

Nutritional and management
strategies to improve reproductive efficiency in beef cattle

by

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Abstract

Fertility in beef cattle is one of the most economically important and biologically complex aspects of the beef production system. Improving reproductive efficiency requires an integrated understanding of the factors that regulate puberty, gamete quality, and the establishment of pregnancy. This thesis research is designed to address the fertility of male and female beef cattle through supplementation of omega-3 fatty acids on reproductive development, fatty acid profiles, oxidative status, and semen quality in developing beef bulls, and the evaluation of anogenital distance as a physiological phenotype as a predictor of reproductive tract maturity and the outcome of pregnancy in beef females. These two studies provide a comprehensive analysis of how nutrition and indicators of reproductive maturity, such as anogenital distance, contribute to the variation in fertility, offering practical insight for herd-level management. In the first experiment, developing beef bulls ($n = 39$) were assigned to one of three dietary treatments for an 80-day supplementation period: a control diet with no omega-3 supplementation (CON), a moderate rate of omega-3 inclusion (MOD; 0.22 kg per head daily), or a high omega-3 rate of inclusion (HI; 0.44 kg per head daily). Supplementation effectively altered circulating fatty acid profiles in serum, increasing omega-3 concentrations in the HI group compared with the CON group throughout the study ($P = 0.02$). Despite the shift in serum fatty acids, no improvements in semen quality traits were observed. Sperm motility during the three breeding soundness exams tended to improve in the HI group compared with the CON group ($P = 0.10$), with no treatment effect on progressive motility or morphology. Omega-3 fatty acid supplementation did not alter the reactive oxygen species in seminal plasma at the end of the supplementation period. Scrotal development and carcass characteristics were unaffected by treatment, but the MOD group had the most favorable G:F ratio and was significantly different

from the CON group ($P = 0.04$). Supplementation neither enhanced nor impaired many semen and performance metrics throughout the 80-day supplementation period. The second experiment explored the fertility of beef cattle females ($n = 2,663$) by investigating the anogenital distance as a predictor of pregnancy and the use of reproductive tract score in a subset of heifers ($n = 933$). Anogenital distance has been proposed as a noninvasive indicator of prenatal androgen exposure and postnatal reproductive maturation in several species, yet limited data are available in beef cattle. Cattle in this study were assigned to quartiles and tertiles based on the anogenital distance for each location, as well as some heifers being assigned a reproductive tract score. Pregnancy outcomes from fixed-time artificial insemination were used to assess the predictive power of anogenital distance and reproductive tract score. Although anogenital distance exhibited natural variation and was associated with developmental traits, the reproductive tract score consistently emerged as the strongest predictor of pregnancy. Heifers with a greater reproductive tract score were more likely to become pregnant through fixed-time artificial insemination. When modeled together, anogenital distance contributed minimal predictive power beyond reproductive tract score, suggesting that anogenital distance alone cannot replace reproductive tract score as a standalone tool for heifer selection. The presence of measurable anogenital distance variation supports the continued exploration of anogenital distance as part of a set of physiological indicators that captures reproductive tract maturation not fully represented by the reproductive tract score. Collectively, these two studies contribute important insight into the biology of fertility in beef cattle. The bull supplementation trial demonstrates that omega-3 fatty acids effectively modify blood lipid profiles, but short-term supplementation did not yield substantial improvements in semen quality or oxidative status. The anogenital distance offers potential for identifying females with a greater probability of becoming pregnant through timed artificial

insemination, but the reproductive tract score remains the most reliable tool available for predicting pregnancy outcomes. Integration of nutritional and physiological assessments across sexes underscores the multifactorial nature of fertility and emphasizes the importance of using validated, biologically meaningful indicators to enhance reproductive efficiency in beef cattle production.

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Dedication

This thesis is dedicated to all the family members, friends, colleagues, students, and anyone else that helped me during my time spent at Kansas State University.

Chapter 1 - Literature Review

Fertility

Fertility is a multifaceted term with numerous interpretations and no universally accepted definitions. The most simplistic definition of fertility can be described as: the quality or state of being fertile, or the birth rate of a population (Merriam-Webster, 2004). While straightforward, this definition oversimplifies the complex biological processes required by both males and females to be physiologically capable of conception, gestation, and parturition. Historically, fertility research has focused on either the male or female in isolation, rather than considering reproductive success as a product of both. Consequently, when conception fails or a pregnancy is not sustained, the cause has often been attributed to one sex being non-fertile.

In males, fertility has been described using various functional measures beyond what is defined by the dictionary. Early studies defined male bovine fertility as the relationship between the number of services and conception when mated with fertile females (Dawson, 1938) or the association between spermatozoa concentration per ejaculate and conception rates (Lasley and Bogart, 1943). Subsequent research demonstrated that ejaculation of bulls at least twice a day, consistent with the routine collection schedules, yields a satisfactory fertility rate (Mercier et al., 1949; Bratton and Foote, 1954; Van Demark et al., 1955). Interestingly, no significant difference was observed in fertility outcomes based on the interval between ejaculations, whether bulls were collected daily or at intervals up to 30 days (Ellenberger and Lohmann, 1946).

Prolonged or excessive collection schedule can impair reproductive performance. Perry (1952) reported that bulls subjected to daily collections for 57 consecutive days ceased sperm production until provided a rest period of approximately one week. This observation led to more extensive work on the overuse or overcollection effects on temporarily infertile males. The

subsequent analyses of the stud facility records were designed to test the impact of collection frequency on fertility (Dawson, 1938; Schmidt et al., 1956). Hafs et al. (1959) examined mature dairy bulls collected daily over a 32-week period and observed a 73% non-return rate of 60-90 days, compared to a 70% non-return rate among bulls collected only once per week. These findings indicate that long-term fertility was not significantly affected by the collection frequency or semen storage temperature (-79°C, 5°C, or 15-25°C), even when using commercially available extenders for approximately 49,000 inseminations.

Although definitions of male fertility vary, it is widely recognized that the bull contributes approximately half of the herd's overall fertility potential (Thomas, 2021). Within breeding populations, about 5% of bulls are classified as infertile, while an additional 25% are considered sub fertile based on outcomes from breeding soundness exams (BSE) (Kennedy et al., 2002).

The age at puberty has been shown to be significantly correlated with the age at semen collection when the first sperm cell was recovered. Similarly, puberty was highly correlated with the age at collection for the first motile sperm obtained using an artificial vagina (Wolf et al., 1965). In developing bulls, electroejaculation at two-week intervals beginning at 25 weeks of age produced the first motile sperm by approximately 43 weeks of age (Wolf et al., 1965). When the collection interval was extended to four weeks, the first motile sperm appeared at 41 weeks in purebred bulls and at 36 weeks in crossbred bulls (Bellows et al., 1964). Weekly semen collections using an artificial vagina yielded motile sperm at around 43 weeks of age (Wolf et al., 1965). Collectively, these findings demonstrate that electroejaculation provides a reliable and practical method for obtaining semen from young bulls, particularly during BSE, as it allows for rapid and safe sample collection.

While male fertility has often been the focus of early reproductive studies, female fertility has also been defined in multiple ways depending on the reproductive objectives. For instance, Dawson (1938) considered a cow fertile up to the time of her last successful conception, regardless of how many services or sires were required. Earlier studies varied in their definitions based on specific reproductive variables, emphasizing that fertility is not a fixed trait but a dynamic measure influenced by management and physiology. In modern beef production systems, fertility is more commonly evaluated using performance metrics such as days open, days postpartum, and calving interval. These measures have become essential tools for herd-level reproductive management and for assessing individual cow productivity within well-managed operations. However, before fertility can be optimized, animals must first reach reproductive maturity. Understanding the attainment of puberty is therefore critical to improving reproductive efficiency and advancing genetic progress within beef herds

Attainment of Puberty

Puberty is a multifactorial process influenced by several interrelated components, including hormonal regulation, growth, nutrition, and genetics. These factors rarely align perfectly in time, yet each contributes to the attainment of reproductive maturity in beef cattle. Puberty is commonly defined as the stage at which an individual becomes capable of producing viable gametes and reproducing successfully. The Merriam-Webster dictionary (2004) describes puberty as the period of first becoming capable of sexual reproduction through the production of sex hormones and maturation of the gonads, while Senger (2012) defines it as the ability of an individual to successfully accomplish reproduction of the species. Although physical signs can indicate that puberty has been reached, it is best viewed as a gradual development process that

requires adequate gonadotropin-releasing hormone (GnRH) secretion at appropriate frequencies to activate the hypothalamic-pituitary-gonadal axis.

In females, puberty is often defined as the age at first estrus, first ovulation, or the age at which a heifer can sustain a pregnancy. The first estrus is typically a silent ovulation, meaning it occurs without visible behavioral signs (Senger, 2012). Ovulation can be identified more precisely through ovarian palpation or ultrasonography, though such methods are labor-intensive. While the mechanisms governing the onset of puberty are not fully understood, adequate body condition and adipose reserves appear to be necessary before cyclicity begins (Schneider, 2004; Senger, 2012).

In males, several definitions of puberty exist. Foote (1969) described it as the stage at which spermatogenesis is complete, libido is expressed, and penile development permits normal ejaculation- collectively allowing a male to successfully impregnate a female. Despite multiple definitions, a widely accepted criterion has emerged: puberty in bulls is defined as the age at which an ejaculate first contains a minimum of 50×10^6 spermatozoa with at least 10% motility is required to classify a bull as pubertal, (Wolf et al., 1965; Killian and Amann, 1972; Barber and Almquist, 1975; Lunstra et al., 1978). Although only 10% motility is required to classify a bull as pubertal, a minimum of 30% motility is needed to pass a BSE. Developing bulls are typically evaluated between 12 and 15 months of age, when physical examination, reproductive tract evaluation, and semen analysis are conducted. While the BSE provides a standardized assessment of reproductive potential, it does not quantitatively measure libido and may not fully capture incomplete penile development. According to Foote (1969), the attainment of puberty in bulls requires viable sperm production, expression of sexual aggression, and completion of penile maturation.

Despite the standardized definition of puberty in bulls, considerable variation exists in the age at which it is achieved across breeds. Lunstra et al. (1978) reported that Brown Swiss bulls reached puberty earlier and at greater body weights than Hereford, Angus, F₁ Hereford x Angus, F₁ Angus x Hereford, and Red Poll bulls, with Herefords being the last to meet all pubertal criteria. Similarly, Wolf et al. (1965) observed that Hereford and Angus bulls attained puberty around 45 weeks of age. Dairy bulls generally reach puberty earlier than purebred or crossbred beef bulls, likely due to the differences in growth rate and metabolic signaling. Although nutritional interventions can hasten sexual development, consistent evidence indicates that dairy breeds inherently attain puberty earlier than beef breeds.

Some bulls experience delayed penile development compared to their peers. Adhesion between the glans penis and preputial mucosa can prevent natural ejaculation, a condition regarded as a sign of immaturity (Foote, 1969). Surgical correction is possible but carries a risk of tearing and infection if not performed carefully. Thus, incomplete separation of the glans from the preputial mucosa is generally indicative of developmental immaturity rather than a permanent defect.

Variation in ejaculate volume or sperm concentration can also influence the apparent age of puberty. When stricter criteria are applied such as 100×10^6 sperm with at least 50% progressively motile, bulls average approximately 48 weeks of age at puberty (Wolf et al., 1965). Bellows et al., (1964) reported that straightbred bulls achieved first ejaculation at 45 weeks of age compared with 41 weeks in crossbred bulls. Lunstra et al. (1978) further observed no significant differences in scrotal circumference at puberty among breeds with measurements ranging from 25.9 to 30.1 cm. The use of a standardized, widely accepted definition of puberty,

therefore, enables consistent comparison of developmental milestones across studies and genetic lines.

Growth patterns also play a key role in reproductive development. While the lifetime growth in cattle follows a sigmoidal curve, the phase between puberty and one year of age tends to be linear (Barber and Almquist, 1975). During this period, rapid changes occur in endocrine function, muscle accretion, and fat deposition. The timing of sexual maturation in bulls is primarily governed by hypothalamic activity and the secretion of GnRH which initiates the downstream release of gonadotropins that stimulate testicular function (Brito, 2024). Although age, genetics, and nutrition all contribute to pubertal development, growth rate exerts a particularly strong influence on the age at which bulls reach reproductive maturity. Given the integral role of development in reproductive function, understanding the factors that influence growth provides valuable insight into how bulls progress toward sexual maturity.

Growth

Because the attainment of puberty is closely tied to physiological development, examining patterns of growth provides insight into the progression toward reproductive maturity in beef bulls. Growth in cattle does not occur in a linear manner throughout life. Management strategies such as ad libitum feeding, limit feeding, and a stair-step nutrition can be used to influence the rate of gain in developing bulls and replacement heifers. The natural growth curve of cattle follows a curvilinear pattern. Growth begins at birth when nutrient demand is high, but the rate of growth is relatively slow due to limited feed intake and immature digestive capacity. Nutrient requirements increase substantially throughout the prepubertal stage as muscle tissue deposition accelerates and the animal undergoes adolescent physiological changes.

Once puberty is reached, a smaller proportion of nutrients is directed toward growth, resulting in a slower rate of weight gain. Cattle continue to grow until they reach their genetically determined mature size, which is influenced by environmental conditions such as nutrition and management. The proportions of muscle and adipose tissue deposition vary across different stages of the growth curve and are influenced by breed, sex, and plane of nutrition (Berg and Butterfield, 1968).

Following the onset of puberty at approximately 9 months, beef bulls experience a predominantly linear phase of growth lasting until about 12 months, occurring mainly between 7 and 13 months of age (Barber and Almquist, 1975). This steady growth phase reflects the consistent nutrient demands required for continued development up to maturity, within the limits set by the environment. During this period, Hereford bulls tended to weigh less than other beef breeds, whereas Brown Swiss bulls were heavier than beef breeds (Lunstra et al., 1978). Interestingly, bulls that experienced more rapid weight gain during development produced pubertal ejaculates of lower quality compared to those that gained weight more moderately (Barber and Almquist, 1975).

The relationship between growth and reproductive development has also been explored across species. Van Demark (1956) reported that testicular size was directly related to body weight, with approximately 80% of the variation in testicular weight explained by differences in body weight among bulls. This strong association highlights the interdependence of somatic growth and reproductive organ development during maturation. The physiological and endocrine mechanisms driving somatic growth also influence testicular development, marking a critical stage in the progression toward reproductive competence.

Testicular Development

Testicular development begins during gestation with differentiation of the gonads, marked by changes in the genetic tubercle. The majority of testicular growth occurs within the first two years of life (McGowan et al., 2018). Although testicular growth can be described as linear during certain stages, the overall developmental trajectory follows a sigmoid pattern (Abdel-Raouf, 1960; Macmillan and Hafs, 1968a; Amann, 1983).

During the prepubertal period, GnRH secretion and LH pulse frequency remain relatively low. As puberty approaches, increased GnRH secretion stimulates more frequent LH pulses, which, in turn, elevate testosterone secretion from the Leydig cells and initiate spermatogenesis within the seminiferous tubules (Amann, 1983). This hormonal shift coincides with a phase of rapid testicular growth occurring between 6 and 12 months of age (Amann, 1983). The expansion of the testes during this stage reflects an increase in seminiferous tubule diameter and length, along with the growing volume of testicular parenchyma (Curtis and Amann, 1981).

Between 25 weeks of age and puberty, rapid enlargement of the seminiferous tubules occurs, accompanied by proliferation and differentiation of germ cells, which mature into spermatozoa between 32 and 40 weeks of age. Around 30 weeks, Leydig cell populations become sufficient for testosterone production, while Sertoli cell numbers are adequate to support spermatogenesis between 30 and 40 weeks (Rawlings et al., 1972). Although rapid testicular growth continues through approximately 50 weeks of age, the rate slows thereafter as the spermatogenic process stabilizes. Despite this decline in growth rate, the differentiation of pre-spermatogonia, the appearance of adult Leydig cells, and the establishment of mature Sertoli cells continue (Rawlings et al., 1972).

Endocrine and metabolic factors also influence testicular growth. Proliferation of Sertoli cells was observed in 8-week-old calves treated with a combination of insulin-like growth factor-1 (IGF-1) and follicle-stimulating hormone (FSH), suggesting that metabolic status early in life can affect testicular development and possibly have lasting effects beyond puberty (Dance et al., 2017).

Scrotal circumference remains the most practical and widely used indicator of testicular development. Measurements taken between seven and ten months of age can reliably predict age at puberty, and these values appear consistent across breeds and independent of body weight (Lunstra et al., 1978). While the measurement encompasses the scrotum, testes, and epididymides, researchers have extensively evaluated both testicular mass and circumference in relation to sperm production. Scrotal circumference is typically measured at the widest point of the scrotum using a flexible tape, while testes length, width, and depth may be determined using calipers (Foote, 1969; Hahn et al., 1969). The measures are highly repeatable, with coefficients of repeatability exceeding 0.90, indicating minimal variation between technicians and strong reliability for evaluating pubertal progression (Hahn et al. 1969).

Strong correlations have been reported between scrotal circumference and testicular weight, both in situ ($r = 0.94$; Willett and Ohms, 1957) and in mature bulls ($r = 0.92$; Hahn et al., 1969). Among breeds, testis size was smaller in Hereford bulls and larger in Brown Swiss bulls, with no differences detected among Angus, F_1 Hereford x Angus, F_1 Angus x Hereford, or Red Poll bulls from 7 to 13 months of age (Lunstra et al., 1978). Scrotal circumference serves as a useful indicator of sperm production and testicular development in young bulls, although this relationship weakens after approximately 5 years of age as physiological changes in the testes alter the connection between size and spermatogenic capacity (Hahn et al., 1969).

Previous research has consistently demonstrated that testis size or weight at slaughter is positively correlated with sperm reserves and production capacity (Van Demark, 1956; Boyd and Van Demark, 1957; Willett and Ohms, 1957; Almquist and Amann, 1961; Amann and Almquist, 1961a, 1961b; Hahn et al., 1969). Testicular development in beef bulls increases linearly between 7 and 13 months of age and is strongly correlated with age ($r = 0.88$; Lunstra et al., 1978). However, after approximately six years of age, this relationship declines markedly ($r = 0.12$; Hahn et al., 1969). These data suggest that maximum scrotal circumference is achieved between five and six years of age and remains relatively stable throughout mature adulthood. Degenerative changes, including tubular regression and interstitial tissue decline, have been observed in bulls beyond five years of age (Hooker, 1944).

Spermatogenic activity begins early in development. Primary spermatocytes appear as early as 15 weeks of age, secondary spermatocytes by 26 weeks, and mature sperm between 32 and 36 weeks (Phillips and Andrews, 1936). Quantitative studies of spermatogenesis across species – including bulls, rats, and poultry – have identified similar age-related declines in reproductive efficiency (Walton and Edwards, 1938; Jones and Lamoreux, 1942; Clermont and Morgentaler, 1955). Bulls displaying abnormal testicular development or reduced spermatogenesis relative to testis size may exhibit testicular lesions or degenerative changes affecting sperm motility and fertility (Dunn and McEntee, 1964).

The period of rapid testicular growth following 25 weeks of age coincides with the differentiation of mature Leydig and Sertoli cells and rapid epididymal development beginning around 28 weeks (Abdel-Raouf, 1960; Macmillan and Hafs, 1969). Postnatal secretion of FSH and LH before 25 weeks of age is crucial for the differentiation and development of these cells, while a subsequent reduction in gonadotropin levels allows for a marked increase in testosterone

production (Rawlings et al., 1978). Hormonal shifts occurring between 25 and 30 weeks of age thus represent a critical transition point linking growth, metabolism, and sexual development in young bulls. The morphological and structural maturation of the testes is closely regulated by endocrine activity. Thus, understanding the hormonal changes that occur during puberty is essential to fully explain the physiological mechanisms driving testicular development and the cost of sperm production.

Hormones at Puberty

A primary driver of puberty and sexual development in beef cattle is the endocrine system, which regulates the production and release of hormones that coordinate reproductive and metabolic processes. Hormonal concentrations fluctuate throughout the estrous cycle in females and during puberty in both sexes, reflecting the dynamic nature of endocrine control during development. The hormonal mechanisms associated with puberty attainment differ between males and females. One key distinction lies in hypothalamic function – females possess both a surge and tonic center that enable the rapid, simultaneous release of large quantities of hormones, whereas males have only the tonic center (Senger, 2012; Day and Nogueira, 2013). The difference governs sex-specific patterns of hormone secretion and reproductive maturation.

Endocrine signals act throughout the body by stimulating the production and release of hormones into the bloodstream, allowing them to bind to their respective target tissues and elicit physiological responses. These hormones play essential roles in growth, metabolism, and reproduction, with each class contributing uniquely to sexual development and gametogenesis in beef cattle (Brito, 2024). Hormones can be classified in several ways, but the most common method is by chemical structure, categorizing them into neuropeptide or glycoprotein, steroid, amino acid-derived, and eicosanoid categories (Nussey and Whitehead, 2001). This classification

framework helps explain the diversity of hormone action and provides a foundation for understanding how specific hormones regulate puberty onset in bulls.

The initiation of puberty begins with increased secretion of GnRH from the hypothalamus. Gonadotropin releasing hormone enters the hypophyseal portal system and stimulates the gonadotroph cells of the anterior pituitary to release LH and FSH into circulation (Nedresky and Singh, 2022). These gonadotropins act on the testes to promote Leydig and Sertoli cell function, spermatogenesis, and steroid hormone synthesis. The secretion of these hormones is modulated by complex positive and negative feedback loops involving gonadal steroids such as progesterone, estradiol, and testosterone (Schanbacher, 1982). Feedback strength and direction vary depending on developmental stage, hormone concentrations, and physiological context.

During postnatal development, a characteristic rise in LH followed by a decrease in FSH between 20 and 25 weeks of age suggests that negative feedback – mediated by estrogens and possibly androgens – helps terminate the early postnatal LH surge and refine gonadotropin regulation (Kaneko et al., 2001). This endocrine shift represents a critical turning point in male reproductive maturation as the hypothalamic-pituitary-gonadal axis becomes fully functional. To further understand the hormonal regulation of puberty, it is necessary to consider the peptide and protein hormones that first initiate the reproductive endocrine cascade. Among these, the neuropeptide and glycoprotein hormones – including GnRH, LH and FSH – play central roles in activating the hypothalamic-pituitary-gonadal axis coordinating testicular development in bulls.

Neuropeptide or Glycoprotein Hormones

Most peptide or glycoprotein hormones relevant to reproduction are secreted from the anterior pituitary and are regulated through the hypothalamic-pituitary-gonadal axis. The

hypothalamus is inherently female; however, during gestation, testosterone produced by the fetal testes defeminizes the brain, preventing the development of the surge center (Senger, 2012).

The release of GnRH from the hypothalamus into the hypothalamic-hypophyseal portal system enables GnRH to act on gonadotroph cells in the anterior pituitary, stimulating the secretion of LH and FSH. Metabolic cues can influence and regulate the secretion of GnRH based on nutritional and energetic status (Blache et al., 2003). Concentrations of somatotropin decline during puberty in bulls, reflecting the attainment of an advanced stage of somatic development necessary for efficient sperm production (Brito et al., 2007a, 2007d). Additionally, the observed decrease in LH and FSH concentrations around 24 weeks of age coincides with accelerated testicular growth, suggesting that GnRH secretion may, to some extent, regulate the tempo of testicular development (Curtis and Amann, 1981).

Among the gonadotropins, FSH plays a particularly critical role in coordinating testicular growth and spermatogenic development during the pubertal transition, acting in close interplay with LH to regulate Sertoli cell function and germ cell maturation.

Follicle Stimulating Hormone

Follicle-stimulating hormone consists of an alpha subunit containing 92 amino acids and a beta subunit that is 111 amino acids (Sohn et al., 2003). The primary function of FSH in males is to stimulate Sertoli cell activity within the testes, while in females, it promotes follicular development. In males, the pulsatile release of GnRH drives low-amplitude, high-frequency secretion of FSH. Due to the absence of a surge center in the male hypothalamus, GnRH is released in a tonic pattern, resulting in pulsatile secretion of testosterone by the Leydig cells.

The binding of FSH to Sertoli cells within the seminiferous tubules promotes the development of spermatogonia. Concurrently, pulsatile testosterone secretion alleviates the

negative feedback exerted on FSH via GnRH suppression, allowing for proper coordination between the two hormones. Sertoli cells also convert testosterone into dihydrotestosterone and estrogens, in addition to producing inhibin, which provides a separate negative feedback mechanism. In males, circulating inhibin concentrations are high during the early postnatal period but decline by seven to eight months of age (Miyamoto et al., 1989; Matsuzaki et al., 2001). Although the physiological role of estradiol and estrogens produced by Sertoli cells in males remains somewhat unclear, their secretion has been well documented (Senger, 2012).

