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Prepubertal indicators of potential sperm-producing capacity in the beef bull

Arlindo A. Moura

To the Graduate Council:

I am submitting herewith a dissertation written by Arlindo A. Moura entitled "Prepubertal indicators of potential sperm-producing capacity in the beef bull." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

James D. Godkin, Major Professor

We have read this dissertation and recommend its acceptance:

Linda Munson, Arnold M. Saxton, Lilitha C. Mendis-Handagama, F. Neal Schrick

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

Prepubertal Indicators of Potential Sperm-Producing Capacity in the Beef Bull

A Dissertation Presented for the Degree of Doctor of Philosophy

The University of Tennessee, Knoxville

Arlindo A. Moura

August, 1995

Dedication

I would like to dedicate this dissertation to my father, José de Noronha Moura, and my grandfather, Antônio de Alencar Araripe, to whom I owe great respect and admiration. Unfortunately, they are not here anymore to share these moments with me, but I know they would be very proud of me.

To my mother, Moema de Alencar Araripe, to whom I owe most of I am today. I have an immense admiration for her strength, dedication and love, which have inspired me throughout these years.

To my grandmother, Ana, my lovely sister Anabela, my second mother Alzira, and my good friend Carlos Antônio. Thank you for your love and encouragement.

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To all my friends.

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Abstracts

Part II

Study 1. An experiment was carried out to determine whether peripheral hormone concentrations quantified between 2 and 12 months (mo) were meaningfully correlated with testis size and number of Sertoli and germ cells in the yearling beef bull. Twenty four Angus bulls were treated monthly with GnRH (0.05 μg per Kg of body weight) and bled 1.5 and 3 hours (h) later. On the day before the GnRH challenge, three blood samples were taken at 1.5-h intervals to establish basal hormone concentrations. At 12 mo, all bulls were surgically castrated. Concentrations of FSH, LH, testosterone (T), androstenedione (Δ_4A) and 17β-estradiol (E2) were quantified by RIA and number of Sertoli cells per testis and quantitative aspects of spermatogenesis were determined. Analysis of regression and Pearson's partial correlation were used to estimate the degree of association between hormone and testis data. Histology analysis revealed that 3 bulls had no spermatids and, therefore, were infertile. Yearling testis weight (TW) was related to GnRH-stimulated T at 3 mo ($r = 0.48$, $p < 0.05$) and E2 at 2 and 4 mo ($r = 0.40$ and 0.45, $p < 0.10$). Basal and GnRH-stimulated FSH quantified between 2 and 12 mo were correlated with TW ($r = -0.37$, $p < 0.10$ to -0.71, $p < 0.01$), number of Sertoli cells ($r = -0.41$, $p < 0.10$ to -0.68, $p < 0.01$), number of A1 spermatogonia (r = nonsignificant, $p > 0.10$ to $r = -0.59$, $p < 0.05$) and number of round spermatids per testis (r = nonsignificant, $p > 0.10$ to -0.69, $p < 0.01$). Also, FSH between 2 and 12 mo correlated with number of round spermatids per A1 spermatogonia (r =

nonsignificant, $p > 0.10$ to $r = -0.61$, $p < 0.01$), number of round spermatids per Sertoli cell (r=nonsignificant, $p > 0.10$ to -0.64, $p < 0.01$) and percent of seminiferous tubules with round spermatids (r = nonsignificant, $p > 0.10$ to $r = -0.63$, $p < 0.01$) in the 12-mo old testis. Regression analysis showed that FSH and E2 at 2 mo accounted for 45% of the variation in TW. Furthermore, significant variation in TW was accounted by FSH and testis diameter at 6 mo ($R^2 = 0.57$), FSH and testis diameter at 9 mo ($R^2 = 0.67$) and FSH and body weight at 12 mo ($R^2 = 0.63$). Thus, it appears that prepubertal testis size and FSH could be used as indicators of testicular development of yearling beef bulls. However, the inclusion of data from infertile bulls in the correlation and regression analysis may have generated biased results. Three cases of spermatogenic arrest were described in this study. Bull #250 had seminiferous tubules lined by Sertoli cells only and bull #345 had no germ cells that advanced beyond the A1 spermatogonia stage. Testes of these two bulls weighed 60% less than those of normal bulls ($p < 0.01$). Concentrations of T and LH in bulls #250 and #345 were reduced at some ages ($p < 0.10$). Spermatogenic arrest at the level of pachytene spermatocyte was detected in bull #389. Basal FSH and LH were higher in bull #389 than in normal bulls ($p < 0.05$). Testes of this bull weighed 13% of those of normal bulls and secreted less T throughout the year ($p < 0.05$). However, as in the cases of bulls #250 and #345, the decrease in T observed in bull#389 did not appear to be sufficient to interrupt germ cell development. Chromosomal abnormalities and/or deficiency in intratesticular factors may be the causes of infertility in these bulls.

Study 2. The objective of this study was to determine whether concentrations of

gonadotropins and steroids in 2- and 6-month old beef bulls were significantly correlated with testicular development and number of Sertoli cells at the age of 6 months (mo). At 2 mo, 15 crossbred bulls were treated with GnRH (5 μg) and bled 1.5, 3 and 4.5 hours (h) later. One day before the GnRH challenge, three basal samples were taken at 1.5-h intervals. At 6 mo, bulls received 1 mg of estradiol benzoate (E) and were treated with GnRH (10 μg) 12 h later. After 5 days, bulls received GnRH only (10 μg). In both cases, blood samples were taken 1.5, 3 and 4.5 h after GnRH and two basal samples were taken 1 h before the GnRH treatment. Bulls were castrated at 6 mo. Concentrations of FSH, LH, testosterone (T), androstenedione (Δ_4A) and 17β-estradiol (E2) were quantified by RIA. Number of Sertoli cells and A1 spermatogonia per testis and quantitative aspects of spermatogenesis were also estimated. Analysis of regression and Pearson's partial correlation were used to estimate the degree of association between hormone and testis data. Results showed that testis weight at 6 mo (TW) was related to FSH at 2 mo ($r = -0.48$ to -0.62, $p = 0.08$ to 0.02). Also, concentrations of FSH at 2 mo was correlated with number of Sertoli cells (SC; $r = -0.50$ to -0.65, $p = 0.07$ to 0.01), A1 spermatogonia (A1; $r = -0.45$ to -0.58, $p = 0.09$ to 0.03) and number of seminiferous tubules with round spermatids ($r = -0.49$ to -0.60, $p = 0.08$ to 0.02). Regression analysis showed that FSH and body weight at 2 mo accounted for 30% and 72% of the variation in SC and TW in the 6-mo old bull. Concentrations of estradiol at 6 mo were related to SC ($r = 0.62$, $p = 0.02$). At 6 mo, GnRH-stimulated FSH and LH were respectively 2.5- and 3-fold higher when GnRH was given in combination with E than when GnRH was given alone. Correlation between FSH and SC was more pronounced after GnRH+E ($r = -0.68$, $p = 0.008$) than after GnRH only ($r = -0.63$, $p = 0.015$). Regression modeling showed

that FSH and body weight at 6 mo accounted for 48% of the variation in the population of Sertoli cells. Thus, concentrations of FSH at 2 and 6 mo of age appears to be reliable indicators of number of Sertoli cells and testicular development in the 6-mo beef bull.

Part III

Study 3. This study was carried out to determine whether testis size, histology and hormone secretion at the developmental state at which concentrations of testosterone reached 1 ng/ml of serum were related to size of the testis and quantitative aspects of spermatogenesis in the yearling bull testis. Three basal blood samples (at 1.5-hour intervals) were taken from 28 Angus calves since the age of 15 weeks (wk). When concentrations of testosterone (T) reached 1 ng/ml, bulls were unilaterally castrated. On a monthly base, bulls received 0.05 μg of GnRH per Kg of body weight and were bled 1.5 and 3 hours later. At 54 wk, the second testis was excised. RIA's were used to quantify FSH, LH, T, androstenedione (Δ_4A) and 17β-estradiol (E2) in blood samples. Number of Sertoli cells and quantitative aspects of spermatogenesis were estimated in both testes. Analysis of regression and Pearson's partial correlation were used to estimate the degree of association between hormone and testicular development. Results showed that testis weight when T = 1 ng/ml (TW1) was correlated with testis weight (TW2; $r = 0.58$, $p < 0.01$), and number of Sertoli cells and round spermatids per testis at 54 wk ($r = 0.58$, $p < 0.01$). Also, TW1 was related to number of spermatids per Sertoli cell ($r = 0.41$, $p < 0.05$), and number of seminiferous tubules with elongate (STE) and mature (STM) spermatids ($r = 0.47$ and 0.52, respectively; $p < 0.05$) in the second testis.

However, neither STE nor STM were correlated with number of tubule sections with germ cells in the first testis (STG1). Testis diameter averaged between the ages of 30 and 44 wk (AVTD) and TW2 were correlated with AGE1 ($r = -0.58$ and -0.42, $p < 0.01$), TW1 ($r = 0.65$ and 0.58, $p < 0.01$) and STG1 ($r = 0.43$ and 0.41, $p < 0.10$). Regression analysis showed that AGE1 and body weight when T = 1 ng/ml accounted for 41% of the variation in testis diameter between 30 and 44 wk. GnRH-stimulated FSH at 11 wk was related to STG1, TW1, TW2 and AVTD ($r = -0.42$ to -0.53, $p < 0.05$). GnRH-stimulated T at 15 wk was related to TW1 ($r = 0.55$, $p < 0.01$) and AVTD ($r = 0.48$, $p < 0.05$). Also, bulls that secreted more E2 after a GnRH treatment at 15 wk had larger AVTD ($r = 0.62$, $p < 0.62$, $p < 0.01$) and TW2 ($r = 0.45$, $p < 0.10$). Thus, differences between slow and fast growing bulls are affected by the ability of the testis to secrete testosterone early in life. Also, the existence of correlations between FSH, steroids and characteristics of the testis at AGE1 and prepuberty points toward a role played by these hormones at distinct phases of germ cell development.

Part IV

Study 4. A study was conducted to identify the cell types that express retinol-binding protein (RBP) in the bovine testis and to evaluate the profiles of RBP mRNA levels as related to testicular development. Furthermore, the hypothesis that RBP could serve as a marker of state of development of the seminiferous epithelium was evaluated. At the ages of 10 (n=3), 20 (n=8) and 34 (n=7) weeks, Angus bulls were bled three times and surgically castrated afterwards. Blood samples were assayed for LH and testosterone (T) and the degree of

seminiferous tubule development was evaluated in tissue samples stained with hematoxylin and eosin. Immunolocalization of RBP was detected according to the biotin-strepavidin-horseadish peroxidase method (samples from 10-wk old testes could not be analyzed). Nylon membranes containing isolated total RNA were hybridized with a bovine cDNA (bcRBP-700) and a β-actin probe and slot blots were carried out to determine the abundance of RBP and β-actin transcripts. Age-related changes in testis weight, hormone concentrations and relative quantity of RBP transcripts were determine by analysis of variance. Results showed that testis weight and levels of LH and T increased with age ($p < 0.05$). Also, seminiferous tubules at 10 wk had immature Sertoli cells and gonocytes but, at 20 wk, spermatogonia and few spermatocytes were detected. At 34 wk, Sertoli cells appeared differentiated and spermatids were observed. As never shown before in the bovine species, RBP was detected in Sertoli, Leydig and peritubular cells at the ages of 20 and 34 wk, but no immunoreactivity was present in the germ cells. Furthermore, no difference in staining between Sertoli cells from tubules with or without germ cells was detected. Thus, as determined by immunohistochemistry, RBP does not appear to be a distinct marker of Leydig and Sertoli cell differentiation. Northern hybridization of testicular RNA revealed the presence of a mRNA of 1.1 Kb, which was similar to previous RBP transcripts found in bovine conceptuses and extraembryonic membranes. Densitometric scanning of slot blots indicated that the fraction of total RNA coding for RBP was 50 % higher at 10 wk than at 20 and 34 wk. Thus, it is possible that testicular RBP mRNA is down-regulated by testosterone and/or LH, but further studies are needed to verify this hypothesis.

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List of Abbreviations

PART I

Study 1

Characteristics of the testis of 12-month old Angus bulls:

TW: testis weight (g).
TD: testis diameter (mm).
SC: number of Sertoli cells per testis (x 10^9).
A1: number of A1 spermatogonia per testis (x 10^8).
RST: number of round spermatids per testis (x 10^{10}).
AS: number of A1 spermatogonia per Sertoli cell in seminiferous tubule cross sections.
RS: number of round spermatids per Sertoli cell in seminiferous tubule cross sections.
RA: number of round spermatids per A1 spermatogonia in seminiferous tubule cross sections.
ST0: number of seminiferous tubule cross sections without germ cells.
STA: number of seminiferous tubule cross sections with A1 spermatogonia as the most advanced germ cell type.
STB: number of seminiferous tubule cross sections with Intermediate or B spermatogonia as the most advanced germ cell type.
STP: number of seminiferous tubule cross sections with pachytene spermatocytes as the most advanced germ cell type.
STD: number of seminiferous tubule cross sections with round spermatids as the most advanced germ cell type.
STE: number of seminiferous tubule cross sections with elongate spermatids as the most advanced germ cell type.
STM: number of seminiferous tubule cross sections with mature spermatids.

Cases of spermatogenic arrest:

Bull #250: bull with Sertoli cell-only syndrome.
Bull #345: bull showing spermatogenic arrest at the level of A1 spermatogonia.
Bull #389: bull showing spermatogenic arrest at the level of pachytene spermatocytes.

Study 2

Characteristics of the testis of 6-mo old crossbred bulls:

TW: testis weight (g).
TD: testis diameter (mm).
SC: number of Sertoli cells per testis (x 10^9).

A1: number of A1 spermatogonia per testis (x 10^8)
AS: number of A1 spermatogonia per Sertoli cell in seminiferous tubule cross sections.
ST0: number of seminiferous tubule cross sections without germ cells.
STA: number of seminiferous tubule cross sections with A1 spermatogonia as the most advanced germ cell type.
STB: number of seminiferous tubule cross sections with Intermediate or B spermatogonia as the most advanced germ cell type.
STP: number of seminiferous tubule cross sections with pachytene spermatocytes as the most advanced germ cell type.
STD: number of seminiferous tubule cross sections with round spermatids as the most advanced germ cell type.
STE: number of seminiferous tubule cross sections with elongate spermatids as the most advanced germ cell type.
STM: number of seminiferous tubule cross sections with mature spermatids.

PART III (Study 3)

Aspects of testicular development in Angus bulls:

AGE1: age (in days) at which peripheral concentrations of testosterone reached 1 ng/ml.
TW1: testis weight (g) at the developmental stage defined as AGE1.
ST01: number of seminiferous tubule cross sections without germ cells at the developmental stage defined as AGE1.
STG1: number of seminiferous tubule cross sections with germ cells (gonocytes or spermatogonia) at the developmental stage defined as AGE1.
TW2: testis weight (g) at age of 54 weeks.
AVTD: average testis diameter (mm) between the ages of 30 and 44 weeks.
SC2: number of Sertoli cells per testis at the age of 54 weeks.
RST: number of round spermatids per testis at the age of 54 weeks.
AS: number of A1 spermatogonia per Sertoli cell in seminiferous tubule cross sections at 54 weeks.
RS: number of round spermatids per Sertoli cell in seminiferous tubule cross sections at 54 weeks.
RA: number of round spermatids per A1 spermatogonia in seminiferous tubule cross sections at 54 weeks.
ST0: number of seminiferous tubule cross sections without germ cells at 54 weeks.
STA: number of seminiferous tubule cross sections with A1 spermatogonia as the most advanced germ cell type at 54 weeks.
STB: number of seminiferous tubule cross sections with Intermediate or B spermatogonia as the most advanced germ cell type at 54 weeks.
STP: number of seminiferous tubule cross sections with pachytene spermatocytes as the most advanced germ cell type at 54 weeks.

STD: number of seminiferous tubule cross sections with round spermatids as the most advanced germ cell type at 54 weeks.
STE: number of seminiferous tubule cross sections with elongate spermatids as the most advanced germ cell type at 54 weeks.
STM: number of seminiferous tubule cross sections with mature spermatids at 54 weeks.

PART I

(Justification and Literature Review)

Justification

It is well recognized that bulls vary considerably in their ability to produce sperm (Amann and Almquist, 1962; Amann, 1970). The causes of this variation are not fully understood but, in the adult male, they are closely related to differences in scrotal circumference and testis weight (Amann, 1970; Coulter and Foote, 1979), total Sertoli cell numbers (Erickson and Blend, 1976; Berndtson et al., 1987a,b) and some indicators of Sertoli function, such as transferrin mRNA levels measured in extracts of testes (Gilmont et al., 1990).

In the bull, Sertoli cell mitosis occurs mostly during fetal and early postnatal life, and no increase in Sertoli cell population is observed after the age of 10 weeks (Sharpe, 1994). Number of Sertoli cells has been shown to account for a large proportion of the variability (R^2=68.2%) in daily sperm production (DSP) between adult dairy bulls (Berndtson et al., 1987b) and to significantly correlate ($r > 0.65$) with number of A1 spermatogonia (Hochereau-de-Reviers et al., 1987). Thus, these correlations suggest that spermatogenesis, and as an end point DSP, are affected by any factor that may influence Sertoli cell proliferation.

Besides being correlated with DSP, testis size measured after puberty is negatively correlated with age at puberty in males (Lunstra et al., 1978). Breeds with large testes have also high pregnancy rate and early age at puberty in females (Martin et al., 1992). Moreover, scrotal circumference is heritable (h^2=0.46) and is a highly repeatable measurement (Bourdon and Brinks, 1986). Hence, prepubertal indexes of testis size and Sertoli cell numbers and

function could be useful indicators of adult sperm producing capacity and age at puberty in the bull.

For the economy of the beef farm, reproductive performance is 5 and 10 times more important than growth performance and carcass quality, respectively (Trenkle et al., 1977). Moreover, it has been estimated that between 20 and 40 % of the beef bulls raised in range conditions have subnormal ability to reproduce (Elmore et al., 1976; Coulter et al., 1989). Therefore, from the perspective of the beef industry, reliable prepubertal indicators of sperm producing capacity and age at puberty would be of great economic advantage.

Literature Review

Structural organization of the testis

The adult mammalian testis can be divided into different compartments. The interstitial compartment is located between the seminiferous tubules and contains the blood and lymph vessels and nerves of the testicular parenchyma, as well as the androgen-producing Leydig cells (Fawcett, 1973; Setchell et al., 1994; figure 1). The basal and adluminal compartments of the seminiferous tubules are divided by the blood-testis barrier, which is mainly formed by the junctional complexes of the Sertoli cells. The basal compartment of the seminiferous tubules is separated from the interstitial space by a semipermeable lamina propria, on which the spermatogonia rest. The adluminal compartment contains the meiotic germ cells (spermatocytes) and the spermatids, which are in close contact with the Sertoli cell cytoplasm (Fawcett, 1975; Setchell et al., 1994; figure 2).

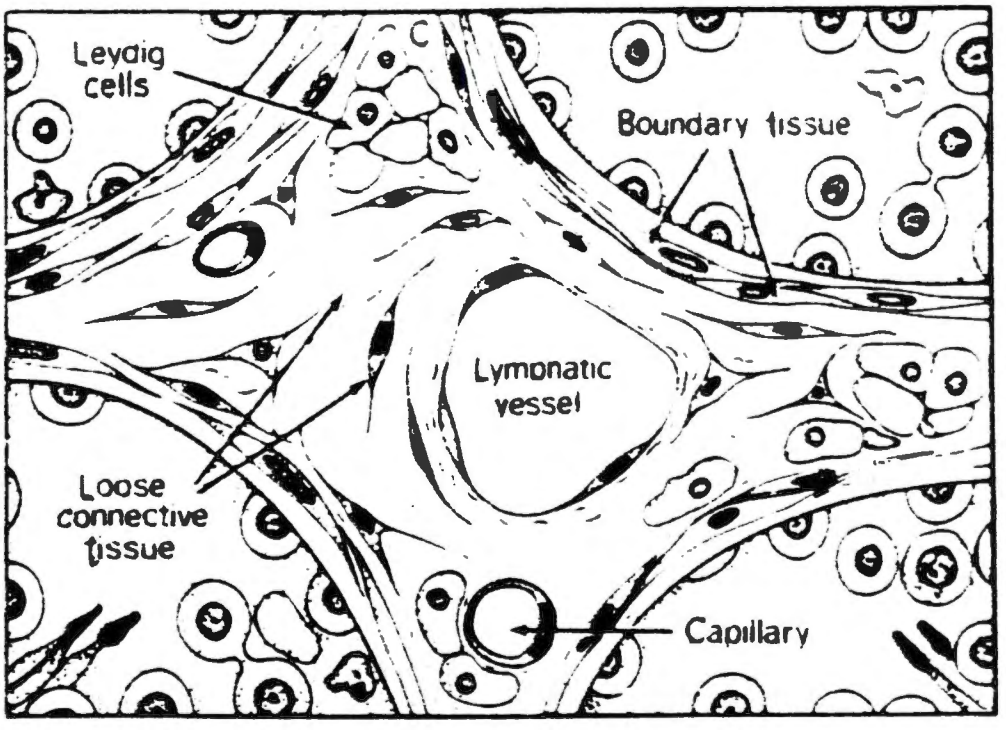

Figure 1: Diagram of the interstitial tissue of the bull, sheep and man, with the Leydig cells either in groups near a blood vessel or in a loose connective tissue (from Fawcett, 1973).

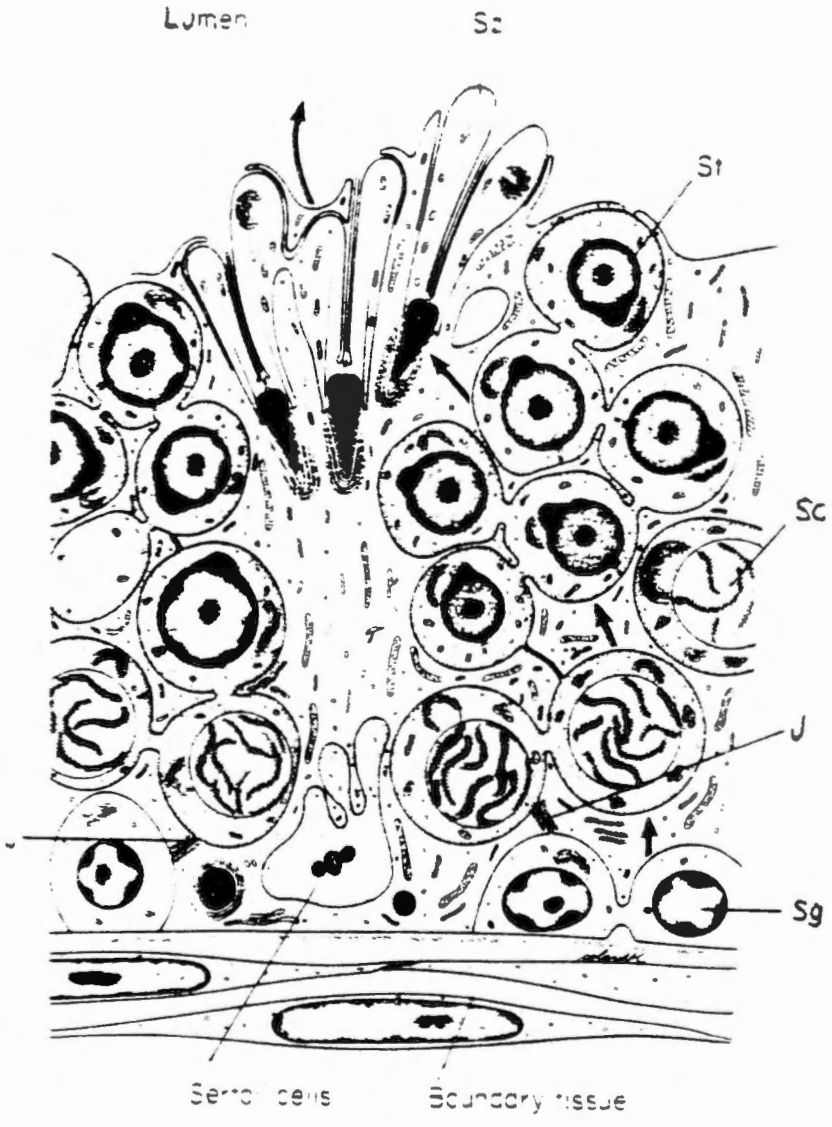

Figure 2: Diagram of the arrangement of the germinal cells in the seminiferous tubule. The diploid spermatogonia (Sg) are confined between the Sertoli cell cytoplasm and the bondary tissue of the tubule. The spermatocytes (Sc) and early spermatids (St) are sandwiched between pairs of Sertoli cells, on the adluminal side of the especialized junctions (J) between adjacent Sertoli cells. The late spermatids (Sz) are embedded in the luminal surface of the Sertoli cell cytoplasm, until they are finally extruded into the lumen and leave the testis (from Fawcett, 1975).

The Leydig cell

The postnatal development of the Leydig cell is controlled by follicle-stimulating hormone (FSH), luteinizing-hormone (LH) and growth factors. Khan et al. (1992) showed that, in vitro, LH in combination with insulin-like growth factor I (IGF-I) and transforming growth factor α (TGF-α) stimulated DNA synthesis in rat Leydig cells. IGF-I mRNA is expressed by both rat Sertoli and Leydig cells (Lamb, 1993) and its synthesis can be induced by FSH and LH, respectively (Dombrowicz et al., 1992; Naville et al., 1990). Moreover, unidentified factors secreted by Sertoli cells induced Leydig cell mitosis. Ojeifo et al. (1990) reported that a 30-kilodalton protein secreted by cultured Sertoli cells enhanced the uptake of [^{3}H]-thymidine by purified Leydig cells obtained from immature and adult rats. Conversely, administration of 17β-estradiol (E2) to rats that had been hypophysectomized (Abney and Meyers, 1991) or treated with EDS - ethane dimethylsulfonate (Hsueh et al., 1978) prevented Leydig cell growth under FSH stimulation.

The differentiation of the Leydig cell also involves both LH and FSH. LH induces mesenchymal cells to differentiate into adult-type Leydig cells (deKretser and Kerr, 1992). FSH in turn can stimulate synthesis of LH receptors (deKretser and Kerr, 1992), secretion of testosterone (T), and induce hypertrophy and functional enhancement of Leydig cells in immature hypophysectomized rats (Kerr and Sharp, 1985a; 1985b). Since the Sertoli cell is the main target for FSH in the mammalian testis (Amann and Schanbacher, 1983), the maturational effect of FSH on the immature Leydig cell is mediated by the Sertoli cell.

The main function of the differentiated Leydig cell is the synthesis and secretion of testosterone. Before puberty, these cells secrete mainly androstenedione, but at the

completion of differentiation, testosterone becomes the major steroid produced under LH stimulation (Amann and Schanbacher, 1983). Leydig cell steroidogenesis can also be induced by factors secreted by the Leydig cell itself, such as IGF-I (Lamb, 1993), or by products of the Sertoli cell, such as TGF-β (Lamb, 1993; Skinner, 1991), IGF-I and inhibin (Skinner, 1991). Thus, it seems that the effects of LH on the growth and secretory activity of the Leydig cell are regulated by the Sertoli cell.

The Sertoli cell

The Sertoli cell is the only somatic cell present within the seminiferous tubules and provides structural and functional support for spermatogenesis (Bardin et al., 1994). Proliferation of Sertoli cells occurs mostly during fetal life and their numbers do not increase after puberty in mammals (Kluin et al., 1984; Steinberger and Steinberger, 1971; Hochereau-de-Reviers et al., 1987), with the possible exception of the horse (Johnson and Nguyen, 1986).

The control of Sertoli cell mitosis involves FSH, which has been shown to stimulate Sertoli cell proliferation in both fetal (Orth, 1984) and prepubertal (Steinberger and Steinberger, 1971) rats. FSH receptors have been detected in rats as early as 17 days postconception (Pelliniemi et al., 1992). Moreover, treatment of immature nonhuman primates with highly purified FSH promoted Sertoli cell mitosis (Arslan et al., 1993), and hemicastration of immature rats and other species resulted in an enhancement of Sertoli cell numbers in the remaining testis coincident with elevated plasma levels of FSH (Orth et al., 1984).

LH also seems to influence Sertoli cell proliferation. Kilgour et al. (1985) reported that lambs treated with anti-LH serum had lower Sertoli cell numbers than controls. Also, de-Reviers et al. (1980) showed that total numbers of Sertoli cells in immature hypophysectomized lambs could be maintained by LH alone, but mitosis occurred only when LH and FSH were given together. Thus, since LH binding is confined to the interstitial cells (Gondos et al., 1992), any possible effect of LH on Sertoli cell mitosis must be mediated by Leydig-cell factors.

The Sertoli cell originates from mesenchymal-like cells during fetal life, and the process of maturation continues throughout the prepubertal period (Pelliniemi et al., 1992; Gondos et al., 1992). At the age of 4 weeks, the bovine Sertoli cell had a round or oval nucleus containing large aggregates of heterochromatin associated with a small nucleolar-like structure (Sinowatz and Amselgruber, 1986). At 20 weeks, most of the primitive Sertoli cells had acquired attributes characteristic of the mature Sertoli cell, such as an elongated nucleus without clumps of chromatin, and at the age of 24 weeks, Sertoli cell junctions made a functional blood-testis barrier. The process of Sertoli cell maturation is characterized by decreases in FSH-induced inhibin secretion (MacDonald et al., 1991), aromatase, mitotic activity and cAMP production, and increases in synthesis of androgen-binding protein (ABP) and FSH receptors (Ritzen et al., 1981). Control of Sertoli cell differentiation is exerted by FSH, testosterone, PModS (an androgen-regulated factor secreted by the peritubular cells) and growth factors (Gondos et al., 1992; Skinner, 1992). Solari and Fritz (1978) have shown that morphological features of the mature Sertoli cell such as the pattern of chromatin condensation, large nucleoli, nuclear infolding and smooth endoplasmic reticulum are affected

by FSH.

The appearance of the first mature Sertoli cells in the bovine is coincident with a decline in mitotic activity (Sinowatz and Amselgruber, 1986) and an increase in testosterone concentrations (Amann, 1983). Sertoli cells express androgen receptors (Sar et al., 1992) and Orth et al. (1984) showed that treatment of hemicastrated rats with testosterone prevented Sertoli cell proliferation in the remaining testis. However, it is still possible that this effect was mediated via a negative feedback by T on FSH synthesis and secretion. Also, Walker et al. (1984) showed that Holstein calves immunized against testosterone from 1 to 6 mo, had significant increases in testis weight (21 %, $p < 0.07$) and DSP (35 %, $p < 0.04$) at the age of 18 mo. The fact that neither LH nor FSH was increased in the immunized bulls, suggested that low levels of testosterone may have delayed the differentiation of the Sertoli cells and allowed them to proliferate for a longer period of time, ultimately causing an increase in sperm numbers.

Cooke et al. (1991) reported that hypothyroidism in immature rats prolonged the period of Sertoli cell proliferation and caused significant increases in testis size and sperm production. Since hypothyroidism can delay the prepubertal rise in plasma LH in rats (Kirby et al., 1992), decrease both LH and testosterone in rams (Chandrasekhar et al., 1985) and retard Sertoli cell maturation (Francavilla et al., 1990), it appears that the regulation of the Sertoli cell differentiation is dependent upon testosterone. However, studies have suggested that thiiodothyronine (T_3) can act directly on the Sertoli cell. Jannini et al. (1990) reported that Sertoli cells of fetal and prepubertal rats expressed high affinity T_3-binding sites. Cooke et al. (1995) showed that T_3 was able to prevent FSH-stimulated DNA synthesis by cultured

rat Sertoli cells. Also, secretion of ABP, which is a marker of Sertoli cell differentiation, was inhibited by T_3. Thus, there is strong evidence that thyroid hormones are directly involved in the process of Sertoli cell development, acting either by itself or modulating the effects of testosterone and FSH.

Skinner (1992) has provided evidence that Sertoli cell differentiation can also be mediated by PmodS (Peritubular cell-modulating substance). Peritubular cells contain as many androgen receptors as do Sertoli cells and undergo differentiation under the influence of androgens and FSH at early stages of puberty. PModS synthesis is stimulated by testosterone and affects several functions associated with Sertoli cell maturation such as increases in ABP and transferrin gene expression and a decrease in aromatase activity (Skinner, 1992).

During postnatal life, most Sertoli cell functions show age-related changes. Under FSH stimulation, the Sertoli cell is able to produce estradiol 17-β (E2) from androgens secreted by the Leydig cell (Bardin et al., 1994). In rats, peripheral concentrations of E2 are highest early in life but gradually decrease as puberty approaches (Ritzen et al., 1981). FSH-induced estradiol synthesis can be controlled directly by androgens, growth factors and cytokines, PModS or even substances secreted by spermatocytes and spermatids (Dorrington and Khan, 1992). In the rat, the source of E2 is shifted from the Sertoli cell to the Leydig cell, as the latter differentiates (Dorrington and Khan, 1992). In the postpubertal bull, Amann and Ganjam (1976) showed that exogenous hCG increased concentrations of E2 in the peripheral blood, suggesting that the Leydig cell was the source of estrogen in the testis. Moreover, Purohit et al. (1992) reported that human osteoblast cell lines express aromatase activity and other enzymes involved in estrogen metaboism and Nitta et al. (1993) used

immunocytochemistry and Western and Northern blots to detect P450 aromatase in round, elongate and late spermatids in 8-week old mouse testes. Thus, besides the Sertoli and Leydig cell, other cell types in the testis or in peripheral tissues are able to express aromatase. However, it is still unknown how much E2 is secreted by these newly identified sources.

Estradiol plays an important role in the function of the hypothalamus-pituitary axis, since it affects both GnRH and gonadotropin release (Johnson and Everitt,1984; Nett,1990). In the rat, E2 decreases testicular responsiveness to exogenous LH in vivo (Morger, 1976). Also, Nakhala et al. (1986) and Miyashita et al. (1990) reported the presence of estrogen receptors in mouse Leydig cell lines (TM3 and B-1). Intratesticular injections of E2 prevented, via a local pathway, Leydig cell proliferation in rats subjected to EDS treatment or hypophysectomy (Abney and Meyers, 1991; Moore et al., 1992; respectively). Thus, E2 is a potential intratesticular regulator of both T production and Leydig cell numbers.

Estradiol also appears to play a role in Sertoli cell proliferation. Nakhala et al. (1986) found that mouse and rat Sertoli cell lines (TM4 and TR-ST, respectively) expressed estrogen receptors. In primary Sertoli-cell cultures, E2 receptors were almost undetectable for the first 3 days. However, when cells were maintained in culture for 15 days, E2 receptors increased more than 4-fold. Thus, production of E2 receptors by the Sertoli cell is dependent upon cell density and expression of E2 receptors in vivo is controlled by Sertoli-Sertoli cell interactions. Benhamed et al. (1988) showed that E2 increased secretion of TGF-β by Sertoli cells of immature pigs, and Dorrington et al. (1993) reported that FSH acted synergistically with TGF-β to enhance uptake of [H^3]thymidine by Sertoli cells isolated from 10-day old rats. Also, rat Sertoli cells secreted TGF-β before puberty (Mullaney and Skinner, 1992). Thus,

E2 appears to influence Sertoli cell mitosis by increasing TGF-β secretion. It can be suggested that once E2 concentrations diminish at the end of the prepubertal phase, TGF-β also decreases, slowing the mitotic rate of the Sertoli cell. Hence, estradiol not only influences hypothalamic and pituitary activity but also Leydig and Sertoli cell maturation and growth.

Both Sertoli and Leydig cells produce inhibin (Bardin et al., 1994; Tilbrook et al., 1992) and this dimeric protein selectively suppresses FSH secretion. Immunoneutralization of inhibin in young bulls caused an increase in FSH secretion, but LH was not affected (Kaneko et al., 1992). Basal and GnRH-stimulated FSH secretion of cultured rat pituitary cells was diminished by inhibin (Bardin et al., 1994). Also, the pituitary has binding sites for inhibin, which are tissue specific, saturable, dose and time related, and correlated with inhibin activity as well (Seethalakshmi et al., 1984).

Besides having a specific effect on FSH synthesis, inhibin has also been reported to act as an intratesticular paracrine factor. In cultures of Leydig cells isolated from 20-day old rats (Chen et al., 1992), inhibin augmented androstenedione secretion in response to human chorionic gonadotropin (hCG), and antagonized the inhibitory effect of activin on the sensitivity of Leydig cells to LH in young and adult hypophysectomized rats. Intratesticular injections of inhibin have been reported to reduce the numbers of A3, A4, In and B spermatogonia, but A1 and A2 spermatogonia were not affected (van-Dissel et al., 1989). Whether this effect is direct or mediated by other factors is not known, but points to a potential role for inhibin in regulating spermatogenesis.

Production of inhibin by the rat Sertoli cell is stimulated by FSH and epidermal growth factor (EGF) (Chen et al., 1992; Morris et al., 1988) and unaffected by either testosterone or

estradiol in vitro (Morris et al., 1988). Chen et al. (1992) reported that hypophysectomy decreased testicular inhibin α-subunit mRNA and that FSH treatment restored it to normal levels in the rat. FSH, but not testosterone, upregulated expression of the immunoassayable inhibin α-subunit (Morris et al., 1988) but had no effect on beta-subunit mRNA in Sertoli cell-enriched cultures (Chen et al., 1992). Also, Morris et al. (1988) have shown that androstenedione suppressed inhibin secretion by prepubertal rat Sertoli cells in vitro. This unexpected result can not be fully explained but deserves attention since androstenedione is the major androgen secreted by the immature Leydig cell (Bedair and Thibier, 1979), and may thus may play a role in interactions between the interstitial and tubular compartments of the testis.

Secretion of inhibin by Leydig cells has been shown to occur in men and rats (Tilbrook et al., 1992). McLachlan et al. (1988) reported that normal serum concentrations of inhibin in men required both FSH and LH since suppression of FSH caused a 30% decrease in inhibin and suppression of both FSH and LH with exogenous testosterone caused a 60% decrease in inhibin. Studies in rats revealed that inhibin concentrations in serum (Sharpe et al., 1988) and interstitial fluid (Drummond et al., 1989) could be stimulated by hCG, but this effect was suppressed when Leydig cells were destoryed by EDS. Also, Risbridger et al. (1989) reported that Leydig cells isolated from adult rats produced inhibin and expressed mRNA for the α-subunit of inhibin. Thus, the Leydig cell is also a source of inhibin, but some authors have argued that the inhibin secreted by Leydig cells does not significantly contribute to the pools of inhibin measured in intratubular fluid or peripheral blood (Maddocks and Sharpe, 1989).

In Holstein bulls, levels of α-subunit inhibin peak at 3 months of age and gradually

decline to a nadir at 8 months (MacDonald et al., 1991). Inhibin concentrations are inversely related to testosterone and to the pattern of germ cell multiplication in the bull (MacDonald et al., 1991; Curtis and Amann, 1981). Thus, given the inhibin-related events mentioned above and the fact that bulls immunized against inhibin showed higher DSP/g of testicular parenchyma (Martin et al., 1991), and that serum inhibin levels were correlated with both testis size and sperm count in men (Plymate et al., 1992), age at which peripheral inhibin concentration is highest may be related to testicular growth and germ cell numbers.

Retinol and testicular development

Vitamin A is essential for normal germ cell development (Kim and Wang, 1992). Cessation of spermatogenesis and a decrease in testis size has been observed in rats fed diets deficient in vitamin A. After 7 weeks of being fed a Vitamin A-deficient diet, rats decreased in body weight by 25% and testis weight, as a proportion of body weight, decreased by 25% one week later. Eight weeks following the beginning of the experiment, rats also had necrotic spermatocytes and spermatids. After 11 weeks, the spermatogonial population was 25% of that found in controls, and spermatids degenerated before becoming spermatozoa (Mitramond et al., 1979; Sobhorn et al., 1979).

It has also been shown that differentiating spermatogonia are absent in rats fed diets without vitamin A (Griswold et al., 1989; Ismail et al., 1990). Wang and Kim (1993) reported that A1 spermatogonia in vitamin A deficient seminiferous tubules are arrested at the S phase of the cell cycle. When vitamin A-deficient rats were fed retinol, normal body growth and testicular morphology and function were restored (Kim and Wang, 1992; Wang and Kim,

1993). Type A1 spermatogonia were reported to resume mitosis 3 h following retinol replacement (Ismail et al., 1990). However, retinoic acid, a normal metabolite of retinol, restored normal body growth, but spermatogenesis remained impaired (Mitramond et al., 1979) perhaps due to a lack of a mechanism to transport retinoic acid across the blood-testis barrier (see below).

Vitamin A deficiency in rats also resulted in FSH hypersecretion, low peripheral testosterone concentrations and unchanged LH plasma levels (Huang et al., 1983). Vitamin A deficiency did not affect FSH binding and cAMP production under FSH stimulation in rat Sertoli cells (Huang et al., 1989). Attempts to restore normal spermatogenesis using testosterone treatment were unsuccessful, whereas retinol replacement alone resulted in normal germ cell development (Huang et al., 1983). Thus, Vitamin A apparently affects the normal course of spermatogenesis and this effect is not mediated by deficiencies in gonadotropin or androgen levels.

The effect of retinol on Sertoli cell structure and function has also been described. Sobhon et al. (1979) reported that in vitamin A-deficient rats, the upper part of the Sertoli cell cytoplasm had disintegrated but the rest of the cell was unaffected. Retinol stimulates secretion of androgen-binding protein (ABP) (Karl and Griswold, 1980), transferrin (Skinner and Griswold, 1982; Hugly and Griswold, 1987) and cellular retinol- binding protein (CRBP) mRNA synthesis (Faraonio et al., 1993) in cultures of Sertoli cells from rats deficient in vitamin A. Retinol can also alter the pattern of protein phosphorylation in testis of vitamin A deficient rats (Kim and Wang, 1992). Hence, retinol affects some functions that are associated with Sertoli-cell maturation such as secretion of ABP and transferrin.

