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SERUM HORMONE CONCENTRATIONS, IN VITRO RESPONSE TO LUTEINIZING HORMONE AND TESTICULAR GONADOTROPIN BINDING IN BULLS DURING SEXUAL DEVELOPMENT

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SERUM HORMONE CONCENTRATIONS, *IN VITRO* RESPONSE TO LUTEINIZING HORMONE AND TESTICULAR GONADOTROPIN BINDING IN BULLS DURING SEXUAL DEVELOPMENT

By

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ABSTRACT

SERUM HORMONE CONCENTRATIONS, IN VITRO RESPONSE TO LUTEINIZING HORMONE AND TESTICULAR GONADOTROPIN BINDING IN BULLS DURING SEXUAL DEVELOPMENT

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The objectives of these experiments were to describe serum hormone changes in bulls from birth through puberty, to examine negative feedback relationships between testes and hypothalamus/pituitary and to examine testicular changes during the peripubertal period.

In the first experiment serum growth hormone, testosterone, prolactin and luteinizing hormone (LH) concentrations were measured over a 9-hr period in bulls and steers before and after puberty. Steers had higher serum LH concentrations (9.9 ng/ml) than bulls (2.1 ng/ml) and a greater frequency of LH peaks (6.8/9 hr) than bulls (2.3/9 hr), but LH did not change with age. Testosterone was greater in pubertal than prepubertal bulls (5.4 vs 1.4 ng/ml). Prepubertal bulls had higher growth hormone concentrations than other groups.

In experiment 2, two-week-old bulls were assigned to be bulls, steers, or short scrotum bulls (SS bulls) and blood samples from a 24-hr period were collected monthly to measure follicle stimulating hormone (FSH), LH, testosterone and androstenedione. Treatment did not affect

growth rates. By 10 months of age bulls had epididymidal sperm, but spermatogenesis was abolished in SS bulls. Serum LH and FSH concentrations were greater in steers than bulls or SS bulls from 3 to 10 months and LH was greater in SS bulls than bulls at 5 and 6 months. Episodic LH peaks occurred more frequently in bulls than in steers from 2 to 10 months and in SS bulls at 3 and 4 months. FSH was not released episodically, did not differ between bulls or SS bulls, and did not change with age in bulls or SS bulls. Serum testosterone concentrations were < 1 ng/ml until 4 months in bulls and 5 months in SS bulls. Increased frequency and amplitude of testosterone peaks and increased baseline concentrations contributed to increased mean concentrations. Serum androstenedione increased transiently at 4 months in bulls and SS bulls.

In Experiment 3 blood samples were collected frequently for 24 hr from 1-,3-,4-,5-,7- and 9-month-old bulls on a single day to measure the effect of age on serum hormones independent of season or photoperiod. Serum LH concentrations and frequency of LH peaks increased from 1 to 4 months and then declined. Mean concentrations, peak frequency and height of testosterone peaks increased from 5 to 9 months. Androstenedione increased transiently in 4-month-old bulls. FSH did not change with age and had no episodic peaks. Average serum prolactin increased from 1 to 5 months while peak frequency increased from 1 to 4 months. Testicular tissue from bulls of different ages produced equivalent amounts of testosterone in response to LH <u>in vitro</u>. Androstenedione production was greater in 1-,3- and 4- than 5-,7- and 9-month-old bulls. LH and FSH binding to testicular homogenates was greater in 1- to 3-month-old bulls than in 5- to 9-month-old bulls, but total gonadotropin binding/testis increased from 1 to 9 months.

Limited *in vivo* responsiveness of testes to gonadotropins is not due to absence of gonadotropin binding sites.

In conclusion, the major hormonal change during puberty is increased testosterone production. Increased frequency of LH peaks at 4 months may induce maturation of testicular steriodogenesis. Growth hormone, prolactin and FSH may participate in puberty but the role of FSH is difficult to assess because serum concentrations do not change during puberty. Testicular products control gonadotropin secretion as early as 2 months of age, because FSH and LH concentrations were greater after castration and LH was slightly greater in SS bulls than the normal bulls. Sertoli cells, the principal occupant of seminiferous tubules in SS bulls, apparently produced inhibin since serum FSH did not differ in SS bulls and bulls.

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INTRODUCTION

Economic and social factors encourage increased efficiency in animal agriculture. Major advances in animal production can be made using genetically superior sires available through artificial insemination (AI) and by manipulating hormonal and nutritional factors which result in greater efficiency and rate of growth.

AI organizations require proven sires; sires with known superior genetic potential for a particular trait. Proving dairy sires requires 5 to 6 years and costs AI organizations up to $100,000/bull. Any reduction in the interval required to prove bulls would result in savings to the AI organization and to producers. It also would decrease generation interval, allowing more rapid genetic progress. Induction of early puberty in bulls could reduce the interval needed to prove sires. At present, however, the mechanisms involved in the onset of puberty are not clear and attempts to advance age of puberty have failed. Increasing our understanding of the physiological events occurring around the time of puberty in bulls, may lead to ways to induce early puberty.

Nutritional, metabolic and hormonal factors influence the rate and efficiency of growth. By increasing our basic understanding of hormonal changes during development in bulls, we may be able to manage pituitary hormones or anabolic testicular steroids to increase growth.

Studies described within this thesis were designed to increase understanding of the endocrine regulation of sexual maturation in bulls. The objectives were to describe serum hormonal changes in bulls from birth until after puberty; to examine negative feedback relationships between the testes and the hypothalamus/pituitary using bulls, steers and bulls in which the scrotum had been shortened; and to examine testicular changes which probably affect spermatogenesis.

REVIEW OF LITERATURE

A. Introduction

The aim of this literature review is to discuss reproductive development in bulls, accompanying hormonal events and hormonal control of testicular function. Information cited will be predominately from studies in bulls, but data from other species will be used where information on bulls is unavailable.

B. Factors Influencing the Onset of Puberty in Bulls

During the first year of life bulls develop from reproductive quiescence, incapable of spermatogenesis to near maturity with sperm production per gram of testis equal to mature rats (Macmillan and Hafs, 1968a). This is puberty.

Puberty has been defined in many different ways in males. These include the time of appearance of the first sperm in the seminiferous tubules or in an ejaculum, first motile sperm in an ejaculum, first sexual interest and first completed mating (Lunstra et al., 1978). Donovan and Van der Werff ten Bosch (1965) defined puberty as "the phase of bodily development during which the gonads secrete hormones in amounts sufficient to cause accelerated growth of genital organs and appearance of secondary sex character." Foote (1969) suggested that puberty in the fullest sense occurs when spermatogenesis is complete, when libido is present, and when penile development is adequate for

intromission. Probably the most widely used definition for puberty in bulls is the presence of 50 x 10^6 total spermatozoa in an ejaculum (Wolf et al., 1965; Killian and Amann, 1972; Barber and Almquist, 1975). The bull's capacity to impregnate a female is most important in a functional definition of puberty (Foote, 1969).

The age at which bulls reach puberty depends upon genetic (Foote, 1969, Lunstra et al., 1978) and nutritional factors (Bratton et al., 1959; Abdel-Raouf, 1960). Beef breeds generally mature more gradually and reach puberty later than dairy breeds (Abdel-Raouf, 1960; Macmillan and Hafs, 1968a; Swanson et al., 1971; Lunstra et al., 1978). The species also influences the rate of sexual maturation. Bos indicus mature later than Bos taurus cattle (Plasse et al., 1968). In addition, specific lines within a breed show wide variations in the age at puberty.

The level of nutrition influenced the age at puberty in Holstein bulls (Bratton et al., 1959); average age at onset of sperm production (500 x 10^6 sperm/ml with 50% mobility) was 37, 43 and 51 weeks for bulls receiving high, medium or low levels of nutrition, respectively. According to Abdel-Raouf (1960), low nutrition retarded puberty by 8 weeks. Level of nutrition during puberty did not affect subsequent fertility when bulls were fed normally after 1 year of age, but prolonged underfeeding can prevent bulls from ever achieving normal fertility (VanDemark et al., 1964).

Other than nutrition, environmental influences on age at puberty in bulls have been investigated little and information on environmental effects on fertility in cattle is limited. Amann et al. (1966) reported monthly variation in sperm output in bulls while Mercier and Salisbury

(1947) demonstrated highest fertility in bulls in summer and fall, decreased fertility in winter and gradually increasing fertility in the spring. High temperatures (85 F) for 5 weeks decreased spermatogenesis (Casady, 1953) in young bulls. Jersey bulls raised at 35 to 36 C and 80 to 90% humidity for 8 hr daily beginning at 26 weeks of age, reached puberty at 55 weeks, 7 weeks later than controls (DeAlba et al., 1966).

C. Body Growth and Development of Reproductive Organs

Rate of body growth to 1 year of age is linear in Holstein bulls (Bratton et al., 1959; Macmillan and Hafs, 1968a). The relationship $\hat{Y} = 22.5 + 26.5X$, where Y is body weight (kg) and X is age (mo), described by Macmillan and Hafs (1968a) agrees with the growth rate of bulls fed a high plane of nutrition by Bratton et al. (1959). A reduced level of nutrition decreased the slope of this relationship (Bratton et al., 1959).

In contrast to the linear relationship described above, Abdel-Raouf (1960) found a concave quadratic growth curve in Swedish red and white cattle. This difference was probably due to Swedish red and white cattle attaining mature size earlier than Holsteins.

Seminal vesicular growth does not parallel body growth; after an initial lag period (birth to 2 months), seminal vesicles grow more rapidly than the rest of the body from 2 to 4 and from 7 to 9 months, with an intervening plateau. Seminal vesicular RNA and DNA follow similar patterns, but RNA increased more rapidly until 4 months, after which both increased at the same rate. The early increase in RNA demonstrates a proportionately greater increase in protein synthesizing potential as compared to hyperplasia (DNA content). Seminal vesicular fructose and citric acid content, reflecting androgen production (Mann

et al., 1949), increased slowly until 6 months and then increased markedly to 9 months (fructose) and 12 months (citric acid; Macmillan, 1967).

The length of the penis increased linearly until 9 months when it nears mature size (Abdel-Raouf, 1960; Macmillan, 1967). The separation of the penis from the preputial sheath at 34 to 40 weeks of age (Ashdown, 1962) is an important change occuring during puberty because it permits intromission (Foote, 1969).

Epididymidal growth is curvilinear, with a slow initial growth rate from birth to 4 to 5 months and a more rapid growth rate to 12 months (Macmillan, 1967). Testicular growth follows a similar pattern; a quadratic growth curve to 9 months followed by decreased growth rate until 12 months (Abdel-Raouf, 1960; Macmillan, 1967).

D. Testicular Development

At birth the seminiferous tubules are solid chords about 40 to 50 microns in diameter (Abdel-Raouf, 1960; Macmillan, 1967). The diameter increases at a rate of approximately 5 microns per month and lumina become apparent at 4 to 6 months.

The progressive development of germ cells in the seminiferous tubules of young bulls has been studied (Hooker, 1944; Santamarina and Reece, 1957; Abdel-Raouf, 1960, 1961; Fossland and Schultze, 1961; Attal and Courot, 1963). At 1 week of age the primary components of the tubules are precursor supporting cells (bis-cells), abundantly distributed around the periphery, and gonocytes located more centrally (Abdel-Raouf, 1961). Work by Abdel-Raouf established that by 4 weeks some bis-cells have given rise to more advanced supporting cells (cis-cells). The

gonocytes double in size and undergo nuclear changes suggestive of cellular degeneration. Both types of supporting cells increase in number by 8 weeks, the gonocytes disappear and spermatogonia, larger than the gonocytes from which they arise, are present. Few changes take place from 8 to 12 weeks, although the number of gonocytes is reduced. By 16 weeks, lumen formation usually begins, no gonocytes remain and the cell types present are cis-cells, bis-cells and spermatogonia. At 20 and 24 weeks, primary spermatocytes and more spermatogonia and cis-cells are present, and few bis-cells exist. Primary spermatocytes are more numerous and small round spermatids and Sertoli cells are present by 28 weeks. By 32 to 44 weeks all eight cell types are present, including bis-cells, cis-cells, Sertoli cells, spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and sperm. However, during this period there is a progressive decrease in the numbers of the two types of precursor support cells and an increase in Sertoli cells.

An important change that takes place in seminiferous tubules during the onset of spermatogenesis is the formation of tight junctions between adjacent Sertoli cells (Vitale-Calpe et al., 1973). This phenomenon has not been studied in bulls but has been extensively investigated in rats. Tight junctions result in formation of the blood-testis barrier and create basal and adluminal compartments within the tubule. These compartments contain different germinal elements and provide two separate environments for germ cell development.

The developmental changes in the intertubular tissue have not been so carefully documented. Abdel-Raouf (1960) stated that it was too complicated to estimate the degree of metamorphosis of Leydig cells or to

estimate their absolute or relative numbers. Hooker (1944) described differentiation of mesenchymal cells into Leydig cells and fibroblasts. Few Leydig cells are present from 1 to 3 months in bulls; subsequently the number increases due to division of precursor cells. Leydig cells appear fully functional and secretory by 4 months (Hooker, 1944).

E. Sperm Production

Sperm are absent in testicular homogenates from bulls between birth and 4 months of age (Macmillan and Hafs, 1968a). Few sperm are present between 5 to 7 months, while a pronounced increase occurs between 7 and 8 months (4×10^6/g of testis to 28×10^6/g). By 12 months the concentration (57×10^6/g) is equivalent to that in mature bulls (55×10^6/g) reported by Almquist and Amann (1961). Increases in testicular weight between 12 months and full maturity account for a two-fold increase in total sperm/bull. Total daily sperm production calculated from testicular homogenates was 0, 168×10^6, 3230×10^6 and 5900×10^6 at 0 to 4, 5 to 7, 8 to 10 and 11 to 12 months, respectively, and continued to increase until 7 years (Amann, 1970).

F. Hormonal Control of Spermatogenesis

Hypophysectomy causes gonadal atrophy in rats while replacement therapy with pituitary extracts restores testicular function (Smith, 1930). Zondek (1930) discovered "Follicle ripening factor" (FSH) and "Luteinizing factor" (LH) and knowledge of these two factors led Greep *et al.* (1936) to postulate FSH controls spermatogenesis and LH controls testicular steroidogenesis. The effects of androgens on spermatogenesis confused this originally neat hypothesis. Androgens maintain

spermatogenesis in hypophysectomized rats (Walsh et al., 1934; Nelson and Merckel, 1938), but do not reinitiate the process after atrophy of the germinal epithelium following hypophysectomy (Nelson, 1937; Simpson et al., 1942).

The roles of gonadotropins were re-evaluated in hypophysectomized rats (Woods and Simpson, 1961) using replacement therapy with more purified materials than were available previously. LH at "low doses" could maintain spermatogenesis but testicular weight was reduced. At "low doses," FSH with no significant LH contamination produced only slight effects on testes weight and development. High FSH doses with significant LH contamination produced heavier testes than either hormone alone, indicating that the two hormones acted synergistically.

The hormonal requirements for spermatogenesis have been reviewed by Steinberger (1971) and Lostroh (1976). FSH in male rats functions primarily during sexual development. It is synergistic with LH in initiating androgen production and in development of spermatids and immature spermatozoa. FSH also stimulates Sertoli cell function. LH is required for androgen production by Leydig cells, synergistic with FSH in initiating androgen production, required for maturation of spermatozoa and synergistic with growth hormone in stimulating testosterone production. Apparently the only actions of LH on spermatogenesis are via testosterone production. Testosterone is required to stimulate meiosis of primary spermatocytes and for spermiogenesis.

Madhwa Raj and Dym (1976) and Dym and Madhwa Raj (1977) selectively withdrew FSH or LH using highly specific antisera. FSH antiserum treatment for 14 days reduced testes weight, tubular diameter and spermatid and spermatocyte numbers without interfering with testosterone

production (Madhwa Raj and Dym, 1976). LH antiserum injections decreased testosterone in rete testis fluid, Leydig cell area and Leydig cell endoplasmic reticulum. In the seminiferous tubules, LH antiserum caused late spermatids to be retained by Sertoli cells, pachytene spermatocytes to degenerate and Sertoli cell nuclei to become sperical, a sign of degeneration.

Woods and Simpson (1961) found that prolactin increased the rate of spermatogenesis in LH treated rats. In agreement with these studies, prolactin treatment of hypophysectomized rats potentiated LH action on spermatogenesis, but did not potentiate spermatogenesis when given with testosterone propionate (Bartke, 1971a). These results suggest that prolactin may augment LH effects on steroidogenesis, but not testosterone effects on spermatogenesis. This was confirmed when Hafiez et al. (1972) showed that hypophysectomized rats had higher plasma testosterone after prolactin plus LH than after LH alone.

Woods and Simpson (1961) also observed that growth hormone potentiates the action of gonadotropins on spermatogenesis in hypophysectomized rats. Later Lostroh (1976) indicated that the stimulatory effect of growth hormone was caused by increased androgen production. Growth hormone plus LH resulted in larger testes and greater accessory gland weights (an indirect measure of androgen production) than LH alone.

G. Hormone Action in the Testis

Few studies have been conducted on the mechanism of hormone action in the bovine testis. Most information in this section is from experiments in rats.

1. LH

The mechanism of LH action on testicular steroidogenesis has been reviewed recently by Catt and Dufau (1976). Dufau et al. (1976) and Cooke (1976). Summarizing these reviews, LH first binds specifically to Leydig cells on a membrane bound receptor. This receptor has been purified to 50% homogeneity and is composed of two subunits of 90,000 molecular weight (Dufau et al., 1975). Binding to the receptor results in activation of adenylate cyclase at the cell membrane and in most cases, production of cyclic adenosine monophophate (cAMP) in the Leydig cells. The fate of the hormone-receptor complex has been determined in luteal cell preparations and Leydig tumor cells. After binding of HCG to the plasma membrane of ovine luteal cells, significant quantities of hormone are internalized, localized in lysosomes (Chen et al., 1977a) and degraded by acid hydrolases (Chen et al., 1978). In Leydig tumor cells the fate of the bound LH is similar to that of HCG in luteal cells. Blockade of lysosomal activity does not interfere with HCG-induced steroidogenesis, indicating that lysosomal degradation is not prerequisite for steroid production (Ascoli and Puett, 1978). The LH internalization phenomenon may explain decreased responsiveness of testicular tissue (testicular desensitization) that occurs transiently after LH or HCG (Hsueh et al., 1977).