Pituitary FSH concentrations decline at puberty and are more closely associated with reproductive tract development than LH concentrations in Holstein bulls (Macmillan and Hafs, 1968b). In Hereford bulls, those classified as low sperm producers ($0.18 \mu\text{g}/\text{mg}$) exhibited nearly double the pituitary FSH concentrations compared to high sperm producers ($0.10 \mu\text{g}/\text{mg}$; Swanson et al., 1971). Given that anterior pituitary LH concentrations remained relatively constant with age, FSH may play a more critical role in the initiation of puberty in males (Macmillan and Hafs, 1968b). The negative correlation between pituitary FSH and gonadal sperm concentrations ($r = -0.45$) further suggests that as spermatogenesis progresses, pituitary FSH declines, highlighting the hormone's role in testicular function (Swanson et al., 1971).

Reports describing circulating FSH concentrations during bull development are inconsistent, with some studies observing no clear pattern (McCarthy et al., 1979; Amann and Walker, 1983), while others report elevated concentrations that decline by approximately 25 weeks of age, appearing less pulsatile than LH (Miyamoto et al., 1989; Evans et al., 1996; Bagu et al., 2006).

While FSH primarily supports Sertoli cell development and spermatogenesis, LH plays a complementary and equally critical role by stimulating Leydig cell differentiation and

testosterone secretion, processes essential for the completion of puberty and maintenance of testicular function.

Luteinizing Hormone

Luteinizing hormone is a glycoprotein composed of the common alpha subunit shared with FSH, consisting of 92 amino acids, and a beta subunit unique to LH with 121 amino acids and several oligosaccharides attached throughout its structure. Luteinizing hormone is produced by gonadotroph cells in the anterior pituitary, and its secretion is stimulated by the pulsatile release of GnRH. Leading up to puberty, LH secretion gradually increases, transitioning to a more defined pulsatile release pattern after puberty is attained.

In females, LH plays a critical role in initiating ovulation and maintain the corpus luteum. The female hypothalamus contains both the surge and tonic centers, allowing of the characteristic preovulatory LH surge that occurs approximately every 21 days. This surge, preceded by rising estradiol concentrations from the dominant follicle, triggers ovulation and luteinization of the follicle. Progesterone exerts a strong negative feedback effect on GnRH and LH secretion during the luteal phase; however, when progesterone is removed, either naturally or artificially, pulse frequency and amplitude of GnRH increase. Once estradiol reaches a threshold level, it induces positive feedback on the hypothalamus, generating the LH surge necessary for ovulation (Senger, 2012).

In males, LH acts primarily on the Leydig cells of the testes to stimulate testosterone production. Unlike females, males lack an LH surge due to the absence of a hypothalamic surge center, resulting in continuous, pulsatile LH release. Each LH pulse stimulate Leydig cells to synthesize progesterone, which is rapidly converted to testosterone. Circulating LH concentrations remain elevated for approximately 30- 75 minutes, while testosterone synthesis

and secretion occur within 30 minutes of LH release (Senger, 2012). Testosterone and estradiol exert negative feedback on GnRH secretion, but the sensitivity of GnRH neurons to this feedback diminishes as bulls approach puberty, allowing for increased gonadotropin release and stimulation of testicular growth and function.

Breed differences in LH concentrations have also been reported. Red Poll bulls (3.2 ng/mL) exhibited significantly greater concentrations compared to Hereford bulls (2.3 ng/mL), while Angus (2.7 ng/mL), Brown Swiss (2.8 ng/mL), F1 Herford x Angus (2.5 ng/ml), and F1 Angus x Hereford (2.4 ng/mL) bulls did not differ significantly (Lunstra et al., 1978). The weight of the anterior pituitary and LH concentration in Hereford bulls were similar to those of yearling Holstein bulls (Macmillan and Hafs, 1968b; Swanson et al., 1971). Serum LH concentrations of Hereford bulls increased from 1.93 ng/mL at 10 months of age to 3.22 ng/mL at 12 months, followed by a decline to 2.22 ng/mL at 12.5 months (Swanson et al., 1971). The decrease was attributed to stress associated with transportation and slaughter, while the overall increase with age underscores the role of LH in reproductive maturation. Furthermore, Hereford bulls displayed more than double the serum LH concentrations of Holstein bulls at 12 months of age (Swanson et al., 1971; Rawlings et al., 1972).

Across developmental stages, LH secretion patterns reveal that concentrations are low at 4-5 weeks of age, peak between 12-16 weeks, and then decline until approximately 25 weeks, after which levels remain low but pulsatile (Jones and Lamoreux, 1942; Rawlings et al., 1978; Lacroix and Pelletier, 1979; McCarthy et al., 1979; Amann and Walker, 1983; Rodriguez and Wise, 1989; Rawlings and Evans, 1995; Aravindakshan et al., 2000; Bagu et al., 2006). The first LH peak can be attributed to increased GnRH pulse frequency (Rodriguez and Wise, 1989; Rawlings and Evans, 1995).

Blood LH concentrations vary considerably during the prepubertal period and stabilize around 40 weeks of age (Rawlings et al., 1978). Bulls reaching puberty earlier at approximately 42 weeks exhibit greater LH concentrations between 10-20 weeks than those maturing later (Evans et al., 1995; Aravindakshan et al., 2000). Serum LH levels have been positively correlated with age, body weight, and scrotal circumference ($r = 0.39, 0.37, \text{ and } 0.44$; Lunstra et al., 1978). In contrast, heifer pituitary LH concentrations decrease by approximately 54% between 7 and 12 months of age, suggesting that LH may have a more defined regulatory role in pubertal onset in females than in males.

While LH and other glycoprotein hormones act as upstream regulators of reproductive function, their biological effects are largely mediated through the synthesis and action of steroid hormones. Steroid hormones – including testosterone, estradiol, and progesterone – serve as critical mediators of sexual differentiation, gametogenesis, and feedback regulation within the hypothalamic-pituitary-gonadal axis. The transition from peptide-based signaling to steroidal regulation represents a key step in the coordination of reproductive development and function in both sexes.

Steroid Hormones

Steroid hormones are lipid-soluble compounds derived from the precursor cholesterol. Through a series of enzymatic conversions, cholesterol is metabolized to form corticoids, androgens, estrogens, and progestins. These reactions occur within steroidogenic tissues, including the adrenal cortex, testes, and ovaries, where enzymes catalyze each step of steroidogenesis. Steroid hormones exert broad regulatory effects, influencing metabolic processes, stress responses, reproductive function, and sexual differentiation. Their ability to diffuse across cell membranes and bind to intracellular receptors allows for precise modulation

of gene transcription, making them essential regulators of both reproductive development and physiological homeostasis.

Among steroid hormones, androgens represent the primary class responsible for the development and maintenance of male reproductive function. These hormones serve as key intermediates in the steroidogenic pathway and precursors for estrogen synthesis, with testosterone being the most biologically significant androgen in male reproduction.

Androgens

Androgens are the principal class of steroid hormones produced by the testis and play a central role in the regulation of puberty and male fertility. These hormones are primarily synthesized by the Leydig cells, which become active at an early developmental stage and contribute to the maturation of the seminiferous tubules, growth of accessory sex glands, and the emergence of secondary sexual characteristics (Foote, 1969). Among the androgens, testosterone is the predominant of the male reproductive tract, and expression of reproductive behavior in adult bulls.

Foote (1969) proposed that during the prepubertal stage in bull calves the biosynthetic activity of the testes transitions from androstenedione dominance to testosterone production as Leydig cell function matures. This shift corresponds with the enlargement of seminiferous tubules and seminal vesicles, both androgen-dependent processes stimulated by rising testosterone concentrations within Leydig cells. Supporting this developmental transition Lindner and Mann (1960) report that the androstenedione to testosterone within the testes of bulls changes from approximately 1:1 at four months of age to 1:10 by nine months. In some bulls older than nine months, androstenedione levels declined below detectable limits, indicating a maturation shift toward testosterone as the principal androgen.

During early postnatal development, low concentrations of androstenedione and dihydrotestosterone are also secreted from the testes. Although these levels are substantially lower than testosterone, they are believed to influence aspects of testicular development, potentially through paracrine effects that remain incompletely understood (Rawlings et al., 1972; McCarthy et al., 1979; Sundby et al., 1984; Rawlings and Cook, 1986). This period aligns with the rapid proliferation of Leydig cells and the transient elevation of LH secretion from the anterior pituitary, highlighting a coordinated endocrine mechanism supporting gonadal maturation.

Comparative studies in rodents show a similar pattern. In rats, androstenedione was undetectable in plasma before 30 days of age; however, by 30 to 40 days, testicular concentrations of androstenedione and testosterone were approximately equal (Resko et al., 1968). Notably, androstenedione was detectable within testicular tissue during the neonatal period, even when plasma levels remained below the assay detection limits, suggesting localized steroidogenic activity (Resko et al., 1968).

Additional hormones may interact with androgens to support testicular development and spermatogenesis. Prolactin, for instance, although not yet quantified in bulls during early research, has been shown in rodents to enhance testosterone-induced seminal vesicle growth (Peyre et al., 1968) and stimulate androgen synthesis by testicular interstitial tissue (Bartke and Lloyd, 1970). These findings imply that prolactin may act synergistically with LH and androgens to optimize testicular function.

Overall, androstenedione appears to play a transient but important role during early sexual differentiation, with testosterone gradually assuming dominance as the primary androgen through puberty and into sexual maturity (Resko et al., 1968). The developmental transition from

androstenedione to testosterone synthesis reflects a critical step in the activation of the hypothalamic-pituitary-gonadal axis and the onset of reproductive competence in bulls.

Building upon the androgenic foundation, testosterone represents the principal steroid responsible for the initiation and maintenance of male reproductive capacity. Its production, regulation, and physiological actions define the hallmark endocrine events of puberty and fertility in the bull.

Testosterone

The synthesis of testosterone within the testes is initiated by the binding of LH to the membrane bound LH receptors on Leydig cells. This interaction stimulates the conversion of cholesterol to progesterone, which is subsequently transformed into testosterone and other androgens through a series of enzymatic reactions. Testosterone synthesis and secretion occur rapidly, typically within thirty minutes of LH stimulation, and follow a pulsatile pattern lasting approximately 20 to 60 minutes. This pulsatile pattern allows Leydig cells to remain responsive to continuous LH signaling. However, chronic or excessive LH exposure can lead to reduced testosterone secretion due to increased aromatization of testosterone to estrogens within Sertoli cells (Senger, 2012).

Once released into systemic circulation, testosterone concentrations are diluted to approximately to $1/500^{\text{th}}$ of the concentrations in the testes. This steep gradient is essential for maintaining sufficient localized androgen concentrations to support spermatogenesis while preventing excessive negative feedback on the hypothalamus, thus preserving basal GnRH secretion and subsequent LH and FSH release (Senger, 2012). Lindner (1963) observed that testosterone concentrations in testicular lymph were substantially higher than in arterial blood,

indicating that the testicular microenvironment retains adequate testosterone availability for seminiferous tubule function despite low systemic levels.

During development, testosterone concentrations vary with age and physiological maturation. In rats, testicular testosterone concentrations are greatest at one day of age and decline thereafter (Resko et al., 1968). In bulls, serum testosterone increases linearly from approximately 7 to 13 months of age, showing positive correlations with body weight ($r = 0.26$) and scrotal circumference ($r = 0.51$; Lunstra et al., 1978). Similar trends have been documented across beef breeds and Brown Swiss, with testosterone levels rising steadily as bulls approach puberty (Lunstra et al., 1978). Holstein bulls, in contrast, exhibit more erratic increases in testosterone from birth to 11 months, followed by a decline near one year of age. Plasma androstenedione concentrations also fluctuate during early development, remaining detectable until approximately six months of age before declining to undetectable levels by one year (Rawlings et al., 1972).

Multiple studies have reported gradual increases in testosterone concentrations from birth until 20 weeks of age, followed by rapid elevations between 20 and 35 weeks that coincide with accelerated testicular growth (Secchiari et al., 1976; Lacroix et al., 1977; Sundby and Velle, 1980; Miyamoto et al., 1989; Rawlings and Evans, 1995; Evans et al., 1996; Rawlings et al., 2008; Byrne et al., 2018). Bulls with higher testosterone concentrations typically achieve puberty earlier than those with lower concentrations (Lunstra et al., 1978) reinforcing testosterone's central role in testicular maturation and reproductive readiness.

Breed differences in testosterone secretion have been observed. Lunstra et al. (1978) found that Hereford (4.0 ng/mL), F₁ Hereford x Angus (4.7 ng/mL), and F₁ Angus x Hereford (5.1 ng/mL) bulls exhibited linear increases in testosterone from seven to twelve months of age,

where as Angus (5.7 ng/mL), Red Poll (7.5 ng/mL), and Brown Swiss (6.4 ng/mL) bulls displayed greater fluctuations and more complex temporal patterns. Moreover, testosterone concentrations exceeding 1 ng/mL at 20 weeks of age were strongly correlated ($r = 0.67$) with larger testes and greater Sertoli cell numbers at 54 weeks, indicating that early androgen production supports long term testicular growth and spermatogenic capacity (Moura et al., 2011).

Beyond its endocrine functions, testosterone plays critical roles in sexual differentiation and behavior. It facilitates separation of the glans penis from the preputial mucosa, a developmental event required for libido and ejaculation (Foote, 1969) and exerts masculinizing effects on the hypothalamic-pituitary axis and peripheral tissues (Resko et al., 1968). Evidence from freemartin cattle confirms testosterone's role in establishing permanent male behavioral and physiological characteristics through early life exposure. Collectively suggesting that testosterone may be the hormone responsible for the masculinization of the hypothalamic-pituitary axis as well as the masculinization of tissues in adults, which has been confirmed in freemartins.

Testosterone serves a crucial role as an endocrine signal in the initiation and maintenance of spermatogenesis. Acting through androgen receptors within Sertoli cells, testosterone promotes the maturation of germ cells, supports the structural integrity of the seminiferous epithelium, and facilitates sperm release into the lumen of the seminiferous tubules. Thus, the synthesis and regulation of testosterone directly govern sperm production and overall testicular function.

Sperm Production

Sperm production in bulls is influenced by an intricate interplay between endocrine function, testicular development, environmental conditions, and nutritional management. These

components work in concert to regulate spermatogenesis within the testes through highly coordinated hormonal and cellular interactions. In bulls, one complete cycle of spermatogenesis requires approximately 61 days, followed by an additional 14 days for epididymal transit and maturation (Amann and Almquist, 1962). The process begins with the action of gonadotropins on the Leydig and Sertoli cells, which together initiate and sustain spermatogenic activity.

Spermatogenesis is defined as the sequential mitotic and meiotic divisions of spermatogonia leading to the formation of specialized spermatozoa. A high intratesticular testosterone concentration typically 100 – 500 times greater than found in peripheral blood is required to initiate and maintain this process (Senger, 2012). Within the seminiferous tubules, Leydig cells primarily secrete testosterone, with small quantities of estrogen, while the Sertoli cells support germ cell development.

The spermatogenic process can be divided into three major phases. The proliferation phase involves mitotic divisions of A-spermatogonia, generating B-spermatogonia while maintaining a population of stem cells for continued renewal. The meiotic phase begins with the primary spermatocyte, during which DNA replication and genetic recombination occurs. Finally, the differentiation phase transforms round spermatids into mature spermatozoa through the development of an acrosome, flagellum, and mitochondrial sheath. Mature spermatids are released into the lumen of the seminiferous tubules and transported to the epididymis, where final maturation and motility acquisition occur over approximately 14 days (Senger, 2012; Staub and Johnson, 2018).

Understanding the rate of spermatogenic production is essential to maximize the reproductive potential of breeding bulls. Knowledge of sperm output enables optimization of semen collection schedules to ensure that adequate sperm numbers are obtained without

impairing subsequent collections. Studies in dairy cattle have reported that the rate of spermatogenesis at one year of age is comparable to that of mature bulls, with later increases in sperm output primarily reflecting continued testicular growth (Boyd and Van Demark, 1957; Macmillan and Hafs, 1968a). However, Amann and DeJarnette (2012) observed that bulls entering their first breeding season at stud may only produce 35-50% of the semen volume of mature sires. Similarly, Hereford bulls averaging 12.5 months of age demonstrated substantial sperm output, possessing significantly greater gonadal and extragonadal reserves than expected for their age (Swanson et al., 1971).

Under natural mating conditions, bulls are capable of high mating frequencies, with reports of six to eleven copulations per day over an eight to nine-day period (Verekina and Smirnov-Ugrjumov, 1940). Early investigations into the total sperm production utilized depletion or exhaustion tests, first described in bulls by Walton and Edwards (1938) and adapted from similar work in rabbits. These tests and subsequent refinements formed the basis for estimating sperm production capacity through repeated collections, varying schedules, and evaluations of storage and output potential.

The extra-gonadal reserves (EGR) of a bull's reproductive tract represent the sperm stored primarily within the epididymis and vas deferens. Estimating EGR provides insight into the number of sperm available before testicular production must replenish the supply, serving as an indirect predictor of daily sperm production (DSP). Daily sperm production is defined as the total number of spermatozoa produced by both testes in one day (Senger, 2012). The rate of DSP is influenced largely by the number of Sertoli cells within the testes (Berndtson et al., 1987). However, predicting DSP in live animals remains challenging, as most early studies validated estimates through exhaustion or depletion trials followed by slaughter to quantify testicular

output but is challenging to predict in live animals, as several studies have measured the rate of semen production in bulls through exhaustion trials followed by slaughtering bulls to validate their findings (Almquist and Hale, 1956; Van Demark, 1956; Boyd and Van Demark, 1957).

Holstein bulls were estimated to produce approximately 2.88 million sperm per gram of testis (Boyd and Van Demark, 1957). Across trials, the average ejaculate contained around 37.3 billion sperm (Almquist and Hale, 1956; Almquist et al., 1958; Almquist and Amann, 1961; Amann and Almquist, 1961a). The earliest reported EGR estimates in bulls were provided by Ortavant (1956), while gonadal sperm reserves were later described in detail by Frederick (1958a), and Almquist et al. (1958). In Holstein bulls, Van Demark (1956) reported production rates of approximately 20×10^6 sperm per gram of testicular and epididymal tissue per week.

The relationship between testis size and sperm output is especially pronounced in younger bulls, but this correlation tends to decline with age (Willett and Ohms, 1957; Hahn et al., 1969). After puberty, both sperm quality and total output continue to increase for a period before reaching a plateau (Almquist and Cunningham, 1967; Lunstra and Echtenkamp, 1982). When scrotal circumference was held constant, a negative correlation between age and total sperm output ($r = -0.60$) was reported, suggesting a gradual decline in spermatogenic capacity with advancing age (Willett and Ohms, 1957; Hahn et al., 1969).

Scrotal circumference is a reliable indicator of testicular volume and sperm production potential, with strong correlations reported between scrotal circumference and testicular volume ($r = 0.96$) and with the exclusion of the epididymis ($r = 0.95$) and between testicular weight and testicular volume ($r = 0.997$; Willett and Ohms, 1957). Although the left testis was, on average, 8.1 g heavier than the right, paired testes weight was significantly correlated with age ($r = 0.51$), but not with body weight ($r = 0.15$; Almquist and Amann, 1961). Testis and epididymis were

closely associated ($r = 0.78$), as were testis weight and testicular sperm content ($r = 0.62$) and sperm concentrations in the cauda and corpus epididymis ($r = 0.65$; Almquist and Amann, 1961). Sperm output across ten ejaculates was highly correlated ($r = 0.80$) with combined testis and epididymal weight at slaughter, accounting for 64% of the variation in total sperm obtained among bulls (Van Demark, 1956).

Corrections for testicular volume that exclude the tunica albuginea (~8.7% of total volume) have been recommended to improve the accuracy of sperm production estimates (Amann and Almquist, 1961a). Despite strong correlations between scrotal circumference and testicular traits, it accounted for only 9% of the variation in spermatozoa production ($r = 0.30$; Willett and Ohms, 1957). Suggesting that testicular efficiency is influenced by additional biological and hormonal factors.

Early studies employed sperm depletion and exhaustion tests to estimate total sperm output per gram of testis—an approach later viewed as limited but historically valuable (Boyd and Van Demark, 1957; Willett and Ohms, 1957; Almquist and Amann, 1961; Amann and Almquist, 1962; Hahn et al., 1969). Edwards (1940) removed 56% of the EGR through depletion in rabbits, while Almquist and Amann (1961) observed a similar 53% reduction in dairy bulls that were sexually rested for 30 days. In bulls, young animals averaged 48.5×10^6 and 63.4×10^6 spermatozoa per gram of tissue in two consecutive exhaustion series, compared with 46.7×10^6 for mature bulls (Willett and Ohms, 1957).

Daily sperm production in mature dairy bulls has been reported to range from 32 to 34 billion sperm per week (Hale et al., 1953; Hafs et al., 1959; Willett and Ohms, 1957). Amann and Almquist (1962) estimated daily production at 12.8 billion for mature Holstein bulls and 5.3 billion for young Shorthorn bulls per testis. Collectively, these studies suggest that maximum

sperm output in mature bulls, regardless of breed, approximates 34 billion sperm per week. Testicular sperm concentrations in Hereford bulls were comparable to those of yearling and mature dairy bulls, averaging 20.6 billion sperm per testis (Almquist and Amann, 1961), which was intermediate between the 8.4 billion reported by Frederick (1958b) and the 29.9 billion reported by Ortavant (1956). Total gonadal sperm reserves in Hereford bulls (21 billion) were lower than in Holsteins (40.6 billion), primarily due to smaller testes (Swanson et al., 1971).

Extragonadal reserves play an important role in reproductive capacity. Yearling Holsteins had approximately 16.6 billion EGR sperm, comparable to 14 billion in Herefords, with ~60% localized in the cauda epididymis awaiting ejaculation (Macmillan and Hafs, 1968a; Swanson et al., 1971). Total EGR ranged from 37.7 to 118.2 billion sperm, with dairy bulls rested for 30 days containing ~37.6 billion sperm in the cauda epididymis – accounting for 54% of the total EGR – while the ductus deferens and ampulla contained 7.6 billion (Almquist and Amann, 1961). Bulls that underwent depletion and were rested for 30 days before slaughter exhibited EGR values ranging from 19.2 to 65.5 billion sperm, with 85% of the total sperm removed during depletion originating from the cauda epididymis, ductus deferens, and ampulla. Rested bulls averaged 45.2 billion sperm in the reproductive tract compared with the 15.8 billion in previously depleted bulls (Almquist and Amann, 1961).

Mature bulls have been reported to contain an average 72.6 billion EGR sperm and 54.8 billion gonadal sperm (Almquist and Amann, 1961; Amann and Almquist, 1961a). When compared across species, sperm production per gram of testis has been estimated at 134 million in boars, 105 million in boars, 105 million in breeding rams, 80 million in nonbreeding rams, and 90 million in bulls (Ortavant, 1956; Amann, 1961). Histological evaluations, however, have yielded lower estimates for bulls at approximately 55 million (Almquist and Amann, 1961).

Testes achieve full spermatogenic activity shortly after puberty, continuing to enlarge until maturity as responsiveness to gonadotropins increases (Hooker, 1937). In young bulls, scrotal circumference explained up to 85% of the variation in sperm production capacity from exhaustion procedures, where as older bulls deviated from linear trends, reflecting a decline in production efficiency with advancing age (Willett and Ohms, 1957).

Collection frequency and management significantly influence semen characteristics. Collecting semen four times per week yielded an estimated 36-44% of total daily sperm production, with mature bulls producing more sperm per ejaculate than younger bulls (Amann and Almquist, 1962; Hahn et al., 1969). Patrick et al., (1949) and Boyd and Van Demark, (1957) demonstrated that at least one night of sexual rest was adequate to restore semen reserves, though Almquist et al., (1958) noted that frequent ejaculation can deplete epididymal reserves. The gonadal reserve represents approximately 3.27 days of semen production (Amann, 1961). Although results differ among studies, seminal plasma appears to aid in the removal of protoplasmic droplets within the epididymis, potentially reducing the numbers ejaculated regardless of collection frequency (Bialy and Smith, 1958). Bulls collected daily for 32 weeks exhibited greater sperm motility than those collected weekly, despite lower sperm concentrations (0.81 vs 1.89 billion sperm/mL; Hafs et al., 1959). Exhaustion trials at 1, 4, and 7 day intervals suggest that sperm filling of the reproductive tract occurs most rapidly within 24 hours (Van Demark, 1956).