Retinol is transported in plasma by a 21 K Da protein, retinol-binding protein (RBP), which specifically binds to only one molecule of retinol (Goodman, 1984). RBP is mainly secreted by the liver but it can also be synthesized by other tissues that are affected by vitamin A (for review, see Soprano and Blaner, 1994). For example, RBP has been found in cultured adipocytes and homogenate from whole eye of the rat (Davis and Ong, 1992), and in uterine, conceptus and placental tissues of pregnant cows (Liu and Godkin, 1992). McGuire et al. (1981) and Kato et al. (1985), using immunocytochemical techniques, found RBP in the interstitial space but not inside of the seminiferous tubules of rat testes. However, Davis et al. (1992) reported the isolation of RBP from the media of cultured rat Sertoli cells.

Currently, it is thought that the Sertoli cell acquires retinol from a retinol-RBP complex without internalization of RBP (Kim and Wang, 1992, Bishop and Griswold, 1987; Shingleton et al. 1989). Once in the cytoplasm, retinol binds to a cellular retinol-binding protein (CRBP) and can either be esterified for storage, transported to adluminal germ cells or be metabolized to retinoic acid, and activates transcription of vitamin A-dependent genes (Kim and Wang, 1992).

The exact functions of retinol esters in the Sertoli cell and meiotic and post-meiotic germ cells are not known. Retinoic acid (RA), which can be generated from retinol, is a putative candidate for vitamin A-dependent gene expression in Sertoli and adluminal germ cells. The Sertoli cell expresses two types of retinoic acid receptors (RARα and RARβ), A1 spermatogonia express RARα (deRooij et al., 1994) and RARγ spermatids express RARα (Huang et al. 1994). Cellular retinoic acid binding protein (CRABP) was present primarily in spermatids and, as well as CRBP, was also associated with spermatozoa in the epididymis

(Porter et al., 1985). The expression of CRBP-I has been shown to correlate with the expression of RA-responsive genes in embryonic mouse tissues (Ruberte et al., 1992) and RA-inducible reporter genes in transgenic mice (Rossant et al., 1991; Balkan et al., 1992). Thus, it seems that retinol-binding proteins play important roles in the metabolism and fate of retinol in the Sertoli cell. Moreover, these proteins may control the delivery of retinol and/or its derivatives to the adluminal germ cells.

Synthesis of cellular retinol-binding protein by Sertoli cells in vitro is positively regulated by FSH, testosterone and retinol (Faraonio et al. 1993) and concentrations of CRBP mRNA have been shown to vary with stage of the spermatogenic cycle in the rat (Rajan et al., 1990). Although the Sertoli cell seems to be the major site of CRBP synthesis in the testis (Kim and Wang, 1992), peritubular cells also appear to contain mRNA for CRBP (Eskild et al., 1991). Data regarding the presence of CRBP in spermatogonia is, however, contradictory. Porter et al. (1985) failed to detect CRBP immunoreactivity in rat spermatogonia, but Blaner et al. (1987) and Eriksson et al. (1987) found CRBP in germ cells by means of immunocytochemistry and radioimmunoassay.

In conclusion, retinol is essential for testicular development, and even though the exact mechanism by which it affects both somatic and germ cells in the testis is not completely understood, it is evident that metabolism and transport of retinol are mediated by proteins such as RBP and CRRB. These binding proteins can be synthesized by the Sertoli cell and their expression is regulated by FSH, androgens, retinol and possibly paracrine factors secreted by germ cells. Since Sertoli cells are in close contact with peritubular and germ cells, have receptors for androgens and FSH (Sar, 1992; Griswold, 1992), and respond to retinol,

they may play an important role in making vitamin A available to germ cells.

Despite the amount of research focused on the effect of vitamin A on the mammalian testis, no information on the pattern of RBP and CRBP expression in the bovine testis has been reported. Quantitation of RBP and CRBP expression by the testis during prepubertal development could provide insight into their effect on germ cell proliferation and quality and the degree to which they are hormonally dependent. Studies of interactions between gonadotropins, steroids, RBP and CRBP could also lead to finding useful markers of spermatogeneis and Sertoli cell function.

Spermatogenesis

Spermatogenesis can be defined as the sequential process of proliferation and differentiation of spermatogonia and derived cells that result in the formation of spermatozoa and renewal of stem cells (Courot et al., 1970; Ortavant et al., 1977; Amann and Schanbacher, 1983). In the bull, gonocytes multiply after birth to give rise to the A1-spermatogonia, which in turn undergoes mitotic division to generate more differentiated cells termed A2, A3, In, B1 and B2-spermatogonia. The latter give rise to spermatocytes, which undergo meiosis resulting in the haploid spermatid, which subsequently becomes the spermatozoa (Courot et al., 1970). According to Ortavant et al. (1977), the number of spermatozoa greatly depends on the efficiency at which the A1-spermatogonia are produced.

In the embryonic bovine testis, number of gonocytes increased from nearly zero on day 32 to 13 million on day 270. Mitotic activity was at a high between days 37 and 55, slowed down from 65 to 110 days but increased between days 230 and 270 (Matschke and

Erickson, 1969). In the ram, proliferation of gonocytes also occurred extensively during embryonic life but no differentiation was observed by Hochereau-de Reviers et al. (1995). Moreover, because the number of primordial germ cells (PGC) did not change after either hemicastration or hypophysectomy, these authors suggested that the dependence of germ cell growth on gonadotropins in the fetal testis is not as relevant as it is in the prepubertal or adult testis.

Based on the studies conducted in mice, proliferation of mouse PGC can be maintained in culture medium containing several growth factors, including basic fibroblast growth factor (bFGF) (Matsui et al., 1992; Resnick et al., 1992). Also, De-Felici and Pesce (1994) found that Steel factor prevented apoptosis of cultured mouse PGC. Steel factor (SF) is the ligand for the c-kit transmembrane tyrosine kinase receptor, which is expressed in PGC of 12.5-day old mouse embryos, type A and B spermatocytes and Leydig cells. SF is synthesized by Sertoli cells in a soluble or membrane-bound form (for review, see Kierszenbaum, 1994). Also, FSH and cAMP has been shown to stimulate the synthesis of mRNA for both forms by Sertoli cells isolated from immature rats (Rossi et al., 1993).

In the rat, gonocytes are quiescent at birth but resume meiosis 3 days after birth. Upon differentiation into A spermatogonia, germ cells become responsive to SF (Kierszenbaum, 1994). Rossi et al. (1991) showed that SF induced DNA synthesis in A spermatogonia and Tajima et al. (1994) reported that, in 5-day old mice, an anti-c-kit antibody was able to block the SF-induced proliferation of A1 spermatogonia. In 2-day old mice, however, this antibody had no effect on A1 spermatogonia, suggesting that germ cells differentiated from a non-responsive to a SF-responsive state between 2 and 5 days after birth. Thus, it appears that the

SF-c-kit interaction is part of the mechanism by which the Sertoli cells control germ cell growth in the fetal and neonatal testis.

Several other families of growth factors are expressed in the testis but the determination of their effects has been limited to in vitro studies only. In the testis of the adult rat, TGF-β1 expression has been detected in Sertoli cells, spermatocytes and spermatids, at the periods when meiosis occurs, but TGF-β2 was present in post-meiotic germ cells only, suggesting that these growth factors may function as regulators of meiosis and spermiation (Teerds and Dorrington, 1992). Also, both Sertoli and Leydig cells, but not germ cells, possess EGF receptors (Suarez-Quiarret et al., 1989) and EGF stimulates in cultured neonatal mouse Sertoli cells the synthesis of ornothine decarboxylase, an enzyme involved in cell growth and differentiation (Smith et al., 1987). Jaillard et al. (1987) has found that bFGF stimulated DNA synthesis in cultured Sertoli cells and this effect was even more pronounced than that of FSH. Insulin-like growth factor I (IGF-I) has been immunocytochemically detected in both Sertoli and Leydig cells (Handelsman et al., 1985) and IGF-I receptors are present in secondary spermatocytes and early spermatids (Tres et al., 1986). It is possible, therefore, that both EGF and bFGF play important roles in the process of Sertoli cell development and that IGF-I mediates some of the actions of Sertoli cells on spermatogenesis.

Studies conducted in the rat have shown that both spermatogonia and meiotic germ cells express specific proto-oncogene transcripts at different phases and stages the speramtogenic process. Spermatogonia, spermatocytes and spermatids express proteins that belong to the protein kinase family (c-kit, c-raf, c-mos, etc.), GTP-binding proteins with GTPase activity (c-ras H, K and N) and nuclear transcription factors such as c-fos and c-jun.

These proteins mediate the intracellular signal of growth factors and are thought to be synthesized during specific phases of sperm formation and mitosis and meiosis of germ cells.

In conclusion, it is clear that the effects of gonadotropins and steroids on the germ cells are mediated by factors secreted by Leydig and Sertoli cells. Despite the large amount of information on the sites of synthesis of growth factors and their receptors in the testis, it remains to be precisely determined what genes, and consequently what physiological responses are modulated by such intratesticular factors.

The effects of gonadotropins and steroids on spermatogenesis has been studied extensively. Courot et al. (1970) reported that hypophysectomy of rats caused a significant reduction in spermatogonial numbers, and exogenous FSH and LH restored spermatogonial divisions to about 80% of normal rates after 24 hours of treatment. Russel et al. (1987), studying spermatogenesis in young and mature rats, showed that LH and FSH treatment of hypophysectomized rats reduced numbers of degenerating germ cells in comparison to that found in intact animals. Also, these authors found that neither FSH nor LH given separately reversed effects of hypophysectomy, but effiency was much greater when the hormones were used in combination. Since LH receptors are present on Leydig cells (Chowdhury, 1979), it appears that the action of LH on spermatogenesis is mediated by testosterone and/or LH-induced growth factors.

In hypophysectomized rats treated with testosterone, spermatogenesis occurred normally, although quantitatively reduced when compared to intact animals (Chowdhury, 1979). Marshall et al. (1983) reported that spermatogenesis in the hypophysectomized rhesus monkey could be maintained by testosterone alone. These results reveal that testosterone is

crucial for germ cell development but other factors must also be involved in that process. Van Alphen et al (1988) reported that intact adult monkeys treated for 28 days with exogenous FSH had normal testosterone levels and a greater number of A- and B-spermatogonia, spermatocytes and spermatids than untreated controls. Treatment of these monkeys with LH alone did not affect germ cell proliferation. Haneji et al. (1984) reported that, in the rodent, FSH increased the number of spermatogonia entering mitosis.

As reported by Sum et al. (1990), both FSH and LH seem to act synergistically at crucial phases of spermatogenesis. These authors, studying germ cell development in hypophysectomized rats, concluded that FSH and testosterone had an additive stimulatory effect on conversion of spermatogonia to spermatocytes and of spermatocytes to round spermatids, but the effect of FSH was more significant on the latter. In summary, both FSH and testosterone are currently accepted as being required for normal spermatogenesis, but their effects, as mentioned above, are mediated by Sertoli cell-secreted factors.

The hypothalamic-pituitary-gonadal axis in the male

The hypothalamus is the major regulator of testicular function (Schanbacher, 1982). and contains neurons that secrete GnRH into the portal circulation connecting the hypothalamus to the anterior pituitary. GnRH stimulates the synthesis and secretion of both LH and FSH (Bond and Adelman, 1993), apparently by separate cell types (Bastings et al., 1991). Gonadotropin-releasing hormone is secreted in a pulsatile pattern, which is essential for optimal endocrine function (Bond and Adelman, 1993).

In the adult male, luteinizing hormone, but not FSH, is secreted in pulses (Price,

1991), which are coincident with the GnRH pulses (Jackson et al., 1991). Also, it is currently accepted that LH secretion is more dependent on GnRH than is FSH (Amann and Schanbacher, 1983; Price, 1991). Experimental results showed that exogenous infusion of GnRH induced a much greater release of LH than FSH from the pituitary (Convey et al., 1975), and that immunoneutralization of GnRH caused a less dramatic fall in FSH than in LH peripheral concentrations (Lincoln and Fraser, 1987). Ultimately, FSH secretion will cease in the absence of GnRH.

GnRH stimulation of LH and FSH is mediated by specific receptors located on the plasma membrane of gonadotrophs (Nett, 1990). Synthesis of GnRH receptors can be induced by GnRH itself (Nett, 1990), and this seems to be the major factor controlling the number of GnRH receptors in the male. Limonta et al. (1986) reported that castration induced and testosterone treatment prevented the decrease in the number of GnRH receptors. However, whether this effect was caused by the inhibitory effect of testosterone on the GnRH pulse generator or on the pituitary is not clear. Also, E2 can induce the synthesis of GnRH-receptors and enhance the pituitary response to GnRH through a postreceptor mechanism (Miller, 1993)

To better understand the role of testosterone in the control of GnRH secretion, Jackson et al. (1991) assayed blood collected from both portal vessels and the jugular vein, and found that testosterone treatment of castrated rams caused a significant reduction in the mean concentration of GnRH, and both GnRH and LH pulse frequencies, but not amplitudes. Testosterone tended to diminish LH release caused by exogenous GnRH injections, but this effect was not statistically significant. Although there is evidence for androgenic control of

the GnRH pulse generator, the possibility that testosterone can also control gonadotropin secretion at the pituitary can not be ruled out. Thieuland and Pelletier (1979) and Takeda et al. (1990) detected the presence of androgen receptors in gonadotrophs, and Crewman and Baldwin (1988) reported that testosterone directly inhibited synthesis of LH by male pituitary cells incubated with GnRH. Thus, it is likely that androgens can regulate LH secretion by means of its effects on both the hypothalamus and pituitary. Since FSH secretion is also dependent on GnRH, testosterone can have a significant effect on its regulation, as shown by D'Occhio et al. (1982).

The GnRH neuron is also affected by estradiol. Although GnRH neurons do not have estrogen receptors (Fink, 1988), estradiol can suppress GnRH release in the male (Johnson and Everitt, 1984) possibly through stimulation of dopaminergic and adrenergic neurons (Fink, 1988). Estradiol also has a direct negative effect on FSH and LH secretion by gonadotrophs, which express E2 receptors (Amann et al., 1986). An estrogen response element (ERE) is present in genes for gonodotropin beta-subunits, but not for the common alpha-subunit, and binding of the estrogen receptor to these DNA sequences (ERE's) results in inhibition of transcription of the β-subunit gene (Nett, 1990). Thompson et al. (1984) found that physiological doses of E2 given to castrated bulls decreased the gonadotropin response to GnRH injections. Thus, the ability of the pituitary to secrete gonadotropins may be determined, among other factors, by the amount of estradiol and/or by the sensitivity of the hypothalamus and pituitary to the inhibitory effect of this steroid.

As previously mentioned, the testis secretes inhibin. Miller (1993) reported that inhibin decreases FSH mRNA content in ovine pituitary cell cultures. Also, based on in vitro studies

in rats, it has been shown that inhibin can decrease numbers of GnRH-receptors on the gonadotrophs by 50% (Wang et al., 1989). Although Steinberger and Ward (1988) reported that inhibin caused a decrease in GnRH content in rat hypothalami, there is no evidence that this affects gonadotropin secretion. Since immunization of animals against inhibin affects only FSH secretion (Martin et al., 1991), and since a separate FSH releasing factor has not been identified (Steinberger and Ward, 1988), it is likely that inhibin regulates FSH release only at the gonadotroph.

Hence, the hypothalamic-pituitary-gonadal axis is a complex system where GnRH positively regulates the synthesis and secretion of both LH and FSH, and these hormones in turn stimulate gonadal function. The testis secretes steroids, which regulates both GnRH and gonadotropin synthesis, and inhibin, which is specifically involved in the control of FSH release. Even though development of the hypothalamic-pituitary-gonadal axis in the bull has been studied, it is not known how the components of this system interact early in postnatal life to determine Sertoli and germ cell numbers, testis size and age at puberty.

Hormonal regulation of puberty in the bull

Generally, puberty is defined as the age at which the male can produce enough sperm to affect fertilization. In the bull, puberty is defined as that time when the bull is able to produce a minimum of $50x10^6$ spermatozoa per testis with at least 10% progressive motility, or when DSP reaches $0.5x10^6$/g of testicular parenchyma (Lunstra et al., 1978). Pubertal development is primarily controlled by GnRH. As GnRH pulse frequency increases so does LH pulse frequency, which in turn accelerates testicular growth (Amann and Schanbacher,

1983).

In the dairy bull, Rodriguez and Wise (1989) detected pulsatile GnRH secretion as early as two weeks after birth. At this age, a GnRH pulse was not followed by a detectable pulse of LH. Between 2 and 12 weeks, there was a significant increase in GnRH pulse frequency. Also, between 6 and 10 weeks of age, Amann et al. (1986) reported a 4-fold increase in numbers of GnRH receptors in the gonadotrophs. However, Rodriguez and Wise (1984) showed that LH pulse frequency remained minimal prior to 6 weeks, but frequency increased more than 2-fold between this age and 12 weeks. Therefore, the pituitary initially may not be highly sensitive to GnRH, but becomes more responsive as gonadotrophs differentiate and accumulate GnRH receptors. Pieper et al. (1984) and Cayton et al. (1982) showed that the rate of GnRH release is the principal regulator of the number of GnRH receptors on the gonadotroph.

Prepubertal development is controlled by GnRH pulse frequency, and regulation of the GnRH neuron involves a decrease in its sensitivity to the inhibitory effect of gonadal steroids (the "gonadostat hypothesis"). Maturation of the GnRH neuron is important in this regard as is its response to stimulatory and inhibitory inputs from other neurons (Rodriguez et al., 1993). In the anterior pituitary of the dairy bull, increases in number of receptors for estradiol and GnRH occur between the ages of 1 and 18 weeks (Amann et al., 1986). Concentration of E2 receptors in the pituitary increases five-fold between the age of 1 and 10 weeks and declines two-fold at the age of 18 weeks. The amount of estradiol present in serum is maximal at 6 weeks, but six-fold lower at 10 weeks (Amann et al., 1986). Synthesis of E2 receptors may be stimulated by E2 itself but the fact that the decrease in E2 receptors

in the pituitary does not parallel the fall in serum levels of E2 suggests that the pituitary becomes more sensitive to E2 and/or additional factors regulating the expression of these receptors may be present.

Both LH and FSH stimulate testicular steroid synthesis. Before puberty, increasing levels of LH induce the Leydig cell to secrete androstenedione (Amann and Schanbacher, 1983). Plasma concentrations of this androgen increase up to 530 pg/ml at the age of 16 weeks and declines to a plateau of 130 pg/ml at the age of 24 weeks. Meanwhile, differentiation of the Leydig cell induced by both LH and FSH is coincident with a change in the Leydig cell's major secretory product from androstenedione to testosterone. Concentrations of testosterone increase constantly until the bull reaches puberty (Amann and Schanbacher, 1983). The amount of FSH in the dairy bull is high until the age of 2.5 months, declines at the age of 3 months and increases again at the age of 5 months. After this age, FSH secretion declines reaching a nadir at 9 months which is 70% of that at the age of 5 months (MacDonald et al., 1991).

Inhibin production increases until the age of 3 months and gradually declines to a low at 8 months (MacDonald et al., 1991). Concentrations of FSH and inhibin are parallel until the age of 2.5 months and become inversely related at postpubertal ages (MacDonald et al., 1991). Estradiol secretion increases until the age of 3 months and declines somewhat thereafter (Amann et al., 1986). These hormonal patterns seem to be in agreement with the hypothesis that some aspects of Sertoli cell function change as the Sertoli cell evolves toward its differentiated state.

Early aspects of testosterone-induced Sertoli cell maturation are coincident with

differentiation of gonocytes into prespermatogonia and A1-spermatogonia but full Sertoli cell differentiation does not occur until spermatocytes give rise to spermatids. In the dairy bull, these events occur between the ages of 18 and 24 weeks. Also, in the dairy bull, the end of Sertoli cell mitosis occurs before the age of 20 weeks (Sinowatz and Amselgruber, 1986; Curtis and Amann, 1981), in association with a testosterone concentration of 1 ng per ml of plasma. Further events in testicular development include a four-fold increase in numbers of A1-spermatogonia and the appearance of the first spermatocytes and spermatids between 20 and 28 weeks. A 50-fold increase in the number of elongated spermatids follows in company with the appearance of spermatozoa at 28 to 32 weeks. Finally, puberty is reached at approximately 32 weeks of age in the well-nurtured dairy bull. At this age, scrotal width was 94±3 mm (Curtis and Amann, 1981).

In the beef bull, testicular development occurs at a slower rate than in the dairy bull. Amann (1970) reported that, between the ages of 1 and 3 years, Angus and Hereford bulls had consistently less weekly sperm production than did Holstein bulls. Lunstra et al. (1978) found that Angus bulls did not attain puberty until the age of 42 weeks, when their scrotal width was 91 mm.

As the bull matures toward puberty, amplitude and frequency of LH pulses tend to diminish possibly due to an increase in sensitivity of the hypothalamic-pituitary axis to the inhibitory effect of gonadal steroids (Amann and Schanbacher, 1983). Hence, the net result of the hormonal changes that take place early in postnatal life is the onset of testicular endocrine function followed by germ cell development and sperm production. Thus, the rate at which these hormonal changes occur at prepuberty may significantly affect the rate and

extent of testicular growth.

Testicular development after puberty

The establishment of puberty is a process associated with a large increase in testis size due mainly to germ cell mitosis and maturation, which leads to enlargement and lengthening of the seminiferous tubules and, consequently, to an increase in the proportion of the testicular parenchyma occupied by the seminiferous tubules (Curtis and Amann, 1981). By the age of 32 weeks, the proportion of testicular parenchyma occupied by the tubular compartment reaches 81%, which is the same as in the adult dairy bull. Thus, further testis growth is due to lengthening of the seminiferous tubules only (Curtis and Amann, 1981).

The efficiency of sperm production in Holstein bulls, as evaluated by DSP/g of testicular parenchyma, increases six-fold between the ages of 6 and 9 months, but only 1.4-fold between 9 and 12 months, the latter being the age at which it reaches a plateau. However, testis weight and DSP increase up to the age of 5 years (Amann, 1970).

Physiological aspects of spermatogenic arrest

Spermatogenic arrest is an interruption of the process of germ cell proliferation and/or differentiation, but not of testis formation, resulting in either oligospermia (partial arrest) or azoospermia (total arrest), as described by Martin-du Pan and Campana (1993). Impairment of germ cell development can be caused by genetic or hormonal anomalies and external agents, such as drugs, radiation, infections, heat, etc. In this report, however, only cases of infertility related to genetic or physiological causes will be reviewed.

Sertoli cell-only syndrome

This syndrome is characterized by the complete absence of germ cells in the seminiferous tubules, although both Sertoli and Leydig cell populations do develop. Sertoli cell-only syndrome has been described in bulls, mice and humans, and in some cases the causes have been identified. Russel (1979) described that mutations in the gene that codes for the c-kit tyrosine kinase receptor (W-locus) prevented the primordial germ cells from migrating to the gonadal ridges during embryonic development in the mouse. Also, germ cells that did reach the newly formed testicular cords underwent degeneration. Mutations in the W-locus or its allele, the Sl locus (bearing the SF gene), lead to abnormalities in stem cell mitosis during haematopoiesis and melanogenesis (Russel, 1979). De-Franca et al. (1994), studying W-mutant mice, observed that seminiferous tubules lacking germ cells contained Sertoli cells with reduced nuclear, cytoplasmic and smooth endoplasmic reticulum volumes. Levels of FSH were elevated but testosterone synthesis and Leydig cells were not affected in these mice. In men, similar defects occur as a consequence of a mutation in the c-kit proto-oncogene (Fleischman et al., 1991). Also, men with Sertoli cell-only syndrome frequently have high levels of FSH in peripheral blood (Bergman et al., 1994). Rao Veeramachanemi et al. (1986) have described a case of Sertoli cell-only syndrome in the bovine. According to these authors, the infertile bull had small testis and reduced T and LH pulse frequencies, but normal FSH. Thus, it appears that there are significant differences regarding the effects of germ cell depletion on the pituitary-gonadal axis of bulls and other species such as mice and men.

Although mutations in the W and Sl loci are recognized as causes of impaired germ

cell-development, other genetic abnormalities also result in the formation of a testis without germ cells. Bulls with Klinefelter's syndrome (60, XXY) have small seminiferous tubules lined only by Sertoli cells (Logue et al., 1979). Also, this syndrome (47, XXY) accounts for 14 % of the cases of azoospermia in men, which are characterized by reduced testis size, Leydig cell hyperplasia and absence of spermatogenesis (Jaffe and Oates, 1994). Men that are 46,XX present a male phenotype but lack spermatogenesis (Page et al., 1985). The reason for maleness is probably related to a translocation of the Sry gene but the cause of infertility is not certain. Jaffe and Oates (1994) suggested that 46,XX individuals lacked the Azoospermia Factor Gene (AZF). This gene has been located in the long arm of the Y chromosome (Anderson et al., 1988) and it is believed to play a role in the migration and differentiation of the PGC during embryonic development (Chandley et al., 1986). Finally, Chubb (1986) reported that mice homologous for the atrichosis gene had small testis due to the absence of spermatogenesis, but T secretion was normal. In summary, Sertoli cell-only syndrome occurs in several species and appears that, in the cases studied so far, to be caused by genetic abnormality as affecting the germ cells instead of hormone secretion.

Speramtogenic arrest at the levels of spermatogonia, spermatocytes and spermatids

Beamer et al. (1988) described a phenotypically normal male mouse that was homozygous for the juvenile spermatogonial depletion gene (jsd). These mice developed a first wave of spermatogenesis but failed to continue due to impaired spermatogonial proliferation. At maturity (9 weeks of age), jsd/jsd mutants had small seminiferous tubules, most of which had only Sertoli cells and very few had A1 spermatogonia, but no other

advanced germ cell type. Since FSH was elevated and T was normal, it was suggested that an abnormal Sertoli cell failed to provide support for the germ cells to develop. However, aspects of the Sertoli cell function altered by genetic mutation remain to be determined. Such cases of spermatogenic arrest have not been described in the bovine or primate.

Studies have shown that arrest of the meiotic division in human germ cells is related to anomalies during chromosome pairing at zygotene, interrupted separation of paired homologues at late pachytene or disfunction of the synaptonemal complex (Martin-du Pan and Campana, 1993). In the bovine, a case of meiotic arrest has been described by Ansari et al. (1993). The bull was phenotypically normal with testes that were slightly smaller than the average of fertile bulls but the seminiferous tubule diameter was not decreased. However, histological analysis revealed that most of the pachytene spermatocytes degenerated, and no spermatids were present in the testis. This bull also had two extra chromosomes, a large one derived by fusion of chromosomes 8 and 13 and a small one formed by the centromere of chromosome 8 and a piece of chromosome 13. Arrest of meiosis was probably caused by nonhomologous synapses that occurred at pachytene of prophase I and the attachment of the abnormal chromosomes to the X and Y chromosomes, preventing the formation of a fully developed XY body. Hormone profiles in this case were not provided. Another case of spermatogenic arrest at the level of spermatocytes has been reported by Gargiulo et al. (1991). In this case, two 18-mo old brown Swiss bulls had reduced seminiferous tubule diameter and small testes, in which degenerating primary spermatocytes were detected. Although neither hormonal nor genetic analysis was conducted to determine the origin of infertility, the authors suggested that interruption of germ cell meiosis may have been caused

by abnormal function of the Sertoli cells.

In rats, different cases of meiotic arrest have been described. Gill III and Kunz (1979) reported the existence of a strain of rats (B1) that carried mutations in a group of genes designated the growth and reproduction complex (Grc). Rats that were recessive for both mutant genes had small body and testis size, Leydig cell hyperplasia and spermatogenesis arrested at the level of pachytene spermatocyte. Also, peripheral concentrations of T were reduced, FSH and LH were elevated and growth hormone and Somatomedin C were normal. Noguchi et al. (1993) described another type of infertility in the rat that was characterized by meiotic arrest at pachytene, reduced testis weight, but normal body growth. As mentioned above, these rats had high FSH and LH, but decreased T levels. Intratesticular implants with testosterone did not have any effect on the status of germ-cell development, suggesting that the cause of azoospermia was related to genetic defects.

In humans, low production of spermatozoa has been associated with subnormal pituitary function, diabetes and aging (Martin-du Pan and Campana, 1993). Also, complete arrest of spermatogenesis at the spermatid has been found in men as a consequence of either defects in the use of glycogen by the Sertoli cell (Aumuller et al., 1987) or lack of microtubules of the axial filaments and of the central body in the spermatids (Re et al., 1979). Both cases of infertility were probably caused by genetic anomalies, according to the respective authors. In mice, a mutation termed symplastic spermatids (sys) caused azoospermia due to a deffect in the intercellular bridges connecting the round spermatids, which resulted in formation of symplasts and multinucleated cells. In this case, elongation of the spermatid nuclei was impaired (Russel et al., 1991). Such cases however, have not been

reported in bulls or men.

Testicular hypoplasia can also be caused by insufficiency of trophic hormones. Chubb (1989) reported that mice homozygous for either the Ames dwarf (df) or dwarf (dw) gene mutations presented low sperm numbers due to reduced secretion of growth hormone, prolactin, thyroxin, LH and testosterone. FSH, however, was not affected despite a reduced cell population in the pituitary. In another instance, Cattanach et al. (1977) described a mouse with a gene mutation (hpg) resulting in hypogonadism. These animals had a mutation in the GnRH gene (Mason et al., 1986), which led to a deficiency in FSH and LH, and ultimately, testosterone. Also, weight of the testis and seminal vesicles was < 3 % of normal, diameter of the seminiferous tubules was decreased and no spermatids were generated (Chubb, 1989). Liven et al. (1992) reported that the hpg mouse was able to show complete spermatogenesis and copulatory behavior upon receiving a transplant of normal GnRH neurons and treatment with T before puberty. Also, O'Shaughnessy et al. (1992) showed that, when adult hpg mice received exogenous FSH, they responded with germ cell mitosis and differentiation, Leydig cell differentiation and an increase in T secretion under LH stimulation.

In summary, mutations affecting either the germ cells or the soma do result in infertility and have been described in most mammals. Despite its potential importance, the genetic and endocrine aspects, as well as the frequency and mode of inheritance of such conditions, have not been studied in detail in the bull.

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PART II

(Studies 1 and 2)

Abstracts

Study 1. An experiment was carried out to determine whether peripheral hormone concentrations quantified between 2 and 12 months (mo) were meaningfully correlated with testis size and number of Sertoli and germ cells in the yearling beef bull. Twenty four Angus bulls were treated monthly with GnRH (0.05 μg per Kg of body weight) 1.5 and 3 hours (h) later. On the day before the GnRH challenge, three blood samples were taken at 1.5-h intervals to establish basal hormone concentrations. At 12 mo, all bulls were surgically castrated. Concentrations of FSH, LH, testosterone (T), androstenedione (Δ_4A) and 17β-estradiol (E2) were quantified by RIA and number of Sertoli cells per testis and quantitative aspects of spermatogenesis were determined. Analysis of regression and Pearson's partial correlation were used to estimate the degree of association between hormone and testis data. Histology analysis revealed that 3 bulls had no spermatids and, therefore, were infertile. Yearling testis weight (TW) was related to GnRH-stimulated T at 3 mo ($r = 0.48$, $p < 0.05$) and E2 at 2 and 4 mo ($r = 0.40$ and 0.45, $p < 0.10$). Basal and GnRH-stimulated FSH quantified between 2 and 12 mo was correlated with TW ($r = -0.37$, $p < 0.10$ to -0.71, $p < 0.002$), number of Sertoli cells ($r = -0.41$, $p < 0.10$ to -0.68, $p < 0.002$), number of A1 spermatogonia (r = nonsignificant, $p > 0.10$ to $r = -0.59$, $p < 0.05$) number of round spermatids per testis (r = nonsignificant, $p > 0.10$ to -0.69, $p < 0.002$). Also, FSH between 2 and 12 mo correlated with number of round spermatids per A1 spermatogonia (r = nonsignificant, $p > 0.10$ to $r = -0.61$, $p < 0.01$), number of round spermatids per Sertoli cell (r=nonsignificant, $p > 0.10$ to -0.64, $p < 0.005$) and percent of seminiferous tubules with

round spermatids (r = nonsignificant, $p > 0.10$ to $r = -0.63$, $p < 0.005$) in the 12-mo old testis. Regression analysis showed that FSH and E2 at 2 mo accounted for 45% of the variation in TW. Furthermore, significant variation in TW was accounted by FSH and testis diameter at 6 mo ($R^2 = 0.57$), FSH and testis diameter at 9 mo ($R^2 = 0.67$) and FSH and body weight at 12 mo ($R^2 = 0.63$). Thus, it appears that prepubertal testis size and FSH could be used as indicators of testicular development of yearling beef bulls. However, the inclusion of data from infertile bulls in the correlation and regression analysis may have generated biased results. Three cases of spermatogenic arrest was described in this study. Bull #250 had seminiferous tubules lined by Sertoli cells only and bull #345 had no germ cells that advanced beyond the A1 spermatogonia stage. Testes of these two bulls weighed 60% less than those of normal bulls ($p < 0.01$). Concentrations of T and LH in bulls #250 and #345 were reduced at some ages ($p < 0.10$). Spermatogenic arrest at the level of pachytene spermatocyte was detected in bull #389. Basal FSH and LH were higher in bull #389 than in normal bulls ($p < 0.05$). Testes of this bull weighed 13% of those of normal bulls and secreted less T throughout the year ($p < 0.05$). However, as in the cases of bulls #250 and #345, the decrease in T observed in bull#389 did not appear to be sufficient to interrupt germ cell development. Chromosomal abnormalities and/or deficiency in intratesticular factors may be the causes of infertility in these bulls.

Key words: Bull, Testis, Sertoli, Germ Cell, Infertility, FSH.

Study 2. The objective of this study was to determine whether concentrations of gonadotropins and steroids in 2- and 6-month old beef bulls were significantly correlated with testicular development and number of Sertoli cells at the age of 6 months (mo). At 2 mo, 15 crossbred bulls were treated with GnRH (5 μg) and bled 1.5, 3 and 4.5 hours (h) later. One day before the GnRH challenge, three basal samples were taken at 1.5-h intervals. At 6 mo, bulls received 1 mg of estradiol benzoate (E) and were treated with GnRH (10 μg) 12 h later. After 5 days, bulls received GnRH only (10 μg). In both cases, blood samples were taken 1.5, 3 and 4.5 h after GnRH and two basal samples were taken 1 h before the GnRH treatment. Bulls were castrated at 6 mo. Concentrations of FSH, LH, testosterone (T), androstenedione (Δ_4A) and 17β-estradiol (E2) were quantified by RIA. Number of Sertoli cells and A1 spermatogonia per testis and quantitative aspects of spermatogenesis were also estimated. Analysis of regression and Pearson's partial correlation were used to estimate the degree of association between hormone and testis data. Results showed that testis weight at 6 mo (TW) was related to FSH at 2 mo ($r = -0.48$ to -0.62, $p = 0.08$ to 0.02). Also, concentrations of FSH at 2 mo was correlated with number of Sertoli cells (SC; $r = -0.50$ to -0.65, $p = 0.07$ to 0.01), A1 spermatogonia (A1; $r = -0.45$ to -0.58, $p = 0.09$ to 0.03) and percent of seminiferous tubules with round spermatids ($r = -0.49$ to -0.60, $p = 0.08$ to 0.02). Regression analysis showed that FSH and body weight at 2 mo accounted for 30% and 72% of the variation in SC and TW in the 6-mo old bull. Concentrations of estradiol at 6 mo were related to SC ($r = 0.62$, $p = 0.02$). At 6 mo, GnRH-stimulated FSH and LH were respectively 2.5- and 3-fold higher when GnRH was given in combination with E than when GnRH was given alone. Correlation between FSH and SC were more pronounced after GnRH+E ($r =$

−0.68, p = 0.008) than after GnRH only (r = − 0.63, p = 0.015). Regression modeling showed that FSH and body weight at 6 mo accounted for 48% of the variation in the population of Sertoli cells. Thus, concentrations of FSH at 2 and 6 mo of age appears to be reliable indicators of number of Sertoli cells and testicular development in the 6-mo beef bull.

Key words: Bull, Testis, Sertoli, FSH, Estradiol.

Introduction

In the bull, testis size is closely related to daily sperm output (r = 0.80 to 0.90, reviewed by Coulter and Foote 1979; and Foote, 1984) and daily sperm production (r = 0.65 to 0.72, Amann and Alquimist, 1962; Berndtson et al., 1987; Palasz et al., 1994). Testis size is heritable (h^2 = 0.41, Lunstra et al., 1988) and correlates with age at puberty (R^2 = − 0.85, Lunstra et al., 1978) and breeds with greater scrotal circumference have earlier age at puberty and higher pregnancy rate in female offsprings (r = 0.90, Martin et al., 1992).

The Sertoli cell is the only somatic cell present in the seminiferous tubules and provides morphological, nutritional and hormonal support for the germ cells (Bardin et al., 1994). Sertoli cells proliferate during fetal and neonatal life and, in the bovine, become amitotic between 6 and 10 weeks (Sharpe, 1994). Decrease in the Sertoli cell population caused by immunization against GnRH (Orth et al., 1988) or radiation (Erickson and Blend, 1976) leads to reduced testis size in rats. Moreover, it is believed that each Sertoli cell can support only a limited number of spermatids (Berndtson et al., 1987a,b). Therefore, the

number of Sertoli cells per testis ultimately determines both potential sperm-producing capacity and testis size. In accordance with this hypothesis, the total number of Sertoli cells in the bull testis is related to daily sperm production ($R^2 = 0.70$) and testis weight ($R^2 = 0.56$, Berndtson et al., 1987a).

Age-related changes in testicular development are regulated mainly by the interaction between gonadotropins and sex steroids (Schanbacker, 1982). Important aspects of Sertoli cell function, such as mitosis, differentiation, synthesis of 17β-estradiol (E2) and androgen-binding protein, secretion of tubular fluid and growth factors are dependent on FSH and testosterone (reviewed by Bardin et al., 1994). Also, the phase of growth and subsequent maturation of the Leydig cell that precedes the onset of spermatogenesis is controlled by LH and FSH (reviewed by Khan et al., 1995). Furthermore, both FSH and testosterone are generally accepted as being essential for normal proliferation and differentiation of germ cells (Sharpe, 1994). Cases of infertility reported in men (Hauser et al., 1995; Martin-du Pan and Campana, 1993), bulls (Veeramachanemi et al., 1986), rats (Noguchi et al., 1993) and mice (de-França et al., 1994) have been associated with abnormal blood concentrations of FSH, LH and/or testosterone.

In conclusion, although the development of the hypothalamic-pituitary-gonadal axis and the hormonal control of spermatogenesis have been studied in the bull and other species, it is still unclear how gonadotropins and steroids interact before puberty to determine Sertoli and germ cell numbers and testis size. Thus, a series of studies were conducted with the following objectives:

Study 1: to evaluate changes in peripheral concentrations of basal and GnRH-

stimulated-FSH, LH, testosterone, androstenedione (Δ_4A) and E2 in Angus bulls between 2 and 12 months (mo) of age and to determine whether concentrations of these hormones were correlated with testicular development and number of Sertoli and germ cells at 12 mo.

Study 2: to determine whether gonadotropin or steroid concentrations in 6-mo-old crossbred bulls (Charolois x Angus and Charolois x Hereford) challenged with estradiol benzoate and GnRH were meaningfully correlated with testicular development and number of Sertoli or germ cells.

Materials and Methods - Study 1

General Procedures

Twenty four Angus bulls born between January 6th and March 30th were used. Calves were pastured with their dams until weaning (8 months) and thereafter, maintained in feedlots with free access to hay and corn silage.

Starting at 2 mo and continuing to 12 mo, all bulls were treated monthly with 0.05 μg of GnRH (des-gly^{10}, [D-ala^6]-GnRH-ethylamide, Sigma Co., St. Louis, MO) and blood samples were collected from the jugular vein 1.5 and 3 hours (h) later. Doses of GnRH were given based on a ratio of 0.05 μg per Kg of body weight. One day before the GnRH challenge, bulls were bled three times at 1.5-h intervals to establish basal hormone concentrations. On a monthly basis, animals were weighed and the diameter of the right testis of each bull was measured using a caliper. At the age of 12 mo (361 ± 15 days), all bulls were surgically castrated (body weight = 433.3 ± 6.5 Kg).

Histological analysis

At castration, both testes were weighed and measured (diameter and length) after the removal of the tunica vaginalis and epididymis. Two 4-mm thick segments were taken near the poles of one testis and placed in Bouin's fixative for 24 h, rinsed with water and washed in three changes of 70% ethanol. Thereafter, the tissue was dehydrated in alcohol, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. Two slides, separated by 100 µm, of each testicular segment were prepared.

It has been shown in the bovine that there is uniformity between different regions within the testis (Amann, 1962; Swierstra, 1966). Also, Berndtson et al. (1987) have estimated that the use of 10 cross sections of seminiferous tubule per bull is adequate for determination of Sertoli and germ cell numbers. To check these findings, 10 bulls were chosen at random and cell numbers were estimated in either 10 or 20 sections. Results showed that differences in Sertoli and germ cell numbers per testis were not significant when either 10 or 20 cross sections were used ($p < 0.10$). Therefore, calculation of Sertoli and germ cell numbers and ratios presented in this study were based on samples of 10 sections selected from different regions of one slide from each bull. These sections were all at stages I through VI, as described by Berndtson and Desjardins (1974). When the bull did not have elongated or mature spermatids, the sections chosen were those with the most advanced germ cell type.

Numbers of Sertoli cells, A1 spermatogonia, pachytene spermatocytes and round spermatids containing an intact nucleolus were counted in each of the 10 sections. These crude counts were converted to true counts according to Abercrombie's formula (Abercrombie, 1946): True# = Crude# × (section thickness / (section thickness + average

nuclear diameter in microns). Section thickness in this case was 5 μm. Based on the true counts, the following cell ratios per cross section was estimated: # of A1 spermatogonia (A1)/Sertoli cell (SC), and # of round spermatids per A1 and per SC.

