN	53.33	53.29	3.06	0.99
ELONG	46.00	44.48	2.08	0.48
AREA	4.86	4.60	0.22	0.27
LIVE	76.70	75.29	4.63	0.76
MAJAB	10.07	13.48	2.45	0.19
MINAB	13.11	20.62	4.08	0.09
TOTAB	23.19	34.10	5.73	0.08

[a] Least square means

Table 14: Effect of SNP T412A on mean[a] semen quality measurements for Brahman influenced bulls

	SNP T412A			
Variable	TT	TA	SE	P Value
MOT	65.11	58.81	7.77	0.43
PROG	49.30	45.14	7.25	0.58
RAP	57.81	52.86	7.95	0.54
VAP	113.68	111.43	9.02	0.81
VSL	97.24	95.69	8.47	0.86
VCL	186.94	180.88	13.47	0.66
ALH	7.24	6.73	0.44	0.28
BCF	28.41	25.86	1.61	0.14
STR	83.74	80.86	3.70	0.45
LIN	53.33	53.29	3.06	0.99
ELONG	46.00	44.48	2.08	0.48
AREA	4.86	4.60	0.22	0.27
LIVE	76.70	75.29	4.63	0.76
MAJAB	10.07	13.48	2.45	0.19
MINAB	13.11	20.62	4.08	0.09
TOTAB	23.19	34.10	5.73	0.08

[a] Least square means

Table 15: Effect of INDEL CC413-414CAC on mean[a] semen quality measurements for Brahman influenced bulls

	INDEL CC413-414CAC			
Variable	CC	CAC	SE	P Value
MOT	65.11	58.81	7.77	0.43
PROG	49.30	45.14	7.25	0.58
RAP	57.81	52.86	7.95	0.54
VAP	113.68	111.43	9.02	0.81
VSL	97.24	95.69	8.47	0.86
VCL	186.94	180.88	13.47	0.66
ALH	7.24	6.73	0.44	0.28
BCF	28.41	25.86	1.61	0.14
STR	83.74	80.86	3.70	0.45
LIN	53.33	53.29	3.06	0.99
ELONG	46.00	44.48	2.08	0.48
AREA	4.86	4.60	0.22	0.27
LIVE	76.70	75.29	4.63	0.76
MAJAB	10.07	13.48	2.45	0.19
MINAB	13.11	20.62	4.08	0.09
TOTAB	23.19	34.10	5.73	0.08

[a] Least square means

Table 16: Effect of SNP[a] G783A upon maturity in Brahman heifers

	SNP G783A					
Puberty	GG	GA	AA	PAF[b]	MAF[c]	P Value
Early (n = 18)	0	5	13	13.89	86.11	0.02
Late (n = 17)	0	0	17	0.00	100.00	

[a]SNP, Single nucleotide polymorphism occurred at the base pair represented. The first letter represents the dominant allele according to the bovine FSHβ published sequence (GenBank: M83753.1 GI:163063, NCBI) while the letter following the base pair number is the resulting minor allele.

[b]PAF, Percent Primary Allele Frequency

[c]MAF, Percent Minor Allele Frequency

Table 17: Effect of SNP[a] T171C upon maturity in Brahman heifers

	SNP T171C					
Puberty	TT	TC	CC	PAF[b]	MAF[c]	P Value
Early (n = 18)	0	4	14	11.11	88.89	0.08
Late (n = 17)	4	2	11	29.41	70.59	

[a]SNP, Single nucleotide polymorphism occurred at the base pair represented. The first letter represents the dominant allele according to the bovine FSHβ published sequence (GenBank: M83753.1 GI:163063, NCBI) while the letter following the base pair number is the resulting minor allele.

[b]PAF, Percent Primary Allele Frequency

[c]MAF, Percent Minor Allele Frequency

Table18: Genotype and allele frequencies of SNP found in the FSHβ promoter region of Angus, Balancer, Brahman influenced, and Brahman cattle.