Genetic and environmental influences also affect semen traits. In rabbits, semen volume has been shown to be heritable, while sperm concentration is more strongly affected by environmental conditions (Venge and Frölich, 1951). In bulls, however, semen production is less predictable, as body weight and testis size exhibit wide variation (Bane, 1954; Willett and Ohms

1957; Almquist and Amann 1961). Throughout the growth period, body weight and testicular development remain closely associated with sperm production potential (Van Demark, 1956; Boyd and Van Demark, 1957; Willett and Ohms, 1957). Early studies in rabbits also revealed weak relationships between body weight and semen volume (Branton and Salisbury, 1946; Frölich and Venge, 1948; Venge and Frölich, 1951). Finally, methodological limitations such as sperm loss during collection must be considered; Foote and Heath (1963) estimated that up to 12% of sperm from the original ejaculate could remain trapped within the artificial vagina following collection.

Once spermatogenesis and epididymal transit are complete, spermatozoa must acquire the capacity for progressive movement to ensure fertilization success. This acquisition of motility reflects the culmination of morphological maturation and extensive biochemical remodeling within the epididymis, including changes in membrane composition, surface protein expression, and mitochondrial activation. Thus, while spermatogenesis determines the quantity of sperm produced, the development and regulation of motility ultimately dictate sperm functionality and fertilizing potential.

Motility

Once spermatozoa complete maturation within the epididymis, motility becomes a key determinant of their fertilizing potential. Although motility is often used the primary indicator of sperm viability, it does not necessarily confirm that a sperm cell is alive or capable of fertilization. However, it provides an essential assessment of functional competence, reflecting both cellular energy metabolism and membrane integrity (Foote 1969).

Motility evaluation encompasses several parameters, including the proportion of sperm exhibiting progressive movement, the velocity of that motion, and the presence of abnormal

motility patterns. Traditionally, these assessments were conducted manually under light microscopy, but advancements in computer-assisted sperm analysis (CASA) systems have enabled more precise and objective quantification of sperm kinetics.

Gross motility is typically evaluated under low magnification to assess the collective movement of sperm cells within a sample. The Society for Theriogenology uses a descriptive classification system - poor, fair, good, and very good – to characterize sperm motion pattern (Koziol and Armstrong, 2018). This evaluation is influenced by several factors, including sperm concentration, the proportion of cells moving progressively in a forward direction, and the velocity of those cells. External factors such as temperature, dilution, and the type of equipment used can influence observed motility patterns (Barth, 2013).

While gross motility provides a useful overview, it should be interpreted with caution. A more accurate measure of fertilizing potential is obtained through individual assessment of progressive motility. This evaluation must occur promptly after collection, as sperm viability and motility rapidly decline over time unless preserved with an extender. During microscopic examination at 400x magnification, progressively motile sperm are identified as those moving in a linear or near-linear fashion, while round or immotile cells may represent immature germ cells or leukocytes (Koziol and Armstrong, 2018).

Although The Society of Theriogenology does not employ a percentage-based scoring system, it is generally accepted that bulls must exhibit at least 30% progressively motile sperm to meet the minimum threshold for a satisfactory BSE (Koziol and Armstrong, 2018).

While motility provides insight into the functional capacity of sperm, it does not reveal structural integrity or potential defects that may impair fertilization. Therefore, evaluating sperm morphology is essential to complete motility assessment and provide a more complete

understanding of semen quality. Morphological evaluation identifies abnormalities in the head, midpiece, and tail that can affect motility, viability, and the sperm's ability to penetrate the oocyte.

Morphology

Morphology focuses on the percentage of normal cells in the ejaculate, which has been associated with fertility potential in bulls and other species (Barth and Oko 1989; Ostermeier et al. 2001; Kubo-Irie et al. 2005; Enciso et al. 2011; Koziol and Armstrong 2018). Morphological assessment serves as a reliable predictor of mating success, regardless of whether conception occurs through artificial insemination, natural service, or in vitro fertilization (Wiltbank and Parish, 1986; Barth and Oko, 1989; Saacke et al., 2000). A consistent relationship has been established between morphologically abnormal sperm and reduced DNA integrity, with morphology below 70% normal cells being the most common reason bulls fail a BSE or are classified as unsatisfactory breeders (Carson and Wenzel, 1997; Enciso et al., 2011).

The use of stains such as eosin-nigrosin and eosin-aniline blue enables differentiation of viable and nonviable sperm cells. Sperm with damaged membranes absorb the stain, indicating injury or loss of membrane integrity. Notably, staining outcomes have been shown to correlate significantly with pronuclear formation during in vitro fertilization, as non-stained cells were more likely to contribute to normal fertilization (Hancock, 1951; Tanghe et al., 2002).

A widely accepted classification system exists for identifying sperm abnormalities, though some variation may occur due to technician interpretation. Primary abnormalities originate during spermatogenesis, while secondary abnormalities develop during epididymal transit. Tertiary defects, in contrast, arise from environmental or iatrogenic factors (Barth, 2013; Koziol and Armstrong, 2018). Within this framework, all defects affecting sperm heads are

classified as primary, as the Dag effect, mitochondrial sheath defects, and coiled principal pieces (Barth, 2013; Koziol and Armstrong, 2018). It is also not uncommon for a sperm cell to display both primary and secondary abnormalities, in which case it is classified as having both (Barth and Oko, 1989).

Spermatogenesis and sperm quality are influenced by metabolic and endocrine processes; nutritional status plays a critical role in supporting testicular function and overall reproductive efficiency. Nutrients and dietary supplements can influence hormone production, oxidative balance, and membrane integrity, all of which directly affect sperm morphology and motility.

Nutrition

Nutrition represents a complex network of microbial interactions with feedstuffs that supply essential nutrients to the body. Among the many components that define ruminant nutrition, the most critical dietary elements are energy, protein, and essential fatty acids. Nutritional inputs influence physiological processes through a metabolic regulatory system that modulates the hypothalamic-pituitary-gonadal axis, particularly under conditions when the animal's metabolic status is challenged (Schneider, 2004). As a result, nutrient intake can be reflected in changes to metabolic hormones that, in turn, affect the secretion patterns of reproductive hormones, especially during the developmental stages in bulls (I'Anson et al., 1991).

The foundation of modern ration formulation is the net energy system, first established by Lofgreen and Garrett (1968). Their work provided the basis for the National Research Council (NRC) to adopt and publish the net energy equations in 1978, which have since undergone several revisions to reflect the changing genetics, physiology, and growth potential of cattle over the past five decades. Prior to this advancement, energy evaluation systems relied primarily on

TDN and CP. While TDN remains a useful measure of overall energy content – representing the sum of digestible carbohydrates, protein, lipids, and fiber – it fails to account for the various stages of energy loss inherent to the net energy cycle. In ruminants, energy is primarily derived from VFA, which are produced through microbial fermentation of feedstuffs within the rumen and reticulum. This process relies on the coordinated activity of bacteria, archaea, protozoa, and fungi that degrade complex carbohydrates and fiber into VFA available for metabolic use.

Protein nutrition in ruminants is equally intricate. Nitrogen serves as the building block for protein synthesis, and ruminants possess a unique ability to utilize low-quality protein sources or non-protein nitrogen to meet their amino acid requirements. Through the urea cycle, nitrogen can be converted into amino acids, peptides, and ammonia, which are then incorporated into microbial protein by rumen microbes or absorbed post-rationally in the small intestine. Although CP remains the standard metric for expressing dietary protein, it does not accurately reflect the amount of protein truly available to the animal. Instead, the nutritional value of protein depends on its digestibility and partitioning into rumen-degradable (RDP) and rumen-undegradable protein (RUP). The actual utilization of dietary protein can be estimated through measurements of nitrogen excretion in urine and feces (NASEM, 2016).

Lipids encompass all compounds soluble in fat-solubilizing solvents and represent a diverse group of molecules that are approximately 2.25 times more energy-dense than carbohydrates. In beef cattle, lipids are primarily composed of SFA, which contain no double bonds between carbon atoms (Ladeira et al., 2012). In contrast, PUFA contain one or more double bonds, influencing their physical properties and biological roles.

A specific and biologically important subclass of lipids is the EFA that are critical for normal cellular and reproductive function. Mammals cannot synthesize EFA – specifically

linoleic acid (LA; 18:2 n-6), and α -linolenic acid (ALA; 18:3 n-3) because they lack the Δ^{12} and Δ^{15} desaturase enzymes required to introduce double bonds at the 12th or 15th carbon from the carboxyl end. The designation between n-6 and n-3 refers to the location of the first double bond to the methyl end of the fatty acid chain (NASEM, 2016).

The dietary balance of between LA and ALA plays a critical role in regulating long chain PUFA synthesis. Higher dietary ALA suppresses the metabolism of LA to arachidonic acid (ARA), while an increase in LA intake has the opposite effect, reducing the conversion of ALA to longer-chain omega-3 fatty acids (Holman, 1998). Both EFA serve as precursors for eicosanoids, which contribute to cell membrane composition, fluidity, and selective permeability, and give rise to bioactive compounds such as prostaglandins. These metabolites influence numerous physiological processes, including growth, immune function, inflammation, vascular tone, and reproduction.

The EFA composition of feedstuffs varies widely depending on the feed source. Historically, much of the research summarized in the (NASEM, 2016) has focused on the role of essential fatty acids in female reproduction, with comparatively fewer studies exploring their impact in impact. In cattle nutrition, fatty acids have traditionally been viewed primarily as energy sources; however, growing evidence suggests they exert important regulatory effects on reproductive performance. Supplementation with specific fatty acids can also include palatability, enhance feed efficiency, and reduce the physiological effects of heat stress.

Although dose-response data remain limited, variation among published studies can often be attributed to differences in FA source, inclusion rate, duration of supplementation, basal diet composition, timing of feeding, and environmental factors (NASEM, 2016).

Polyunsaturated fatty acids are not synthesized *de novo* in the rumen. Because ruminants lack the necessary enzymes to synthesize or maintain omega-3 and omega-6 PUFA, these FA must be supplied through the diet. Conjugated linoleic acid is produced as an intermediate during the incomplete biohydrogenation of LA and has been reported to exert beneficial effects, including anticarcinogenic, antiatherogenic, and immunomodulatory properties, as well as influences on protein and energy metabolism (Belury, 2002; Pariza, 2004; Palmquist et al., 2005; Chilliard et al., 2007).

The ratio of omega-6 to omega-3 FA in ruminant tissues can be manipulated through grazing management and dietary supplementation strategies (Enser et al., 2000). The World Health Organization recommends a dietary omega-6 to omega-3 ratio between 4:1 and 5:1 to maintain optimal physiological function (World Health Organization, 2003).

Supplementation of PUFA, particularly omega-3 and omega-6 FA, has yielded variable results on reproductive function despite their critical role in cell membranes, which comprise approximately 30-50% of bovine spermatozoa (Poulos et al., 1973; Wathes et al., 2007; Gholami et al., 2010; Fair et al., 2014; Byrne et al., 2017). Supplementation with docosahexaenoic acid (DHA) has been shown to improve sperm motility after nine weeks, as assessed both subjectively and by a CASA, and to enhance sperm membrane integrity, as indicated by a higher proportion of positive hypo-osmotic swell test results (Gholami et al., 2010). Similarly, supplementation with ALA as an omega-3 source, or palmitic acid (PA) as a saturated fatty acid, increased the proportion of plasma membrane – and acrosome–intact sperm cells post–thaw, though no differences were observed in motility, volume, or concentration of ejaculate (Gürler et al., 2015). However, ALA supplementation was associated with elevated lipid peroxidation

(LPO) after post-thaw incubation, suggesting increased susceptibility to reactive oxygen species (ROS) attack on membrane lipids.

Other studies have reported comparable improvements in semen quality with PUFA supplementation. Holstein bulls fed flaxseed oil for 7 weeks demonstrated a greater concentration of live, motile sperm and a reduced incidence of morphological abnormalities (Bastos et al., 2021). Likewise, supplementation of rams with a fish oil extract increased sperm concentration per milliliter, although other semen parameters remained unaffected (Fair et al., 2014). In yearling bulls, omega-6 or omega-3 based supplementation altered sperm PUFA composition but did not affect semen quality traits in either fresh or frozen-thawed samples (Byrne et al., 2017). Notably, omega-3 supplementation improved fresh semen motility in dairy bulls subjected to heat stress (Gholami et al., 2011), and prolonged flaxseed or fish oil supplementation enhanced the proportion of DHA, ALA and total omega-3 FA in sperm (Moallem et al., 2015).

Despite these positive findings, detrimental effects have also been reported. Long-term PUFA supplementation of 10 months in Nellore bulls reduced sperm motility, membrane integrity, and altered lipid proportions (Bastos et al., 2021). Conversely, short-term supplementation with fish or flaxseed oil reduced ROS concentrations during and after treatment without affecting viability or mitochondrial membrane potential (Kalo et al., 2022). These mixed results highlight that the reproductive response to PUFA may depend on factors such as supplementation duration, fatty acid source, metabolic status, and environmental conditions.

Functionally, fatty acids play essential roles in maintaining sperm membrane composition and flexibility, both of which are critical for capacitation, the acrosome reaction, and sperm-oocyte fusion during fertilization (Wathes et al., 2007; Van Tran et al., 2017; Ahmad et al.,

2019). However, interspecies variability and differences in experimental design contribute to inconsistencies across studies (Yeste et al., 2011; FAO of the United Nations, 2012; Byrne et al., 2017). Even so, omega-3 fatty acid concentrations appear closely linked to phosphatidylserine translocation, a process influencing sperm DNA integrity in both humans and cattle (Moustafa, 2004; Komsky-Elbaz et al., 2020).

Feed intake, plane of nutrition, and rate of growth have long been recognized as key regulators of the age at puberty attainment in bulls (Van Demark, 1956). Lower energy intake generally slows sexual development, whereas greater energy intake accelerates the onset of puberty; however, these differences tend to diminish as animals age (Bratton, 1961; Flipse and Almquist, 1961; Foote, 1969). Postweaning nutritional plane is particularly influential—diets deficient in nutrients can hinder sexual development, while those exceeding requirements can promote it (Mann et al., 1967; Pruitt et al., 1986).

Beyond energy balance, the hormonal responses to nutrition play a crucial role. Research on LH and insulin-like growth factor-1 (IGF-1) has shown that these hormones act synergistically to promote Leydig cell proliferation, differentiation, and testosterone secretion. Evidence suggests that the composition of the diet can influence steroidogenesis within the testes by modulating LH and IGF-1 concentrations (Brito et al., 2007b, 2007c, 2007d; Dance et al., 2015; Byrne et al., 2018; English et al., 2018). Thus, the nutritional environment prior to the postnatal surge in LH can alter testicular growth and the timing of puberty though its effects on metabolic hormones and nutrient availability.

Classic studies demonstrated that even short-term reductions in energy intake can delay puberty. Holstein bulls with temporarily restricted feed intake reached puberty later than controls, though they showed no long term impairment in fertility or semen production once

normal feeding resumed (Bratton, 1959, 1961; Flipse and Almquist, 1961). However, prolonged nutrient restriction can have more permanent consequences. Van Demark and Mauger (1964), reported that bulls fed only 60% of the recommended TDN from 8 weeks to 46 months of age exhibited irreversible reductions in bone, endocrine, and testicular growth, even after switching to full nutritional intake for 12 additional months.

Overall, increasing nutrient availability has consistently been linked with faster growth rates and earlier puberty. Bulls receiving higher levels of TDN not only grew more rapidly but also produced their first motile sperm at a younger age, indicating a direct link between nutrient intake and reproductive development (Van Demark, 1956; Flipse and Almquist, 1963). In contrast, low now nutrition diets during the prepubertal period delayed puberty by approximately 3-4 weeks and resulted in lighter paired testes weights – by 50 to 120 grams – at 1.5 years of age compared with bulls receiving a higher plane of nutrition (Brito et al., 2007b, 2007c; Byrne et al., 2017, 2018).

Together, these findings emphasize the importance of maintaining adequate nutrition during prepubertal development, particularly in bulls being prepared for breeding. While rapid growth supported by high-concentrate diets can advance puberty, excessive energy intake may also impair semen quality later in life, leading to reductions in motility and an increase in morphological abnormalities (Coulter et al., 1997; Smith et al., 2025). Therefore, the optimal nutritional strategy must balance growth promotion with long-term reproductive capacity.

Sperm output increased with higher TDN intake; however, by two years of age, differences between high and low intake bulls were no longer apparent (Bratton, 1961). In a long term study spanning from 8 weeks to 58 months of age, Van Demark and Mauger (1964) evaluated the effects of energy intake on reproductive performance, growth, and puberty in

Holstein bulls. Animals were fed either 60% or 100% of the recommended TDN levels outlined by Morrison (1956). Bulls on the 60% TDN diet exhibited reduced growth across several tissues and glands, including the body, testes, and accessory sex glands. Although these bulls were later switched to a 100% TDN diet for the final 12 months of the study and achieved near-normal body weight, testicular development and endocrine function did not fully recover.

This partial recovery highlights that while compensatory growth can restore overall body condition, early postnatal nutritional restriction exerts lasting effects on reproductive development. In particular, a higher plane of nutrition during or after puberty cannot completely offset deficiencies that occur during the early postnatal period (Brito et al., 2007b; Byrne et al., 2018). Breed differences also contribute to variation in nutrient requirements and developmental timing. Angus and Hereford bulls fed either a timothy-alfalfa hay mix (8.6% CP and 57% TDN) and a concentrate mix (16.4% CP and 84% TDN) reached puberty at approximately 45 weeks of age, compared with Holstein bulls that attained puberty earlier, at around 40 weeks, on a 100% TDN diet (Almquist and Amann, 1962; Wolf et al., 1965).

Dietary protein concentration is another key factor influencing reproductive performance. Flipse and Almquist (1963) reported that semen quality remained normal in bulls consuming diets containing 10-22% CP. In contrast, severe protein restriction produced detrimental outcomes: half of the bulls consuming a diet containing only 1.5% CP survived for just 151 days (Shirley et al., 1963). Similarly, limiting protein intake to 1.5-8% CP for 3-6 months resulted in significant reductions in the weights of the testes, epididymides, and seminal vesicles compared with bulls fed a diet containing 14% CP (Meacham et al., 1963, 1964).

While energy and protein restriction can have detrimental effects on livestock performance, prolonged growth restriction also limits sexual development due to reduced body

weight and inadequate nutrient supply for metabolic function. For instance, cattle restricted to an average daily gain of 0.3 kg/day for one year exhibited delayed sexual development compared to those restricted to 0.8 kg/day (Tegegne et al., 1992). Conversely, excessive weight gain during the pubertal and developmental phases can have the opposite effect, impairing sperm production and semen quality – some of which may result from compromised thermoregulation (Skinner, 1981; Smith et al., 2025).

Nutritional enhancement during early development can influence testicular output, as bulls fed at a higher nutritional plane until 31 weeks of age produced approximately 30% more harvestable spermatozoa (Dance et al., 2016). However, studies have shown that improving nutritional status during the prepubertal stage alone does not necessarily increase postpubertal sperm yield (Harstine et al., 2015; Byrne et al., 2018). High rates of gain have also been associated with reduced DSP and epididymal growth rate, along with higher frequencies of sperm abnormalities (Coulter and Kozub, 1984; Coulter et al., 1987, 1997; Coulter and Bailey, 1988).

Collectively, these studies indicate that nutritional management during the prepubertal period can manipulate testicular growth and endocrine function, primarily through changes in metabolic hormone concentrations such as IGF-1 and leptin. Both hormones have been positively associated with testicular size in growing beef bulls, suggesting that they may play roles in GnRH-independent regulation of testicular development (Brito et al., 2007a, 2007d). Leptin secretion is directly correlated with adipose tissue mass (Landry et al., 2013). Although leptin receptors were initially thought to mediate reproduction through direct effects on GnRH neurons, their absence in rodent GnRH neurons suggests that leptin acts indirectly in reproductive regulation (Quennell et al., 2009). As an endocrine regulator, leptin influences

multiple target tissues, while adiponectin – another adipocyte-derived hormone has been detected in sperm, implying potential involvement in sperm metabolism or function (Kasimanickam et al., 2013).

Environmental stressors further modulate reproductive development by influencing nutrient intake and metabolic balance. High ambient temperatures, in particular, can reduce feed intake and delay puberty attainment. Jersey bulls exposed to heat stress conditions (35°-36°C and 80-90% relative humidity for 8 hours daily) from 26 weeks of age reached puberty approximately seven weeks later than control bulls and exhibited reduced testicular size and spermatogenic activity (Alba and Riera 1966). Such findings highlight the interaction between environment, nutrition, and endocrine regulation in determining the onset of puberty, sperm quality, and overall reproductive performance.

While nutrition and metabolic status strongly influence testicular growth, spermatogenesis, and endocrine function, reproductive success ultimately depends on the expression of normal sexual behavior. Even when semen quality is adequate, inadequate libido or failure to complete mating behaviors can limit fertility in natural service systems. Therefore, understanding the physiological and environmental factors influencing sexual behavior is critical for interpreting the relationship between nutrition, endocrine regulation, and overall reproductive efficiency in bulls.

Current Challenges for Reproductive Efficiency in Beef Systems

Despite extensive research on the endocrine, morphological, and behavioral regulation of reproduction in bulls, variability in fertility remains a major constraint to reproductive efficiency within beef production systems. Differences in nutritional management, developmental environment, and physiological maturity contribute to inconsistent semen quality, testicular

development, and libido expression among young bulls. These sources of variation underscore the importance of identifying nutritional and physiological factors that influence male fertility. Of interest are developmental nutrition strategies and measurable predictors of reproductive potential, such as anogenital distance (AGD) and reproductive tract score (RTS) in females. Addressing these challenges provides the foundation for the two studies conducted in this thesis, which examine the effects of omega-3 FA based supplementation during bull development and the potential use of AGD along with RTS as predictors of fertility in beef females.

Improving reproductive efficiency remains a major challenge for beef production systems and is central to the sustainability and profitability of the industry. Although reproductive management traditionally focuses on the female, male fertility is equally critical, as a single sub fertile bull can substantially reduce conception rates and extend the calving interval (Burns et al., 2010; Barth, 2013; Chenoweth, 2015). Approximately 10 – 25% of bulls fail or are deferred from a BSE, leading to economic losses and decreased reproductive success (Parkinson, 2004). Nutritional management during bull development plays a vital role in optimizing testicular growth, sperm production, and libido (Brito et al., 2007d; Dance et al., 2016; Byrne et al., 2018). In particular, dietary lipids – especially omega-3 PUFA have received increased attention for their potential to enhance semen quality and reduce oxidative stress by altering sperm membrane composition and improving cellular resilience (Gholami et al., 2010; Fair et al., 2014; Kalo et al., 2022). However, results remain inconsistent across studies due to variations in FA source, duration, and supplementation rate. These gaps in knowledge underscore the need to evaluate nutritional strategies that can support male fertility under practical beef production conditions.

Addressing these issues aligns with the objectives of this thesis, “Nutritional and Management Strategies to Improve Reproductive Efficiency in Beef Cattle”, which encompasses

two studies designed to explore key aspects of reproductive performance. The first study investigates the effects of omega-3 FA supplementation during bull development as a nutritional approach to enhance semen quality and oxidative balance. The second study evaluates anogenital distance (AGD) and reproductive tract score (RTS) as management based predictors of fertility potential in beef heifers. Together, these studies aim to improve our understanding of how nutrition and management interventions can be applied to increase reproductive efficiency in beef cattle.

Although nutritional supplementation can influence male reproductive development, improving efficiency within cow-calf systems also requires early and accurate identification of heifer fertility potential. Reproductive tract scoring is used to estimate pubertal status, but it provides only a physiological snapshot rather than a developmental predictor (Anderson et al., 1991). Anogenital distance is a sexually dimorphic trait influenced by prenatal androgen exposure, and has recently gained attention as a potential indicator of reproductive capacity in females (Wathes, 2022). Research has identified animals with naturally greater androgen concentrations within cattle herds (Abedal-Majed et al., 2022). However, few studies have combined AGD with conventional reproductive assessments such as RTS to determine whether these measures can be used together to predict breeding outcomes. Exploring these indicators may provide producers with low-cost, non-invasive tools to improve selection decisions and overall herd reproductive performance.

Conclusion

Although increasing attention has been given to male fertility in recent years, there remains a notable lack of comprehensive research focused specifically on beef bulls. Much of the foundational knowledge surrounding bull fertility originates from studies in the mid-20th century,

predominantly using dairy breeds. While these data have provided valuable insights, the genetic progress and production environments of modern beef and dairy cattle differ substantially from those of animals studied decades ago. Longitudinal studies that follow beef bulls from birth through multiple breeding seasons are exceedingly rare, and existing investigations on young bulls often differ in design, limiting comparability across studies. Furthermore, research exploring the influence of nutrition on neuroendocrine regulation of testicular development and spermatogenesis remains limited, though recent reviews have outlined potential mechanisms linking metabolic status to reproductive function (Plant, 2015). In contrast to the extensive research available on female fertility and developmental physiology, male reproductive biology in beef cattle remains underexplored. Future research integrating nutritional, physiological, and emerging omics-based approaches will be critical for improving the understanding of male fertility and identifying biomarkers to distinguish sub fertile bulls within the populations.