Cyclic AMP formed as a result of LH stimulation binds to two protein kinase holoenzymes (4S and 6.5S; Dufau et al., 1976). Each of these holoenzymes dissociates into two regulatory and one catalytic subunit. The catalytic subunit of protein kinase phosphorylates a variety of protein substrates, but the function of phosphorylation is unclear at present. Active protein kinase translocated to the nucleus has been

demonstrated to phosphorylate histones (Walsh et al., 1968) and ribosomal RNA's in other tissues (Barden and Labrie, 1973). These events may alter transcription or translation processes. Changes in total protein synthesis in Leydig cells could not be detected after LH treatment, but treatment with the protein synthesis inhibitor, cycloheximide, prevented increased testosterone synthesis (Cooke et al., 1974).

Since LH stimulates increased conversion of cholesterol to pregnenolone, protein phosphorylation probably influences the activity of cholesterol-side-chain-cleavage-enzyme complex or increases cholesterol transport into mitochondria. Subsequent conversion of pregnenolone to testosterone requires at least five enzymatic reactions in the smooth endoplasmic reticulum. Prolonged LH treatment can stimulate these reactions (Dufau et al., 1976).

Direct involvement of cAMP in the mechanism of LH action has been questioned. Treatment of Leydig cells with low levels of LH can increase steroidogenesis without detectable changes in cAMP (Catt and Dufau, 1973). Catt and Dufau (1976) suggested that LH may enhance steroidogenesis through mechanisms other than cAMP, but they also point out that small (undetectable) increases in cAMP, or cAMP translocated within intracellular compartments as a result of LH stimulation may stimulate steroidogenesis. Treatment of Leydig cells with cAMP or dibutyryl cAMP results in steroidogenesis, additional proof of the importance of cAMP (Dufau et al., 1976).

2. FSH

FSH action in the testes has been reviewed by Hansson et al. (1976), Means et al. (1976) and Bartke et al. (1978). Sertoli cells are the

primary target for FSH in the testis and specific binding sites for FSH are found on Sertoli cell plasma membranes (Means and Vaitukaitis, 1972; Castro et al., 1972; Steinberger et al., 1974; Orth and Christensen, 1977). Events following FSH binding resemble those after binding of LH to Leydig cells. The interaction of FSH with its receptor results in activation of adenylate cyclase, production of cAMP and activation of cAMP-dependent protein kinase in Sertoli cells (Means, 1973; Means and Huckins, 1974). After rats are 21 to 24 days old, testicular response to FSH is diminished, but hypophysectomy results in renewed sensitivity to FSH. Decreased sensitivity to FSH was thought to be due to specific isoenzyme of phosphodiesterase, appearing during development and preventing cAMP accumulation in response to FSH. This was proven incorrect, but an inhibitor of protein kinase under the control of FSH increases rapidly during the first 20 days of age in rats, and may be one factor responsible for the loss of sensitivity of Sertoli cells to FSH (Tash et al., 1978). Another possibility is that decreased sensitivity to FSH in mature rats is due to decreased adenylate cyclase activity (Van Sickle et al., 1977).

The specific function of protein kinase in Leydig and Sertoli cells is unknown, but following activation by cAMP, protein phosphorylation occurs in every subcellular fraction. Protein and RNA synthesis increase as do RNA polymerase I and II (Means et al., 1976).

A specific protein induced by FSH is androgen binding protein (ABP). ABP is produced by Sertoli cells (Dorrington et al., 1974), secreted into the lumen of seminiferous tubules and transported through efferent ducts to the epididymis. The function of ABP is not known but it is suspected to concentrate androgens in the tubule and epididymis for

spermatocytogenesis and spermiogenesis (Ritzen et al., 1971; French and Ritzen, 1973). Hypophysectomy causes a loss of ABP, but replacement of FSH restores its production (Sanborn et al., 1975).

An age difference in ABP production in response to FSH has been reported (Kotite et al., 1978). Fourteen-day-old rats produced ABP after FSH treatment whereas adult rats did not respond to FSH with ABP production. Age-related refractoriness to FSH in terms of ABP production resembles FSH effects on cAMP.

Testosterone alone maintains spermatogenesis for extended periods and this led some to suggest that the primary action of FSH is on steriodogenesis (Bartke et al., 1978). FSH treatment of immature hypophysectomized rats increased testicular responsiveness to LH (Odell et al., 1973; Catt, 1977) and increased LH binding per testis (Chen et al., 1976; Catt, 1977). These findings suggest that the role of increasing serum FSH concentrations during puberty is to increase steroidogenesis in response to LH. Payne et al. (1977) postulated that FSH might affect steroidogenesis by stimulating 17β-hydroxysteroid dehydrogenase activity.

The inability of FSH to bind to Leydig cells complicates hypotheses of its action on steroidogenesis. FSH possibly alters steroidogenesis indirectly via Sertoli cell products such as estradiol (Fritz, 1977). FSH stimulates aromatase synthesis in Sertoli cells and subsequent conversion of testosterone to estradiol (Dorrington and Armstrong, 1975). Part of the steroidogenic stimulation induced by FSH may be its action on steroidogenesis within the seminiferous tubules (Bell and Vinson, 1975; Welsh and Wiebe, 1976).

3. Prolactin

Demonstration of prolactin binding to isolated Leydig cells (Rajaniemi et al., 1974) and autoradiographic localization of prolactin around Leydig cells (Charreau et al., 1977) suggests that prolactin acts specifically at Leydig cells. Suggestions of several actions of prolactin on Leydig cells include regulation of LH binding and mobilization of cholesterol for steroidogenesis. Decreasing serum prolactin concentrations in hamsters by shortening the photoperiod resulted in a loss of LH receptors (Bex and Bartke, 1977a). When rats were given ergocryptine to reduce prolactin secretion, LH binding to Leydig cells was reduced (Aragona et al., 1977). Prolactin administration restored LH binding in dwarf mice (Bohnet and Friesen, 1976), hypophysectomized rats (Zipf and Payne, 1977) and hamsters on reduced photoperiod (Bex and Bartke, 1977a).

Prolactin increased Leydig cell cholesterol esters (Bartke, 1969, 1971) which serve as a precursor pool for steroidogenesis (Bartke, 1971, Bartke et al., 1973a). Prolactin also increases conversion of acetate to cholesterol in vitro and increases 3β- and 17β-dehydrogenase activity (Hafiez et al., 1971; Musto et al., 1972).

4. Growth Hormone

Growth hormone treatment of testes-regressed hamsters increased testicular and seminal vesicular weights (Bex and Bartke, 1977b), and treatment of hypophysectomized rats with growth hormone plus LH stimulated greater reproductive organ growth than LH alone (Lostroh, 1976). LH binding, plasma testosterone and testicular responsiveness to LH treatment increased after growth hormone treatment (Bex and Bartke,

1977b; Zipf and Payne, 1977; Swerdloff and Odell, 1977).

5. Androgens

The ability of androgens to maintain spermatogenesis is undisputed in rodents (Steinberger, 1971). How androgens are involved in spermatogenesis is less clear. Leydig cells are the main source of testosterone in the testes (Cooke et al., 1976), but Sertoli cells have also been suggested to be capable of de novo synthesis of testosterone (Lacy, 1973). Several investigators have demonstrated conversion of pregnenolone, progesterone and dehydroepiandrosterone to testosterone in seminiferous tubular preparations (Christensen and Mason, 1965; Bell et al., 1968), and many have shown that the seminiferous tubules or isolated Sertoli cells are effective in converting testosterone to 3α-androstanediol, 3β-androstanediol, dihydrotestosterone and estradiol (Rivarola and Podesta, 1972, Folman et al., 1973; Dorrington and Armstrong, 1975; Welsh and Wiebe, 1976; Tence and Drosdowsky, 1976).

The androgen target tissue in the testis is primarily Sertoli cells (Sanborn et al., 1976), but androgen binding to germ cells has also been demonstrated (Sanborn, 1977). In a nuclear exchange assay, the androgen-receptor complex in germ cells was not translocated to the nucleus, which questions the biological significance of germ cell binding of androgens. In Sertoli cells, androgens bind to a cytoplasmic receptor (Mainwaring and Mangen, 1973; Galena et al., 1974; Mulder et al., 1974; Hansson et al., 1974; Bardin et al., 1973; Means et al., 1976). The cytoplasmic androgen receptor has been determined to be different than ABP by a variety of physical and chemical properties (Means et al., 1976). After binding of androgens to the receptor, the androgen-receptor complex is

translocated to the nucleus where it stimulates synthesis of numerous proteins, a major one being ABP.

Both FSH and testosterone can independently increase ABP synthesis. The relative importance of these two hormones in regulating ABP levels in normal animals remains to be elucidated.

6. Estrogens

The testes were known to produce estrogens as early as 1934 (Zondek, 1934). Since then many studies have been conducted to determine the testicular compartment producing estrogens, as well as a testicular role for estrogens. Interstitial tissue has been shown to be a major site of aromatization and estrogen production after HCG stimulation in human and rat testes (Payne et al., 1976; Valladares et al., 1978). In addition, Dorrington and Armstrong (1975) demonstrated that isolated-cultured Sertoli cells produce estradiol in response to FSH when testosterone was added as a precursor. Estrogen production in response to FSH was age-dependent and decreased from maximum at 5 days to almost undetectable concentrations by 30 days of age (Dorrington et al., 1978).

The role of estrogen in testicular function is unknown. However, estradiol decreased testosterone production in vitro (Samuels et al., 1964; Sholiton, 1975; Bartke et al., 1977) and in vivo (Danutra et al., 1973; Tcholakian et al., 1974). Estrogen effects may occur by direct action on cytoplasmic estrogen receptors found in isolated Leydig cells (Mulder et al., 1976).

Some are skeptical of evidence that estrogens act directly on Leydig cells. Van Beurden et al. (1977) showed that estrogens affected testicular steroidogenesis indirectly by negative feedback on gonadotropins.

Also, Bartke et al. (1977) stated that non-physiological estrogen doses (1000 x physiological) were used to alter steroidogenesis in vitro. Estradiol affected testicular function in hypophysectomized immature rats; a model in which estradiol can not influence gonadotropin secretion. In these animals estradiol inhibited FSH-increased testicular responsiveness to LH (Van Beurden et al., 1976; Chen et al., 1977b). From the age-dependent decline in estrogen production in response to FSH by Sertoli cells (Dorrington et al., 1978), lower concentrations of testicular estrogens in 30-vs 16-day-old rats (Chen et al., 1977b) and the inhibitory action of estrogens on FSH-increased responsiveness to LH; it is tempting to suggest that high intratesticular estrogen concentrations early in sexual maturation may inhibit testicular development.

H. Concentrations of Pituitary Hormones in Serum During Puberty

Changes in serum LH, FSH and prolactin in the peripubertal period have been extensively investigated in rats (Swerdloff et al., 1971; Dohler and Wuttke, 1975; Dussault et al., 1977a; Dussault et al., 1977b; Payne et al., 1977). Various authors disagree about LH concentrations during this period. Payne et al. (1977) and Dussault et al. (1977a) found no change in LH concentrations with advancing age, but characterized the LH pattern as highly variable during puberty in rats. Dohler and Wuttke (1975) found increased LH concentrations between 40 and 50 days, while Swerdloff et al. (1971) found a progressive rise between 21 and 91 days of age. In contrast to highly varied results found in LH concentrations, most workers have found an increase in FSH at 32 to 35 days of age and then a decline coincident with the onset of spermatogenesis (Swerdloff et al., 1971; Payne et al., 1977; Dussault et al.,

1977a). Serum prolactin concentrations increase from the neonatal period to about 35 days of age and then decline with maturity (Dohler and Wuttke, 1975; Dussault et al., 1977b).

In bulls, serum LH concentrations during sexual maturation vary between investigations similar to rat studies. No changes in serum LH were observed between birth and 1 year in Brown Swiss (Karg et al., 1976) and 3/4 Limousin cattle (Schanbacher, In press). However, Lacroix et al. (1977) found Charolais bulls had low LH concentrations between 1 to 5 weeks, higher and more variable from 5 to 20 weeks, and lower and stable after 20 weeks. Holstein bulls had a similar LH profile; 2.9 ng/ml at 1 month, 6.7 at 3 months and 4.2 at 15 months (Barnes et al., 1976). Macmillan and Hafs (1968b), also using Holstein bulls, reported slightly lower and more variable LH concentrations between 2 to 7 months and higher more stable concentrations from 8 to 12 months. They also converted LH concentrations to total LH content per bull and saw a marked increase from 2 to 4 months, a plateau to 6 months and a gradual increase to 12 months. The increase in LH content with age is expected. It reflects increased body weight and blood volume but not necessarily increased LH available at the testes. Lunstra et al. (1978) in several beef breeds, Gombe et al. (1973) in Angus, Holsteins and Guernseys, and Mori et al. (1974) in Holsteins found that LH concentrations gradually increased from 7 months to maturity. So, similar to data in rats, reported LH data during the peripubertal period are inconsistent in bulls.

Technical problems with radioimmunoassay have prevented extensive investigations of serum FSH concentrations in bulls. Only recently has FSH been measured in bulls of different ages (Karg et al., 1976; Schanbacher, In press). Both these studies reported no significant

elevations in serum FSH between birth and 1 year of age.

Season influences prolactin concentrations in cattle (Koprowski et al., 1972; Karg and Schams, 1974; Tucker et al., 1974). More specifically, increased ambient temperatures and photoperiods raise serum prolactin concentrations while reduced temperatures and photoperiods decrease prolactin (Wetteman and Tucker, 1974; Bourne and Tucker, 1975). Therefore, studies conducted across seasons should not be used to determine age effects on prolactin. Two such studies determined prolactin concentrations in bulls from birth to 20 and 12 months, respectively (Schams and Reinhardt, 1974; Lacroix et al., 1977). Prolactin concentrations were high in summer and low in winter, but were not affected by age. These studies did not definitively assess age effects on prolactin in bulls because they confounded age with season.

Plasma growth hormone in bulls from 1 to 12 months of age was high at birth, decreased in the first month and was constant to 12 months. Plasma growth hormone was not related to growth criteria (Purchas et al., 1970).

I. Concentrations of Steroid Hormones During Puberty

The concentration of androgenic steroids in testicular tissue, spermatic vein plasma and peripheral plasma is low in bull calves less than 4 months of age (Lindner and Mann, 1960; Lindner, 1961; Rawlings et al., 1972; Karg et al., 1976; Seechiari et al., 1976). As bulls increase in weight and age, there is a shift in type and amount of steroids produced. The androstenedione to testosterone ratio in testicular tissue was greater than one before 4 months, less than one from 4 to 6 months and less than one-tenth from 6 months onward (Linder and Mann, 1960).

The spermatic vein concentration of these steroids followed a similar pattern (Lindner, 1961). In bulls 4 to 6 months old, androstenedione secretion rate (μg/hr) increased from 62 for an 89-kg bull to 217 for a 123-kg bull, and then decreased to 4 for a 200-kg bull. Testosterone secretion rate (μg/hr) for these animals was 14 (89-kg), 30 (123-kg) and 55 (200-kg). The same type of change in androstenedione:testosterone ratio seen in testes tissue was observed in spermatic vein effluent.

Peripheral concentrations of testosterone and androstenedione have been studied more completely during maturation in bulls (Rawlings et al., 1972; Karg et al., 1976; Seechiari et al., 1976). Plasma testosterone increased erratically to 11 months, and androstenedione was high and variable until 6 months, then declined (Rawlings et al., 1972). In other reports (Karg et al., 1976; Seechiari et al., 1976), testosterone remained <1 ng/ml for 5 months and increased markedly to 3 to 5 ng/ml by 12 months.

Changes in the activity of enzymes related to steroidogenesis during maturation have not been studied in bulls. Experiments in rats have shown that the activity of enzymes essential to steriodogenesis increase with age, especially during puberty. The enzymes 3β-hydroxysteroid dehydrogenase, 17β-hydroxylase and C_{17}-C_{20} lyase all increase sharply in male rats from 20 to 30 days of age while 17β-hydroxysteroid dehydrogenase increases more gradually from 20 to 60 days of age (Inano et al., 1967). Similarly, Payne et al. (1977) found increased 3β-hydroxysteroid dehydrogenase between 10 and 30 days of age and 17β-hydroxysteroid dehydrogenase from 20 to 60 days. In conclusion, maturation of enzyme systems in the testes is required for mature levels of steroid production. The mechanisms responsible for maturation of these pathways

are unknown, but gonadotropins (LH and FSH) have been implicated (Payne et al., 1977; Wiebe, 1978). Payne et al. (1977) suggested that FSH induces 17β-hydroxysteroid dehydrogenase production while Wiebe (1978) demonstrated that HCG induced 3β-hydroxysteroid dehydrogenase.

In mature bulls the major steroid product of the testes is testosterone (Schanbacher, 1976; Amann and Ganjam, 1976). Schanbacher (1976) reported that bull plasma had 3.9 ng/ml of testosterone and 0.1 ng/ml of dihydrostestosterone (dihydrotestosterone equalled approximately 3% of testosterone). Testosterone, androstenedione, progesterone, estrogen (estradiol plus estrone), 5-androstenediol, 3α-androstanediol, and 3β-androstanediol were present in spermatic vein blood (Amann and Ganjam, 1976). The presence of androstenedione and androstenediol indicates that both the Δ_4 and Δ_5 steroid pathways are functional and contribute to testosterone production. Presence of 3α- and 3β- androstanediol and estrogen indicate that considerable metabolism of testosterone occurs in bulls. Progesterone, androstenedione, 5-androstenediol, 3α-androstanediol and 3β-androstanediol each represented from 3 to 7% of testosterone (78 ng/ml) while estrogens were only 15 pg/ml.

J. Hormones in the Pituitary and Hypothalamus During Puberty

Pituitary LH concentration and content increase in bulls from birth to 1 month, then fluctuate randomly, not associated with the onset of puberty. Pituitary FSH concentration and content increase from 1 to 2 months, then decline at 8 to 9 months and remain low (Macmillan, 1967). Pituitary growth hormone content and concentration peaked at 3 and 4 months, declined, and remained constant to 12 months (Purchase et al., 1970).