SNP[a]	Breed	HPA[b]	HET[c]	HMA[d]	PAF[e]	MAF[f]	P Value
A169G	ANG (n=5)	1	0	4	20.00	80.00	< 0.01
	BAL (n=13)	9	0	4	69.23	30.77	
	BI (n=16)	16	0	0	100.00	0.00	
	BRAH (n=35)	35	0	0	100.00	0.00	
G170T	ANG (n=5)	1	0	4	20.00	80.00	< 0.01
	BAL (n=13)	9	0	4	69.23	30.77	
	BI (n=16)	16	0	0	100.00	0.00	
	BRAH (n=35)	35	0	0	100.00	0.00	
T171C	ANG (n=5)	5	0	0	100.00	0.00	< 0.01
	BAL (n=13)	13	0	0	100.00	0.00	
	BI (n=16)	9	7	0	78.12	21.88	
	BRAH (n=35)	4	6	25	20.00	80.00	
C225G	ANG (n=5)	5	0	0	100.00	0.00	< 0.01
	BAL (n=13)	13	0	0	100.00	0.00	
	BI (n=16)	9	7	0	78.12	21.88	
	BRAH (n=35)	3	11	21	24.29	75.71	
C353A	ANG (n=5)	5	0	0	100.00	0.00	< 0.01
	BAL (n=13)	13	0	0	100.00	0.00	
	BI (n=16)	9	7	0	78.12	21.88	
	BRAH (n=35)	2	8	25	17.14	82.86	
G410T	ANG (n=5)	5	0	0	100.00	0.00	< 0.01
	BAL (n=13)	13	0	0	100.00	0.00	
	BI (n=16)	9	7	0	78.12	21.88	
	BRAH (n=35)	3	12	20	25.71	74.29	
G411T	ANG (n=5)	5	0	0	100.00	0.00	< 0.01
	BAL (n=13)	13	0	0	100.00	0.00	
	BI (n=16)	9	7	0	78.12	21.88	
	BRAH (n=35)	3	11	21	24.29	75.71	

Table18: Continued

SNP[a]	Breed	HPA[b]	HET[c]	HMA[d]	PAF[e]	MAF[f]	P Value
T412A	ANG (n=5)	5	0	0	100.00	0.00	0.02
	BAL (n=13)	13	0	0	100.00	0.00	
	BI (n=16)	9	7	0	78.12	21.88	
	BRAH (n=35)	24	11	0	84.29	15.71	
G783A	ANG (n=5)	5	0	0	100.00	0.00	< 0.01
	BAL (n=13)	13	0	0	100.00	0.00	
	BI (n=16)	11	5	0	84.37	15.63	
	BRAH (n=35)	0	5	30	7.14	92.86	
C1702T	ANG (n=5)	5	0	0	100.00	0.00	0.01
	BAL (n=13)	13	0	0	100.00	0.00	
	BI (n=16)	9	6	1	75.00	25.00	

[a]SNP, Single nucleotide polymorphism occurred at the base pair represented. The first letter represents the dominant allele according to the bovine FSHβ published sequence (GenBank: M83753.1 GI:163063, NCBI) while the letter following the base pair number is the resulting minor allele.
[b]HPA, Homozygous Primary Allele
[c]HET, Heterozygous
[d]HMA, Homozygous Minor Allele
[e]PAF, Percent Primary Allele Frequency
[f]MAF, Percent Minor Allele Frequency

Table19: Genotype and allele frequencies of INDEL GT411-413GAACTT found in the FSHβ promoter region of Angus, Balancer, Brahman influenced, and Brahman cattle.

INDEL[a]	Breed	PS[b]	INS[c]	HET[d]	PSF[e]	INSF[f]	HETF[g]	P Value
GT411-412GAACTT	ANG (n=5)	5	0	0	100.00	0.00	0.00	< 0.01
	BAL (n=13)	13	0	0	100.00	0.00	0.00	
	BI (n=16)	16	0	0	100.00	0.00	0.00	
	BRAH (n=35)	3	21	11	8.57	60.00	31.43	

[a]INDEL, Insertion/deletion occurred at the base pair represented. Letters preceeding the base paid number represents the published sequence (GenBank: M83753.1 GI:163063, NCBI) while the letter following the base pair number is the resulting insertion/deletion.
[b]PS, Published sequence
[c]INS, Insertion of a four base pairs (AACT) compared to the published sequence
[d]HET, No INDEL but heterozygous at the base pair 411 & 412
[e]PSF, Frequency of published sequence in percentage
[f]INSF, Frequency of the four bp insertion frequency in percentage
[g]HETF, Frequency of heterozygous at bp 411 & 412 with no INDEL in percentage

