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Early prepubertal testis criteria, seminiferous epithelium and hormone concentrations as related to testicular development in beef bulls

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ABSTRACT

The present study was conducted to evaluate testis size, spermatogenesis and hormone concentrations before and when peripheral testosterone reached 1 ng/ml as related to further gonad development of beef bulls ($n = 28$). Blood samples were taken weekly starting at 10 weeks (wk) and when testosterone reached 1 ng/ml (AGE1), the left testis was surgically excised. From AGE1 until 54 wk, blood samples were collected to follow basal and GnRH-stimulated hormone profiles. At 54 wk, the second testis was removed. Testosterone reached 1 ng/ml at 20 ± 0.6 wk and, at this developmental state, the seminiferous tubules occupied 57 ± 1.1% of the testis parenchyma. At this phase, 79.3 ± 1.4% of tubule sections had no germ cells and only 2.4 ± 0.3% of the remaining tubules had spermatocytes as the most advanced germ cell type. Also at AGE1, testis size was correlated with the number of Sertoli cells per testis ($r = 0.67$; $P < 0.05$), but not ($P > 0.05$) with the percentage of tubules with germ cells. There was a consistent increase in body weight and testis size throughout the study showing that hemicastration did not impair the development of the bulls. At 54 wk, seminiferous tubules represented 76 ± 0.7% of the testis parenchyma and 72.3 ± 1.7% of tubule sections were found with either round or elongated spermatids. Quantitative criteria of spermatogenesis in the second testis (excised at 54 wk) were not correlated ($P > 0.05$) with the percentage of seminiferous tubules with germ cells in the first testis (excised at AGE1). As determined by regression analysis, testis diameter measured between 30 and 44 wk (AVTD) was associated with AGE1 and testis diameter averaged at 12 wk and AGE1 ($R^2 = 0.77$; $P < 0.01$). Also, AVTD was related to AGE1, testis diameter at 12 wk and concentrations of 17β-estradiol (estradiol; basal + GnRH-stimulated) averaged between 10 wk and AGE1 ($R^2 = 0.79$; $P < 0.01$). Yearling testis weight, in turn, was linked to AGE1 and testis weight at AGE1 ($R^2 = 0.49$, $P < 0.01$). In conclusion, early detection of 1 ng of testosterone/ml, larger testis size and greater estradiol before and at that developmental period positively relate to future testis attributes. When testosterone reached 1 ng/ml, the seminiferous tubules had Sertoli cells, spermatogonia and a few spermatocytes and events occurring before and at that phase are potential markers of testis growth and sperm-producing capacity of sires.

1. Introduction

In bull calves, there is a transient increase in the peripheral concentrations of both LH and FSH between 1 and 4 months of age (Rawlings et al., 1978; MacDonald et al., 1990; Evans et al., 1996; Moura and Erickson, 1997), which coincides with immature Leydig cells secreting great amounts of androstenedione. As differentiation of

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these cells takes place at later ages, more testosterone is produced and androstenedione secretion by the testis is rapidly diminished (Amann, 1983; Moura and Erickson, 1997). This shift in the type of androgen secretion occurs around the age of 4 months in the well-fed *Bos taurus* bull (Moura and Erickson, 1997) and is timely coincident with a decrease in proliferation and start in maturational changes of the Sertoli cell (Sharpe, 1994; Rawlings et al., 2008), as well as with the presence of renewing stem cells and A1 or differentiating spermatogonia in the seminiferous tubules (Curtis and Amann, 1981; Wrobel, 1990, 2000; Bagu et al., 2006). The efficiency by which these A1 spermatogonia are produced may thus determine the number of haploid cells in the testis (Attal and Courot, 1963; Ortavant et al., 1977), suggesting that attributes of the young testis are related to the sperm-producing capacity of adult males. Also, changes in LH, FSH and testosterone at prepubertal ages are important for Leydig and Sertoli cell proliferation and differentiation, which, in turn, establish the necessary structural and biochemical conditions for spermatogenesis to advance until production of spermatozoa in the seminiferous tubules (Amann and Almquist, 1962; Sinowazt and Amselgruber, 1986; Huhtaniemi, 1993; Jégou and Sharpe, 1993; Walker, 2003; Petersen and Söder, 2006). Although testis development and attainment of breeding capacity take several months in bulls, it is possible that events related to testosterone secretion and, therefore, Leydig cell differentiation early in life are potential indicators of testicular growth at later ages. Thus, the present study was conducted to determine if testis size, histology and hormone concentrations before and when testosterone becomes the dominant androgen secreted by the gonads are related to testis criteria and quantitative aspects of spermatogenesis at more advanced developmental states of the beef bull.

2. Materials and methods

2.1. Experimental design

Twenty-eight Angus bulls, born between 8 January and 7 February, were used in the present study. Calves were kept on pasture with their dams until weaning (8 months) and thereafter raised in a pen with access to hay, corn silage and concentrate. Animals were raised in the same location throughout the experiment (Knoxville, TN, USA). According to previous results (Moura and Erickson, 1997), peripheral concentrations of testosterone in Angus bulls increased from 0.2 ng/ml at 12 wk to 1.0 ng/ml at an average of 20 wk of age. Thus, to monitor the changes in testosterone secretion occurring at prepubertal ages, three blood samples were taken from the jugular vein (at 1-h intervals) weekly, starting at 10 wk and continuing until concentrations of testosterone reached 1.0 ng/ml. At this developmental state (AGE1), the left testis was surgically excised. From AGE1 to 54 wk, three blood samples from all bulls were taken monthly (at 1-h intervals) to follow basal hormone profiles. On the day following basal sampling, bulls also received a subcutaneous injection of GnRH, at a dose of 0.05 mg per kg of body weight (des-gly^{10}, [D-ala^{6}]-GnRH-ethylamide, Sigma Co., St. Louis, MO), and blood samples were taken 1.5 and 3 h later. Before AGE1, GnRH treatment was conducted only every other week. Animals had the diameter of the right testis measured throughout the experiment and at 54 wk, the second testis was surgically removed. All bulls were surgically castrated by a veterinarian using approved animal care practices, as previously reported (Moura and Erickson, 1997, 1999; Aguiar et al., 2006).

