Factors Relating to the Attainment of Puberty in Bulls

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Introduction

The use of selective breeding in cattle, especially within the last 50 to 60 years, has allowed for impressive increases in milk and meat production from the commonly used breeds (Ruaw et al., 1998). These increases in production efficiency coincide with increased consumer demand, a growing population, and increasing production costs for farmers. Companies providing genetics to the cattle industries (i.e. AI companies) are cognizant of the demands of cattle breeders, and they strive to supply ever-improving genetics. Two main factors affecting genetic improvement are the ability to identify animals of high genetic merit at young ages and the physical ability of these animals to provide genetic material in terms of oocytes and sperm. Regarding the first of these obstacles, the use of genomics to predict an animal's future productivity has become increasingly common. The genetic worth of a bull can be calculated or determined using genomics far before the bull reaches sexual maturity (Schefers and Weigel, 2012; Humblot, 2010). Regarding the second barrier, at present, bulls must reach reproductive maturity, marked by the attainment of puberty, in order to pass along their genes (Blood and Studdert, 1999). An increase in semen supply earlier in the productive life of a genomically-prominent sire allows greater accessibility to these superior genetics. The attainment of puberty in bulls has been an area of increasing interest several decades, and various methods to hasten puberty have been attempted by other groups. Within recent years, our laboratory's interests in cattle genetics, breeding, and fertility have led us to also investigate methods to advance puberty in bulls.

Why the Recent Desire to Hasten Puberty?

Before the use of genomics, the genetic worth of a bull was determined initially by the animal's pedigree and individual physical characteristics. As a bull obtained daughters, their

performance in terms of milk production (dairy) and growth rate, birth weight, etc. (beef) increased or decreased his desirability amongst producers accordingly. Today, the use of genomics is changing the industry by adding powerful tools to select bulls that will be used for AI. The industry of cattle genetics is undergoing a "paradigm shift," according to Amann and DeJarnette (2012). The genomic era of cattle reproduction shows no signs of slowing in its implementation or utility.

Due to genomic selection, stud companies acquire bulls at earlier ages and desire to sell their genetics as soon as possible. Hence, there is a recent favor towards collecting bulls as early as possible in order to disseminate their genetics. It is due to the relatively recent changes within the AI industry that bull puberty has become a major area of interest. Before presenting work performed by our lab and others, it is useful to summarize the endocrine and physiological changes that occur in the prepubertal bull which lead to attainment of spermatogenesis.

Endocrinology of the Prepubertal Bull

Postpubertal endocrinology underlies many of the physiological processes leading up to puberty in males. The hypothalamo-pituitary-gonadal (HPG) axis controls much of the postnatal maturation and subsequent postpubertal function of the reproductive tract and spermatogenesis. Gonadotropin-releasing hormone (GnRH) is synthesized within the arcuate nuclei of the hypothalamus and released in a pulsatile manner from specific GnRH-releasing neurons in mature males. GnRH's target receptors are in close proximity, in the anterior pituitary, and secreted GnRH travels through the hypophyseal portal system to the receptors (Schwanzel-Fukuda and Pfaff, 1989). During a pulse of GnRH in the *postpubertal* male, the hormone is released into the portal vasculature and binds GnRH receptors of gonadotropes in the anterior pituitary, prompting the release of FSH and luteinizing hormone (LH) from the gonadotropes (Campbell et al., 2009). In postnatal animals, a "priming" of the hypothalamus and pituitary (6 – 10 weeks of age) is required before the previously mentioned release of LH can be achieved. It is hypothesized that estrogen is an inhibitor to postnatal LH release. In cattle, it is well established that the increase in peripubertal LH secretion is the result of not only increased GnRH secretion (Rodriguez and Wise, 1989), but also because of a decline in the negative feedback of estradiol on GnRH and LH secretion (Day and Anderson, 1998). However, estrogen of gonadal origin is

unlikely the block to the pituitary's LH secretion in the prepubertal bull before 10 weeks of age since castration does not induce or increase LH pulsatility (Wise et al., 1987). Furthermore, the pituitary may have stores of LH postnatally but be unable to respond to GnRH before 10 weeks of age due to a lack of GnRH receptors (GnRH-R; Rodriguez and Wise, 1989). Coincidentally, there is a significant increase (314%) in GnRH-R in the anterior pituitary between 6 – 10 weeks of age and a decrease (70%) in estradiol-R in the hypothalamus during this same time (Amann et al., 1986). Other studies suggest that estradiol originating locally via neuronal production (McEwen, 1980; Dickson and Clark, 1981) and from the adrenal glands (Henricks et al., 1988) can act as a hormonal block to GnRH during the neonatal and early prepubertal stages.

Regardless of the postnatal control of LH, once the pituitary gains responsiveness to GnRH it acquires the ability to secrete LH and FSH, and studies consistently report a transient increase in LH beginning at 6 weeks of age that plateaus and remains until approximately 20 weeks of age (Amann et al., 1986; Evans et al., 1995; Madgwick et al., 2008). Most attribute this transient increase in LH to an increased pulse frequency (McCarthy et al., 1979; Amann, 1983; Rawlings and Evans, 1995). The decrease of LH at approximately 20 weeks is likely caused by negative feedback from rising androgen levels being produced within the testes (Bagu et al., 2006).

In postnatal bulls, the concentrations of testosterone are low before 12 weeks of age (Lacroix and Pelletier, 1979; Amann and Walker, 1983). Testosterone does not show marked increases until after the gonadotropin rise, and this coincides with other species such as rams (Wilson and Lapwood, 1979) and rats (Kolho et al., 1987). Testosterone is sequestered within the testes by androgen-binding protein (ABP), which also increases after 20 weeks of age, causing extremely high intra-testicular testosterone concentrations necessary to maintain spermatogenesis (Dadoune and Demoulin, 1993; Gilula et al., 1976).

Pituitary-derived FSH has a similar prepubertal secretion to that of LH. The majority of studies report a transient increase beginning at approximately 4 weeks of age that lasts until 25 weeks of age (Evans et al., 1993; Rawlings and Evans, 1995; Aravindakshan et al., 2000; Bagu et al., 2006). FSH is a hormone of focus in this dissertation research because of its proliferative effect on Sertoli cells within the seminiferous tubules (Orth, 1984). This will be discussed in further detail later.

Lastly, inhibins and activins regulate FSH production and action. Inhibin is initially detectable at 8 weeks of age (MacDonald et al., 1991) and is produced by Sertoli cells in response to pituitary FSH. Notably, the concentration of inhibin begins to relate inversely to FSH levels beginning at 10 weeks of age. Activins, in contrast to the narrower range of actions of inhibins, have a broader range of effects on the body. For example, it is believed that activins play a large role in embryonic development and the establishment of functioning reproductive ability (Matzuk et al., 1995). In normal males, activin counteracts inhibin by stimulating basal and GnRH-induced secretion of FSH from the pituitary (de Kretser and Phillips, 1998). Males experience their highest circulating levels of activin after birth (Barakat et al., 2008), but these elevated concentrations decrease around the same time the transiently-elevated gonadotropins begin their decline in concentration at 20 weeks of age (Buzzard et al., 2004).