Few have studied hypothalamic hormone content in bulls during puberty. Macmillan (1967) found increased hypothalamic luteinizing hormone-releasing factor (LRF) activity at 5 months. LRF decreased at 6 months, increased until 8 months and was stable thereafter.

K. Hormonal Influences on Testicular Function in Bulls

LH is released into peripheral circulation in bulls in short secretory surges or episodes, each persisting 1 to 2 hr (Katongole et al., 1971; Mongkonpunya et al., 1974a; Kiser et al., 1976). Mongkonpunya et al. (1974a) concluded that there were 3.7 episodic LH surges per 24 hr in 9-month-old bulls, while Katongole et al. (1971) found from 4 to 9 per 24 hr in mature bulls. Each increase in LH was followed within 1 hr by increased serum testosterone. The existence of a temporal relationship does not causally relate LH to testosterone secretion. However, studies using HCG (Lindner, 1961; Katongole et al., 1971) and LH injections (Smith et al., 1973; Kiser et al., 1978) demonstrated a direct cause and effect relationship between LH and testosterone secretion. Also, treatment of bovine testicular tissue in vitro with LH promotes synthesis of testosterone (Kiser et al., 1974).

Gonadotropin releasing hormone (GnRH) injections also demonstrated tha causative role of gonadotropins in testosterone production. Treatment of bulls with GnRH increased serum FSH and LH, followed by increased serum androstenedione and testosterone at 4 hr post-injection (Zolman and Convey, 1973). Kiser et al. (1978) demonstrated that by elevating serum LH concentrations with prostaglandin $F_2\alpha$ ($PGF_2\alpha$), serum testosterone increased approximately 1 hr following LH peaks.

The testicular response to gonadotropins is age-dependent (Lindner, 1961; Mongkonpunya et al., 1975a). HCG or pregnant mare serum gonadotropin (PMSG) administered to immature calves resulted in more androstenedione than testosterone production, whereas HCG resulted primarily in testosterone production in mature bulls (Lindner, 1961). Kesler and Garverick (1977) reported that testosterone increased more in 10- to 24-day-old calves than in 2- to 5-day-old calves after GnRH treatment. A similar age difference in testicular response was seen by Mongkonpunya et al. (1975a) after treatment of 2-, 4- and 6-month-old bulls with GnRH. The increase in LH following GnRH did not differ between ages, but serum testosterone concentrations were significantly greater in 6-month-old bulls compared with 2- and 4-month-old bulls. The androstenedione to testosterone ratio was greatest in 2-month-old bulls. In vitro results (Kiser et al., 1974) agree with in vivo experiments. Testes explants from 1- and 3-month-old bulls produced predominately androstenedione in response to LH whereas explants from 5-month-old bulls produced predominately testosterone.

The role of FSH in steroidogenesis in bulls has not been investigated. In other species, FSH increases androgen production in response to an LH stimulus (Bartke et al., 1978).

Few studies have been conducted to investigate prolactin's role in testicular function in bulls. Smith et al. (1973) determined that a single prolactin injection did not increase serum testosterone in bulls, and prolactin plus LH did not increase testosterone more than LH alone. Serum prolactin is related with seminal vesicular fructose, an indirect measure of androgen secretion, and to sperm numbers in yearling bulls (Swanson et al., 1971). Extended prolactin treatment can stimulate

androgen production in rats by increasing the sensitivity of the testes to LH (Bartke et al., 1978). Whether a similar mechanism exists in bulls has not been studied.

The effect of growth hormone on testicular function in bulls also has not been studied. In other species growth hormone increased the sensitivity of the testes to LH (Woods and Simpson, 1961; Swerdloff and Odell, 1977).

Attempts to stimulate early testicular function and cause early puberty have been unsuccessful. Treatment with GnRH (Mongkonpunya et al., 1975b) and GnRH plus thyrotropin releasing hormone (Haynes et al., 1977a) caused pituitary hormone release but failed to increase testicular size or sperm numbers in young bulls.

L. Hypothalamic-Pituitary-Gonadal Interactions

1. Castration and Cryptorchidism

Castration results in increased serum gonadotropin concentrations in a number of species including rats (Gay and Midgley, 1969; Swerdloff et al., 1971), monkeys (Atkinson et al., 1970), rams (Pelletier, 1968; Crim and Geschwind, 1972) and bulls (Odell et al., 1971; Mongkonpunya et al., 1974a; McCarthy and Swanson, 1976; Tannen and Convey, 1977). As early as 1 month of age, castration results in increased serum LH concentrations (Odell et al., 1971; Tannen and Convey, 1977). Five- to 6-month-old (McCarthy and Swanson, 1976) and 9-month-old bulls responded similarly (Mongkonpunya et al., 1974a).

Induced cryptorchidism also results in increased LH and FSH in rats (Swerdloff et al., 1971; Rager et al., 1975) and rams (Schanbacher

and Ford, 1977), but these LH and FSH increases are not as pronounced as in castrates.

2. Effects of Gonadal Steroids

Testicular steroids are involved in negative feedback on gonadotropins in intact males (Gay and Dever, 1971). Testosterone injections reduce LH concentrations in rams (Bolt, 1971) and castrate rams (Pelletier, 1970, 1973; Crim and Geschwind, 1972). Bolt (1971) found that estradiol and progesterone also reduced LH concentrations in intact rams. Schanbacher and Ford (1977) demonstrated that dihydrotestosterone was ineffective in castrates and cryptorchid rams, while testosterone reduced LH and FSH in castrates only. Estradiol reduced gonadotropins in castrate and cryptorchid rams. FSH was reduced much less than LH and it was suggested that factors other than estradiol alone are required for maintenance of normal serum FSH concentrations.

Likewise, steroids reduce LH concentrations in bulls. Estradiol was very effective in reducing LH levels in steers, while testosterone propionate influenced LH concentrations only when administered at high doses for several days (McCarthy and Swanson, 1976). Testosterone treatment also reduced LH concentrations in 1- and 9-month-old steers (Mongkonpunya et al., 1974a; Tannen and Convey, 1977).

Not only do castrates have higher basal LH concentrations, but they also have a heightened response to GnRH. One- and 9-month-old steers released more LH in response to GnRH than bulls of similar ages (Mongkonpunya et al., 1974a; Tannen and Convey, 1977).

Testosterone treatment initiated 21 days post-castration and continued for 6 days failed to return the heightened GnRH-incuded LH

response to normal intact levels (Mongkonpunya et al., 1974a). One- and 9-month-old bulls castrated and immediately given testosterone responded with less LH secretion to a series of three GnRH injections than did bulls. However, initiation of testosterone treatment to steers at 21 days post-castration failed to reduce the exaggerated LH response to GnRH (Mongkonpunya et al., 1974a; Tannen and Convey, 1977). Thus, testosterone treatment started immediately post-castration prevents castration-induced increase in LH biosynthesis, while testosterone treatment started later after castration cannot reduce LH biosynthesis to levels in intact bulls. In agreement with these conclusions testosterone propionate treatment of chronically castrate steers failed to decrease LH response to GnRH (McCarthy and Swanson, 1976).

Twelve-hour continuous infusion of testosterone reduced LH concentrations and inhibited episodic LH surges (Haynes et al., 1977b). GnRH or $PGF_2\alpha$ injection during testosterone infusion increased serum LH concentrations. These results support the concept that acute testosterone treatment inhibits LH release by action at the hypothalamus or higher centers and that the block is not at the pituitary. In this same study, aspirin, a known prostaglandin synthesis inhibitor, administered for 12 hr, blocked episodic LH and testosterone secretion. Administration of $PGF_2\alpha$ overcame the block and caused LH release. Haynes et al. (1977b) suggested that normal circulating testosterone concentrations may inhibit LH by inhibiting prostaglandin production.

3. Inhibin

McCullagh (1932) postulated that a testicular substance from the seminiferous tubules suppresses FSH production. Since then, numerous

observations have supported the idea that seminiferous tubular elements produce "inhibin." Serum concentrations of FSH but not LH are elevated in conditions in which the germinal epithelium has been destroyed as after irradiation, induced cryptorchidism, viral infections, antifertility agents or idiopathic infertility with normal Leydig cells (Franchimont et al., 1975).

Water soluble rather than lipid soluble testicular extracts prevent castration-induced changes in FSH. Extracts of bull and rat testes (Lee et al., 1974; Braunstein and Swerdloff, 1977), ram rete testis fluid (Setchell and Sirinathsinghji, 1972), human and bull seminal plasma (Franchimont et al., 1975) and media from Sertoli cell cultures (Steinberger and Steinberger, 1977) reduced FSH concentrations in several bioassay systems including castrate male rats, rabbits and rams and pituitary cell cultures.

Chemical properties of inhibin have not been fully characterized. Steinberger and Steinberger (1977) have shown that the Sertoli cell product capable of decreasing FSH production from pituitary cell cultures is a heat-labile molecule with a molecular weight greater than 12,000. This compound has inhibin-like properties, suggesting that Sertoli cells are a source of inhibin. This does not exclude other tubular elements as possible inhibin sources.

4. A Possible Explanation for the Onset of Puberty

Reduction in the sensitivity of hypothalamus and pituitary to negative feedback by gonadal products has been suggested as a possible cause for increased serum gonadotropins and the initiation of puberty in rats (Ramirez, 1973). Ramirez and McCann (1965) showed that smaller dosages

of testosterone on a body weight basis were capable of inhibiting LH release in prepubertal than in adult rats. Negro-Vilar et al. (1973) also demonstrated that testosterone propionate (10 μg per 100 g body weight) suppressed elevated post-castrational LH concentrations in 15- and 28-day-old rats, but not in 58- to 88-day-old rats.

The previous experiments did not insure that circulating testosterone levels achieved by treatment were similar between animals in different age groups. A recent study was conducted to fulfill that objective (Negri and Gay, 1976). Adult and immature rats were implanted at orchidectomy with polydimethylsiloxane capsules of varying size containing enough testosterone to bring circulating testosterone levels to equivalent concentrations in all age groups. These circulating levels of testosterone suppressed LH concentrations in immature but not mature rats. These findings substantiate the theory that reduced sensitivity to negative feedback occurs as animals mature. The importance of this mechanism in bulls has not been assessed.

MATERIALS AND METHODS

A. General Methods

1. Animals

All animals used in these experiments were Holstein bull calves purchased locally at about 1 week of age or obtained from the Michigan State University dairy herd. Except during experiments, the bulls were housed in an enclosed calf barn until about 3 months of age and in a dry lot thereafter. Bulls were tied with halters or stanchioned in a cold, enclosed barn for 2 to 3 days to allow them to adjust to the surroundings before each experiment or blood collection period.

2. Blood Collection Procedures

Cannulae were inserted in the jugular vein 2 to 24 hr before starting blood sample collection. Cannulae 30 inches in length (SLV-105#-18, PCV cannula, ICO Rally Co., Palo Alto, Calif.) were inserted into the jugular vein through a 2 inch-12 ga thin-walled needle (Becton and Dickinson and Co., Rutherford, N.J.). The outside free end was plugged and placed in a 2 inch by 2 inch Elastoplast envelope (Beiersdorf, Inc., South Norwalk, Conn.), secured to the neck with branding cement at the exit point of the cannula from the epidermis. To prevent blood coagulation, cannulae were filled with sodium citrate (3.5% in sterile water) between blood collections.

After blood samples were collected they were allowed to clot at room temperature for 4 to 6 hr and at 4 C for 24 hours. Blood was centrifuged at 2000 x g for 20 to 30 min; serum was decanted and frozen at -20 C until radioimmunoassays were completed.

3. Radioimmunoassays

Serum prolactin, growth hormone, luteinizing hormone and testosterone were estimated by previously described double antibody radioimmunoassays (Koprowski and Tucker, 1971; Purchas et al., 1970; Convey et al., 1976; Haynes et al., 1977c).

Androstenedione was determined as described earlier (Mongkonpunya et al., 1975b), except a second antibody was used to separate free from bound androstenedione similar to the testosterone assay described by Haynes et al. (1977c). Briefly, 200 µl of rabbit anti-androstenedione[1] serum diluted to 1:2000 in .1M phosphate buffered (pH 7.4) saline with .1% gelatin (PBS-G) and 1:100 normal rabbit serum was added to the benzene:hexane (1:3) extract from each serum sample. Then 200 µl of 1,2,6,7-^{3}H-androstenedione (5000 cpm) in PBS-G was added, the samples were vortexed briefly and incubated for 24 hr at 4 C. Sheep anti-rabbit gamma globulin serum (400 µl) at a dilution of 1:20 in PBS-G was added to each tube. After incubation for 24 to 48 hr at 4 C, tubes were centrifuged for 30 min at 2000 x g and a .5-ml aliquant was taken for assessment of radioactivity by liquid scintillation spectrometry. The

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[1]Rabbit #866-8-17-70 anti-androstenedione serum prepared against 6β-succinyl androstenedione conjugated to bovine serum albumin generously supplied by Dr. G. D. Niswender; Dept. Physiology and Biophysics, Colorado State University, Fort Collins, Colo.

useful range of the androstenedione standard curve was from 10 to 500 pg. Fifty picograms of standard androstenedione reduced ^{3}H-androstenedione binding by 50%. The average intra- and inter-assay coefficients of variation calculated from standard serum assayed six times in each of nine assays were 21.3% and 10%, respectively.

Bull and steer serum with added androstenedione (10 ng/ml) were assayed in quadruplicate with and without isolation by column chromatography. Benzene:hexane (1:3) extracts were chromatographed on Lipidex 5000 columns, 11 cm in length. Petroleum ether:chloroform (95:5) solvent eluted androstenedione at 5 to 8 ml. After correction for procedural losses, assay values for direct extraction were in good agreement with chromatographed extracts. The bull serum averaged .15 $\pm$ .01 ng/ml without and .16 $\pm$ .06 ng/ml with chromatography. Comparable values for steer serum with 10 ng/ml added androstenedione were 10.11$\pm$.42 and 9.05$\pm$.55 ng/ml. When .5, 1.0, 2.0 and 5.0 ng/ml androstenedione were added to steer serum, assays (quadruplicate) detected 101%, 107%, 107% and 87% of the added mass.

Two different radioimmunoassays for FSH were used in Experiment 2 for comparative purposes and to obtain more confidence in results. Results from both assays were similar so only one assay was used in Experiment 3. The first was a heterologous assay using anti-rat FSH serum, highly purified rat FSH for iodination and bovine FSH for standard.[2]

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[2]Rabbit anti-rat FSH serum (S-7), rat FSH (I-3) and NIH-FSH-B1 (.49 x NIH-FSH-S1) were obtained from NIAMD of NIH.

Rat FSH was iodinated by the chloramine-T method (Greenwood et al., 1963). Ten micrograms FSH in 5 µl of double distilled water was mixed with 1.0 mCi $Na^{125}I$ (10 µl NaOH, pH 7 to 11) in 25 µl .5M phosphate buffer (pH 7.5). Chloramine-T (5 µg/5 µl .05M phosphate buffer, pH 7.5) was mixed vigorously with FSH and ^{125}I for 2 minutes. The reaction was stopped with sodium metabisulfite (12.5 µg/5 µl .05M phosphate buffer, pH 7.5). After adding 25 µl PBS-G and 100 µl of 1% potassium iodide the mixture was placed on a 15 cm x .8 cm Biogel P-60 column to separate free ^{125}I from FSH-^{125}I.

FSH assays were conducted as follows: 200 µl rabbit anti-rat FSH serum (1:2000 in 1:400 normal rabbit serum-.05M EDTA-PBS, pH 7.0) was added to 500 µl standard FSH (NIH-B1) or 500 µl sample (serum plus PBS-G) and incubated for 24 hr at 4 C. FSH-^{125}I (20,000 cpm) in 100 µl PBS-G was added to each tube and incubated at 4 C, followed in 24 hr by 200 µl sheep anti-rabbit gamma globulin serum diluted 1:20 in .05M EDTA-PBS (pH 7.0). After 72 hr, 3 ml PBS was added and tubes were centrifuged at 2000 x g for 30 minutes.

The second FSH radioimmunoassay was a slight modification of the homologous bovine FSH assay developed by Cheng (1978). This assay utilized an antiserum prepared in rabbits against highly purified bovine FSH, both prepared by Cheng (1976, 1978).[3] Iodination of bovine FSH was conducted similar to that for rat FSH (above), not by the lactoperoxidase method as used by Cheng (1978).

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[3]Highly purified bovine FSH (160 x NIH-FSH-S1) and rabbit anti-bovine FSH serum were kindly provided by Dr. K. W. Cheng; University of Manitoba, Winnipeg, Manitoba, Canada.

Two hundred microliters of rabbit anti-bovine FSH serum (1:120,000 in 1:400 normal rabbit serum-1% bovine serum albumin (BSA) -PBS) was added to 500 µl standard FSH (NIH-B1) in BSA-PBS or 500 µl of sample (serum plus BSA-PBS) and incubated for 24 hr at 4 C. FSH-^{125}I (20,000 cpm) in 100 µl BSA-PBS was added to each tube and incubated at 4 C, followed in 24 hr by 100 µl sheep anti-rabbit gamma globulin serum diluted 1:20 in BSA-PBS. After 48 to 72 hr, 3 ml of cold PBS was added and tubes were centrifuged at 2000 x g for 30 minutes.

Cross-reaction with prolactin (NIH-B4), growth hormone (NIH-B12), TSH (NIH-B6), LH (NIH-B8); recovery of FSH-B1 added to serum and parallelism of serum curves with FSH-B1 standard curve were tested for both FSH radioimmunoassays.

Also luteinizing hormone-releasing hormone (500 µg Beckman Lot # D1018) was administered iv to four 10-month-old bulls (300 kg body weight) and serum samples were collected (-20, 0, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240 min) to measure FSH response as an additional validation step for both assays.