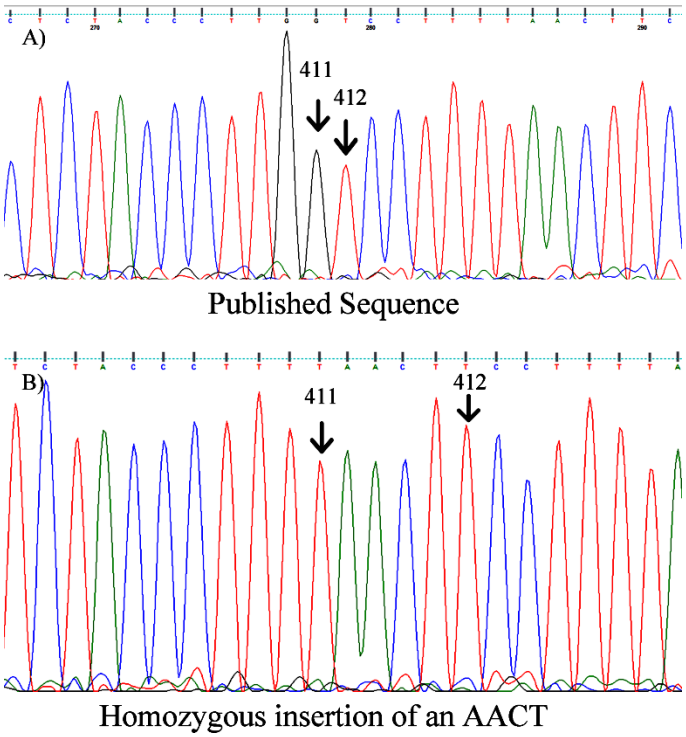
Table20: Genotype and allele frequencies of INDEL CC413-414CAC found in the FSHβ promoter region of Angus, Balancer, Brahman influenced, and Brahman cattle.

INDEL[a]	Breed	PS[b]	INS[c]	PSF[d]	INSF[e]	P Value
CC413-414CAC INDEL	ANG (n=5)	5	0	100.00	0.00	0.02
	BAL (n=13)	13	0	100.00	0.00	
	BI (n=16)	9	7	56.25	43.75	
	BRAH (n=35)	24	11	68.57	31.43	

[a]INDEL, Insertion/deletion occurred at the base pair represented. Letters preceeding the base paid number represents the published sequence (GenBank: M83753.1 GI:163063, NCBI) while the letter following the base pair number is the resulting insertion/deletion.
[b]PS, Published sequence
[c]INS, Insertion of an adenine between two cysteine bases compared to the published sequence
[e]PSF, Frequency of published sequence in percentage
[f]INSF, Frequency of the four bp insertion frequency in percentage