Changing Testicular Physiology of the Prepubertal Bull

The abovementioned changing hormonal milieu plays an integral role in the establishment of the testicular environment needed for spermatogenesis. The general definition of puberty for bulls was dictated by Wolf and Lunstra in the 1980's. The attainment of puberty in bulls is marked using two criteria: the first being the animal's ability to produce an ejaculate containing 50 million sperm with 10% motility, and the second being the attainment of 28 centimeter scrotal circumference (SC; Wolf et al., 1965; Lunstra, 1982). Often, the attainment of 28 cm scrotal circumference precedes the animal's ability to produce the required amount of sperm (Wolf et al., 1965).

Regarding LH and testosterone and their effect on developing Leydig cells, the testes begin a period of rapid growth at or soon after 20 weeks of age (Amann and Walker, 1983; Bagu et al., 2006). This is most likely because of the prior, transient increase in LH from 4 to 25 weeks of age, which has a proliferative and maturing effect on Leydig cells in the testicular interstitium (Curtis and Amann, 1981). The Leydig cells derive from mesenchymal stem cells that have migrated to the interstitium, and thyroid hormone is believed to be one the main factors causing differentiation of mesenchymal cells into progenitor cells and then into Leydig cells (Mendis-Handagama and Ariyaratne, 2001).

The interplay between Leydig cells, testosterone, and LH are dynamic during development. The concentration of LH-R on Leydig cells increases at the time of birth in males, and concentrations are probably near their highest at, or soon after, birth (Purvis et al., 1997; Hardy et al., 1990; Bagu et al., 2006). Concentration of LH-R decreases from 13 to 21 weeks of age as the number of undifferentiated progenitor and fetal Leydig cells undergo differentiation. While the number of LH-R are decreasing, their receptor (LH) affinity is maintained from birth through puberty. This is crucial in allowing Leydig cells to begin testosterone production in response to LH beginning at 28 weeks of age (Bagu et al., 2006). After this time, Leydig cells have gained mature function and will serve as the main source of androgens in the mature male.

Sertoli cells lining the seminiferous tubules are responsible for supporting the maturation of spermatogonia to elongated spermatids. Specifically, they regulate the biochemical surroundings of germ cells because they enclose the cells, thus forming a distinct environment within the seminiferous tubules (Griswold, 1998). Sertoli cells aid in sperm production while also maintaining high intra-tubule testosterone concentrations via the production of ABP and the formation of the blood-testis barrier (Gilula et al., 1976).

In postnatal animals, Sertoli cells are often referred to as being "undifferentiated" (Bagu et al., 2006; Amann, 1983). These early Sertoli cells, which migrated to the testis from the mesonephros (Skinner and Griswold, 2005), are undifferentiated in the sense that they are not located in a specific position within the tubule, have not formed the blood-testis barrier, and do not yet have proximal relationships with developing germ cells. These early cells do have FSH-R, but the concentration of FSH-R receptor decreases per mg of testis tissue rapidly after birth until 8 weeks of age (Dias and Reeves, 1982). FSH-R concentration may continue to decrease slightly after 8 weeks of age until 25 weeks of age (Bagu et al., 2006) or remain relatively unchanged (Dias and Reeves, 1982). It is important to note that receptor affinity for the FSH ligand is consistent regardless of FSH concentration during the prepubertal period (Bagu et al., 2006), which is crucial because Sertoli cells must remain sensitive to FSH as spermatogenesis commences (Orth, 1984). The transition from undifferentiated supporting cells to Sertoli cells is rapid between 13 and 25 week of age, with rapid testicular growth afterwards (Bagu et al., 2006; Amann, 1983).

Pituitary-derived FSH, which exerts its effects on developing germ cells via the FSH-R on Sertoli cells (Senger, 1997), is a major focus for the proposed research. There are temporal

changes in FSH production during the prepubertal period in bulls. Mainly, there is a transient increase in FSH beginning at 4 weeks of age that lasts until approximately 25 weeks of age (Evans et al., 1993; Rawlings and Evans, 1995; Aravindakshan et al., 2000; Bagu et al., 2006). During this transient FSH increase, Sertoli cells also undergo many proliferations under the stimulatory effects of FSH until they reach their final numbers in the adult population (Orth, 1984). The number of sperm/germ cells that each mature Sertoli cell 'hosts' is fixed in the bull (Curtis and Amann, 1981; Hochereau-de Reviers et al., 1987). This phenomenon is also observed in humans but not in seasonally breeding species such as the hamster and the stallion (Blanchard and Johnson, 1997; Leal et al., 2004). Furthermore, the ability of Sertoli cells to replicate under the stimulatory effects of FSH is lost in adulthood (Sharpe et al., 2003). Therefore, the number of Sertoli cells present upon the attainment of puberty can be linked to adult sperm production. As the testes equip themselves for spermatogenesis, activin and FSH concentrations decrease. This coincides with the cessation of Sertoli cell proliferation and may indicate these two main factors regulate the postnatal increase in Sertoli cell numbers (Buzzard et al., 2004).

Attainment of Spermatogenesis

The combination of endocrine changes and physiological development culminates in the bull's ability to produce and ejaculate viable sperm. The early stages of spermatogenesis begin once spermatogonia occupy the spaces along the basement membrane of the seminiferous tubules, and this can be visualized in testis histological sections beginning between 3 to 4 months of age in the bull (Chandolia et al., 1997a). Spermatocytes can be seen by 6 months of age, and elongated spermatids by 8 months of age (Chandolia et al., 1997a; Barth, 2004). Early ejaculations may contain sperm that have visual abnormalities if puberty has not been finalized, since the testicular components necessary for sperm maturation (i.e. epididymis) may not be fully developed (Evans et al., 1995). These abnormalities may include the presence of proximal droplets or compromised motility, but there is a marked decrease in sperm abnormalities just prior to puberty (Evans et al., 1995). The decrease in visible sperm abnormalities coincides with the attainment in puberty as testosterone concentrations in the gonad are increasing and are known to be important for the maturation of sperm in the epididymis (Martig and Almquist, 1969).

The testes experience rapid growth between 25 and 28 weeks of age (Lunstra et al., 1978), and this is most attributable to the changes in individual functions and numbers of somatic and germ cells (Bagu et al., 2004). Overall, the composition of the testis changes as the seminiferous tubules rapidly develop and fill with sperm cell-types of varying maturity. From 12 to 32 weeks of age the percentage of testis comprised of seminiferous tubules increases from 44 to 81% (Curtis and Amann, 1981), and the testes will continue to grow in size before they will be 90% of their final size by 24 months of age (Coulter, 1986).