Androstenedione and testosterone in media from *in vitro* incubations were assayed by assays as described above. Before assay of tissue testosterone, samples were homogenized and testosterone was isolated chromatographically (Bartke *et al.*, 1973). Testicular tissue was homogenized in PBS (10 to 20 mg tissue/ml buffer) with a Brinkman Polytron for two 10-sec blasts. To account for procedural losses, 25,000 cpm ^{3}H-testosterone was added to 200 µl testicular homogenate. Five milliliters benzene:hexane (1:3) was added to homogenates and mixed for 1 minute. After freezing the aqueous phase, the solvent phase was decanted and evaporated in a vacuum oven. The residues were dissolved in

200 µl of column solvent (iso-octane:benzene:methanol, 90:5:5) and transferred to 11 x .5 cm Sephadex LH-20 columns with two 200 µl-solvent rinses. Ten milliliters column solvent was added to the column and discarded. Then 10 ml was added, saved, evaporated, and the residue was redissolved in PBS-G. Aliquants were taken for testosterone assay and calculation of procedural losses. Testosterone was assayed as described by Haynes et al. (1977c) except 1% dextran-.5% charcoal was used to separate bound from free testosterone.

4. Histological Preparation

Immediately after castration, testes were cut into 1 mm^3 pieces with a razor blade and fixed for 2 hours in a modified Karnovsky's fixative (Karnovsky, 1965) containing .5% formaldehyde and 1.3% glutaraldehyde in .1M phosphate. Post-fixation was done in phosphate buffered osmium tetroxide and samples were dehydrated in a series of alcohols, washed in propylene oxide and embedded in Epon 812-Araldite 502 Resin (50:50). Sections of .5 to 1.0 µ were made and stained with Azure II for examination under a light microscope.

5. Statistical Analyses

Data were analyzed by split plot analysis of variance (Gill and Hafs, 1971). Logarithmic transformations were made where heterogeneous variance existed. Specific comparisons of means were by orthogonal contrasts and Scheffe's interval. Regression procedures were used to test for linear, quadratic or cubic trends in data with advancing age (Gill, 1978).

An objective method to select episodic peaks was developed and used to evaluate these data. A standard serum sample containing concentrations

that we judged to represent basal concentrations of each hormone was assayed eight times in each assay. Values obtained were used to estimate a 95% confidence interval around basal concentrations. An increase greater than two 95% confidence intervals above a preceding value in a hormone pattern was considered to be a peak. Two 95% confidence intervals were .64 ng, .28 ng, .12 ng and 2.54 ng for LH, testosterone, androstenedione and prolactin, respectively. The frequency of hormone peaks, mean peak height (peak height = highest value in each peak) and mean baseline concentration (lowest value between peaks) were calculated for each animal and each age group. The objective of this method was to insure that peaks reflected actual hormonal changes in animals, not assay variation. This procedure was used to evaluate hormone profiles in Experiments 2 and 3, but not in Experiment 1. Subjective evaluation of graphs of hormone data was used to determine peaks in Experiment 1.

B. Experiment 1

The objective was to determine patterns of LH, prolactin, growth hormone and testosterone in serum before and after puberty in bulls and steers. Eight prepubertal and eight pubertal bulls were balanced for age and weight within groups, four to be left intact and four to be castrated 2 weeks before the experiment (age and body weight in Table 1). Jugular blood was collected via cannulae at .5-hr intervals from 0800 to 1700 hr for determination of growth hormone, prolactin, testosterone and luteinizing hormone concentrations.

Table 1. Age and weight for bulls and steers in Experiment 1.

	Treatment Groups			
	Prepubertal		Pubertal	
Parameter	Bulls	Steers	Bulls	Steers
Age (days)	113 ± 24[a]	157 ± 6	270 ± 32	286 ± 35
Weight (kg)	80 ± 12	100 ± 7	229 ± 36	184 ± 16

[a]Mean ± SE of four animals.

C. Experiment 2

The objective was to compare at montly intervals from 1 to 10 months of age, patterns of LH and FSH secretion in bulls, steers and bulls in which the scrotum had been shortened (SS bulls).

Twelve bulls were 2 weeks of age on April 1 when assigned randomly four each to be left intact, castrated, or made short scrotum. Bulls were made short scrotum by retaining the testes at the top of the scrotum with an elastic band. The purpose of making bulls short scrotum was to interfere with seminiferous tubular function without effecting Leydig cell function.

Body weight for all animals and scrotal circumference for bulls were recorded at montly intervals from 1 to 10 months. Each month blood was taken at .5-hr intervals from 1000 hr until 0930 hr of the following day. All samples were assayed for LH and testosterone. Androstenedione was measured in samples from the last 8 hr of each monthly bleeding in bulls and SS bulls. FSH was measured by both radioimmunoassays in three

samples taken at hourly intervals (0700, 0800, 0900) from each animal at each month. In addition, FSH concentrations were determined using the rat FSH assay in two animals from each group at 3, 6 and 9 months in all serum samples from the last 8 hr of the collection period. These samples were measured to estimate FSH variability between groups and ages.

At 10 months of age, testes and epididymides were removed and weighed; and testes were fixed for histological evaluation to compare seminiferous tubular development in normal and SS bulls. Epididymides were homogenized in .9% saline-Triton X-100 buffer and spermatozoa were counted in a hemocytometer with phase contrast microscopy (Kirton et al., 1967).

D. Experiment 3

The objectives were to describe serum hormone profiles, testicular responsiveness to LH and binding of gonadotropins to testicular tissue in bulls of different ages. Serum samples were collected from all bulls on a single day to permit determination of age effects independent of season.

1. Blood Collection

Bulls at 1, 3, 4, 5, 7 and 9 months of age and weighing 55, 79, 132, 176, 237 and 269 kg, respectively, were used in this experiment. Jugular cannulae were installed 24 hr before the experiment. On September 29 at 0800 hr, blood samples were collected at 20-min intervals for 24 hours.

Testosterone, androstenedione, prolactin and FSH were assayed in samples collected for an 8-hr period (1200 hr to 2000 hr) and LH was quantified in samples collected over 24 hours. FSH was estimated with the rat FSH radioimmunoassay.

2. Testis Incubations

Testicular tissue from bulls of different ages was incubated *in vitro* to measure testosterone and androstenedione production after LH treatment. Testicular tissue from each bull was cut into explants (1 mm^3) in a petri dish containing Media TC 199. Five testis explants were placed in each of 15 flasks for each bull. Tissue was also frozen immediately to serve as unincubated control. Three milliliters Media TC 199 was added to each flask and treatments resulting in 0, 5, 50 ng LH/ml of media were added to five flasks each in 300 µl .1% gelatin-PBS. Flasks were incubated for 3 hr at 34 C in a Dubnoff Metabolic Shaker while being gassed with 95% O_2-5% CO_2. After 3 hr, tissue and media were separated and frozen until assayed.

3. Testicular Gonadotropin Binding

The amount of specific ^{125}I-FSH and ^{125}I-HCG binding to testicular tissue homogenates from bulls of different ages was determined. Three to five 1-cm^3 pieces of testicular tissue were placed in 15 ml of 20% glycerol-PBS buffer, frozen in dry ice-methanol and stored at -60 C. Later, testes tissue was thawed, blotted dry, weighed, placed in 20% glycerol-PBS at a concentration of 500 mg tissue/ml and homogenized in a Sorvall Omnimixer for 5 to 10 sec at top speed at 4 C. The homogenate was filtered through two layers of cheese cloth, dispensed into 2-ml

aliquots, frozen in a dry ice-methanol bath and stored at -60 C until assayed.

Human chorionic gonadotropin (HCG) and rat FSH were iodinated by the chloramine-T method of Greenwood et al. (1963).[4] HCG or FSH (10 µg/5 µl double distilled water) was added to 1.0 mCi $Na^{125}I$ in 10 µl NaOH (pH 7 to 11) containing 20 µl of .5M phosphate buffer (pH 7.5). Chloramine-T (5 µg/5 µl double distilled water) was added and allowed to react for 2 min with gentle mixing. Sodium metabisulfite (10 mg/5 µl double distilled water) was added, followed by 100 µl of 1% potassium iodide and 25 µl of PBS-G. The contents were placed on a 16 cm x .8 cm Biogel P-60 column which had been equilibrated with .05M phosphate buffer (pH 7.5, column buffer). One rinse of 100 µl potassium iodide was added to the iodination vial, mixed, and added to the column. Free ^{125}I was isolated from hormone-bound ^{125}I by elution with column buffer. Fractions of 1 ml were collected into tubes containing 1 ml PBS-G. Aliquots of 20 µl of the collected fractions were counted for .1 min in a gamma counter to characterize the elution pattern (Figure 1).

Iodinated hormone preparations were evaluated as described by Ireland and Richards (1978). Specific activity of labeled hormones was estimated to be 150 cpm/pg for HCG and 55 cpm/pg for FSH. This was determined using labeled and unlabeled HCG (CR 119) or rat FSH (I-3) and bovine testes homogenates as a source of binding sites. A constant amount of receptor (35 to 70 µg of protein/tube for FSH and 1 to 1.5 mg protein/tube for HCG) was used to generate two types of parallel

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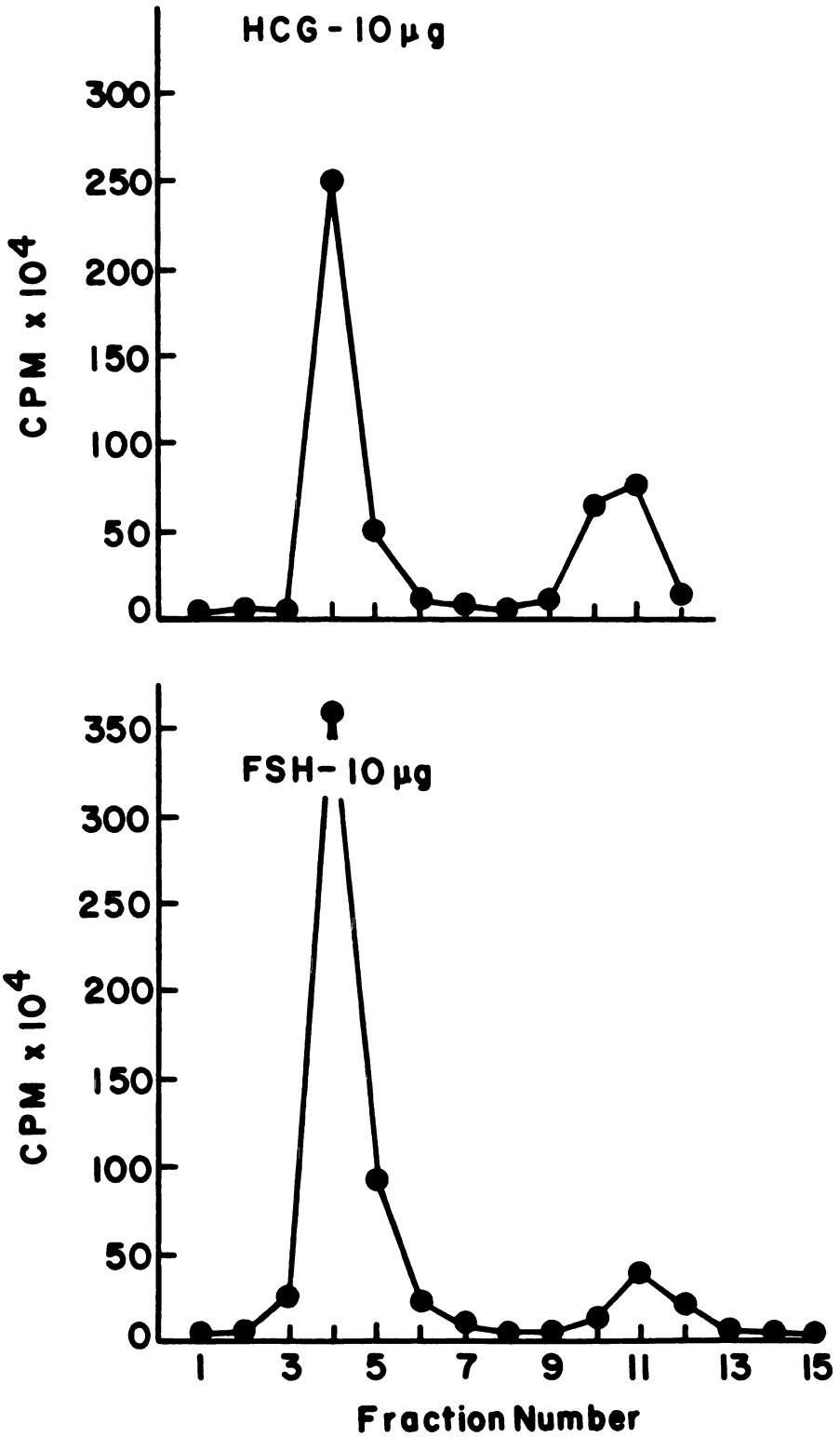
[4]HCG (CR 119; 11,600 IU/mg) and rat FSH (I-3; 150 x NIH-FSH-S1) were obtained from NIAMD of NIH.

Figure 1.--Elution profiles of HCG (top) and FSH (bottom) iodination reaction mixtures on Bio-Gel P-60 columns. Each point represents the radioactivity in 20-µl aliquots of 1-ml column fractions. The first peak (left) represents hormone bound ^{125}I and the second (right) represents free ^{125}I.


HCG - 10 µg
CPM x 10^4
300
250
200
150
100
50
0
FSH - 10 µg
CPM x 10^4
350
300
250
200
150
100
50
0
1
3
5
7
9
11
13
15
Fraction Number

standard curves. In one type increasing concentrations of labeled hormone were used. In the other, counts per min of labeled hormone were kept constant and the concentration of unlabeled hormone was increased. The 50% inhibition point on each curve was used to calculate total cpm/μg of active hormone. The active hormone or "active fraction" was defined as the portion of labeled preparation which was specifically bound in the presence of excess receptor. This was 30 to 40% for HCG and 20 to 30% for FSH, thus the specific activity of active labeled HCG was 52.2 cpm/pg and labeled FSH was 14 cpm/pg.

Binding assays were conducted in polypropylene tubes (12 x 75 mm) precoated with 5% BSA-PBS. Specific binding was determined by subtracting non-specific binding (binding in the presence of 1000-fold excess of cold hormone) from total binding for each tissue sample. Ninety microliters (total binding tubes) or 60 μl of PBS (non-specific binding) were added to each tube. Thirty microliters of PBS containing unlabeled hormone (LH-NIH-B8, 30 μg; FSH-NIH-B1, 75 μg) was added to non-specific binding tubes. Saturating amounts of iodinated hormones in 40 μl PBS were added to each tube including "total count tubes" (HCG-^{125}I, 250,000 cpm/tube; FSH-^{125}I, 500,000 cpm/tube). Homogenized tissue samples in 100 μl PBS were added, mixed and incubated in a shaker at room temperature for 24 hours (time determined from time-study described below). At the end of the incubation, 1 ml cold PBS was added to each tube and the contents were centrifuged at 2000 x g for 30 minutes. The supernatant was discarded; the pellet was resuspended in 1 ml cold PBS by vigorous mixing; and tubes were recentrifuged, supernatant discarded and the ^{125}I in the tubes containing the pellet was quantified in a gamma counter.

A time-study was conducted to determine the period required for binding to reach equilibrium. Testicular tissue, homogenized as previously described, was incubated with iodinated hormone at room temperature for .17, .5, 1, 2, 6, 12, 24, 36 and 48 hours. Tubes were centrifuged and the specific binding was determined. From results in Figure 2, maximum binding occurred in 12 to 36 hours.

Saturation studies were conducted to determine the amount of iodinated hormone required to saturate a given amount of tissue. Increasing amounts of iodinated hormone were added to a constant amount of tissue and specific binding was determined (Figure 3). Fifty milligrams of tissue required approximately 40,000 cpm HCG-^{125}I for saturation. One and 5 mg of tissue required approximately 125,000 cpm and 600,000 cpm of FSH-^{125}I for saturation, respectively.

Before determining specific binding of testes homogenates from Experiment 3, samples were thawed in cold water (4 C) and centrifuged at 2000 x g for 10 minutes. The supernatant was discarded, the pellet was weighed and resuspended in PBS at a concentration of 20 mg of pellet/ml (1 to 1.5 mg protein) for HCG binding assays and 1 mg of pellet/ml (35 to 70 µg protein) for FSH binding assays. Resuspended tissue was rehomogenized with 20 strokes in a glass homogenizer and 100 µl was used to assess binding. During resuspension and rehomogenization, tissues and buffers were maintained at 4 C. Specific binding was determined as described above and expressed as hormone bound per µg of protein, per µg of DNA or per testis x 2. Protein was measured with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.) and DNA was estimated by the method of Burton (1956).

Figure 2.--Time-course of ^{125}I-HCG (top) and ^{125}I-FSH (bottom) binding to testicular tissue. A weighed amount of tissue pellet obtained from centrifugation of crude testicular homogenates for 10 min at 2000 x g was rehomogenized and used in these studies.