2.2. Histological analysis

Collection of testis samples, tissue fixation, preparation of slides and methods for evaluation of cell counts and seminiferous tubules were conducted according to procedures previously published (Moura and Erickson, 1997, 1999, 2001; Aguiar et al., 2006). After the first and second castration, testes were weighed and measured after the removal of the *tunica vaginalis* and epididymis. Two 4-mm thick segments were taken near the poles of the testis and placed in Bouin's fixative for 24 h, rinsed with water and washed in three changes of 70% ethanol. Thereafter, tissue was dehydrated in alcohol, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. Calculation of Sertoli cell numbers in the first testis was based on counts done in 10 round tubule cross sections selected at random from different regions of one slide from each bull. In the second testis, number of Sertoli cells, A1 spermatogonia, pachytene spermatocytes and round spermatids containing an intact nucleolus were counted in each of 10 tubule cross sections, at stages I through VI of the seminiferous epithelium (Berndtson and Desjardins, 1974). These counts, here defined as crude counts, were converted to true counts according to Abercrombie's formula (Abercrombie, 1946): true cell number = crude cell number × (section thickness/(section thickness + average nuclear diameter in microns)). Based on the true counts, the following cell ratios per cross section were estimated: number of round spermatids and spermatocytes per A1 spermatogonia and per Sertoli cell; number of A1 spermatogonia, spermatocytes and spermatids per Sertoli cell.

To estimate the degree of testicular development in the first testis, 600 tubule cross sections per animal were chosen at random and placed in one of the following categories based on the most advanced germ cell type: tubules without germ cells, tubules with gonocytes, A1 spermatogonia, intermediate or B type, and with spermatocytes. In the second testes, sections were evaluated and placed in one of the following categories, based on the most advanced germ cell type: tubules without germ cells, with A1 spermatogonia, intermediate or B spermatogonia, pachytene spermatocytes, round spermatids, elongated and mature spermatids.

Total number of Sertoli cells per testis was estimated in both testes (Berndtson et al., 1987; Moura and Erickson, 1997). Testicular volume (V) was determined by the formula $V = TW/D$, where TW and D represented testicular weight (g) and density (1.052 g/cm^3), respectively. The volume occupied by 10 seminiferous tubule cross sections (V_{st}) was calculated by the formula: $V_{st} = \pi \times h \times (d^2/4)$, where "$h$" is the section thickness (5 μm) and "d" represents the tubule diameter (μm). The percentage of testicular volume occupied by seminiferous tubules (%ST)

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was determined as described by Chalkley (1943), i.e., the percentage of 600 "hits" taken at random within a cross section of a testis. Crude numbers of Sertoli cells per testis were determined by the following formula: crude cell number = $(V \times \%ST \times C)/V_{st}$, where C represented the number of Sertoli cells actually counted in the tubule cross sections. The resulting crude numbers were converted to true counts according to Abercrombie's formula (Abercrombie, 1946).

2.3. Radioimmunoassays

Blood samples were collected from the jugular vein and immediately placed on ice. At the laboratory, samples were allowed to warm for 2 h at room temperature and then centrifuged at $1764 \times g$ for 25 min. Serum was harvested and stored at −20° C until radioimmunoassayed for FSH, LH, testosterone, androstenedione and 17β-estradiol (estradiol), as previously described (Moura and Erickson, 1997). Briefly, concentrations of FSH were determined using a double antibody radioimmunoassay (Bolt and Rollins, 1983). Both the first antibody (USDA-5-0122) and the purified FSH used for iodination and reference curve (USDA-bFSH-I-1) were provided by Dr. D.J. Bolt (USDA, Beltsville, MD). The sensitivity of the FSH RIA was 0.25 ng/ml and the intra- and inter-assay coefficient of variation (CV) were <6% and <13%, respectively. Peripheral concentrations of LH were also quantified using a double-antibody RIA method (Niswender et al., 1969; Bolt, 1981). The anti-LH antibody (# 15 anti-ovine LH) was obtained from Dr. G. Niswender (CSU, Fort Collins, CO) and the purified hormone used for the reference curve and iodination was provided by Dr. L.E. Reichert (Rochester Medical School, Albany, NY). The assay sensitivity was 31.3 pg/ml and the intra- and inter-assay CV were <5% and <10%, respectively. Serum samples (150 μl) were analyzed for steroid concentrations after extraction with benzene (1.5 ml). Concentrations of testosterone and androstenedione were estimated based on a single-antibody method (Cox et al., 1987). The androstenedione antibody (X – 322 Rao) was purchased from Dr. P.N. Rao (Southwest Foundation for Biomedical Research, San Antonio, TX) and the testosterone antibody was provided by Dr. G. Niswender. Assay sensitivity for the testosterone and androstenedione assays were 10 pg/ml and 2.5 pg/ml, respectively. The intra- and inter-assay CV were <8% and <14%, respectively, for the testosterone assay, and <7% and <15%, respectively, for the androstenedione assay. Concentrations of estradiol were quantified according to a procedure described by Cox et al. (1987) and the antibody was supplied by Dr. N. Manson (Lilly Research Laboratories, Indianapolis, IN). The sensitivity of the assay was 0.15 pg/ml and the intra- and inter-assay CV were <6% and <15%, respectively. Validation of the assays has been described before (Moura and Erickson, 1997).

2.4. Statistical analysis

Age-related changes in body weight, testis size and hormone concentrations were determined by repeated measure design. This analysis was conducted in a group of 14 animals that had been hemicastrated within a period of 2 wk (from 19 to 21 wk) so that the effect of hemicastration could be evaluated without the bias of age at which this procedure was performed. Information about such animals is shown in Figs. 1–3. Using the entire group of 28 bulls, Pearson's method was used to estimate the correlations among AGE1, testis size, Sertoli and germ cell numbers and hormone concentrations. Only correlations with P-values <0.05 were considered as significant. We conducted regression analysis to establish the extent by which testis size (right testis) averaged between 30 and 44 wk of age and at 54 wk was associated with variables such as AGE1, hormone concentrations, body weight and testis size (right testis) measured from 10 wk until AGE1. Criteria employed to evaluate the regression models were R^2, Mallow's C(P) value and multicollinearity (SAS, 2003).