The accessory sex glands must develop in synchrony with the gonads, so the vesicular glands and prostate begin to increase in size rapidly after 34 weeks of age (Chandolia et al., 1997b). Select Sires, Inc. begins sperm collection attempts in bulls at 40 weeks of age, or at 28 to 30 cm scrotal circumference for some high genomic bulls. Few bulls will mount a teaser at this age, but as time progresses most will gain the ability to mount and ejaculate into an artificial vagina by 12 months of age, and it is estimated that 95% of bulls have gained the ability to mount and ejaculate sperm of acceptable quality by 12 – 13 months of age (personal correspondence, Mel DeJarnette). Sperm numbers per collection will also continue to increase during the initial year of collection, and most bulls will reach a peak of semen production around 48 months of age (personal correspondence, Don Monke).

Interventions to Hasten Puberty in Cattle

Bulls owned by AI companies fail to provide income until they are able to produce semen as saleable product. To reduce the loss associated with the time prior to puberty—and to potentially elevate income once maturity is attained—several types of intervention have been experimentally tested in hopes of hastening puberty, increasing mature sperm production, or both. Experiments have been done in several mammalian species, and a few have showed promise in humans with sexual-development disorders. Overall, interventions fall into one of three broad categories: increasing gonadotropin concentrations via exogenous gonadotropin therapy, increasing gonadotropin concentrations by immunologically blocking their respective inhibitors, and/or dietary enhancements.

Beginning with exogenous gonadotropin addition, GnRH, FSH, and LH have been extensively studied. Madgwick et al. (2008) treated prepubertal bulls with 120 ng/kg GnRH

twice daily from 4 to 8 weeks of age. Bulls experienced an LH pulse after each injection, and the bulls receiving GnRH had more rapid testicular growth from 22 to 44 weeks of age, and puberty was hastened by 6 weeks relative to controls. Puberty advancement was attributed to the early, induced transient increase in LH that is normally initiated at 6 weeks of age. Similarly, Chandolia et al. (1997c) treated bulls with 200 ng LH-releasing hormone (GnRH) intravenously every 2 hours for 14 days from 4 to 6 weeks of age and reported increased GnRH levels in prepubertal bulls cause increased LH and FSH secretion. This reportedly caused permanent positive effects on testicular function by increasing the numbers of Sertoli and germ cells within the testes.

Mainly due to its proliferative effects on Sertoli cells, FSH has gained popularity in studies. One week of age is the earliest FSH concentrations have been experimentally altered in bulls (Kaneko et al., 2001). Bagu el al. (2004) treated bulls from 4 to 8 weeks of age with exogenous FSH (10 mg NIH-FSH-S1 equivalent) every other day, which caused a transient increase in systemic FSH concentrations. In this study puberty was hastened, and histological evaluation of the testes at 56 weeks of age revealed that FSH-treated bulls had greater numbers of Sertoli cells, elongated spermatids, and spermatocytes. Myers et al. (1983) administered 5 mg FSH to bulls twice daily (10 mg/day) for 10 days beginning at 4 months of age. This was repeated for two groups of animals, one in summer and the other in winter. Strangely, the winter animals experienced no detectable physiological changes, while the summer group responded with an increased (38%) testicular weight and intratesticular testosterone concentration. In rats, FSH immunizations at various, early postnatal ages consistently result in decreased Sertoli cell proliferation and increased germ cell apoptosis (Meachem et al., 2005). Similarly, treatment with GnRH antagonists (FSH suppression) causes a 45 – 52% reduction in the number of Sertoli cells and a 46% decrease in testis mass (Atanassova et al., 1999). Also in rats, postnatal treatment with recombinant FSH (FSH addition) can increase the number of adult Sertoli cells by as much as 149% and cause testicular hypertrophy of up to 124% (Meachem et al., 1996). Lastly, in humans, gonadotropin treatment is common in boys diagnosed with hypogonadotropic hypogonadism. These patients do not produce FSH, and are thus at risk for poor testis growth and impaired spermatogenesis. In one study, boys received FSH injections (r-hFSH, 1.5IU/kg) thrice weekly anywhere from 2 to 34 months *preceding* puberty (induced by hCG regimen). Treatment with FSH induces prepubertal testicular growth, increases inhibin-beta, and allows the majority of

study participants to ejaculate motile sperm at the conclusion of treatment (Raivio et al., 2007). This list of studies demonstrates the effect of addition (or in some cases, suppression) of exogenous gonadotropins in prepubertal animals. Another approach to increase FSH concentrations involves removal of its main inhibitors, as highlighted in the following experiments.

A variety of immunological manipulations have been performed in rodent models and have progressed into domestic livestock studies. One such comprehensive study in rats used post-pubertal immunization against GnRH to effectively shut down testis function in adulthood (McLachlan et al., 1995). These treated rats are deficient in FSH, LH, testosterone, and inhibin production, and they are an excellent model to study the independent effects of each hormone using replacement studies. In GnRH-immunized rats, administration of rFSH for 7, 14, or 21 days after the cessation of spermatogenesis consistently caused increases in testis weight by up to 43% that accompanies an increase in seminiferous tubule volume and the proportion of testis comprised of interstitium. Furthermore, rFSH supplementation results in a resumption of spermatogenesis up to the stage of round spermatids as well as a restoration or serum inhibin concentration to normal. Serum or testicular androgen concentrations were not restored via rFSH treatment, however. This treatment showcases FSH's ability to maintain spermatogenesis up to the stage of round spermatids, yet this progression is telling because testosterone was absent in this system, and it was noted by the authors that testosterone is most likely the crucial, missing factor for normal spermatogenesis to occur (McLachlan et al., 1995). The main target of immunological manipulation in bulls has been inhibin. Early studies immunizing bulls against inhibin at 6 months of age reported increased FSH concentrations, but a link between inhibin immunizations early in life and mature concentrations and sperm production were not performed (Kaneko et al., 1993). Subsequent studies (Kaneko et al., 2001; Bame et al., 1999; Martin et al., 1991) immunized bulls against inhibin at early postnatal ages with boosters given periodically thereafter. All demonstrated that immunization against inhibin elevates serum/plasma FSH concentrations. A successive study using the bulls from the Martin et al. (1991) study showed that the immunizations increased the number of elongated spermatids per gram of testicular tissue (Lunstra et al., 1993). Although lacking in consistency, removal of gonadotropin inhibitors using immunological methods circumvents the more direct route of directly administering gonadotropins.