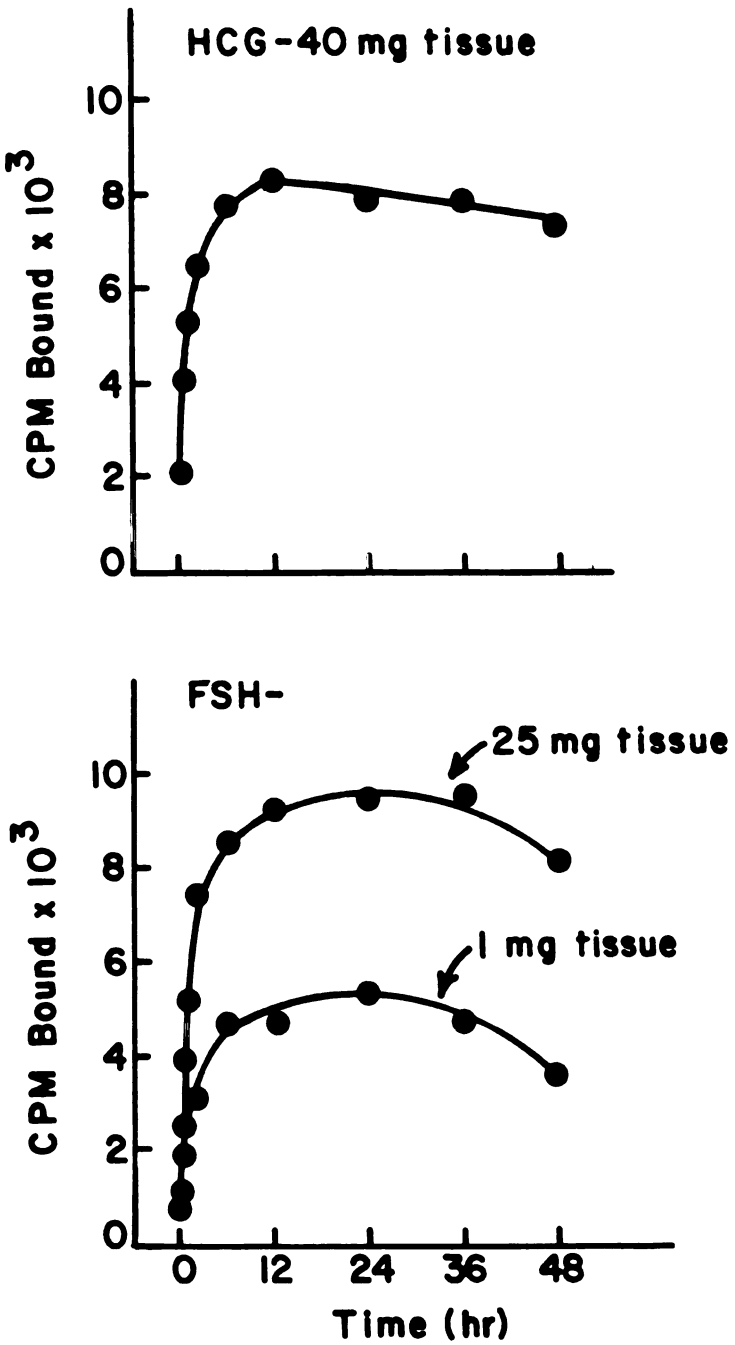
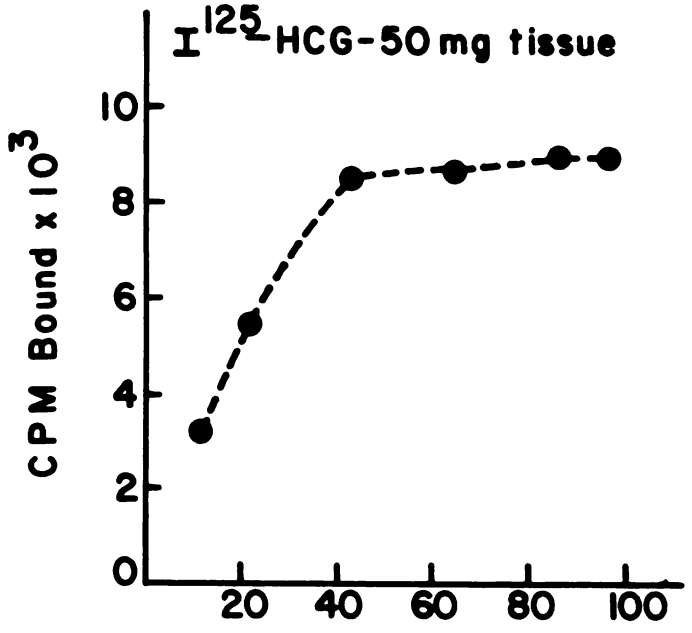
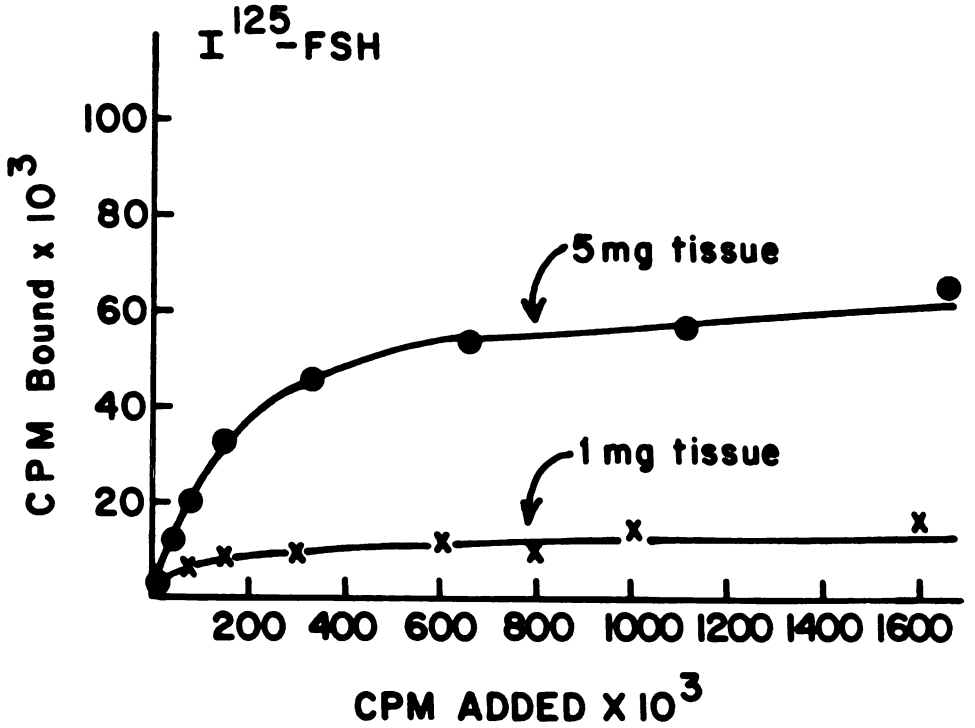

HCG-40 mg tissue
CPM Bound x 10^3
0
2
4
6
8
10
FSH-
25 mg tissue
1 mg tissue
0
12
24
36
48
Time (hr)

Figure 3.--Saturation curves for ^{125}I-HCG and ^{125}I-FSH binding to bovine testicular tissue. A weighed amount of tissue pellet obtained from centrifugation of crude testicular homogenates for 10 min at 2000 x g was rehomogenized and used in these studies.


I125 HCG-50 mg tissue
CPM Bound x 10^3
0
2
4
6
8
10
20
40
60
80
100


I125-FSH
CPM Bound x 10^3
20
40
60
80
100
5 mg tissue
1 mg tissue
200
400
600
800
1000
1200
1400
1600
CPM ADDED X 10^3

In addition to determining specific binding, saturation curves using tissue from one to three bulls in each age group were used to determine the affinity of hormone binding. These assays required a constant amount of tissue (same as used previously) and varying amounts of iodinated hormone. Results were evaluated by the method of Scatchard (1949) and the affinities were estimated from the slope of these plots.

RESULTS AND DISCUSSION

A. FSH Radioimmunoassays

Both radioimmunoassays were suitable to determine concentrations of FSH in bovine serum (Table 2). The homologous FSH assay was 3 to 4 times more sensitive than the heterlogous FSH assay, but exhibited high apparent cross-reactivity with NIH-TSH-B6. TSH cross-reaction was not unlike that reported previously for bovine gonadotropin assays (Oxender et al., 1972; McCarthy and Swanson, 1976). It is suspected that the TSH standard preparation was contaminated with gonadotropins rather than FSH antiserum reacting with TSH. The contamination left in TSH preparations after purification has been suggested to be immunologically active, but not biologically active gonadotropins (Niswender et al., 1969). To prove that this phenomenon is not true TSH cross-reaction, serum containing low and high concentrations of TSH (pre- and post-injection of a 50 μg, iv dose of thyrotropin-releasing hormone) were assayed and did not have different LH concentrations (McCarthy and Swanson, 1976). More highly purified bovine TSH, as well as bovine TSH-α and TSH-β preparations obtained from Dr. J. G. Pierce (University of California, Los Angeles, Calif.) did not cross-react significantly in the homologous FSH assay (Cheng, 1978). Consequently, the observed TSH cross-reaction originates from immunological contamination of NIH-TSH-B6 standard, not nonspecificity of FSH antiserum.

Table 2. Comparison of two radioimmunoassays for FSH in bovine serum.

Criterion	Heterologous rat FSH assay	Homologous bovine FSH assay
Antisera (rabbit)	Anti-Rat-FSH serum (S-7; NIAMD)	Anti-Bovine-FSH-serum (Cheng)
Antisera dilution	1:2000	1:120,000
Hormone for iodination	Rat FSH (I-3; NIAMD)	Bovine FSH (Cheng)
CPM ^{125}I-FSH added	20,000	20,000
% of ^{125}I-FSH bound	25-30%	20-25%
Standard	NIH-FSH-B1	NIH-FSH-B1
Cross-reaction (%)[1]		
TSH-NIH-B6	3	60.7
LH-NIH-B8	<1	4.3
Prolactin-NIH-B4	<1	<1
Growth hormone-NIH-B12	<1	<1
Sensitivity[2]	9.5 ng	2.5 ng
Coefficient of variation		
Interassay (%)	4.4	13.5
Intra-assay (%)	8.2	14.2
Recovery (%)[3]	100.3	91.6
GnRH response[4]		
Preinjection FSH (ng/ml)	100.8	44.0
Maximum FSH (ng/ml)	132.9	180.3
Time to peak (min)	60	45

[1]A ratio of amount of FSH required to reduce binding of ^{125}I-FSH equivalent to reduction achieved by 1000 ng of other hormone was multiplied by 100 to get percent cross-reaction.

[2]Sensitivity (ng) is defined as the amount of hormone required to displace ^{125}I-FSH binding equivalent to two standard deviations from zero.

[3]Mean recovery (%) of 1 µg/ml of FSH (NIH-B1) added per ml of bull serum from 50, 100 and 200 µl serum.

[4]Mean preinjection FSH concentration and FSH response of four bulls to a 500 µg iv dose of luteinizing hormone-releasing hormone (GnRH).

Several pools of bovine serum and media from pituitary cell cultures displaced ^{125}I-FSH parallel to that of NIH-FSH-B1 in both assays (Figure 4).

B. Experiment 1

Serum LH concentrations and number of secretory peaks per 9 hr (Table 3) were greater in steers than bulls at both ages ($P<.05$), but average serum LH did not differ significantly between ages. These results demonstrate that, in 4-month-old as well as 9-month-old bulls, testicular products normally maintain low serum LH concentrations, since removal of the testes at both ages causes increased serum LH.

Number of episodic peaks and mean testosterone concentrations (Table 3) were four-fold greater in pubertal than in prepubertal bulls, but these changes only approached significance ($P\simeq.10$). Wide variation in the testosterone secretion profiles between bulls within an age-group prevented these groups from differing significantly. One bull in the prepubertal group had serum testosterone concentrations as high as the mean of bulls in the pubertal group and one pubertal bull had low testosterone concentrations. There is no question through, that testosterone concentrations increase with advancing age (Rawlings et al., 1972; Karg et al., 1976).

Average prolactin concentrations and episodic release patterns were not affected significantly by castration or by age, although prolactin tended to be higher and more variable in older animals. Growth hormone concentrations and number of peaks were greater in prepubertal bulls than in pubertal bulls ($P<.07$), but this age effect was not evident in steers.

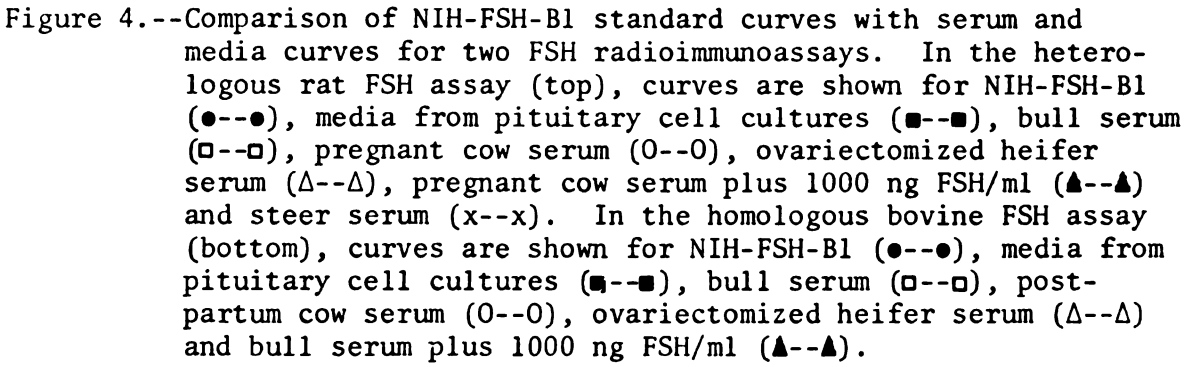


Figure 4.--Comparison of NIH-FSH-B1 standard curves with serum and media curves for two FSH radioimmunoassays. In the heterologous rat FSH assay (top), curves are shown for NIH-FSH-B1 (●--●), media from pituitary cell cultures (■--■), bull serum (□--□), pregnant cow serum (O--O), ovariectomized heifer serum (Δ--Δ), pregnant cow serum plus 1000 ng FSH/ml (▲--▲) and steer serum (x--x). In the homologous bovine FSH assay (bottom), curves are shown for NIH-FSH-B1 (●--●), media from pituitary cell cultures (■--■), bull serum (□--□), postpartum cow serum (O--O), ovariectomized heifer serum (Δ--Δ) and bull serum plus 1000 ng FSH/ml (▲--▲).

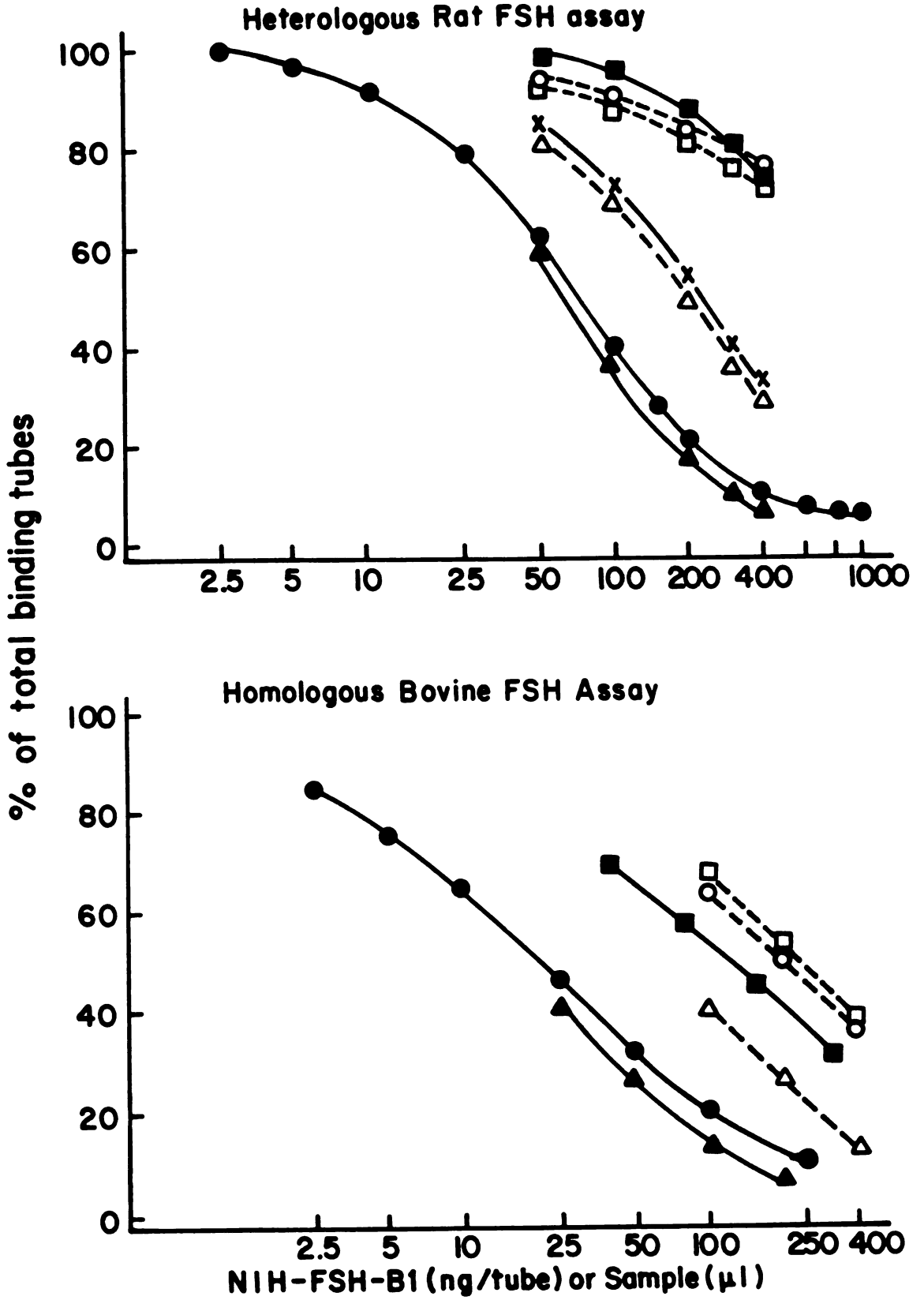

Heterologous Rat FSH assay
100
80
60
40
20
0
2.5
5
10
25
50
100
200
400
1000
Homologous Bovine FSH Assay
100
80
60
40
20
0
2.5
5
10
25
50
100
250
400
% of total binding tubes
NIH-FSH-B1 (ng/tube) or Sample (µl)

Table 3. Serum hormone characteristics for prepubertal and pubertal bulls and steers.

Hormone		Prepubertal		Pubertal	
		Bulls	Steers	Bulls	Steers
LH	Concentration[1]	2.9±1.7	10.5±7.3	3.3±1.8	9.2±3.1
	Episodic peaks[2]	2.0± .0	6.8± .3	2.5± .5	6.8± .9
Testosterone	Concentration	1.4±1.1	--	5.4±3.5	--
	Episodic peaks	.5± .5	--	2.3± .3	--
Prolactin	Concentration	7.7±3.5	8.4±2.8	16.6±7.9	18.0±8.8
	Episodic peaks	1.5± .7	2.0± .7	2.5± .8	3.0± .8
GH	Concentration	14.9±4.0	8.0±1.9	6.7±2.4	9.8±4.2
	Episodic peaks	4.3± .3	3.3±1.0	2.0± .0	3.0± .7

[1]Mean ± SE of hormone concentration (ng/ml) for all samples taken over 9 hr for four animals.