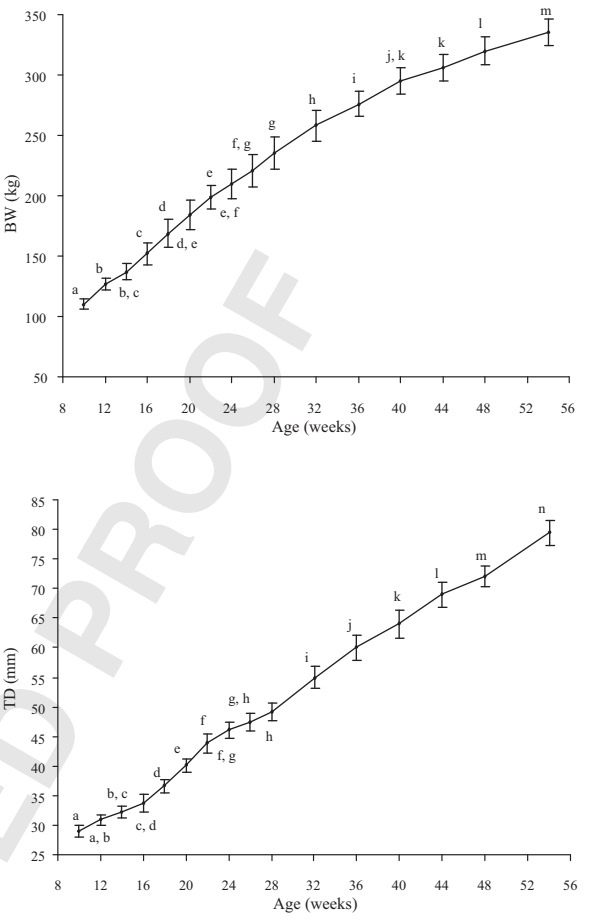

Fig. 1. Body weight (BW) and testis diameter (TD) of Angus bulls (mean ± s.e.m.). Graphs represent the averages obtained from a group of 14 animals that were hemicastrated between 19 and 21 wk. Values followed by the same letters do not differ ($P > 0.05$).

3. Results

3.1. Age-related changes in testis size and hormone concentrations before and after hemicastration

Bulls were hemicastrated when peripheral concentration of testosterone reached 1 ng/ml and at this

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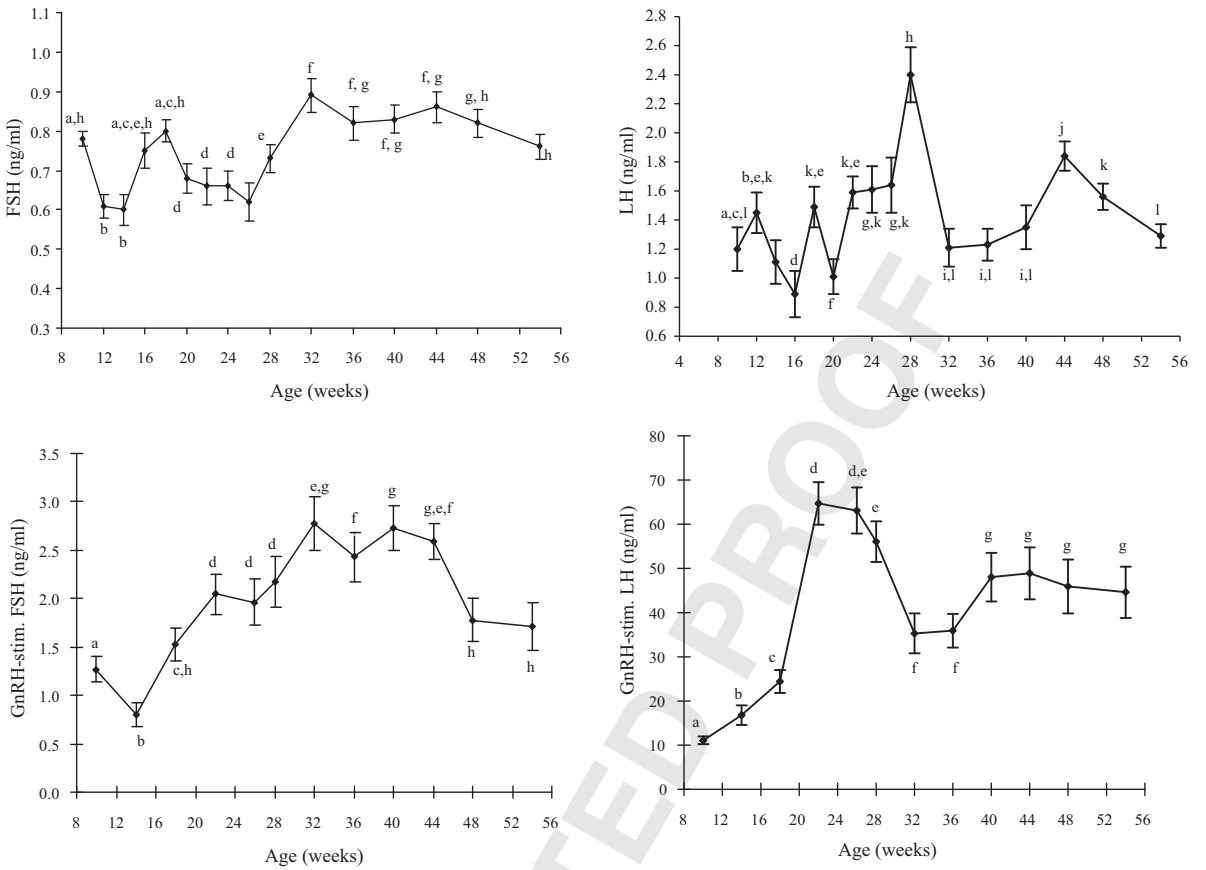

Fig. 2. Peripheral concentrations of basal and GnRH-stimulated FSH and LH in Angus bulls (mean ± s.e.m.). Basal values shown as the average of three samples collected at 1 h intervals and GnRH-stimulated was quantified 1.5 and 3 h after a GnRH injection. Graphs represent the averages obtained from a group of 14 animals that were hemicastrated between 19 and 21 wk. Values followed by the same letters do not differ ($P > 0.05$).

developmental state, the average age and body weight of all calves were 20 ± 0.6 wk and 189 ± 7.6 kg, respectively. After hemicastration, the size of the remaining testis increased ($P < 0.05$) with age and paralleled changes in body weight (Fig. 1). Concentrations of basal FSH decreased ($P < 0.05$) between 10 and 14 wk, increased ($P < 0.05$) from 26 to 32 wk of age, and was reduced ($P < 0.05$) after 44 wk (Fig. 2). Across ages, GnRH-stimulated FSH decreased ($P < 0.05$) between 10 and 14 wk, consistently increased ($P < 0.05$) from 14 to 32 wk, but diminished ($P < 0.05$) after 44 wk of age (Fig. 2). Basal LH changed ($P < 0.05$) between 10 and 26 wk, and reached greater ($P < 0.05$) concentrations at 28 wk. LH decreased ($P < 0.05$) again from 28 to 40 wk, increased ($P < 0.05$) at 44 wk and appeared to diminish ($P < 0.05$) afterwards. Across ages, GnRH-stimulated LH increased ($P < 0.05$) between 10 and 26 wk, but decreased ($P < 0.05$) at 32 and 36 wk. It was amplified ($P < 0.05$) again at 44 wk and remained with small variations ($P > 0.05$) thereafter (Fig. 2).