Using diet to regulate the attainment of puberty is usually affecting the underlying endocrinology associated with the process. Prepubertal dietary manipulations in heifers are well documented. For example, Gasser et al. (2006a, b, c, d) demonstrated that early weaning of *Bos taurus* beef heifers and utilizing a high concentrate diet can advance puberty by 3 to 4 months. In bulls, particularly those destined for use in AI, studies examining the effects of prepubertal nutrition are less common. It has been demonstrated that limiting nutrition during sexual maturation delays puberty in bulls (Flipse and Almquist, 1961; Pruitt et al., 1986), results in smaller testes (VanDemark and Mauger, 1964), decreases SC (Pruittet al., 1986), lowers intratesticular testosterone (Mann et al., 1967), and lowers total sperm with ejaculates of lower volume (VanDemark et al., 1964). A recent study utilizing high energy diets in Holstein bulls by Dance, et al. (2015) reported that feeding a high energy diet from 2 to 31 weeks of age resulted in hastened puberty, larger testes, a substantial early rise in LH, and increased concentrations of IGF-1. Similarly, our laboratory (Harstine et al., 2015) fed Holstein bulls a high-energy diet from 58 to 230 days of age. Bulls experienced increased SC, an earlier rise in LH pulsatility, increased testosterone concentration, and increased testicular weight and volume. Overall, the effects of nutrition, particularly those using a high energy diet to increase prepubertal ADG, are known to hasten puberty. Oftentimes, high energy diets may accelerate the function of the HPG-axis, and thereby underlie the hastening of puberty.

Effects of Dietary Energy on Sexual Maturation and Sperm Production in Holstein Bulls

Our laboratory did several studies in heifers demonstrating that puberty can be precociously induced with targeted high energy diets which activate the endocrine mechanisms related to puberty attainment such as increased LH pulse frequency of greater intensity (Gasser et al., 2006a,b,c,d). In bulls, the early gonadotropin rise can also be affected by diet. High energy diets initiated at 10 weeks of age can result in larger testes, greater SC, and greater total daily sperm production compared to bulls fed control rations (Brito et al., 2007). It was also recently demonstrated that Holstein bulls fed high energy diets from 2 to 31 weeks of age experienced hastened puberty and an increase in LH pulse frequency of greater magnitude at a younger age (Dance et al., 2015).

We hypothesized that feeding bulls a high energy diet beginning at 8 weeks of age would advance and increase the prepubertal increase in LH, leading to advanced testes maturation and puberty. Thus, the objective of our experiment was to evaluate the effect of a high energy diet initiated early in life on the initial transient increase in LH secretion, testosterone concentrations, scrotal circumference (SC), age at puberty, mature sperm production, and characteristics of the mature testes in Holstein bulls.

To summarize the experimental methods, fifteen Holstein bulls were raised on the same feeding program designed for replacement dairy heifers where they received 1.9 liters of milk replacer twice daily until they were 50 days of age. The bulls were weaned at 50 days of age, moved to The OSU Beef and Sheep Center, and allowed 7 days to adapt before they were randomized by age, body weight, and pedigree into one of two treatments. The control group (CONT, n = 7) received a diet designed to support an average daily gain of 0.75 kg/day, and the high energy treatment group (HE, n = 8) received a diet designed to support 1.5 kg/day average daily gain. Both diets were isonitrogenous (18.2% crude protein), but they differed in energy intake (Table 1). The diets were fed at 2.5% of body weight, which was measured biweekly. At 230 days of age, bulls were transported to Select Sires, Incorporated. Once at Select Sires all bulls received the same ration that consisted of corn silage and hay total mixed ration.

Regarding data collection, body weight and scrotal circumference were measured approximately every 30 days. For blood hormone analysis, serum to measure LH was collected at 10 minute intervals for 8 hours at 69, 97, 125, 156, 181, and 210 days of age on a subset of six bulls from each treatment. On the same days, blood samples to obtain plasma were also collected hourly in order to measure testosterone concentration. To determine when puberty occurred, attempts to collect the bulls by trained Select Sires technicians began at 241 days of age. Collections were attempted every 14 days using a teaser animal, two false mounts, and an artificial vagina. The semen from two consecutive ejaculates was analyzed, and puberty was defined as the day the bull was able to ejaculate more than 50 million spermatozoa with at least 10% progressively motile sperm (Wolf et al., 1965). Bulls were removed from semen collection upon attainment of puberty, with the last bull attaining puberty at 396 d of age. Next, mature sperm production was assessed three times per week when the bulls were 552 to 569 days of age using the same collection technique previously described. The mature sperm production was determined using collections from the final six days of collection.

To examine the effect of the dietary treatments on testicular size and histology, the bulls were slaughtered at 569 days of age and their testes collected. The epididymis and pampiniform plexus were removed, and volume and weight of each testis was determined. Histological sections from each testis were fixed for subsequent immunohistochemical analysis for seminiferous tubule diameter and the percentage of the testis comprised of seminiferous tubules.

During feeding of experimental diets (before being transported to Select Sires), average daily gain (ADG) was 1.51 ± 0.14 kg/d for bulls in the HE which differed from ADG of 1.00 ± 0.15 kg/d in the CONT treatment ($P < 0.01$). After being moved to Select Sires and placement on a common diet, ADG was 0.87 ± 0.16 kg/d for the HE bulls, which was less than the 1.16 ± 0.1 kg/d ADG for the CONT treatment ($P < 0.05$). Despite the substantial shift in ADG between treatments once bulls were at Select Sires, BW remained greater ($P < 0.05$) in the HE than CONT treatments to 360 d of age (Figure 1).

A major finding of the study was that the HE diet caused an enhanced and prolonged increase in LH pulse frequency (Figure 2). Testosterone concentrations were greater in the HE than CONT treatment at 181 and 210 days of age. Scrotal circumference increased over time in both treatments albeit at a different rate and by 146 d of age (and thereafter to 360 d of age), SC was greater in the HE than CONT treatment. Age at puberty did not differ between the HE (323.3 ± 11.5 d of age) and CONT (301.9 ± 13.0 d of age) bulls, and neither did the number of collections needed before 50 million spermatozoa were produced between the HE (16.3 ± 6.7 collections) and CONT (14.1 ± 3.0 collections) bulls. Mature daily sperm production also did not differ between the HE (6.5 ± 0.7 billion sperm cells/d) and CONT (6.1 ± 0.8 billion sperm cells/d) treatment. At slaughter (569 d of age) the testis weight, epididymal weight, and testis volume were greater in the HE than CONT treatment (Table 2). However, percentage of the testis comprised of seminiferous tubules and average diameter of seminiferous tubules did not differ between the HE and CONT treatments (Table 2).

The results of this study demonstrate not only the need for proper nutrition early in life for bulls destined for use in the AI industry and the possibility of utilizing a high energy diet to enhance the testicular growth of bulls. The positive effects of the high energy diet did not translate to a hastened puberty in this study, however, and this may be due to the decreased weight gain of the HE bulls after their move to Select Sires. The positive effects on the

endocrinology of the HE bulls, especially their LH secretion compared to controls, was similar to the results of the recent study by Dance et al. (2015).