To estimate the degree of seminiferous tubule development, 400 cross sections from each of the testicular segments were evaluated and placed in one of the following categories, based on the most advanced germ cell type: tubules without germ cells (ST0), tubules with A1 spermatogonia (STA), Intermediate or B spermatogonia (STB), pachytene spermatocytes (STP), round spermatids (STD), elongated spermatids (STE) and mature spermatids (STM). The percentage of tubules in each of these categories was used in the analysis of correlation.

Total number of Sertoli cells per testis was calculated based on the method described by Erickson and Blend (1971). Testicular volume (V) was determined by the formula $V = TW \div D$, where TW and D represented testicular weight (g) and density (1.052 g/cm^3, Johnson and Neaves, 1981), respectively. The volume occupied by 10 seminiferous tubule cross sections (Vst) was calculated by the formula $Vst = \prod \times h \times (d^2/4)$, where h was the section thickness (5 μm) and d was tubule diameter in microns. The percentage of testicular volume occupied by seminiferous tubules (%ST) was determined as described by Chalkley (1943), i. e., the percentage of 600 "hits" taken a random within a cross section of a testis. A "hit" was defined as the object at the point of each of 6 pointers mounted in the ocular of the microscope. The ocular was passed over the surface of a testicular cross section in a random manner and the number of times each pointer fell on either tubular or interstitial tissue was recorded.

Crude numbers of Sertoli cells, A1 spermatogonia and round spermatids per testis was

determined by the following formula: Crude# = (V × %ST × C) / (Vst x 10), where C represented the true number of Sertoli cells or A1 spermatogonia counted in 10 cross sections. The resulting crude numbers were converted to true counts according to Abercrombie's formula (Abercrombie, 1946): True# = Crude# × (section thickness / (section thickness + average nuclear diameter in microns). The final estimate of the number of A1 spermatogonia per testis was obtained by multiplying the True# by: (1 - % ST0). The population of round spermatids per testis was estimated by multiplying the True# by: (1 - (ST0+STA+STB+STP)).

The volume of the interstitial space was obtained multiplying the percent of the testis occupied by the interstitium by total testicular volume. Estimation of the average volume of a Leydig cell was based on the formula: $Vlc = (1/6) \times \pi \times D^3$, where D is the average diameter (in μm) taken from 25 Leydig cells. Dividing the volume of the interstitium space by the average volume of a Leydig cell gave the total number of Leydig cells per testis (Lc) and dividing Lc by testis weight (in g) gave the numerical density of the Leydig cell population.

Radioimmunoassays

Blood samples were collected at the farm and immediately placed on ice. At the laboratory, samples were allowed to warm for 2 h at room temperature and then centrifuged at 1764 x g for 25 minutes. Serum was harvested and stored at -20 C until assayed for FSH, LH, testosterone, androstenedione and 17β-estradiol.

Concentrations of FSH were determined in 200 μl of serum using a double antibody

radioimmunoassay (RIA), as described by 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 samples collected during the present study were analyzed in two different assays. The sensitivity of the FSH RIA was 0.25 ng/ml and the intra- and interassay coefficient of variation (CV) were <7 % and < 12 %, respectively. Peripheral concentrations of LH were quantified in 100 μl of serum using a double-antibody RIA method described by Niswender et al. (1969) and modified by Bolt et al. (1981). The LH first 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 interassay CV were < 7% and < 10%, respectively.

Samples of serum (150 µl) were extracted with 1.5 ml of benzene before being analyzed for testosterone (T), Δ_4A and E2. Concentrations of T and Δ_4A were estimated based on a single-antibody method described by Jackson et al. (1989). The Δ_4A antibody (X - 322 Rao) was purchased from Dr. P. N. Rao (Southwest Foundation for Biomedical Research, San Antonio, TX) and the T antibody was provided by Dr. G. Niswender. Assay sensitivities for the T and Δ_4A assays were 10 pg/ml and 2.5 pg/ml, respectively. The intra- and interassay CV were <8% and <12%, respectively, for the T assay, and <8% and <12%, respectively, for the Δ_4A assay. Concentrations of E2 were quantified according to a procedure described by Cox et al. (1987) and modified by Britt (1990). 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 interassay CV were <8% and <15%, respectively.

Statistical Analysis

The effect of exogenous GnRH on peripheral concentrations of FSH, LH, E2, T and Δ_4A was evaluated by analysis of variance using the GLM procedure of SAS (1990). Treatments were defined as basal versus GnRH-stimulated. Within each age period, if significant, body weight was used as a covariate. Across ages, differences in basal and GnRH-stimulated hormone concentrations were determined by Tukey's mean separation. Also, the z statistical test was used to determine if single observations from infertile bulls were different from the average of a sample of 17 normal bulls (as described by Dowdy and Wearden, 1991).

Pearson's partial correlations (Corr procedure, SAS, 1990) were used to determine the strength of the linear relationships involving testis size, Sertoli and germ cell numbers and hormone concentrations. Body weight was used as the partial variable so that its effect on the correlation between other variables could be controlled. Regression analysis (REG procedure, SAS, 1990) was used to model yearling testicular weight and diameter. The independent variables included hormone concentrations, testis diameter and body weight recorded throughout the study. Because histological samples from 4 animals were lost, correlations between hormone data and cell counts were estimated with 20 bulls, while those between hormone levels and testis size were determined in a sample of 24 bulls.

Results - Study 1

Age-related changes in testis size and basal and GnRH-stimulated peripheral hormone concentrations

Bulls were weaned at 8 mo, and the increase in body weight between 4 and 6 mo (34 %) was higher than that between 6 and 8 mo (27 %; figure 1.1). Testis diameter did not change significantly from 2 to 4 mo. From 4 to 8 mo, the diameter of the average testis increased by 30 % (from 33 mm to 43 mm, $P < 0.05$) and between 8 mo and 12 mo, testicular diameter increased by 71 % (from 43 to 74 mm, $p < 0.01$). As presented in table 1.1, the correlation between yearling testis weight and testis diameter was low when diameter was measured before 4 mo ($r < 0.47$, $p > 0.10$). However, r values increased ($r = 0.55$ to 0.91, $p < 0.05$ to 0.01) when testis diameter was measured at later ages (5 to 11 mo). After removal of the tunica vaginalis and epididymis, the average testis diameter (TD) and weight (TW) at 12 mo were 58.9 ± 2.0 mm and 229.7 ± 16.2 g, respectively. Also, values for TD and TW ranged from 29 mm to 78 mm and from 32 g to 418.5 g, respectively. When age was held constant, correlations between TD and body weight were high between 4 and 6 mo ($r = 0.70$ to 0.75, $p < 0.001$) but decreased thereafter ($r = 0.54$, $p < 0.01$; $r = 0.44$, $p < 0.05$; at 9 and 12 mo, respectively). At 12 mo, TW was correlated with body weight ($r = 0.56$, $p < 0.01$) but not with age (in days).

Both basal and GnRH-stimulated concentrations of FSH changed with age ($p < 0.05$, figure 1.2). Basal FSH decreased from 0.45 ± 0.04 ng/ml at 2 mo to 0.26 ± 0.03 ng/ml at 4 mo. After the age of 5 mo, FSH increased to 0.36 ± 0.01 ng/ml but reached a low of 0.22 ±

0.02 ng/ml at 8 mo. Between 8 and 10 mo, serum FSH increased 2-fold (to 0.45 ± 0.03 ng/ml), decreased to 0.34 ± 0.02 ng/ml at 11 mo and returned to 0.44 ± 0.04 ng/ml at 12 mo. Across ages, basal and GnRH-stimulated FSH was correlated ($r = 0.79$ to 0.90, $p < 0.01$) and, within ages, concentrations of FSH measured 1.5 h (T1.5) and 3 h (T3) after GnRH were higher ($p < 0.01$) than and correlated with ($r = 0.60$ to 0.92, $p < 0.01$) basal FSH.

Between 2 and 4 mo, LH increased significantly ($p < 0.05$) from 0.47 ± 0.03 ng/ml to 0.66 ± 0.06 ng/ml, but remained practically unchanged for the next two months (figure 1.3). Concentrations of basal LH increased from 0.70 ± 0.07 ng/ml to 1.09 ± 0.05 ng/ml ($p < 0.05$) between 6 and 9 mo, decreased to 0.90 ± 0.04 ng/ml at 10 mo ($p < 0.05$), but increased again to levels that were not significantly different from those at 9 mo. During the course of the study, the average increase in LH was 27 ± 2.1-fold and 35 ± 2.14-fold at T1.5 and T3, respectively (figure 1.3). Although basal and GnRH-stimulated LH changed with age ($p < 0.05$), neither were correlated ($p > 0.10$) within age-periods. Across ages, between 2 and 12 mo, basal and GnRH-stimulated LH were correlated ($r = 0.65$) and concentrations of LH at T1.5 were correlated with both basal FSH ($r = 0.48$, $p < 0.10$) and FSH at T1.5 ($r = 0.69$, $p < 0.01$). Also, LH at T3 showed a significant correlation with FSH at T3 ($r = 0.75$, $p < 0.01$).

Both basal T and its response to GnRH changed with age ($p < 0.05$; figure 1.4), starting from 0.10 ± 0.01 ng/ml and 0.19 ± 0.02 ng/ml at 2 mo, respectively, reaching peak concentrations of 6.5 ± 0.6 ng/ml and 12.4 ± 0.71 ng/ml at 10 mo, and declining to 5.0 ± 0.85 ng/ml and 9.5 ± 0.65 ng/ml at 12 mo, respectively. Within and across ages, basal and GnRH-stimulated T were correlated ($r = 0.45$ to 0.56, $p < 0.10$; $r = 0.53$ to 0.81, $p < 0.05$). As

shown in figure 1.5, the highest concentrations of basal and GnRH-stimulated Δ_4A in serum were detected between 3 (394 ± 41 and 657 ± 78 pg/ml, respectively) and 4 mo (401 ± 50 and 630 ± 58 pg/ml, respectively). Basal Δ_4A changed between the ages of 6 and 12 mo. Concentrations increased from 172 ± 16 pg/ml at 6 mo to 256 ± 19 pg/ml at 9 mo and 342 ± 26 pg/ml at 10 mo ($p < 0.05$), and declined to 230 ± 15 pg/ml at 12 mo ($p < 0.05$). The changes in Δ_4A between 6 and 12 mo coincided with those observed in T. For all ages, basal and GnRH-stimulated Δ_4A were correlated and the r value averaged 0.78 ($p < 0.01$). Also, within ages, both Δ_4A and T (basal) were correlated with each other ($r = 0.45$ to 0.71, $p < 0.10$) and with LH ($r = 0.40$ to 0.60, $p < 0.10$). Across ages, Δ_4A and T were correlated with GnRH-stimulated, but not basal, LH ($r = 0.42$ to 0.77, $p < 0.10$).

Basal E2 increased significantly ($p < 0.05$) between 2 mo (2.58 ± 0.11 pg/ml) and 3 mo (3.71 ± 0.31 pg/ml) and remained unchanged at 4 mo (3.28 ± 0.29 pg/ml; figure 1.6). Estradiol declined to 2.37 ± 0.13 pg/ml ($p < 0.05$) at 5 mo but increased between 6 to 12 mo (3.70 ± 0.22 pg/ml at 6 and 8 mo, 7.63 ± 0.55 pg/ml at 10 mo and 9.91 ± 0.70 pg/ml at 12 mo; $p < 0.05$). Concentrations of E2 increased in response to exogenous GnRH at all ages ($p < 0.05$). However, differences between basal and GnRH-stimulated E2 evaluated between 2 and 4 mo (average basal of 3.19 ± 0.24 pg/ml versus 3.49 ± 0.23 and 3.97 ± 0.27 pg/ml for T1.5 and T3, respectively) were lower than those evaluated between 5 and 8 mo (average basal of 3.25 ± 0.21 pg/ml versus 5.22 ± 0.33 and 5.22 ± 0.35 pg/ml for T1.5 and T3, respectively) and between 9 and 12 mo(average basal of 8.18 ± 0.57 pg/ml versus 10.84 ± 0.83 and 11.9 ± 0.82 pg/ml for T1.5 and T3, respectively). Across ages, concentrations of basal and GnRH-stimulated E2 were correlated with FSH (0.64 to 0.85, $p < 0.05$), LH ($r =$

0.66 to 0.89, $p < 0.05$) and testosterone ($r = 0.77$ to 0.86, $p < 0.01$). Within all age-periods, basal and GnRH-stimulated E2 were correlated (average $r = 0.63$, $p < 0.01$). Positive correlations were found between basal and GnRH-stimulated E2 and T ($r = 0.35$ to 0.54 from 2 to 4 mo; 0.41 to 0.81 from 5 to 12 mo). Also, negative correlations between E2 and FSH were observed from 2 to 6 mo ($r = -0.41$ to -0.61, $p < 0.10$), but not at other ages. Conversely, starting at the age of 6 mo, but not before, there were positive correlations between GnRH-stimulated concentrations of E2 and LH ($r = 0.44$ to 0.50, $p < 0.05$).

Correlations between testis size, numbers of Sertoli cells, quantitative aspects of spermatogenesis and degree of seminiferous tubule development

Table 1.2 shows the averages, standard errors and ranges of number of A1 spermatogonia (A1), round spermatids (RST) and Sertoli cells (SC) per testis and the following cell ratios: number of A1 spermatogonia per Sertoli cell (AS), and number of round spermatids per Sertoli cell (RS) and per A1 spermatogonia (RA) present in ST cross sections. The average number of Sertoli cells per testis was $4.59 \pm 0.32 \times 10^9$, with values ranging from 1.63 to 8.47×10^9. Also, the average yearling testis had $5.85 \pm 0.74 \times 10^8$ A1 spermatogonia, and $0.85 \pm 0.58 \times 10^{10}$ round spermatids, with 1 bull without A1 and 3 bulls without spermatids. The average Sertoli cell supported 6.31 ± 1.41 round spermatids and 0.13 A1 spermatogonia and there were 42.21 ± 9.45 round spermatids per each A1 spermatogonium in the seminiferous tubules (table 1.2). The average bull had 20.6 ± 6.45 % of the ST sections without any germ cells (ST0) and 68.35 ± 8.85 % of ST sections with at least round spermatids as the most advanced germ cell type (table 1.3). Only nine bulls out of the group

of 20 had less than 5 % of ST sections without germ cells and 3 bulls had no spermatids, being therefore infertile.

As shown in table 1.4, TW was correlated with SC ($r = 0.85$, $p < 0.01$), A1 ($r = 0.80$, $p < 0.01$) and RST ($r = 0.79$, $p < 0.01$). Also, bulls with heavier testis had more round spermatids generated per A1 spermatogonia ($r = 0.73$, $p < 0.01$) and more A1 spermatogonia and round spermatids supported by each Sertoli cell ($r = 0.54$, $p < 0.05$; $r = 0.69$, $p < 0.01$, respectively). Also, larger populations of Sertoli cells, A1 spermatogonia and round spermatids per testis were associated with higher numbers of RA, RS and AS ($r = 0.43$ to 0.85, $p < 0.10$). Correlations between TD and total cell counts per testis and cell ratios per seminiferous tubule sections were also significant ($r = 0.57$ to 0.84, $p < 0.05$). By means of regression analysis, TW and TD were estimated according to the following models:

a) $TW = -31.71 + 39.32 \times SC + 81.31 \times RS$, $R^2 = 0.87$; p-values for SC and RS were less than 0.002.

Testes with higher percentage of ST sections without germ cells weighted less ($r = -0.65$, $p < 0.01$) and had fewer SC ($r = -0.45$, $p < 0.10$), A1 ($r = -0.80$, $p < 0.01$), RST ($r = -0.79$, $p < 0.01$) and RS ($r = -0.80$, $p < 0.01$; table 1.5). However, testes with more ST sections with elongate or mature spermatids were heavier ($r = 0.79$, $p < 0.01$) and had larger number of SC ($r = 0.64$, $p < 0.01$), A1 ($r = 0.88$, $p < 0.01$), RST ($r = 0.85$, $p < 0.01$) and RS ($r = 0.84$, $p < 0.01$).

Correlations between TW and TD and basal and GnRH-stimulated hormone concentrations

By analysis of partial correlations, TW and TD were determined to be not significantly correlated with either basal or GnRH-stimulated concentrations of Δ_4A and LH between 2 and 12 mo. However, TW and TD were related to peripheral concentrations of GnRH-stimulated testosterone measured at 3 mo ($r = 0.48$ and 0.42, $p < 0.05$, respectively) and E2 measured at 2 and 4 mo ($p = 0.40$ and 0.45, $p < 0.10$; respectively).

Correlations between TW, TD and concentrations of basal and GnRH-stimulated FSH were always negative, as presented in tables 1.6A and 1.6B. The linear relationships between TW and FSH were stronger when FSH was measured at 2 and 3 mo ($r = -0.54$ to -0.62, $p < 0.01$), and from 9 to 12 mo ($r = -0.47$ to -0.71, $p < 0.05$ and $p < 0.01$) than from 4 to 8 mo ($r < -0.47$, $p < 0.05$). Also, correlations between TD and FSH tended to be higher ($r = -0.62$) than those between TW and FSH ($r = -0.53$) and no significant differences in r values were apparent between basal and GnRH-stimulated FSH when either TW ($r = -0.53$ and -0.52. respectively) or TD ($r = -0.64$ and -0.59, respectively) was used in the calculations.

Based on the correlations presented above, regression analysis was used to model yearling testis weight (TW, in g) based on the average between basal and GnRH-stimulated FSH (in ng/ml), GnRH-stimulated E2 (in pg/ml), testis diameter (in mm) and/or body weight (in Kg) and the following equations were generated:

a) $TW = 253.1 - 124.3 \times FSH2 + 26.0 \times E2$, with $R^2 = 0.45$ and p-values for FSH and E2 were 0.007 and 0.09, respectively. FSH2 and E2 represent concentrations of FSH and estradiol quantified at 2 mo. Scatter plots are shown in figures 1.7a and 1.7b.

b) $TW = -125.8 - 46.8 \times FSH6 + 10.3 \times TD6$, with $R^2 = 0.57$, and p-values for FSH and

TD6 were 0.067 and 0.002, respectively. FSH6 and TD6 represent values of FSH and testis diameter at 6 mo. When the average basal and GnRH-stimulated FSH measured between 2 and 6 mo and TD6 were used in the model presented above, the R^2 did not reach values above 0.55. Scatter plots are shown in figures 1.8a and 1.8b.

c) TW = - 9.2 - 99.8 x FSH9 + 6.2 x TD9, $R^2 = 0.67$, and p-values for FSH and TD9 were 0.013 and 0.0005, respectively. FSH9 and TD9 represent values of FSH and testis diameter at 9 mo. Scatter plots are shown in figures 1.9a and 1.9b.

d) When testis diameter at 12 mo (TD12) was included in the regression model, the p-values for FSH became nonsignificant ($p > 0.10$). Models with TD12 as the only independent variable gave $R^2 = 0.85$ for TW. However, when FSH and body weight at 12 mo (FSH12 and BW12, respectively) were used, the following equation was generated: TW = - 147.8 - 112.8 x FSH12 + 1.08 x BW12, with $R^2 = 0.63$, and p-values for FSH and BW12 were 0.0006 and 0.005, respectively (scatter plots are shown in figures 1.10a and 1.10b). When basal and GnRH-stimulated FSH quantified between 2 and 12 mo were averaged and included in the regression model with BW12, the R^2 found was 0.57.

Correlations between concentrations of FSH and number of Sertoli cells, quantitative aspects of spermatogenesis and degree of seminiferous tubule development

Concentrations of either Δ_4A, T or LH measured between 2 and 12 mo were not significantly related to histological criteria of the yearling testis. However, as presented in tables 1.7A and 1.7B, correlations between SC and FSH were significant when FSH was measured as early as 2 mo ($r = -0.51$, $p < 0.05$) and 3 mo ($r = -0.54$, $p < 0.05$), but the

highest correlations were found at 11 ($r = -0.68$, $p < 0.01$) and 12 mo ($r = -0.63$, $p < 0.01$). Number of A1 in the yearling testis was correlated with FSH only when FSH was quantified in samples taken after 9 mo. Again, the highest r values were detected at the ages of 11 ($r = -0.59$, $p < 0.01$) and 12 mo ($r = -0.55$, $p < 0.01$). Correlations involving FSH and RA and RS were very similar ($r = -0.53$ and -0.52, respectively) at the ages of 3, 6 and 8 mo. Also, r values for both RA and RS tended to be higher from 9 to 12 mo ($r = -0.52$ to -0.61, $p < 0.05$, respectively) than from 2 to 8 mo ($r = -0.40$ to -0.57, $p < 0.10$, respectively). The percentage of ST cross sections with round spermatids as the most advanced cell type (STD) was correlated with both basal and GnRH-stimulated FSH at all ages after 3 mo. Correlation coefficients increased from an average of -0.47, between 2 and 8 mo, to -0.63 at ages after 9 mo. Finally, basal and GnRH-stimulated FSH at 9 mo were related to STE, with r values ranging from -0.50 ($p < 0.05$) to -0.64, ($p < 0.01$).

Testicular development and peripheral hormone concentrations of infertile bulls: the case of bulls #250, #345 and #389 as compared to normals bulls

Among the three bulls with abnormal spermatogenesis, bull #250 had no germ cells and bull #345 had 90 % of the tubules without germ cells and only A1 spermatogonia were present in the remaining cross sections. Volume and total number of Leydig cells per testis and number of Leydig cells per g of testicular parenchyma in these bulls were not significantly different from those in normal bulls. Also, Sertoli cell numbers in bull #250 and 345 were 3.75×10^9 and 4.27×10^9, respectively, but these values were not different from the average SC count for bulls with normal spermatogenesis ($4.93 \pm 0.30 \times 10^9$; $p > 0.10$). In this case, the

group of normal bulls was formed by 17 bulls that had all the 12 stages of the cycle of the seminiferous epithelium, as described by Berndtson and Desjardins (1974). Testes of bulls #250 and 345 weighed only 40% (99 and 105 g, respectively) of those of normal bulls (251.5 ± 55.8 g). Also, in comparison with the body weight of normal bulls (437 ± 31.7 Kg), that of bull #345 (434.3 Kg) was not significantly different but that of bull #250 was (396 Kg, $p < 0.10$). Cross sections of the seminiferous tubules of these two animals were vacuolated and had diameters of 169 μm and 174 μm, respectively, which were smaller than ($p < 0.05$) the average tubule diameter of normal bulls (200 ± 3.08 μm). The nuclear diameter of Sertoli cells of animals #250 and 345 (11 μm) was not different from that of normal bulls (11 μm). As observed by light microscopy, the Sertoli cell nuclei of bull #250 and #345 did not show major morphological differences from that of normal bulls, i. e., they were located near the basement membrane, irregularly-shaped with indentations and the nucleolus appeared as a single structure with little chromatin surrounding it.

In the other case (bull #389), numbers of SC and A1 (1.63×10^9 and 1.04×10^8, respectively) and testis weight (32 g) were 33 %, 14 % and 13 % of those measured in normal animals. The number of Leydig cells per g of testicular parenchyma was 4.5-fold higher ($p < 0.01$) in bull #389 than in normal bulls and the percentage of the testis volume occupied by the interstitial tissue was 58 %, while in normal bulls this value was 25 %. However, Leydig cells in bull #389 did not show any signs of hypertrophy. At the age of 12 mo, body weight of bull #389 (392 Kg) was 10 % lower than that of normal bulls ($p < 0.10$). Analysis of 800 ST cross sections of this animal revealed that 32 % of them had no germ cells. Also, 30 % of these sections were placed in the STA, 25 % in the STB and 12 % in the STP category. Some

of the spermatocytes observed in the ST sections showed signs of degeneration. In normal testes, 11 ± 2.8 % of the ST sections had no germ cells, less than 3 % were placed in either the STA or STB, and 5.5 ± 1.41 % in the STP category. The average diameter of the tubules and Sertoli cell nucleus in bull # 389 was 98.51 μm and 8.62 μm, respectively, which were lower ($p < 0.01$) than those found in normal bulls. Also, the Sertoli cell nuclei were round or oval with few of them resembling those of mature Sertoli cells. The nucleoli appeared to be split and often associated with large aggregates of heterochromatin.

Testicular size of infertile bulls measured between 2 and 12 mo was lower than that of normal bulls (figure 1.11). Starting at the age of 4 mo and continuing to 12 mo, testis diameter of bull #389 was smaller ($p < 0.05$) than that of normal bulls. Testis size of bulls #250 and 345 was smaller than those of normal bulls from the age of 4 mo, but differences became significant only after the age of 8 mo ($p < 0.10$ at 8 mo; $p < 0.05$ after 11 mo). Between 8 and 12 mo, testis diameter of normal bulls increased at an average rate of 9.33 ± 3.66 mm per mo, while those of bull #389, 250 and 345 grew at rates of 3.75, 6.75 and 5.75 mm per mo, respectively. Testis diameter of bulls # 389, 250 and 345 were 47 %, 70 % and 71 % of those of normal bulls, these differences decreased to 13 %, 39 % and 41 %, respectively, when comparisons were made between testis weights.

Patterns of FSH secretion of normal bulls and bulls #250, 345 and 389 are shown in figures 1.12 and 1.13. Average concentrations of basal and GnRH-stimulated FSH (1.74 and 2.37 ng/ml, respectively) in bull #389 were 5-fold and 3.2-fold higher ($p < 0.01$) than the respective values for normal bulls (0.35 ± 0.02 and 0.74 ± 0.07 ng/ml, respectively). Also, the difference between the average basal and GnRH-stimulated FSH was 36 % for bull #389 and

110 % for normal bulls. In the case of bull # 250, values of basal FSH at 9 and 10 mo (0.56 and 0.59 ng/ml, respectively) and GnRH-stimulated at 11 and 12 mo (1.31 and 1.50 ng/ml, respectively) were higher ($p < 0.05$) than those of normal bulls (0.34 ± 0.01 and 0.35 ± 0.03 ng/ml at 9 and 10 mo; 0.94 ± 0.08 and 1.19 ± 0.12 ng/ml at 11 and 12 mo, respectively). However, the average basal and GnRH-stimulated FSH secretion of normal bulls and bull #250 (0.38 and 0.74 ng/ml, respectively) were not different ($p > 0.10$). At 11 and 12 mo, GnRH-concentrations of FSH in bull #345 (1.35 and 1.55 ng/ml, respectively) were higher ($p < 0.05$) than those measured in normal bulls. On average, basal (0.45 ng/ml) and GnRH-stimulated FSH (0.82 ng/ml) secreted throughout the year were higher in bull #345 than in normal bulls, but differences were significant ($p < 0.05$) only for basal values.

From 2 to 12 mo, basal LH in bull #389 increased with age ($r = 0.94$, $p < 0.01$; figure 1.14). Not only were differences in basal LH secretion between bull #389 and normal ones significant at 12 mo (4.1 ng/ml versus 2.85 ± 0.15 ng/ml, $p < 0.01$, respectively), the difference was also significant at 2 mo (0.76 ng/ml versus 0.49 ± 0.03 ng/ml, $p < 0.05$, respectively). However, at none of the ages studied was basal LH in bulls #250 and 345 significantly different from that of normal bulls. Throughout the year, the average GnRH-stimulated LH in bull #389 (12.59 ng/ml) was lower than that in normal bulls (25.5 ± 2.1 ng/ml, $p < 0.01$; figure 1.15).

GnRH-stimulated LH secreted by bull #250 at ages from 3 to 6 mo was lower ($p < 0.10$) than that found in normal bulls (figure 1.15). Although from 8 to 12 mo the LH response to GnRH in bull #250 remained, on average, 36 % lower than that one in normal bulls, differences did not reach statistical significance ($p > 0.10$). At 2 and 3 mo,

concentrations of GnRH-stimulated LH in bull #345 (11.44 and 17.26 ng/ml, respectively) were lower ($p < 0.10$) than those detected in normal bulls (15.4 ± 1.6 and 22.0 ± 1.65 ng/ml, respectively), but became normal after the age of 5 mo.

Concentrations of basal testosterone (figure 1.16) in bull #389 measured from 4 to 10 mo (0.10 to 3.11 ng/ml) were lower ($p < 0.10$) than those in normal bulls (0.96 ± 0.22 to 6.5 ± 0.67 ng/ml). However, while testosterone in normal bulls decreased after 10 mo, those in bull #389 continued to increase, reaching a peak of 6.08 ng/ml at 12 mo. Basal testosterone in bull #250 was, on average, 37 % lower than that of normal bulls but differences were significant only at the ages of 4 and 5 mo ($p < 0.10$). Basal testosterone in bull #345 was lower ($p < 0.10$) than that of normal bulls at 4 mo, but after 6 mo, it averaged 22 % higher, although this increase was not significant ($p > 0.10$). From 4 to 12 mo, GnRH-stimulated testosterone in bulls #389, 250 and 345 was lower than that in normal bulls, but differences were significant only after 5 mo in bulls #389 and 250 ($p < 0.10$) and between 8 and 11 mo for bull #345 ($p < 0.10$; figure 1.17). Also, while GnRH-stimulated testosterone in normal bulls numerically decreased from 12.2 to 10 ng/ml between 11 and 12 mo, its change in any of the infertile bulls was minimal (1 %) during the same period.

At all ages, basal concentrations of Δ_4A (figure 1.18) in bull #250 were lower than those detected in normal bulls ($p < 0.10$). The average Δ_4A measured between 2 and 11 mo was lower ($p < 0.05$) in bull #250 (200 pg/ml) than in normal bulls (288 ± 34 pg/ml). Although at 3 mo, basal Δ_4A in bull #345 (640 pg/ml) was higher ($p < 0.10$) than that in normal bulls (443 ± 52 pg/ml), Δ_4A secreted at other ages was not different from normal values and the average Δ_4A measured from 2 to 11 mo in bull #345 (309 pg/ml) did not differ

from normal ($p > 0.10$).

At 3,4 and 5 mo, bull #389 had values of Δ_4A (242, 302 and 231.5 pg/ml, respectively) that were lower ($p < 0.10$) than those in normal bulls (429 ± 52, 443 ± 62 and 311 ± 37 pg/ml, respectively; figure 1.18). Between 5 and 11 mo, basal Δ_4A in bull #389 increased with age ($r = 0.98$, $p < 0.01$), going from 231.5 to 767.5 pg/ml, respectively, and reaching a peak of 801 pg/ml at 10 mo. However, during the same period, concentrations of Δ_4A in normal bulls were lower ($p < 0.05$), with values of 181 ± 15, 349 ± 22 and 243 ± 15 pg/ml at 6, 10 and 11 mo, respectively.

Within age-periods, GnRH-stimulated concentrations of Δ_4A in bull #250 (figure 1.19) were lower than those in normal bulls, but differences did not reach significance ($p > 0.10$) at the ages of 6 and 8 mo. Also, the average Δ_4A in bull #250 (288 pg/ml) between 2 and 11 mo was lower ($p < 0.05$) than that in normal bulls (443 ± 54 pg/ml). At the ages of 2, 3, 4, 8 and 9 mo, GnRH-stimulated Δ_4A in bull #345 was higher ($p < 0.10$) than that in normal bulls at the ages of 2, 3, 4, 8 and 9 mo. At other ages, differences were only numerical and less than 10 %.

From 2 to 11 mo, the Δ_4A response to GnRH in bull #389 increased with age ($r = 0.90$, $p < 0.01$; figure 1.19). At 3 and 4 mo, Δ_4A in bull #389 (363.5 and 492.5 pg/ml, respectively) was lower ($p < 0.10$) than in normal bulls (683 ± 75 and 664 ± 62 pg/ml, respectively) but from 6 to 11 mo, it became, on average, 85 % higher (638 to 802 pg/ml) than in normal bulls (436 ± 53 to 416 ± 41 pg/ml, $p < 0.10$).

In the case of bull #345, basal E2 was not different from normal values at any age (figure 1.20). However, basal concentrations of E2 between 2 and 5 mo were lower in bull

#250 (1.77 to 2.15 pg/ml) than in normal bulls (2.63 ± 0.11 to 2.46 ± 0.13 pg/ml), and at ages from 9 to 12 mo E2 was also lower in bull #250 (4.21 to 4.91 pg/ml) than in normal bulls (6.2 ± 0.55 to 9.26 ± 0.50 pg/ml), but differences were significant ($p < 0.10$) only at 11 and 12 mo. At 2 and 3 mo, basal E2 in bull #389 (1.35 and 1.3 pg/ml) was lower ($p < 0.10$) than in normal bulls (2.63 ± 0.11 and 3.6 ± 0.27 pg/ml). Also, from 8 to 12 mo, basal E2 was significantly ($p < 0.10$) lower in bull #389 (2.13 to 6.0 pg/ml) than in normal bulls (3.79 ± 0.29 to 9.26 ± 0.64 pg/ml).

Throughout the year, GnRH-stimulated E2 was between 14 % and 58 % lower in bull #250 than in normal bulls (figure 1.21). However, only at 10, 11 and 12 mo of age, were concentrations of E2 in bull #250 (5.64, 5.87 and 4.49 pg/ml, respectively) significantly ($p < 0.10$) different from those detected in normal bulls (12.2 ± 1.0, 11.18 ± 1.68 and 10.68 ± 1.5 pg/ml, respectively). GnRH-stimulated E2 in bull #345 was not different from that of normal bulls at any age, although at 10, 11 and 12 mo concentrations of E2 in bull #345 (8.99, 8.7 and 7.18 pg/ml, respectively) were 26 % to 33 % lower than in normal bulls. Between 2 and 12 mo, concentrations of GnRH-stimulated E2 measured in bull #389 (1.48 to 5.70 pg/ml) were lower than those in normal bulls (2.88 ± 0.15 to 10.68 ± 1.5 pg/ml), but differences were not significant ($p > 0.10$) at 4, 5 and 9 mo.

Discussion - Study 1

Age-related changes in testicular development and peripheral hormone concentrations

Results of this study show that testis diameter at ages less than 4 mo were poorly associated with yearling testis size but correlations became higher as the interval between measurements taken at early ages (4 to 11 mo) and at 12 mo decreased. In young bulls, the fact that testes were small and difficult to confine within the scrotum led to inaccurate measurements. Also, spermatogenesis has not started at 4 mo and this may be required to accentuate interanimal differences. It was found that correlations between testis size and body weight decreased with age but they were still significant at 12 mo ($r = 0.56$). Similar results have been found in pre and postpubertal rams ($r = 0.69$, Yarnay et al., 1990) and in bulls ($r = 0.30$ to 0.55, Sadowski et al., 1965, cited by Amann, 1970). Also, Lunstra et al. (1981) found that body weight was related to scrotal circumference ($r = 0.73$), but not to testis weight, in yearling beef bulls. Thus, there is a correlation between body weight and testis size in young bulls, where there is a wide variation in growth rates.

Angus bulls used in this study had FSH levels ranging from 0.26 to 0.44 ng/ml, which are similar to those found in Holstein bulls (0.35 to 0.55 ng/ml) at ages from 1 to 9 mo (MacDonald et al., 1990). Also, FSH was high at 2 mo, low at 4 mo and increased again at 5 and 6 mo. MacDonald et al. (1990) found that FSH was high at 2 mo in Holstein bulls, but decreased earlier than in Angus bulls (3 mo). These authors also reported an increase in FSH between 3 and 5 mo and a fall to intermediary levels between 6 and 9 mo. In addition to promoting Sertoli cell mitosis in the 2-mo old testis, FSH could also induce the synthesis of

LH receptors on the Leydig cells and thereby enable them to respond to LH. The second increase in FSH between 4 and 6 mo is coincident with the process of mitosis of A1 spermatogonia and differentiation of the Sertoli cell. At 8 mo, the decrease in FSH may be a consequence of both the stress and the reduction in body weight that occurred when the bulls were weaned. In fact, the rate of body growth was decreased at 8 mo (figure 1.1). This hypothesis can be supported by the results of Printz et al. (1970), which showed that stress can cause inhibition of FSH secretion in cyclic hamsters. Finally, the increase in FSH after 8 mo was coincident with similar increases in T, LH and testis size and is possibly related to the proliferation of germ cells, differentiation of spermatids, secretion of ABP by the Sertoli cell and enhancement of LH-stimulated testosterone production by the Leydig cell through Sertoli cell-secreted factors (MacDonald et al., 1990; Jégou and Sharpe, 1993; Bardin et al., 1994; Sharpe, 1994). Within and across ages, there were high correlations between basal and GnRH-stimulated FSH, even when only one blood sample was used to estimate basal hormone concentrations. Thus, peripheral FSH 1.5 and 3 h after a GnRH challenge are reliable indicators of age-related changes in basal secretion in young bulls.

In the Angus bulls of this study, basal LH showed an initial increase at 2 mo and a second one at 8 mo. There was, however, a transient decrease in LH at 10 mo, but the reason for such change is not known. These results are in agreement with studies performed by Rawlings et al. (1978). These authors reported that peripheral levels of LH in Angus x Hereford bulls increased form 0.40 ng/ml at 2.5 mo to 1.0 ng/ml at 3.5 mo, decreased to 0.50 ng/ml at 5 mo, returned to 1.0 ng/ml at 7.5 mo, and increased further to 1.2 ng/ml at 10 mo. Evans et al. (1993) also showed that basal LH in Hereford bulls was around 0.70 ng/ml at 2

mo, decreased to as low as 0.25 ng/ml at 7 mo and increased again to 0.75 ng/ml at 11 mo. Thus, there appear to be two major changes in the pattern of LH secretion in the prepubertal beef bull. The first one early in life, at the time when there is a large increase in both number and mass of the Leydig cell per testis (Wrobel, 1990), and the second between the ages of 6 and 8 mo, which is coincident with the appearance of meiotic cells in the seminiferous tubules, increase in testis growth, and continued increase in Leydig cell mass (Wrobel, 1990). The mean Leydig cell mass in the bovine testis increases 25-fold between 2 and 4 mo of age, remains constant from 4 to 5 or 6 mo, but resumes growth until 12 mo (Wrobel, 1990).

The response of LH to GnRH was always greater than that of FSH, in accordance with the fact that the former is more dependent on GnRH activity than the latter (Price, 1991). Across ages, patterns of GnRH-stimulated LH resembled those of basal LH, with the highest levels being detected at 2 mo and after 8 mo. However, in contrast to what was found for FSH, within-age correlations between basal and GnRH-stimulated LH were not significant. In other words, bulls with high basal did not have high GnRH-stimulated LH. Causes for this are not known, but these results emphasize the differences that exist between the response of LH- and FSH-secreting gonadotrophs to changes in GnRH.

Age-related changes in basal T paralleled those of LH. Curtis and Amann (1981) reported that testosterone in Holstein bulls reached a peak at an earlier age than did in Angus bulls used in this study (6.5 mo versus 10 mo) but the magnitude of the peaks were similar in these two breeds (7 - 8 ng/ml and 6.5 ng/ml). Since high levels of T are required for the initiation of spermatogenesis (Amann, 1983), the results presented here suggest that Angus bulls reached puberty at an older age than did Holstein bulls (Curtis and Amann, 1981).

Moreover, testosterone concentrations in Hereford bulls studied by Evans et al. (1993) reached peaks of 5 and 5.5 ng/ml at the ages of 10 and 11 mo, suggesting that these bulls also attained puberty at an older age than did dairy bulls.

Exogenous GnRH caused significant increases in T secretion at all ages. The difference between basal and GnRH-stimulated concentrations of T increased with age probably as a consequence of maturation of the Leydig cells (Wrobel, 1990). Contrary to the results of this study, an early report suggested that exogenous GnRH did not cause significant increases in T secretion if bulls were 4 mo or younger (Mongkonpunya et al., 1975). This difference may be caused by differences in RIA methods or specificity of antibodies.

The highest concentrations of Δ_4A were detected when bulls were 3- to 4-mo old, in accordance with what was found by Malak and Thibier (1975). However, these authors did not report a second increase in Δ_4A between 8 and 10 mo, coinciding with increases in T, FSH and LH (figures 1.1 through 1.5). Also, the fact that Δ_4A at early ages correlated with T at 10 mo, and, within ages, basal and GnRH-stimulated Δ_4A were related to each other and to T and LH suggest that most of the Δ_4A measured in peripheral blood is of gonadal origin and that prepubertal Δ_4A concentrations are indicators of T secretion at puberty.

Basal concentrations of E2 in Angus bulls changed from 2.58 pg/ml at 2 mo, 3.71 pg/ml at 3 mo to 3.79 pg/ml at 8 mo. Also, E2 in these bulls increased significantly after 8 mo, reaching nearly 10 pg/ml at the age of 12 mo (figure 1.5). Amann et al. (1986) found similar values in Holstein bulls at the ages 2.5, 3.5 and 4.5 mo (1.8, 3.0 and 2.0 pg/ml, respectively). Moreover, the results reported here are in perfect agreenment with those by Evans et al. (1993). These authors showed that, in Hereford bulls, E2 concentrations remained at 2 to 5

pg/ml between the ages of 1 and 7 mo, but increased thereafter reaching 11 ng/ml at the age of 12.5 mo.

The reason for the increase in E2 after the age of 8 mo is not known, but it is possible that there is a shift in the control of E2 secretion in the prepubertal bull. Dorrington and Armstrong (1979) reported that rat Sertoli cells secreted E2 in response to FSH at ages between 5 and 30 days, but not thereafter. Also, Dorrington and Khan (1993) suggested that, once the Sertoli and Leydig cells differentiate and spermatogenesis starts in the rat, the Sertoli cell loses its ability to aromatize androgens and Leydig cells decrease the synthesis of 5α-reduced androgens (such as dehydrotestosterone) and increase that of testosterone, which in turn can be partially converted to estrogens. Detailed studies about the source of E2 in the immature bull are not available but Amann and Ganjam (1976) reported that an injection of hCG in mature Holstein bulls caused significant increases in E2 measured in the spermatic vein. These results suggest that the Leydig cell could be the source of E2 in the mature bull. Also, in this study, E2 was negatively correlated with FSH from 2 to 6 mo and positively correlated with LH from 6 to 12 mo. Moreover, basal and GnRH-stimulated E2 was related to T at all ages. From such results, it can be concluded that E2 measured in the peripheral blood of Angus bulls was of testicular origin and, before 6 mo of age, E2 secretion is regulated by FSH and probably released by the Sertoli cell. After 6 mo, E2 secretion could be shifted to the Leydig cell and controlled by LH. However, because aromatase is expressed by germ cells isolated from the mouse testis (Nitta et al., 1993), it is possible that part of the E2 detected in serum could have been secreted by germ cells. Also, there is evidence that human osteoblasts express aromatase (Purohit et al., 1992), and therefore, the possibility that

peripheral production of E2 in the bull may occur can not be ruled out. Given that E2 can be produced by cells other than the Sertoli and Leydig, the identification of the precise source(s) of E2 in the bull awaits studies of cell types that express the aromatase enzyme at different phases of development.