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Chapter 2 - Effects of Dietary Inclusion of an Omega-3 Fatty Acid-Based Supplement on Biological Parameters and Sperm Characteristics of Developing Beef Bulls

Abstract

The objective of this study was to evaluate the effects of a dietary omega-3 fatty acid-based supplement on growth performance, semen quality, and oxidative status of seminal plasma in developing beef bulls. A total of 39 bulls were assigned to one of three dietary treatments for 80 d: a control diet with no added supplement (CON), a moderate level of omega-3 supplement (MOD; 0.22 kg per d on a DM basis), or a high level of omega-3 supplement (HI; 0.44 kg per d on a DM basis). Body weight, scrotal circumference, and semen traits were measured on d 0, 53 or 55, 80, and 101. Blood samples were collected to analyze serum fatty acid (FA) concentrations, and seminal plasma was evaluated for reactive oxygen species (ROS). Omega-3 supplementation did not affect BW, ADG, or scrotal circumference ($P > 0.05$). However, bulls in the MOD group demonstrated improved feed efficiency ($P < 0.05$) compared with the CON and HI bulls. Supplementation increased ($P < 0.05$) the concentrations of total omega-3 FA in serum and also decreased the omega-6 : omega-3 ratio, confirming that the supplement was adequately incorporated into the diet. Semen quality parameters, including motility and morphology, did not differ ($P > 0.05$) but were numerically improved in omega-3 supplemented bulls, suggesting that there are potential benefits to sperm function. Following incubation for 3 h, ROS concentrations in seminal plasma were similar among treatment ($P > 0.05$) but numerically decreased for the supplemented groups. However, 21 d after cessation of supplementation, progressive motility decreased to levels that were equivalent to d 53 or 55. The results indicate that dietary omega-3

fatty acid supplementation during the developmental period can enhance systemic omega-3 status and oxidative stress numerically decreased in seminal plasma after incubation without negatively impacting growth performance. Moderate inclusion levels may optimize feed efficiency while maintaining semen quality in young beef bulls. Further research is warranted to evaluate the long-term reproductive outcomes of omega-3 supplementation leading into the first breeding season and the impacts on fertility.

Introduction

Reproductive efficiency in beef cattle operations is a major determinant of the overall herd productivity. Bulls provide the largest contribution to the herd's genetics, and it is imperative for the bull to be fertile (Gifford et al., 2023). The genetics from the bull are passed down to the offspring only when the coordination of testicular growth, spermatogenesis, and endocrine functions occur during sexual development. The nutritional management of bulls plays a critical role in supporting the physiological processes, particularly through the provision of EFA that influence the membrane composition of sperm, hormone synthesis, and the antioxidant balance within the testes and sperm cells (Yuan et al., 2023).

Omega-3 FA have been used as dietary supplements to enhance human and reproductive performance of male livestock for their antioxidant properties (Kalo et al., 2022; Yuan et al., 2023). There are three long-chain PUFA that are classified as omega-3 FA: α -linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). These FA are integral components of the sperm cell membrane, and research has found that supplementation can improve sperm motility, morphology, and overall sperm viability in several species (Kalo et al., 2022; Yuan et al., 2023). In bulls that were not supplemented with FA, the seminal plasma protein profiles changed among a number of ejaculates collected on the same day, indicating that

protein expression in seminal plasma was related to oxidative stress, membrane integrity, and sperm function (Udoekong et al., 2025). The proteomic shifts reflect the biological impact of fatty acid-driven modulation of antioxidants, particularly during periods of rapid development when bulls are susceptible to high metabolic stress and oxidative damage.

From the post-weaning phase through one yr of age, there is a period of rapid growth of the body and testes, in bulls, marking a period particularly sensitive to metabolic stress (Barber and Almquist, 1975; Amann, 1983). Excess concentrations of ROS in seminal plasma can impair sperm motility, damage DNA, and trigger apoptosis (Hussain et al., 2023). Because of this, dietary strategies that reduce oxidative damage while supporting reproductive development and efficiency may enhance male fertility. In support of this, elevated oxidative stress markers such as malondialdehyde and carbonyl proteins have been negatively associated with sperm motility and viability, whereas higher antioxidant enzyme activity, particularly catalase and superoxide dismutase, has been positively correlated with sperm function (Udoekong et al., 2025). Similarly, omega-3 supplementation in Holstein bulls has been shown to improve sperm motility and viability, particularly under heat stress conditions (Gholami et al., 2011). Omega-3 FA incorporation into the phospholipids of the sperm membrane, and modulation of antioxidant pathways represent a nutritional strategy to mitigate oxidative damage during the developmental period.

Excessive nutrient intake can negatively impact fertility. Smith et al. (2025) reported that yearling beef bulls that were in the top 10% for subcutaneous backfat thickness in a retrospective analysis exhibited a lower proportion of morphologically normal sperm (79.60%) and were more likely to be deferred at their initial breeding soundness examination (BSE) compared to the bottom 10% bulls (87.63%). The results highlight the potential consequences of over-

conditioning and emphasize the importance of balancing nutritional inputs to support reproductive development without compromising semen quality.

The primary objectives of this study were threefold: first, to evaluate the impact of the supplement on growth performance and feed efficiency; second, to determine its effects on testicular development and semen quality traits; and third, to assess whether supplementation alters oxidative status within seminal plasma. The objectives were designed to address both production-level outcomes and physiological indicators of reproductive function. It was hypothesized that omega-3 supplementation would reduce ROS concentrations in the seminal plasma and improve semen quality characteristics, while exerting minimal effects on growth performance and feed efficiency. This hypothesis reflects the proposed antioxidant and membrane-stabilizing roles of omega-3 fatty acids during a critical window of reproductive development in beef bulls.

Materials and Methods

Animals and Experimental Procedures

Weaned beef bull calves ($n = 39$, initial BW = 334 ± 50 kg, initial age = 282 ± 25 days) of Angus ($n = 16$), Hereford ($n = 12$), or Simmental ($n = 11$) breeds from the Kansas State University Purebred Beef Unit in Manhattan, Kansas. Animals were handled and cared for in accordance with the procedures approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC) #4912. Calves were weaned from dams in September of 2024 and moved to open dry lot pens at the Kansas State University Precision Intake Facility where they were offered native grass hay and a commercial creep feed for *ad libitum* consumption. On October 18, 2024 bulls were processed and fitted with an electronic identification tag (Allflex, Dallas, TX). Bulls were vaccinated for protection against respiratory

and clostridial diseases according to the manufacturer's directions with Pyramid 5 + Presponse SQ (Boehringer Ingelheim Animal Health USA, Duluth, GA), Bovilis 20/20 Vision 7 with Spur (Merck, Rahway, NJ), and Bar-Vac CD/T (Boehringer Ingelheim Animal Health USA, Duluth, GA). Bulls were also treated for internal and external parasites with a topical anthelmintic Cydectin (Elanco, Indianapolis, IN). Following processing, bulls were moved into two conjoined partially covered dry lot pens equipped with Insentec Roughage Intake System bunk modules (Hokogarm Group, Emmeloord, Netherlands). Bulls underwent an adaptation period of 10 d with the pneumatic gates locked open to allow for unlimited access to feed from the bunk modules. Following the initial adaptation period, the pneumatic gates were then set to an automatic function for 21 d to allow bulls to learn how to consume feed from bunks by reaching into the modules, allowing the electronic identification tags to be read by the radiofrequency reader equipped on each bunk to unlock the pneumatic gates.

Treatments and Management

This experiment utilized a randomized complete design. Bulls were randomly assigned to treatment groups, with treatment groups subsequently assigned to specific bunk modules. A total of 8 bunks was assigned to each treatment in each of the two pens that were conjoined along the bunk line. In total, there were 24 bunks across the two pens that were randomly assigned to treatments. Each treatment had access to their 8 assigned bunks, which equates to 1.625 bunks per bull for each treatment group. Bulls were stratified into three treatment groups by age and BW (Table 2.1). A base diet was fed to all treatments with the omega-3 supplement and a corn germ meal to be isonitrogenous and thoroughly hand mixed into feed bunks. The omega-3 based supplement was a proprietary blend of flaxseed and algae (NBO3 Technologies, Manhattan, KS). The control group (CON; n = 13, no omega-3 supplementation), moderate (MOD; n = 13, 0.22

kg per day of the omega-3 FA based supplement on a DM basis) or the high (HI; n = 13, 0.44 kg per day of the omega-3 FA based supplement on a DM basis). Diet composition and analysis are presented in Table 2.2 along with the ration feed before starting treatments (PRIOR). Treatments were randomly assigned to bunks on d –1 with treatment diets beginning on d 0. The study began in late November 2024 and concluded in early February 2025 after an omega-3 supplementation period of 80 d.

Ration samples of the base, CON, MOD, and HI diets, along with each ingredient, were sampled weekly. Samples were compiled by week into 3 periods. Samples from the compiled periods were shipped to SDK Laboratories (Hutchinson, KS) for wet-chemistry analysis.

Body Weight and Body Condition Score

Body weight was measured on three consecutive d BW and was collected on d –6 through –4, before feeding occurred in the morning. Weights from the first 2 d were averaged to assign bulls to treatments, with the average of all 3 d considered as the initial weight. A full body weight was taken on d 55 of the trial and designated at the midpoint. Ending BW was calculated by averaging daily weights taken from d 78 through d 80. Body condition scores (BCS; 1-9 scale) were recorded multiple times throughout the study by the same evaluator on d -5, 53, and 80 following release from the chute.

Blood Collection

Blood was collected in 10 mL silicone-coated red stopper tubes with no additives (Cardinal Health, Dublin, OH) using an 18-gauge X 38.1 mm needle (Greiner Bio-One North America, Monroe, NC). Blood samples were collected via coccygeal venipuncture on d –6, 53 or 55, and 80. Bulls were sampled at random order on d 53 by the first 20 through the chute, and sampled from the remaining bulls were collected on d 55 such that blood samples and breeding

soundness exams collected on the same d. Blood samples were immediately placed on ice after collection. When the collection was finished, blood samples were immediately taken to the lab and centrifuged at 2,850 RPM for 15 min without the brake applied. The serum was then pipetted into 6.5 mL scintillation vials (Globe Scientific, Mahwah, New Jersey) in triplicate and immediately placed into a -20°C freezer until analysis.

Carcass Ultrasonographs

Individual bull carcass ultrasonography was performed on d -5 and again at the end of the feeding period on d 80. Measurements collected included ribeye area, subcutaneous back fat thickness, and intramuscular fat. A technician licensed by the Ultrasound Guidelines Council performed the ultrasounds, and the data were submitted to the Centralized Ultrasound Processing, and raw data were received for analysis.

Thermal Images

Infrared thermography images were obtained on d -6 and 79 using a research-grade infrared camera (Fluke TIX580, Fluke Corp.). Testicular images were obtained by pointing the camera at the posterior aspect of the scrotum at a 90° angle and a distance of 1 m. Eye medial cantus images were obtained by pointing the camera frontally at the cattle's face at a 90° angle and a distance of 1 m. Images were saved, and the corresponding image file numbers were recorded for later analysis. Ambient average temperature and relative humidity were recorded. Day -6 had an average temperature of 17.15 °C with a relative humidity of 68.18%, while day 79 had an average temperature of 13.40 °C with a relative humidity of 37.75%. The average ambient temperature throughout the study was -0.54 °C. Weather data was obtained from the National Oceanic and Atmospheric Administration weather station at the Manhattan Regional Airport in Riley County, Kansas, located 11.6 kilometers from the Kansas State Precision Intake Facility.

Infrared images were analyzed using research-specific computer software (SmartView v. 4.3, Fluke Thermography). The maximum, median, and average temperature (°C) of the scrotum, inguinal region of the scrotum, and medial canthus of the eye were recorded and analyzed. The temperatures were determined by centering a circular window over the target area.

Breeding Soundness Exams and Semen Analysis

Scrotal circumference (SC) was collected by pulling the testes firmly down into the lower part of the scrotum and placing a metal scrotal tape around the widest portion of the scrotum on day -5 and during each breeding soundness exam (BSE). Two BSEs were performed during the supplementation period, and a final BSE was conducted 21 d after the supplementation period ended d 101. Examinations occurred on d 53 or 55, 76, and 101, with the final BSE occurring 21 d post supplementation. Bulls were transported to the Kansas Artificial Breeding Services Unit, which is located 1.77 km from the Kansas State Precision Intake Facility. The first BSE was performed on day 53 for the first randomly selected 20 bulls. Due to the schedule of KABSU, only 20 bulls were collected on d 53, with the remaining bulls collected on d 55. Prior to shipping bulls to KABSU on d 53 and 55 for BSE, individual BW was recorded, and blood samples were collected as described above. Breeding soundness exams were performed by the collection facility, with the electroejaculation technique used to collect semen samples. Upon collection of the ejaculate, approximately 0.25 mL of the ejaculate was pipetted into microcentrifuge tubes, placed in a Styrofoam cooler with warm pads and taken to a computer-assisted sperm analysis (CASA) machine (Sperm Vision, Minitube, Verona, WI) located at the Kansas State College of Veterinary Medicine for analysis on d 53, 55, and 80. The samples were evaluated for motility, progressive motility, and morphology. Morphology slides were prepared using Eosin-Nigrosin stain and read on a 100X oil immersion with a phase contrast microscope.

One hundred cells were counted per slide, and abnormalities were recorded for each bull. Any of the 100 cells with multiple abnormalities were counted for each abnormality, and the total may have exceeded 100, but each cell with multiple abnormalities was still counted as 1 cell.

Ejaculates from d 101 were analyzed with a CASA machine located at KABSU. The CASA machine analyzed the percent of sperm cells that were morphologically normal, the percent of motile sperm, and the percent that were progressively motile. The CASA was an IVOS II (Hamilton Thorne, Beverly, MA), and the settings and sample dilutions can be found as described by Menegatti Zoca et al. (2020). The samples that required dilution were diluted to the concentration required for the CASA standard setting. The remaining ejaculate was split equally into two separate aliquots and placed in 6.5 mL scintillation vials (Globe Scientific, Mahwah, New Jersey) as depicted in Figure 2.1. One vial was immediately centrifuged for fifteen minutes at 3,500 rpm. The last semen sample was placed in a 35 °C water bath for three hours. At the end of the three hours, the sample was centrifuged for 15 minutes at 3,500 rpm. Upon centrifugation, the seminal plasma was pipetted into a new scintillation vial and snap-frozen in liquid nitrogen. After all seminal plasma samples were collected, they were stored in a -80°C freezer until analysis for ROS.

Reactive Oxygen Species

Day 80 seminal plasma samples were analyzed for ROS with the In Vitro ROS/RNS Assay (Cell Biolabs Inc, San Diego, CA). Standards were prepared in accordance with the product manual. Seminal plasma samples were validated to verify that the ROS was within the linear range of the standards. Samples were stored in a -80 °C freezer for seven months until analysis. All samples and standards were run in duplicate. Samples were removed from the freezer and sorted so that samples from hours 0 and 3 were run on the same plate. Samples were

thawed in a bead bath at 37°C for 10 min. After thawing, 50 µL of each individual sample was pipetted into a black 96-well plate suitable for fluorescence. Then 50 µL of the catalyst was added to each well, mixed well, and incubated at room temperature for 5 min. Then 100 µL of the 2', 7' – dichlorodihydrofluorescein (DCF) was pipetted last into the wells, along with the DCF standards to limit exposure to light. Plates were then incubated at room temperature in the dark for 15 minutes before being placed in the plate reader. The Gen 5 Software (Agilent Technologies, Santa Clara, CA) was used to measure the relative fluorescence units (RFU). The Synergy HTX multi-mode reader (Agilent Technologies, Santa Clara, CA) was set to 480 nm excitation / 528 nm emission.

Gas Chromatography

Gas Chromatography (GC) of the fatty acid (FA) composition of blood was performed by NBO3 Technologies (Manhattan, KS) for FA analysis. A Supelco Sp-2560 capillary column of 100m x 0.25mm x 0.20µ film (Supelco, Bellefonte, PA) was used with an injection and detection temperature of 250°C. A temperature gradient of 3 °C/min was applied from 20 °C to 240 °C, with the final oven temperature maintained at 240 °C for 20 minutes. A gas flow rate of 1 ml/min was maintained with a split ratio of 80:1.

Samples (200 µl) were pipetted into a 10 ml screw cap tube and combined with 1 ml of a benzene-containing internal standard and 4 ml of BF₃ – Methanol reagent (Supelco #B1252 Supelco, Bellefonte, PA). Tubes were flushed with nitrogen, capped, and incubated at 60°C for 60 min with mixing at 20 min. After cooling to room temperature, 4 mL of water and 1 mL of hexane were added, and tubes were vortexed and centrifuged (1,000 x g for 10 min). The hexane layer containing FA methyl esters were transferred to screw cap GC vials for analysis.

Statistical Analysis

Daily Collection Data

Data were analyzed using the GLIMMIX procedure of SAS version 9.4 (SAS Institute, Cary NC). All models included breed as a random effect and treatment group as a fixed effect. Normally distributed data were analyzed by least squares means with a Tukey adjustment. Semen data for morphology, motility, and progressive motility were coded as a beta distribution, with the logit link, and were back-transformed using ilink. Data collected at least twice throughout the trial were also analyzed as a repeated measure, using the Kenward-Rogers adjustment in SAS. Since semen characteristics were analyzed with two different CASA machines depending on the day of collection, CASA was included as a random effect for morphology, motility, and progressive motility. The repeated measures model for motility and progressive motility included the individual bull nested in breed.

Thermal Image Data

Thermal images were analyzed using the GLIMMIX procedure of SAS for temperatures of the eye, scrotal neck, and testes. The models included random effects for the intercept and residuals due to the two collection days. The Kenward–Roger adjustment was used for the denominator degrees of freedom.

Blood Data

For the analysis of blood data, the Mixed procedure of SAS was used. Models included the Kenward–Roger and Tukey adjustment. Day was included in the models as a repeated variable, with individual bull and breed as a random variable. A treatment and day interaction was tested, and only the effects are reported if there was no significant interaction.

Reactive Oxygen Species Data

For analysis of the ROS data, the Mixed procedure of SAS was used. A repeated measure for time was used, with breed and bull nested within breed. For the least square means of ROS, a Tukey adjustment was used. Due to limited sample availability, any sample that had a coefficient of variation (CV) of over 10% was excluded from the analysis.

Results and Discussion

Prior to the beginning of the supplementation period, bulls were stratified into treatments based on age and BW. The descriptive statistics of age, BW, BCS, and SC are included in Table 2.3, prior to the beginning of the supplement period. The BW of bulls for stratifying them into treatment groups has a wider range due to the deaths of two bulls during the study. Both bulls died of unrelated causes to the treatment, with one bull from each supplementation group dying. Removal of the two mortalities maintained a balance and statistical integrity with 13 bulls per treatment. The carcass ultrasonography data for the ribeye area ($P = 0.95$), intramuscular fat ($P = 0.76$), and subcutaneous backfat ($P = 0.84$) are reported in Table 2.4, with no treatment effects observed, confirming uniformity among groups prior to supplementation.

By d 53 or 55, bulls had no difference in BW or BCS, and the values by treatment are presented in Table 2.5. Due to the schedule of the stud facility and the time required for semen analysis, 20 bulls were collected with electroejaculation on d 53 at random, while the remaining 20 were collected on d 55. Semen from the ejaculate was analyzed 0 hrs after collection, and no samples were saved for later analysis of freeze-thaw characteristics (Table 2.6) The semen collected on these two days showed a tendency for increased motility of sperm cells ($P \leq 0.10$), for the HI group (51.52%) compared to the CON group (33.70%). No other significance or

tendencies were observed at this time for performance or semen traits, although progressive motility approached a tendency ($P = 0.12$).

With the length of spermatogenesis in bulls being 61 days (Staub and Johnson, 2018), therefore, the sperm collected at d 53 or 55 largely developed prior to the start of supplementation. The increase in motility for the HI bulls may reflect early systemic metabolic changes in the lipid composition of the sperm membrane, rather than direct incorporation of the supplement into developing sperm cells. The observed results agree with early supplementation studies, suggesting gradual integration of long-chain FAs into sperm membranes over multiple spermatogenic cycles (Ngcobo et al., 2021).

Performance data collected on d 80, marking the end of the supplementation period, are presented in Table 2.7. There was no difference for BW, BCS, ADG, and DMI. There was a significant difference ($P = 0.04$) in G:F, with the MOD group being the most efficient at converting feed into weight compared to the CON group, which was the least efficient group. The result of the MOD group being the most efficient was unexpected; some of this could be attributed to one of the Simmental bulls, which was a breed leader for the \$Gain index of the Simmental Association. Although this bull was not an outlier for ADG or DMI, it could be possible that he could skew the data in favor of a greater G:F ratio. The enhanced feed efficiency in the MOD group suggests that inclusion of an omega-3 fatty acid at 0.22 kg per d may optimize nutrient utilization without depressing intake. Carcass ultrasound data revealed no difference between treatments and are reported in Table 2.8. With breed as a random effect, no effects for carcass traits at the end of the 80 d supplementation period were observed for ribeye area ($P = 0.74$), intramuscular fat ($P = 0.65$), or subcutaneous back fat ($P = 0.74$).

Semen characteristics for the bulls at the end of the supplementation period are presented in Table 2.9. Bulls were collected on d 76 instead of d 80 due to the scheduling ability of KABSU. No differences were observed in fresh semen; however, motility and progressive motility numerically improved with increasing supplementation levels. Interestingly, the MOD group had the greatest number of morphologically normal sperm, followed by the HI group and then the CON group. Although this was not significant, it does not align with the observed results of motility and progressive motility increasing based on the supplement inclusion level.

Although differences in semen traits were not observed on d 80, the consistent numeric improvement aligns with prior studies suggesting that dietary omega-3 fatty acids improve sperm membrane fluidity and reduce the oxidative susceptibility (Kalo et al., 2022; Eidan et al., 2024). These data indicate that omega-3 supplementation did not affect growth or carcass composition while potentially supporting spermatogenic health. Requirements to pass a BSE from the Society of Theriogenology are 30% progressively motile sperm, 70% morphologically normal sperm, and a SC of 30 cm for bulls $\leq$ 15 months. All treatment groups would pass the BSE requirements for SC, and progressively motile sperm (Koziol and Armstrong, 2018). For morphology, the MOD group (78.54%) would be the only group to pass a BSE as the morphology of HI (68.15%) and CON (57.46%) is less than 70%.

Supplementation officially ended on d 80, and all bulls were fed the BASE diet over the next 21 days. At 21 days after supplementation ended, a final BSE collection was performed on all study bulls. The semen characteristics and SC of bulls on d 101 are reported in Table 2.10. No treatment effects were observed for SC, morphologically normal sperm, motile sperm, or the number of progressively motile sperm. Progressively motile sperm decreased on d 101 compared to the observed values on d 76.

Since several of the performance, carcass, and semen variables were collected multiple times during the duration of the study and on d 101, the averages for each treatment over all 101 days can be found in Table 2.11. For the 3 BSE collections during the study, there was a tendency ($P \leq 0.10$) for improved motile sperm in the HI group (61.86%) compared to the CON group (44.79%). The semen that was collected on d 53 or 55 would have matured before the beginning of supplementation due to the length of spermatogenesis being 61 days in the bull. The d 76 ejaculate would have undergone one complete cycle of spermatogenesis and completed epididymis transit time from the start of treatment on d 80. Although the supplementation period ended on d 80, the collection on d 101 allowed for the ability to see any carryover effects from the supplement. Even though no treatment differences were observed on this day, it is interesting to note a decrease in progressively motile sperm after the end of supplementation based on the observed progressively motile sperm on d 80 for all treatment groups.

With data collected on multiple days throughout the study, a repeated measure model for day was also ran to represent the change in variables throughout the 80-day supplementation period, and a 21-day period without supplementation can be found in Table 2.12. A day effect was observed for each variable and biologically aligns with the age and stage of growth of the bulls. The progressively motile sperm on d 101 were significantly different from d 80 but were not different from the first collection on d 53 or 55. The results imply that supplementation benefits on sperm function may be transient, likely requiring continuous dietary inclusion through breeding to maintain enhanced motility and membrane integrity. A previous report noted a short carryover persistence of omega-3 FA in sperm membranes, lasting approximately one cycle of the seminiferous epithelium (Kalo et al., 2022). The morphology or motile sperm on d

101 did not differ from d 80. The actual fertility of the bulls remains unclear, as all bulls were evaluated for semen quality and not breeding trials.

Blood serum from d -6, 53 or 55, and 80 was analyzed for FA composition and concentrations using gas chromatography. The total FA concentrations ($\mu\text{g/mL}$) are presented in Figure 2.2. No treatment effect was observed across the 3 blood collection days for total FA concentrations. However, a significant day effect was detected: total FA concentrations differed between d 0 and d 53 or 55 TFA ($P = 0.05$), while d 80 concentrations did not differ from either time point. Due to the d 0 samples collected prior to the initiation of treatment diets, the increase in total FA at d 53 or 55 may reflect the dietary transition. From d 53 or 55 through d 80, total FA concentrations remained stable, suggesting that only the FA profile was altered by treatment.