Figures

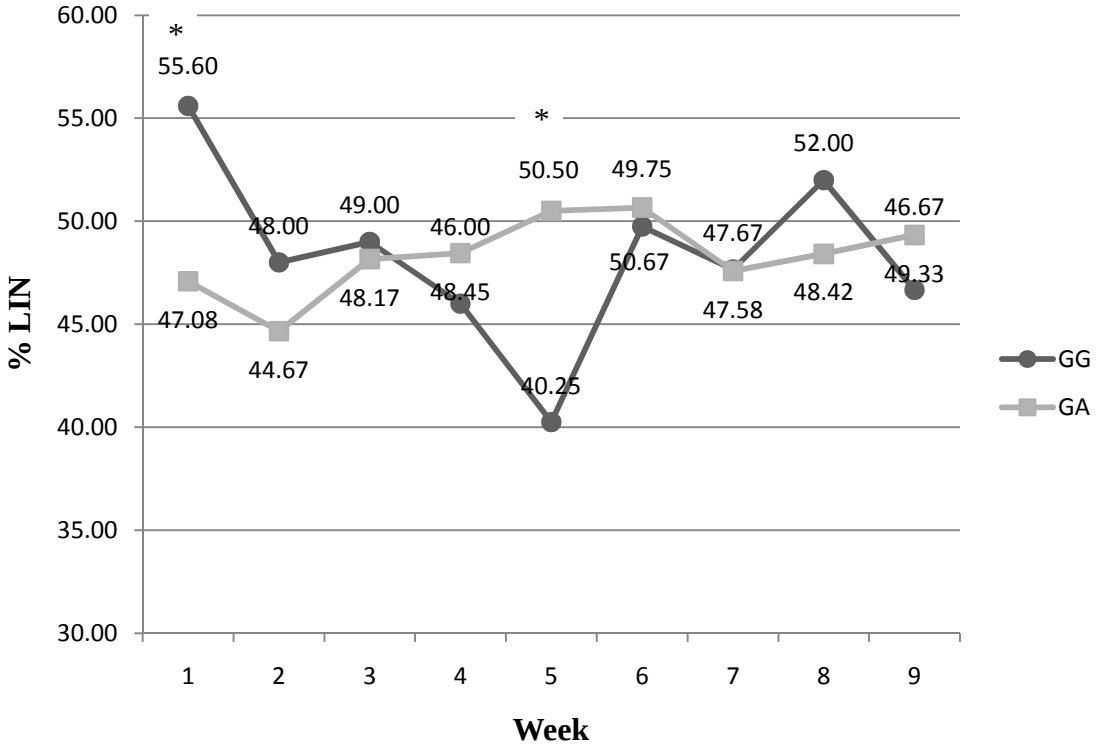
Figure 1: INDEL GT411-412GAACTT



Published Sequence: TCTACCCTTGG - - - - TCCTTTTAAC
Homozygous insertion of 4 bp: TCTACCCTTTTAACTTCCTTTTAAC

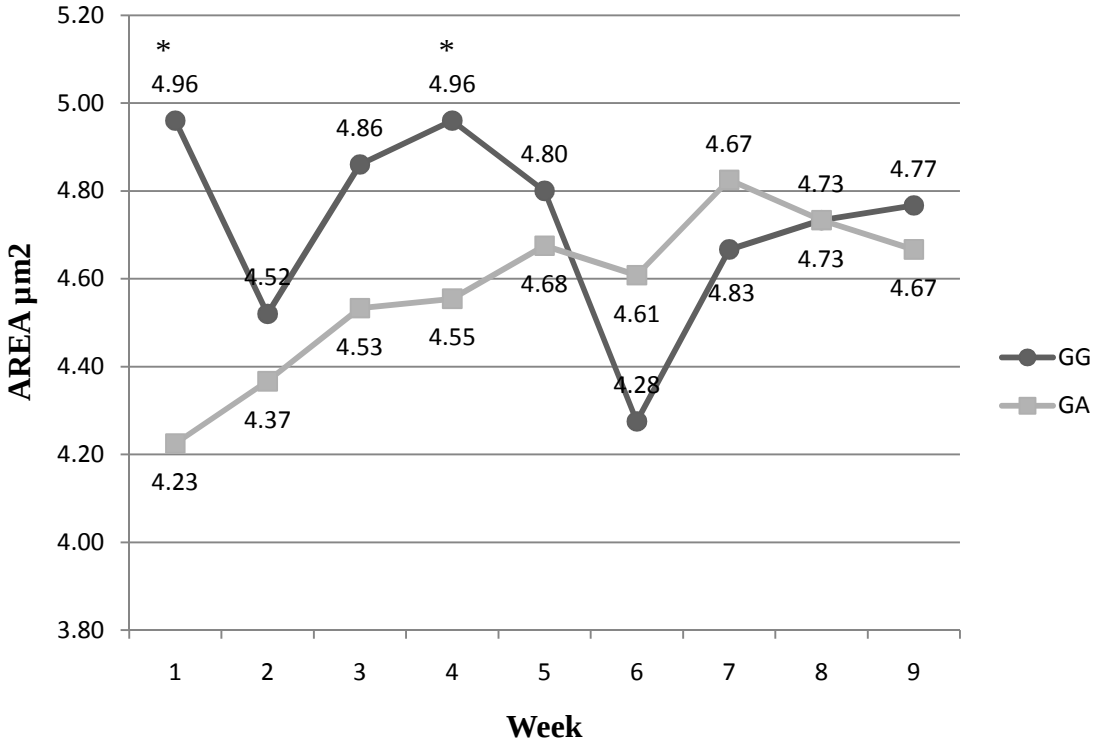
A) Picture of an electropherogram depicting the most recent FSHβ published sequence (GenBank: M83753.1 GI:163063; NCBI). B) Homozygous insertion of four bp (AACT) between bp 411 and 412.