Relationships between yearling testis size, number of Sertoli and germ cells and degree of seminiferous tubule development

The average number of Sertoli cells per testis estimated in this study was $4.59 \pm 0.32 \times 10^9$, ranging from 1.63 to 8.47×10^9 (table 1.1), which is similar to that found in Holstein bulls (4 to 13×10^9 per testis, Berndtson et al., 1987a), Normand bulls (average = $5.4 \pm 3.6 \times 10^9$, DeReviers et al., 1987) and Hereford and Angus bulls (3 to 11.5×10^9, Berndtson et al., 1987b). Also, in this study, an estimated average of 6.3 spermatids per Sertoli cell and 42 spermatids per A1 spermatogonium was observed. Berndtson et al. (1987a, b) found 4 to 12 spermatids per Sertoli cell and 18 to 46 spermatids A1 per spermatogonium in mature bulls and 2 to 8 spermatids per Sertoli cell in 1.5-year old beef bulls.

Erickson and Blend (1976) found a close relationship between Sertoli cell numbers and testis size in the rat by showing that irradiation-induced loss of Sertoli cells caused an irreversible decrease in testis weight and sperm-producing capacity. Later, other studies showed positive correlations between number of Sertoli cells and testis size and DSP in men (Johnson et al., 1984) and bulls (Berndtson et al., 1987a,b). In the present study, number of Sertoli cells was related to testis size, and number of A1 spermatogonia and round spermatids per testis. Bulls with large testes had a high number of spermatids per Sertoli cell and a low

rate of germ-cell degeneration, as evidenced by the high spermatid / A1 ratio. Thus, in addition to a high number of Sertoli cells, the ability of each Sertoli cell in bulls with large testes to support the development of germ cells was also significant and, when combined, these two criteria accounted for 90 % of the variation in yearling testis weight. Berndtson et al. (1987a, b) reported a negative correlation between number of round spermatids per Sertoli cells ($r = -0.40$) and total number of Sertoli cell per testis, while in this study such correlations were positive ($r = 0.43$). The reason for these differences might be related to the fact that Berndtson and colleagues used older bulls (> 1.5 years).

As expected, heavier testes had more sections with spermatids and fewer sections without germ cells or with A1 spermatogonia only. Also, the fact that Sertoli and germ-cell numbers per testis and cell ratios were negatively correlated with ST0 and positively related to STD or STE demonstrate the existence of a high degree of association between testis size and distribution of the germ cells within the seminiferous tubules.

Relationships between hormonal concentrations, testis size, Sertoli and germ cell numbers and degree of seminiferous tubule development

Among the group of bulls used in this study, 3 bulls had no spermatids at the age of 12 mo and, therefore, were infertile (for more details, see next section below). However, data from these bulls were kept in the analysis of regression and correlation because the original objective of this study was to identify the potential sperm production of yearling bulls while they were still immature. In other words, the objective of this study included the identification of infertile and/or low sperm-producing bulls by analyzing prepubertal hormone

concentrations while bulls were still at some ages when abnormalities in the spermatogenic cycle could not be detected accurately. Based on my previous results (unpublished), body weight, and size and number of germ cells (gonocytes and spermatogonia) of one testis surgically removed were normal in a 5-mo old bull and, thus, did not serve to indicate that this bull had no elongate spermatids in the remaining testis at the age of 12 mo. Also, the existence of low correlations ($r < 0.47$) between testis diameter at ages earlier than 5 mo and testis weight at 12 mo (table 1.1) suggests that differences between infertile and fertile bulls can not be detected with reliability before 5 mo and, therefore, testicular development in bulls of this study may be considered normal at young ages.

Among the hormones quantified, FSH was the one that accounted for the most variation in testis weight of the yearling bull. As evidenced by regression analysis, concentrations of FSH and E2 at 2 mo accounted for 45 % of the variation in testis weight. Moreover, when FSH and testis diameter at 6 mo were combined, the R^2 obtained increased to 0.57 and a model with FSH and testis diameter at 9 mo accounted for 67 % of the variation in yearling testis weight. Also, FSH and body weight at 12 mo gave an R^2 of 63 %. Since testis size was correlated with Sertoli and germ cell numbers, cell ratios and degree of ST development, it was not surprising that these criteria were also inversely related to FSH. Therefore, prepubertal concentrations of FSH could be used to predict the potential sperm-producing capacity of the yearling bull. However, because the group of bulls used in this study had infertile ones, it is not known if results presented here will also be found in samples with normal bulls only.

Swanson et al. (1971) observed that the pituitary content of FSH in 12-mo old

Hereford bulls was negatively correlated with epididymal sperm numbers. Also, Malak and Thibier (1985) showed that FSH responses to exogenous GnRH was related to quantitative and qualitative aspects of semen production in yearling Montbéliarde bulls ($r = -0.40$ to -0.70). Schanbacher (1990) reported that, within a group of bulls that had been immunized against inhibin, FSH was inversely related to testis weight ($r = -0.70$). Erickson and Whaley (1988) showed that yearling bulls destined to have heavier testis at 12 mo secreted 51 % less FSH in response to GnRH at 2 and 6 mo than did those destined to have small testis but no correlation between FSH and Sertoli or germ cell numbers was detected. Thus, results presented here are the first to show that FSH measured between 2 and 12 mo was not only negatively related to yearling testis size but also to Sertoli cell numbers, yield of germ cells per testis and per Sertoli cell and rate of germ cell degeneration.

The reason for such negative correlations is not known but it can be hypothesized that heavier testes secrete higher concentrations of steroids, which in turn control the synthesis and secretion of gonadotropins and GnRH and promote the activity of the Sertoli cell. In this regard, results from this study showed that concentrations of T and E2 at 2, 3 and 4 mo were positively correlated with yearling testis size, although the r values were low ($r = 0.40$ to 0.45). Also, since inhibin specifically regulates the secretion of FSH (Vale et al., 1994) and there is an inverse relationship between those two hormones in bulls after the age of 10 weeks (MacDonald et al., 1990), it is possible that yearling bulls with heavier testis secreted higher concentrations of inhibin throughout the prepubertal phase of development. Finally, the fact that FSH accounted for only part of the variation in testis size suggests that other systemic or intratesticular hormones or growth factors may also play crucial roles in determining the

number of Sertoli and germ cells.

Testicular development and hormone profiles of infertile bulls

In this study, three distinct cases of spermatogenic arrest are reported. The case of bull #250 was characterized by the complete absence of germ cells and that of bull #345 by the presence of only Sertoli cells in the majority of the seminiferous tubules and impaired differentiation of A1 spermatogonia in the remaining. At the age of 1 year, the diameter of the seminiferous tubules in these bulls was 80 % of the normal values, but testis weight was more significantly reduced and represented only 40 % of that of normal bulls. Bull #389 had spermatogenic arrest at the level of the pachytene spermatocyte, reduced diameter of the tubules and testes that weighed only 12 % of normal.

Morphological features and size of the nucleus and nucleolus of Sertoli cells in bulls #250 and 345 resembled those present in testes with normal spermatogenesis. More detailed analysis, however, could reveal other structural changes due to the absence of germ cells. In this regard, deFrança et al. (1994) showed that, in mutant mouse testes lacking germ cells, the volume of the cytoplasm, nucleus and smooth endoplasmic reticulum of the Sertoli cell was significantly reduced. Also, several studies have shown that loss of germ cells caused a decrease in the surface area of the apical region of the Sertoli cell (Sinha Hikin et al., 1989; Chosh et al., 1992). Thus, although the Sertoli cells in the testis of bulls #250 and 345 did not resemble immature cells, it is possible that they were not fully developed and ultrastructural changes occurred as a consequence of the absence of germ cells. Neither individual volume nor numerical density of the Leydig cells in bulls #250 and 345 were affected by seminiferous

tubule damage, showing that the local and systemic mechanisms involved in the control of Leydig cell growth were not affected.

These cases of infertility were unlikely to be caused by deficiency of gonadotropins because concentrations of FSH and LH in these bulls were either normal or elevated at ages from 2 to 12 mo. Concentrations of T increased slowly prior to 5 mo, but came within the normal range afterwards. In rats immunized against GnRH, spermatogenesis can proceed with 20 - 40 % of the normal concentrations of intratesticular testosterone and, in the presence of FSH, the requirements for testosterone are much lower (reviewed by Weinbauer and Nieschalg, 1993). Thus, the change in the pattern of T secretion in bulls #250 and #345 did not appear to be sufficient to cause a dramatic process of germ-cell failure.

In addition to low testosterone levels before the age of 5 mo, bull #250 had lower LH- and T-responses to GnRH at almost all ages. In the case of bull #345, GnRH-stimulated concentrations of LH were normal, but those of T were reduced after the age of 8 mo. Given that the number of Leydig cells was normal in these bulls, such results suggest that some impairment of the Leydig cell's ability to optimally respond to a GnRH-mediated increase in LH had occurred. Treatment of rats with FSH increased testosterone production under LH stimulation (reviewed in Sharpe, 1982). Also, FSH can increase the number of LH receptors in the testis and Sertoli cells secrete factors that are stimulated by FSH and able to enhance steroidogenesis in rat and human Leydig cell cultures (Sharpe, 1993). Thus, because the absence of germ cells potentially causes structural (deFrança et al., 1994) and functional (Sharpe, 1993) change in the Sertoli cell, low T-response to GnRH in bull #250 and 345 could be related to abnormal function of Sertoli cells.

Besides the reduction in T secretion, GnRH-stimulated Δ_4A in bull #250 tended to be lower and in bull #345 higher than normal values, which suggests that steroidogenesis was affected differently by lack of germ cells in these two bulls. However, because the magnitude of the changes in Δ_4A was low more studies would be needed to test this hypothesis.

It is unclear why, at the ages of 4, 5 and 6 mo, the LH response to GnRH was low in bull #250. This could be related to lower levels of T detected at 4 and 5 mo. Also, since production of FSH and LH is carried out by separate cells (Bastings et al., 1991), it is possible that this bull had a defective population of LH secreting cells.

As mentioned above, the case of Sertoli cell-only syndrome described in bull #250 was characterized by normal levels of LH and FSH and reduced T at some ages. Such results are in agreement with those reported by Rao Veeramachanemi et al. (1986). They showed that a 2-year old Hereford bull with no germ cells had normal peripheral concentrations of FSH but reduced frequency of LH and T pulses. However, Sertoli cell-only syndrome described in mice (deFrança et al., 1994) and humans (Bergman et al., 1994) correlated with high FSH and normal T. Thus, results presented here suggest that the effects of germ cell absence on the testis-mediated control of pituitary activity in the bull are different from those in the mouse or human.

Agametic seminiferous tubules have been linked to mutations in the mouse w and sl genes, which code for the c-kit tyrosine kinase receptor and its ligand, the Steel Factor, respectively. The Steel Factor is secreted by the Sertoli cell and essential for the survival of primordial germ cells (PGC) and proliferation of A1 spermatogonia (Kierszenbaum, 1994; Tajima et al., 1994). Mintz (1957) and Mintz and Russel (1957) showed that some of the

mutations in the w-locus caused impairment of the migration of the PGC to the gonadal ridges and degeneration of those cells that did reach the testis. Moreover, Logue et al. (1979) reported that a Friesian bull with Klinefelter's syndrome (61 XXY) had small testes and seminiferous tubules devoid of germ cells. In male mice that were homozygous for the mutant gene termed juvenile spermatogonial depletion (jsd), testes weighted 1/3 to 1/4 of the normal ones and the few germ cells present in the seminiferous tubules failed to advance beyond the A1 spermatogonial stage. Normal T levels were detected in those mice but FSH was elevated. Thus, based on the similarities between the infertility cases described in mice and bulls in the present study, it is possible that spermatogenic arrest in bull #250 and 345 could be caused by either gene mutations or chromosomal abnormality.

Histological analysis of the testis of bull #389 revealed that spermatogenesis was interrupted at the level of pachytene spermatocytes. In this bull, the size of the Leydig cell and its number per testis were normal, but the numerical density was 3 times higher than that in normal bulls. Also, reduced number of Sertoli cells and A1 spermatogonia, testis size, seminiferous tubule diameter and body weight were found. The case of bull #389 is similar to those reported by Gargiulo et al.(1991). These authors described two 18-mo old Brown Swiss bulls that had small testes and seminiferous tubules and few germ cells detected did not develop beyond the primary spermatocyte. However, neither morphological and quantitative aspects of the Sertoli cell nor hormone profiles of these animals were provided.

Concentrations of basal FSH and LH were consistently higher in bull #389 than in normal bulls. The reason why only LH increased linearly with age is uncertain but it may reflect the lack of steroid negative feedback at the level of the hypothalamus, which would

lead to an increase in the activity of the GnRH neuron. This way, the pattern of LH secretion, more than that of FSH, would follow the increase in GnRH.

High levels of FSH and LH may have resulted from low levels T and E2 that were detected at almost all ages. It has been shown in rats and humans that the absence of elongate spermatids was associated with a decrease in inhibin and an increase in FSH (Sharpe, 1993; Bergman et al., 1994). Also, in the rat, high FSH is associated with cases of Sertoli cell-only syndrome (deFrança et al., 1994) and spermatogenic arrest at the level of primary spermatocytes (Gill III et al., 1979; Noguchi et al., 1993). Schanbacher (1991) showed that bulls immunized against inhibin (at prepuberty) produced more spermatozoa per g of testicular parenchyma than did control bulls at the age of 1 year. Thus, it is possible that bull #389, besides having reduced serum levels of steroids, also secreted less inhibin. However, bulls #250 and 345 did not have spermatids and had normal concentrations of FSH and LH. Consequently, the absence of spermatids in the bull can not be always linked to an increase in FSH.

GnRH-stimulated LH and the difference between basal and GnRH-stimulated FSH were lower in bull #389 than in normal bulls. High basal and low GnRH-stimulated gonadotropins may indicate that, due to low levels of T and E2, most of the hormone synthesized was promptly released and less was stored in the gonadotrophs.

From 2 to 5 mo, peripheral levels of basal and GnRH-stimulated Δ_4A in bull #389 were normal but those of T were significantly reduced. Because there was no deficiency of gonadotropins at any age, such results suggest that, in the calf, interactions among immature Leydig and Sertoli cells and early germ cells (gonocytes and spermatogonia) were impaired.

After the age of 5 mo, concentrations of T continued to be lower in bull #389 than in normal bulls but those of Δ_4A became substantially higher, suggesting that sufficient expression of the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) in the Leydig cell had not occurred. In the normal bulls used in this study, levels of Δ_4A decreased after 4-5 mo of age while those of T continually increased. It is accepted that this shift from Δ_4A to T as the main androgen produced by the testis is the most important indicator of Leydig cell maturation (Amann, 1981). Proliferation of adult-type Leydig cells is controlled by FSH and differentiation of the Sertoli cell and testosterone synthesis by the Leydig cell can be enhanced by Sertoli cell factors stimulated by FSH (Jégou and Sharpe, 1993; Sharpe, 1993). Also, in the rat, E2 is able to inhibit Leydig cell proliferation via a local pathway (Abney and Meyers, 1991). Thus, based on these pieces of evidence, the following cascade of events are suggested in the case of bull #389: 1) the Sertoli cells failed to undergo complete differentiation, as shown by their morphological aspects (discussed below); 2) Sertoli cells may have responded poorly to FSH and not secreted the factors required to trigger the differentiation of the Leydig cells, and as a consequence, the latter continued to proliferate; 3) these immature Leydig cells failed to convert the Δ_4A into testosterone and released high levels of Δ_4A in response to high basal or GnRH-stimulated LH; 4) considering the hypothesis about the sites of E2 synthesis discussed above, it is possible that as the result of a low supply of T, these Leydig cells secreted less E2; and 5) Because it has been shown that E2 can inhibit Leydig cell proliferation (Abney and Mayers, 1991; Hsueh et al., 1978), low E2 in bull #389 may have contributed to abnormal growth of the Leydig cell population.

Morphological features of the Sertoli cells of bull #389 resembled those of immature

ones. The round or oval shape of the nucleus, the limited occurrence of indentations on the nuclear membrane and the presence of a split nucleolus are characteristics of undifferentiated bovine Sertoli cells, as described by Sinowartz and Amselgruber (1986). It is known that FSH is important for Sertoli cell mitosis and differentiation (Bardin et al., 1988) but studies have suggested that T is also involved in this process. Walker et al. (1984) showed that Holstein calves immunized against T had significant increases in testis size and sperm production at the age of 18 mo, probably because low levels of T delayed the differentiation of the Sertoli cells and allowed them to proliferate for a longer period of time. Orth et al. (1984) showed that, in hemicastrated neonatal rats, exogenous T prevented the mitosis of Sertoli cells in the remaining testis. Although cessation of Sertoli cell proliferation could be affected by a T-mediated negative feedback on FSH, a direct effect of T on the Sertoli cells can not be ruled out because these cells express androgen receptors (Sar et al., 1993). Thus, the case of bull #389 provides new evidence that T is involved in the process of Sertoli cell differentiation.

Gill III and Kunz (1979) and Kunz et al. (1980) reported that rats homozygous for the growth and reproduction complex (grc) had reduced testis size and body weight (12 % and 65 % of normal values, respectively) and were sterile because of an arrest of spermatogenesis at the level of primary spermatocytes. Also, both FSH and LH were higher but T was lower in affected rats than in normal ones. Given the similarities between this case and the one reported for bull #389, it is possible that the latter is related to the same genetic defects. Also, these similarities provide directions for future studies dealing with genetic markers of infertility in the bull.

In summary, this study describes, for the first time, age-related changes in peripheral

hormone concentrations of bulls with the Sertoli cell-only syndrome and spermatogenic arrest at the level of A1 spermatogonia and pachytene spermatocyte. A deficiency in gonadotropin and steroid secretion did not appear to be the primary cause of infertility, which points towards other genetic abnormalities as the cause of impaired germ cell development. Also, such cases of seminiferous tubule anomaly could be useful models for the study of cell-cell interactions in the testis.

Table 1.1: Pearson's correlations between yearling testis diameter (TD) and weight (TW) and testis diameter measured in Angus bulls at ages from 2 to 11 months (n=24).

	Age at which testis diameter was measured								
	2 mo	3 mo	4 mo	5 mo	6 mo	8 mo	9 mo	10 mo	11 mo
TW	NS	NS	0.42 a	0.55 b	0.61 c	0.56 b	0.77 c	0.87 c	0.91 c
TD	NS	NS	0.47 b	0.59 c	0.67 c	0.53 c	0.77 c	0.85 c	0.92 c

NS: nonsignificant ($p > 0.10$); a, b and c : $p < 0.10$, $p < 0.05$ and $p < 0.01$, respectively.

Table 1.2: Averages, standard errors and ranges of cell counts per testis and cell ratios per seminiferous tubule (ST) cross sections in yearling Angus bulls (n=20).

	Total cell counts per testis			Cell ratios per ST sections		
	SC ($x10^9$)	A1($x10^8$)	RST($x10^{10}$)	RA	RS	AS
Average	4.59	5.85	0.85	42.26	6.31	0.13
S.E.M.	0.32	0.74	0.58	9.45	1.41	0.03
Range	1.63 - 8.47	0 - 10.6	0 - 5.38	0 - 80.91	0 - 10.04	0 - 0.21

SC, A1 and RST: Sertoli cells, A1 spermatogonia and round spermatids per testis, respectively.
RA, RS and AS: number of round spermatids per A1 spermatogonia, and number of round spermatids and A1 spermatogonia per Sertoli cell, respectively.

Table 1.3: Averages, standard errors and ranges of degrees of seminiferous tubule development in yearling Angus bulls (n=20).

	Percent of tubule cross-sections *						
	ST0	STA	STB	STP	STD	STE	STM
Average	20.6	3.81	1.86	5.27	31.75	28.05	8.55
S.E.M.	6.45	1.59	1.25	1.31	3.49	3.62	1.74
Range	0 - 100	0 - 30	0 - 5	0 - 25	0 - 47	0 - 50	0 - 32

*Percent of tubule sections: without germ cells (ST0), with A1 spermatogonia (STA), Intermediate or B spermatogonia (STB), pachytene spermatocytes (STP), round spermatids (STD), elongated spermatids (STE) or mature spermatids (STM).

Table 1.4: Pearson's partial correlations between yearling testis weight (TW) and diameter (TD), number of Sertoli cells (SC), A1 spermatogonia (A1) and round spermatids (RST) per testis, and cell ratios* per seminiferous tubule (ST) cross-sections in Angus bulls (n=20).

		Total cell counts per testis			Cell ratios per ST section		
	TW	SC	A1	RST	RA	RS	AS
TW	-	0.85 c	0.80 c	0.79 c	0.73 c	0.69 c	0.54 b
TD	0.96 c	0.84 c	0.79 c	0.79 c	0.69 c	0.73 c	0.57 b
SC	-	-	0.69 c	0.72 c	0.62 c	0.43 a	-
A1	-	-	-	0.89 c	0.54 b	0.76 c	0.81 c
RS	-	-	-	-	0.83 c	-	0.85 c

*Cell ratios: RA=number of round spermatids per A1 spermatogonia; RS and AS=number of round spermatids and A1 spermatogonia per Sertoli cell.
Partial variable: body weight at the age of 12 months.

Table 1.5: Pearson's partial correlations between yearling testis weight (TW), total number of Sertoli cells (SC), A1 spermatogonia (A1) and round spermatids (RST) per testis, number of round spermatids per Sertoli cel (RS) and degree of seminiferous tubule (ST) development* in Angus bulls (n=20).

	Degree of seminiferous tubule development *						
	ST0	STA	STB	STP	STD	STE	STM
TW	-0.65 c	-0.52 b	-0.51 b	-0.23	0.70 c	0.79 c	0.47 b
SC	-0.45 a	-0.48 b	-0.47 b	-0.34	0.55 b	0.64 c	0.31
A1	-0.80 c	-0.44 a	-0.35	-0.27	0.79 c	0.88 c	0.47 b
RST	-0.79 c	-0.53 b	-0.41 a	-0.49 b	0.80 c	0.85 c	0.56 b
RS	-0.81 c	-0.56 b	-0.46 b	-0.23	0.85 c	0.84 c	0.46 b

*Degree of ST development: ST0 = no germ cells; STA = A1 spermatogonia; STB = intermediate B type spermatogonia; STP =pachytene spermatocytes; STD = round spermatids; STE = elongate spermatids; STM = mature spermatids.
Partial variable: body weight at the age of 12 months.
a, b and c: $p < 0.10$, $p < 0.05$ and $p < 0.01$, respectively.

Table 1.6: Pearson's partial correlations between peripheral concentrations of basal and GnRH-stimulated FSH measured from 2 to 12 mo and yearling testis weight (TW) and diameter (TD) in Angus bulls (n=24).

1.6A	Age at which FSH concentration was measured									
	2 mo		3 mo		4 mo		5 mo		6 mo	
FSH/	Basal	GnRH	Basal	GnRH	Basal	GnRH	Basal	GnRH	Basal	GnRH
TW	-0.56 c	-0.54 c	-0.62 b	-0.57 c	-0.45 b	-0.36	-0.39 a	-0.37 a	-0.42 b	-0.47 b
TD	-0.67 c	-0.57 c	-0.70 c	-0.67 c	-0.60 c	-0.47 b	-0.55 c	-0.53 c	-0.59 c	-0.60 c

1.6B	Age at which FSH concentration was measured									
	8 mo		9 mo		10 mo		11 mo		12 mo	
FSH/	Basal	GnRH	Basal	GnRH	Basal	GnRH	Basal	GnRH	Basal	GnRH
TW	-0.47 b	-0.45 b	-0.57 c	-0.71 c	-0.62 c	-0.47 b	-0.56 c	-0.64 c	-0.61 c	-0.64 c
TD	-0.63 c	-0.59 c	-0.53 c	-0.58 c	-0.73 c	-0.50 b	-0.70 c	-0.67 c	-0.74 c	-0.70 c

For each age-period, body weight was used as a partial variable in the analysis of correlation.
a, b and c : $p < 0.10$, $p < 0.05$ and $p < 0.01$, respectively.
FSH/Basal: the average of 3 blood samples taken at 1.5-h intervals.
FSH/GnRH: the average of FSH concentrations measured 1.5 and 3 h after GnRH treatment.

Table 1.7: Pearson's partial correlations between peripheral concentrations of basal and GnRH-stimulated FSH measured from 2 to 12 mo and number of Sertoli cells (SC) and round spermatids per testis (RST), number of round spermatids per Sertoli cell (RS), number of round spermatids per A1 spermatogonia (RA) and number of A1 spermatogonia per Sertoli cell, and degree of seminiferous tubule (ST) development* in Angus bulls (n=20).

1.7A	Age at which FSH concentration was measured									
	2 mo		3 mo		4 mo		5 mo		6 mo	
FSH/	Basal	GnRH	Basal	GnRH	Basal	GnRH	Basal	GnRH	Basal	GnRH
SC	-0.51 b	NS	-0.54 b	-0.54 b	-0.43 a	-0.43 a	NS	NS	-0.41 a	-0.46 b
RST	NS	NS	-0.43 a	-0.40 a	NS	NS	NS	NS	NS	NS
RA	NS	NS	-0.57 b	-0.45 a	-0.48 b	NS	NS	NS	-0.45 a	-0.46 b
RS	NS	NS	-0.50 b	-0.45 a	-0.40 a	NS	NS	NS	-0.44 a	-0.43 a
STD	-0.40 a	NS	-0.52 b	-0.43 a	-0.46 b	NS	-0.44 a	-0.45 a	-0.51 b	-0.46 b

1.7B	Age at which FSH concentration was measured									
	8 mo		9 mo		10 mo		11 mo		12 mo	
FSH/	Basal	GnRH	Basal	GnRH	Basal	GnRH	Basal	GnRH	Basal	GnRH
SC	-0.47 b	-0.53 b	-0.50 b	-0.56 b	-0.48 b	-0.56 b	-0.51 b	-0.68 c	-0.53 b	-0.63 c
A1	NS	NS	-0.43 a	-0.42 a	-0.51 b	-0.50 b	-0.43 a	-0.59 b	-0.49 b	-0.55 b
RST	NS	NS	-0.45 a	-0.55 b	-0.55 b	-0.61 c	-0.48 b	-0.66 c	-0.53 b	-0.69 c
RA	-0.49 b	-0.48 b	-0.55 b	-0.61 c	-0.60 c	-0.60 c	-0.52 b	-0.56 b	-0.52 b	-0.57 b
RS	-0.45 a	-0.41 a	-0.56 b	-0.60 c	-0.64 c	-0.61 c	-0.56 b	-0.54 b	-0.59 c	-0.57 c
STD	-0.52 b	-0.48 b	-0.65 c	-0.57 c	-0.72 c	-0.63 c	-0.60 c	-0.62 c	-0.62 c	-0.60 c
STE	NS	NS	-0.50 b	-0.55 b	-0.59 c	-0.64 c	-0.51 b	-0.56 b	-0.57 b	-0.64 c

*Degree of ST development: STD = round spermatids; STE = elongate spermatids.
For each age-period, body weight was used as a partial variable in the analysis of correlation.
NS: nonsignificant ($p > 0.10$); a, b and c: $p < 0.10$, $p < 0.05$ and $p < 0.01$, respectively.
FSH/Basal: average of 3 blood samples taken at 1.5-h intervals.
FSH/GnRH: average of FSH concentrations measured 1.5 and 3 h after GnRH treatment.

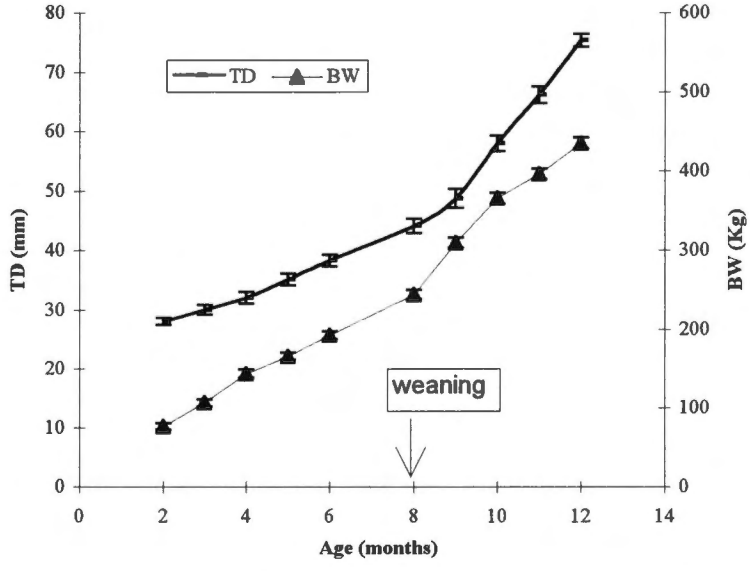


Figure 1.1: Age-related changes in testis diameter (TD) and body weight (BW) in Angus bulls (n=24)

Error bars represent the standard error of the mean within each age-period.

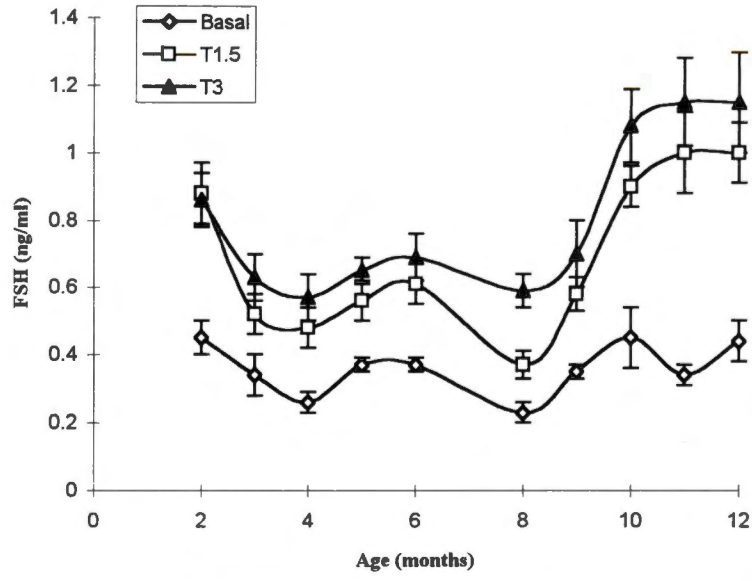


Figure 1.2: Peripheral concentrations of basal and GnRH-stimulated FSH in Angus bulls (n=24)

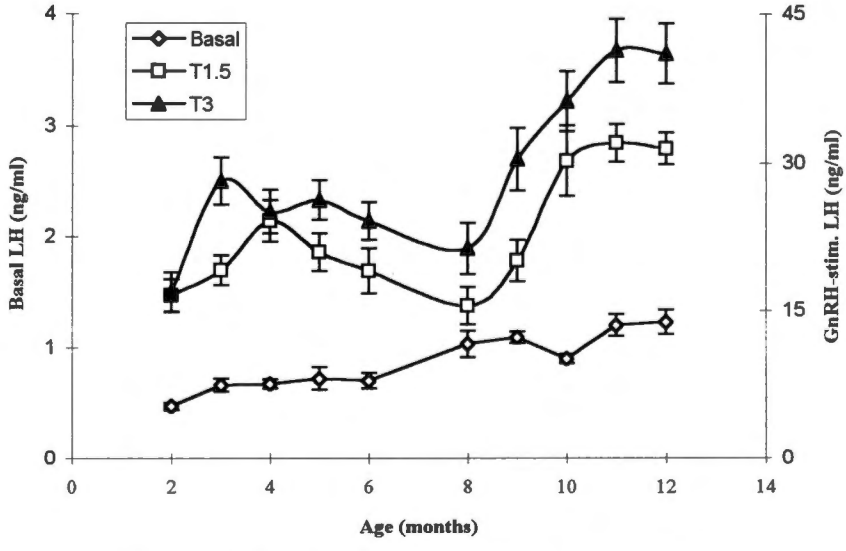


Figure 1.3: Peripheral concentrations of basal and GnRH-stimulated LH in Angus bulls (n=24)

Basal: average of 3 blood samples taken at 1.5-h intervals.
T1.5 and T3: hormone concentrations 1.5 and 3 h after GnRH treatment.
Error bars represent the standard error of the mean within each age-period.

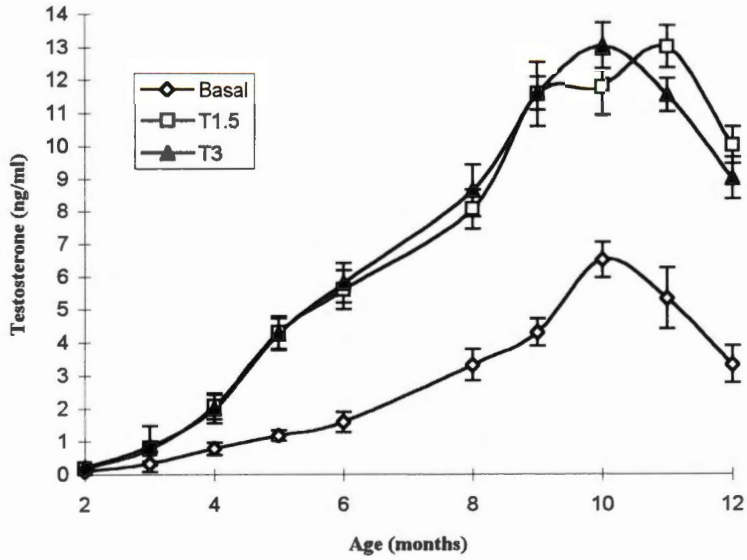


Figure 1.4: Peripheral concentrations of basal and GnRH-stimulated testosterone in Angus bulls (n=24)

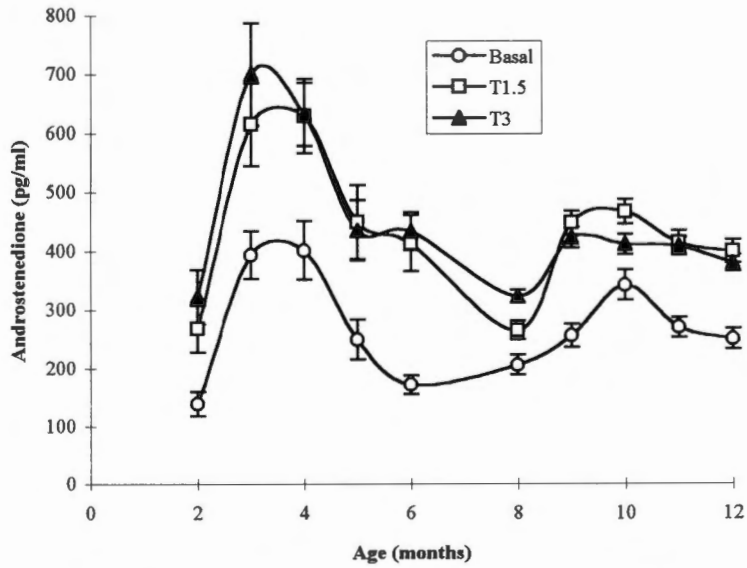


Figure 1.5: Peripheral concentrations of basal and GnRH-stimulated androstenedione in Angus bulls (n=24)

Basal: average of 3 blood samples taken at 1.5-h intervals.
T1.5 and T3: hormone concentrations 1.5 and 3 h after GnRH treatment.
Error bars represent the standard error of the mean within each age-period.

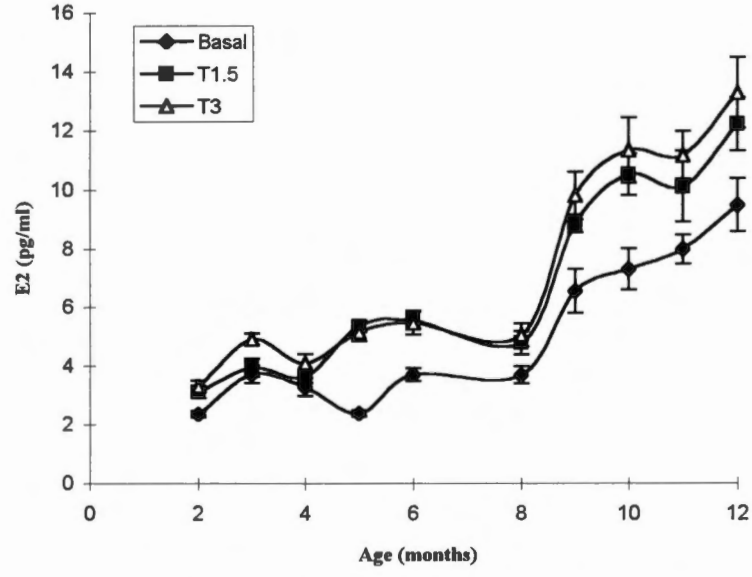
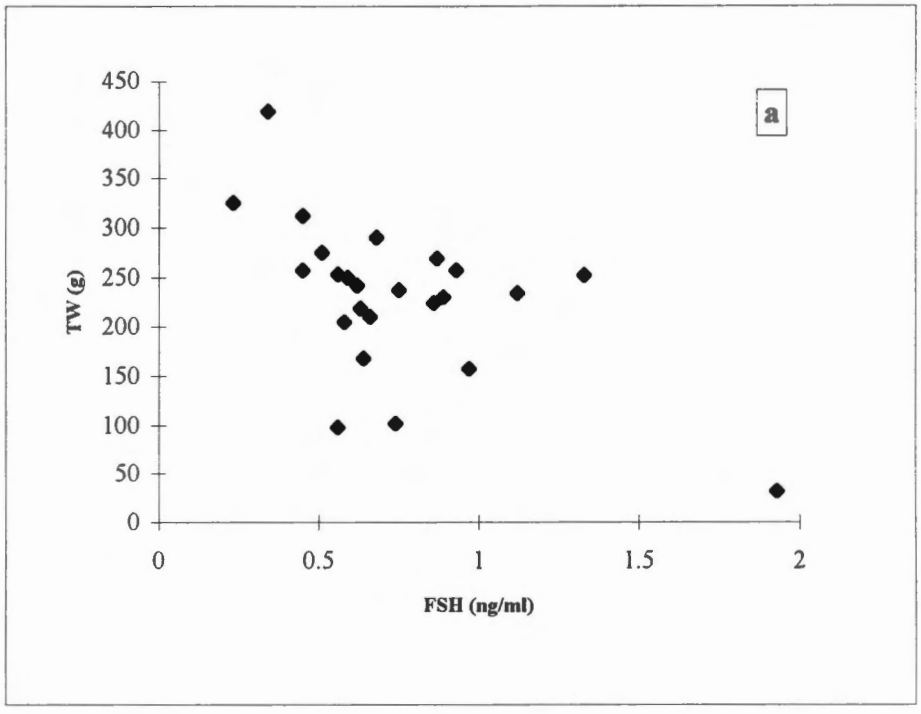


Figure 1.6: Peripheral concentrations of basal and GnRH-stimulated of E2 in Angus bulls (n=24)

Basal: average of 3 blood samples taken at 1.5-h intervals.
T1.5 and T3: hormone concentrations 1.5 and 3 h after GnRH treatment.
Error bars represent the standard error of the mean within each age-period.



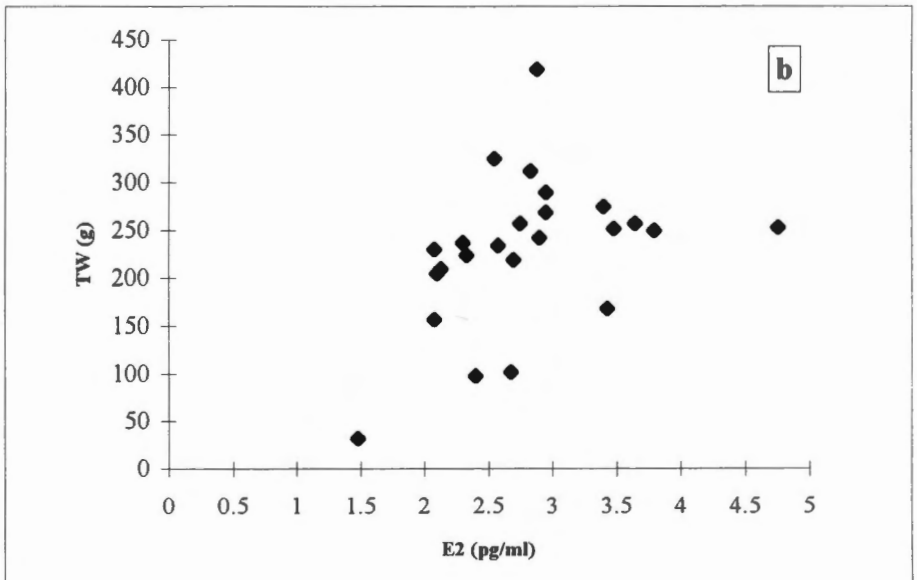
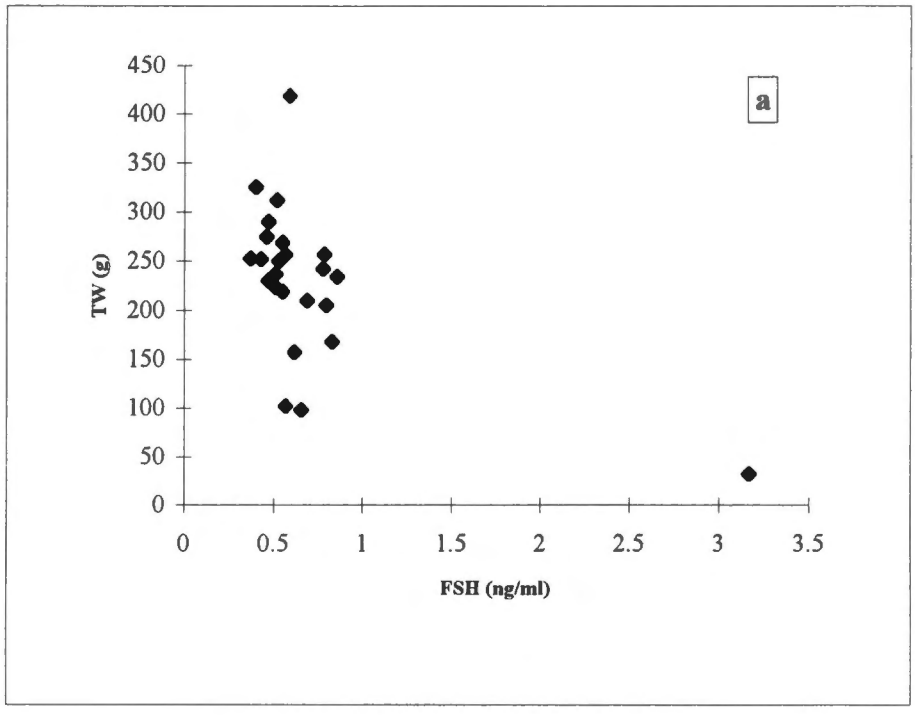


Figure 1.7: Relationship between yearling testis weight (TW) and a) FSH and b) estradiol (E2) at 2 mo in Angus bulls (n=24).