Development and Implementation of a Slow-Release FSH Treatment in Prepubertal Bulls

We sought to develop a system for treating prepubertal bulls with exogenous FSH in order to positively affect their testes development. The FSH treatment was administered in the form of the drug Folltropin-V, which is purified pituitary-derived porcine FSH (Bioniche Animal Health, Ontario, Canada). A novel portion of the experiment included the addition of hyaluronic acid (HA, 2%) as a vehicle for the Folltropin-V. HA is considered to extend the release of a drug at its site of absorption by acting a mucoadhesive (Surini et al., 2003). To attain similar hormone concentrations achieved by prior researchers in prepubertal bulls, we administered 60 mg NIH-FSH-S1 in the form of Folltropin-V weekly. Based upon the estimated performance of 2% HA's time-release abilities, in this experiment we administered 30 mg Folltropin-V in 2% HA twice weekly (every 3.5 days) with the aim to achieve supraphysiological levels of FSH within the bulls (Bo and Mapletoft, 2012; Tribulo et al., 2012). Two experiments were completed. The objective of the first was to classify the serum concentrations of FSH caused by the exogenous FSH treatment. The objective of the second experiment was to implement the FSH treatment into an 8 week long treatment period in prepubertal bulls and examine its effect on the development of the testes.

To clarify the methods of the first experiment, ten Angus-cross bull calves of similar ages were randomized into two treatments. Beginning at 50 ± 6.5 days of age, the bulls were either injected with either 30 mg NIH-FSH-P1 (Folltropin-V) in a 2% hyaluronic acid solution (FSH, n = 5) or saline (control, n = 5) every 3.5 days for one week. The intramuscular injections were given in the neck, being sure to deliver the entire bolus of the injection in one area and alternating between sides. Blood samples to assess FSH were collected immediately before each treatment and every 6 hours thereafter for a 24 hour period. After 24 hours, blood samples to assess FSH and testosterone were collected every 12 hours. The FSH in 2% hyaluronic acid treatment transiently elevated FSH concentrations (Figure 3), and this treatment was implemented into a subsequent study to examine its effect on testes development.

In the second experiment, twenty-two Angus cross bull calves were randomized and evenly allocated into two treatment groups based on birth date and pedigree. Beginning at 35 ± 2.0 days of age, the bulls were injected intramuscularly with 30 mg NIH-FSH-P1 (Folltropin-V) in a 2% hyaluronic acid solution (FSH, n = 11) or saline (control, n = 11) every 3.5 days until they were 91 days of age. The treatments were given in the same manner as in the previous experiment. Blood samples to assess FSH were collected immediately before each treatment every 3.5 days from 35 to 91 ± 2.0 days of age of age for a total of 17 collections. Blood samples to assess testosterone were collected in the same manner and at the same times as FSH samples. Bulls were castrated at 93 ± 2 days of age and their testes were collected in order to measure several linear measurements as well as testis and epididymides weights and volume. At castration, three histological sections per testis were collected to measure seminiferous tubule diameters using an ocular micrometer, as well as to measure the percentage of the testes comprised of seminiferous tubules. In order to quantify the number of Sertoli cells per seminiferous tubule cross section, a GATA-4 immunohistochemical stain was optimized and used to stain testis histological sections for each bull as described by McCoard et al. (2001). The numbers of stained Sertoli cell nuclei present in a circular monolayer within the round tubule were counted. The GATA-4 stained prepubertal bull histological sections were compared to GATA-4 stained postpubertal 18 month old bulls to verify that GATA-4 was indeed selectively staining Sertoli cells (Figure 4). Lastly, intratesticular testosterone concentrations were determined for each bull using pieces of homogenized testicular parenchyma. Intratesticular testosterone (ITT) concentrations were measured from samples after an appropriate dilution was chosen so that all samples fell on the standard curve of the RIA.

During the treatment from 35 to 91 days of age, animals differed in the concentrations of systemic testosterone only on day 84, with control animals having higher concentration. FSH concentrations did not differ between treatments from 35 to 67.5 days of age but increased ($P < 0.05$) in the FSH treatment at 70 days of age and remained elevated over previous levels through 91 d of age. This increase in FSH at 70 days of age in bulls administered pFSH was also greater ($P < 0.05$) than control levels at any time during the study. Control levels did not change during the time of blood sampling (Figure 5). There were no differences in post mortem testis weight or volume across treatments. Histology revealed no differences in the percent of parenchyma comprised of seminiferous tubules or seminiferous tubule diameter between treatments (Table 3).

Immunohistochemistry showed that FSH treated bulls had more Sertoli cells per round seminiferous tubule cross section than control (Table 3). Concentration of intratesticular testosterone was not different among treatment groups (Table 3).

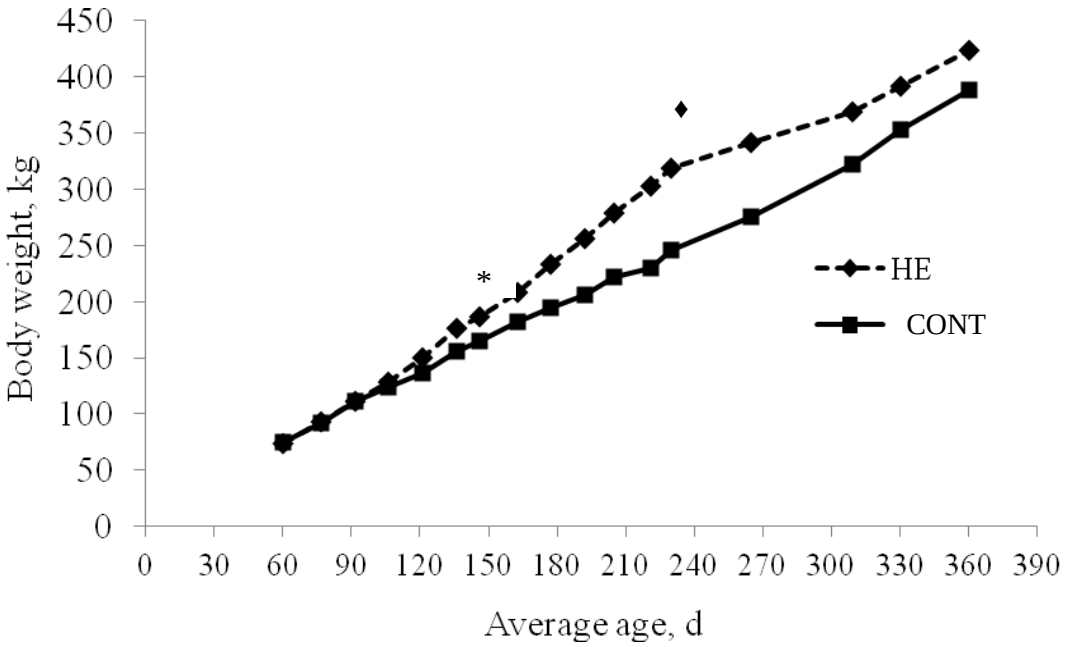
An interesting finding of this experiment was that the exogenous FSH treatment resulted in elevated endogenous FSH concentrations. It is important to note that the samples analyzed for FSH were collected before FSH injections at each 3.5 day interval, and based upon findings of the preliminary experiment, elevated concentrations at this time were not the result of the preceding FSH treatment but rather were due to elevation of FSH concentrations from endogenous sources. Currently, we are performing analyses that may provide insight into how exogenous FSH supplementation was able to augment endogenous FSH concentrations in the treated bulls. The FSH-HA treatment also positively affected development of the testes through enhancing the number of Sertoli cells. Importantly, if the increase in the number of Sertoli cells was a permanent effect of the FSH-HA treatment, we hypothesize that this could translate to an increased mature sperm production. Accordingly, it would be interesting to examine in future experiments if the positive effects on the testes caused by prepubertal FSH supplementation early in life persist into adulthood and if these changes translate into hastened puberty or increased mature sperm production.