[2]Mean number (± SE) of episodic peaks observed per animal per 9 hours.

Bartke (1976) and Bartke and Dalterio (1976) demonstrated the importance of prolactin in testicular function, especially steroidogenesis in several rodent species. Prolactin was greater in postpubertal bulls than prepubertal bulls. However, we cannot determine from this investigation whether prolactin caused increased testosterone production in older animals, or if testosterone stimulated prolactin synthesis (Nolin et al., 1977).

Prepubertal bulls had greater growth hormone concentrations than did postpubertal bulls. Whether high prepubertal growth hormone concentrations participate in testicular maturation in bulls cannot be determined from this investigation. However, growth hormone has recently been reported to increase testosterone production by immature male rats in response to LH (Swerdloff and Odell, 1977).

C. Experiment 2

Body weights for bulls, steers and SS bulls did not differ significantly. Averaged over treatments they were 50 $\pm$ 3 ($\bar{X}$ $\pm$ SE) kg at 1 month and 273 $\pm$ 12 kg at 10 months. Scrotal circumference increased linearly ($P<.05$) with age in intact bulls from 106 mm at 1 month to 277 mm at 10 months. Testicular weight (including epididymides) of bulls and SS bulls at 10 months did not differ significantly (186 $\pm$ 29 vs 140 $\pm$ 19 g).

At 10 months, bulls had spermatogonia, Sertoli cells, spermatocytes and spermatids, whereas short scrotum bulls had Sertoli cells and a few spermatogonia. Epididymidal sperm were present in bulls (7×10^8/epididymis) but not SS bulls.

LH increased from 1 (1.1 ± 1.6 ng/ml) to 3 months (5.6 ± 3.3 ng/ml) in steers and from 1 (.8 ± 1.1 ng/ml) to 2 months (2.4 ± 3.1 ng/ml) in SS bulls ($P<.05$), and remained unchanged thereafter (Figure 5). In normal bulls, LH was greater ($P<.05$) at 4 months (2.7 ± 2.6 ng/ml) than at 1 month (.6 ± .5 ng/ml), but did not differ significantly among other months.

Serum LH concentrations were greater ($P<.05$) in steers than in bulls or SS bulls from 3 to 10 months. Also, SS bulls had higher serum LH than bulls at 5 months (2.9 ± 1.1 vs 1.5 ± 1.7 ng/ml) and 6 months (2.2 ± 1.2 vs 1.2 ± 2.6 ng/ml), but not thereafter. Interestingly, only at months (Figure 5), was testosterone less in SS bulls (.3 ± .2 ng/ml) than bulls (1.2 ± .2 ng/ml).

Serum testosterone concentrations in steers were low (100 pg/ml) and did not change with time of day or age, thus they were excluded from statistical analysis of results. The first significant increase in serum testosterone ($P<.05$) occurred between 4 (.5 ± .2 ng/ml) and 5 months (1.2 ± .9 ng/ml) in normal bulls but not until 5 (.3 ± .2 ng/ml) to 6 months (1.4 ± 1.4 ng/ml) in SS bulls. Average androstenedione concentrations (Figure 5) did not differ significantly between bulls and SS bulls; it increased ($P<.05$) transiently at 4 months in both groups.

Frequency of LH peaks increased ($P<.05$) from 1 to 4 months in bulls, steers and SS bulls (Table 4; Figure 6). Thereafter the frequency of LH peaks declined ($P<.05$) in bulls and SS bulls, but not steers. This decreased frequency occurred coincident with increased testosterone secretion (Figure 5). Frequency of LH peaks did not differ between treatments at 1 month, but at 2 months steers had a higher ($P<.05$) frequency

Figure 5.--Mean LH, testosterone and androstenedione concentrations in bulls (O--O), steers (Δ--Δ) and short scrotum bulls (●--●). Values are means of 48 samples from a 24-hr period for LH and testosterone and 16 samples from an 8-hr period for androstenedione for four animals in each group.

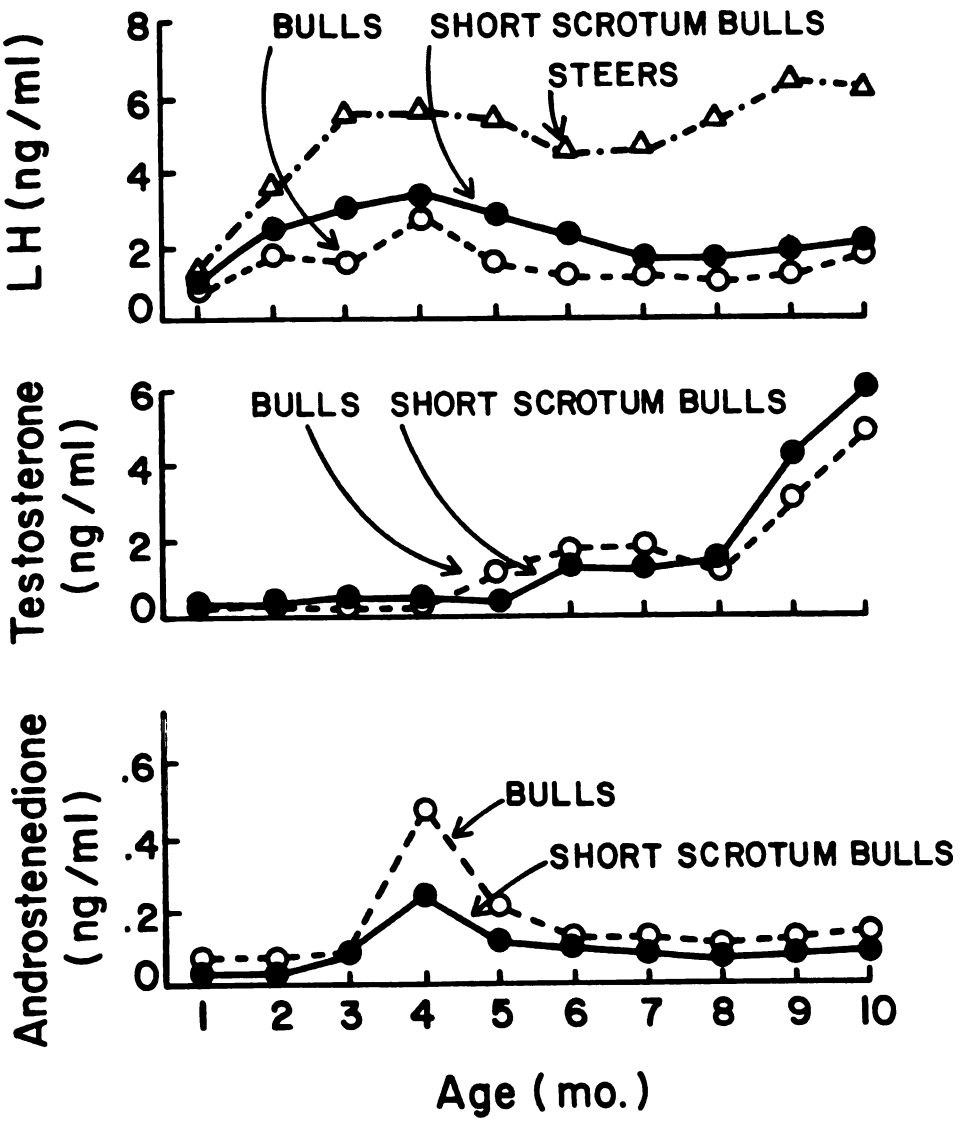
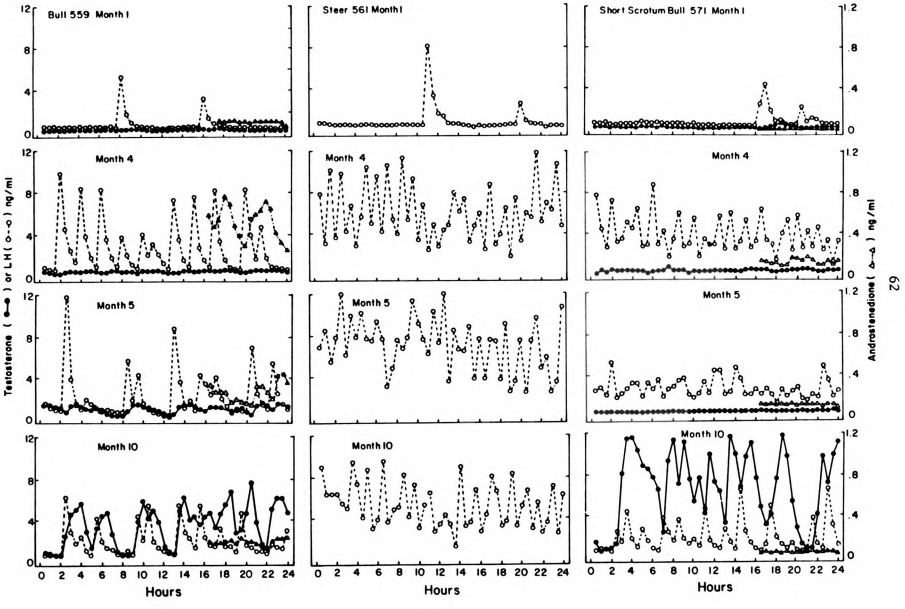

LH (ng/ml)
8
6
4
2
0
BULLS
SHORT SCROTUM BULLS
STEERS
Testosterone (ng/ml)
6
4
2
0
BULLS
SHORT SCROTUM BULLS
Androstenedione (ng/ml)
.6
.4
.2
0
BULLS
SHORT SCROTUM BULLS
1 2 3 4 5 6 7 8 9 10
Age (mo.)

Table 4. Frequency and height of episodic peaks and basal concentrations of LH in bulls, steers and short scrotum bulls (SSbulls).[a]

Age (Mo)	Frequency			Peak Height			Basal		
	Bulls	Steers	SSbulls	Bulls	Steers	SSbulls	Bulls	Steers	SSbulls
	-(No. peaks/24hr)-			-(ng/ml)-			-(ng/ml)-		
1	1.3	3.8	3.3	3.3	5.1	4.4	.5	.6	.6
2	4.3	9.5	6.8	8.4	9.4	8.1	.6	1.3	.7
3	5.3	16.3	10.0	5.5	8.6	7.6	.5	3.1	1.1
4	11.0	17.5	15.5	6.1	7.5	4.8	1.1	3.9	2.1
5	8.8	14.5	10.0	4.2	7.1	4.4	.5	3.6	2.0
6	4.8	10.0	7.8	4.6	5.9	3.4	.5	3.1	1.5
7	6.8	10.8	6.0	4.4	6.2	3.5	.6	3.0	1.0
8	5.0	10.3	3.8	4.0	6.8	5.1	.4	3.8	.9
9	7.3	15.5	7.3	3.9	9.2	4.0	.5	4.3	1.0
10	7.0	13.3	8.3	6.4	8.1	5.1	.7	4.0	1.0

[a]Values are the means of four animals.

Figure 6.--LH (O--O), testosterone (●--●) or androstenedione (Δ--Δ) concentrations in a representative bull, steer and short scrotum bull at 1, 4, 5 and 10 months of age.


Bull 559 Month 1
Steer 561 Month 1
Short Scrotum Bull 571 Month 1
Month 4
Month 5
Month 10
Testosterone (●—●) or LH (o--o) ng/ml
Androstenedione (△--△) ng/ml
Hours
0 2 4 6 8 10 12 14 16 18 20 22 24

of LH peaks than bulls (Table 4). At 3 and 4 months steers and SS bulls had more ($P<.05$) peaks than bulls and from 5 to 10 months steers had more ($P<.05$) peaks than bulls.

LH peak height doubled ($P<.05$) from 1 to 2 months (Table 4) in each treatment. When averaged over all months of the experiment, steers had higher ($P<.05$) LH peaks than bulls or SS bulls (7.4 ng/ml vs 4.9 ng/ml and 5.0 ng/ml). From 3 to 10 months, steers had higher ($P<.01$) basal LH concentrations (lowest value between peaks) than bulls and from 8 to 10 months basal LH in steers exceeded ($P<.05$) that in SS bulls. Basal LH concentrations were elevated in steers because peaks occurred so frequently that new peaks were initiated before serum LH declined from the previous peak.

Increased ($P<.05$) frequency and amplitude of testosterone peaks and increased ($P<.05$) baseline concentrations (Table 5; Figure 6) contributed to increasing average testosterone (Figure 5). Only a few small testosterone peaks (.3 to .6 ng/ml) occurred in bulls from 1 to 3 months and in SS bulls from 1 to 5 months. After this age, the number and size of testosterone peaks increased ($P<.05$). In bull 559 (Figure 6) at 5 months, testosterone peaks of 2 ng/ml followed each LH peak and by 10 months peaks of 5 ng/ml occurred. Frequency of surges differed ($P<.05$) between bulls and SS bulls only at 5 months.

Basal testosterone concentrations in both normal and SS bulls increased ($P<.05$) linearly with age, approximately 10- and 30-fold, respectively (Table 5). Basal testosterone did not differ significantly between bulls and SS bulls.

The increase in mean concentrations of androstenedione in bulls at 4 months (Figure 5) resulted from an increase ($P<.05$) in frequency and

Table 5. Frequency and height of episodic peaks and basal concentrations of testosterone in bulls and short scrotum bulls (SSbulls).[a]

Age (Mo)	Frequency		Peak Height		Basal	
	Bulls	SSbulls	Bulls	SSbulls	Bulls	SSbulls
	-(No. peaks/24hr)-		-(ng/ml)-		-(ng/ml)-	
1	.3	.0	.4	--	.1	.0
2	1.3	1.8	.5	.4	.1	.1
3	.5	.3	.6	.4	.1	.1
4	2.3	1.8	1.8	.7	.2	.2
5	7.5	1.0	2.1	.8	.5	.2
6	4.5	2.8	4.2	2.1	.4	1.3
7	6.3	3.5	3.7	2.3	.4	.5
8	4.5	3.0	3.4	2.4	.5	.9
9	6.3	9.0	6.5	7.4	.8	2.3
10	6.8	8.5	9.3	10.1	1.0	3.1

[a]Values are the means of four animals.

amplitude of peaks and increased ($P<.05$) baseline (Table 6; Figure 6). In SS bulls, an increase ($P<.05$) in peak height at 4 months contributed to the increased mean. However, frequency of peaks or baseline did not differ significantly at 4 months from other months.

These results support those of Rawlings et al. (1972) and Karg et al. (1976) who also found that increased serum testosterone concentration was the most pronounced hormonal change during sexual maturation in bulls. Concomitant changes in gonadotropins or other pituitary hormones have not been previously observed in bulls (e.g., Karg et al., 1976). In this study, mean serum LH concentrations increased at 4 months, associated with an increase in frequency of LH episodic peaks. Androstenedione also increased at 4 months, 1 month prior to the onset of significant testosterone production. Exposure of the testes to more LH around 4 months may have been the stimulus that resulted in greater androstenedione production at 4 months and increased testosterone production at 5 months.

Frequency of episodic LH peaks increased in bulls, steers and SS bulls from 1 to 4 months, in agreement with results reported in rams (Foster et al., 1978). What caused the increased frequency of LH is not known, but it may be related to maturation of the hypothalamus. Since increased frequency of LH peaks occurred in all treatments, irrespective of the steroid milieu, hypothalamic maturation presumably occurs independent of circulating steroid concentrations.

The frequency of LH peaks was greater in steers than in bulls throughout the experiment. Factors involved in negative feedback on LH before puberty may not be testosterone or androstenedione, because

Table 6. Frequency and height of episodic peaks and basal concentrations of androstenedione in bulls and short scrotum bulls (SSbulls).[a]

Age (Mo)	Frequency		Peak Height		Basal	
	Bulls	SSbulls	Bulls	SSbulls	Bulls	SSbulls
	-(No. peaks/8 hr)-		-(ng/ml)-		-(ng/ml)-	
1	.0	.3	--	.1	--	.1
2	.0	.0	--	--	--	--
3	.0	.8	--	.2	--	.1
4	1.8	.8	.7	.6	.3	.2
5	1.0	.3	.4	.2	.2	.1
6	1.0	.0	.3	--	.1	--
7	.8	.8	.3	.2	.1	.1
8	.3	.5	.2	.4	.1	.1
9	.5	.8	.4	.2	.1	.1
10	.5	.8	.4	.2	.1	.1

[a]Values are the means of four animals.

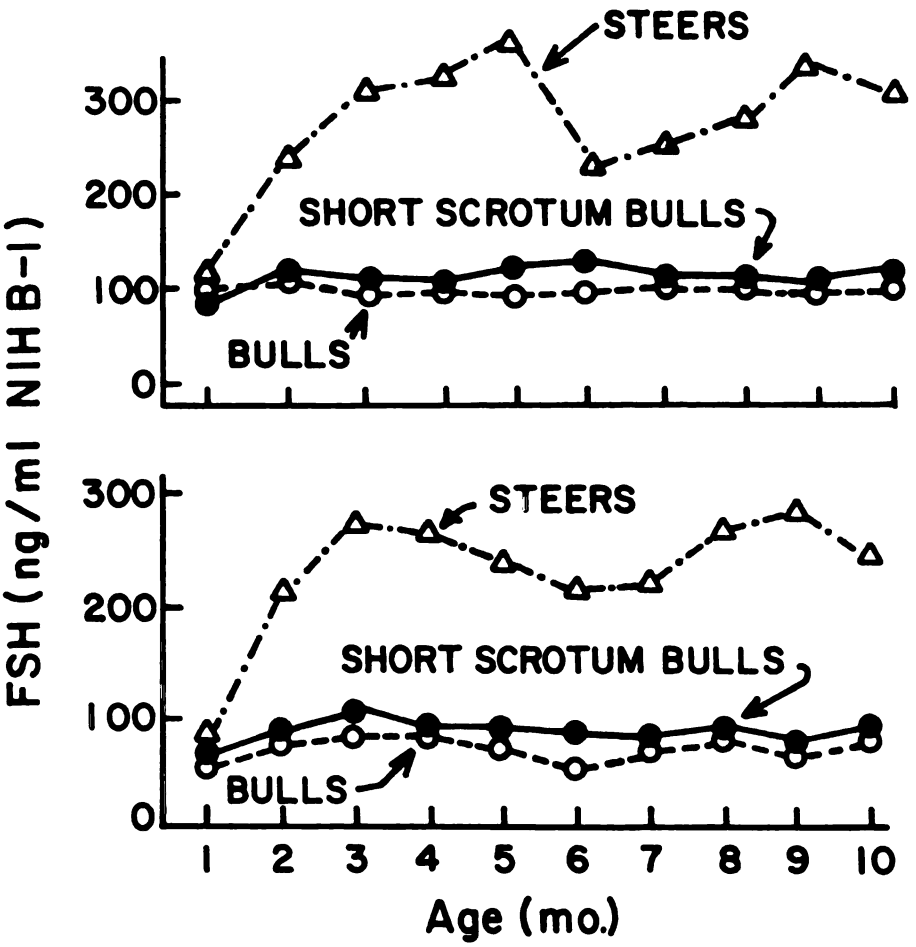
even at 2 months when these steroids were low, bulls had fewer LH peaks than steers.