Regression model: TW = 253.1 - 124.3 x FSH + 26 x E2; with $R^2 = 0.45$ and p-values of 0.007 (FSH) and 0.09 (E2).
FSH: average between basal and GnRH-stimulated FSH at 2 mo.
E2: average GnRH-stimulated estradiol at 2 mo.



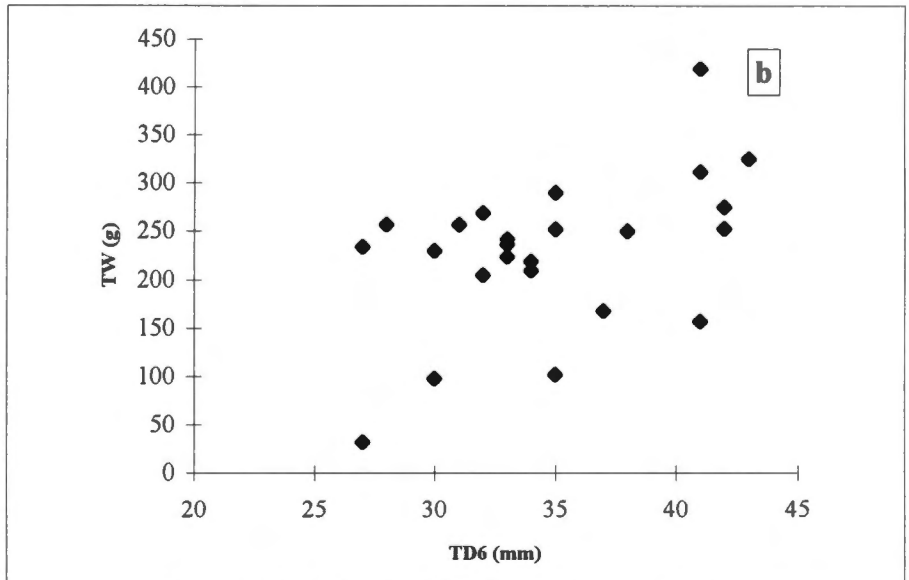
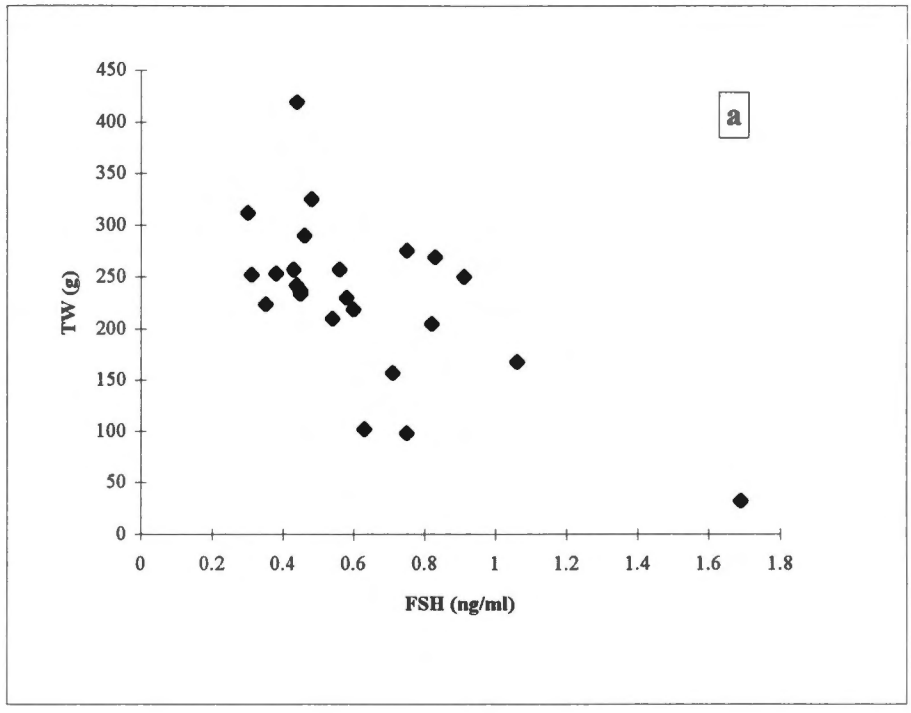


Figure 1.8: Relationship between yearling testis weight (TW) and a) FSH and b) testis diameter at 6 mo (TD6) in Angus bulls (n=24).

Regression model: TW = - 125.8 - 46.8 x FSH + 10.3 x TD6; with $R^2 = 0.57$ and p-values of 0.067 (FSH) and 0.002 (TD6).
FSH: average between basal and GnRH-stimulated FSH at 6 mo.



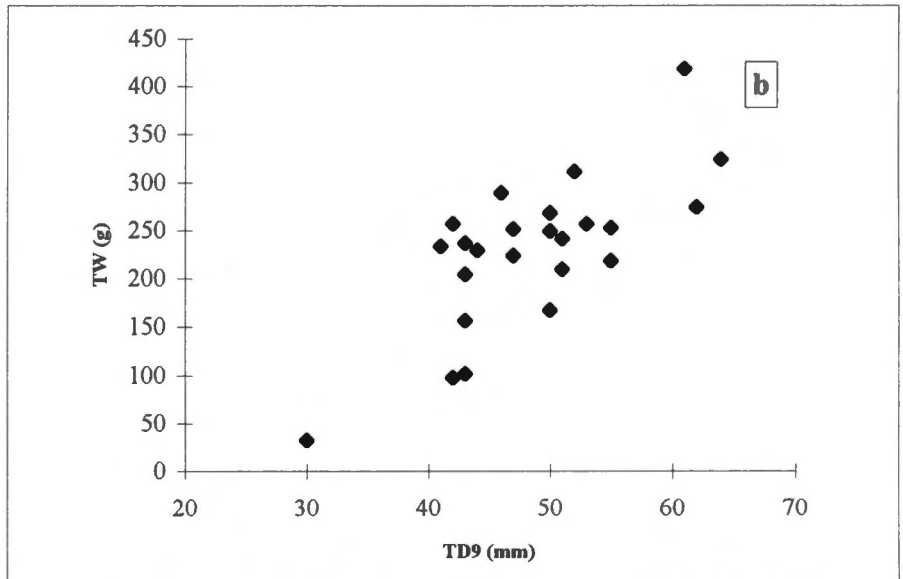
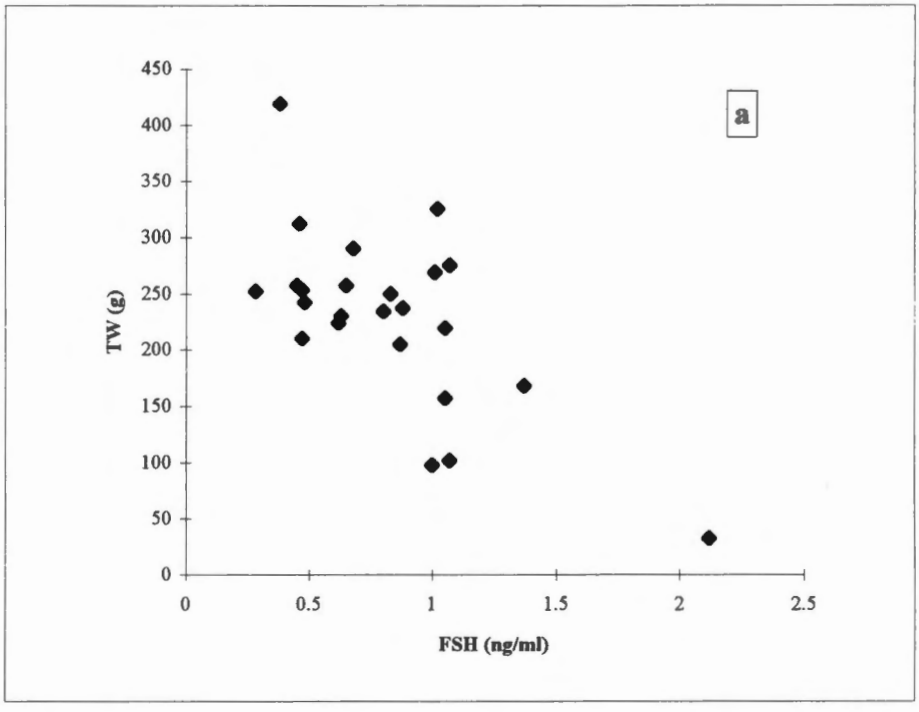


Figure 1.9: Relationship between yearling testis weight (TW) and a) FSH and b) testis diameter at 9 mo (TD9) in Angus bulls (n=24).

Regression model: TW = - 9.20 - 99.8 x FSH + 6.23 x TD9; with $R^2 = 0.67$ and p-values of 0.013 (FSH) and 0.0005 (TD9).
FSH: average between basal and GnRH-stimulated FSH at 9 mo.



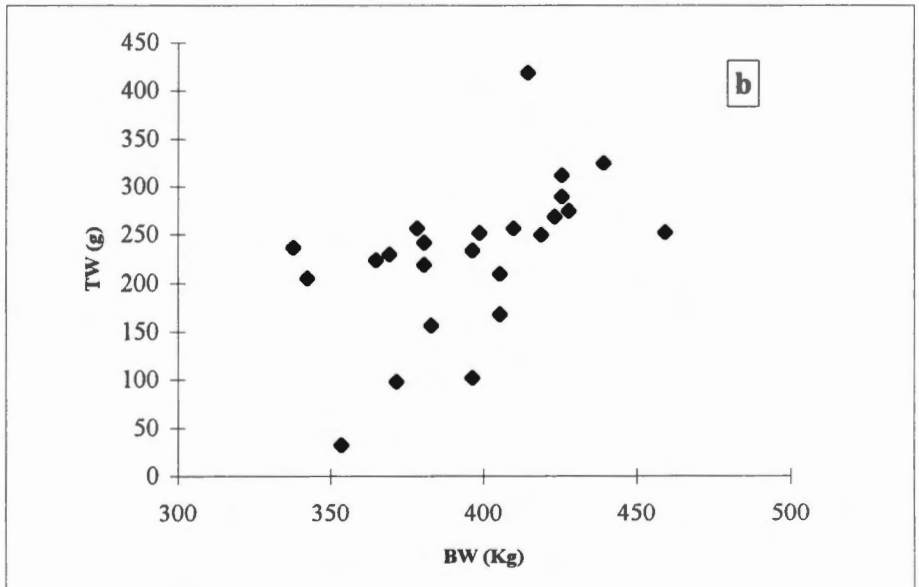


Figure 1.10: Relationship between testis weight (TW) and a) FSH and b) body weight (BW) in yearling Angus bulls (n=24).

Regression model: TW = - 147.8 - 112.8 x FSH + 1.08 x BW; with $R^2 = 0.63$ and p-values of 0.0006 (FSH) and 0.005 (BW).
FSH: average between basal and GnRH-stimulated FSH at 12 mo.

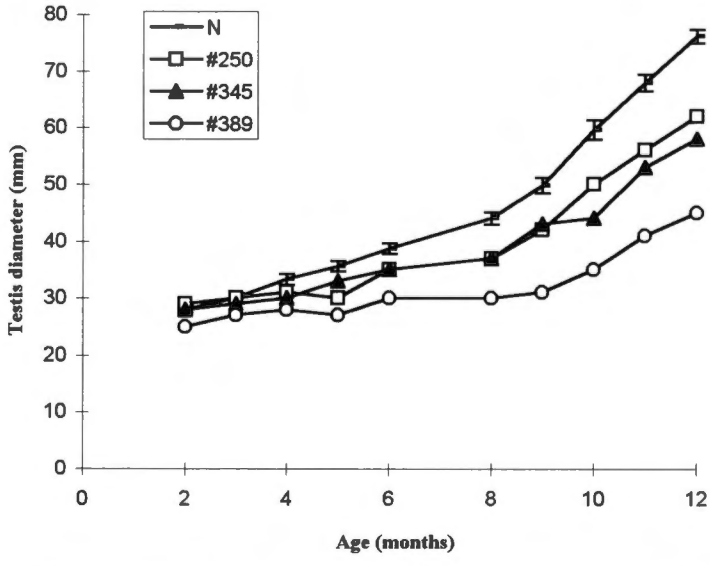


Figure 1.11: Age-related changes in testis diameter of normal (N) bulls and bulls #250, #345 and #389

Normal bulls (N): average of 17 bulls with all 12 stages of the cycle of the seminiferous epithelium (as defined by Berndtson and Desjardins, 1974). Error bars are the standard error of the mean calculated from the group of normal bulls within each age-period.

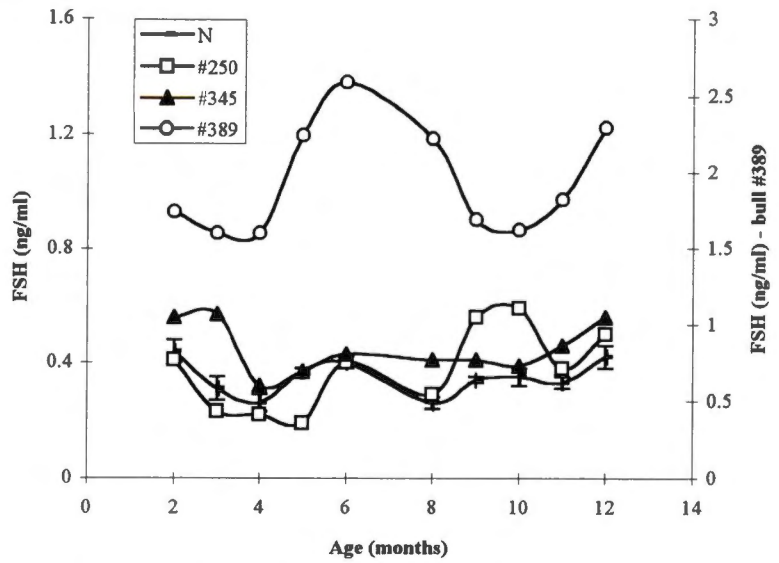


Figure 1.12: Basal FSH in normal bulls (N) and bulls #250, #345 and #389.

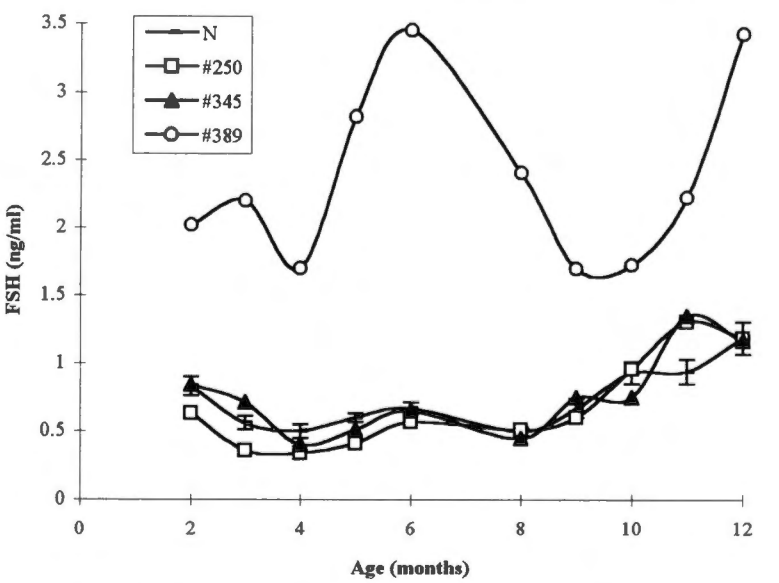


Figure 1.13: GnRH-stimulated FSH in normal bulls (N) and bulls #250, #345 and #389.

Normal bulls (N): average of 17 bulls that had all 12 stages of the cycle of the seminiferous epithelium (as defined by Berndtson and Desjardins,1974).
Basal hormone concentrations are the average of 3 blood samples taken at 1.5-h intervals.
GnRH-stimulated concentrations represent the average of T1.5 and T3.
Error bars are the standard error of the mean calculated from the group of normal bulls within each age-period.

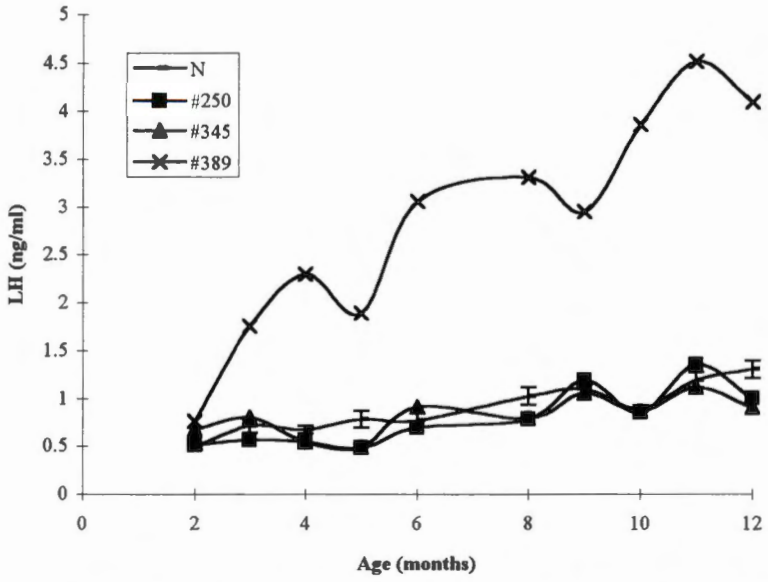


Figure 1.14: Basal LH in normal bulls (N) and bulls #250, #345 and #389.

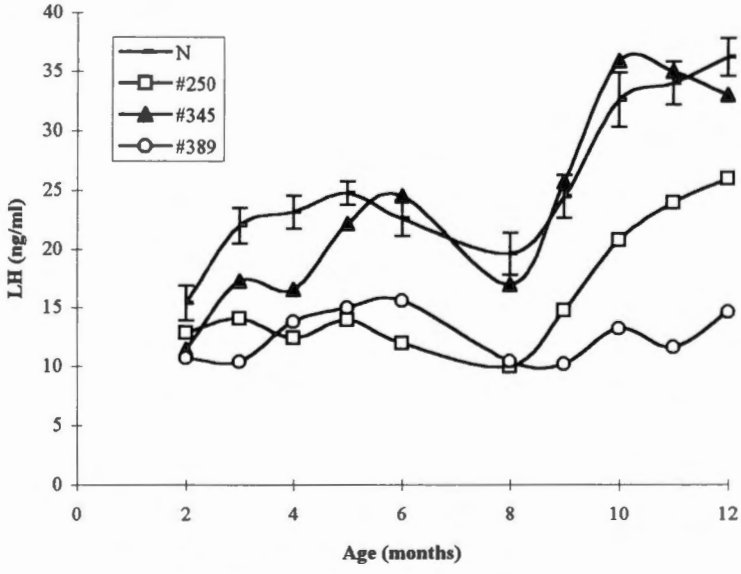


Figure 1.15: GnRH-stimulated LH in normal bulls (N) and bulls #250, #345 and #389.

Normal bulls (N): average of 17 bulls that had all 12 stages of the cycle of the seminiferous epithelium (as defined by Berndtson and Desjardins,1974).
Basal hormone concentrations are the average of 3 blood samples taken at 1.5-h intervals.
GnRH-stimulated concentrations represent the average of T1.5 and T3.
Error bars are the standard error of the mean calculated from the group of normal bulls within each age-period.

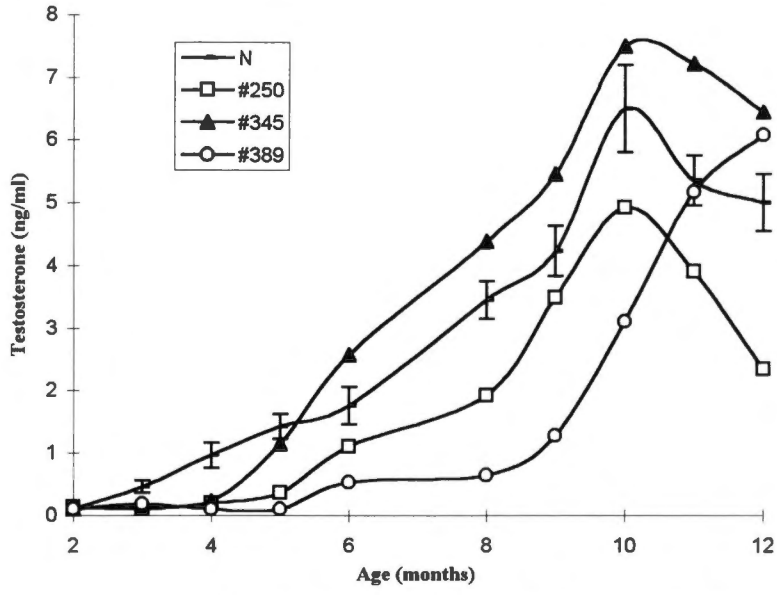


Figure 1.16: Basal testosterone in normal bulls (N) and bulls #250, #345, #389.

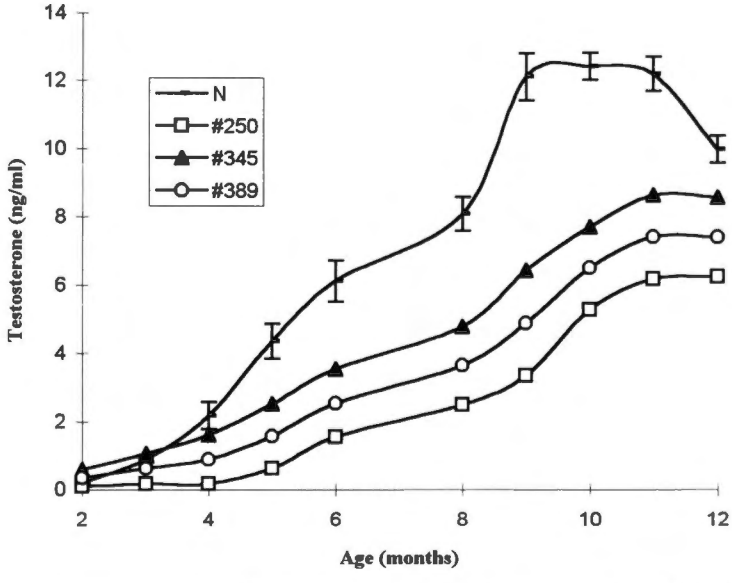


Figure 1.17: GnRH-stimulated testosterone in normal bulls (N) and bulls #250, #345, #389.

Normal bulls (N): average of 17 bulls that had all 12 stages of the cycle of the seminiferous epithelium (as defined by Berndtson and Desjardins,1974).
Basal hormone concentrations are the average of 3 blood samples taken at 1.5-h intervals.
GnRH-stimulated concentrations represent the average of T1.5 and T3.
Error bars are the standard error of the mean calculated from the group of normal bulls within each age-period.

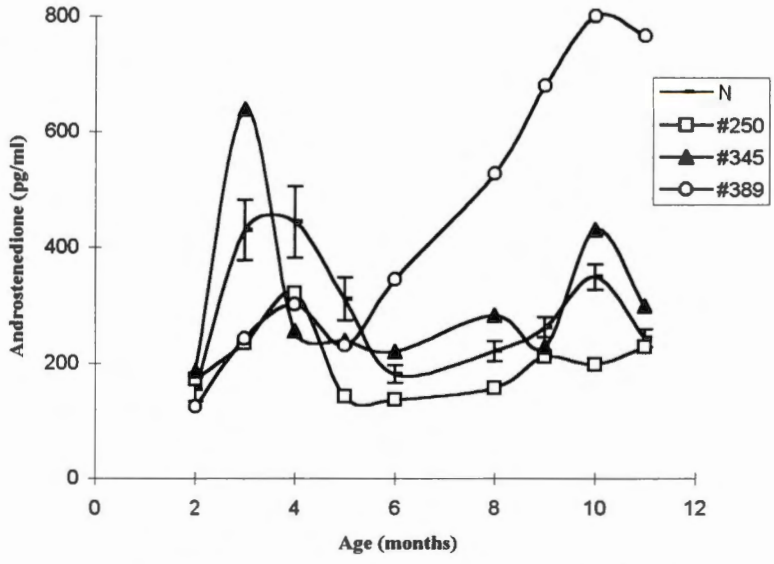


Figure 1.18: Basal androstenedione in normal bulls (N) and bulls #250, #345, #389

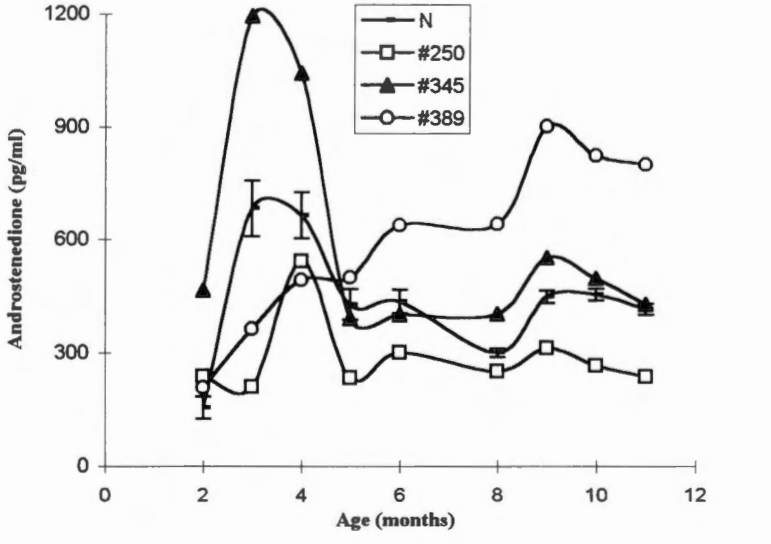


Figure 1.19: GnRH-stimulated androstenedione in normal bulls (N) and bulls #250, #345, #389

Normal bulls (N): average of 17 bulls that had all 12 stages of the cycle of the seminiferous epithelium (as defined by Berndtson and Desjardins,1974).
Basal hormone concentrations are the average of 3 blood samples taken at 1.5-h intervals.
GnRH-stimulated concentrations represent the average of T1.5 and T3.
Error bars are the standard error of the mean calculated from the group of normal bulls within each age-period.

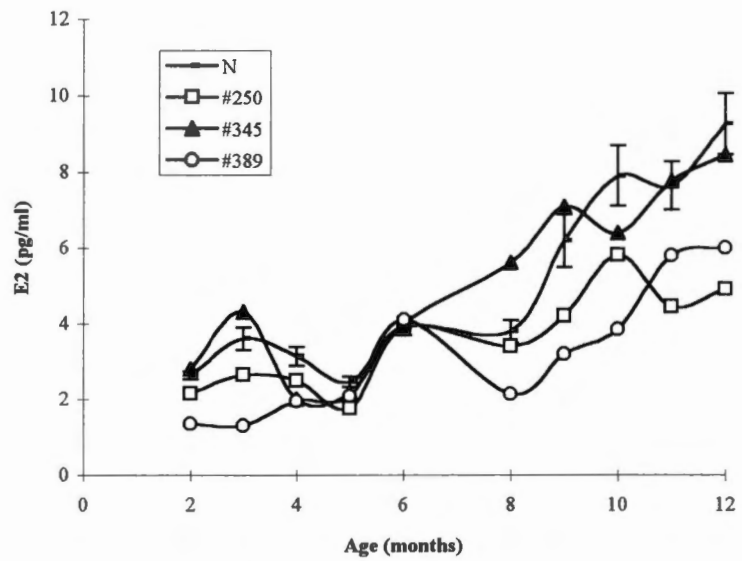


Figure 1.20: Basal E2 in normal bulls (N) and bulls #250, #345 and #389

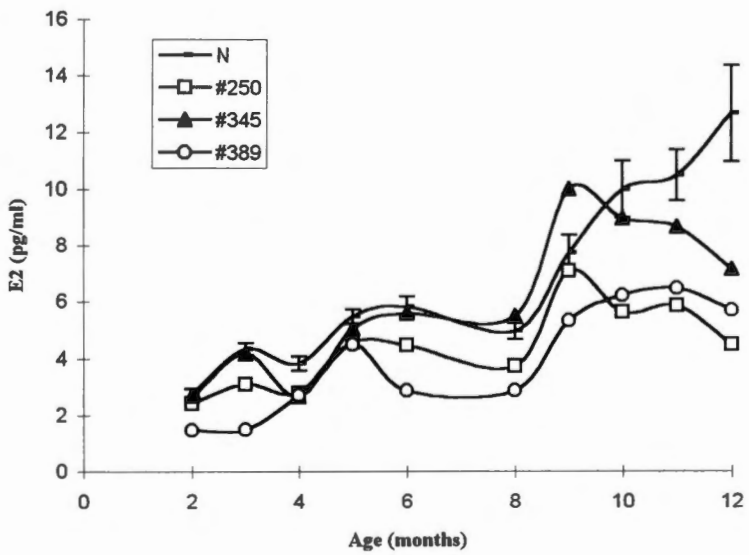


Figure 1.21: GnRH-stimulated E2 in normal bulls (N) and bulls #250, #345, #389

Normal bulls (N): average of 17 bulls that had all 12 stages of the cycle of the seminiferous epithelium (as defined by Berndtson and Desjardins,1974).
Basal hormone concentrations are the average of 3 blood samples taken at 1.5-h intervals.
GnRH-stimulated concentrations represent the average of T1.5 and T3.
Error bars are the standard error of the mean calculated from the group of normal bulls within each age-period.

Materials and Methods - Study 2

General procedures

Fifteen crossbred bulls (Charolois x Hereford and Charolois x Angus) born between February 6th and March 25th were pastured with their dams. At the age of 2 months (mo), all bulls were treated with 5 μg of GnRH (des-gly^{10},[D-ala^{6}]-GnRH-ethylamide, Sigma Co., St. Louis, MO) and blood samples were taken 1.5, 3 and 4.5 hours (h) later. One day before the GnRH challenge, animals were bled three times at 1.5-h intervals to establish basal hormone concentrations.

At 6 mo of age, bulls were treated with 1 mg of estradiol benzoate (E) and 12 h later received a subcutaneous injection of 10 μg of GnRH. Five days later, another 10 μg of GnRH was administered. In both cases, blood samples (from the jugular vein) were taken 1.5, 3 and 4.5 h after the GnRH injection and basal hormone concentrations were establish as before. Two basal samples were taken 1 h before the GnRH challenge to establish the effect of E on basal hormone secretion. All bulls were surgically castrated at 6 mo of age (193 ± 2.45 days; body weight = 215 ± 9.89 Kg).

Histological analysis

Fixation and staining of tissue as well as estimation of number of Sertoli cells and A1 spermatogonia per testis and number of A1 spermatogonia per Sertoli cell were performed as described in study 1. Because most of the bulls were prepubertal and did not have all the 12 stages of the spermatogenic cycle (as described by Berndtson and Desjardins, 1974), cells

were counted in sections of seminiferous tubules with the most advanced germ cell type.

To estimate the degree of seminiferous tubule development, 400 cross sections from each of the testicular segments were evaluated and placed in one of the following categories based on the most advanced germ cell type: tubules without germ cells (ST0), tubules with A1 spermatogonia (STA), Intermediate or B spermatogonia (STB), pachytene spermatocytes (STP), round spermatids (STD) and elongate spermatids (STE). The percentage of tubules in each of those categories was used in analysis of correlation.

Radioimmunoassays

Serum was radioimmunoassayed for FSH, LH, testosterone (T), androstenedione (Δ_4A) and 17β-estradiol (E2), as described in study 1. All the hormones, with the exception of E2, were quantified in the same assay in order to avoid interassay variation.

The sensitivity of the FSH RIA was 0.25 ng/ml and the intra-assay coefficient of variation (CV) was <7 %. In the case of LH, assay sensitivity was 31.3 pg/ml and intra-assay CV was < 7%. Assay sensitivity for the T and Δ_4A assays were 10 pg/ml and 2.5 pg/ml, respectively and intra-assay CVs were <10 %. The sensitivity of the assay was 0.15 pg/ml and the intra- and interassay CV were <8% and <15%, respectively.

Statistical Analysis

The effect of exogenous GnRH and E on peripheral concentrations of FSH, LH, E2, testosterone and Δ_4A was evaluated by analysis of variance using the GLM procedure of SAS (1985). Within each age period, body weight was used as a covariate whenever it became

significant. Pearson's partial correlations (Corr procedure, SAS, 1990) were used to determine the strength of the linear relationships among testis size, cell numbers and hormone concentrations. Body weight was used as the partial variable. Regression analysis (REG procedure, SAS, 1990) was used to model yearling testicular size and number of Sertoli cells by using hormone data and body weight as independent variables.

Results - Study 2

Correlations between testis size, cell numbers and degree of seminiferous tubule development in the 6-mo old testis

After the removal of the epididymis and tunica vaginalis, the averages of testis weight (TW) and diameter (TD) of 15 bulls were 48.0 ± 6.14 g and 34.3 ± 1.42 mm, respectively. While values for TD ranged from 25 to 45 mm, those of TW showed a much broader variation, ranging from 14.3 to 112.4 g. Number of Sertoli cells per testis (SC) averaged 9.14 ± 0.37 x 10^9, with a minimum of 6.54 x 10^9 and a maximum of 11.58 x 10^9.

Based on the analysis of 800 cross sections of seminiferous tubules (ST) from each animal, it was found that, on average, 16.6% of the sections did not have germ cells. Also, 31.5% of the sections had spermatocytes as the most advanced germ cell type and only 9.4% and 7.5% of the sections contained round and elongate spermatids, respectively. Out of the 15 bulls, only 6 had less than 5% of the ST sections without germ cells, while 7 and 5 bulls had more than 5% of the sections with round and elongate spermatids, respectively. The mean diameter of the seminiferous tubules was 42.3 ± 2.24 μm and that of the Sertoli cell nucleus

was 8.0 ± 0.25 μm. Also, on average, 65 ± 0.01% of the volume of the testis was occupied by the seminiferous tubules, while 35% was occupied by the interstitial space, including cells and vessels.

By means of analysis of partial correlation, it was found that A1 was correlated with TW ($r = 0.50$, $p < 0.10$), TD ($r = 0.61$, $p < 0.05$) and SC ($r = 0.64$, $p < 0.05$). However, the correlation between TW or TD and SC ($r = 0.41$) did not reach statistical significance. The percentage of STO was inversely related to A1 ($r = -0.58$, $p < 0.05$) and TW ($r = -0.48$, $p < 0.10$). Also, the percentage of STD or STE was correlated with TW and TD ($r = 0.70$ to 0.80, $p < 0.01$). Bulls with larger number of SC and A1 had less ST sections without germ cells ($r = -0.45$, $p < 0.10$; $r = -0.58$, $p < 0.05$) and more sections with either pachytene spermatocytes ($r = 0.59$, $p < 0.05$; $r = 0.49$, $p < 0.10$, respectively) or round spermatids ($r = 0.46$, $p < 0.10$). Number of A1 per SC (AS) was correlated with TD ($r = 0.52$, $p < 0.10$), STE ($r = 0.52$, $p < 0.10$) and STO ($r = -0.57$, $p < 0.05$).

Hormonal response to GnRH and GnRH + E in 2-mo and 6-mo old bulls

As shown in figure 2.1, peripheral concentrations of FSH in 2-mo old bulls increased from a baseline of 0.82 ± 0.06 ng/ml to 1.59 ± 0.15 ng/ml ($p < 0.01$) 1.5 h (T1.5) after an injection of 5 μg of GnRH. However, further changes from 1.67 ± 0.19 to 1.63 ± 0.18 ng/ml 3 h (T3) and 4.5 h (T4.5) after GnRH were not significant. While FSH increased only 1.94 times at T1.5, concentrations of LH (figure 2.2) went from 0.79 ± 0.06 ng/ml to 12.69 ± 1.43 ng/ml ($p < 0.01$) within the same period of time, representing an increase of 16-fold. Concentrations of LH remained high at T1.5 (13.37 ± 1.4 ng/ml, not different from T1.5, p

> 0.10) but, in contrast to what happened to FSH, dropped to 7.3 ± 1.1 ng/ml ($p < 0.01$) at T4.5.

The response of testosterone (figure 2.3) to GnRH-stimulated gonadotrophin release showed a pattern that was similar to that observed for LH. Testosterone increased from 0.25 ± 0.05 ng/ml to 0.52 ± 0.04 ng/ml ($p < 0.01$) at T1.5, remained practically unchanged at T3 and decreased to 0.35 ± 0.04 ng/ml at T4.5.

In the case of Δ_4A (figure 2.4), GnRH caused a significant response at T1.5 (from 156 ± 23 to 304 ± 41 pg/ml, $p < 0.01$), but concentrations remained high and did not change significantly thereafter. As shown in figure 2.5, concentrations of E2 at the age of 2 mo increased from a basal value of 3.3 ± 0.12 pg/ml to 3.8 ± 0.14 pg/ml at T1.5 ($p < 0.10$) and to 4.5 ± 0.27 pg/ml at T3 ($p < 0.05$). At T4.5, E2 decreased to 3.9 pg/ml, which was different from E2 at T3 ($p < 0.05$), but not at T1.5. GnRH-stimulated concentrations of E2 at 2 mo were correlated with FSH ($r = -0.48$, $p < 0.10$), but not with LH.

Figure 2.6 shows the changes in FSH following treatments with GnRH alone (GnRH) and GnRH given 12 h after an injection of E (GnRH + E). Across sampling periods (basal, T1.5, T3 and T4.5), the patterns of FSH secretion were different when GnRH and GnRH + E treatments were applied to the bulls. Although concentrations of FSH went from 0.40 ± 0.02 ng/ml to 0.63 ± 0.06 ng/ml (a 0.6-fold increase) 1.5 h after GnRH, this difference was significant only when α was set at 0.10. Further changes from 0.63 ± 0.06 to 0.83 ± 0.10 ng/ml at T3 was significant at $\alpha = 0.10$, but from 0.83 ± 0.10 to 0.77 ± 0.08 ng/ml at T4.5 was not. Also, FSH at T3 and T4.5 was different from basal values ($p < 0.05$). When GnRH + E was used, concentrations of FSH increased from 0.39 ± 0.02 ng/ml (measured 1 h before

the GnRH challenge) to 1.21 ± 0.21 ng/ml (a 3-fold increase) at T1.5 and to 2.41 ± 0.30 ng/ml at T3, but decreased 33% to 1.86 ± 0.20 ng/ml at T4.5. In comparison to basal values, FSH at T1.5, T3 and T4.5 was significantly higher ($p < 0.05$). However, FSH at T4.5 was not different ($p > 0.10$) from that at either T1.5 or T3.

Basal concentrations of FSH measured 1 h before estradiol benzoate was injected (0.49 ± 0.03 ng/ml), 12 h later (0.39 ± 0.02 ng/ml) or 5 days later (0.40 ± 0.02 ng/ml) were not different ($p > 0.10$). However, within T1.5, T3 and T4.5 (figure 2.6), FSH was significantly ($p < 0.01$) higher after GnRH + E (1.21 ± 0.21, 2.41 ± 0.30 and 1.85 ± 0.20 ng/ml, respectively) than after GnRH alone (0.63 ± 0.06, 0.83 ± 0.10 and 0.77 ± 0.08 ng/ml, respectively).

Across sampling times, the pattern of LH response to GnRH and GnRH + E treatments was similar (figure 2.7). When GnRH was used alone, LH assayed at T1.5 (8.33 ± 0.89 ng/ml), T3 (16.9 ± 1.62 ng/ml) and T4.5 (9.10 ± 0.91 ng/ml) was significantly higher ($P < 0.01$) than basal levels (0.82 ± 0.05 ng/ml). However, LH at T4.5 was significantly lower than LH at T3 ($p < 0.05$), but not at T1.5.

After treating bulls with GnRH + E, concentrations of LH at T1.5 (33.9 ± 4.15 ng/ml), T3 (44 ± 2.78 ng/ml) and T4.5 (18.9 ± 1.61 ng/ml) were significantly higher ($p < 0.01$) than basal (0.79 ng/ml). Although basal LH measured at any time was not affected by E treatment, within T1.5, T3 and T4.5, GnRH-stimulated LH was 2 to 4 times higher ($p < 0.05$) when GnRH + E were used than when GnRH alone was injected.

Within the GnRH group, concentrations of testosterone (figure 2.8) increased from 0.90 ± 0.08 ng/ml to 1.25 ± 0.17 ng/ml at T1.5 ($p < 0.10$) and 2.26 ± 0.21 ng/ml at T3 ($p <$

0.05). After 4.5 h, testosterone decreased to 1.76 ± 0.16 ng/ml, which was different from the concentration detected at T3 ($p < 0.05$), but not at T1.5. When the GnRH + E combination was used, testosterone increased from a basal value of 0.89 ± 0.16 ng/ml to 1.77 ± 0.18 ng/ml at T1.5 ($p < 0.05$) and 3.2 ± 0.21 ng/ml at T3 ($p < 0.05$). At T4.5, testosterone decreased to 2.62 ± 0.17 ng/ml, which was different from T3 ($\alpha = 0.10$), but not from T1.5. As observed for LH, basal testosterone was not affected by E. However, at T1.5, T3 and T4.5, testosterone levels were 51% higher ($p < 0.05$) when GnRH was given in combination with E than when GnRH was given alone.