Given that the supplement was omega-3-based, serum omega-3 FA concentrations were also analyzed and are reported in Figure 2.3. As expected, no differences were observed among treatments on d 0, prior to supplementation. By d 53 or 55, blood serum omega-3 concentrations began to diverge, with a tendency for increased levels in the HI group compared to the CON group. Although no significant differences were detected on d 80, a treatment effect was observed across the supplementation period ($P \leq 0.05$), with both MOD and HI groups differing from the CON group. A significant day effect was also noted for d 0 ($P \leq 0.05$), consistent with the timing of diet initiation. Although omega-3 supplementation began early in the feeding period, concentrations initially declined from the d -6 concentrations, likely reflecting ruminal biohydrogenation and delayed incorporation into tissues. Early oxidative metabolism may also increase peroxidation of these highly UFA, temporarily reducing measurable levels (AbuGhazaleh and Jenkins, 2004). As adaptation occurs, microbial stabilization and membrane turnover typically improve omega-3 retention and utilization (Kalo et al., 2022).

To further characterize fatty acid dynamics, serum omega-6 concentrations were analyzed and are presented in Figure 2.4. No treatment effect was observed ($P > 0.05$), but a significant day effect was detected ($P \leq 0.05$). Day 0 samples had the lowest omega-6 concentrations and differed significantly from both d 53 or 55 and d 80. This pattern was expected, given the omega-3 based nature of the supplement, although a shift in overall fatty acid composition was anticipated.

The omega-6:omega-3 ratio was also evaluated and is depicted in Figure 2.5. All bulls began with similar ratios on day -6. A significant increase in the ratio was observed in the CON group over the 80-day supplementation period, while the MOD and HI groups exhibited a noticeable decrease, consistent with the higher proportion of omega-3 FA in the supplemented diets. This interaction ($P < 0.01$) between treatment and day supports the expected shift in serum fatty acid balance.

Finally, total PUFA concentrations over the feeding period are shown in Figure 2.6. No differences were detected among treatments or across days. The CON group received no supplemental FA, while the MOD and HI groups were provided with differing doses of the omega-3 supplement. Despite these differences in dietary input, PUFA concentrations remained stable throughout the study period.

Despite no differences in TFA concentrations among treatments, the observed changes in omega-3 FA concentrations and omega-6: omega-3 ratios suggest that dietary supplementation successfully altered the serum lipid profile of developing bulls. Since all bulls were fed the base diet with supplements mixed in per bunk, the diet provided equal amounts of corn silage and chopped hay, which are known sources of PUFA, particularly linoleic acid, an omega-6 FA (Palmquist, 2009). However, the proprietary supplement, composed of flaxseed and algae, was

not fully rumen-protected, meaning that a portion of the supplemented PUFA may have undergone biohydrogenation before absorption. The microbes of the rumen converted the unsaturated fatty acids into saturated forms, limiting the systemic availability of omega-3 FAs (Rojas-González et al., 2024)

Nevertheless, serum omega-3 concentrations were significantly higher in the MOD and HI groups compared to the CON group on d 53 or 55, and 80, and the omega-6: omega-3 ratio was markedly reduced in supplemented bulls. These findings indicate that despite potential ruminal biohydrogenation, a sufficient quantity of omega-3 FAs was absorbed and circulated, altering the balance of serum lipids. This shift is biologically relevant, as elevated omega-6:omega-3 ratios in sperm and seminal plasma have been associated with increased oxidative stress and sperm functions in humans (Abdollahzadeh et al., 2023). Although direct comparisons between species and other biofluids must be made cautiously, the reduction of the ratio observed in this study and our study may reflect a more favorable oxidative environment for reproductive performance.

The lack of change in the PUFA concentrations across treatments further supports the idea that supplementation influenced the FA composition rather than overall abundance with the fat among diets ranging from 4.76% for the CON group, 4.93% for the Mod group, and 5.76% for the HI group. Similar outcomes have been reported in beef heifers, where the dietary omega-3 PUFA supplementation increases EPA and DHA concentrations in plasma and tissues without altering the total PUFA concentrations (Hennessy et al., 2021). In bulls, Kalo et al., (2022) demonstrated that flaxseed and fish oil as omega-3 supplements reduced the ROS levels and DNA fragmentation in spermatozoa, while improving acrosome integrity and membrane fluidity.

These effects were observed two weeks after the end of the feeding period, suggesting a carryover benefit of omega-3 incorporation into the sperm membranes.

Studies in Holstein bulls have shown that the FA composition of seminal plasma is associated with sperm motility and freezability, with greater omega-3 concentrations linked to improved morphology and reduced DNA damage (Eidan et al., 2024). These findings support the hypothesis that dietary manipulation of FA profiles can influence reproductive outcomes, even in the absence of changes in total lipid concentrations.

One limitation of the current study was the absence of a blood serum collection shortly after the initiation of supplementation. Including a sample approximately one week after diet transition would have provided valuable insight into the early kinetics of fatty acid absorption and adaptation. Such data could clarify the timing of serum lipid changes and help distinguish between transient dietary effects and the sustained incorporation of these changes. Studies in dry beef cows and mares have demonstrated a rapid increase in omega-3 FA concentrations within 2 weeks of supplementation utilizing Eicosapentaenoic acid and Docosahexaenoic acid (King et al., 2008; White et al., 2012). There are a limited number of studies that have published blood FA concentrations in livestock species, while the FA composition of semen, seminal plasma, and other body tissues is more readily available.

Thermal image data for testes temperature showed no significant difference among treatments ($P > 0.05$) on either day. For d -6 the HI group had the greatest testes temperature (32.53°C) compared to the CON (32.32°C) and MOD (32.45°C) groups. On d 79 the MOD group had the testes' greatest temperature (28.30°C), followed by HI (28.27°C) and then CON (27.12°C). With no treatment differences, the difference for lower temperatures at the end of the trial could be attributed to the ambient temperature being colder on d 79. The neck of scrotum

temperature follows the same pattern as the testes' temperature. There was a tendency ($P < 0.08$) for the CON group to have a decreased temperature at the scrotal neck. The scrotal neck temperature at d -6 was colder in the CON group (33.81°C), followed by MOD (32.39°C), and warmest in HI (32.15°C). At d 79, the temperature of the scrotal neck followed the same pattern as the testes' temperature. The CON (30.82°C) was the coldest, and the MOD (32.39°C) was the warmest, with the HI (32.15°C) following closely behind.

Although knowing the testicular and scrotal neck temperatures may help determine scrotal temperature for thermoregulation, there is little we can do with only scrotal neck and testicular infrared thermal images. Infrared eye temperatures of cattle have been associated with body temperature and stress-related behaviors (Macmillan et al., 2019; Mota-Rojas et al., 2021; Idris et al., 2024). Eye temperatures measured by infrared thermal imaging at d -6 or 79 were not different among treatments ($P > 0.05$). Eye temperature in the CON group was 32.29°C at d -6 and 79; in the MOD group, 32.62°C and 31.48°C; and in the HI group, 32.03°C and 31.52°C. While there was no difference among treatments for testes, scrotal neck, and eye temperatures, temperatures decreased by the end of the trial. Weather data from the thermal imaging camera and the Manhattan Regional Airport does show a colder ambient temperature on d 79 compared to d -6. We speculate that the change in ambient temperature is reasonable for the difference between the 2 collection days in testes and scrotal temperature. Although scrotal temperature needs to be kept 2 - 6°C below the body temperature of bulls, there has been work on different ways to measure the testicular temperature and thermoregulation. The work has shown that increased temperature has detrimental effects on spermatogenesis, but less work has been done on the effects of cold stress on spermatogenesis (Kastelic et al., 1997). Since there were consistent temperature patterns across treatments, it indicates that supplementation did not impair

heat dissipation. The ability to maintain the thermal gradient supports the findings that reproductive differences are likely metabolic or oxidative rather than thermogenic (Kastelic et al., 1997).

Reactive oxygen species have been reported in bulls in several studies using flow cytometry. There is limited information on the ROS concentrations in just the seminal plasma of livestock species. Total ROS concentrations in the ejaculate were analyzed with chemiluminescence by using the fluorescent probe 2',7'-Dichlorofluorescein (DCF), where the light emitted from the sample is related to the concentration of total ROS. The polynomial standard curve is displayed in Figure 2.7. The equation to convert relative fluorescence units (RFU) to DCF concentration was $y = -0.0004x^2 + 9.9805x + 56.55$. The CON bulls had the lowest concentration at the 0 h collection of 1,335.60 nM of DCF. There were no treatment differences at h 0 or after the 3 h water-bath stress test, and this is presented in Figure 2.8. The MOD (1,638.65 nM) and HI (1,675.62 nM) groups had higher DCF concentrations at h 0. After the stress test, the 3 h CON (1,577.69 nM) group had the highest DCF concentration, while the MOD (1,343.80 nM) and HI (1,554.20 nM) treatment groups showed decreased DCF concentrations. Concentrations of DCF in seminal plasma utilizing luminol chemiluminescence are not easily obtained in the literature, especially in livestock species. In a study using DCF with flow cytometry on sperm cells, cells with intact plasma membranes generated H₂O₂ over 24 hours, whereas those with compromised plasma membranes did not (Ahmad et al., 2015). Studies have reported the number of reflective light units per second by sperm concentration in humans (Castleton et al., 2022). A study in infertile men utilizing intracytoplasmic sperm injection for infertility only published the ROS means for sperm abnormalities, but no concentrations were reported (Alkhafaji et al., 2023). The use of chemical luminescence in

human seminal plasma for ROS quantification has been validated (Vessey et al., 2014), in humans, with most work in livestock species utilizing flow cytometry (Kalo et al., 2022; Giaretta et al., 2025).

Hydrogen peroxide (H_2O_2) concentrations were also analyzed to determine the concentration of H_2O_2 ROS in seminal plasma. There was no difference between treatment groups for H_2O_2 concentration in seminal plasma 0 h or 3 h after collection. The polynomial standard curve for the H_2O_2 standards can be found in Figure 2.9. The equation used to convert RFU to μM of H_2O_2 was $y = -22.454x^2 + 1711.9x + 1341.6$. Seminal plasma from d 76 BSE collections at 0 h for the CON group had the least amount of H_2O_2 at 7.32 μM while the MOD (9.31 μM) and HI (9.56 μM) groups had greater concentrations. Following the same trend as the DCF concentrations, the CON group H_2O_2 concentration increased to 8.91 μM after a 3 h stress test, while the MOD (7.38 μM) and HI (8.75 μM) groups' concentrations decreased and are portrayed in Figure 2.10.

Reactive oxygen species produced in sperm mitochondria and cytoplasm can damage proteins, lipids, and DNA, impairing overall sperm function (Krishnamurthy and Wadhwani, 2012). Oxidative protein markers such as advanced oxidation protein products reflect increased ROS activity and seminal plasma damage in men (Celi et al., 2011; Bordinon et al., 2014; Kratz et al., 2016; Kratz and Piwowar, 2017). Seminal plasma provides antioxidant protection, and total antioxidant status offers a more consistent measure of oxidative balance than individual enzyme activities (Ünal and Uysal, 2024). Key seminal Plasma proteins, including peroxiredoxin 5, spermadhesin-1, and BSP5, have been linked to sperm freezability and fertility potential (Gomes et al., 2020; Giaretta et al., 2025).

While lesser ROS concentrations are essential for spermatogenesis and sperm-oocyte binding, excessive concentrations contribute to up to 80% of male infertility cases (Hussain et al., 2023). Sperm cells are particularly vulnerable because they lack cytoplasmic antioxidant defenses and have PUFA-rich membranes prone to lipid peroxidation (Aitken et al., 2006; Hwang and Lamb, 2012). The FA composition of sperm influences motility and oxidative balance; disturbances in omega-3 metabolism can disrupt ROS homeostasis and DNA integrity (Pang et al., 2025). Reduced omega-3 and docosahexaenoic acid concentrations have been associated with lower sperm viability and morphology, suggesting that omega-3 supplementation can mitigate oxidative stress and enhance membrane stability (Collodel et al., 2020; Kogan et al., 2021).

Our results from seminal plasma ROS show that supplementation with an omega-3 supplement numerically improves the seminal plasma resistance to oxidative stress, which has been consistently linked to reducing lipid peroxidation (Gürler et al., 2015; Moallem et al., 2015). Elevated ROS disrupts sperm membrane integrity, motility, and DNA (Dutta et al., 2022). Although there were no significant treatment effects, the consistent biological reduction in ROS for the treatment groups over time strengthens the mechanistic evidence for omega-3 mediated antioxidant protection.

Conclusion

Omega-3 supplementation effectively altered the circulating lipid composition, increasing omega-3 and decreasing the omega-6 : omega-3 in the blood serum of bulls. Omega-6 FA did not differ among treatments throughout the supplementation period, while omega-3 FA differed between the CON and HI group indicating that the supplement amount was able to increase the omega 3 concentrations in blood serum. Growth and carcass traits remained stable, but moderate

supplementation improved feed efficiency and coincided with numerical improvements in the percent of motile sperm and the percent of sperm that were morphologically normal.

A tendency ($P = 0.10$) for an increase in motility was observed in the repeated measures model between the HI and CON group, suggesting that incorporation of the omega-3 FA-based supplement improved motility. Progressive motility of sperm declined after supplementation was withdrawn, indicating potential short-lived benefits that could be partially influenced by CASA machine analysis and suggesting that continual inclusion maybe needed to sustain improvements during breeding. Three weeks after the supplementation period on d 101, the progressive motility of sperm decreased to the same amount as d 53 to 55. This suggests that omega-3 supplementation had potential effects on the progressive motility of sperm but needs to be investigated further as d 101 was analyzed with a different CASA than d 80. Together with reduced ROS accumulation and altered lipid profiles, these results support the hypothesis that omega-3 supplementation enhances the antioxidant capacity of seminal plasma and the stability of sperm membranes. These findings complement previous work demonstrating that omega-3 enrichment improves sperm structure, function, and fertilizing potential across species (Ngcobo et al., 2021; Giaretta et al., 2025). Continued supplementation through the breeding season may sustain these benefits and improve overall reproductive efficiency.

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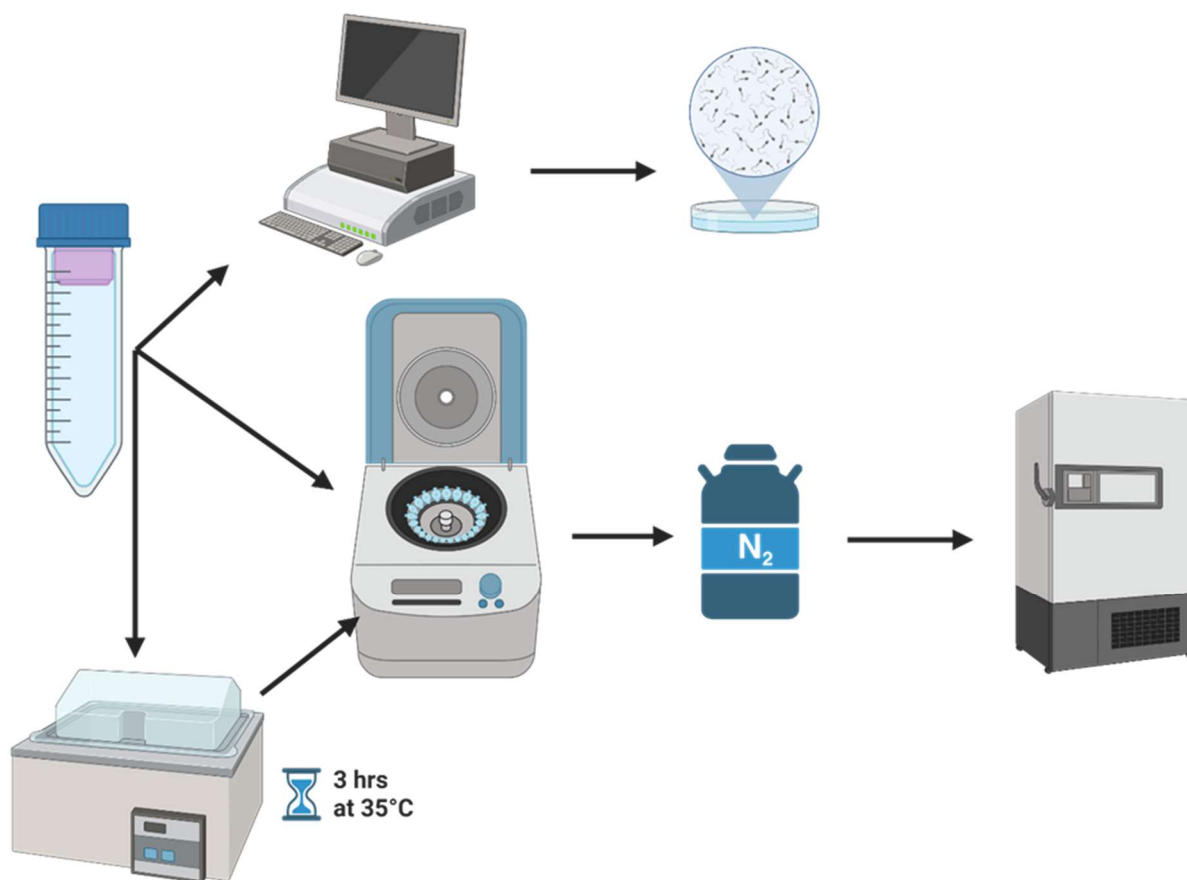


Figure 2.1 Seminal plasma collection procedure.

Semen samples from the ejaculate were collected, and approximately 0.25mL of the sample was pipetted and placed into a clean microcentrifuge tube for analysis with a computer-assisted sperm analysis (CASA) machine. Approximately half of the remaining sample was pipetted into a clean vial and centrifuged for 15 minutes at 3,500 rpm. Seminal plasma was pipetted off, snap-frozen in liquid nitrogen, and stored at -80°C until analysis for reactive oxygen species. The remaining sample was placed in a water bath at 35°C for 3 hours as a stress test. Then centrifuged for 15 minutes at 3,500 rpm. Seminal plasma was pipetted off, snap-frozen in liquid nitrogen, and stored at -80°C until analysis for reactive oxygen species.

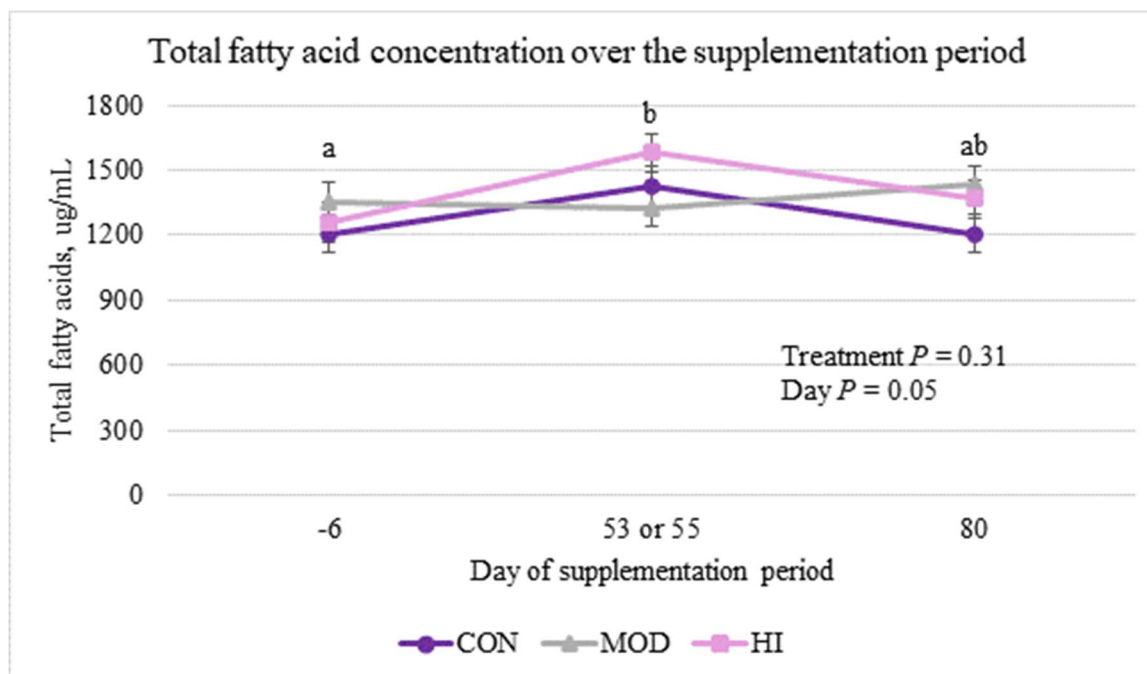


Figure 2.2 Total fatty acid concentration over the supplementation period.

Total fatty acid concentration (mean $\pm$ standard error ug/mL) in blood serum of beef bulls during the supplementation period (day -6, 53 or 55, and 80). Different superscripts (a,b) indicate differences among days ($P \leq 0.05$). There was no interaction between treatment and day ($P = 0.18$); no treatment effect on total fatty acids ($P = 0.31$); a day effect was observed ($P \leq 0.05$).

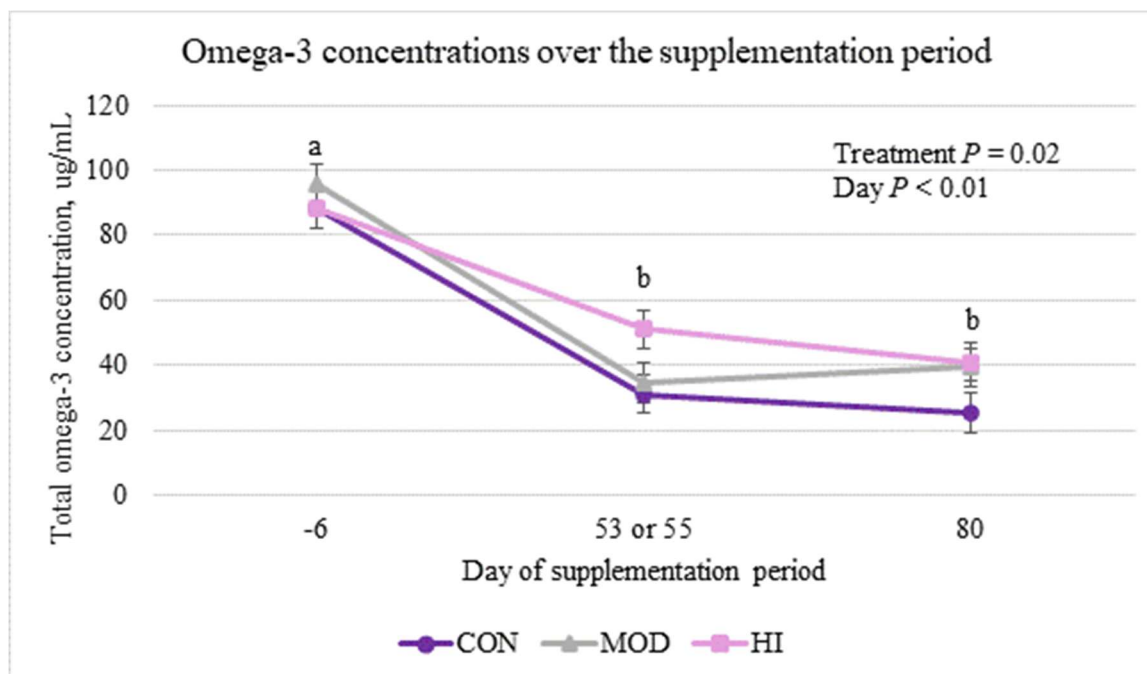


Figure 2.3 Omega-3 concentrations over the supplementation period.

Omega-3 concentrations with standard error of the mean (ug/mL) in blood serum of developing bulls during the 80-day supplementation period. Different superscripts (a,b) indicate differences among days ($P < 0.01$); a treatment effect was observed with the CON and HI groups being significantly different ($P < 0.05$), and no interaction for treatment and day was observed ($P = 0.08$).