Until 6 months, frequency of LH peaks in SS bulls resembled that in steers more closely than that in bulls, but after testosterone secretion commenced in SS bulls the LH pattern resembled that in bulls. Testosterone and androstenedione did not differ between bulls and SS bulls until 5 months, so some factor other than these steriods, lacking in young SS bulls must be involved in negative feedback on LH. Reduction in seminiferous tubular function in SS bulls could be the cause of greater frequency of LH peaks in this group. Inhibin or steroids produced by the tubules (estradiol or 5α-reduced androgens) might be involved (Dorrington and Armstrong, 1975; Dorrington and Fritz, 1975; Franchimont et al., 1977).

The increased frequency of LH peaks which occurred in all three treatment groups around 4 months of age occurred during months with the longest photoperiod. Changes in secretion of other pituitary hormones due to photoperiod and temperature have been reported in cattle (Peters and Tucker, 1978). Whether increased LH secretion at 4 months was due to seasonal hormone variation or age was unknown. This question was investigated in Experiment 3.

Both radioimmunoassays were used to estimate serum FSH concentrations (Figure 7). Absolute hormone concentrations varied slightly between assays, but this did not alter the physiological interpretation of the data. Data from each assay were analyzed separately by analysis of variance. Treatment and age effects were similar between assays. FSH increased ($P<.01$) in serum of steers from 1 to 2 months then did not change significantly through 10 months. Serum FSH did not change

Figure 7.--Serum FSH concentrations in bulls (O--O), steers (Δ--Δ) and short scrotum bulls (●--●) in two FSH radioimmunoassays; heterologous rat FSH assay (top) or homologous bovine FSH assay (bottom). Values are means of three samples collected at hourly intervals from four animals in each group.


STEERS
SHORT SCROTUM BULLS
BULLS
300
200
100
0
STEERS
SHORT SCROTUM BULLS
BULLS
300
200
100
0
1 2 3 4 5 6 7 8 9 10
Age (mo.)
FSH (ng/ml NIH B-1)

with age in normal or SS bulls. Steers had higher ($P<.05$) serum FSH concentrations than bulls or SS bulls from 2 to 10 months.

Serum samples collected at .5 hr-intervals for 8 hr from bulls, steers and SS bulls (two each at 3, 6 and 9 months) were assayed in the heterologous FSH assay to determine if episodic FSH peaks occurred. Episodic peaks were not observed, but FSH concentrations did fluctuate within an animal at a particular month (Table 7).

These results agree with Karg et al. (1976), who also observed no change in serum FSH concentrations during puberty. This does not mean that FSH plays no role during sexual maturation in bulls! Serum FSH increases before the onset of spermatogenesis in lambs and rats (Lee et al., 1976; Payne et al., 1977). Also, FSH sensitizes the testes to respond to LH with testosterone production in rats (Swerdloff and Odell, 1977). This effect may occur as a result of FSH stimulation of Sertoli cell secretions which affect the interstitium (Bartke et al., 1978). Constant serum FSH concentrations might accomplish these functions in bulls.

Serum FSH concentrations were greater in steers than in bulls or SS bulls by 2 months, demonstrating the importance of negative feedback on FSH during the first few months of life. Although the factors involved are not known, inhibin or steroids other than testosterone or androstenedione produced by the prepubertal testes could be important.

The short scrotum procedure stopped spermatogenesis, yet did not alter serum FSH levels by comparison with bulls. Only Sertoli cells and scattered spermatogonia remained in the tubules. Sertoli cells are a source of inhibin and estradiol, both of which are capable of reducing serum FSH concentrations (Dorrington and Fritz, 1975; Schanbacher and

Table 7. Serum FSH concentrations (ng/ml) in bulls, steers and bulls with shortened scrotums (SSbulls) at 3, 6 and 9 months of age.[a]

Age (Mo)	Parameter	Treatment					
		Bulls		Steers		SSbulls	
		567	570	573	579	569	571
3	$\bar{X} \pm SE$	110 ± 2.5	66 ± 2.6	274 ± 3.3	433 ± 4.7	121 ± 2.7	135 ± 3.3
	Range	94-124	45-85	231-290	402-470	100-138	116-157
6	$\bar{X} \pm SE$	112 ± 5.5	117 ± 3.4	299 ± 5.4	304 ± 1.3	113 ± 2.8	129 ± 1.8
	Range	54-145	93-131	245-357	296-315	91-129	114-143
9	$\bar{X} \pm SE$	110 ± 3.2	161 ± 1.8	348 ± 2.6	349 ± 4.6	130 ± 2.1	125 ± 2.3
	Range	81-132	150-171	327-364	291-367	118-148	109-142

[a]Serum FSH concentrations were determined with the heterologous FSH assay on blood samples taken at .5-hr intervals for 8 hr.

Ford, 1977; Steinberger and Steinberger, 1977). The short scrotum procedure may not have altered Sertoli cell function sufficiently to reduce production of these compounds, hence low serum FSH concentrations were maintained.

D. Experiment 3

1. Body and Testicular Growth

Body and testicular weight increased as a third degree polynomial with age (Table 8). The curves for body weight and testicular weight differed ($P<.001$), primarily due to slower testicular growth from 1 to 4 months and more rapid testicular growth from 5 to 7 months. The changing ratio of testicular weight to body weight reflected the greater relative increase in testicular growth from 5 to 7 months (Table 8).

Changes in testicular weight and body weight for these Holstein bulls agree with similar observations reported by Macmillan and Hafs (1968a). The rapid increase in testicular weight occurring after 4 months probably reflects increased seminiferous tubular diameter and proliferation of spermatogenic cell types (Macmillan and Hafs, 1968a) as well as Leydig cells hyperplasia and hypertrophy (Hooker, 1970).

2. Serum Hormone Concentrations

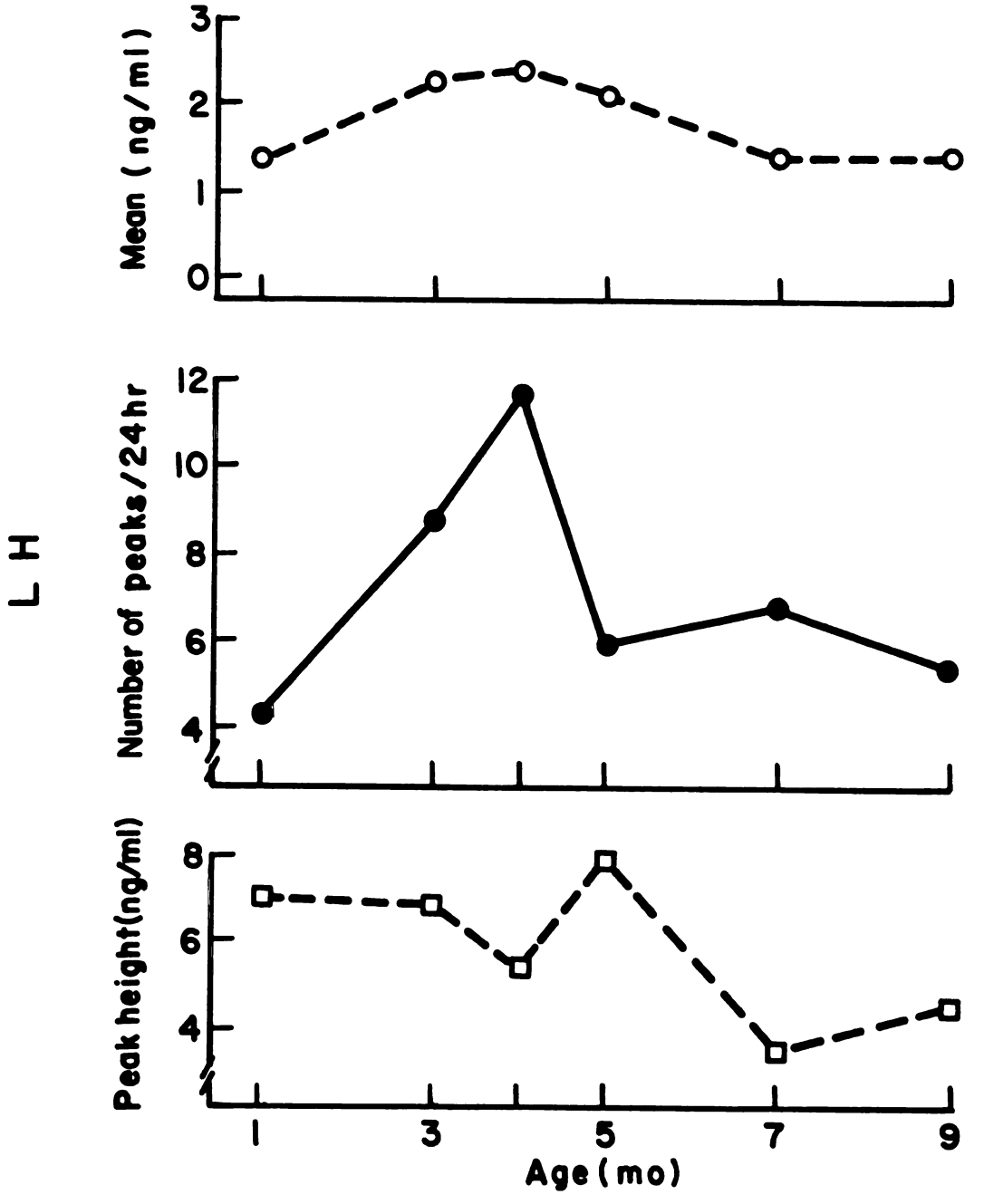
Mean LH concentration (Figure 8) increased ($P<.05$) from 1.4 ng/ml at 1 month to <2 ng/ml at 3, 4 and 5 months and then decreased to 1.4 ng/ml at 7 and 9 months. This increase in mean LH resulted from an increase ($P<.05$) in the number of LH peaks/24 hr (Figure 8), from 4.4 at 1 month to 11.8 at 4 months; then LH peak frequency decreased to 6.0 at 5 months. LH peak height (Figure 8) was relatively constant in 1-,

Table 8. Body and testicular weight in 1-, 3-, 4-, 5-, 7- and 9-month-old bulls.[a]

Age (Mo)	Body weight (kg)	Testicular weight (g)	Testicular weight: body weight ratio (g/kg)
1	55.4 ± 3.9	12.8 ± 1.2	.23
3	79.0 ± 3.8	25.4 ± 2.1	.32
4	131.5 ± 5.6	48.3 ± 2.5	.37
5	176.3 ± 6.5	96.5 ± 2.1	.55
7	236.6 ± 10.3	247.9 ± 9.1	1.05
9	268.5 ± 11.4	304.8 ± 16.0	1.14

[a]Values are the mean ± SE of five bulls per group.

Figure 8.--Serum LH averaged for 72 samples/bull (top), mean number of LH peaks/24 hr (middle) and mean LH peak height in five bulls at each age (bottom).


L H
Mean (ng / ml)
3
2
1
0
Number of peaks / 24 hr
12
10
8
6
4
Peak height (ng/ml)
8
6
4
1
3
5
7
9
Age (mo)

3-, 4- and 5-month-old bulls and then decreased ($P<.05$) from 5 months (7.6 ng/ml) to 7 months (3.5 ng/ml). Baseline concentrations (lowest point between peaks) varied from .8 ng/ml at 1 month to 1.3 ng/ml at 4 months, but did not differ significantly between bulls of different ages.

The increase in mean LH concentrations and frequency of peaks preceded increased testosterone production (Figure 9). Mean testosterone was <1 ng/ml at 1, 3 and 4 months and then increased markedly at 5, 7 and 9 months. Frequency and peak height (ng/ml) increased linearly ($P<.05$) from .4 and .9 at 1 month to 3.0 and 13.2 at 9 months, respectively (Figure 9). Baseline concentrations followed a similar pattern; a linear increase ($P<.05$) from 1 month (.2 ng/ml) to 9 months (2.8 ng/ml). Decreased LH concentrations, number of peaks and peak height from 4 to 7 months may have been due to increased testosterone secretion at this time.

Mean serum concentrations of androstenedione, frequency of peaks, height of peaks or baseline did not change significantly with age. However, there was a trend for an increase ($P\simeq.10$) in mean concentration at 4 months (Figure 9).

In contrast to LH, mean serum FSH (Figure 10) did not change with age, and remained between 99 and 120 ng/ml. In addition, FSH profiles within a bull did not have distinct episodic releases similar to those observed for LH. Only small changes in FSH were seen during an 8-hr period.

Mean prolactin concentrations and peak height increased ($P<.01$) from 1 to 5 months, while frequency increased ($P<.01$) from 1 to 4 months (Figure 10). Baseline prolactin concentrations increased ($P<.01$) from

Figure 9.--Androstenedione (0--0) and testosterone (●--●) concentrations averaged for 25 samples/bull (top); mean number of androstenedione and testosterone peaks/8 hr (middle); and mean androstenedione and testosterone peak height of five bulls at each age (bottom).

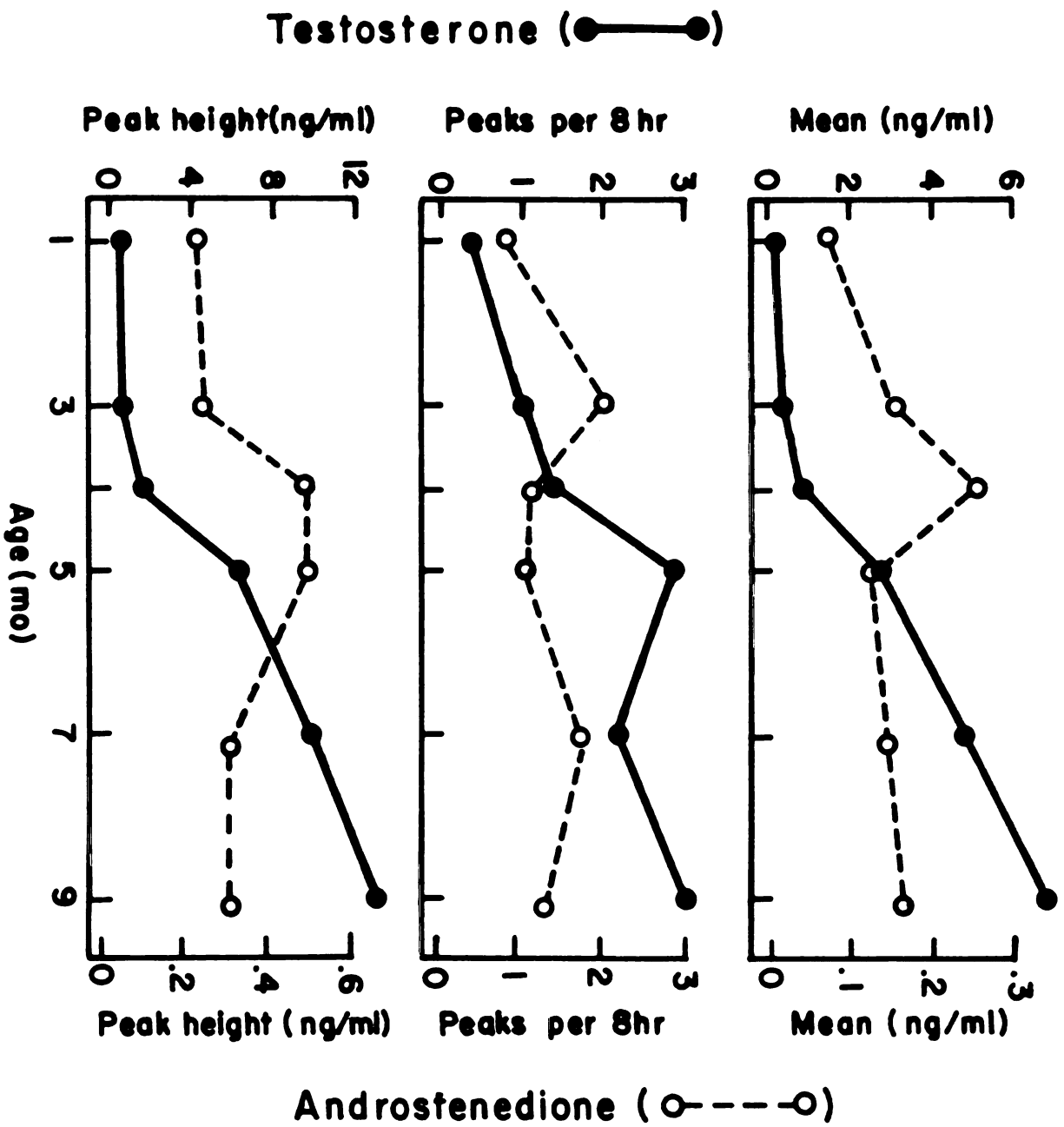
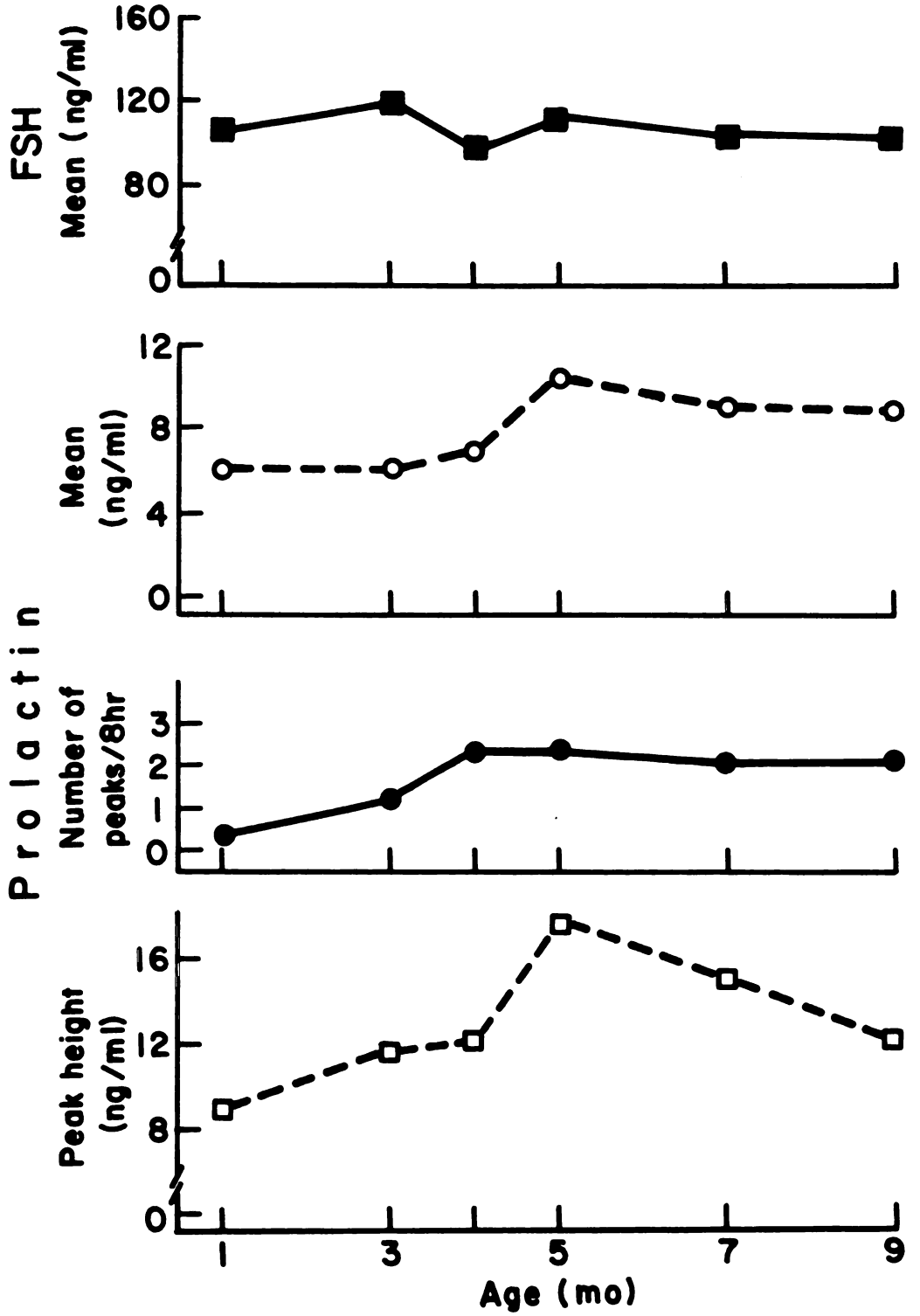