The pattern of Δ_4A response to GnRH was similar to that observed for testosterone, with the exception that testosterone decreased and Δ_4A continued to increase from T3 to T4.5 (figure 2.9). Also, basal Δ_4A measured before (120 pg/ml) or any time after the E treatment (106 ± 8 pg/ml and 109 ± 9 pg/ml) was not different. Within bleeding times, however, Δ_4A was higher ($P < 0.05$) after GnRH + E than after GnRH alone at T1.5 (208 ± 11 and 170 ± 11 pg/ml, respectively), T3 (284 ± 15 and 239 ± 10 pg/ml, respectively) and T4.5 (330 ± 15 and 288 ± 8 pg/ml, respectively).

Peripheral concentrations of estradiol measured in all bulls were still above 67 pg/ml 12 h after the injection of estradiol benzoate. Therefore, the effect of GnRH could not be compared between the GnRH + E and the GnRH groups because it was not possible to determine how much of the E2 detected in blood came from endogenous or exogenous source. However, when GnRH was used alone (5 days after the GnRH + E treatment, figure 2.10), basal concentrations of E2 (3.08 ± 0.24 pg/ml) significantly increased to 5.08 ± 0.20 pg/ml at T1.5 and to 5.85 ± 0.32 pg/ml at T3 ($p < 0.05$). At T4.5, E2 levels decreased to 5.35

± 0.31 pg/ml, but this change was not significantly different ($p > 0.10$) from T3 or T4.5 (figure 2.10). At 6 mo, E2 was negatively correlated with FSH ($r = -0.46$, $p < 0.10$), but positively correlated with LH ($r = 0.55$, $p < 0.05$).

Correlations between hormone concentrations, testis size, cell numbers and degree of seminiferous tubule development

Testis weight at 6 mo (TW; table 2.1) was correlated with basal FSH ($r = -0.48$, $p < 0.10$) and with GnRH-stimulated FSH measured at 2 mo ($r = -0.61$, -0.60 and -0.62, $p < 0.05$, at T1.5, T3 and T4.5, respectively). TD was also related to FSH, but values of r and p were higher than those found for TW ($r = -0.70$, -0.75 and -0.74, $p < 0.01$, at T1.5, T3 and T4.5, respectively). As also presented in table 2.1, bulls with higher basal and GnRH-stimulated FSH at 2 mo had more SC ($r = -0.50$ to -0.65, $p < 0.10$) and AS ($r = -0.45$ to -0.58, $p < 0.10$) at the age of 6 mo. Also, FSH at 2 mo was positively related to the percentage of ST sections without germ cells ($r = 0.54$ to 0.66, $p < 0.05$) and inversely related to the percentage of ST sections with round spermatids as the most advanced germ cell type ($r = -0.49$ to -0.60, $p < 0.10$).

Concentrations of GnRH-stimulated E2 at the age of 6 mo was correlated with SC ($r = 0.62$, $p < 0.05$). GnRH-stimulated FSH at 6 mo was not related to either testis size, A1, AS or degree of ST development, regardless of the effects of GnRH and/or E. However, basal FSH at 6 mo was correlated with SC ($r = -0.54$, $p < 0.05$). Also, correlations between FSH and SC were significant when animals were treated with either GnRH + E ($r = -0.62$, -0.66 and -0.62, $p < 0.02$, at T1.5, T3 and T4.5, respectively) or GnRH alone ($r = -0.65$, -0.61

and −0.59, $p < 0.03$, at T1.5, T3 and T4.5, respectively). The average GnRH-stimulated FSH was more highly correlated with SC when bulls were pretreated with E2 ($r = -0.68$, $p = 0.008$) than when bulls received GnRH only ($r = -0.63$, $p = 0.015$).

Regression analysis was used to estimate TW (in g), TD (in mm) and SC (billions) based on the average GnRH-stimulated FSH (in ng/ml) and body weight (in Kg) recorded at the following ages:

a) 2 mo:

a1) SC = 11.3 − 1.30 x FSH, with $R^2 = 0.30$ and $p = 0.035$ (scatter plot in figure 2.11).

a2) TW = − 17.4 − 17.3 x FSH + 0.70 x BW2, with $R^2 = 0.72$. The p-values for FSH and BW2 were 0.017 and 0.002, respectively (scatter plots in figures 2.12a and 2.12b).

b) 6 mo:

b1) when GnRH was used alone: SC = 7.9 − 2.85 x FSH + 0.02 x BW6, with $R^2 = 0.48$. The p-values for FSH and BW6 were 0.015 and 0.072, respectively.

b2) when GnRH was combined with E2: SC = 8.80 − 1.06 x FSH, with $R^2 = 0.45$ and p-value = 0.006 (scatter plots in figures 2.13a and 2.13b).

Discussion -Study 2

Relationships between testis size, cell numbers and degree of seminiferous tubule development in the 6-mo old testis

Analysis of the seminiferous epithelium of the 6-mo old testis revealed that most of the cross sections had either spermatogonia (35 %) or spermatocytes (31.5 %), while only

few of them had spermatids (16.9 %), showing that the bulls were prepubertal. Curtis and Amann (1981) showed that testes of 5.5 to 6.5-mo old dairy bulls had differentiating spermatogonia and pachytene spermatocytes, but very few round spermatids. Thus, if it is true that postpubertal beef bulls have smaller testes and produce less sperm than do dairy bulls (Amann, 1970), such differences are not related to the state of germ cell development at the age of 6 mo.

In this study, there was a close relationship between the numbers of A1 and SC ($r = 0.64$), as has been reported by Hochereau-de Reviers et al. (1989). The population of A1 spermatogonia was also associated with testicular growth ($r = 0.50$ to 0.61). The correlation between SC and testis size, however, was found to be lower in the 6-mo old testis ($r = 0.41$) than in the yearling testis ($r = 0.84$, see Results - Study 1), and this is probably an age-related event. In the bull, the population of Sertoli cells becomes amitotic by the age of 10 weeks (Sharpe, 1994), but testicular growth is a continuous process that goes on for about 4 years in the Angus (Amann, 1970). Some of the bulls used in this study had an above-average SC numbers but relatively small testes because spermatogenesis had just started. Thus, discrepancies in the time at which Sertoli and germ cells proliferate contributed to a low correlation between testis size and SC numbers in the prepubertal bull.

At the age of 6 mo, number of SC was inversely related to percentage of seminiferous tubules without germ cells and positively related to that of tubules with spermatids. Thus, the existence of this relationship suggest that the Sertoli cell exerts control over germ-cell proliferation and differentiation even during the early stages of spermatogenesis.

Hormonal responses to GnRH and GnRH + E in 2-mo and 6-mo old bulls

The response of LH to GnRH in the 2-mo old bull was much higher (16-fold) than that of FSH (2-fold), but while FSH remained high at T4.5, LH decreased 45 % between T3 and T4.5. The sharp increase in LH occurred because it is well known that LH is more dependent on GnRH than is FSH (Price, 1991). The decrease in LH after T3 was probably a consequence of the rapid depletion of LH storages in the gonadotrophs. Besides being less dependent on the GnRH stimulus, FSH also has a longer half-life in the circulation than LH (170 versus 60 minutes; Johnson and Everitt, 1984) and, because of these facts, the reduction in FSH after T3 was minimal.

Concentrations of T increased at T1.5 and T3, but decreased at T4.5, showing a pattern similar to that of LH. Levels of Δ_4A also increased with GnRH but, unlike T, the decrease in Δ_4A between T3 and T4.5 was not significant. The cause for such differences is not clear but, since the adrenal secretes large quantities of Δ_4A in the circulation (Vermeulen, 1979), it is possible that part of the Δ_4A detected in the crossbred bulls was secreted in response to stress during blood collection.

At 6 mo, the FSH-response to GnRH was lower than that observed at the age of 2 mo (figures 2.1 and 2.6). Thus, given that samples collected at both ages were analyzed in the same assay, the changes in FSH are age-related and, therefore, probably caused by changes in factors that control FSH, such as inhibin. Moreover, the fact that GnRH-stimulated LH at 6 mo was not reduced in comparison to that observed at 2 mo suggests that the decrease in FSH at 6 mo was not caused by difference in dose of GnRH.

Twelve hours after bulls were injected with estradiol-benzoate, E2 in the peripheral

blood was at least 10-fold higher than physiological levels, but basal concentrations of FSH and LH were not altered. However, GnRH-stimulated levels of these hormones were significantly higher when GnRH was given in combination with E than when it was given alone. Estrogen can negatively control the synthesis of both FSH and LH (Miller, 1993; Nett et al., 1990). However, high concentrations of E2, such as those detected immediately before the LH surge in females, can enhance GnRH-stimulated gonadotropin secretion by increasing the number of GnRH receptors and the sensitivity of the gonadotrophs to GnRH by a postreceptor mechanism (reviewed by Miller, 1993). Thus, the increased GnRH-stimulated FSH and LH 12 h after the E treatment may have been caused directly by the estrogen's effect on the pituitary through the mechanisms described above. Since basal levels of gonadotropins did not increase, this aspect of the function of the gonadotroph was apparently not affected by estrogen in the 6-mo old bull.

GnRH-stimulated concentrations of T and Δ_4A were also increased when bulls were pretreated with E probably as a consequence of the increase in LH. Also, as a result of unchanged basal levels of LH, basal T and Δ_4A were not changed 12 h after the estrogen injection. As observed at 2 mo, T decreased between T3 and T4.5 while Δ_4A remained high. Again, the reason why responses of T and Δ_4A to GnRH-mediated LH increase were similar between 0 and 3 h but different between 3 and 4.5 h after the GnRH injection is not known but it may be related to contribution from the adrenal.

At 2 mo, concentrations of E2 increased significantly in response to GnRH treatment. At 6 mo and 5 days after bulls were injected with 1 mg of estradiol benzoate, basal levels of E2 averaged 3.2 ± 0.2 pg/ml, which were similar to those found in Angus bulls at the same

age (3.92 ± 0.24 pg/ml; see Results - Study 1). Thus, it appears that the estradiol benzoate that had been injected was metabolized and excreted after 5 days. E2 measured at 2 mo was correlated with FSH (r = - 0.48) but E2 at 6 mo was correlated with both FSH and LH (r = - 0.46 and 0.55, respectively). This may indicate that the Sertoli cell is involved in the secretion of E2 at 2 mo, but the Leydig cell (under LH stimulation) may contribute to the secretion of E2 in the 6-mo old testis.

Relationships between hormone concentrations, testis size and degree of seminiferous tubule development

Concentrations of FSH at 2 mo were positively related to the percentage of ST sections without germ cells in the 6-mo old testis, but there were negative correlations between FSH and testis size, number of SC, number of A1 per SC and percentage of ST cross sections with round spermatids. Since concentrations of steroids did not correlate significantly with testis size, it is possible that the inverse relationship involving FSH and testis size may be due to the fact that heavier testis were secreting more inhibin at 2 mo.

FSH at 6 mo was not significantly related to testis size. The reasons FSH and testis size were more closely related at 2 than at 6 mo may be linked to the fact that levels of FSH were higher at 2 than at 6 mo and that, based on studies performed in rats, the neonatal Sertoli cell is more responsive to FSH than it is the prepubertal Sertoli cell (Dorrington and Khan, 1993). Concentrations of E2 at 6 mo were positively related to SC numbers and this may account for the negative correlations between FSH and number of Sertoli cell. As never reported before, concentrations of FSH was correlated with Sertoli cell numbers per testis (r

= - 0.63 versus r = - 0.68), when bulls were treated with either GnRH alone (r = - 0.63) or GnRH combined with estradiol (r = - 0.68). However, regression analysis showed that, given body weight at 6 mo, pretreatment with E increased the amount of gonadotropin secreted in response to GnRH but did not strengthen the relationship between FSH and SC.

In conclusion, FSH at 2 mo seems to be a reliable indicator of testicular development because, in combination with body weight, it accounted for as much as 72 % of the variation in testis weight at 6 mo. Moreover, results obtained from studies 1 and 2 suggest that peripheral levels of FSH, more than those of LH or steroids, closely reflect developmental aspects of the prepubertal and yearling testis in bulls of different breeds.

Implications

Results from studies 1 and 2 indicate the existence of a significant association between peripheral concentrations of FSH and histology and size of the prepubertal and yearling testis of bulls from different breeds. It is suggested that, before any practical application of the regression models presented in this study is recommended, these models need to be tested in larger samples with bulls of the same and/or other breeds. It is possible, however, that the potential sperm-producing capacity of bulls could be predicted based on hormonal secretion at prepuberty. Also, the comparative study of hormone profiles as related to testicular development of normal and infertile bulls help to better understand the interactions occurring in the hypothalamus-pituitary-gonadal axis as well as the causes and consequences of impaired germ cell proliferation and differentiation in the bull.

Table 2.1: Pearson's partial correlations between FSH concentrations measured at 2 mo and testis weight (TW) and diameter (TD), number of Sertoli cells (SC) per testis, the A1/SC ratio (AS) and degree of seminiferous tubule (ST) development of the 6-mo old testis (n=15).

	FSH /			
	Basal	T1.5	T3	T4.5
TW	-0.48 a	-0.61 b	-0.60 b	-0.62 b
TD	-0.46 a	-0.70 c	-0.75 c	-0.74 c
SC	-0.55 b	-0.50 a	-0.65 b	-0.60 b
AS	-0.58 b	-0.50 a	-0.45 a	-0.46 a
ST0	0.66 c	0.60 b	0.59 b	0.54 b
STD	-0.49 a	-0.60 b	-0.55 b	-0.53 a

FSH/Basal: average of 3 basal bleedings was used to estimate the correlations.
FSH/T1.5, T3, T4.5: concentrations of FSH measured 1.5, 3 and 4.5 h after GnRH.
ST0: percent of ST sections with no germ cells; STD: percent of ST sections with round spermatids as the most advanced cell type.
Partial variable: body weight at the age of 2 mo.
a, b and c: $p < 0.10$, $p < 0.05$ and $p < 0.01$, respectively.

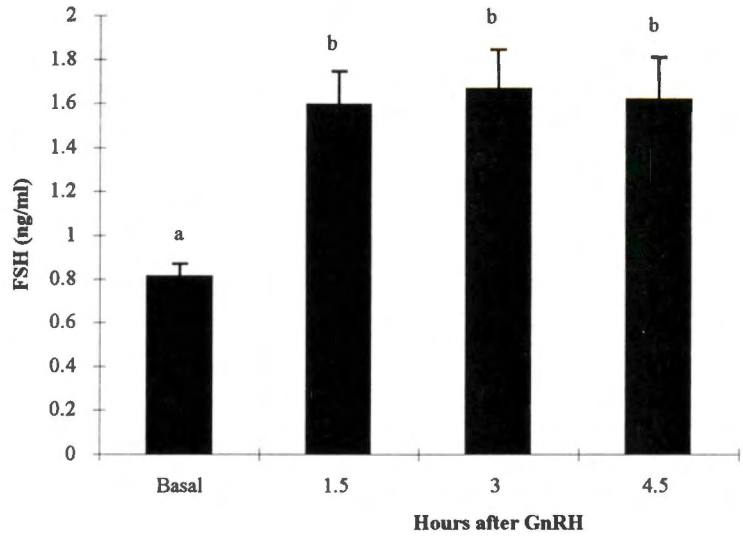


Figure 2.1: Concentrations of FSH in 2-mo old crossbred bulls treated with GnRH (n=15)

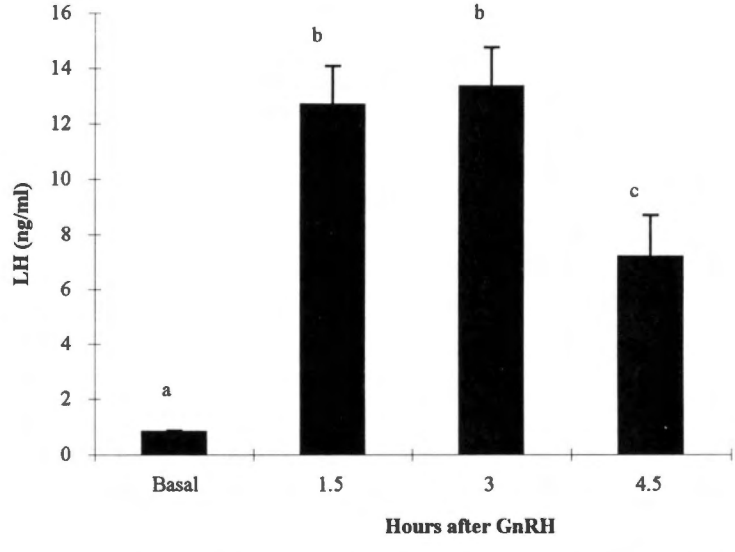


Figure 2.2: Concentrations of LH in 2-mo crossbred old crossbred bulls treated with GnRH (n=15)

Basal: average of 3 blood samples taken at 1.5-h intervals.
Across sampling times, values with the same letters are not significantly different ($p > 0.05$).
Error bars represent the standard error of the mean.

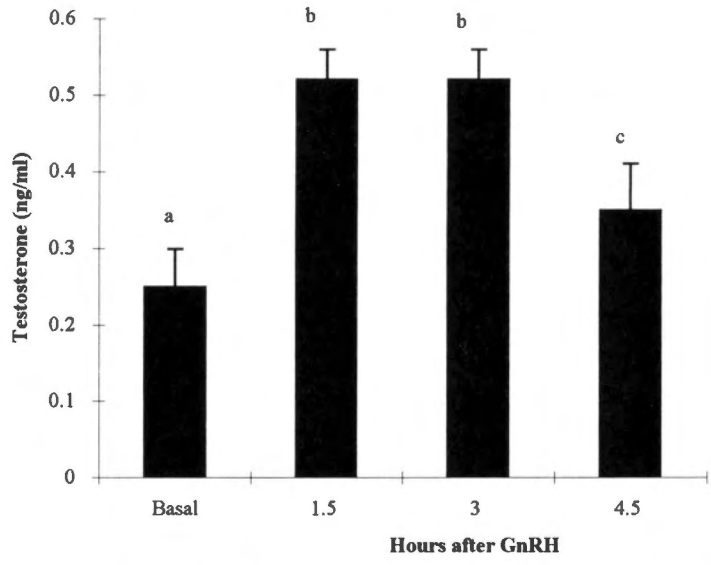


Figure 2.3: Concentrations of testosterone in 2-mo old crossbred bulls treated with GnRH (n=15)

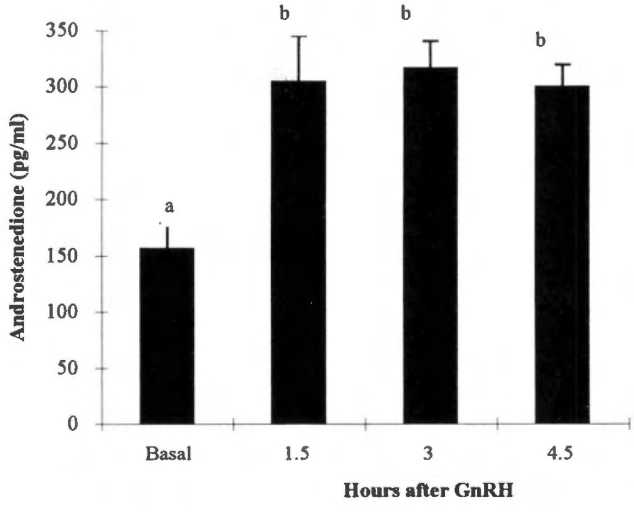


Figure 2.4: Concentrations of androstenedione in 2-mo old crossbred bulls treated with GnRH (n=15)

Basal: average of 3 blood samples taken at 1.5-h intervals.
Across sampling times, values with the same letters are not significantly different ($p > 0.05$).
Error bars represent the standard error of the mean.

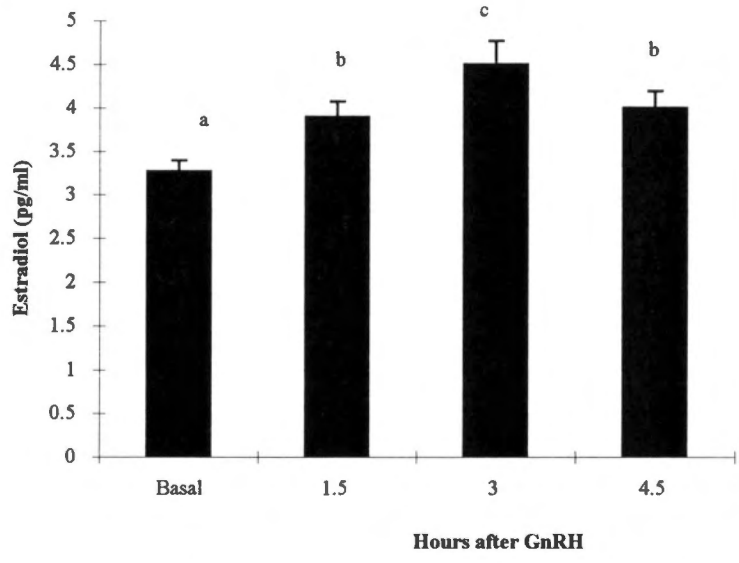


Figure 2.5: Concentrations of estradiol in 2-mo old crossbred bulls treated with GnRH (n=15)

Basal: average of 3 blood samples taken at 1.5-h intervals.
Across sampling times, values with the same letters are not significantly different ($p > 0.10$).
Error bars represent the standard error of the mean.

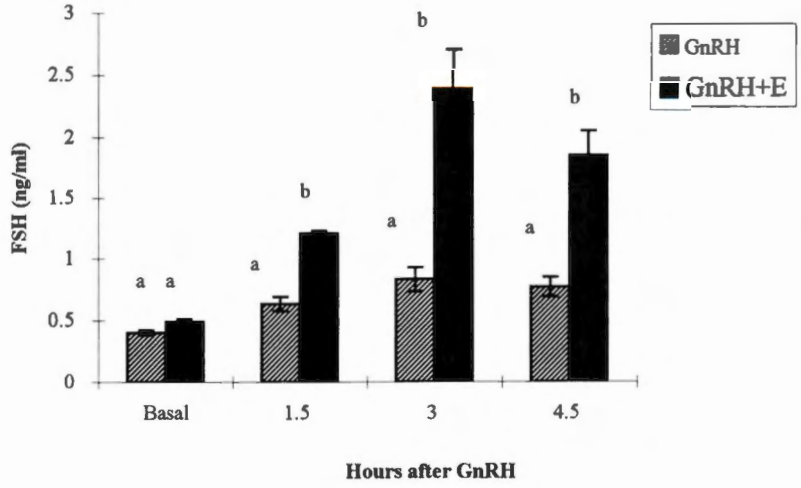


Figure 2.6: Concentrations of FSH in 6-mo old crossbred bulls treated with GnRH and GnRH+E (n=15)

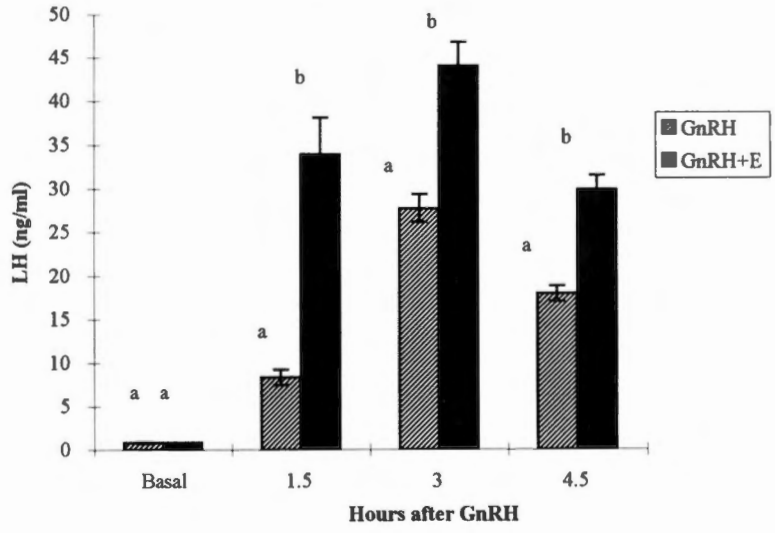


Figure 2.7: Concentrations of LH in 6-mo old crossbred bulls treated with GnRH and GnRH+E (n = 15)

Bulls were injected with 1 mg of estradiol benzoate (E) 12 h before the first GnRH challenge (GnRH+E). Five days later, a second GnRH injection was given without pretreatment with E (GnRH).
Within sampling times, values with the same letters are not significantly different ($p>0.05$).
Error bars represent the standard error of the mean.

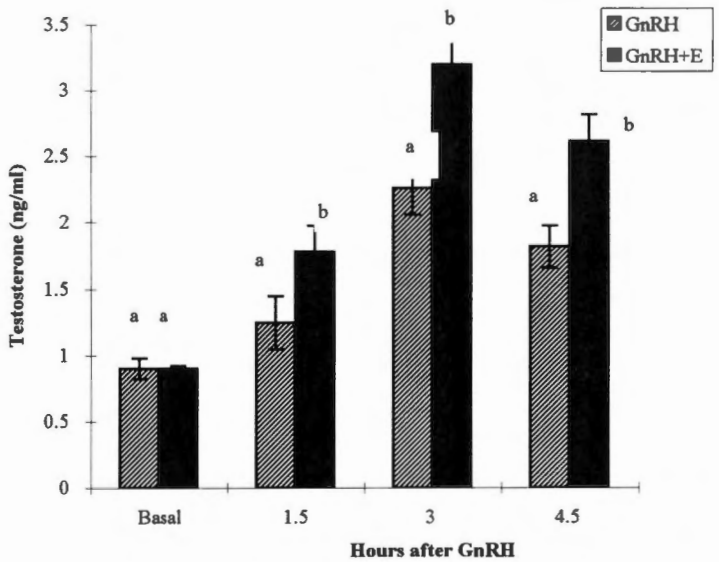


Figure 2.8: Concentrations of testosterone in 6-mo old crossbred bulls treated with GnRH and GnRH+E (n=15)

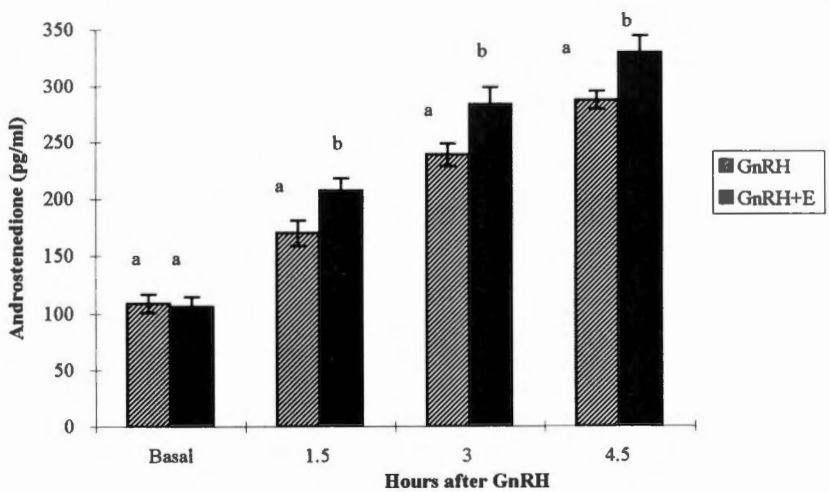


Figure 2.9: Concentrations of androstenedione in 6-mo old crossbred bulls treated with GnRH and GnRH+E2 (n=15)

Bulls were injected with 1 mg of estradiol benzoate (E) 12 h before the first GnRH challenge (GnRH+E). Five days later, a second GnRH injection was given without pretreatment with E (GnRH).

Within sampling times, values with the same letters are not significantly different ($p > 0.05$).

Error bars represent the standard error of the mean.

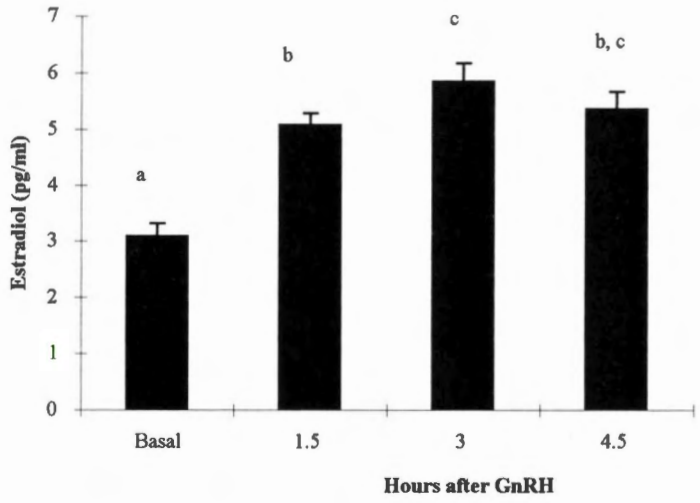


Figure 2.10: Concentrations of estradiol in 6-mo old crossbred bulls treated with GnRH (n=15)

Concentrations of E2 five days after the GnRH+E treatment.
Across sampling times, values with the same letters are not significantly different ($p > 0.10$).
Error bars represent the standard error of the mean.

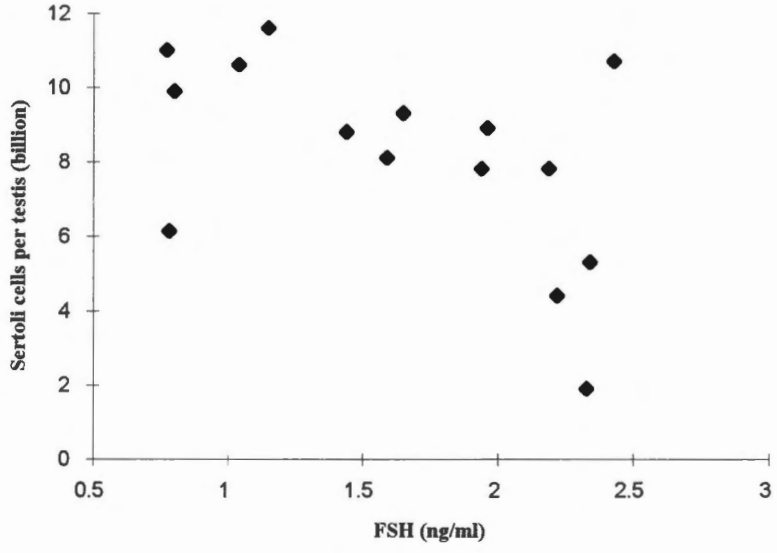
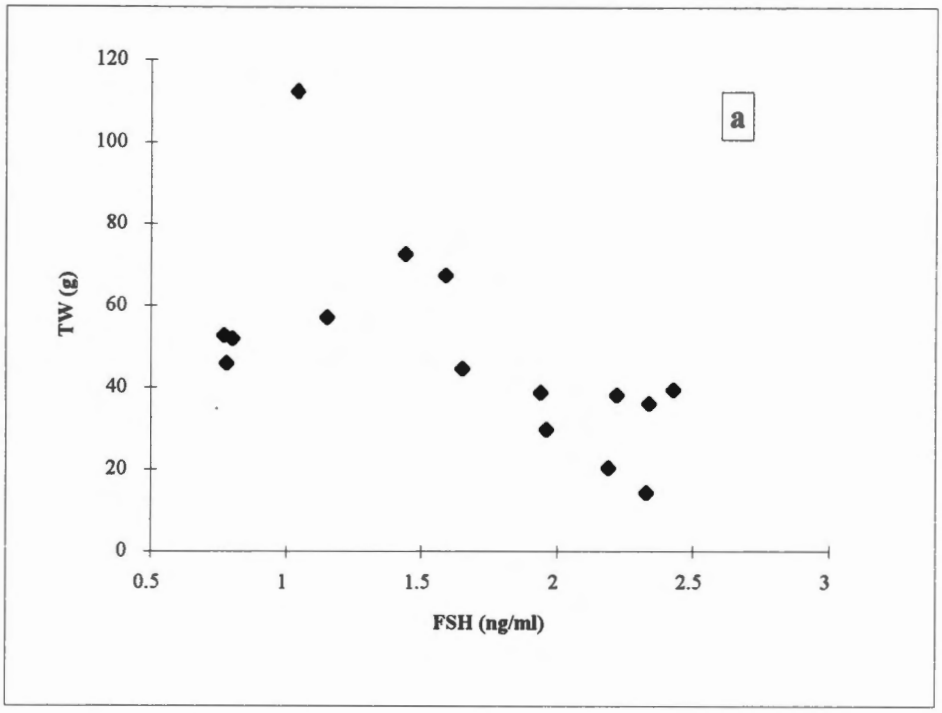


Figure 2.11: Relationship between FSH at 2 mo and number of Sertoli cells measured in 6-mo old crossbred bulls (n=15)

Regression model: SC = 11.3 - 1.3 x FSH, with $R^2 = 0.30$ and p-value=0.035.
FSH: average GnRH-stimulated FSH at 2 mo.
SC: number of Sertoli cells (billion) in the 6-mo old testis.



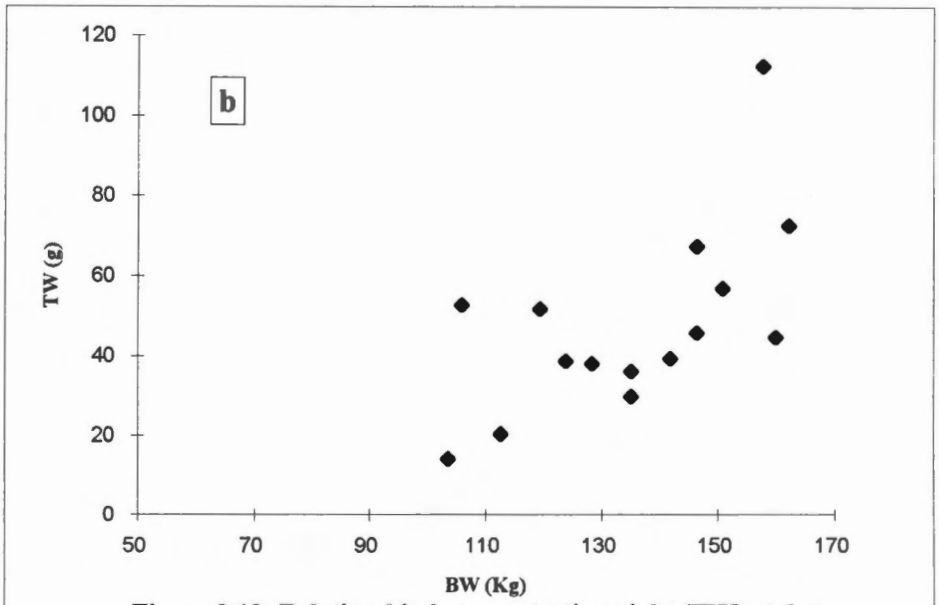
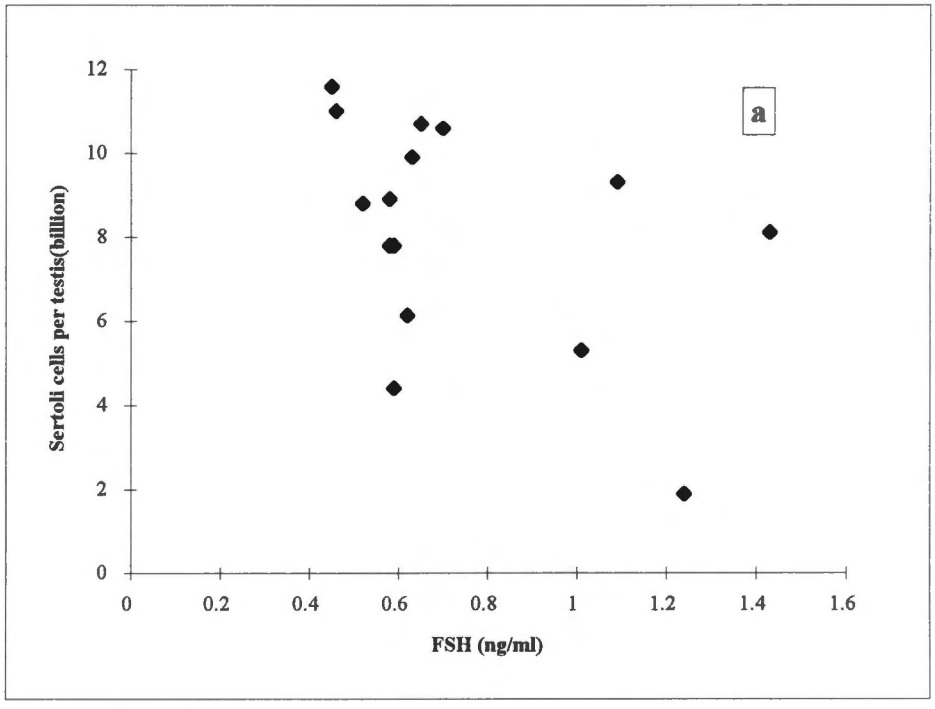


Figure 2.12: Relationship between testis weight (TW) at 6 mo and a) FSH and b) body weight (BW) at 2 mo in crossbred bulls (n=15)

Regression model: TW = - 17.4 - 17.3 x FSH + 0.7 x BW, with $R^2 = 0.72$ and p-values of 0.017 (FSH) and 0.002 (BW).
FSH: average GnRH-stimulated FSH at 2 mo.



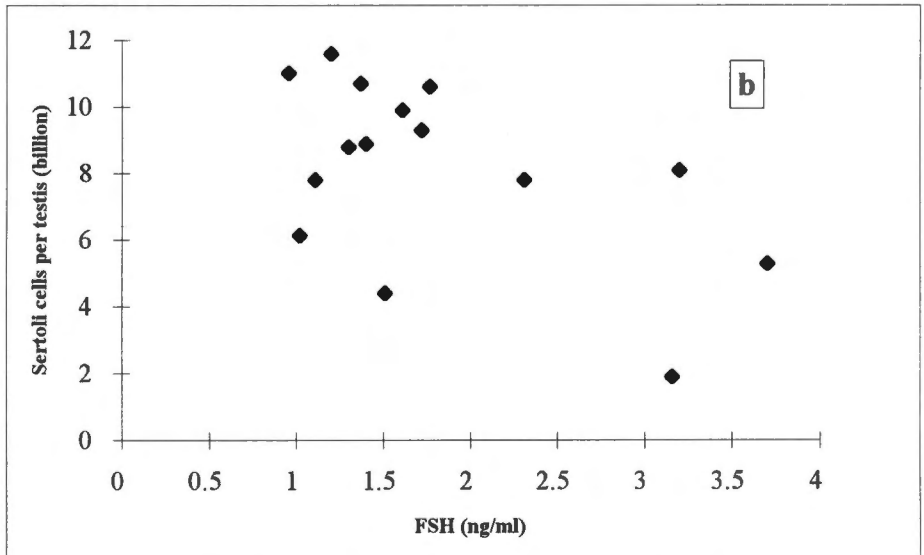


Figure 2.13: Relationship between number of Sertoli cells and FSH in 6-mo old crossbred bulls treated with a) GnRH and b) GnRH + estradiol* (n=15)

Model (2.13a): SC = 7.9 - 2.85 x FSH + 0.02 x BW6, with R^2 = 0.48 and p-values of 0.015 (FSH) and 0.072 (BW6).
BW6: body weight at 6 mo (Kg).
Model (2.13b): SC = 8.8 - 1.6 x FSH, with R^2 = 0.45 and p-value = 0.006.
*Bulls were treated with 1 mg of estradiol benzoate 12 h before the GnRH challenge.
FSH: average GnRH-stimulated FSH at 2 mo.
SC: number of Sertoli cells per testis (billion) in the 6-mo old bull.

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PART III

(Study 3)

Abstract

This study was carried out to determine whether testis size, histology and hormone secretion at the developmental state at which concentrations of testosterone reached 1 ng/ml of serum were related to size of the testis and quantitative aspects of spermatogenesis in the yearling bull testis. Three basal blood samples (at 1.5-hour intervals)were taken from 28 Angus calves since the age of 15 weeks (wk). When concentrations of testosterone (T) reached 1 ng/ml, bulls were unilaterally castrated. On a monthly base, bulls received 0.05 μg of GnRH per Kg of body weight and were bled 1.5 and 3 hours later. At 54 wk, the second testis was excised. RIA's were used to quantify FSH, LH, T, androstenedione (A) and 17β-estradiol (E2) in blood samples. Number of Sertoli cells and quantitative aspects of spermatogenesis were estimated in both testes. Analysis of regression and Pearson's partial correlation were used to estimate the degree of association between hormone and testicular development. Results showed that testis weight when T = 1 ng/ml (TW1) was correlated with testis weight (TW2), and number of Sertoli cells and round spermatids per testis at 54 wk ($r = 0.58$, $p < 0.01$). Also, TW1 was related to number of spermatids per Sertoli cell ($r = 0.41$, $p < 0.05$), and number of seminiferous tubules with elongate (STE) and mature (STM) spermatids ($r = 0.47$ and 0.52, respectively; $p < 0.05$) in the second testis. However, neither STE nor STM were correlated with number of tubule sections with germ cells in the first testis (STG1). Testis diameter averaged between the ages of 30 and 44 wk (AVTD) and TW2 were correlated with AGE1 ($r = -0.58$ and -0.42, $p < 0.01$), TW1 ($r = 0.65$ and 0.58, $p < 0.01$) and STG1 ($r = 0.43$ and 0.41, $p < 0.10$). Regression analysis showed that AGE1 and body weight when T

= 1 ng/ml accounted for 41% of the variation in testis diameter between 30 and 44 wk. GnRH-stimulated FSH at 11 wk was related to STG1, TW1, TW2 and AVTD ($r = -0.42$ to -0.53, $p < 0.05$). GnRH-stimulated T at 15 wk was related to TW1 ($r = 0.55$, $p < 0.01$) and AVTD ($r = 0.48$, $p < 0.05$). Also, bulls that secreted more E2 after a GnRH treatment at 15 wk had larger AVTD ($r = 0.62$, $p < 0.62$, $p < 0.01$) and TW2 ($r = 0.45$, $p < 0.10$). Thus, differences between slow and fast growing bulls are affected by the ability of the testis to secrete testosterone early in life. Also, the existence of correlations between FSH, steroids and characteristics of the testis at AGE1 and prepuberty points toward a role played by these hormones at distinct phases of germ cell development.