Basal testosterone increased ($P < 0.05$) between 10 and 20 wk, decreased ($P < 0.05$) at 22 wk, but continuously increased ($P < 0.05$) thereafter, reaching the highest concentrations at 48 wk (Fig. 3). GnRH-stimulated testosterone showed a steady increase ($P < 0.05$) between 10 and 48 wk, without significant ($P > 0.05$) changes afterwards (Fig. 3). Basal androstenedione, in turn, was greatest ($P < 0.05$) in the period from 12 to 14 wk but decreased ($P < 0.05$) at 18 and 20 wk, remaining low at the following ages (Fig. 3). Similarly, the greatest ($P < 0.05$) concentrations of GnRH-stimulated androstenedione were detected at 12 wk, with a sharp decrease ($P < 0.05$) at 18 wk and small changes beyond that period (Fig. 3). Concentrations of basal estradiol in the Angus bulls had small but significant ($P < 0.05$) increases from 10 to 14 wk, were less ($P < 0.05$) at 20 wk but increased again ($P < 0.05$) until the age of 54 wk (Fig. 3). GnRH-stimulated estradiol decreased ($P < 0.05$) between 10 and 22 wk, but consistently increased ($P < 0.05$) thereafter (Fig. 3).

3.2. Gonadal development and associations between testis size, histological criteria and hormone concentrations

When concentrations of testosterone reached 1 ng/ml in the peripheral blood of the Angus bulls, testis diameter and weight averaged 32 ± 0.6 mm and 36 ± 1.8 g, respectively, the seminiferous tubules occupied 57 ± 1.1% of testicular parenchyma and a population of $5.2 \pm 0.4 \times 10^9$ Sertoli cells was estimated per testis. Also, 79.3 ± 1.4% of the tubules had no germ cells and the remaining tubule cross sections

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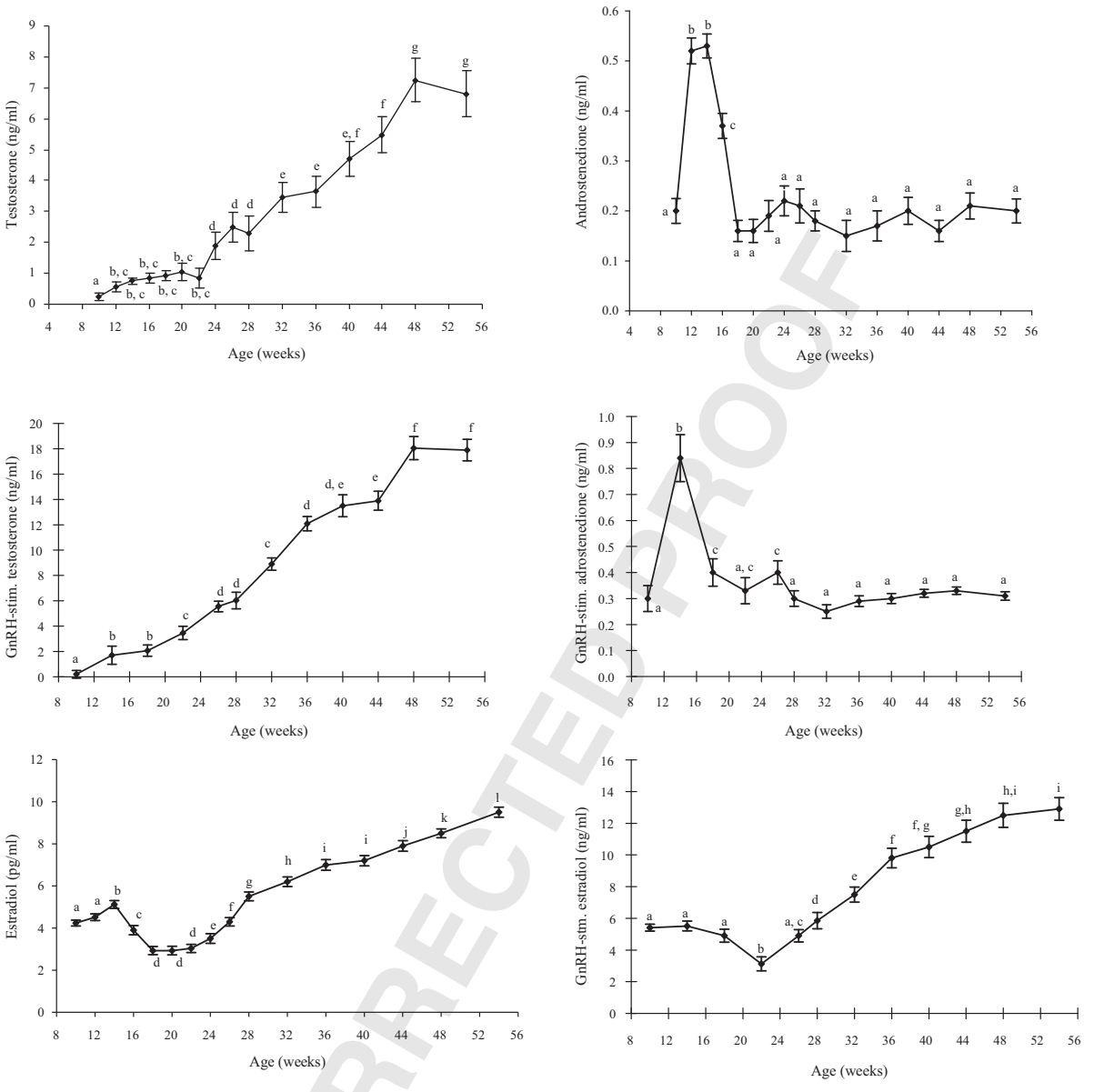

Fig. 3. Peripheral concentrations of basal and GnRH-stimulated testosterone, androstenedione and 17β−estradiol (estradiol) in Angus bulls (mean ± s.e.m.). Basal values shown as the average of three samples collected at 1 h intervals and GnRH-stimulated were quantified 1.5 and 3 h after a GnRH injection. Graphs represent the averages obtained from a group of 14 animals that were hemicastrated between 19 and 21 wk. Values followed by the same letters do not differ ($P > 0.05$).

were found with gonocytes (2.4 ± 0.2%), A1 spermatogonia (13.4 ± 0.7%), intermediate or B type spermatogonia (2.5 ± 0.3%) and spermatocytes (2.4 ± 0.3%), as the most advanced germ cell type. At AGE1, testis size correlated with the number of Sertoli cells per testis ($r = 0.67$), but there were no significant ($P > 0.05$) correlations between testis weight or diameter and the percentage of tubules with any germ cell type.