Figure 2: Interaction between week and SNP G485A for LIN



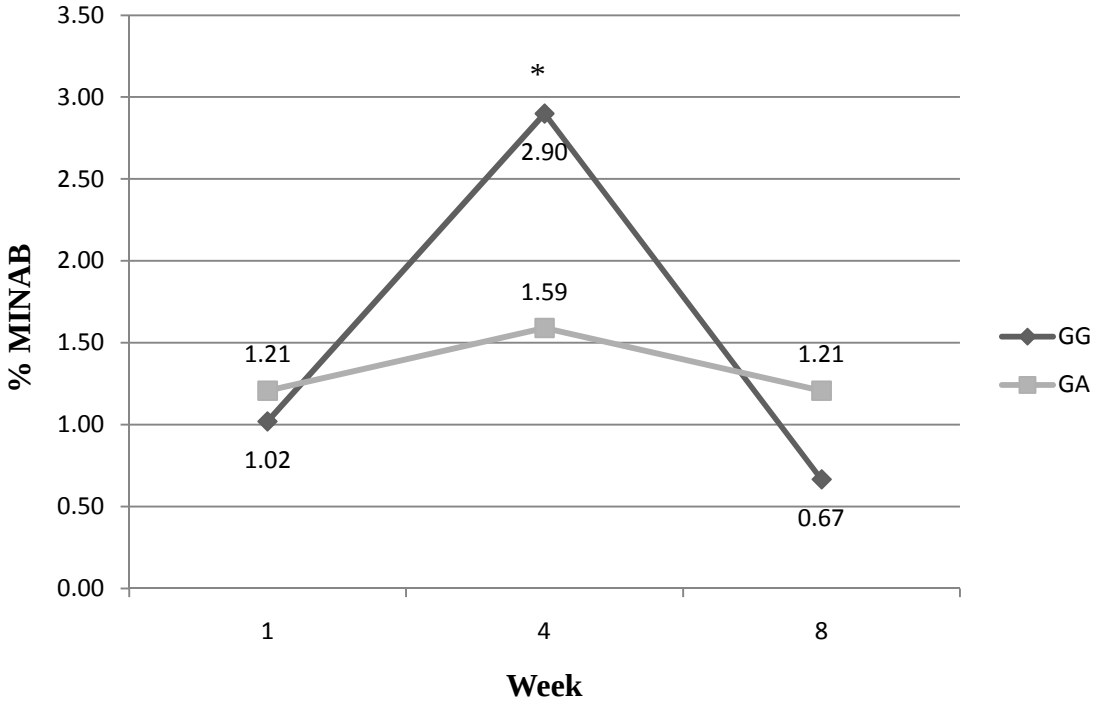
*, indicates $P \leq 0.05$

Figure 3: Interaction between week and SNP G485A for AREA



*, indicates $P \leq 0.05$

Figure 4: Interaction between week and SNP G485A for MINAB



*, indicates $P \leq 0.05$

Appendices

Appendix A: Description of sperm quality variables measured

Measured by a Hamilton-Thorne Sperm Analyzer

% Motile (MOT) - The population of cells that is moving at or above a specific speed. It is a ratio of the motile to total cells counted. Measured as millions/ml.

% Progressive (PROG) - Fraction of all motile cells moving with a path velocity (VAP) greater than the progressive cell VAP. VAP ≥ 50 micrometers per second (µm/s) and STR ≥ 70%.

% Rapid (RAP) - Percent PROG with a path velocity > 50µm/s.

% Live (LIVE) - The percent live sperm. Measured as millions/ml.

Path Velocity (VAP) - The average velocity of the smoothed cell path measured in µm/s.

Progressive Velocity (VSL) - The average velocity measured in a straight line from the beginning to end of the track in µm/s.

Track Speed (VCL) - The average velocity measured over the actual point-to-point track followed by the cell. Total distance traveled by the sperm measured in µm/s.

Lateral Amplitude (ALH) - Displacement corresponds to the mean width of the head oscillation as the sperm swims.

Beat/Cross Frequency (BCF) - The frequency at which the sperm heads move back and forth in its track across the path measured in microns/second.

% Straightness (STR) - A ratio of the progressive velocity (VSL) to the path velocity (VAP) for the selected track, expressed as a percent.

% Linearity (LIN) - A ratio of the progressive velocity (VSL) to the track speed (VCL) for the selected track, expressed as a percent.

% Elongation (ELONG) - The ratio of sperm head width to length for the selected track, expressed as a percent.

Average size of sperm heads (AREA) - The calculated head area of the selected cell in μm^2.