Key words: Bulls, Testes, Testosterone, FSH.

Introduction

In the bovine, testis size and Sertoli cell numbers are reliable indicators of daily sperm production (Amann and Alquimist, 1962; Berndtson et al., 1987). Also, scrotal circumference of the 7- to 10-mo old beef bull is closely associated with age at puberty ($R^2 = 0.85$, Lunstra et al., 1978).

In bull calves, there is a transient increase in concentrations of LH between the ages of 1 and 3 mo (Rawlings et al., 1978; Evans et al., 1993) and serum FSH is also high before 4 mo (MacDonald et al., 1990; Evans et al., 1993). The immature Leydig cell secretes primarily androstenedione (Δ_4A) but testosterone becomes the primary androgen produced

as differentiation takes place (Amann and Schanbacher, 1983). Both LH and FSH are involved in the process of Leydig cell proliferation and differentiation (Hardy et al., 1991; Jégou and Sharpe, 1993). In the bull, the shift in androgen secretion occurs around the age of 4 mo and coincides with the cessation of Sertoli cell mitosis (Sharpe, 1994). Also, at this state of development there are gonocytes, renewing stem cells and A1 or differentiating spermatogonia, but no more advanced germ cell types in the seminiferous tubules (Curtis and Amann, 1981).

It has been shown that the number of stem cells per testis increase from neonatal to adulthood in bulls (Attaal and Courot, 1963). Also, the efficiency by which the A1 spermatogonia are produced greatly determines the number of spermatozoa in the testis (Ortavant et al., 1977). Bulls with higher LH secretion between 2 and 4.5 mo reach puberty earlier than those with lower concentrations of LH (Evans et al., 1993). Thus, the objective of this study was to determine whether testis size, histology and hormone secretion at the developmental state at which concentrations of testosterone reached 1 ng/ml of serum were related to size of the testis and quantitative aspects of spermatogenesis at 12 months.

Materials and Methods

General Procedures

Twenty eight Angus bulls born between January 19th and April 2nd were raised on pasture and weaned at the age of 8 mo. According to previous results (see study 1, figure 1.4), peripheral concentrations of T in Angus bulls increased from 0.2 ng/ml at 12 weeks to

1.0 ng/ml at an average of 20 weeks. In order to monitor the rapid changes in T levels that occur at prepubertal ages, 3 blood samples (at 1.5 h intervals) were taken biweekly starting at 12 weeks and continuing until concentrations of T reached 1.0 ng/ml. At this state of development (AGE1), the first testis was excised. On a monthly base, bulls were treated with GnRH - 0.05 μg per Kg of body weight (des-gly^{10}, [D-ala^{6}]-GnRH-ethylamide, Sigma Co., St. Louis, MO) and bled 1.5 and 3 hours (h) later. Also, animals were weighed every month and the diameter of the right testis of each bull was measured using a caliper. At 54 wk (373 ± 12 days; 277 ± 7.5 Kg), the second testis was surgically excised.

Histological analysis

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, the tissue was dehydrated in alcohol, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. Two slides, separated by 100 μm, of each testicular segment were prepared.

Studies have shown that there is uniformity between different regions within the bovine testis (Amann, 1962; Swierstra, 1966). Also, Berndtson et al. (1987) have estimated that the use of 10 cross sections of seminiferous tubule per bull is adequate for determination of Sertoli and germ cell numbers. To check this finding, 10 bulls were chosen at random and cell numbers were estimated in either 10 or 20 sections. Results showed that differences in Sertoli and germ cell counts were not significant ($p < 0.10$) and less than 10 %. Therefore,

calculation of Sertoli and germ cell numbers presented in this study were based on samples of 10 sections selected from different regions of one slide from each bull. These sections were all at stages I through VI, as described by Berndtson and Desjardins (1974). When the bull did not have elongate or mature spermatids, the sections chosen were those with the most advanced germ cell type.

In the second testis, numbers of Sertoli cells, A1 spermatogonia and round spermatids containing an intact nucleolus were counted in each of the 10 sections. These crude counts were converted to true counts according to Abercrombie's formula (Abercrombie, 1946): True# = Crude# × (section thickness / (section thickness + average nuclear diameter in microns). Section thickness in this case was 5 μm. Based on the true counts, the following cell ratios per cross section was estimated: # of round spermatids per A1 spermatogonia and per Sertoli cell.

To estimated the degree of testicular development in the first testis, 800 cross sections were chosen at random and placed in one of two categories: sections without germ cells (ST01) and sections with either gonocytes or spermatogonia (STG1). 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 (ST02), tubules with A1 spermatogonia(STA), Intermediate or B spermatogonia (STB), pachytene spermatocytes (STP), round spermatids (STD), elongated spermatids (STE) and mature spermatids (STM). The percentage of tubules in each of those categories was used in the analysis of correlation.

Total number of Sertoli cells per testis was estimated in both testes and the calculation was based on the method described by Erickson and Blend (1971). 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, Johnson and Neaves, 1981), respectively. The volume occupied by 10 seminiferous tubule cross sections (Vst) was calculated by the formula Vst = $\prod \times h \times (d^2/4)$, where h was the section thickness (5 μm) and d was tubule diameter in microns. The percentage of testicular volume occupied by seminiferous tubules (%ST) was determined as described by Chalkley (1943), i. e., the percentage of 600 "hits" taken at random within a cross section of a testis. A "hit" was defined as the object at the point of each of 6 pointers mounted in the ocular of the microscope. The ocular was passed over the surface of a testicular cross section in a random manner and the number of times each pointer fell on either tubular or interstitial tissue was recorded.

Crude numbers of Sertoli cells, A1 spermatogonia and round spermatids per testis were determined by the following formula: Crude# = (V × %ST × C) / Vst, where C represented the true number of Sertoli cells or A1 spermatogonia counted in 10 cross sections. The resulting crude numbers were converted to true counts according to Abercrombie's formula (Abercrombie, 1946): True# = Crude# × (section thickness / (section thickness + average nuclear diameter in microns). The final estimate of the number of A1 spermatogonia per testis was obtained by multiplying the True# by: (1 - % ST0). The population of round spermatids per testis was estimated by multiplying the True# by: (1 - (ST0+STA+STB+STP)).

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 x g for 25 minutes. Serum was harvested and stored at -20 C until assayed for FSH, LH, testosterone (T), androstenedione (Δ_4A) and 17β-estradiol (E2).

Concentrations of FSH were determined in 200 μl of serum using a double antibody radioimmunoassay (RIA), as described by 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 samples collected during the present study were analyzed in two different assays. The sensitivity of the FSH RIA was 0.25 ng/ml and the intra- and interassay coefficient of variation (CV) were <6 % and < 13 %, respectively. Peripheral concentrations of LH were quantified in 100 μl of serum using a double-antibody RIA method firstly described by Niswender et al. (1969) and modified by Bolt et al. (1981). The LH first antibody (# 15 antiovine 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 interassay CV were < 5% and < 10%, respectively.

Samples of serum (150 µl) were extracted with 1.5 ml of benzene before being analyzed for T, Δ_4A and E2. Concentrations of T and Δ_4A were estimated based on a single-antibody method described by Jackson et al. (1989). The Δ_4A antibody (X - 322 Rao) was purchased from Dr. P. N. Rao (Southwest Foundation for Biomedical Research, San Antonio,

TX) and the T antibody was provided by Dr. G. Niswender. Assay sensitivity was for the T and Δ_4A assays were 10 pg/ml and 2.5 pg/ml, respectively. The intra- and interassay CV were <8% and <14%, respectively, for the T assay, and <7% and <15%, respectively, for the Δ_4A assay. Concentrations of E2 were quantified according to a procedure described by Cox et al. (1987) and modified by Britt (1990). 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 interassay CV were <6% and <15%, respectively.

Statistical Analysis

Age-related changes in hormone concentrations were determined by analysis of variance and Tukey's statistical test (Proc GLM, SAS, 1990). Pearson's partial correlations (Corr procedure, SAS, 1990) were used to determine the strength of the linear relationships involving AGE1, testis size, Sertoli and germ cell numbers and hormone concentrations. Body weight was used as the partial variable so that its effect on the correlation among the other variables could be controlled. Regression analysis (REG procedure, SAS, 1990) was used to estimate prepubertal testis size. The independent variables tested for statistical significance in the regression models included hormone concentrations, testis diameter and body weight.

Results

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

Bulls were hemicastrated when peripheral concentration of testosterone was 1 ng/ml. At this state of development, the average age and body weight of the calves were 20 ± 0.6 weeks (AGE1) and 189 ± 7.6 Kg, respectively. In some animals, concentrations of T reached 1 ng/ml as early as 14 and 15 weeks (wk) while in other bulls such concentrations of T were not detected before the ages of 25 and 27 wk. Because of these differences in age at the first castration, the effects of hemicastration and age-related changes in testis weight and hormone concentrations were analyzed in a group of 14 bulls only (figures 2 through 5). These bulls were castrated at 20 ± 0.3 wk.

After hemicastration, the remaining testis increased with age ($p < 0.01$) and paralleled the increase in body weight (figure 1). Between the ages of 44 and 54 wk, body weight decreased ($p < 0.05$) from 306 ± 13 Kg to 288 ± 11 Kg, but the rate of testicular growth was not significantly affected. The decrease in body weight after 44 wk was caused by adverse weather conditions in the winter and reduced pasture quality.

Concentrations of basal FSH were high at the age of 11 wk (0.78 ± 0.09 ng/ml) but decreased significantly ($p < 0.05$) to 0.61 ± 0.06 and 0.60 ± 0.04 ng/ml at 15 and 17 wk, respectively (figure 2). Two and four weeks after hemicastration, FSH increased to 0.75 ± 0.07 and 0.80 ± 0.05 ng/ml ($p < 0.05$), resepctively. Also, levels of FSH increased ($p < 0.05$) from 0.62 ± 0.02 ng/ml (at 30 wk) to 0.89 ± 0.1 ng/ml (at 36 wk) and 0.86 ± 0.86 (at 50 wk), but decreased ($p < 0.10$) to 0.76 ± 0.08 ng/ml at 54 wk. Concentrations of basal LH (figure

2) were as high as 1.45 ± 0.15 ng/ml at 15 wk but decreased to 0.89 ± 0.14 ng/ml ($p < 0.05$) at 19 wk. However, LH continuously increased ($p < 0.05$) between 19 and 32 wk, when it reached 2.4 ± 0.19 ng/ml, decreased to 1.21 ± 0.14 ng/ml at 36 wk but peaked again at 50 wk (1.84 ± 0.12 ng/ml).

As shown in figure 3, concentrations of GnRH-stimulated FSH continuously increased ($p < 0.05$) between 19 and 36 wk (from 1.52 ± 0.17 ng/ml to 2.77 ± 0.28 ng/ml), remained practically unchanged between 36 and 50 wk and decreased ($p < 0.05$) to 1.79 ± 0.19 ng/ml at 54 wk of age. GnRH-stimulated LH increased ($p < 0.01$) from 24 ± 2.6 ng/ml at 19 wk to 65 ± 4.8 ng/ml at 23 wk and 56 ± 4.6 ng/ml at 32 wk, but decreased ($p < 0.01$) to 35 ± 4.5 ng/ml at 36 wk. Coincidentally with basal levels, there was a second increase in GnRH-stimulated LH after the age of 40 wk.

Peripheral concentrations of basal T varied from 1.0 ± 0.15 to 0.88 ± 0.11 ng/ml between the ages of 17 and 21 wk, but continuosly increased thereafter, reaching 8 ± 1.02 ng/ml at 54 wk (figure 4). Concentrations of basal E2 decreased ($p < 0.05$) from 5.12 ± 0.3 pg/ml at 17 wk to 3.03 ± 0.24 pg/ml at 23 wk, but increased with age afterwards (figure 4). Levels of basal Δ_4A increased ($p < 0.05$) from 0.20 ± 0.03 ng/ml at 11 wk to 0.53 ± 0.05 ng/ml at 15 and 17 wk, but decreased to 0.16 ± 0.09 ng/ml at 21 wk and thereafter (figure 4). GnRH-stimulated T increased from 1.7 ± 0.4 to 3.5 ± 0.1 ng/ml ($p < 0.10$) between the ages of 15 and 23 wk. There was a transient peak in T between 36 and 40 wk, which was coincident with decreased levels of basal and GnRH-stimulated LH (figure 5). Concentrations of GnRH-stimulated E2 decreased ($p < 0.05$) from 4.9 ± 0.3 pg/ml to 3.1 ± 0.14 pg/ml between 19 and 23 wk, but consistently increased with age thereafter.

Correlations between histological aspects of the first and second testis and peripheral hormone concentrations

At the state of development at which concentration of T was 1 ng/ml , diameter and weight of the testis averaged 32 ± 0.6 mm and 36 ± 1.8 g, respectively. Seminiferous tubules occupied 57 ± 1.1 % of the testicular volume. Sixty-nine percent (S.E.M. = 9 %) of the tubule cross sections had no germ cells and only 31 ± 9 % contained gonocytes and spermatogonia (A1, Intermediate and B types). At 20 wk, testis weight (TW1) was negatively correlated with ST01 ($r = -0.57$, $p < 0.01$) and positively correlated with STG1 ($r = 0.62$, $p < 0.01$).

At 54 wk of age (12 mo), the average testis weighed 264 ± 19 g and the seminiferous tubules occupied 76 ± 0.7 % of their volume. Also, there were 50 ± 4 round spermatids per A1 spermatogonia (RA) and 7.7 ± 0.4 round spermatids supported by each Sertoli cell (RS) and 3.4 ± 0.4 x 10^{10} round spermatids per testis (RST). The number of Sertoli cells (SC) estimated in the yearling testis (5.2 ± 0.4 x 10^{9}) was not different ($p > 0.10$) from that counted in the 20-wk old testis (4.9 ± 0.4 x 10^{9}). Also, both testis weight (TW2) and number of Sertoli cells per testis (SC) were related to number of A1 spermatogonia (A1, $r > 0.58$, $p < 0.01$) and round spermatides per testis (RST, $r = 0.84$, $p < 0.01$). Moreover, TW and SC were correlated with RA ($r = 0.50$, $p < 0.01$), RS ($r = 0.60$ and 0.43, respectively) and number of ST sections without germ cells (ST02, $r = -0.52$ and 0.62, $p < 0.01$) and number of sections with round (STD), elongate (STE) or mature spermatids (STM; $r = 0.53$ to 0.73, $p < 0.01$).

As shown in table 1, testis weight at AGE1 was correlated with TW2, SC and RST ($r = 0.58$, $p < 0.01$), number of spermatids per Sertoli cell ($r = 0.41$, $p < 0.05$), STE ($r = 0.47$,

$p < 0.05$) and STM ($r = 0.52$, $p < 0.05$). Number of seminiferous tubules with elongate or mature spermatids in the second testis were not correlated with either number of ST sections without or with germ cells in the first testis. Testis diameter (AVTD) averaged between the ages of 30 and 44 wk (7 and 10 mo) or testis weight at 54 wk (12 mo) were correlated with AGE1 ($r = -0.58$ and -0.42, $p < 0.01$), TW1 ($r = 0.65$ and 0.58, $p < 0.01$), ST01 ($r = -0.45$ and -0.40, $p < 0.10$) and STG1 ($r = 0.43$ and 0.41, $p < 0.10$). Regression analysis using AVTD (in mm) as the dependent variable and age (AGE1, in days) and body weight (BW, in Kg) when T = 1 ng/ml as the independent variables generated the following model: AVTD = 68.7 - 0.33 x AGE1 + 0.19 x BW, with $R^2 = 0.41$. The p-values for AGE1 and BW were 0.0016 and 0.0007, respectively.

Basal hormone concentrations measured at 11 and 15 wk were not correlated with AGE1. However, as shown in table 2, GnRH-stimulated FSH at 11 wk was related to ST01 ($r = 0.55$, $p < 0.01$) and to STG1, TW1, TW2 and TD ($r = -0.42$ to -0.53, $p < 0.05$). FSH at 15 wk was weakly correlated with ST01 ($r = 0.47$, $p < 0.05$) and STG1 ($r = -0.50$, $p < 0.05$). GnRH-stimulated T at 15 wk was related to TW1 ($r = 0.55$, $p < 0.01$) and TD ($r = 0.48$, $p < 0.05$). Bulls that secreted more E2 after a GnRH treatment at 15 wk had larger TW1 ($r = 0.60$, $p < 0.01$), TD ($r = 0.62$, $p < 0.62$, $p < 0.01$) and TW2 ($r = 0.45$, $p < 0.10$). Also, E2 at 15 wk was related to ST01 and STG1 ($r = -0.50$ and 0.53, respectively, $p < 0.05$).

Discussion

Age-related changes in testis size and hormone concentrations and the effects of hemicastration

Results of this study show that unilateral castration at the state of development at which T = 1 ng/ml does not impair the growth of the remaining testis (figure 1). The testis of the yearling hemicastrated bull (264 ± 19 g) was heavier than the testis of intact bulls (230 ± 16 g) used in study 1 (see Results - study 1). Number of Sertoli cells was not affected by hemicastration and the round spermatid/A1 spermatogonia ration (50 ± 4) was not significantly different from that in intact bulls (42 ± 9). However, the number of round spermatids per testis ($3.4 \pm 0.4 \times 10^{10}$) and per Sertoli cell (7.7 ± 0.4) were higher in the castrated than in intact bulls ($0.85 \pm 0.58 \times 10^{10}$ and 6.31 ± 1.41, respectively). These findings are similar to those of Mirando et al. (1989) and Barnes et al. (1980) reporting that unilateral castration of rams and bulls increased daily sperm production and the number of spermatids supported by each Sertoli cell. Thus, results presented here support the concept that the Sertoli cell population in the bovine remains stable after the age of 20 wk. Also, as it seems to happen in the ram (Mirando et al., 1989), greater number of spermatids per testis in hemicastrated bulls was not related to a decrease in the rate of germ cell degeneration, as revealed by the unchanged spermatid/A1 spermatogonia ratio.

Correlations between testis weight, Sertoli cell and germ cell numbers and degree of seminiferous tubule development were as significant as those estimated in intact Angus bulls (see Results - Study 1, tables 1.4 and 1.5). Thus, hemicastration induced germ cell

proliferation in the remaining testis but, as in the intact bull, such event was closely regulated by the Sertoli cell population.

Unilateral castration was followed by significant increases in GnRH-stimulated LH and FSH (figure 3). Studies have suggested that hemicastration in prepubertal bulls leads to enhanced basal FSH, but not LH secretion (Barnes et al., 1981; Schanbacher et al., 1987). However, although intact bulls showed a 30 % increase in LH between 19 and 32 wk (4.4 and 7.3 mo; see Results - Study 1, figure 1.3), such increase was not as great as that (2.8-fold) detected in hemicastrated bulls (figure 2). Also, reduced levels of E2 at the same period strongly suggest that, contrary to what had been found before, hemicastration did account for elevated basal LH secretion.

Although E2 significantly decreased as early as 2 wk after hemicastration, concentrations of T were not reduced (figures 4 and 5). Brown et al. (1987) showed that serum testosterone was lower 48 h after unilateral castration in rams, but levels returned to normal after 1 wk. Thus, it is possible that if there was a decrease in peripheral T concentration, such decrease was small and rapidly overcome by the growth of the second testis. In this case, high LH and FSH appeared to induce the second testis to grow and maintain normal T concentration and such effects are related to increases in testicular LH and FSH receptor numbers (Schanbacher et al., 1987). Also, because the Leydig cell is regulated by gonadotropins (Jégou and Sharpe, 1993), it is possible that high FSH and LH increased T secretion by Leydig cells of the single testis.

The increase in FSH and LH after 36 and 30 wk, respectively, was coincident with similar increases in testis size and steroid secretion (T and E2). Such events were also

detected in intact beef bulls (Evans et al., 1993; Study 1, figures 1.1 through 1.6) and suggest that, 10 to 16 wk after hemicastration, the function of the pituitary-gonad axis had returned to normality.

Correlations between histological aspects of the first and second testis and hormone concentrations

Bulls with larger testis between 7 and 10 mo reached T = 1 ng/ml at earlier ages. As determined by regression analysis, the age and body weight at which T reached 1 ng/ml accounted for 41 % of the variation in testis size of the prepubertal bull (7- to 10-mo old). Thus, significant differences between slow and fast growing bulls are determined by the ability of their Leydig cells to differentiate and secrete testosterone early in life. Histological criteria of the first testis (when T = 1 ng/ml) were related to characteristics of the second one (table 1). However, because the correlations were of low magnitude ($r < 0.50$), it is possible that events occurring at later stages of testicular development are also crucial for determining sperm production rates in the yearling bull.

The control the neonatal stages of testicular development involves the actions of gonadotropins and local factors (Huhtaniemi, 1993; Robertson et al., 1993; Kierszenbaum, 1994). In support of this concept, results from this study revealed that FSH secreted as early as 11 wk and testosterone and estradiol at 15 wk were related to testis weight at AGE1 and the average testis diameter between 7 to 10 wk (TD). Moreover, bulls with more ST sections with germ cells at AGE1 and larger TD had a tendency to secrete less FSH at 11 wk and more E2 at 15 wk. The existence of correlations between testicular size and GnRH-stimulated

T suggests an effect by LH, although correlations involving LH were not significant. Evans et al. (1995) reported that early maturing Hereford bulls secreted more LH between 12 and 17 wk than did late maturing bulls. However, as found in the present study, these authors did not show differences in basal T between early and late maturing bulls.

Correlations between FSH and aspects of the testis at prepuberty and AGE1 had not been reported before and points toward a role played by FSH in the early stages of germ cell development. It is known that FSH stimulates spermatogonial proliferation (Sharpe, 1994) and survival of gonocytes in the neonatal rat testis is dependent on a factor secreted by the Sertoli cell (Steel factor, Kierszenbaum, 1994), which in turn can be regulated by FSH (Rossi et al., 1993). Because FSH also induces estradiol and inhibin secretion by the Sertoli cell (Dorrington and Kahn, 1993; Vale et al., 1994) is probably the reason for negative correlations between FSH and testicular development. Finally, the fact that the correlations involving FSH were of low magnitude ($r = -0.42$ to -0.55) suggests that factors unrelated to FSH and its control play important roles in germ cell proliferation and differentiation.

Implications

According to the results presented in this study, bulls with larger testis at prepuberty (30 to 44 weeks) and at the age of 1 year had faster rate of testicular development early in life, as revealed by the age at which levels of testosterone reached 1 ng/ml. Because prepubertal testis size is closely related to age at puberty, it possible that the pattern of testosterone secretion early in life can be used as a reliable criteria for selecting early maturing

bulls. However, since the results from regression analysis were of low magnification (R^2 = 0.41), other important factors may also contribute to determine testicular development and, as a consequence, age at puberty. The existence of correlations between FSH and steroids at 11 and 15 weeks and characteristics of the immature and mature testis may help researchers to determine models for selecting bulls with high sperm-producing capacity based on the hormone profiles.

Table 1: Pearson's partial correlations between testis size and histology at T = 1 ng/ml and degree of testicular development in the prepubertal and yearling Angus bull (n=28).

	AVTD	TW2	SC2	RST	RS	STE	STM
AGE1	- 0.58 c	- 0.42 b	NS	NS	NS	NS	NS
TW1	0.65 c	0.58 c	0.58 c	0.59 c	0.41 b	0.47 b	0.52 b
ST01	- 0.45 b	- 0.40 a	- 0.44 b	- 0.45 b	NS	NS	NS
STG1	0.43 b	0.41 a	0.40 a	0.41 b	NS	NS	NS

AGE1 and TW1: age (in days) and testis weight when T = 1 ng/ml.
ST01 and STG1: percent of seminiferous tubule cross sections without germ cells and with gonocytes or spermatogonia when T = 1 ng/ml, respectively.
AVTD: average diameter of testis between 30 and 44 wk (7 to 10 mo).
TW2, SC2 RST and RS: testis weight, number of Sertoli cells and round spermatids per testis and number of round spermatids per Sertoli cell at the age of 54 wk (12 mo).
STE and STM: percent of seminiferous tubule cross sections with elongate and mature spermatids at the age of 54 wk (12 mo).
Partial variable: body weight when T = 1 ng/ml.
NS: nonsignificant ($p > 0.10$); a, b, and c: $p < 0.10$, $p < 0.05$ and $p < 0.01$, respectively.

Table 2: Pearson's partial correlations between GnRH-stimulated FSH, testosterone (T) and estradiol (E2) at the ages of 11 and 15 wk and size and histology of the bull testis at AGE1 and 54 wk (n=28).

	Ages at which hormone concentrations were estimated:					
	11 wk			15 wk		
	FSH	T	E2	FSH	T	E2
TW1	- 0.43 b	NS	0.36	NS	0.55 c	0.60 c
AVTD	- 0.42 b	0.41 b	0.33	NS	0.48 b	0.62 c
ST01	0.55 c	NS	NS	0.47 b	NS	- 0.43 b
STG1	- 0.53 c	NS	NS	- 0.51 b	NS	0.46 b
TW2	- 0.42 a	NS	NS	NS	NS	0.45 b

TW1: testis weight when T = 1 ng/ml.
AVTD: average diameter of the testis between 30 and 44 wk (7 and 10 mo).
ST01 and STG1: percent of seminiferous tubule cross sections without germ cells and with gonocytes or spermatogonia when T = 1 ng/ml, respectively.
TW2: testis weight at 54 wk (12 mo).
Partial variable: body weight at 11 and 15 wk.
NS: nonsignificant ($p > 0.10$); a, b and c: $p < 0.10$, $p < 0.05$ and $p < 0.01$, respectively.

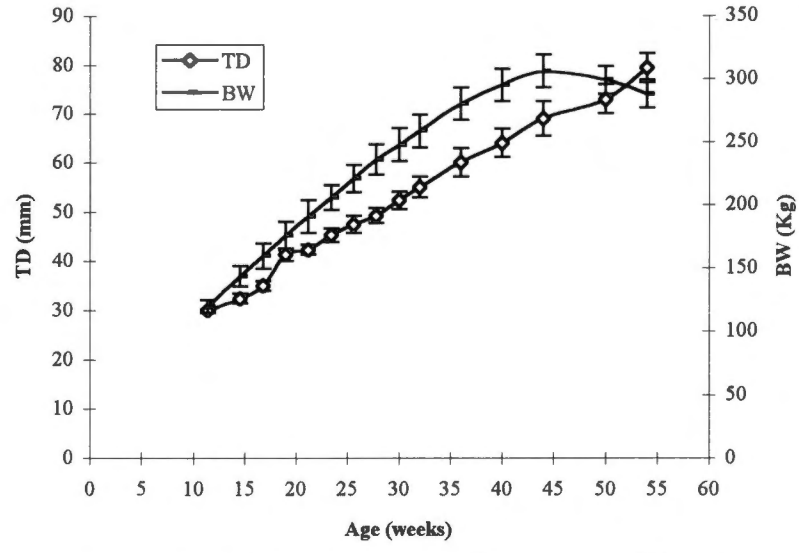


Figure 1: Age-related changes in testis diameter (TD) and body weight (BW) in Angus bulls (n=14).

Bulls were unilaterally castrated when concentrations of testosterone reached 1 ng/ml (average age of 20 +/- 0.3 wk).
Error bars represent the standard error of the mean within age-periods.

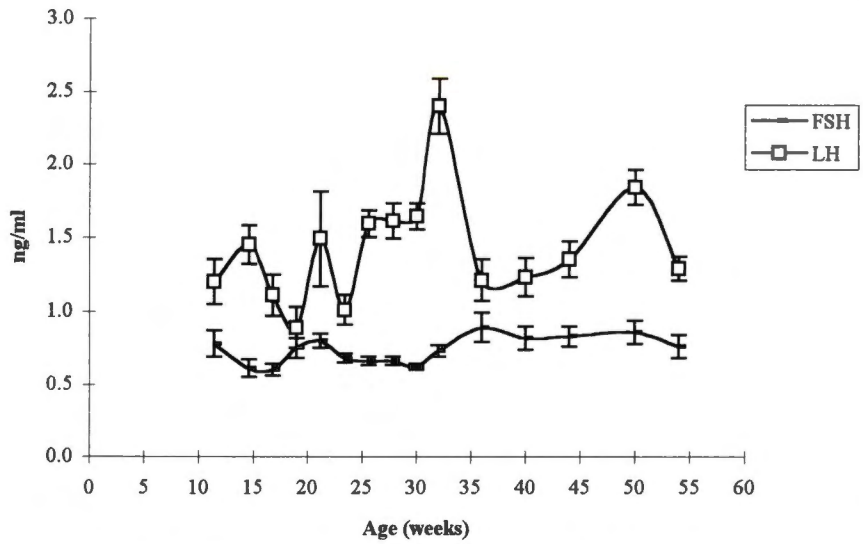


Figure 2: Concentrations of basal FSH and LH in Angus bulls (n=14).

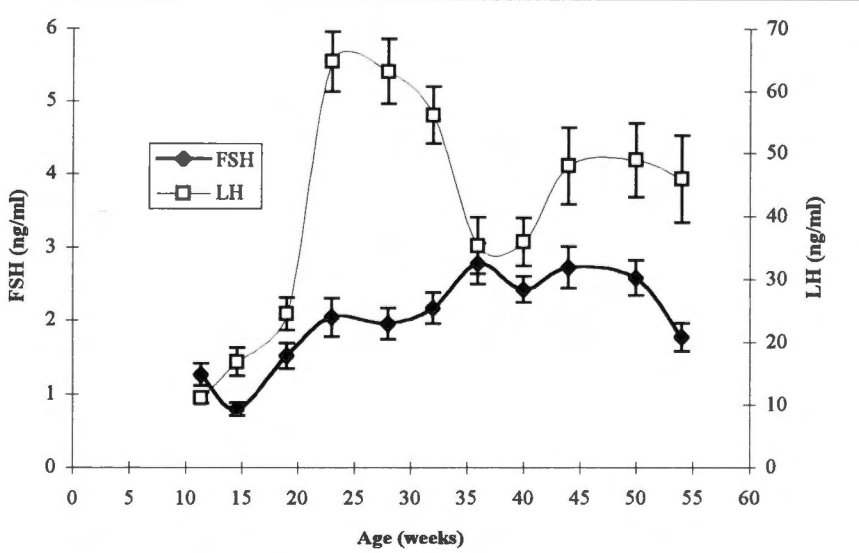


Figure 3: Concentrations of GnRH-stimulated FSH and LH in Angus bulls (n=14).

Bulls were unilaterally castrated when concentrations of testosterone reached 1 ng/ml (average age of 20 +/- 0.3 wk).
Basal values: average of 3 blood samples taken at 1.5-h intervals.
GnRH-stimulated values: average of blood samples taken 1.5 and 3 h after the GnRH treatment.
Error bars represent the standard error of the mean within age-periods.

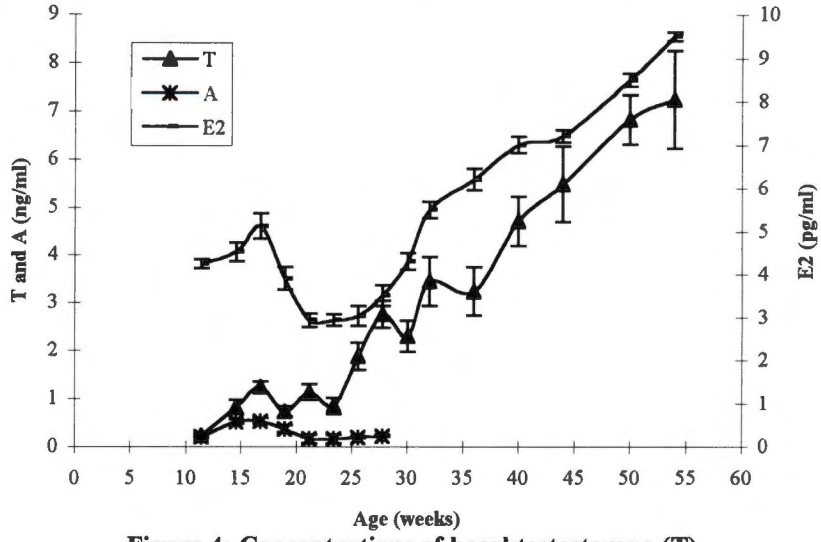


Figure 4: Concentrations of basal testosterone (T), androstenedione (A) and estradiol (E2) in Angus bulls (n=14).

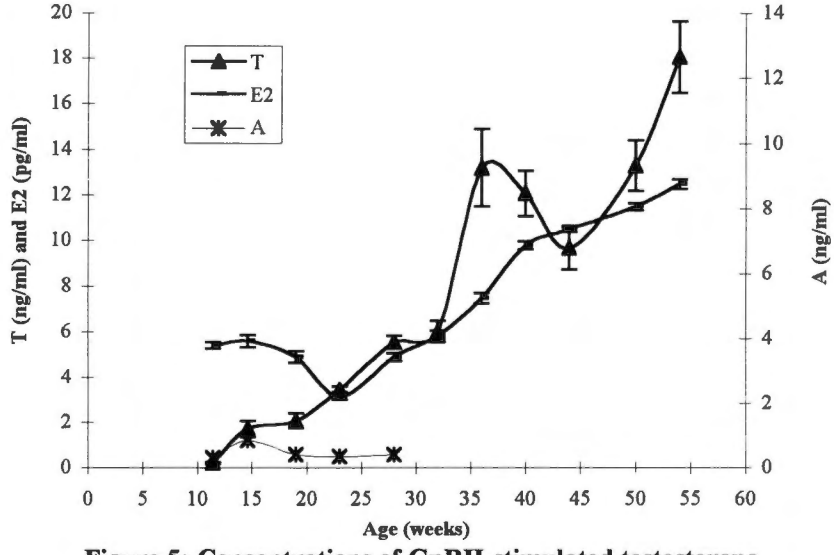
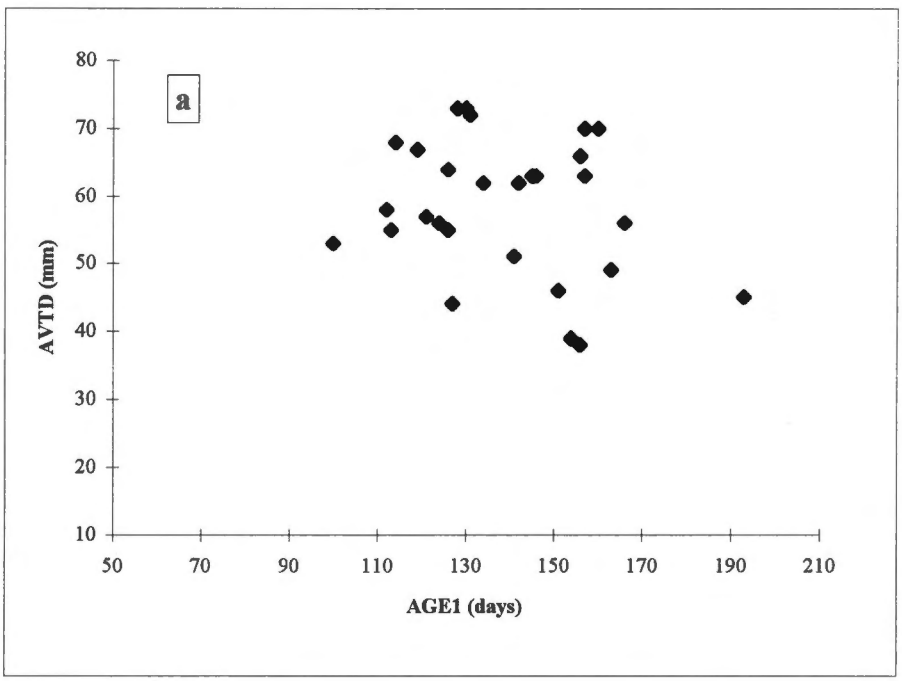


Figure 5: Concentrations of GnRH-stimulated testosterone (T), androstenedione (A) and estradiol (E2) in Angus bulls (n=14).

Bulls were unilaterally castrated when concentrations of testosterone reached 1 ng/ml (average age of 20 +/- 0.3 wk).
Basal values: average of 3 blood samples taken at 1.5-h intervals.
GnRH-stimulated values: average of blood samples taken 1.5 and 3 h after the GnRH treatment.
Error bars represent the standard error of the mean within age-periods.



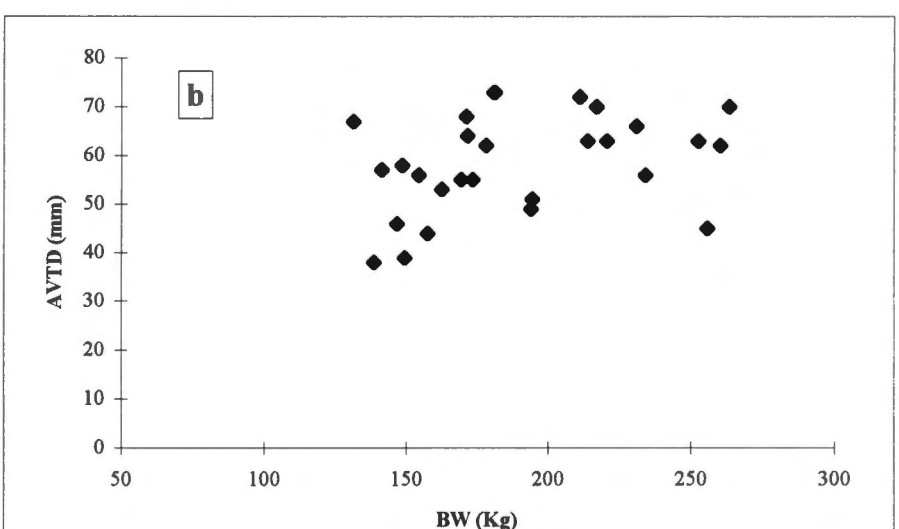


Figure 6: Relationship between average testis diameter between 30 and 44 wk (AVTD) and a) AGE1 and b) body weight (BW)* in Angus bulls (n=28).

Regression model: AVTD = 68.7 - 0.33 x AGE1 + 0.19 x BW, with $R^2 = 0.41$ and p-values of 0.0016 (AGE1) and 0.0007 (BW).
AGE1: age at which concentrations of testosterone reached 1 ng/ml.
* Body weight recorded at AGE1.

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PART IV

(Study 4)

Abstract

A study was conducted to determine the sites of expression of retinol-binding protein (RBP) in the bovine testis and to evaluate the profiles of RBP mRNA levels before spermatogenesis had initiated (10 weeks), when A1 spermatogonia were being differentiated from gonocytes (20 weeks) and when spermatids were being produced at prepuberty (34 weeks). Furthermore, the hypothesis that RBP could serve as a marker of state of development of the seminiferous epithelium was evaluated. At the ages of 10 (n=3), 20 (n=8) and 34 (n=7) weeks, Angus bulls were bled three times and surgically castrated afterwards. Blood samples were assayed for LH and testosterone (T) and the degree of seminiferous tubule development was evaluated in tissue samples stained with hematoxylin and eosin. Immunolocalization of RBP was detected according to the biotin-strepavidin-horseadish peroxidase method (samples from 10-wk old testes could not be analyzed). Nylon membranes containing isolated total RNA were hybridized with a bovine cDNA (bcRBP-700) and a β-actin probe and slot blots were carried out to determine the abundance of RBP and β-actin transcripts. Age-related changes in testis weight, hormone concentrations and relative quantity of RBP transcripts were determine by analysis of variance. Results showed that testis weight and levels of LH and T increased with age ($p < 0.05$). Also, seminiferous tubules at 10 wk had immature Sertoli cells and gonocytes but, at 20 wk, spermatogonia and few spermatocytes were detected. At 34 wk, Sertoli cells appeared differentiated and spermatids were observed. As never shown before in the bovine species, RBP was detected in Sertoli, Leydig and peritubular cells at the ages of 20 and 34 wk, but no immunoreactivity was present in the

germ cells. Furthermore, no difference in staining between Sertoli cells from tubules with or without germ cells was detected. Thus, as determined by immunohistochemistry, RBP does not appear to be a distinct marker of Leydig and Sertoli cell differentiation. Northern hybridization of testicular RNA revealed the presence of a mRNA of 1.1 Kb, which was similar to previous RBP transcripts found in bovine conceptuses and extraembryonic membranes. Densitometric scanning of slot blots indicated that the fraction of total RNA coding for RBP was 50 % higher at 10 wk than at 20 and 34 wk. Thus, it is possible that testicular RBP mRNA is down-regulated by testosterone and/or LH, but further studies are needed to verify this hypothesis.

Key words: Retinol-binding Protein, Bull, Testis, Sertoli, Leydig.

Introduction

It is well known that vitamin A is essential for the development of the seminiferous epithelium in mammals (Thompson et al., 1964, Huang et al., 1979). Decrease in testis size, cessation of spermatogenesis due to reduction in the spermatogonial population and degeneration of spermatocytes and spermatids have been observed in rats fed diets deficient in vitamin A (Mitramond et al., 1979; Sobhorn et al., 1979). Furthermore, Wang and Kim (1993) reported that A1 spermatogonia in vitamin A deficient rats were arrested at the S phase of the cell cycle. In the rodent, retinol deficiency resulted in FSH hypersecretion, low testosterone and unchanged LH plasma levels (Huang et al., 1983). Attempts to restore

normal spermatogenesis with testosterone (Huang et al., 1983) or retinoic acid were unsuccessful, whereas retinol replacement alone resulted in normal germ cell development (Huang et al., 1983; Thompson, 1969; Ahluwalia and Bieri, 1971). Thus, vitamin A has been shown to affect the normal course of spermatogenesis and by means other than deficiencies in gonadotropin or androgen secretion.