At 54 wk of age, testis weighed 264 ± 19 g and seminiferous tubules occupied 76 ± 0.7% of the testis parenchyma. Only 15.5 ± 1.9% of the tubule sections had no germ cells and 72.3 ± 1.7% were found with either round or elongate spermatids as the most advanced germ cell type. Also, there were 55.8 ± 4 round spermatids per A1 spermatogonium and 7.4 ± 0.4 round spermatids per Sertoli cell in each tubule cross section, as well as a population of $4.9 \pm 0.4 \times 10^9$ Sertoli cells per testis. Both testis weight and Sertoli cell numbers/testis at 54 wk correlated with the number of round spermatids per A1 spermatogonium ($r = 0.42$ and 0.45), per Sertoli cell ($r = 0.60$ and 0.42; respectively) and number of tubule cross sections containing round or elongate spermatids ($r = 0.53$–0.73). Quantitative criteria of spermatogenesis in the second testis (excised at 54 wk) were not correlated ($P > 0.05$) with the percentage of seminiferous tubules with germ cells in the first testis (excised at AGE1).

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Table 1
Regression equations explaining the variations in variables associated with testicular development in the beef bull.

Regression model	R^2
$AVTD = 27.6 - 0.12 \times AGE1 + 1.65 \times (TD_{12}+TD_{AGE1})/2$	0.77
$AVTD = 12.17 - 0.13 \times AGE1 + 1.24 \times TD_{12} + 6.46 \times [estradiol]$	0.79
$TW_2 = 328.06 - 2.57 \times AGE1 + 7.55 \times TW_1$	0.49

AVTD: testis diameter (mm) averaged between 30 and 44 wk of age; AGE1: age (days) at which basal concentrations of testosterone reached 1 ng/ml in the peripheral circulation; TD_{12}: testis diameter (mm) at 12 wk of age; TW_{AGE1}: testis weight (g) at AGE1; [estradiol]: average of basal and GnRH-stimulated concentrations (pg/ml) of estradiol between 10 wk and AGE1; TW_1: testis weight (g) at AGE1; TW_2: testis weight (g) at 54 wk of age.

Concentrations of GnRH-stimulated FSH averaged in the period between 10 wk and AGE1 were related to testis weight at AGE1 ($r = -0.45$), testis diameter averaged between 30 and 44 wk (AVTD; $r = -0.58$) and the number of spermatids per A1 spermatogonium per Sertoli cell at 54 wk ($r = -0.54$). Peripheral concentrations of GnRH-stimulated testosterone between 10 wk and AGE1 correlated with testis size at 12 wk ($r = 0.76$), testis weight at AGE1 ($r = 0.65$) and AVTD ($r = 0.55$). Moreover, the sum of basal and GnRH-stimulated estradiol quantified from 10 wk and AGE1 were correlated with testis size at 12 wk ($r = 0.80$), testis weight at AGE1 ($r = 0.81$) and AVTD ($r = 0.73$), as well as with the following criteria of the yearling testis: percentage of tubules without germ cells ($r = -0.50$), percentage of tubules with either round or elongate spermatids ($r = 0.58$) and the number of Sertoli cells/testis ($r = 0.52$). Concentrations of LH and androstenedione did not correlate ($P > 0.05$) with any testicular variables.

Regression equations were generated using testis diameter averaged between 30 and 44 wk and testis weight at 54 wk as dependent variables (Table 1). The variation in AVTD was associated with AGE1 and testis diameter averaged at 12 wk and AGE1 ($R^2 = 0.77$; $P < 0.01$). Also, AVTD was related to AGE1, testis diameter at 12 wk and concentrations of estradiol (basal + GnRH-stimulated) averaged between 10 wk and AGE1 ($R^2 = 0.79$; $P < 0.01$). Another regression model showed that testis weight of the 54-wk old bull related to AGE1 and testis weight at AGE1 ($R^2 = 0.49$; $P < 0.01$).

4. Discussion

In the present study, hormone secretion, testis growth and quantitative aspects of spermatogenesis at early prepuberty were evaluated as related to further testis criteria in the beef bull. Hemicastration was performed when testosterone increased and androstenedione reduced in the peripheral blood because this transition is crucial to gonad development and closely linked to Leydig and Sertoli cell function. More specifically, testosterone reached 1 ng/ml at 20 ± 0.6 wk of age, when only 57% of the testis parenchyma was occupied by seminiferous tubules. At this developmental state, most tubule sections had no germ cells and only a few contained A1 spermatogonia or spermatocytes, implying that meiosis had just started in the testis. Still, at AGE1, testis measurements were correlated with Sertoli cell numbers, but not with the percentage of tubules with germ cells, suggesting that gonad size was mainly determined by the Sertoli cell population. Probably, the mitotic activity of the germ cells and their pool at that state (testosterone = 1 ng/ml) were not sufficient yet to fill the intra-tubular spaces and significantly affect testis size. This is also likely the reason why the percentage of tubules populated by germ cells (any type) in the first testis was not correlated with any criteria of the second testis. As previously shown, when animals become older and proliferation of germ cells increases, the number of these cells starts to affect testis weight. In 26-wk old bulls, where only $24.8 \pm 8.9\%$ of the tubules had no germ cells, testis weight was indeed correlated with the percentage of tubules containing germ cells ($r = 0.76$; Moura and Erickson, 1999). Also, in the present study, both testis weight and Sertoli cell numbers were significantly correlated with quantitative aspects of spermatogenesis when bulls became yearling.

As evidenced by the consistent increase in both body weight and testis size throughout the study, it was concluded that hemicastration did not impair the development of the bulls. In support of this concept, only a small percentage of the seminiferous tubules were devoid of germ cells and most of them had either round or elongate spermatids in the remaining testis at 54 wk. Also at this age, the spermatid/A1 spermatogonium ratio (55.8 ± 4) and the number of round spermatids per Sertoli cell (7.4 ± 0.4) in the tubule sections were greater ($P < 0.05$; analysis not shown) than what we had reported before in intact bulls at the same age (42 ± 9 and 6.3 ± 1.4, respectively; Moura and Erickson, 1997). These findings were expected and similar to those reported by Mirando et al. (1989) and Barnes et al. (1980), who showed that unilateral castration of rams and bulls caused a compensatory growth of the remaining testis associated with amplification of both the spermatid/Sertoli cell ratio and daily sperm production. Despite the increase in those criteria, both testis weight and Sertoli cells/testis at 54 wk were correlated with the number of round spermatids/A1 spermatogonium and per Sertoli cell and the number of tubule sections with spermatids, results also equivalent to what has been reported for intact yearling bulls (Moura and Erickson, 1997). Moreover, Sertoli cell numbers/testis were not different when compared between the two testes, implying that compensatory growth of the gonad affected only the population of germ cells and that Sertoli cells may have lost their ability to divide after the developmental state at which hemicastration was performed.