Sperm morphology determined using Orcein stain

% Major Abnormalities (MAJAB) - Includes sperm with a malformed/misshapen, cratered, pyriform, and small or giant head; abnormal acrosome; proximal droplet; midpiece defect; and sperm with a strongly folded tail.

% Minor Abnormalities (MINAB) - Includes sperm with a distal droplet, coiled/bent tail, and those that are headless or tailless.

% Total Abnormalities (TOTAB) - The total percent of sperm with abnormalities.

Appendix B: Puregene DNA Purification Kit (cat. no. 158445, Quiagen, Valencia, CA).

Protocol: DNA purification from whole blood or bone marrow using the GentraPuregen Blood Kit.

1. Add 900 µl RBC Lysis Solution into a 1.5 ml tube.
2. Add 300 µl whole blood, and mix by inverting 10 times.
3. Incubate 1 minute at room temperature inverting at least once during incubation.
4. Centrifuge for 20 seconds at 13,000 x g to pellet the white blood cells.
5. Carefully discard the supernatant by pipetting off the residual liquid leaving ~ 10 µl of the residual liquid and the red blood cell pellet.
6. Vortex the tube vigorously to resuspend the pellet.
7. Add 300 µl Cell Lysis Solution and vortex vigorously for 10 seconds.
8. Add 100 µl Protein Precipitation Solultion, and vortex vigorously for 20 seconds at high speed.
9. Centrifuge for 1 minute at 13,000 x g allowing the precipitated proteins for form a tight dark pellet.
10. Pipet 300 µl isopropanol into a clean 1.5 ml tube and carefully pour off the supernatant from the previous step.
11. Mix by inverting gently 50 times until the DNA is visible as threads or a clump.
12. Centrifuge for 1 minute at 13,000 x g.
13. Carefully discard the supernatant and drain the tube by carefully inverting the tube on a clean piece of absorbent paper.
14. Add 300 µl 70% ethanol and invert several times to wash the DNA pellet.
15. Centrifuge for 1 minute at 13,000 x g.

16. Carefully discard the supernatant and drain the tube by carefully inverting the tube on a clean piece of absorbent paper and allow to air dry for 5 minutes.
17. Add 100 µl DNA Hydration Solution and vortex for 5 seconds at medium speed.
18. Incubate sample in a bio-oven for 5 minutes at 65˚C to dissolve the DNA.
19. Incubate overnight at room temperature then store at -20˚C until ready for use.

Appendix C: QiagenQIAquick PCR Purification Kit (Quiagen, Valencia, CA).

Protocol: QIAquick PCR purification kit protocol using a microcentrifuge

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.
    a. First add PCR sample to a 1.5 ml microcentrifuge tube followed by Buffer BP and pipette up and down to mix.
2. Place a QIAquick spin column in a provided 2 ml collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 minute at 13,000 x g.
4. Discard flow-through. Place the QIAquick column back into the same tube.
5. To wash, add 750 µl Buffer PE to the QIAquick column and centrifuge for 1 minute.
6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge again for an additional 1 minute.
7. Place the QIAquick column in a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, add 50 µl Buffer EB to the center of the QIAquick membrane, let stand for 1 minute, and centrifuge the column for 1 minute.
9. Store purified PCR products at -20˚C until ready for use.

Appendix D:QIAamp DNA Mini Kit (Quiagen, Valencia, CA).

Protocol: DNA purification from blood or body fluids (spin protocol).

1. Pipet 20 µl Qiagen Protease into a 1.5 ml tube.
2. Add 200 µl whole blood to the tube.
3. Add 200 µl Buffer AL to the sample and mix by pulse-vortexing for 15 seconds.
4. Incubate in a bio-oven for 10 minutes at 56˚C.
5. Briefly centrifuge the 1.5 ml tube to remove drops from inside the lid.
6. Add 200 µl ethanol (96 – 100%) to the sample, and mix by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
7. Carefully apply the mixture from the previous step to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6,000 x g for 1 minute. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate.
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6,000 x g for 1 minute. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the collection tube with the filtrate.
9. Carefully open the QIAamp Mimi spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at 16,000 x g for 3 minutes.
10. Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at 16,000 x g for 1 minutes.
11. Place the QIAamp Mini spin column in a clean 1.5 ml tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200

µl Buffer AE. Incubate at room temperature for 5 minute, and then centrifuge at 6,000 x g for 1 minute.

12. Store samples at -20˚C until ready for use.