Summary

The use of genomics is a main reason behind the current desire to hasten puberty in bulls destined for use by AI companies. In this review, several decades' worth of research was summarized, and the most recent examples of pertinent work performed by our laboratory were described in detail. Attempts to hasten puberty, and possibly increase mature sperm production, in bulls relies on changing the prepubertal endocrinology and focuses on three methods: immunizations aiming to decrease negative feedback of the gonadotropins, direct addition of exogenous gonadotropins, and the use of high energy diets which seek to enhance or hasten the endocrinology associated with the onset of puberty. Thus far, our laboratory has focused specifically on direct exogenous hormone supplementation (FSH) as well as the use of a high energy diet to try to hasten puberty and effect testicular development. Other laboratories have performed research similar to ours that has resulted in equally promising strides towards

enhancing prepubertal development in bulls. However, we feel further research helping to specify the optimal ages to implement dietary or hormonal treatments still warrants attention. The relatively recent desire to collect semen from bulls at ages as young as possible will likely be the driving force behind such research.

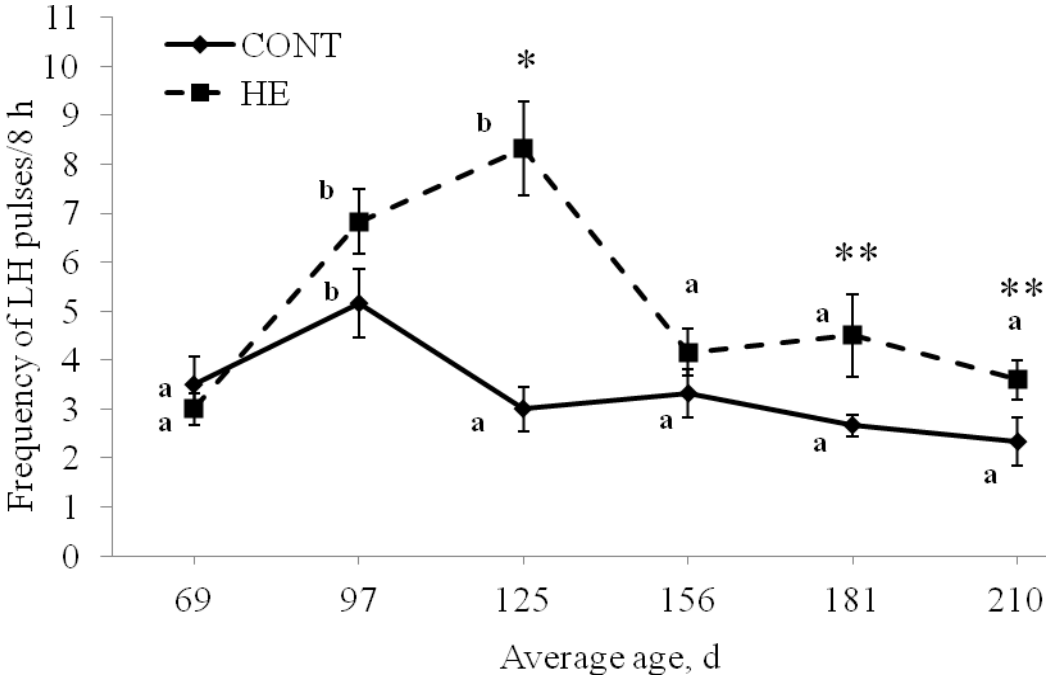
Figure 1.



* Treatments differ at 146 d age and thereafter, ($P < 0.05$)

♦ bulls (HE, n = 8; CONT, n =7) moved to Select Sires, Inc.

Figure 2.



* Treatments differ, (P < 0.05)

** Treatments tend to differ, (P < 0.10)

[ab] Values within treatment differ, (P < 0.05)

Figure 3. The systemic FSH concentrations in bull calves detected by a bovine/ovine double antibody radioimmunoassay after injection of either 30 mg NIH-FSH-P1 in 2% hyaluronic acid (FSH) or saline (control). Time of treatments, indicated by arrows originating from the x-axis, were at 50 ± 6.5 days of age and 53.5 ± 6.5 days of age. Blood was collected every 6 hours following treatment for a 24 hour period, and blood was collected every 12 hours thereafter. Asterisk notes significant difference ($P < 0.05$) between systemic FSH levels between treatments 6 hours after each of the two treatments. Double asterisks indicate a tendency ($P \leq 0.08$) for FSH levels to differ between treatments 12 hours after treatment in both instances. There were no differences in serum concentrations of FSH from 18 to 84 hours after each treatment.