Removal of one testis causes increases in the secretion of basal FSH (Barnes et al., 1981; Schanbacher et al., 1987; Brown et al., 1987) but these changes tend to be transient and an equilibrium in the control of FSH is achieved afterwards because of the compensating growth of the remaining testis and synthesis of steroids at normal rates (Moura and Erickson, 1997; Evans et al.,

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1995; Aravindakshan et al., 2000). The increase in GnRH-stimulated FSH detected between 14 and 32 wk appeared more pronounced than in intact animals (Moura and Erickson, 1997) and this could well have been caused by transient decreases in both testosterone and estradiol that occurred right after hemicastration. The pattern of GnRH-stimulated FSH after 32 wk became comparable to that shown for non-castrated Angus bulls treated with the same doses of GnRH (Moura and Erickson, 1997). Secretion of basal LH showed variations between 16 and 24 wk and some of such variations were consequences of hemicastration and changes in steroid concentrations. Greater concentrations of LH reached at 28 wk may have occurred because steroids had not sufficiently increased yet in the peripheral circulation at that time. Alternate periods of lesser and greater concentrations between 44 and 54 wk appeared again similar to what had been reported for intact bulls (Moura and Erickson, 1997). There was a large increase in the response of LH to GnRH treatments between 18 and 26 wk that seemed more pronounced than what is usually detected in intact bulls but the pattern of GnRH-induced concentrations of LH after that period (32–54 wk) closely resembled those of non-castrated bulls (Moura and Erickson, 1997).

Basal testosterone increased between 10 and 20 wk, showed reductions at 22 wk, but it increased again continuously after that period. Variations at 22 wk of age were probably caused by the absence of one gonad at the average age of 20 wk, and the quick and consistent increase in testosterone after that period reflects the compensatory growth of the remaining testis. Concentrations of testosterone after 22 wk are similar to what has been found in intact bulls (Moura and Erickson, 1997; Evans et al., 1996). In agreement with findings in the present study, Brown et al. (1987) found serum testosterone to be less at 48 h after unilateral castration in rams, but concentrations started to return to normal as early as 1 wk after the removal of the testis. Greater androstenedione concentrations between 12 and 16 wk are typical of early prepuberty (Moura and Erickson, 1997) and after that period its concentrations decreased significantly. This emphasizes that the 1 ng of testosterone/ml mark represented a new developmental state of the gonads, when the Leydig cells were going through a more differentiated state, converting most of the androstenedione to testosterone. Basal estradiol decreased after 14 wk and remained low until 22 wk, a period when there were also variations in testosterone concentrations. Part of this transient decrease in estradiol E2 secretion may have been caused by the absence of the first testis. The pattern of GnRH-stimulated estradiol was closely associated with basal concentrations and after 32 wk the age-related increases in both variables followed the same pattern seen in intact bulls (Moura and Erickson, 1997). Thus, hemicastration of bulls when basal testosterone reached 1 ng/ml in the peripheral circulation may have caused transitory changes in gonadotropins and steroid secretion but eventually allowed normal patterns of hormone secretion and the development of the second testis.

GnRH-stimulated FSH quantified between 10 wk and AGE1 was inversely related to testis size at AGE1, testis diameter averaged between 30 and 44 wk and aspects of the seminiferous tubules at 54 wk. Negative correlations between FSH and testis criteria have been found before in young bulls (Moura and Erickson, 1997) and the fact that FSH induces estradiol and inhibin secretion by the Sertoli cell (Dorrington and Khan, 1993; Vale et al., 1994) is probably the main reason for those correlations. In this regard, there were positive correlations between testosterone and estradiol and testis criteria, which were more significant than those involving FSH. Correlations between testosterone and testis attributes are in agreement with the links between testis development and the age at which animals were first castrated (see below). Total estradiol (basal + GnRH-stimulated) quantified between 10 wk and AGE1 showed marked associations with testis size at 12 wk, testis weight at AGE1 and AVTD, and moderate correlations with the percentage of tubules without germ cells and with spermatids, and with the population of Sertoli cells/testis as well. These correlations between estradiol and attributes of the male gonad had been shown before in intact bulls (Moura and Erickson, 1997) but, to our knowledge, not elsewhere.

The diameter of the remaining testis measured from 30 to 44 wk was related ($R^2 = 0.77$) to the age at which testosterone reached 1 ng/ml and the size of the same testis averaged at 12 wk and at that precise developmental state. Moreover, a comparable R^2 (0.79) was obtained for the same parameter (AVTD) when AGE1, testis diameter at 12 wk and estradiol concentrations between 10 wk and AGE1 were used as independent variables. Yearling testis weight, in turn, was linked ($R^2 = 0.49$) to AGE1 and testis weight at AGE1. Testis size estimated between 7 and 10 months (30–44 wk) correlates with age at puberty (Lunstra et al., 1978) and yearling testis weight is an indicator of sperm producing capacity of bulls (Berndtson et al., 1987). Thus, we conclude from the present study that important attributes of bull reproductive development are influenced by gonad size and estradiol secretion at early prepuberty in conjunction with the animal's ability to release 1 ng of testosterone/ml in the peripheral circulation. Preceding reports have stated that early maturing bulls have greater LH at ages from 10 to 20 wk than late maturing ones (Evans et al., 1995; Aravindakshan et al., 2000) and treatment of sires with GnRH between 4 and 8 wk increased plasma LH, enhanced testis growth and hastened age at puberty (Madgwick et al., 2008). Thus, given that LH is the major modulator of androgen secretion by the Leydig cell, these results are in agreement with the ones described in the present study.