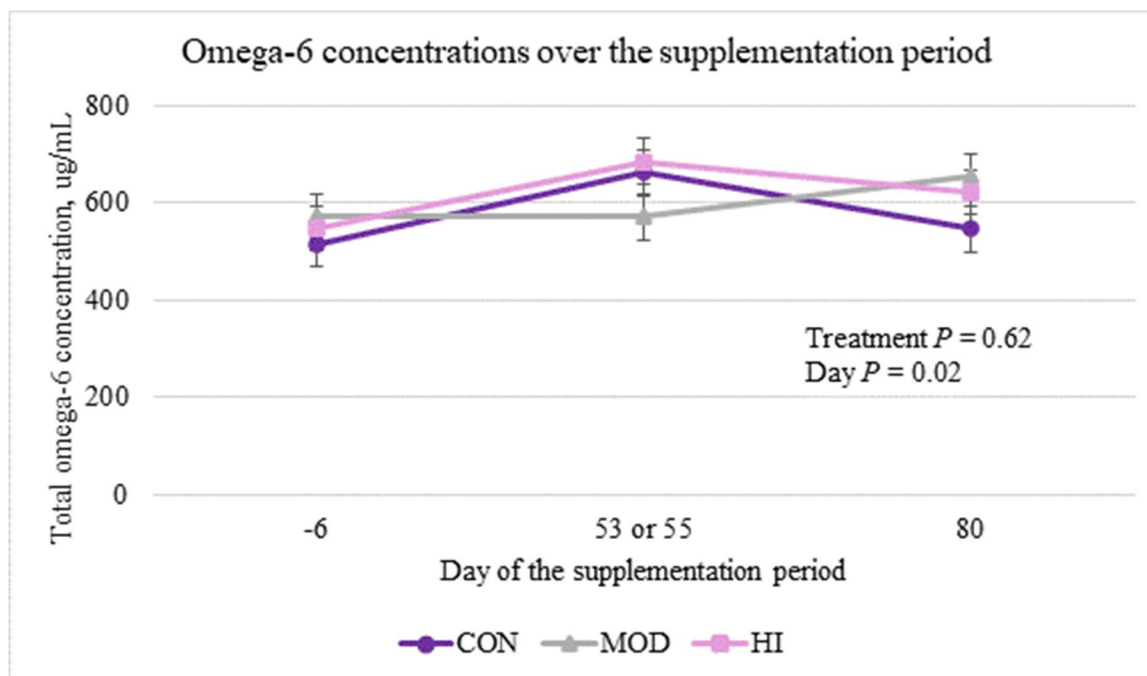


Figure 2.4 Omega-6 concentrations over the supplementation period.

Omega-6 concentrations with standard error of the mean (ug/mL) in blood serum of developing bulls during the 80-day supplementation period. A day effect was observed between day -6 and 53 or 55 ($P \leq 0.05$); a treatment effect was not observed ($P > 0.05$), and no interaction between treatment and day was observed ($P = 0.15$).

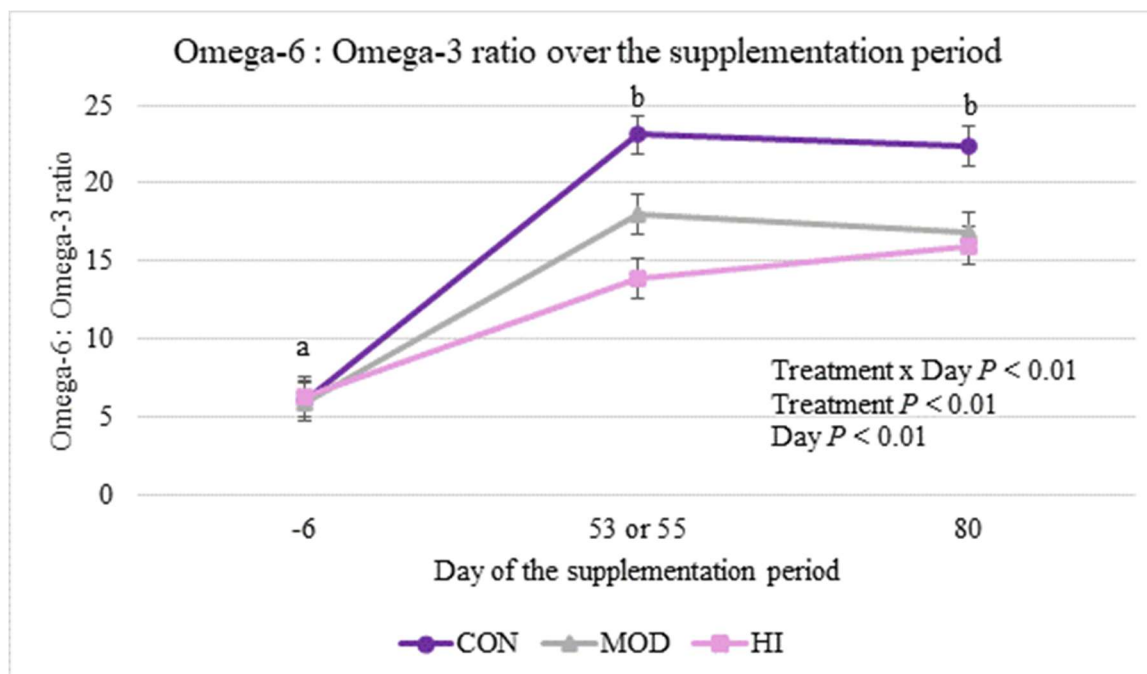


Figure 2.5 Omega-6 : Omega-3 ratio over the supplementation period.

The mean omega-6 : omega-3 fatty acid ratio in the blood serum of developing beef bulls during the supplementation period. An interaction between treatment and day was observed ($P < 0.01$); the CON group showed a treatment effect between the MOD and HI groups ($P < 0.02$); and different superscripts (a,b) denote a difference among days ($P < 0.01$).

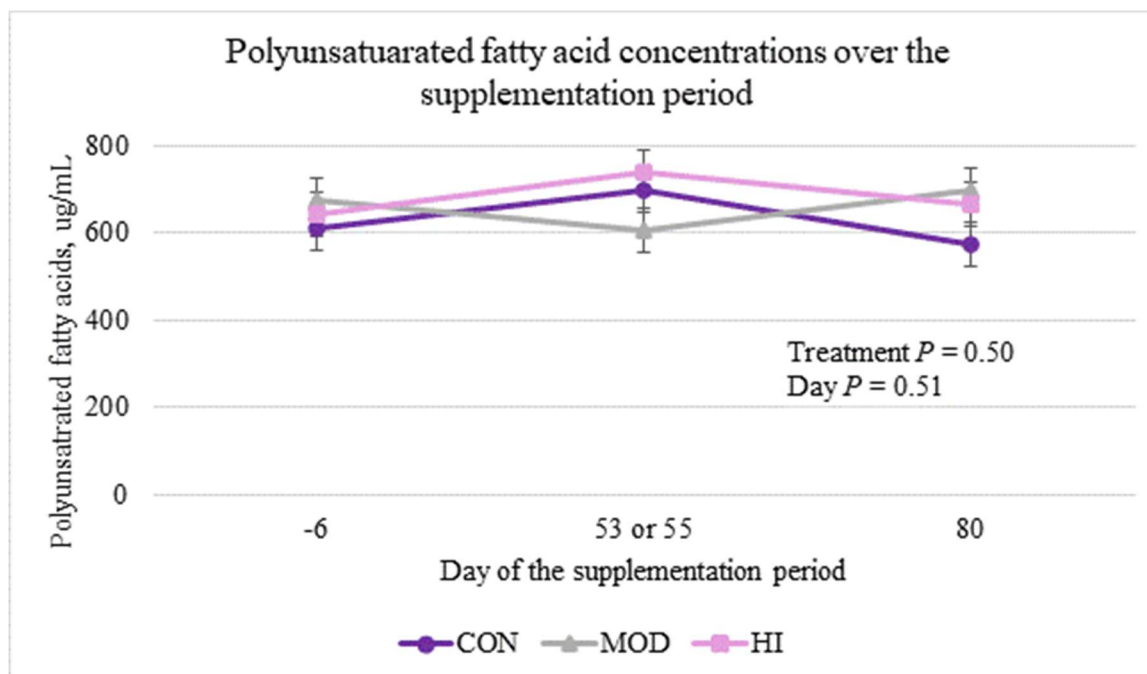


Figure 2.6 Polyunsaturated fatty acid concentrations over the supplementation period.

Polyunsaturated fatty acids concentration (ug/mL) in the blood serum of bulls over the supplementation period. No significant differences were observed among treatments ($P > 0.05$) or day ($P > 0.05$), and no interaction was observed for treatment and day ($P > 0.15$).

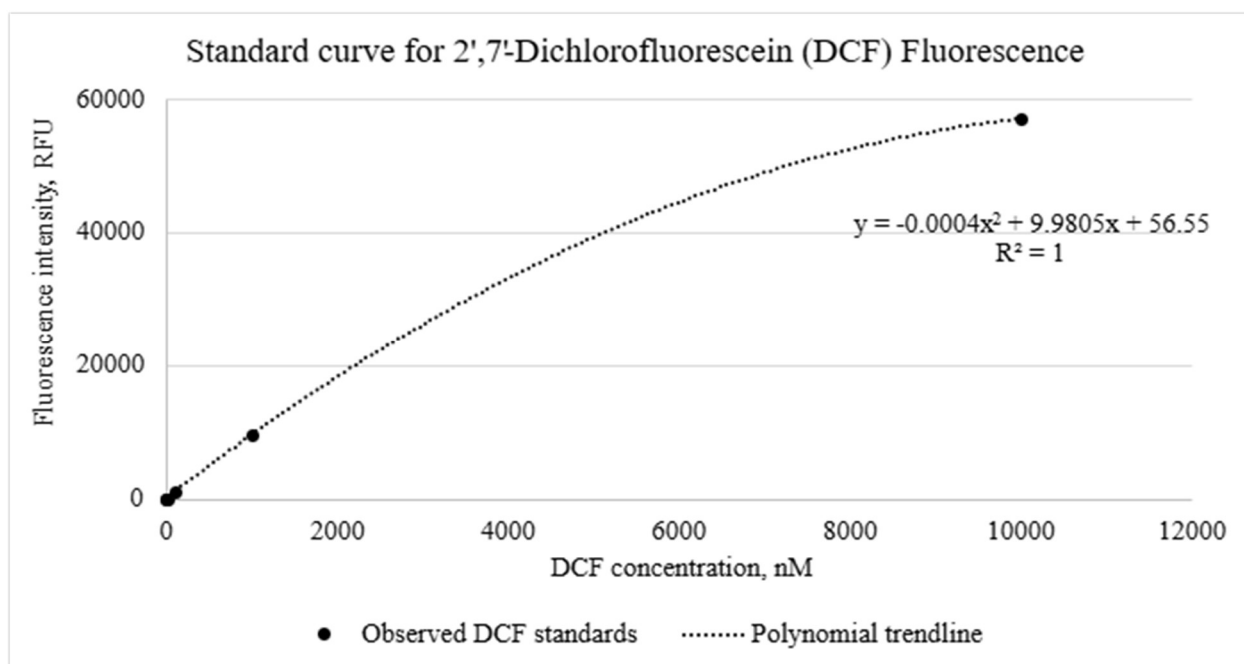


Figure 2.7 Standard curve for 2', 7'-Dichlorofluorescein (DCF) fluorescence.

Black circles represent observed DCF standards (0-10,000 nM). The dotted line indicates the second-order polynomial fit ($y = -0.0004x^2 + 9.9805x + 56.55$; $R^2 = 1$) used to convert fluorescence intensity (relative fluorescence units, RFU) to DCF concentration in nM.

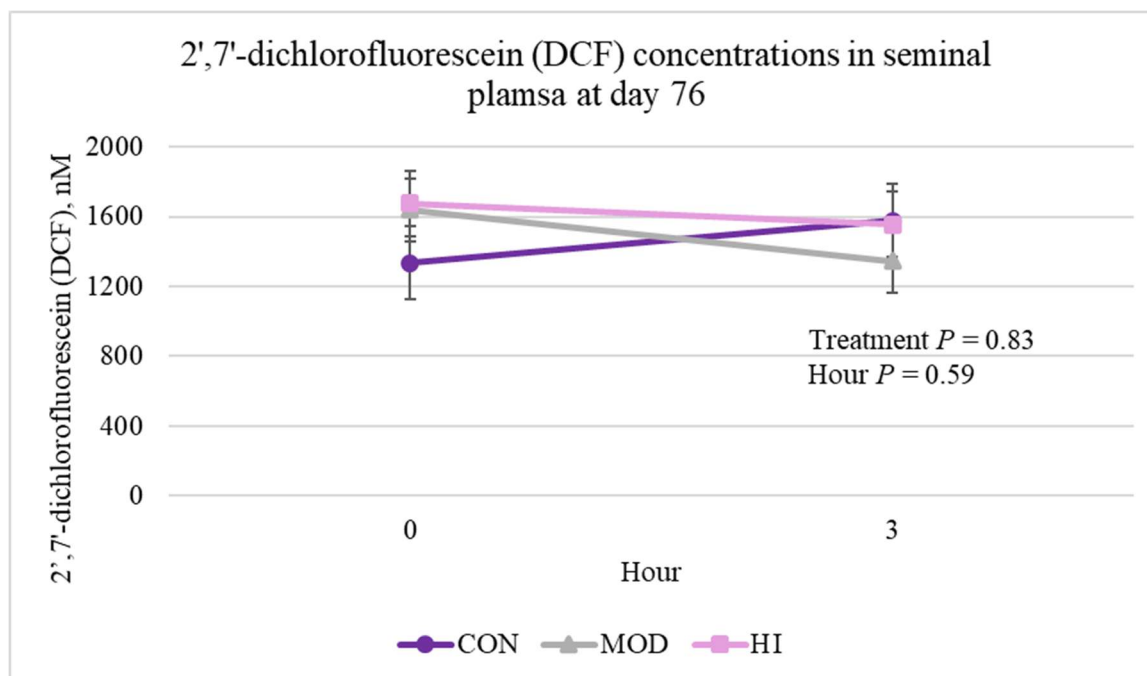


Figure 2.8 2', 7'-dichlorofluorescein (DCF) concentrations in seminal plasma at day 76.

Concentrations of 2',7'-dichlorofluorescein (DCF) in seminal plasma at Day 76 of the supplementation period. The DCF concentrations were measured at 0 and 3 h post-collection to assess reactive oxygen species levels. There was no significant difference observed among treatment groups ($P = 0.83$), hours ($P = 0.59$), or the interaction of treatment and hour, indicating that supplementation of omega-3 did not alter the DCF concentration in seminal plasma.

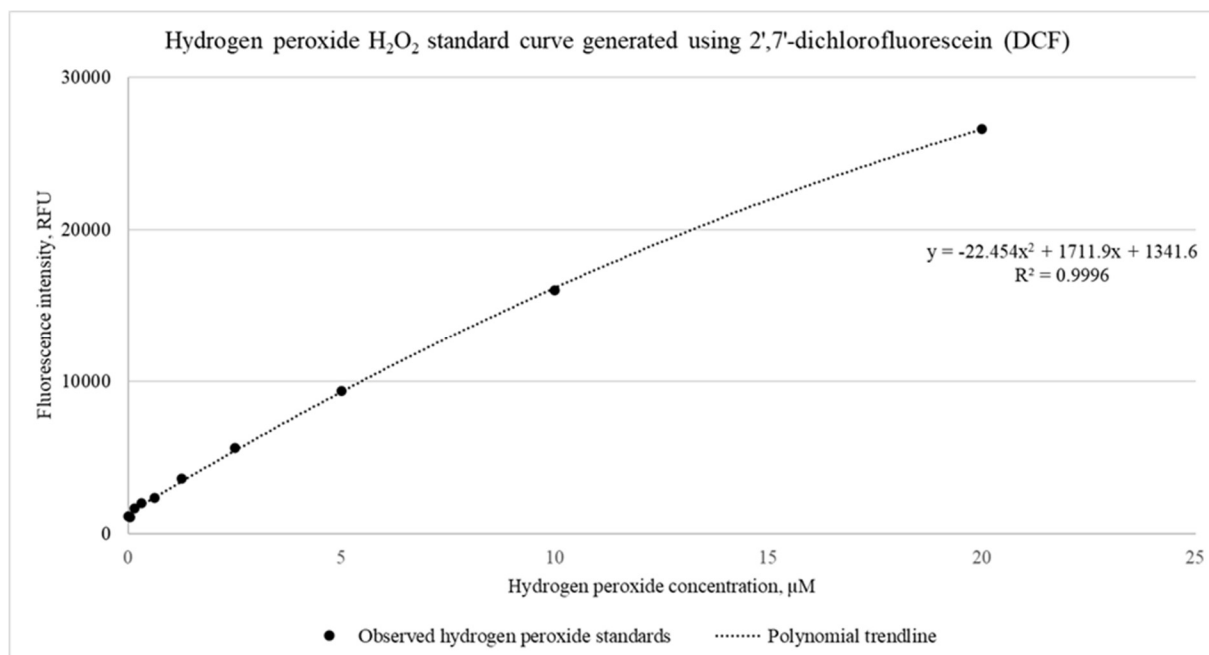


Figure 2.9 Hydrogen peroxide (H_2O_2) standard curve generated using 2', 7'-dichlorofluorescein (DCF).

Standard curve for hydrogen peroxide (H_2O_2) using fluorescence. Black circles represent observed H_2O_2 standards (0-20 μM). The dotted line indicates the second-order polynomial fit ($y = -22.454x^2 + 1711.9x + 1341.6$; $R^2 = 0.99$) used to convert fluorescence intensity (relative fluorescence units, RFU) to H_2O_2 concentration.

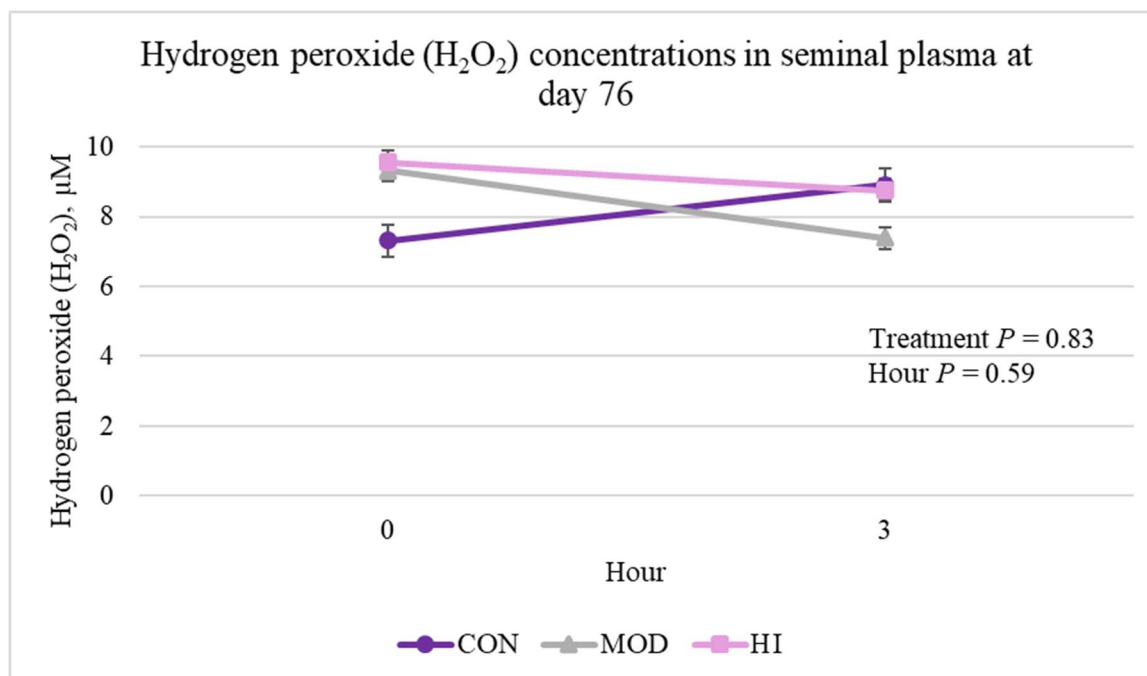


Figure 2.10 Hydrogen peroxide (H_2O_2) concentrations in seminal plasma at day 76.

Hydrogen peroxide (H_2O_2) concentrations in the seminal plasma of bulls at day 76 of the supplementation period. The H_2O_2 concentrations were measured at 0 and 3 h post-collection to assess reactive oxygen species. No differences were observed among treatments ($P = 0.83$) or between hours ($P = 0.59$), and no interaction between h and treatment were observed.

Table 2.1 Breed frequency of bulls in each treatment group.

Treatment	Angus	Hereford	Simmental	Total
CON ¹	4	5	4	13
MOD ²	6	3	4	13
HI ³	6	4	3	13
Total	16	12	11	13

¹CON = Control treatment group.

²MOD = Moderate treatment group.

³HI = High treatment group.

Table 2.2 Ingredient and nutrient composition of diets fed to developing bulls¹.

Ingredient, %	PRIOR ²	BASE ³	CON ⁴	MOD ⁵	HI ⁶	Omega-3 supplement	Corn germ meal
Corn silage	18.4	34.3	34.3	34.3	34.3		
DDGS ⁷	29.0	22.2	22.2	22.2	22.2		
Dry rolled corn	12.46	20.9	20.9	20.9	20.9		
Grass hay	31.7	14.7	14.7	14.7	14.7		
Supplement	8.4	7.9	7.9	7.9	7.9		
Nutrient, %							
Dry Matter		57.40	59.73	58.58	60.53	96.16	91.65
Crude Protein		12.47	13.07	12.61	13.47	32.26	27.23
Acid Detergent Fiber		18.79	17.87	18.08	17.81	11.28	11.40
Neutral Detergent Fiber		31.79	31.01	31.13	32.09	19.76	43.16
Fat		4.95	4.76	4.93	5.76	18.85	5.22
Calcium		0.92	0.87	0.80	0.76	4.04	0.63
Phosphorus		0.47	0.48	0.45	0.47	0.56	0.68
Corn germ meal ^a		-	0.90	0.56	0.54		
Omega-3 supplement ^a		-	-	0.22	0.44		

¹All values on a 100% dry matter basis²PRIOR = Ration feed prior to day 0 or the beginning of the supplementation period³Base ration fed to all treatment groups.⁴CON = Control treatment group.⁵MOD = Moderate treatment group.⁶HI = High treatment group.⁷Corn dried distillers grains with solubles.^aAmounts included on a DM basis per head per day basis.

Table 2.3 Descriptive statistics of developing beef bulls by treatment group at the beginning of the feeding period (Day 0)¹.

Trait	CON ²	MOD ³	HI ⁴	SE ⁵	<i>P</i> – Value ⁶
n ⁷	13	13	13		
Age ⁸ , d	282 ± 24.60	281 ± 26.11	283 ± 26.61	7.15	0.99
Weight ⁹ , kg	332 ± 50.69	334 ± 65.18	338 ± 34.76	14.35	0.96
BCS ¹⁰	4.92 ± 0.76	4.54 ± 0.88	4.62 ± 0.77	0.22	0.44
SC ¹¹ , cm	27.12 ± 2.88	27.38 ± 2.84	28.00 ± 2.61	0.77	0.71

¹Means of treatment groups at Day 0 of the feeding period with the standard deviation.

²CON = Control group with no omega-3 supplementation.

³MOD = Moderate omega-3 supplementation group (0.22 kg per head daily).

⁴HI = High omega-3 supplementation group (0.44 kg per head daily).

⁵SE = Standard error of the mean.

⁶Significance declared at *P* < 0.05.

⁷n = number of bulls per treatment group.

⁸Age of bulls on Day 0 of the feeding period in days.

⁹Weight of the bulls on Day 0 of the feeding period in kilograms.

¹⁰BCS = Body condition score of bulls on Day 0 using a 1-9 scale.

¹¹SC = Scrotal circumference of bulls on Day 0 using a scrotal tape in centimeters.

Table 2.4 Least square means of carcass characteristics on Day 0 of the feeding period by treatment group.

Variable	CON ¹	MOD ²	HI ³	SE ⁴	<i>P</i> – Value ⁵
Longissimus area ⁶ , cm ²	25.20	25.70	25.36	1.30	0.95
Intramuscular fat ⁷ , %	2.12	2.28	2.20	0.18	0.76
Subcutaneous backfat ⁸ , cm	0.37	0.39	0.39	0.03	0.84

¹CON = Control group with no omega-3 supplementation.

²MOD = Moderate omega-3 supplementation group (0.22 kg per head daily).

³HI = High omega-3 supplementation group (0.44 kg per head daily).

⁴SE = Standard error of the mean.

⁵Significance declared at $P < 0.05$.

⁶Longissimus area was measured between the 12th and 13th rib.

⁷Percent of fat between the muscle fibers of the ribeye over the 12th and 13th rib.

⁸Subcutaneous backfat was measured in cm over the 12th and 13th rib.

Table 2.5 Performance data on Day 53 or 55 of the feeding period by treatment group.¹

Variable	CON ²	MOD ³	HI ⁴	SE ⁵	<i>P</i> -Value ⁶
Weight, kg	400	418	422	16.28	0.58
BCS ⁷	5.8	6.0	5.6	0.17	0.28

¹Means by treatment group of performance data of bulls collected on Day 53 or 55 of the feeding period due to the collection schedule of the stud facility.