Vitamin A also plays important roles in the function of somatic cells of the testis. In retinol deficient rats, Sertoli cell cytoplasm appeared disintegrated and exhibited lysosome-like inclusions but other structures of the cell were unaffected (Sobhorn et al., 1979). Retinol stimulated the synthesis of androgen-binding protein (Karl and Griswold, 1980), transferrin (Skinner and Griswold, 1982; Hugly and Griswold, 1987) and cellular retinol-binding protein (CRBP) in cultures of Sertoli cells obtained from rats fed vitamin A-free diets (Faraonio et al., 1993). Also, retinol and retinoic acid can modulate the effect of FSH on the Sertoli cell by decreasing levels of cAMP in vitro (Galdieri and Nisticò, 1994). The effects of retinol on the Leydig cell are less defined, but it has been shown that serum testosterone was significantly lower in vitamin A-deficient rats than in controls. Moreover, retinoic acid did not prevent the lesions in the germinal epithelium in these rats, but did restore normal testosterone concentrations. Thus, there appear to be differences in the response of the seminiferous epithelium and interstitial cells to vitamin A and its derivatives.

In plasma, retinol forms a high-affinity complex with retinol-binding protein (RBP) and transthyretin (Soprano and Blaner, 1994). RBP is primarily secreted by the liver (Soprano et al. 1982), but has also been found in other tissues affected by vitamin A such as the eye (Martone et al., 1988), bovine uterus (Liu and Godkin, 1992) and rat testes (Kim and Wang,

1993). RBP has been detected in the interstitium (McGuire et al., 1981; Kato et al., 1985) and cultures of Sertoli and peritubular cells (Davis and Ong, 1992, 1995; respectively). The function of Leydig cell-secreted RBP is not known, but peritubular and Sertoli cell RBP may be involved in the transport of retinol to the adluminal germ cells.

Despite the importance of vitamin A and its derivatives to testicular development, no studies have focused attention on the role of retinol and retinol-binding proteins in the testis of farm animals. Thus, the objective of this study was to determine the sites of RBP expression in the bovine testis and to evaluate the profiles of RBP mRNA levels before spermatogenesis had initiated (10 weeks), when A1 spermatogonia were being differentiated from gonocytes (20 weeks) and when spermatids were being produced at prepuberty (34 weeks). Furthermore, because the patterns of synthesis of some proteins such as inhibin and aromatase are associated with developmental phases of the Sertoli cell (Bardin et al., 1994), the hypothesis that RBP could serve as a marker of state of development of the seminiferous epithelium was evaluated.

Materials and Methods

General procedures

Angus bulls born in February and March were kept with their dams and raised on pasture. At the ages of 10 (n=3), 20 (n=8) and 34 weeks (n=7)), calves were bled three times at 1.5-hour (h) intervals and surgically castrated afterwards. 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 centrifuged at 1764 x g for 25 minutes. Serum was harvested and stored at - 20 °C until assayed for LH and testosterone. After removal of the tunica vaginalis and epididymis, testes were weighed and two 4-mm thick segments were taken from the same region of one testis, placed in Bouin's fluid for 24 h, and washed in three changes of 70 % ethanol. Also, four grams of testicular parenchyma were obtained for RNA isolation.

Radioimmunoassays

Blood samples collected at 10, 20 and 34 weeks (wk) were analyzed in the same assays to avoid interassay variation. Peripheral concentrations of LH were quantified in 100 μl of serum using a double-antibody RIA method first described by Niswender et al. (1969) and modified by Bolt et al. (1981). The LH first antibody (# 15 antiovine 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-assay coefficient of variation was < 7%. Samples of serum (150 µl) were extracted with 1.5 ml of benzene before being analyzed for testosterone (T). Concentrations of T were estimated based on a single-antibody method described by Jackson et al. (1989) and the T antibody was provided by Dr. G. Niswender. The assay sensitivity was 10 pg/ml and the intra-assay coefficient of variation was <8%.

Histological analysis and immunocytochemical localization of RBP

After fixation, the tissue was dehydrated in graded ethanol, embedded in paraffin, sectioned at 5 μm and finally stained for hematoxylin and eosin. To estimate the degree of seminiferous epithelium development, 200 cross sections of seminiferous tubules from each bull were evaluated and placed in one of the following categories, based on the most advanced germ cell type: tubules without germ cells (ST0), tubules with A1 spermatogonia (STA), Intermediate or B spermatogonia (STB), pachytene spermatocytes (STP), round spermatids (STD), elongated spermatids (STE) and mature spermatids (STM). The diameter of the Sertoli cell nucleolus at each age-group represented the average of nucleoli of bulls with the same age (10 cells were measured per testis).

The procedure used for the immunolocalization of RBP was similar to that described by Stenberger et al. (1970) and Liu et al. (1993). Briefly, tissue was sectioned at 5 μm and adhered on ProbeOn plus slides (Fisher Scientific, Pittsburg, PA). Anti-bcRBP antibody, diluted (1:200) in PBS containing 1 % normal goat serum, was applied to sections and incubated at 37 C for 2 h. Controls were treated with RBP-adsorbed anti-bcRBP serum (1:200 dilution) and incubated at the same conditions. Binding of anti-bcRBP was detected according to instructions for the supersensitive biotin-streptavidin-horseradish peroxidase procedure anti-rabbit IgG immunohistochemical staining kit (Biogenex, San Ramon, CA). Visualization of the specific interaction between the secondary and primary antiserum was achieved by using 3,3'-diaminobenzidine staining for 3 minutes (min.). Samples from 10-wk old testes were not fixed properly and were not used in the immunohistochemical analysis.

RNA isolation and Northern blots

Total RNA was isolated from 10, 20 and 34-wk old testes and bovine liver and spleen according to the method described by Chirgwin et al. (1980). Four grams of tissue was homogenized in 30 ml of a guanidine isothiocyanate solution followed by ultracentrifugation in cesium trifluoroacetate (CsTFA). After centrifugation, the pellet was dissolved in 960 µl of a solution containing water (DEPC-treated), 0.1 % sarcosine, 30 µm Na citrate and 1 % β-mercaptoethanol and centrifuged for 30 min. The supernatant was then mixed with Na acetate and 100 % ethanol and incubated overnight at - 4° C. Next day, samples were centrifuged for 30 min., dried in a speed vacuum, dissolved in 250 µl of a solution containing sarcosine (1 %), β-mercaptoethanol (1 %) and 3-(n-morpholino)propanesulfonic acid (MOPS) buffer and stored at - 80° C.

Northern hybridization was prepared as previously described by Thomas (1980). Based on the analysis of spectrophotometry, the volume of each sample that contained 20 µg of total RNA was estimated and diluted in 10 µl of formamide, 3.5 µl of formaldehyde, 2 µl of 10-strength MOPS and water. Isolated RNA was electrophoresed in 1.4 % (w/v) agarose acid gel containing formaldehyde and MOPS (Sambrook et al., 1989). Since preliminary results showed that the bovine liver contained a large quantity of RBP while the spleen expressed only trace amounts of this protein, samples from these organs were used as positive and negative controls, respectively. Gels were stained with 0.2 µg/ml ethidium bromide to check if the RNA was intact.

Total RNA from bulls at 10 (n=3), 20 (n=4) and 34 (n=4) wk was capillary transferred from the gel to a Nytran positively charged nylon membrane (Schleicher and Schell, Keene,

NH) with 10-strength SSPE (single strength = 0.15 M NaCl, 10 mM $NaPO_4$ [ph 7.4], 1 mM EDTA) for 18 h. RNA was crosslinked to the nylon membrane by UV radiation (0.12 joules; Hoeffer UVC 1000; San Francisco, CA) and hybridized to ^{32}P-labeled bovine RBP cDNA probe (bcRBP-700; Liu et al., 1993). Membranes were prehybridized at 65°C for 3 h with a solution containing 50 % (v/v) formamide, 5-strength SSPE, 5 % (w/v) dextran sulfate, 0.1 % SDS, 5-strength Denhardt's reagent (single strength = 0.1 % (w/v) of BSA, Ficoll and polyvinilpyrrolidone) and 0.1 mg/ml heat-denatured, sheared, single-stranded salmon sperm DNA in a rotatory hybridization oven (Hybaid, Teddington, UK). After discarding this solution, membranes were hybridized with the same solution containing 1.84 x 10^6 cpm/ml for 24 h at 42°C. After hybridization, membranes were washed twice (15 min.) in 5-strength SSPE/0.1 % (w/v) SDS and once in single-strength SSPE/0.1 % (w/v) SDS (15 min.) at 42°C. High stringency washes were carried out twice in 0.1-strength SSPE/0.1 % (w/v) SDS at 65°C for 15 and 30 min. Autoradiographs were obtained by exposing the blots to x-ray film (Kodak XAR, Eastman Kodak Company, Rochester, NY) at - 80°C with Cronex intensifying screens for 72 h. In order to remove the RBP probe off the RNA template, membranes were boiled twice in water containing 0.1 % N-lauryl sarcosine for 15 min. and hybridized with a β-actin probe (2.3 x 10^6 cpm/ml) for 12 h at 42°C. Prehybridization, hybridization and washes were carried out in the same conditions as described above. In this case, autoradiographs were obtained by exposing RNA blots to x-ray films for 24 h. The size of the transcripts were estimated by comparing the distance migrated by the samples with the distance migrated by the 28S and 18S ribosomal RNAs (6333 and 2266 basepairs, respectively).

Slot Blots

Total RNA (20 μg per well) was transferred to nylon membranes using a Minifold slot-blot apparatus (Schleicher and Schuell, Keene, NH) and crosslinked to the membrane by UV radiation (0.12 joules). In this case, samples from all castrated animals were used (n=3 at 10 wk; n=8 at 20 wk; n=7 at 34 wk). Samples were hybridized with labeled RBP and β-actin as described for the Northern analysis. Autoradiographs were obtained by exposing the membranes to X-ray films for 96 h (RBP) and 72 h (β-actin) at - 80°C. The abundance of the RBP and β-actin transcripts was estimated by densitometry and computer integration and data were generated as the area (in arbitrary units) of each band of the slot (GSXL ver. 2 software; LKB). Area of the samples was corrected for differences in total RNA loaded in the wells by comparison with the corresponding area of the β-actin autoradiograph.

Statistical Analysis

Age-related changes in testis weight, hormone concentrations and relative quantity of RBP transcripts were determined by analysis of variance (F test) and Duncan's multiple comparison test (General Linear Models procedure; SAS, 1990). Standard error for age groups was calculated after sample areas were normalized for the respective β-actin area.

Results

Age-related changes in testicular development and peripheral hormone concentrations

Testis weight in Angus bulls increased ($P < 0.01$) from 14 ± 2.5 g at 10 wk to 36 ± 1.6 g at 20 wk and 112 ± 19.6 g at 20 wk (figure 1). Also, basal concentrations of LH and T changed with age (figure 1). Levels of LH started at 0.50 ± 0.1 ng/ml (10 wk), but increased to 0.70 ± 0.1 ng/ml at 20 wk and 1.10 ± 0.1 ng/ml at 34 wk ($p < 0.05$). Concentrations of testosterone increased 5-fold between 10 and 20 wk (from 0.35 ± 0.1 to 1.67 ± 0.5 ng/ml; $p < 0.01$) and 2.6-fold between the ages of 20 and 34 wk (from 1.67 ± 0.5 to 4.34 ± 1.4 ng/ml; $p < 0.01$).

Histological evaluation of the seminiferous epithelium revealed that Sertoli cells at 10 and 20 wk had round or oval nuclei with large amounts of heterochromatin, and no indentations on the nuclear membrane. However, the Sertoli cell nucleus at 10 wk was smaller than that at 20 wk (6.3 ± 0.1 versus 7 ± 0.2 μm; $p < 0.10$). At 34 wk, the nuclear diameter significantly increased to 9.2 ± 0.04 μm ($p < 0.05$), the nuclear membrane contained several indentations and the nucleolus appeared as a single structure with little surrounding chromatin.

In the 10 wk-old testis, 92 ± 1.5 % of the seminiferous tubules had no germ cells and the remaining 8 % had only gonocytes, but no more advanced germ cell types. At 20 wk, an average of 60 ± 6.8 % of the seminiferous tubule sections did not have germ cells, while 34 ± 4.9 % had gonocytes and/or A1 spermatogonia, 5 ± 3.1 % had differentiating spermatogonia (Intermediate or B type) and only 2 ± 1.4 % of the sections had primary

spermatocytes. At 34 wk, the percentage of sections without germ cells decreased to 18 ± 9.9 % and that of sections with spermatocytes as the most advanced germ cell type increased to an average of 24 ± 6.3 %. Also, the average bull had 29 ± 6.7 % of the seminiferous tubule sections with round spermatids, but only 17 ± 6.6 % and 3 ± 1.7 % of the sections with elongate and mature spermatids, respectively.

Immunolocalization of RBP in the developing testis

In the 20-wk old bovine testis, RBP was detected in both the seminiferous epithelium and interstitial tissue (figure 2, letter A). Under the conditions employed in the immunohistochemical analysis, the Sertoli cell exhibited positive staining for RBP, but no apparent staining of germ cells was observed. Also, peritubular myoid cells and Leydig cells showed RBP immunoreactivity. In the 34-wk old testis, RBP was also present in both the seminiferous tubules (Sertoli and peritubular cells) and the interstitium (figure 2, letters C, D and E). There was no detectable difference in staining between Sertoli cells from tubules with or without germ cells (figure 2, letter E). Again, neither spermatogonia nor meiotic germ cells appeared to express RBP at 34 wk. The IgG fraction that was adsorbed with the purified RBP produced no staining in samples from 20- or 34-wk old testes (figure 2, letters B and F).

Developmental Profile of RBP mRNA expression in the bovine testis

Northern hybridization of testicular RNA using the ^{32}P-labeled bcRBP-700 probe revealed the presence of a mRNA of approximately 1.1 Kb (figure 3). Overexposure of the autoradiographs (96 h) allowed the identification of higher molecular weight RBP transcripts,

which probably resulted from partially processed mRNA (data not shown). Liver RNA strongly hybridized with the RBP probe, while spleen RNA showed only trace amounts of RBP (data not shown).

Slot blots were used to quantify the relative amount of RBP mRNA at different phases of testicular development. As shown in figure 4, levels of RBP mRNA in the testis decreased 50 % ($p < 0.05$) between the ages of 10 and 20 wk, but remained practically unchanged as testis size and concentrations of LH and T significantly increased between 20 and 34 wk.

Discussion

Histological analysis of testis samples indicated that the Angus bulls used in this study were at distinct stages of testicular development. At 10 wk of age, the seminiferous tubules contained only immature Sertoli cells and gonocytes. At 20 wk, as concentrations of LH and T increased, the tubules had not only gonocytes but also A1, Intermediate and B spermatogonia, which indicated that spermatogenesis had been initiated. The 34-wk old testis had 53 % of the tubule sections with spermatocytes and round spermatids, but only 19 % of them with elongate and mature spermatids, suggesting that these bulls were at the early stages of puberty.

Morphological features of the nucleus and nucleolus of Sertoli cells at 10 and 20 wk resembled those of immature cells. At 34 wk, increased nuclear diameter, presence of indentations on the nuclear membrane and reduced amounts of heterochromatin associated with the nucleolus of Sertoli cells were characteristics of differentiated cells, as described by

Sinowatz and Amselgruber (1986). The increase in peripheral concentrations of testosterone between 10 and 34 wk (see figure 1) were distinct signs of Leydig cell maturation in prepubertal bulls (Amann, 1983; Wrobel, 1990).

Immunohistochemical analysis showed that retinol-binding protein was expressed by Sertoli, peritubular and Leydig cells from the initial stages of spermatogenesis (20 wk) until early puberty in the testis (34 wk). Under the conditions used in this study, no staining was detected in gonocytes, spermatogonia or meiotic germ cells. Thus, RBP immunoreactivity does not appear to be a distinct marker of the state of differentiation of either the Sertoli or Leydig cell in the bovine.

Localization of RBP in the bovine testis had not been reported before, but these results are in agreement with those by Davis and Ong (1992; 1995), which showed that both Sertoli and peritubular cells isolated from rat testes synthesized and secreted RBP. Also, RBP expression has been detected in the rat interstitium by autoradiographic (McGuire et al., 1981) and immunohistochemical methods (Kato et al., 1985). However, these authors failed to localize RBP in the seminiferous tubules.

The role of RBP secreted by peritubular cells and both immature and differentiated Sertoli and Leydig cells of the bovine testis has not been clearly defined. Based on studies performed in the rat, it is known that CRBP is expressed by peritubular and Sertoli cells and, in lesser amounts, by adluminal germ cells (Porter et al., 1985; Eskild et al., 1991). Cellular retinoic acid-binding protein is primarily synthesized by germ cells (Blaner et al., 1987; Eskild et al., 1991) and both Sertoli and germ cells are capable of expressing retinoic acid receptors (Huang et al., 1994). Because the junctional complexes formed by peritubular cells and Sertoli

cells seem to impose selective barriers to the passage of large molecules to the lumen of the tubules (Waithes and Setchell, 1969; Frawcett et al., 1970; Dym and Fawcett, 1970), it is thought that RBP mediates the transport of retinol through the peritubular space. Once taken up by the Sertoli cells, retinol would be bound by CRBP and either converted to retinyl esters or retinoic acid or released into the seminiferous fluid, where it would be bound by Sertoli cell-secreted RBP and delivered to meiotic germ cells. Although germ cells have CRBP, as mentioned above, it has not been shown if these cells have the ability to oxidize retinol to retinoic acid. Moreover, it is not known if the Sertoli cell secretes RBP through the basal or apical region, or both.

Rat tumor Leydig cells contain CRABP and retinoic acid receptors (Eskild et al., 1991), which suggests that these cells might have the ability to utilize retinol. However, why Leydig cells would synthesize RBP is not known. One possibility is that the Leydig cell, and also the Sertoli and peritubular cells, would secrete RBP to prevent the potentially damaging effects of free retinol. Studies have shown that high concentrations of retinol can destabilize lipoprotein membranes of erythrocytes, causing leaking of lysosomal enzymes and swelling of mitochondria (reviewed by Bielsalski, 1989). It is possible that tissues that require vitamin A secrete RBP not only to transport retinol between neighboring cells but also to prevent the toxic effects of free retinol on these same cells.

At either 20 or 34 wk, there was no apparent variation in RBP immunoreactivity between Sertoli cells in tubules with or without germ cells. However, studies performed in the rat testis (Scimitt and Ong, 1993; Porter et al., 1985) have reported that CRBP staining in Sertoli cells changed according to the stage of the spermatogenic cycle. Although species

differences may exist in this case, it is possible that expression of RBP and CRBP by the Sertoli cell is differentially regulated by germ cells.

In all testis samples, Northern hybridization detected a RBP transcript with the size of approximately 1.1 Kb, which is similar to mRNAs found in bovine conceptuses and extraembryonic membranes (1.4 Kb; Liu et al., 1993). Also, densitometric scanning of slot blot autoradiographs indicated that the fraction of total testicular RNA coding for RBP was significantly higher at 10 wk than at 20 and 34 wk (see figure 2). Similar developmental changes in levels of CRBP have been described in the rat. Eskild et al. (1991) reported that CRBP mRNA in rat testes decreased after the age of 10 days and Schimitt and Ong (1993) showed that CRBP expression in cytosol samples from rat testis was also reduced at ages older than 15 days. Although the age-related decrease in RBP mRNA could be partially affected by a dilution of the RNA as germ cells proliferated, it is possible that RBP is regulated by testosterone and gonadotropins during the early stages of spermatogenesis, but further studies need to confirm this hypothesis.

Implications

Although it is well known that vitamin A is essential for spermatogenesis, no research had focused attention on the profiles of retinol-binding protein expression in the testis of species that have economical relevance, such as the bovine. Results of this study, therefore, are the first to show the cell types that express RBP and the developmental changes in RBP mRNA levels in the bull testis. Understanding of the interactions between hormones, somatic

and germ cell development and RBP synthesis in the testis may help to determine the mechanisms of transport and metabolism of retinol. Furthermore, such knowledge may aid researchers to find out if sperm production can be increased by manipulation of levels of retinol and if patterns of synthesis of retinol-binding proteins reflect other aspects of testicular or epididymal function.

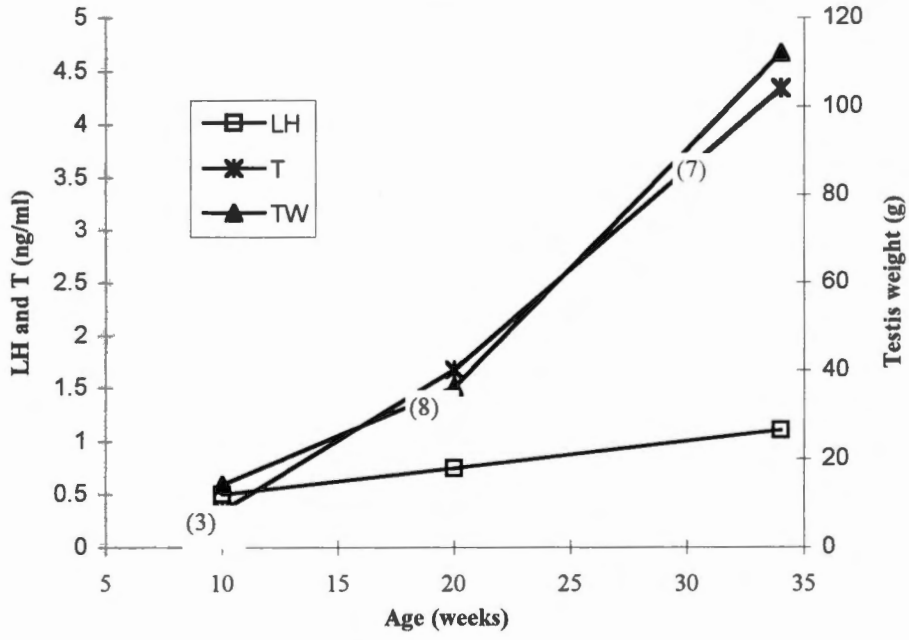
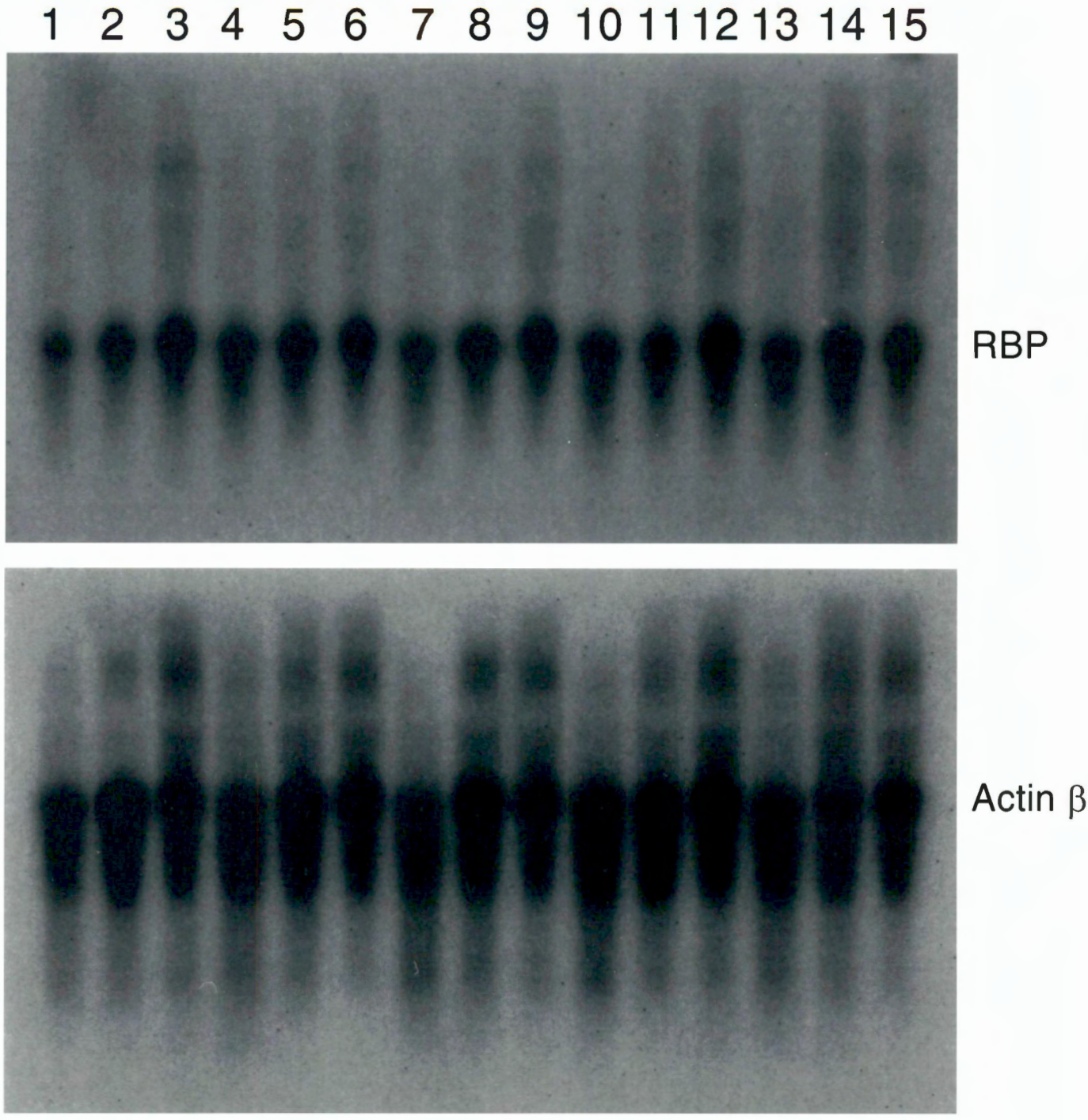


Figure 1: Age-related changes in testis weight and basal concentrations of LH and testosterone (T) in Angus bulls

The number of bulls sampled at each age-period is shown in parenthesis.
Basal hormone concentrations represent the average of 3 blood samples taken at 1.5-h intervals per animal.
S. E. M. for TW: 2.5, 1.6 and 19.6 g at 10, 20 and 34 wk, respectively.
S. E. M. for testosterone: 0.10, 0.50 and 1.4 ng/ml ar 10, 20 and 34 wk, respectively.
S. E. M. for LH: 0.10 at all age-periods.

Figure 2: Immunolocalization of RBP in the bovine testis. Sections (5 μm) of testes were incubated with anti-bcRBP and stained with 3,3'-diaminobenzidine for 3 minutes. Sections were counterstained with hematoxylin. Brown staining indicates the presence of RBP in testes from bulls at the ages of 20 weeks (A) and 34 weeks (C, D and E). Preimmune serum controls are shown for each age (B and F). Magnification: x 200 (C); x 400 (A, B, D, E, F).

Figure 3: Northern blot of bovine testis total RNA probed with bovine RBP cDNA. The RBP transcript (1.1 Kb) is indicated on the right. RNA samples from three different bulls at 10 wk were used in lanes 1, 4 and 7. Sample in lane 10 represent the same bull as in lane 7. Samples from four different animals at 20 wk of age were used in lanes 2, 5, 8 and 11. RNA isolated from four bulls at 34 wk were used in lanes 3, 6, 9 and 12. Blots were exposed to x-ray films for 72 h (RBP) and 24 h (β-actin).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
RBP
Actin β

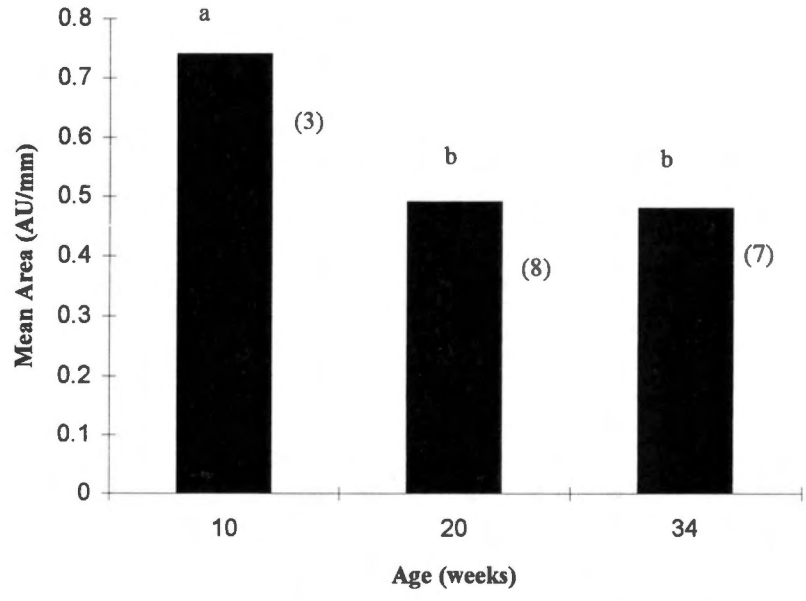


Figure 4: Age-related changes in levels of RBP mRNA in the testis of Angus bulls

The number of bulls sampled at each age-period is shown in parenthesis.
Values with the same letters are not significantly different ($p < 0.05$).
The average areas were calculated from the computer-integrated densitometric scan of the RBP slot blot.
S. E. M.: 0.10, 0.07 and 0.02 AU/mm at 10, 20 and 34 wk, respectively.

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Summary

It is well recognized that the ability of a bull to produce spermatozoa is closely related to scrotal circumference and testis weight. Total Sertoli cell numbers and some indicators of Sertoli cell function, such as transferrin mRNA levels measured in extracts of testes correlate with daily sperm production. Furthermore, testis size at prepuberty is inversely related to age at puberty in the bovine. Thus, prepubertal indexes of testis size and Sertoli cell numbers and function could be useful indicators of adult sperm-producing capacity.

Study 1 was performed to test the hypothesis that peripheral hormone concentrations quantified between 2 and 12 months (mo) were correlated with testis size and number of Sertoli and germ cells in the yearling Angus bull. Twenty four bulls were injected monthly with GnRH and bled 1.5 and 3 hours (h) later. One day before the GnRH challenge, three blood samples were collected at 1.5-h intervals to establish basal hormone concentrations. Bulls were surgically castrated at the age of 12 mo. Serum concentrations of FSH, LH, testosterone (T), androstenedione (Δ_4A) and 17β-estradiol (E2) were quantified by RIA. Number of Sertoli cells per testis and quantitative aspects of spermatogenesis were determined. Analysis of regression and Pearson's partial correlation were used to estimate the degree of association between hormone levels and characteristics of the testis . Histology analysis revealed that 3 bulls had no spermatids and, therefore, were infertile. Yearling testis weight (TW) was related to GnRH-stimulated T at 3 mo ($r = 0.48$, $p < 0.05$) and E2 at 2 and 4 mo ($r = 0.40$ and 0.45, $p < 0.10$). Basal and GnRH-stimulated FSH quantified between 2 and 12 mo were correlated with TW ($r = -0.37$, $p < 0.10$ to -0.71, $p < 0.01$), number of Sertoli cells ($r = -0.41$, $p < 0.10$ to -0.68, $p < 0.01$), number of A1 spermatogonia ($r =$ nonsignificant, $p > 0.10$ to $r = -0.59$, $p < 0.05$) and number of round spermatids per testis

(r = nonsignificant, $p > 0.10$ to -0.69, $p < 0.01$). Also, higher FSH levels between 2 and 12 mo were associated with lower number of round spermatids per A1 spermatogonia (r = nonsignificant, $p > 0.10$ to $r = -0.61$, $p < 0.01$), number of round spermatids per Sertoli cell (r=nonsignificant, $p > 0.10$ to -0.64, $p < 0.01$) and number of seminiferous tubules with round spermatids (r = nonsignificant, $p > 0.10$ to $r = -0.63$, $p < 0.01$) in the 12-mo old testis. Regression analysis showed that FSH and E2 at 2 mo accounted for 45% of the variation in TW. Furthermore, significant variation in TW was accounted by FSH and testis diameter at 6 mo ($R^2 = 0.57$), FSH and testis diameter at 9 mo ($R^2 = 0.67$) and FSH and body weight at 12 mo ($R^2 = 0.63$). Thus, it appears that prepubertal testis size and FSH are potential indicators of sperm-producing capacity of yearling beef bulls. However, because data from infertile bulls were included in the regression analysis, tests with other groups of bulls need to be carried out to validate any practical application of the regression models presented in this study.

Three cases of spermatogenic arrest were described in study 1. Bull #250 had seminiferous tubules lined by Sertoli cells only and bull #345 had no germ cells that advanced beyond the A1 spermatogonia stage. Testes of these two bulls weighed 60% less than those of normal bulls ($p < 0.01$). Concentrations of T and GnRH-stimulated LH in bulls #250 and #345 were reduced at some ages ($p < 0.10$), but basal levels of FSH and LH were normal. Spermatogenic arrest at the level of pachytene spermatocyte was detected in bull #389. Testes of this bull weighed 13% of those of normal bulls ($p < 0.01$) and secreted less basal and GnRH-stimulated T throughout the year ($p < 0.05$). After the age of 6 mo, concentrations of Δ_4A were higher in bull #389 than in bulls with normal spermatogenesis. Also, basal FSH and

LH were higher in bull #389 than in normal bulls ($p < 0.05$) but levels of E2 ($p < 0.10$) were lower at most ages. Thus, the absence of spermatids in bull #389 affected steroid secretion and, as a consequence, levels of gonadotropins. As in the cases of bulls #250 and #345, the decrease in T observed in bull#389 did not appear to be sufficient to interrupt germ cell development. Chromosomal abnormalities and/or deficiency in intratesticular factors may be the causes of infertility in these bulls.

The objective of study 2 was to determine whether concentrations of gonadotropins and steroids in 2- and 6-mo old beef bulls were significantly correlated with testicular development and number of Sertoli cells at the age of 6 mo. At 2 mo, 15 crossbred bulls (Charolois x Hereford and Angus) were treated with GnRH and bled 1.5, 3 and 4.5 h later. Basal samples were taken as in study 1. At 6 mo, bulls received 1 mg of estradiol benzoate (E) and were treated with GnRH 12 h later. After 5 days, bulls received GnRH only. In both cases, blood was collected 1.5, 3 and 4.5 h after GnRH and two basal samples were taken 1 h before the GnRH treatment. Bulls were castrated at 6 mo. Hormone concentrations, histological evaluation of the testis and statistical analysis were carried out as in study 1. Results showed that FSH at 2 mo was correlated with testis weight at 6 mo (TW; $r = -0.48$ to -0.62, $p = 0.08$ to 0.02) as well as with number of Sertoli cells per testis (SC; $r = -0.50$ to -0.65, $p = 0.07$ to 0.01), and number of seminiferous tubules with round spermatids ($r = -0.49$ to -0.60, $p = 0.08$ to 0.02). Regression analysis showed that FSH and body weight at 2 mo accounted for 30% and 72% of the variation in SC and TW in the 6-mo old bull, respectively. Estradiol at 6 mo was related to SC ($r = 0.62$, $p = 0.02$). At 6 mo, GnRH-stimulated FSH and LH were respectively 2.5- and 3-fold higher when GnRH was given in

combination with E than when GnRH was given alone. Correlation between FSH and SC were more pronounced after GnRH+E ($r = -0.68$, $p = 0.008$) than after GnRH only ($r = -0.63$, $p = 0.015$). Regression modeling showed that FSH and body weight at 6 mo accounted for 48% of the variation in the population of Sertoli cells. Thus, concentrations of FSH at 2 and 6 mo of age appears to be reliable indicators of number of Sertoli cells and testicular development in the 6-mo old bull.

Scrotal circumference of the 7- to 10-mo old beef bull is closely related to age at puberty. Immature bovine Leydig cells secrete primarily Δ_4A but testosterone becomes the main androgen produced as differentiation takes place. Both LH and FSH are involved in the process of Leydig cell proliferation and differentiation. Also, previous studies have shown that the shift in androgen secretion occurs around the age of 4 mo and coincides with the cessation of Sertoli cell mitosis. Therefore, to compliment and strengthen the data obtained in studies 1 and 2, an experiment (study 3) was carried out to test the hypothesis that testis size, histology or hormone secretion at the developmental state characterized by the transition from a Leydig cell that secretes mainly Δ_4A to one that secretes T would reflect the degree of testicular development at the age of 12 mo (54 wk). Three basal blood samples were taken from 28 Angus calves since the age of 15 weeks (wk). When peripheral concentrations of T reached 1 ng/ml, bulls were unilaterally castrated. On a monthly base, bulls were treated with GnRH and bled 1.5 and 3 h later. At 54 wk, the second testis was excised. Radioimmunoassays and statistical analysis were performed as in study 1. Number of Sertoli cells and quantitative aspects of spermatogenesis were estimated in both testes. Results showed that testis weight when T = 1 ng/ml (TW1) was correlated with testis weight (TW2;

$r = 0.58$, $p < 0.01$), and number of Sertoli cells and round spermatids per testis at 54 wk ($r = 0.58$, $p < 0.01$). Also, TW1 was related to number of spermatids per Sertoli cell ($r = 0.41$, $p < 0.05$), and number of seminiferous tubules with mature spermatids (STM; $r = 0.52$, $p < 0.01$) in the second testis. However, STM was not correlated with number of tubule sections with germ cells in the first testis (STG1). Testis diameter averaged between the ages of 30 and 44 wk (AVTD) and TW2 were correlated with AGE1 ($r = -0.58$ and -0.42, $p < 0.01$) and STG1 ($r = 0.43$ and 0.41, $p < 0.10$). Regression analysis showed that AGE1 and body weight when T = 1 ng/ml accounted for 41% of the variation in testis diameter between 30 and 44 wk. GnRH-stimulated FSH at 11 wk was related to STG1, TW2 and AVTD ($r = -0.42$ to -0.53, $p < 0.05$). GnRH-stimulated T at 15 wk was related to AVTD ($r = 0.48$, $p < 0.05$). Also, bulls that secreted more E2 after a GnRH treatment at 15 wk had larger AVTD ($r = 0.62$, $p < 0.62$, $p < 0.01$) and TW2 ($r = 0.45$, $p < 0.10$). Thus, differences between slow and fast growing bulls are affected by the ability of the testis to secrete testosterone early in life. Furthermore, such results may aid researchers to select indexes of age at puberty in beef bulls. Correlations between FSH, steroids and characteristics of the testis at AGE1 and prepuberty points toward a role played by these hormones at distinct phases of germ cell development.

Vitamin A (retinol and its derivatives) is essential for spermatogenesis. Retinol modulates the synthesis of proteins by Sertoli cells and can also interfere with the effect of FSH on the Sertoli cell by decreasing levels of cAMP in vitro. The effects of retinol on the Leydig cell are less defined, but it has been shown that serum testosterone is decreased in vitamin A-deficient rats. In plasma, retinol is bound to a retinol-binding protein (RBP), which

is synthesized by the liver. RBP has also been detected in the interstitium and cultures of Sertoli and peritubular cells of rat testes. Thus, the objective of study 4 was to determine the cell types that express RBP in the bovine testis and to evaluate the profiles of RBP mRNA levels during testicular development. Furthermore, because patterns of synthesis of some proteins such as inhibin and aromatase are associated with different phases of Sertoli cell development, the hypothesis that RBP could serve as a marker of state of development of the seminiferous epithelium was evaluated. At the ages of 10 (n=3), 20 (n=8) and 34 (n=7) weeks, Angus bulls were bled three times and surgically castrated afterwards. Blood samples were assayed for LH and T and the degree of seminiferous tubule development was evaluated. Immunolocalization of RBP was detected according to the biotin-strepavidin-horseadish peroxidase method (samples from 10-wk old testes could not be analyzed). Nylon membranes containing isolated total RNA were hybridized with a bovine cDNA RBP (bcRBP-700) and a β-actin probe and slot blots were set up to determine the abundance of RBP transcripts. Age-related changes in testis weight, hormone concentrations and relative quantity of RBP transcripts were determined by analysis of variance. Results showed that testis weight and levels of LH and T increased with age ($p < 0.05$). Also, seminiferous tubules at 10 wk had immature Sertoli cells and gonocytes and, at 20 wk, spermatogonia and few spermatocytes were detected. At 34 wk, Sertoli cells appeared differentiated and spermatids were observed. As never shown before in the bovine species, RBP was detected in Sertoli, Leydig and peritubular cells at the ages of 20 and 34 wk, but no immunoreactivity was present in the germ cells. Moreover, no difference in staining between Sertoli cells from tubules with or without germ cells was detected. Thus, as determined by immunohistochemistry, RBP does

not appear to be a distinct marker of Leydig and Sertoli cell differentiation. Northern hybridization of testicular RNA revealed the presence of a mRNA of 1.1 Kb, which was similar to RBP transcripts found previously in bovine conceptuses and extraembryonic membranes (1.4 Kb). Densitometric scanning of slot blots indicated that the fraction of total RNA coding for RBP was 50 % higher at 10 wk than at 20 and 34 wk. Thus, it is possible that testicular RBP mRNA is down-regulated by testosterone and/or LH, but further studies are needed to verify this hypothesis. Sertoli and peritubular cell RBP probably mediates the transport of retinol to the adluminal germ cells. However, why Leydig cells synthesize RBP is not known, but it is possible that these cells, as well as Sertoli and peritubular cells, may secrete RBP to prevent the potentially damaging effects of free retinol.

Vita

Arlindo A. Moura was born in Fortaleza, Ceará, Brazil. He attended primary school in the city of Aracaju, Sergipe and, after moving back to Fortaleza, went to secondary and high school. He graduated from "Colégio Christus" (high school) in 1981. The following year, he attended The Federal University of Ceará and received a Bachelor Degree in Agronomy in 1987. From 1987 to 1989, he worked for the National Program of Agrarian Reform, providing technical assistance to agricultural projects in areas of agrarian reform in the State of Ceará.

In 1990, he was awarded a Doctoral Scholarship by the Research Council of Brazil (CNPq) and started the graduate program with an emphasis on reproductive physiology, at The University of Tennessee, Knoxville. After graduation, he will return to Brazil and will be working as an assistant professor at The Federal University of Ceará.