At prepuberty, estradiol is secreted by the Sertoli cell (Bardin et al., 1994; Dorrington and Khan, 1993; Hess and Carnes, 2004) and the statistical associations mentioned above leave little doubt that early Sertoli cell function, and not only testis size but also Leydig cell activity, determines the patterns of future testis growth. Indeed, Leydig and Sertoli cells are intrinsically connected and sequential events related to these cells occur since postnatal ages, as described: proliferation of Leydig and Sertoli cells postnataly; subsequent differentiation of Leydig cells and, as a result, increase in testosterone synthesis and reduction in androstenedione secretion by these cells; decrease in mitosis and induction of Sertoli cell differentiation, which

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in turn set the conditions for progression of spermatogenesis and meiosis by the germ cells (Amann, 1983; Moura and Erickson, 1997; Buzzard et al., 2003; Meng et al., 2005; Aguiar et al., 2006; Bagu et al., 2006; Petersen and Söder, 2006; Johnson et al., 2008; Rawlings et al., 2008). In the present study, bulls with larger testes between 30 and 44 wk may have had some of these events, if not all of them, happening earlier in life. In regard to Sertoli cell development and testis growth, valuable information was obtained from experiments about the effects of hypothyroidism in the rat. In these trials, delays in Sertoli cell differentiation caused by suppression of thyroid function prolonged the mitotic phase of those cells and delayed age at puberty as well. When thyroid function returned to normal, Sertoli cells finally differentiated and animals ended up with more Sertoli and germ cell numbers and larger testis at postpuberty (Hess et al., 1993; França et al., 1995; Holsberger and Cooke, 2005). Based on these results, the duration of the mitotic phase of Sertoli cells is one of the determinants of larger testes. However, this does not necessarily imply that a short mitotic phase is always linked to a smaller Sertoli cell population because such number must also be influenced by the rate at which cells divide. In the present study, bulls with larger testes certainly had an early maturation of the Leydig cells because testosterone reached 1 ng/ml earlier. Based on the fact that testis size at AGE1 was correlated with the number of Sertoli cells per testis, it is possible that higher rates of Sertoli cell divisions occurred in those animals to allow larger testis size at that developmental state. However, to confirm such hypothesis, one needs to determine how interactions between the duration and rate of Sertoli cell mitosis affect testicular growth.

In conclusion, the present study summarizes key aspects of the early testis growth in the beef bull and their associations with further gonad development. We showed that early detection of 1 ng of testosterone/ml in the peripheral circulation, larger testis size and greater estradiol before and at that developmental state relate positively to testis attributes later in life. When testosterone reached 1 ng/ml, the seminiferous tubules had basically Sertoli cells, spermatogonia and a few spermatocytes and physiological events occurring before and at that phase are potential markers of precocity and sperm producing capacity of sires.

Q1 Uncited reference

Ortavant (1959).

References

Abercrombie, M., 1946. Estimation of nuclear population from microtome sections. Anat. Rec. 94, 239–247.
Aguiar, G.V., Araújo, A.A., Moura, A.A., 2006. Testicular development, spermatogenesis and hormonal concentrations in Angus bulls. Braz. J. Anim. Sci. 35, 1629–1638.
Amann, R.P., 1983. Endocrine changes associated with onset of spermatogenesis in Holstein bulls. J. Dairy Sci. 66, 2606–2622.
Amann, R.P., Almquist, J.O., 1962. Reproductive capacity of dairy bulls. VIII. Direct and indirect measurements of testicular sperm production. J. Dairy Sci. 45, 774–789.
Aravindakshan, J.P., Honaramooz, A., Bartlewski, P.M., Beard, A.P., Pierson, R.A., Rawlings, N.C., 2000. Pattern of gonadotrophin secretion and ultrasonographic evaluation of developmental changes in the testis of early and late maturing bull calves. Theriogenology 54, 339–354.
Attal, J., Courot, M., 1963. Développment testiculaire et établissement de la spermatogenèse chez le taureau. Ann. Biol. Anim. Biochem. Biophys. 3, 219–241.
Bagu, E.T., Cook, S., Gratton, C.L., Rawlings, N.C., 2006. Postnatal changes in testicular gonadotropin receptors, serum gonadotropin, and testosterone concentrations and functional development of the testes in bulls. Reproduction 132, 403–411.
Bardin, C.W., Cheng, C.Y., Musto, N.A., Gunsalus, G.L., 1994. The Sertoli cell. In: Knobil, E., Neil, J.D. (Eds.), The Physiology of Reproduction. , 2nd ed. Raven Press, New York, pp. 1291–1315.
Barnes, M.A., Riensen, J.W., Woody, C.O., 1980. Influence of unilateral castration and increased plane of nutrition on sexual development of Holstein bulls. II. Histologic development of the testes. Theriogenology 14, 59–1466.
Barnes, M.A., Boockfor, F.R., Bierley, S.T., Kazmer, G.W., Halman, R.D., Dickey, J.F., 1981. Effect of unilateral castration and unilateral cryptorchidism on gonadotropin and testosteroen response to gonadotropin releasing hormone in the bull. Theriogenology 53, 230–241.
Berndtson, W.E., Desjardins, C., 1974. The cycle of the seminiferous epithelium and spermatogenesis in the bovine testis. Am. J. Anat. 140, 167–180.
Berndtson, W.E., Igboeli, G., Parker, W.G., 1987. The numbers of Sertoli cells in mature Holstein bulls and their relationship to quantitative aspects of spermatogenesis. Biol. Reprod. 37, 60–67.
Bolt, D.J., 1981. Development of homologous radioimmunoassay for ovine follicle stimulating hormone: studies of estrous, ovariectomy, estradiol and releasing hormone. J. Anim. Sci. 53, 730–741.
Bolt, D.J., Rollins, R., 1983. Development and application of a radioimmunoassay for bovine follicle stimulating hormone. J. Anim. Sci. 56, 146–154.
Brown, J.L., Stuart, L.D., Chakraborty, P.K., 1987. Endocrine profiles, testicular gonadotropin receptors and sperm production in hemi-castrated ram lambs. J. Anim. Sci. 65, 1563–1570.
Buzzard, J.J., Wreford, N.G., Morrison, J.R., 2003. Thyroid hormone, retinoic acid, and testosterone suppress proliferation and induce markers of differentiation in cultured rat Sertoli cells. Endocrinology 144, 3722–3731.
Chalkley, H.W., 1943. Method for the quantitative morphologic analysis of tissue. J. Natl. Cancer Inst. 4, 47–53.
Cox, N.M., Ramirez, J.L., Matamoros, I.A., Bennett, W.A., Britt, J.H., 1987. Influence of season on estrous and luteinizing hormone responses to estradiol benzoate in ovarioectomized ewes. Theriogenology 27, 395–407.
Curtis, S.K., Amann, R.P., 1981. The establishment of spermatogenesis in the bull. J. Anim. Sci. 53, 1645–1657.
Dorrington, J.H., Khan, S.A., 1993. Steroid production, metabolism, and release by Sertoli cells. In: Russel, L.D., Griswold, M.D. (Eds.), The Sertoli Cell. Cache River Press, Clearwater, FL, pp. 537–545.
Evans, A.C.O., Davies, F.J., Nasser, L.F., Bowman, P.C., Rawlings, N.C., 1995. Differences in early patterns of gonadotrophin secretion between early and late maturing bulls, and changes in semen characteristics at puberty. Theriogenology 43, 569–581.
Evans, A.C.O., Pierson, R.A., Garcia, A., McDougall, L.M., Hrudka, F., Rawlings, N.C., 1996. Changes in circulating hormone concentrations, testes histology and testes ultrasonography during sexual maturation in beef bulls. Theriogenology 46, 345–357.
França, L.R., Hess, R.A., Cooke, P.S., Russel, L.D., 1995. Neonatal hypothyroidism causes delayed Sertoli cell maturation in rats treated with propylthiouracil: evidence that the Sertoli cell controls testis growth. Anat. Rec. 242, 57–69.
Hess, R.A., Cooke, P.S., Bunick, D., Kirby, J.D., 1993. Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli cell and germ cell numbers. Endocrinology 132, 2607–2613.
Hess, R.A., Carnes, K., 2004. The role of estrogen in testis and the male reproductive tract: a review and species comparison. Anim. Reprod. 1, 5–30.
Holsberger, D.R., Cooke, P.S., 2005. Understanding the role of thyroid hormone in Sertoli cell development: a mechanistic hypothesis. Cell Tissue Res. 322, 133–140.
Huhtaniemi, I., 1993. Hormonal control mechanisms of Leydig cells. In: de Kretser, D. (Ed.), Molecular Biology of the Male Reproductive System. Academic Press, New York, pp. 383–397.