²CON = Control group with no omega-3 supplementation.

³MOD = Moderate omega-3 supplementation group (0.22 kg per head daily).

⁴HI = High omega-3 supplementation group (0.44 kg per head daily).

⁵SE = Standard error of the mean.

⁶Significance level was declared at $P < 0.05$.

⁷BCS = Body condition score was measured utilizing the 1-9 scale.

Table 2.6 Semen characteristics data on Day 53 or 55 of the feeding period by treatment group.¹

Treatment	CON ²	MOD ³	HI ⁴	SE ⁵	<i>P</i> -Value ⁶
SC, cm ⁷	35.08	35.35	35.96	0.67	0.63
Morphology ⁸ , %	41.70	50.59	60.54	11.22	0.46
Motility ⁹ , %	33.70 ^a	45.50 ^{ab}	51.52 ^b	5.86	0.09
Progressive motility ¹⁰ , %	22.13	31.72	41.86	7.13	0.12
Passing BSE ¹¹ , %	23.08	38.46	46.15	13.83	0.48

¹Least squares means by treatment group of performance data of bulls collected on Day 53 or 55 of the feeding period due to the collection schedule of the stud facility.

²CON = Control group with no omega-3 supplementation.

³MOD = Moderate omega-3 supplementation group (0.22 kg per head daily).

⁴HI = High omega-3 supplementation group (0.44 kg per head daily).

⁵SE = Standard error.

⁶Significance level was declared at $P \leq 0.05$; tendency declared at $0.05 > P \leq 0.10$.

⁷SC = Scrotal circumference, cm.

⁸Percent of morphologically normal sperm.

⁹Percent of motile sperm.

¹⁰Percent of sperm that were progressively motile.

¹¹Percent of bulls passing a breeding soundness exam (BSE).

^{a,b}Denotes significant difference among treatments or a tendency among treatments.

Table 2.7 Performance data on Day 80 of the feeding period by treatment group.¹

Treatment	CON ²	MOD ³	HI ⁴	SE ⁵	<i>P</i> -Value ⁶
Weight, kg	445	458	462	15.85	0.73
BCS ⁷	5.9	6.2	6.2	0.15	0.28
G:F ⁸	0.14 ^a	0.18 ^b	0.16 ^{ab}	0.01	0.04
ADG ⁹	1.41	1.55	1.56	0.07	0.31
DMI ¹⁰	9.86	9.07	9.77	0.40	0.31

¹Least square means of performance data from bulls collected on Day 80 of the feeding period by treatment.

²CON = Control group with no omega-3 supplementation.

³MOD = Moderate omega-3 supplementation group (0.22 kg per head daily).

⁴HI = High omega-3 supplementation group (0.44 kg per head daily).

⁵SE = Standard error.

⁶Significance level was declared at $P \leq 0.05$; tendency declared at $0.05 > P \leq 0.10$.

⁷BCS = Body condition score was measured utilizing the 1-9 scale.

⁸G:F = Gain to Feed ratio.

^{a-b}Means with unlike superscripts differ ($P < 0.05$).

⁹ADG = Average daily gain in kg.

¹⁰DMI = Daily dry matter intake in kg.

^{a,b}Denotes significant difference among treatments or a tendency among treatments.

Table 2.8 Least square means of carcass characteristics on Day 80 of the feeding period by treatment group.

Variable	CON ¹	MOD ²	HI ³	SE ⁴	<i>P</i> – Value ⁵
Longissimus area ⁶ , cm ²	32.19	33.16	33.02	1.16	0.74
Intramuscular fat ⁷ , %	2.49	2.77	2.52	0.41	0.65
Subcutaneous back fat ⁸ , cm	0.58	0.62	0.58	0.04	0.74

¹CON = Control group with no omega-3 supplementation.

²MOD = Moderate omega-3 supplementation group (0.22 kg per head daily).

³HI = High omega-3 supplementation group (0.44 kg per head daily).

⁴SE = Standard error of the mean.

⁵Significance level was declared at $P \leq 0.05$; tendency declared at $0.05 > P \leq 0.10$.

⁶Longissimus area was measured between the 12th and 13th rib.

⁷Percent of fat between the muscle fibers of the ribeye over the 12th and 13th rib.

⁸Subcutaneous backfat was measured in cm over the 12th and 13th rib.

Table 2.9 Semen characteristics data on day 76 of the feeding period by treatment group. ¹

Treatment	CON ²	MOD ³	HI ⁴	SE ⁵	P-Value ⁶
SC ⁷ , cm	36.38	36.42	37.65	0.66	0.31
Morphology ⁸ , %	57.46	78.54	68.15	8.93	0.24
Motility ⁹ , %	45.27	50.58	57.63	7.27	0.47
Progressive motility ¹⁰ , %	37.89	42.12	50.77	7.14	0.43
Passing BSE ¹¹ , %	38.46	61.54	53.85	13.83	0.50

¹Least squares means by treatment group of performance data of bulls collected on Day 76 of the feeding period.

²CON = Control group with no omega-3 supplementation.

³MOD = Moderate omega-3 supplementation group (0.22 kg per head daily).

⁴HI = High omega-3 supplementation group (0.44 kg per head daily).

⁵SE = Standard error.

⁶Significance level was declared at $P \leq 0.05$; tendency declared at $0.05 > P \leq 0.10$.

⁷SC = Scrotal circumference, cm.

⁸Percent of morphologically normal sperm.

⁹Percent of motile sperm.

¹⁰Percent of sperm that were progressively motile.

¹¹Percent of bulls passing a breeding soundness exam (BSE).

Table 2.10 Semen characteristics data on day 101, 21 days after the conclusion of the supplementation period by treatment group.¹

Treatment	CON ²	MOD ³	HI ⁴	SE ⁵	<i>P</i> -Value ⁶
SC, cm ⁷	37.50	37.58	37.96	0.59	0.84
Morphology ⁸ , %	71.25	74.90	82.44	4.49	0.15
Motility ⁹ , %	57.58	63.78	72.05	6.57	0.27
Progressive motility ¹⁰ , %	24.12	23.13	29.53	5.33	0.65
Passing BSE ¹¹ , %	30.15	21.59	37.42	17.45	0.56

¹Means by treatment group of performance data of bulls collected on Day 101 of the feeding period, or 21 days after the supplementation period.

²CON = Control treatment group.

³MOD = Moderate treatment group.

⁴HI = High treatment group.

⁵SE = Standard error of the mean.

⁶Significance level was declared at $P \leq 0.05$; tendency declared at $0.05 > P \leq 0.10$.

⁷SC = Scrotal circumference, cm.

⁸Percent of morphologically normal sperm.

⁹Percent of motile sperm.

¹⁰Percent of sperm that were progressively motile.

¹¹Percent of bulls passing a breeding soundness exam (BSE).

Table 2.11 Least square means of repeated measures by treatment group averaged through the duration of the 80-day feeding period and 21 days after.

Variable	CON ¹	MOD ²	HI ³	SE ⁴	<i>P</i> – Value ⁵
Weight, kg	392.08	403.18	407.33	15.05	0.76
ADG ⁶ , kg	1.58	1.61	1.60	0.09	0.96
Gain ⁷ , kg	56.46	62.15	62.12	2.99	0.31
BCS ⁸	5.51	5.56	5.46	0.13	0.86
SC ⁹ , cm	34.02	34.18	34.89	0.61	0.57
Morphology ¹⁰ , %	57.83	70.66	73.97	0.34	0.28
Motility ¹¹ , %	44.79 ^a	54.78 ^{ab}	61.86 ^b	5.37	0.10
Progressive motility ¹² , %	25.49	31.23	39.50	5.56	0.17
Passing BSE ¹³ , %	22.20 ^a	36.09 ^b	43.36 ^b	5.37	0.17
Longissimus area ¹⁴ , cm ²	28.62	29.40	29.29	1.41	0.84
Subcutaneous back fat ¹⁵ , cm	0.50	0.47	0.48	0.04	0.78
Intramuscular fat ¹⁶ , %	2.28	2.58	2.43	0.21	0.60

¹CON = Control group with no omega-3 supplementation.

²MOD = Moderate omega-3 supplementation group (0.22 kg per head daily).

³HI = High omega-3 supplementation group (0.44 kg per head daily).

⁴SE = Standard error of the mean.

⁵Significance level was declared at $P \leq 0.05$; tendency declared at $0.05 > P \leq 0.10$.

⁶ADG = Average daily gain in kilograms.

⁷Amount gained over the 80 day supplementation period in kilograms.

⁸BCS = Body condition score on the 1-9 scale.

⁹SC = Scrotal circumference in centimeters.

¹⁰Percent of sperm cells that were morphologically normal.

¹¹Percent of motile sperm.

¹²Percent of sperm that were progressively motile.

¹³Percent of bulls passing a breeding soundness exam (BSE).

¹⁴Longissimus area was measured between the 12th and 13th rib.

¹⁵Subcutaneous back fat was measured in centimeters over the 12th and 13th rib.

¹⁶Percent of fat between the muscle fibers of the ribeye over the 12th and 13th rib.

^{a-b}Means within a row with unlike superscripts differ ($P < 0.05$).

Table 2.12 Least square means of repeated measures by day averaged through the duration of the supplementation period and 21 days after.

Variable	0 ¹	53 or 55 ²	80 ³	101 ⁴	SE ⁵	<i>P</i> – Value ⁶
Weight, kg	334.41 ^a	413.28 ^b	454.90 ^c		8.96	<0.01
BCS ⁷	4.69 ^a	5.79 ^b	6.05 ^b		0.11	<0.01
SC ⁸ , cm	27.50 ^a	35.46 ^b	36.82 ^c	37.68 ^d	0.39	<0.01
Morphology ⁹ , %		50.51 ^a	70.48 ^b	79.40 ^b	5.78	<0.01
Motility ¹⁰ , %		43.34 ^a	52.21 ^a	65.61 ^b	4.11	<0.01
Progressive motility ¹¹ , %		30.74 ^a	41.69 ^b	24.22 ^a	4.17	<0.01
Passing BSE ¹² , %		28.66 ^a	51.58 ^a	22.07 ^b	1.97	0.02
Longissimus area ¹³ , cm ²	25.40 ^a		32.80 ^b		0.60	<0.01
Subcutaneous backfat ¹⁴ , cm	0.38 ^a		0.59 ^b		0.02	<0.01
Intramuscular fat ¹⁵ , %	2.21 ^a		2.65 ^b		0.13	<0.01

¹0 = Day 0 of the supplementation period.

²53 or 55 = Day 53 or 55 of the supplementation period due to the schedule of the stud facility.

³80 = Day 80 or the end of the supplementation period.

⁴101 = Day 101 from the beginning of the supplementation period and includes a 21 day period after the conclusion of the supplement.

⁵SE = Standard error of the mean.

⁶Significance level was declared at $P \leq 0.05$; tendency declared at $0.05 > P \leq 0.10$.

⁷BCS = Body condition score on the 1-9 scale.

⁸SC = Scrotal circumference in centimeters.

⁹Percent of sperm that were morphologically normal,

¹⁰Percent of sperm that are motile.

¹¹Percent of sperm that are progressively motile.

¹²Percent of bulls passing a breeding soundness exam (BSE).

¹³Longissimus area was measured between the 12th and 13th rib.

¹⁴Subcutaneous back fat was measured in centimeters over the 12th and 13th rib.

¹⁵Percent of fat between the muscle fibers of the ribeye over the 12th and 13th rib.

^{a-d}Means within a row with unlike superscripts differ ($P < 0.05$).

Chapter 3 - Relationship of Anogenital Distance and Fertility in Beef

Cattle Females

Abstract

A retrospective analysis evaluating the relationship between anogenital distance (AGD) and pregnancy outcomes in beef cattle females inseminated using fixed-time artificial insemination (TAI) protocols was conducted. Data were collected from 2,661 *Bos taurus* and *Bos indicus* cows and heifers from 16 different locations across the United States and Brazil from 2023 to 2025. All animals were enrolled in a 7-day CO-Synch + CIDR protocol in the United States or an estradiol benzoate-based synchronization protocol in Brazil. Anogenital distance measurements were obtained using digital calipers either during estrus synchronization or at the time of pregnancy diagnosis. A subset of 933 *Bos taurus* heifers was also assigned a reproductive tract score (RTS) prior to the start of the estrus synchronization protocol. Cattle were sorted into AGD quartiles and tertiles based on AGD length for each location using the SAS sort procedure. The GLIMMIX procedure in SAS with contrast statements was used to analyze differences between quartiles or tertiles. No effect was observed for AGD on the probability of pregnancy for *Bos indicus* or *Bos taurus* cows, although *Bos taurus* cows in the shortest AGD quartile tended to have a greater probability of pregnancy than those in the longest AGD quartile. Among *Bos indicus* heifers, the heifers in the shortest AGD tertile had a greater probability of becoming pregnant than those in the longest tertile, with open heifers exhibiting an AGD approximately 2 mm longer than pregnant heifers. In a subset of *Bos taurus* heifers that had AGD and RTS data, RTS was a stronger predictor of pregnancy to TAI ($P < 0.05$) than AGD, with each unit increase in RTS corresponding to a 43% increase in the odds of pregnancy. Conversely, each 1 mm increase in AGD corresponds to an estimated 4% reduction in the odds

of pregnancy. Although AGD was easily and consistently measured, its predictive value for fertility was limited compared to RTS. The observed trends support the literature in dairy and other species, which finds that a shorter AGD is associated with greater reproductive success. However, substantial variability between breeds and locations indicates the need for larger, breed-specific datasets. Future research should focus on AGD within subspecies and breeds, as well as the integration of AGD with other variables, such as RTS, body condition, body weight, and age, to clarify the effectiveness of AGD as a marker for the probability of becoming pregnant.

Introduction

Fertility in beef cattle is a multifaceted and complex interaction among genetics, environment, management, and biology, with fertility being the most economically important trait in beef cattle production (Moorey and Biase, 2020). While artificial insemination (AI) usage in the United States dairy industry accounts for over 60% of the dairy cattle population, the use of AI in beef cattle herds is only about 5% of operations (National Institute of Food and Agriculture 2025). The pregnancy to AI is influenced by physiological, metabolic, and management variables. Even with advances in estrus synchronization (ES) and AI technologies, the pregnancy rate using AI remains variable, with a wide range of pregnancies confirmed to have been conceived using AI. The potential to use non-invasive physiological markers to predict which cattle are more likely to conceive in the first round of AI would enhance the reproductive efficiency of beef herds.

Reproductive efficiency of beef herds relies heavily on the capacity of selecting highly fertile beef replacement heifers, capable of becoming pregnant early during their first breeding season, as these heifers wean heavier calves for several consecutive years and have longer

longevity in the herd, when compared to cohorts that become pregnant later in the breeding season (Cushman et al., 2013). Currently, the tools available for beef producers to identify replacement heifers with greater fertility are limited to evaluating reproductive tract scores (RTS), which assess female reproductive tract maturity and can identify pubertal heifers. While RTS is a reliable and useful tool for selecting replacement heifers, higher RTS scores have been positively associated with improved pregnancy (Holm et al., 2009). Research indicates that heifers with a higher RTS became pregnant earlier in the breeding season, indicating that heifers were more mature at breeding, and allowing for a selection tool to identify heifers that will become pregnant earlier in the breeding season (Gutierrez et al., 2014). Reproductive tract scores currently remain the only phenotypic tool available to beef professionals for assessing potential heifer fertility.

Anogenital distance (AGD) is a sexually dimorphic trait defined as the distance between the center of the anus and the base of the clitoris in females. Previous research has investigated the influence of androgen exposure during fetal development, and this measurement has been shown to reflect the degree of masculinization of external genitalia and serve as a proxy for the prenatal endocrine environment (Thankamony et al., 2016). A study in other women has shown that an excess of androgen exposure in utero has been associated with Polycystic Ovary Syndrome (PCOS; Abbott et al., 2019). Identification of this hormonal disorder requires at least two of the following: high testosterone concentrations, irregular menstrual cycles, and ultrasonography-visualized polycystic ovaries. Work by multiple labs has used other species such as rodents, primates, and sheep as models for PCOS (Sullivan and Moenter, 2004; Foecking et al., 2005; Veiga-Lopez et al., 2011; Abedal-Majed et al., 2022). With differentiation of external genitalia largely complete during mid-gestation, AGD can represent a fixed trait that

remains relatively stable through growth (Van Den Driesche et al., 2011). Consequently, AGD has been adopted as a noninvasive biomarker of prenatal androgen exposure across several different species (Drickamer, 1996; Wolf, 2002; Manikkam et al., 2004; Bánszegi et al., 2012).

In female livestock species, variation in AGD has been associated with development, endocrine function, and fertility. In rodents, a longer AGD is indicative of greater androgen exposure (Hotchkiss et al., 2006; Dean and Sharpe, 2013). In dairy cattle, there has been an interest in AGD and the pregnancy to AI (Gobikrushanth et al., 2017, 2019; Akbarinejad et al., 2019; Carrelli et al., 2022). In Holstein animals an inverse relationship was found between AGD and pregnancy to the first round of AI, with the cattle that have a longer AGD showing a decreased probability of becoming pregnant (Gobikrushanth et al., 2017; Akbarinejad et al., 2019; Carrelli et al., 2022). There is currently no published reports in cattle that follow the AGD from birth through breeding. In swine, the AGD of newborn gilts has been related to the proportion of males in the litter. Gilts born to litters with fewer males had a shorter AGD than gilts with greater percentages of males, and the gilts that failed to become pregnant at the first breeding attempt were from litters with higher proportions of males (Drickamer et al., 1997).

Due to AGD being associated with fertility across multiple species, the objective of this study was to evaluate the relationship between AGD and pregnancy outcomes in beef cows and heifers using fixed-time AI protocols, with both *Bos taurus* and *Bos indicus* genetic backgrounds. In addition, our objective was to compare the accuracy of predicting pregnancy rates to timed artificial insemination (TAI) between AGD and RTS for beef heifers, and whether combining AGD with a RTS enhances the prediction of pregnancy in the first round of TAI. Our hypothesis for this retrospective study was that cattle with a longer AGD would have a lower probability of becoming pregnant to AI than their cohorts with a shorter AGD. To address these

objectives, this study evaluated beef cattle of both *Bos indicus* and *Bos taurus* breeds, as well as the effect of AGD on pregnancy in the first round of a TAI protocol. A subset of the *Bos taurus* heifers also had the RTS collected. The AGD and RTS of the heifers were analyzed to determine the accuracy of these tools in predicting the probability of pregnancy using TAI.

Materials and Methods

Animals and Management

A total of 2,663 beef cows and heifers of both *Bos taurus* and *Bos indicus* subspecies from 16 different locations were enrolled in the study from 2023 through the spring 2025 breeding seasons. Data were collected from 5 locations in Brazil and 11 locations in the United States, including one location in Virginia, three locations in Georgia, one location in Tennessee, and six locations in Kansas. Breed composition varied among locations, but the Brazil locations were predominantly of Nellore lineage and the United States was of commercial beef females predominantly of Angus influence. Cattle utilized in this experiment were handled and cared for in accordance with the Institutional Animal Care and Use Committee (IACUC) of Kansas State University #4970.

All females in the United States were assigned to the 7-d CO-SYNCH + CIDR (Larson et al., 2006; Lamb et al., 2006) protocol, where they received 100 µg of gonadotropin-releasing hormone IM (GnRH; Factrel; Zoetis Animal Health, Parsippany, NJ) and had a CIDR (EAZI-BREED CIDR; 1.38 g of P4; Zoetis Animal Health) inserted intravaginally, followed by 25 mg of prostaglandin F_{2α} IM (PGF_{2α}; Lutalyse; Zoetis Animal Health) at CIDR removal 7 days later. A second dose of 100µg of GnRH was administered IM at TAI, 54 ± 2 hours after CIDR removal for heifers and 63 ± 3 hours after CIDR removal for cows. All females in Brazil received a CIDR (1.9g of P4, CIDR, Zoetis, São Paulo, SP, Brazil) and 2.0 mg of estradiol

benzoate IM (2.0mL of Gonadiol, Zoetis), 12.5 mg of dinoprost tromethamine (PGF; 2.5mL of Lutalyse, Zoetis) 7 days later, 300 IU of equine chorionic gonadotropin for cows, and 200IU for heifers IM (ecG; Novormon, Zoetis), 0.6mg (IM) of estradiol cypionate (ECP; 0.3mL of E.C.P., Zoetis, São Paulo), and CIDR withdrawal 2 days after PGF injection, followed by TAI 48 hours later (Sales et al., 2024).

All females were exposed to bulls that passed a breeding soundness exam 15 days after TAI for the remainder of the breeding season. Transrectal ultrasonography (Ibex portable ultrasound, 5.0-MHz linear multi-frequency transducer, Ibex, E.I. Medical Imaging, Loveland, CO; Easi-Scan portable ultrasound; BCF Technology Ltd., Livingston, UK) was performed between 35 and 45 days after TAI for pregnancy diagnosis.

Anogenital Distance Measurements

A representation of the AGD measurement is shown in Figure 3.1. All females were measured using a digital caliper (Momojia, China), and measurements were taken from the base of the clitoris to the middle of the anus. Due to differences in management and personnel availability between locations, AGD was assessed on different days, either during the estrous synchronization protocol (n = 1,457) or at the time of pregnancy diagnosis (n = 1,206). In Holstein females, no differences in AGD have been observed across stages of the estrous cycle or during gestation, with changes only detected within two weeks before or after parturition (Rajesh et al., 2022).

A subset of *Bos taurus* heifers was assigned a RTS according to the guidelines of Anderson et al. (1991). Reproductive tract score was classified according to (Anderson et al., 1991): RTS of 1 is classified as an immature heifer with uterine horns that are < 20 mm in diameter with no tone, an ovary that is 15 mm in length, 10 mm high, a width of 8 mm, and no

palpable structures. A RTS 2 has uterine horns that are 20 – 25 mm in diameter with no tone, an ovary that is 18 mm in length, 12 mm high, and 10 mm in width, and an 8mm follicle. A RTS 3 has uterine horns with a slight tone and a diameter of 25-30 mm, an ovary with a length of 22 mm, a height of 15 mm, a width of 10 mm, and follicles that are 8 – 10 mm. A RTS 4 has uterine horns with good tone that are 30 mm in diameter, an ovary with a length of 30 mm, a height of 16 mm, and a width of 12 mm, and follicles that are greater than 10 mm and possibly have a corpus luteum. An RTS of 5 is the most mature score with uterine horns that are greater than 30 mm in diameter, with good tone and may be erect, the length of the ovary is greater than 32 mm, a height of 20 mm, and a width of 15 mm, with follicles that are greater than 10 mm and a corpus luteum present. The use of RTS has been primarily utilized in research settings, but it is a tool that, when properly administered, can identify heifers that lack maturity of the reproductive tract.

Statistical Analysis

Data were analyzed based on subspecies and parity to limit variation between parity, subspecies, and estrus synchronization protocols. Data from each group of cattle were analyzed with SAS 9.4 (SAS Institute, Cary, NC). Data observations were sorted with the SORT procedure by location and the variable of AGD. Then, observations on data were equally assigned into quartiles and tertiles by AGD for each location.

The assignment of quartiles was as follows: quartile 1 was 0 to 25% of cattle with the shortest AGD, quartile 2 was 26 to 50%, quartile 3 was 51 to 75%, and quartile 4 was 76 to 100% of cattle that had the longest AGD at each location. The assignment of tertiles was done, with tertile 1 ranging from 0 to 33% of AGD, tertile 2 from 34 to 66% of AGD, and tertile 3 from 67 to 100% of AGD.

The GLIMMIX procedure of SAS was used to analyze the pregnancy rates of cattle by their assigned quartile and tertile, with location as a random variable. Contrast statements were used to compare means for quartiles and tertiles. The MIXED procedure of SAS was used to compare the AGD of open and pregnant heifers with location as a random variable. A probability of pregnancy model for each dataset was developed using GLIMMIX with the Laplace distribution to estimate the relationship between AGD and the probability of pregnancy in the first round of artificial insemination. The model was a pregnancy model with AGD as a binomial predictor, and linear, quadratic, and cubic functions of AGD were included, along with location as a random variable.

Results and Discussion

A total of 2,663 beef females were enrolled in ES protocols and were exposed to TAI. There were 140 *Bos indicus* cows from a single location in Brazil (Table 3.1), 520 *Bos indicus* heifers originating from four locations in Brazil (Table 3.2), 797 *Bos taurus* cows from five locations (Table 3.3), and 1,206 *Bos taurus* heifers from thirteen locations (Table 3.4). Of the 1,206 *Bos taurus* heifers, 933 from six locations had been assigned a RTS before the beginning of the ES protocol (Table 3.5). This subset of heifers was analyzed to compare the effectiveness of RTS and AGD at predicting pregnancy success with TAI. The raw means of each subspecies by parity are reported in Table 3.6. Due to the variation among parities and subspecies in AGD only cattle of the same parity and subspecies were analyzed.