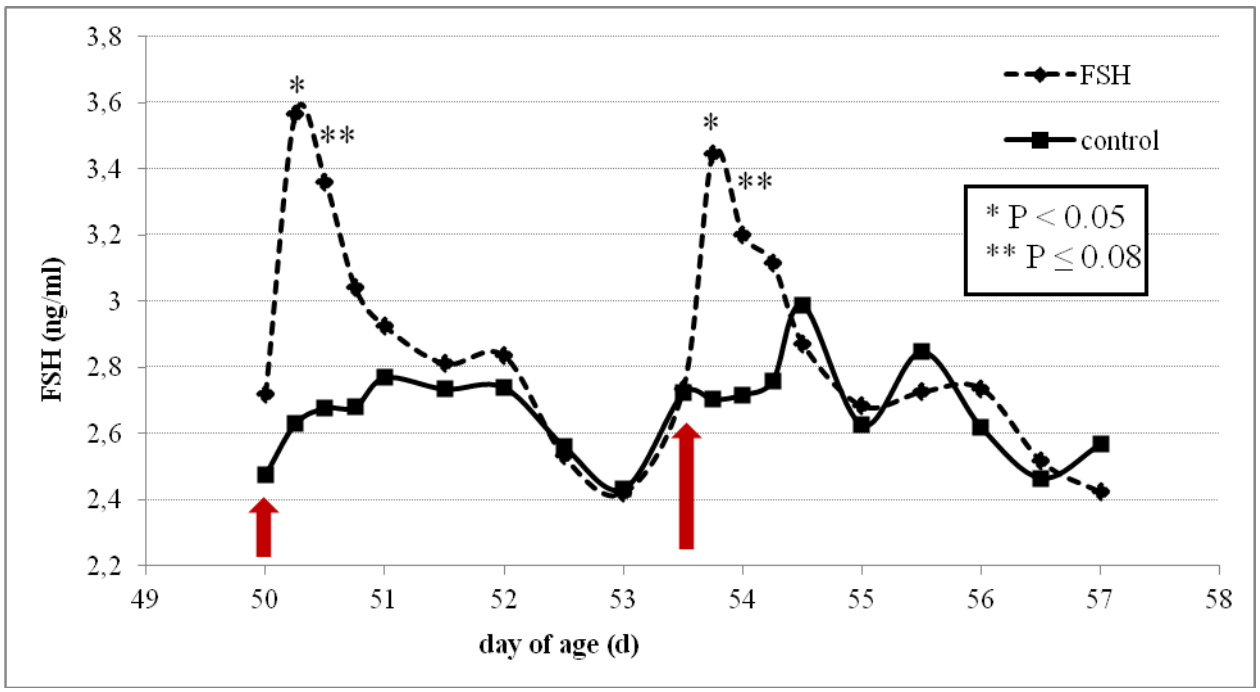


Figure 4. Immunolocalization of GATA-4 in nuclei of Sertoli cells (SC) in bull testes. All slides were counterstained with hematoxylin in order to visualize all cell nuclei present. Both **(B)** Prepubertal and **(D)** postpubertal testis with GATA-4 staining showcasing Sertoli cell nuclei (SC) stained reddish brown within the seminiferous tubules. Germ cells (GC) remained unstained and are noted in both **(B)** prepubertal and **(D)** postpubertal images. It must be noted that Leydig cell nuclei were also non-selectively stained in both prepubertal and postpubertal animals, but since Leydig cells are not found within the seminiferous tubules the immunohistochemistry-based analysis of Sertoli cell numbers was considered valid. Panels **(C)** and **(D)** depict sections in which GATA-4 blocking peptide was used to confirm the specificity of the primary antibody in prepubertal and postpubertal bulls, respectively. The appearance in Panels C and D was identical to sections stained only with hematoxylin, indicating the advantages of using the GATA-4 immunostaining procedures to identify Sertoli cell nuclei in bulls. Magnification bar on each micrograph represents 100 µm.

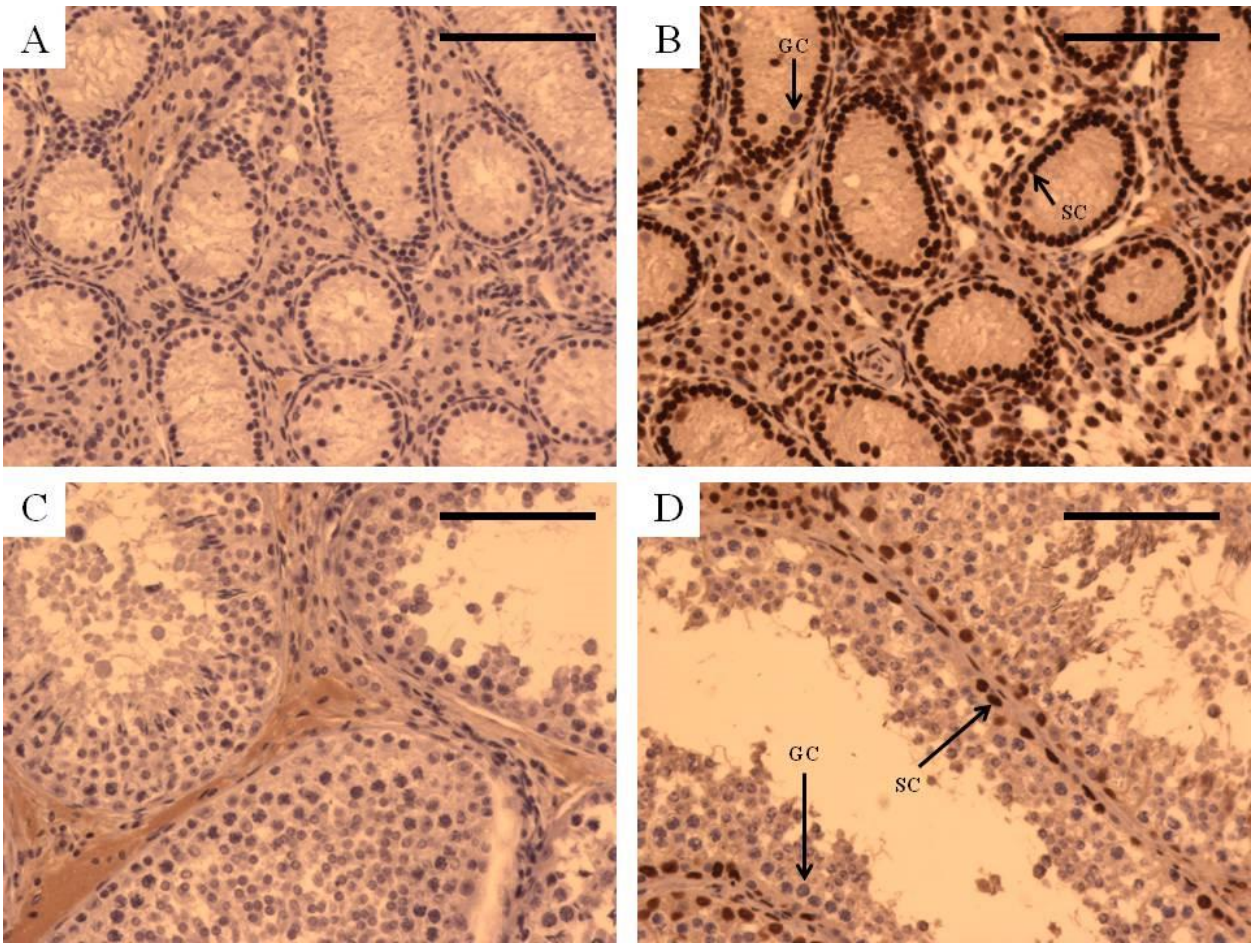


Figure 5. Average systemic concentrations of FSH as measured by a bovine/ovine double antibody radioimmunoassay immediately before bulls were treated with either 30 mg pFSH (FSH, n = 11) or Saline (control, n = 11) every 3.5 days from 35 to 91 days of age. Asterisk denotes an increase ($P < 0.05$) observed in the FSH treatment beginning at 70 days of age, and this increase was maintained and greater than control bulls through 91 days of age.