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Jégou, B., Sharpe, R., 1993. Paracrine mechanisms in testicular control. In: de Kretser, D. (Ed.), Molecular Biology of the Male Reproductive System. Academic Press, New York, pp. 271–279.
Johnson, L., Thompson Jr., D.L., Varner, D.D., 2008. Role of Sertoli cell number and function on regulation of spermatogenesis. Anim. Reprod. Sci. 105, 23–51.
Lunstra, D.D., Ford, J.J., Echternkamp, S.E., 1978. Puberty in beef bulls: hormone concentrations, growth, testicular development, sperm production and sexual aggressviveness in bulls of different breeds. J. Anim. Sci. 46, 1054–1062.
MacDonald, R.D., Deaver, D.R., Schanbacher, B.D., 1990. Prepubertal changes in plasma FSH and inhibin in Holstein bull calves: responses to castration and (or) estradiol. J. Anim. Sci. 69, 276–288.
Madgwick, S., Bagu, E.T., Duggavathi, R., Bartlewski, P.M., Barrett, D.M., Huchkowsky, S., Cook, S.J., Beard, A.P., Rawlings, N.C., 2008. Effects of treatment with GnRH from 4 to 8 weeks of age on the attainment of sexual maturity in bull calves. Anim. Reprod. Sci. 104, 177–188.
Meng, J., Holdcraft, R.W., Shima, J.E., Griswold, M.D., Braun, R.E., 2005. Androgens regulate the permeability of the blood–testis barrier. Proc. Natl. Acad. Sci. U.S.A. 102, 16696–16700.
Mirando, M.A., Hoagland, T.A., Woody Jr., C.O., Riesen, J.W., 1989. The influence of unilateral castration on testicular morphology and function in adult rams. Biol. Reprod. 41, 798–806.
Moura, A.A., Erickson, B.H., 1997. Age-related changes in peripheral hormone concentrations and their relationships with testis size and number of Sertoli and germ cells in beef bulls. J. Reprod. Fert. 111, 183–190.
Moura, A.A., Erickson, B.H., 1999. Hormonal responses to GnRH and estradiol treatments and their correlation with testicular development and number of Sertoli cells in prepubertal Angus × Charolais bulls. Braz. J. Anim. Sci. 28, 35–43.
Moura, A.A., Erickson, B.H., 2001. Testicular development, histology, and hormone profiles in three yearling angus bulls with spermatogenic arrest. Theriogenology 55, 1469–1488.
Niswender, G.D., Richert Jr., L.E., Midgley, A.R., Nalbandov, A.V., 1969. Radioimmunoassay for bovine and ovine luteinizing hormone. Endocrinology 84, 1166–1173.
Ortavant, R., 1959. Spermatogenesis and morphology of the spermatozoon. In: Cole, H.H., Cupps, P.T. (Eds.), Reproduction in Domestic Animals. Academic Press, New York, pp. 1–50.
Ortavant, R., Courot, M., Hochereau de-Revires, M.T., 1977. Spermatogenesis in domestic animals. In: Cole, H.H., Cupps, P.T. (Eds.), Reproduction in Domestic Animals. Academic Press, New York, pp. 203–221.
Petersen, C., Söder, O., 2006. The Sertoli cell—a hormonal target and 'super' nurse for germ cells that determines testicular size. Horm. Res. 66, 153–161.
Rawlings, N.C., Fletcher, P.W., Henricks, D.M., Hill, J.R., 1978. Plasma luteinizing hormone (LH) and testosterone levels during sexual maturation in beef bull calves. Biol. Reprod. 19, 1108–1112.
Rawlings, N., Evans, A.C., Chandolia, R.K., Bagu, E.T., 2008. Sexual maturation in the bull. Reprod. Domest. Anim. 43, 295–301.
SAS Institute Inc., 2003. SAS/STAT® User's Guide, Version 6, vol. 2., 4th ed. SAS Institute Inc., Cary, NC.
Schanbacher, B.D., Fletcher, P.W., Reichert Jr., L.E., 1987. Testicular compensatory hypertrophy in the hemicastrated calf: effects of exogenous estradiol. Biol. Reprod. 36, 1142–1148.
Sharpe, R.M., 1994. Regulation of spermatogenesis. In: Knobil, E., Neil, J.D. (Eds.), The Physiology of Reproduction. Raven Press, New York, pp. 1363–1434.
Sinowazt, F., Amselgruber, W., 1986. Postnatal development of bovine Sertoli cells. Anat. Embriol. 174, 413–423.
Vale, W.W., Bilezikjian, L.M., Rivier, C., 1994. Inhibins and activins. In: Knobil, E., Neil, J.D. (Eds.), The Physiology of Reproduction. Raven Press, New York, pp. 1891–1899.
Walker, W.H., 2003. Molecular mechanisms controlling Sertoli cell proliferation and differentiation. Endocrinology 144, 3719–3721.
Wrobel, K.-H., 1990. The postnatal development of the bovine Leydig cell population. Reprod. Domest. Anim. 25, 51–60.
Wrobel, K.-H., 2000. Prespermatogenesis and spermatogoniogenesis in the bovine testis. Anat. Embryol. 202, 209–222.

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