Data from a single location consisting of *Bos indicus* cows, primarily of the Nellore lineage, were analyzed to evaluate the relationship between AGD and the probability of pregnancy to TAI (Table 3.1). There was no effect of AGD quartile or tertile on the probability of pregnancy (Table 3.7; Table 3.8). This dataset contained only 140 cows and is underpowered

to detect a difference in AGD between the probability of pregnancy and TAI. A numerical difference of just over 0.1 mm was observed between pregnant cows and open cows, and the AGD based on pregnancy status is reported in Table 3.9.

The *Bos indicus* heifer dataset comprised 4 locations in Brazil, and the AGD means for each location, along with the standard deviations are reported in Table 3.2. When heifers were separated into AGD quartiles within a location, no difference in the probability of pregnancy was observed by quartile ($P = 0.41$), and the probability for each quartile is reported in Table 3.10. When *Bos indicus* heifers were separated into tertiles we observed no significant differences ($P = 0.11$). However, contrast statements revealed that tertile 1 differed significantly from tertile 3 ($P = 0.04$) and the average of tertile 2 and 3 ($P = 0.05$). The probability of becoming pregnant following TAI for each AGD tertile is presented in Table 3.11. The pregnancy status of the *Bos indicus* heifers also differed significantly ($P = 0.04$) in AGD, with open heifers averaging 2 mm longer AGD than pregnant heifers (Table 3.12). Despite this difference, AGD would still be extremely challenging to use as a practical selection tool due to the small magnitude of variation. Notably, this distance is greater than any previously reported differences in AGD by pregnancy status in our lab (Dias et al., 2025; Schwartz et al., 2025).

Literature on AGD of *Bos indicus* cattle is extremely limited. Besides the previously cited data published in an abstract from our lab, an undergraduate extension poster from Kansas State University studied AGD as a potential phenotype for selecting donors for *in vitro* embryo production. A large proportion of global cattle production relies on *Bos indicus* breeds, and further research on this subspecies is warranted. Large datasets of AGD for each breed would be beneficial, especially with the number of *Bos indicus* cattle in tropical environments.

Bos taurus cows from 5 locations were enrolled, with the mean AGD of each location presented in Table 3.3. The pregnancy status of *Bos taurus* cows did not differ by AGD, with means for pregnant and open cows presented in Table 3.13. Analysis by AGD quartile revealed no overall differences; however, contrast analysis indicated a difference between quartiles 1 and 4 ($P = 0.04$; Table 3.14). No effect of tertile on the probability of pregnancy to TAI was observed ($P = 0.63$; Table 3.15).

This dataset contains just under 800 *Bos taurus* cows, which is underpowered for the observed results. For the observed results to be significant, approximately 4,120 cows would be required. Increasing the sample size and gaining a better understanding of the breeds of cattle in the dataset would decrease variation and enable analysis within breeds. All current published literature on large data sets of *Bos taurus* cows is from Holstein cattle (Gobikrushanth et al., 2017, 2019; Carrelli et al., 2022; Rajesh et al., 2022).

The total dataset of *Bos taurus* heifers, categorized by location, is summarized in Table 3.4. Anogenital distance was not different between pregnant and non-pregnant *Bos taurus* heifers, as shown in Table 3.16. The heifers with the shortest AGD had significantly greater chances of becoming pregnant to TAI compared to the heifers with the longest AGD. There was no difference for the fixed effects of tertile or quartile without the contrast between heifers with the shortest or longest AGD. The probability of becoming pregnant for TAI based on quartiles can be found in Table 3.17 and Table 3.18, for tertiles. This data set included all the heifers for which AGD was collected during the breeding season. Many of these heifers were also assigned a RTS prior to the breeding season.

The subset of *Bos taurus* heifers assigned a RTS prior to the TAI protocol was evaluated to determine whether RTS or AGD was a better predictor of pregnancy following TAI, as shown

in Table 3.5. There was no effect on pregnancy status based on the AGD of heifers ($P = 0.27$), as shown in Table 3.19; however, a significant difference was observed between open and pregnant heifers for RTS ($P < 0.01$). The effect of RTS on the probability of pregnancy tended to be significant for predicting pregnancy in TAI ($P = 0.08$) and is reported in Table 3.20. There were significant differences between RTS 2 and RTS 4 and 5. For the RTS 3 heifers, there was a significant difference for RTS 5. While RTS of 1 has the greatest probability of pregnancy and is referred to in literature as immature. However, this difference is due to the dataset containing only 3 of the 933 heifers that had a RTS of 1.

Anogenital distance quartiles for the subset of heifers with a RTS were not significant based on the fixed effect of AGD quartile. Numerically, the heifers in quartile 1 had the greatest probability of becoming pregnant to TAI at 51.68%, as reported in Table 3.21.

When analyzed by AGD tertiles, heifers in the first tertile had the highest estimated probability of pregnancy at 52.49% as reported in Table 3.22. Although there was no difference in the probability of pregnancy based on the AGD tertile groups, there were just under 300 heifers per tertile. The limited number of heifers in each tertile may limit the effectiveness of the retrospective analysis of AGD in estimating the probability that beef replacement heifers will become pregnant in the first round of AI.

The RTS was developed as an indicator of reproductive maturity rather than of synchronization response or pregnancy. The use of RTS within the first breeding season allows for a checkpoint in the nutritional development program when conducted 1 or 2 months before breeding (Anderson et al., 1991). Efforts to determine the repeatability of RTS among different technicians have been established by Rosenkrans and Hardin (2003) to allow for uniformity among technicians and locations. It was recommended to use RTS as a screening tool to assess

heifer cyclicity in herds, but not to identify individual animals for culling. There was also an 18% false-negative rate among heifers considered prepubertal that were actually pubertal (Rosenkrans and Hardin, 2003). The RTS utilized before the breeding season was a better predictor of pregnancy in heifers than body weight or body condition score (Holm et al., 2009). The current predictors utilized for pregnancy are RTS, body weight, and body condition score. Body condition score and body weight can fluctuate widely among individuals within a herd, whereas RTS is a subjective measure of the reproductive tract's maturity prior to breeding. The adoption of RTS in herds is just over 1% of producers who utilize this as an indicator of maturity with most of the application used in research herds.

Although the adoption of RTS is low, this assessment requires a technician skilled in transrectal palpation to perform an RTS. Compared to RTS, AGD offers practical advantages in cattle due to its ease and speed of collection, requiring no rectal palpation or reproductive tract manipulation. Research into the heritability and use of AGD as a reproductive phenotype could be further investigated in beef cattle, although large numbers within each breed would be required within a given geographic area. Given that the data set includes cattle from multiple locations and breeds, the ability to separate AGD by breed would allow for a clearer understanding of AGD and the probability of pregnancy for each breed.

The final prediction model for the probability of pregnancy in heifers with RTS and AGD found no difference for AGD ($P = 0.52$). The odds ratio of AGD estimates a 4% decrease in the odds of pregnancy for every 1 mm increase in AGD. For RTS, there was a significant effect ($P < 0.05$) for increasing the odds of pregnancy to TAI. With each increase in RTS, the probability of pregnancy increased by 43%. The reduction in the probability of becoming pregnant to AGD agrees with the 3.4 and 2.4% decrease for every 1 mm increase reported in Canadian Holsteins

(Gobikrushanth et al., 2017). In this subset of *Bos taurus* heifers, RTS was a better predictor of pregnancy when utilizing a TAI protocol.

Conclusions

This retrospective analysis evaluated AGD in relation to pregnancy outcomes following TAI in a diverse population of beef females across two countries. Significant associations between AGD and pregnancy were observed in *Bos indicus* heifers and *Bos taurus* cows; however, results varied by subspecies, and statistical power was limited in smaller populations. Among *Bos taurus* heifers, RTS outperformed AGD in predicting pregnancy, supported by a greater sample size. These findings are consistent with those of Holm et al., (2009), who demonstrated that RTS is a stronger predictor of pregnancy than body weight or body condition score. While AGD offers a practical advantage in ease and speed of collection, its predictive value remains uncertain, especially in *Bos indicus* cattle. However, our results align with previous studies in rodents and cattle, indicating that a shorter AGD is associated with improved pregnancy rates (Drickamer et al., 1997; Gobikrushanth et al., 2017, 2019; Carrelli et al., 2022; Rajesh et al., 2022). Further research with balanced breed representation and integration of physiological metrics such as RTS, body condition score, hormone profiles, and age is needed to clarify AGD's utility as a fertility biomarker in beef cattle.

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Figure 3.1 Anogenital distance measurement.

Anogenital distance was measured from the center of the anus to the base of the clitoris with the use of digital calipers in millimeters

Table 3.1 Means of *Bos indicus* cows by location.

Location¹	Geographic location²	n³	AGD⁴	SD⁵
9	Brazil	140	130.0	14.54

¹The location identifier for the respective location.

²The geographic location.

³Number of cattle for each location.

⁴AGD = Anogenital distance reported in millimeters.

⁵SD = standard deviation that is expressed in millimeters.

Table 3.2 Means of *Bos indicus* heifers by location.

Location ¹	Geographic location ²	n ³	AGD ⁴	SD ⁵
8	Brazil	133	116.60	10.87
10	Brazil	106	116.80	12.32
11	Brazil	146	122.31	11.62
12	Brazil	135	122.48	11.43
Total		520	119.77	11.85

¹The location identifier for the respective location.

²The geographic location.

³Number of cattle for each location.

⁴AGD = anogenital distance in millimeters.

⁵SD = standard deviation that is expressed in millimeters.

Table 3.3 Means of *Bos taurus* cows by location.

Location ¹	Geographic location ²	n ³	AGD ⁴	SD ⁵
3	Georgia	211	105.32	13.97
4	Georgia	204	103.98	12.49
5	Kansas	160	103.88	11.12
6	Georgia	67	109.08	20.70
15	Kansas	155	109.77	11.76
Total		797	105.88	13.56

¹The location identifier for the respective location.

²The geographic location.

³Number of cattle for each location.

⁴AGD = anogenital distance in millimeters.

⁵SD = standard deviation that is expressed in millimeters.

Table 3.4 Means of *Bos taurus* heifers by location.

Location ¹	Geographic location ²	n ³	AGD ⁴	SD ⁵
1	Virginia	49	66.90	12.92
2	Kansas	239	82.39	11.12
3	Georgia	62	79.83	12.79
4	Georgia	34	112.38	10.84
5	Kansas	207	78.06	8.00
6	Georgia	17	82.71	11.64
7	Kansas	118	79.73	11.15
8	Brazil	12	118.46	8.43
10	Brazil	6	117.83	6.55
13	Kansas	65	86.10	7.53
14	Kansas	49	88.91	8.66
15	Kansas	93	87.36	10.19
16	Tennessee	255	90.65	10.06
Total		1206	84.61	13.26

¹The location identifier for the respective location.

²The geographic location.

³Number of cattle for each location.

⁴AGD = anogenital distance in millimeters.

⁵SD = standard deviation that is expressed in millimeters.

Table 3.5 Number of *Bos taurus* heifers that had a reproductive tract score (RTS) and anogenital distance (AGD) by location.

Location ¹	Geographic location ²	n ³
2	Kansas	239
5	Kansas	207
7	Kansas	118
13	Kansas	65
14	Kansas	49
16	Tennessee	255
Total		933

¹The location identifier for the respective location.

²The geographic location.

³Number of cattle for each location.

Table 3.6 Raw mean anogenital distance (AGD) in *Bos indicus* and *Bos taurus* cows and heifers.

Subspecies	Parity	Anogenital distance ¹ , mm	Standard deviation ² , mm
<i>Bos indicus</i>			
	Cows	130.00	14.54
	Heifers	119.77	11.85
<i>Bos taurus</i>			
	Cows	105.88	13.56
	Heifers	84.61	13.26

¹Anogenital distance is reported as the raw mean in millimeters.

²Standard deviation of anogenital distance is reported in millimeters.

Table 3.7 Least squares means of *Bos indicus* cows from location 9, anogenital distance (AGD) quartiles probability of becoming pregnant to artificial insemination.¹

AGD quartile ²	Mean AGD ³ , mm	Probability of pregnancy ⁴ , %	Standard error ⁵ , %
1	112.17	48.57	8.45
2	123.94	58.82	8.44
3	134.31	60.00	8.28
4	148.94	52.78	8.32

¹Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS) with a binary distribution and logit link function with location as a random effect.

²AGD Quartile = Quartiles were derived from the distribution of AGD measurements and represent increasing AGD from Quartile 1 to 4.

³Raw means for each AGD quartile.

⁴Probability values are back-transformed from the logit scale and represent the estimated percentage of cows pregnant to AI.

⁵Standard errors are also back-transformed to the probability scale.

The fixed effect of quartile was not significantly different ($P = 0.75$)

Table 3.8 Least squares means of *Bos indicus* cows from location 9, anogenital distance (AGD) tertiles probability of becoming pregnant to artificial insemination.¹

AGD tertile ²	Mean AGD ³ , mm	Probability of pregnancy ⁴ , %	Standard error ⁵ , %
1	113.91	48.84	7.62
2	128.65	62.75	6.77
3	146.61	52.17	7.37

¹Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS) with a binary distribution and logit link function with location as a random effect.

²AGD Tertile = Tertiles were derived from the distribution of AGD measurements and represent increasing AGD from Tertile 1 to 3.

³Raw means for each AGD tertile.

⁴Probability values are back-transformed from the logit scale and represent the estimated percentage of cows pregnant to AI.

⁵Standard errors are also back-transformed to the probability scale.
The fixed effect of Tertile was not significantly different ($P = 0.37$).

Table 3.9 Least squares means on anogenital distance (AGD) effects on pregnancy status in *Bos indicus* cows from location 9.¹

Pregnancy status ²	n ³	AGD, mm ⁴	SE ⁵
Open	63	130.08	1.84
Pregnant	77	129.97	1.66

¹Least squares means for AGD were estimated using a linear mixed-effects model (Proc Mixed, SAS). The fixed effect of pregnancy status was not statistically significant ($P = 0.97$), indicating no detectable difference in AGD between open and pregnant cows.

²Pregnancy status after artificial insemination was determined by transrectal ultrasonography.

³n = number of cows.

⁴AGD = Anogenital distance in millimeters.

⁵SE = Standard error expressed in millimeters.

Table 3.10 Least squares means of *Bos indicus* heifers from locations 8, 10, 11, and 12, anogenital distance (AGD) quartiles probability of becoming pregnant to artificial insemination.¹

AGD quartile ²	Mean AGD ³ , mm	Probability of pregnancy ⁴ , %	Standard error ⁵ , %
1	104.83	52.00	4.47
2	116.07	49.19	4.49
3	122.55	45.86	4.32
4	133.95	42.03	4.20

¹Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS) with a binary distribution and logit link function with location as a random effect.

²AGD Quartile = Quartiles were derived from the distribution of AGD measurements and represent increasing AGD from Quartile 1 to 4.

³Raw means for each AGD quartile

⁴Probability values are back-transformed from the logit scale and represent the estimated percentage of heifers pregnant to AI.

⁵Standard errors are also back-transformed to the probability scale.

The fixed effect of quartile was not significantly different ($P = 0.41$)

Table 3.11 Least squares means of *Bos indicus* heifers from locations 8, 10, 11, and 12, anogenital distance (AGD) tertiles probability of becoming pregnant to timed artificial insemination (TAI).¹

AGD tertile ²	Raw AGD ³ , mm	Probability of pregnancy ⁴ , %	Standard error ⁵ , %
1 ^a	107.09	53.25	3.84
2 ^{ab}	120.00	46.15	3.70
3 ^b	132.19	42.01	3.80

¹Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS) with a binary distribution and logit link function with location as a random effect.

²AGD Tertile = Tertiles were derived from the distribution of AGD measurements and represent increasing AGD from Tertile 1 to 3.

³Raw means for AGD tertile.

⁴Probability values are back-transformed from the logit scale and represent the estimated percentage of heifers pregnant to AI.

⁵Standard errors are also back-transformed to the probability scale.

The fixed effect of tertile was not significantly different ($P = 0.11$).

^{a-b} Superscripts that differ within a column signify a significant difference among tertiles for the probability of pregnancy ($P \leq 0.05$).

Table 3.12 Least squares means on anogenital distance (AGD) effects on pregnancy status in *Bos indicus* heifers.¹

Pregnancy status	n ²	AGD, mm ³	Standard error, mm
Open	275	120.57	1.71
Pregnant	245	118.45	1.73

¹Least squares means for AGD were estimated using a linear mixed-effects model (Proc Mixed, SAS). The fixed effect of pregnancy status was statistically significant ($P = 0.04$), indicating a detectable difference in AGD between open and pregnant *Bos indicus* heifers.

²n = number of cattle.

³AGD = Anogenital distance in millimeters.

Table 3.13 Least squares means on anogenital distance (AGD) effects on pregnancy in *Bos taurus* cows.¹

Pregnancy status	n ²	AGD, mm ³	Standard error, mm
Open	369	106.97	1.34
Pregnant	428	105.66	1.32

¹Least squares means for AGD were estimated using a linear mixed-effects model (Proc Mixed, SAS). The fixed effect of pregnancy status was not statistically significant ($P = 0.17$), indicating no detectable difference in AGD between open and pregnant *Bos taurus* cows.

²n = number of cattle.

³AGD = Anogenital distance in millimeters.

Table 3.14 Least squares means of *Bos taurus* cows anogenital distance (AGD) quartiles probability of becoming pregnant to artificial insemination.¹

AGD quartile ²	Mean AGD ³ , mm	Probability of pregnancy ³ , %	Standard error, % ⁴
1 ^a	89.70	57.47	5.15
2 ^{ab}	101.95	51.58	5.23
3 ^{ab}	109.16	56.64	5.15
4 ^b	122.36	47.17	5.20

¹Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS) with a binary distribution and logit link function with location as a random effect.

²AGD Quartile = Quartiles were derived from the distribution of AGD measurements and represent increasing AGD from Quartile 1 to 4.

³Raw means for AGD quartile.

⁴Probability values are back-transformed from the logit scale and represent the estimated percentage of cows pregnant to AI.

⁵Standard errors are also back-transformed to the probability scale.

The fixed effect of quartile was not significantly different ($P = 0.14$).

^{a-b} Superscripts that differ within a column signify a significant difference among tertiles for the probability of pregnancy ($P \leq 0.05$).

Table 3.15 Least squares means of *Bos taurus* cows anogenital distance (AGD) tertiles probability of becoming pregnant to artificial insemination.¹

AGD tertile ²	Mean AGD ³ , mm	Probability of pregnancy ⁴ , %	Standard error ⁵ , %
1	92.14	55.35	4.85
2	105.60	53.08	4.86
3	119.86	51.15	4.89

¹Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS) with a binary distribution and logit link function with location as a random effect.

²AGD Tertile = Tertiles were derived from the distribution of AGD measurements and represent increasing AGD from Tertile 1 to 3.

³Raw means for AGD tertile.

⁴Probability values are back-transformed from the logit scale and represent the estimated percentage of cows pregnant to AI.

⁵Standard errors are also back-transformed to the probability scale.
The fixed effect of tertile was not significantly different ($P = 0.63$)

Table 3.16 Least squares means on Anogenital distance (AGD) effects on pregnancy in *Bos taurus* heifers.¹

Pregnancy status	n ²	AGD, mm ³	Standard error, mm
Open	607	90.20	4.40
Pregnant	599	89.62	4.40

¹Least squares means for AGD were estimated using a linear mixed-effects model (Proc Mixed, SAS). The fixed effect of pregnancy status was not statistically significant ($P = 0.33$), indicating no detectable difference in AGD between open and pregnant *Bos taurus* heifers.

²n = number of cattle.

³AGD = Anogenital distance in millimeters.

Table 3.17 Least squares means of *Bos taurus* heifers anogenital distance (AGD) quartiles probability of becoming pregnant to artificial insemination.¹

AGD quartile ²	Mean AGD ³ , mm	Probability of pregnancy ⁴ , %	Standard error ⁵ , %
1	72.21	53.21	4.25
2	80.92	49.81	4.26
3	87.38	50.84	4.21
4	97.52	47.72	4.24

¹Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS) with a binary distribution and logit link function with location as a random effect.

²AGD Quartile = Quartiles were derived from the distribution of AGD measurements and represent increasing AGD from Quartile 1 to 4.

³Raw mean for AGD quartile.

⁴Probability values are back-transformed from the logit scale and represent the estimated percentage of heifers pregnant to AI.

⁵Standard errors are also back-transformed to the probability scale.

The fixed effect of quartile was not significantly different ($P = 0.61$)

Table 3.18 Least squares means of *Bos taurus* heifers anogenital distance (AGD) tertiles probability of becoming pregnant to artificial insemination.¹

AGD tertile ²	Mean AGD ³ , mm	Probability of pregnancy ⁴ , %	Standard error ⁵ , %
1	74.00	53.66	3.99
2	84.18	50.46	4.00
3	95.62	47.08	3.99

¹Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS) with a binary distribution and logit link function with location as a random effect.

²AGD Tertile = Tertiles were derived from the distribution of AGD measurements and represent increasing AGD from Tertile 1 to 3.

³Raw mean for AGD tertile.

⁴Probability values are back-transformed from the logit scale and represent the estimated percentage of heifers pregnant to AI.

⁵Standard errors are also back-transformed to the probability scale.

The fixed effect of tertile was not significantly different ($P = 0.18$); a tendency between tertile 1 and 3 was observed for the probability of pregnancy ($P = 0.06$).

Table 3.19 Least squares means on the subset of *Bos taurus* heifers that had a reproductive tract score (RTS) and anogenital distance (AGD) effects on pregnancy status.¹

Variable	n ²	Open	Pregnant	P-Value
AGD ³ , mm	816	83.63 ± 2.48	82.90 ± 2.48	0.27
RTS, 1-5 ⁴	933	3.73 ^a ± 0.29	3.88 ^b ± 0.29	<0.01
Weight, kg	881	362 ± 8.52	366 ± 8.54	0.19

¹Least squares means were estimated using a linear mixed-effects model (Proc Mixed, SAS).

²n = number of cattle.

³AGD = Anogenital distance in millimeters.

⁴RTS = Reproductive tract score.

^{a-b}Means with unlike superscripts in a row are significantly different ($P < 0.05$).

Location 2 did not have AGD collected during one of the breeding seasons.

Location 14 did not have a weight collected.

Any other missing data points were removed for analysis.

Table 3.20 Least squares means for probability of pregnancy to artificial insemination (AI) in *Bos taurus* heifers with a recorded reproductive tract score (RTS).

RTS ¹	n	Probability of pregnancy ² , %	Standard Error ³ , %
1	3	68.06	27.11
2	69	35.40	6.60
3	321	44.39	4.35
4	264	49.33	4.54
5	276	55.71	5.05

¹RTS = Reproductive tract score (1-5).

²Probability values are back-transformed from the logit scale and represent the estimated percentage of heifers pregnant to AI.

³Standard errors are also back-transformed to the probability scale.

Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS). The fixed effect of RTS was not statistically significant ($P = 0.08$).

Table 3.21 Least squares means for probability of pregnancy to artificial insemination (AI) in *Bos taurus* heifers with a reproductive tract score (RTS), stratified by retrospectively assigned anogenital distance (AGD) quartile.

AGD quartile ¹	Mean AGD ² , mm	Probability of pregnancy ³ , %	Standard error ⁴ , %
1	71.67	51.68	4.76
2	79.88	50.97	4.75
3	85.81	48.90	4.72
4	95.31	44.82	4.69

¹AGD quartiles were assigned retrospectively based on the distribution of AGD measurements.

²Raw mean for AGD quartile.

³Probability of pregnancy values are back-transformed from the logit scale and represent the estimated percentage of heifers pregnant to AI.

⁴Standard errors are also back-transformed to the probability scale.

Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS) with a binary distribution and logit link function. Location was included as a random effect.

The fixed effect of AGD quartile was not statistically significant (P = 0.51).

Table 3.22 Least squares means for probability to artificial insemination (AI) in *Bos taurus* heifers with a reproductive tract score (RTS), stratified by retrospectively assigned anogenital distance (AGD) tertile.

AGD tertile ¹	Mean AGD ² , mm	Probability of pregnancy ³ , %	Standard error ⁴ , %
1	73.58	52.49	4.39
2	82.84	48.55	4.41
3	93.47	46.16	4.38

¹AGD tertiles were assigned retrospectively based on the distribution of AGD measurements.

²Raw mean for AGD tertile.

³Probability of pregnancy values are back-transformed from the logit scale and represent the estimated percentage of heifers pregnant to AI.

⁴Standard errors are also back-transformed to the probability scale.

Least squares means were estimated using a generalized mixed model (Proc Glimmix, SAS) with a binary distribution and logit link function. Location was included as a random effect.

The fixed effect of AGD tertile was not statistically significant ($P = 0.33$).