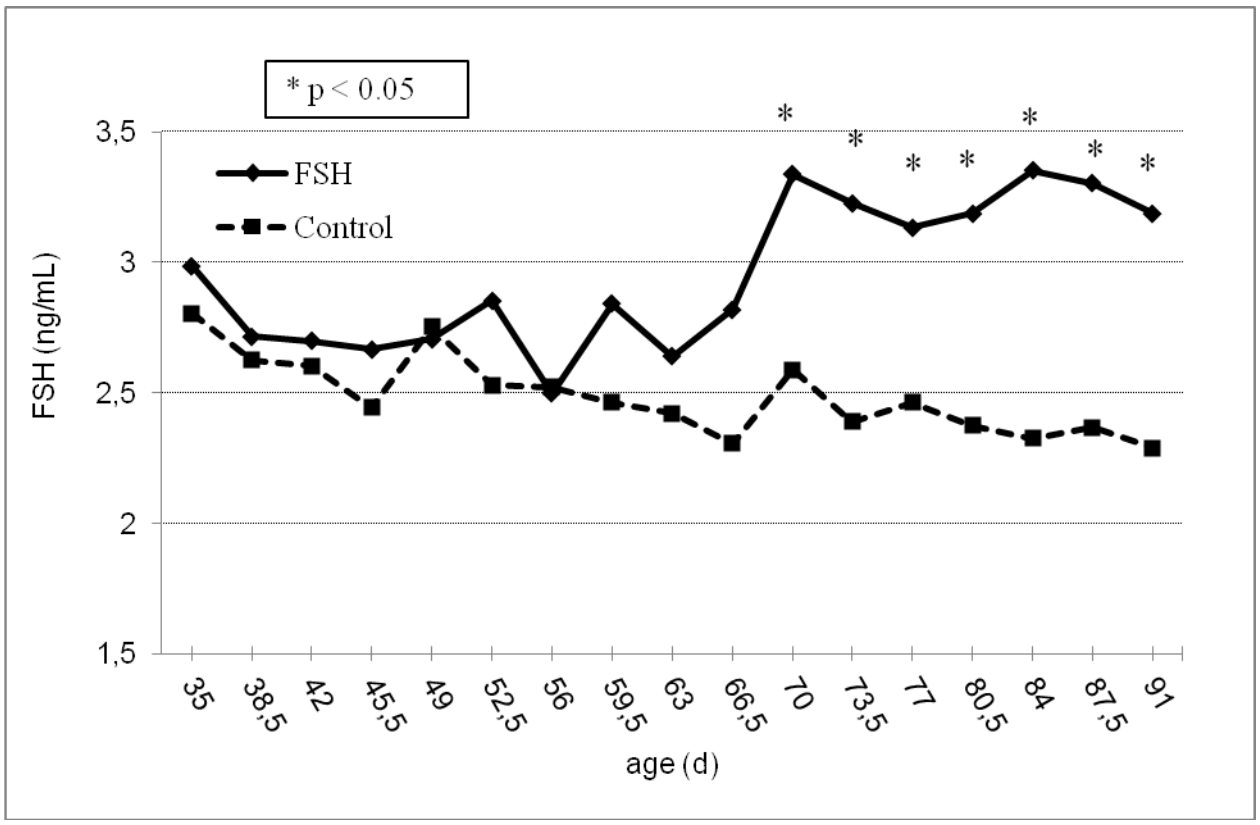

* p < 0.05
FSH
Control
FSH (ng/mL)
3,5
3
2,5
2
1,5
35
38,5
42
45,5
49
52,5
56
59,5
63
66,5
70
73,5
77
80,5
84
87,5
91
age (d)

Table 1. Composition of experimental diets (high energy, HE; control diet, CONT) fed to Holstein bulls from 58 ± 0.3 to 230 ± 0.3 d of age (fed at 2.5 – 3% of BW).

Ingredient	**HE**	**CONT**
Ground Corn (%)	63.4	33.9
Soybean Meal (%)	13.9	15.1
Soybean Hulls (%)	5.7	23.1
Dried Distillers Grains (%)	11.4	0
Alfalfa Meal (%)	0	23.3
Urea (%)	0.9	0.8
Vitamin/Mineral Premix[1] (%)	4.7	3.8

[1]Vitamin/Mineral Supplement meets NRC requirements and includes Limestone, Dicalcium-Phosphate, Trace Mineral Salt, Vitamins A, D, and E, Selenium, Magnesium Oxide, Zinc Sulfate, and Copper Sulfate.

Table 2. Testicular measurements, seminiferous tubule diameter, and percentage of testicular parenchyma comprised of seminiferous tubules (Mean ± SEM; 569 d of age) for Holstein bulls fed either a high energy (HE) or control (CONT) diet from 58 to 230 ± 0.3 d of age (HE, n = 8; CONT, n = 7).

Variable	CONT	HE	Significance
Testis weight (g)	267.5 ± 14.4	318.0 ± 13.5	P < 0.05
Epididymal weight (g)	28.0 ± 1.2	31.6 ± 1.1	P < 0.05
Testis volume (cm^3)	244.9 ± 12.9	305.0 ± 11.9	P < 0.01
Seminiferous Tubule Diameter (µm)	252.8 ± 7.5	251.7 ± 7.0	P = 0.95
Parenchyma Tubule Percentage (%)	71.8 ± 2.1 %	72.3 ± 2.0 %	P = 0.86

Table 3. Post mortem testicular measurements, histological evaluations, and intratesticular testosterone concentration (ITT) of one testis of 93 day old bulls treated with either 30 mg pFSH (FSH, n = 11) or saline (control, n = 11) every 3.5 days from 35 to 91 days of age. No differences were present in any of the measurements between treatment groups except the number of Sertoli cells per round tubule cross section (P = 0.0008). Results are reported as mean ± SE.

Variable	Control	FSH	Significance
Testis Volume (mL)	221.7 ± 1.7	222.9 ± 3.1	0.78
Testis Weight (g)	23.43 ± 2.0	24.14 ± 2.4	0.87
Seminiferous tubule diameter (µm)	78.6 ± 2.1	80.2 ± 2.1	0.6
Percent of testicular parenchyma comprised of seminiferous tubules	51.0%	51.9%	0.18
Sertoli cells per round tubule cross-section	28.27 ± 0.9	33.35 ± 0.9	0.0008
Intratesticular Testosterone (ng/g)	806.1 ± 100.8	738.9 ± 96.1	0.63

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