Testosterone (●——●)
Peak height(ng/ml)
Peaks per 8 hr
Mean (ng/ml)
0 4 8 12
0 1 2 3
0 2 4 6
Age (mo)
1
3
5
7
9
0 .2 .4 .6
0 1 2 3
0 .1 .2 .3
Peak height (ng/ml)
Peaks per 8hr
Mean (ng/ml)
Androstenedione (○- - -○)

Figure 10.--Serum FSH concentrations averaged for eight samples at hourly intervals in each of five bulls at each age (top). Prolactin concentration averaged for 25 samples/bull; mean number of prolactin peaks/8 hr; and mean prolactin peak height of five bulls at each age.


FSH
Mean (ng/ml)
160
120
80
0
Prolactin
Mean (ng/ml)
12
8
4
0
Number of peaks/8hr
3
2
1
0
Peak height (ng/ml)
16
12
8
0
1
3
5
7
9
Age (mo)

4.8 ng/ml at 1 month to 8.9 ng/ml at 5 months and then remained relatively constant.

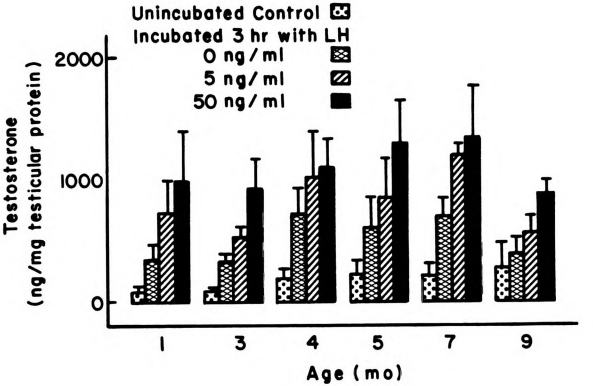
3. Testis Incubations

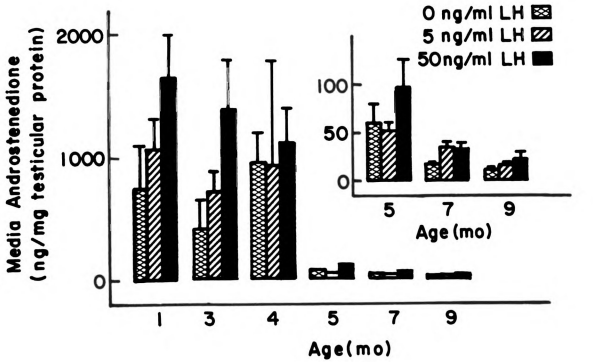
Testosterone concentration in unincubated control tissue increased linearly ($P<.05$) from 75 ng/mg at 1 month to 303 ng/mg testis protein at 9 months (Figure 11). Incubation of testes for 3 hr *in vitro* resulted in accumulation of testosterone (media plus tissue) greater ($P<.05$) than in unincubated control tissue at all ages. When all treatments were considered (0, 5, 50 ng/ml), tissue from 4-, 5- and 7-month-old bulls produced more ($P<.05$) testosterone during incubation than tissue from 1- and 3-month-old bulls. Both LH doses increased testosterone production above incubated controls ($P<.05$) at all ages, and testes from bulls of different ages responded similarly to LH.

LH treatment caused testicular tissue to release androstenedione into media ($P<.05$) when data were pooled over all ages, but large variation among animals prevented detection of significant LH responses within each age (Figure 11). However, androstenedione released into media when all treatments were considered was greater from testicular tissue from 1-, 3- and 4-month-old bulls than from 5-, 7- and 9-month-old bulls ($P<.05$).

Increased frequency of peaks and mean concentrations of LH at 4 months agrees with results from Experiment 2 and with results of Foster *et al.* (1978) with rams. These findings also support the contention that changes in LH peak frequency occur due to age, independent of photoperiod or seasonal changes.

Figure 11.--Testosterone from testicular tissue (tissue plus media) unincubated or incubated for 3 hr with 0, 5 and 50 ng LH/ml of media; five bulls at each age (top). Androstenedione release into media from testicular tissue incubated for 3 hr with 0, 5 or 50 ng LH/ml of media; five bulls at each age (bottom).


Unincubated Control
Incubated 3 hr with LH
0 ng/ml
5 ng/ml
50 ng/ml
Testosterone
(ng/mg testicular protein)
2000
1000
0
1
3
4
5
7
9
Age (mo)


0 ng/ml LH
5 ng/ml LH
50ng/ml LH
Media Androstenedione
(ng/mg testicular protein)
2000
1000
0
100
50
0
5
7
9
Age(mo)
1
3
4
5
7
9
Age(mo)

The mechanisms involved in increasing the frequency of LH peaks prior to puberty in bulls are not known. However, it seems unlikely that increased frequency is due to increased availability of LH. Bulls respond to GnRH treatment with equivalent LH release at 2, 4 and 6 months of age (Mongkonpunya et al., 1975b). In addition, pituitary LH content in bulls is at mature levels by 1 month (Macmillan and Hafs, 1968b). These results suggest that changes in LH release with age may be due to maturation of the hypothalamus, or involve alterations in sensitivity of the brain to negative feedback by gonadal factors.

LH peak height is reduced between 5 to 7 months, indicating less LH released per peak at 7 months than at earlier ages. This change occurred during the period that serum testosterone levels increased. Negative feedback of testosterone or other gonadal steroids may have decreased the rate of LH synthesis or possibly influenced GnRH synthesis or release.

Serum androstenedione was maximal at 4 months and decreased at 5 months, while serum testosterone increased significantly at 5 months and continuously to 9 months. Testosterone peaks following LH peaks increased in size especially between 4 and 5 months, and this increase is attributed to the transient increase in frequency of LH peaks. This outcome is identical to results from Experiment 2.

Decreased androstenedione production between 4 and 5 months in vivo and in vitro is indicative of increasing 17β-hydroxysteroid dehydrogenase activity. Payne et al. (1977) showed increasing activity of this enzyme in male rat testes between 22 and 50 days of age. They suggested that this enzyme is the major factor limiting testicular responsiveness to LH and that the activity of this enzyme may be increased by FSH.

The absence of markedly increased serum testosterone production *in vitro* in response to LH (Figure 11) at puberty does not agree with the marked increased in serum testosterone at 4 to 5 months of age *in vivo* to LH. Furthermore, equivalent testosterone production *in vitro* in response to LH at all ages is surprising. Evidently, a population of mature Leydig cells was present as early as 1 month. In contrast, Hooker *et al.* (1970) in histological studies found that competent secretory cells were not present in the interstitium until 4 months of age. While testicular tissue responds to LH with greatly increased testosterone production *in vitro* as early as 1 month, very little testosterone is released in response to LH *in vivo*. Possibly blood flow to the interstitium is inadequate before 4 to 5 months. Alternatively, inhibitory factors *in vivo* may have been removed *in vitro* or the increase in testicular to body weight ratio with age would give older bulls a greater relative amount of testicular tissue to respond to LH. On the other hand, testicular tissue from older bulls may not have an increased response to LH *in vitro* per mg testicular protein, because the relative volume of the testes occupied by seminiferous tubules increases during puberty (Macmillan and Hafs, 1968a), presumably reflecting a reduced proportion of interstitial tissue.

Serum FSH concentration did not change significantly with age and no distinct episodic FSH peaks were observed. These observations are consistent between Experiments 2 and 3 and with results of Karg *et al.* (1976). Failure to observe changes in FSH is difficult to explain, particularly in view of the known role of FSH during puberty in other species (reviewed by Bartke *et al.*, 1978) and the increase in serum FSH that occurs in male rats prior to initiation of spermatogenesis

(Swerdloff et al., 1971; Dohler and Wuttke, 1975; Payne et al., 1977). Aside from the possibility that FSH is of little importance in bulls, perhaps FSH secretion need not change to affect spermatogenesis or possibly changes in FSH were not detected in this study. These data support the view that constant FSH levels in the presence of changes in other pituitary hormone concentrations may be sufficient to initiate testicular function.

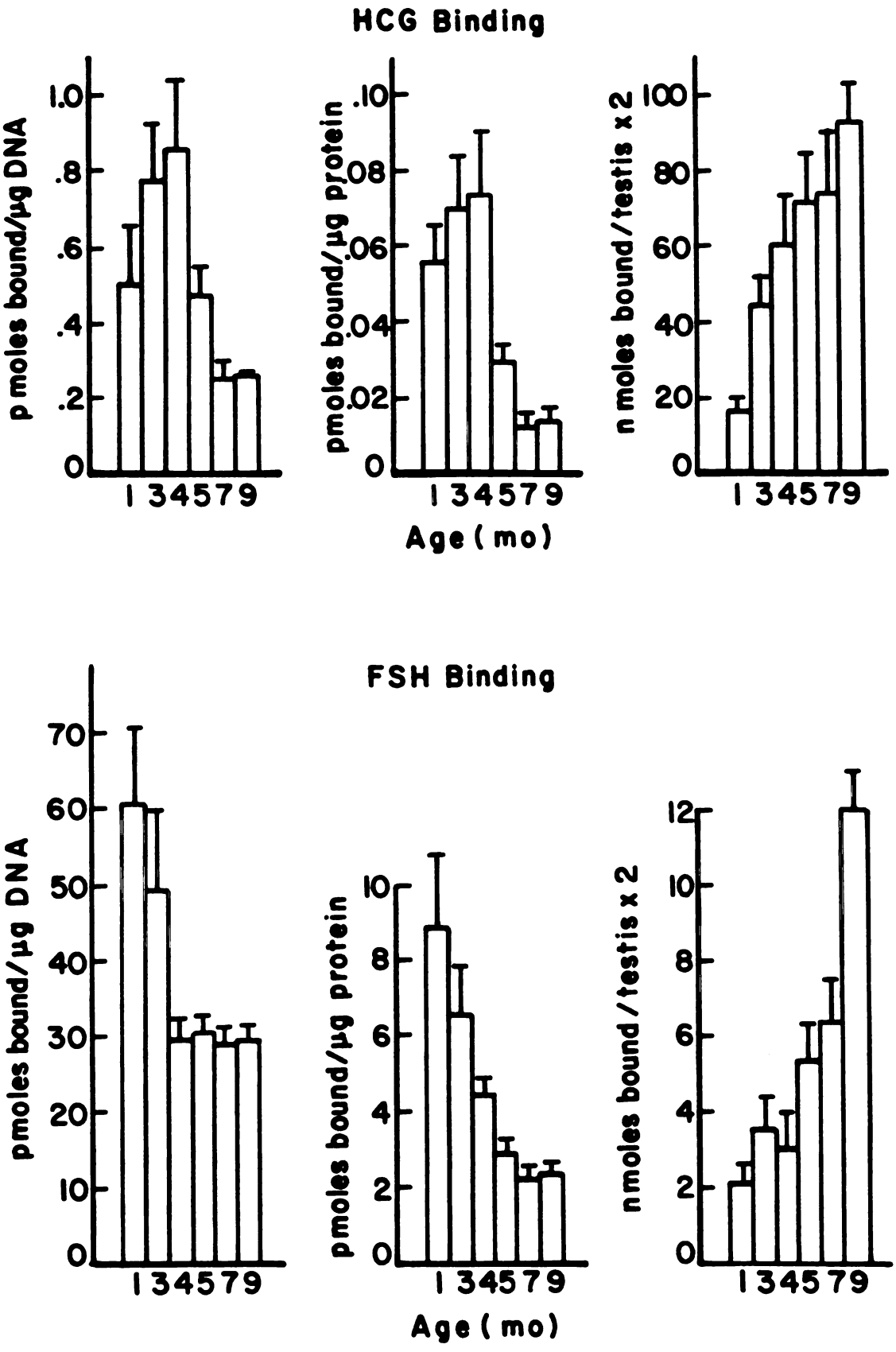
Schams and Reinhardt (1974) reported no change in serum prolactin with advancing age in bulls and heifers. In contrast, Leining et al. (1976) and Davis et al. (1977) reported greater prolactin in serum of heifers at 3 than at 1 month of age. In this study mean prolactin, peak frequency and peak height increased at 4 to 5 months of age. These changes resembled those for LH, but the function of increased prolactin at this age is unknown. It may participate in increased steroidogenesis as described by Bartke et al. (1978) for rodents. However, increased prolactin may be a result of increased testicular androgen production (Nolin et al., 1977).

Increased serum testosterone concentrations and decreased androstenedione at 5 months in bulls may result from more frequent LH peaks at 4 months. In addition, constant FSH levels and increasing prolactin may aid in increasing testicular sensitivity to LH as suggested in other species (Bartke et al., 1978; Swerdloff et al., 1977).

4. Testicular Gonadotropin Binding

^{125}I-HCG binding to testicular homogenates per µg DNA was greater ($P<.05$) in tissue from 3- and 4-month-old than from 7- and 9-month-old bulls (Figure 12). HCG binding per µg protein was greater at 1, 3 and

Figure 12.--Specific binding of ^{125}I-HCG (top) and ^{125}I-FSH (bottom) to testicular tissue. Values are the means of triplicate determinations of five animals at each age.


HCG Binding
p moles bound/µg DNA
1.0
.8
.6
.4
.2
0
1 3 4 5 7 9
pmoles bound/µg protein
.10
.08
.06
.04
.02
0
1 3 4 5 7 9
n moles bound/testis x 2
100
80
60
40
20
0
1 3 4 5 7 9
Age (mo)
FSH Binding
pmoles bound/µg DNA
70
60
50
40
30
20
10
0
1 3 4 5 7 9
pmoles bound/µg protein
10
8
6
4
2
0
1 3 4 5 7 9
n moles bound/testis x 2
12
10
8
6
4
2
0
1 3 4 5 7 9
Age (mo)

4 months than at 5, 7 and 9 months. These data are difficult to interpret because testes contain a heterogeneous cell population and LH binds specifically to Leydig cells (DeKretser et al., 1971; Castro et al., 1972). Thus changes in number and size of other cell types could alter LH binding per μg DNA or protein in the absence of a change in the number of LH binding sites per Leydig cell. The decreased binding capacity of testicular tissue after puberty was probably due to increased seminiferous tubular diameter and proliferation of germ cells that occur after 5 months in bulls (Macmillan, 1967), causing Leydig cells to represent a smaller percent of the total cell population. Total ^{125}I-HCG bound per testis x 2 increased with age and was greater ($P<.05$) in 9-month-old bulls than in 1-month-old bulls (Figure 12). The increase in total LH (HCG) binding sites per bull could be due to more receptor sites per cell or an increase in the number of Leydig cells. Hooker (1970) found an increase in the number and size of Leydig cells in bulls during puberty. Thus, it is possible that both a greater number of cells and an increased number of sites per cell could contribute to increased ^{125}I-HCG binding/testis in older bulls. In contrast to these results, several studies using rat testicular tissue found that tissue from rats at 50 days or older bound more ^{125}I-HCG or LH (per mg testis) than tissue from 10-, 20- and 30-day-old rats (Frowein and Engel, 1975); Thanki and Steinberger, 1976). The reason for the discrepancy between changes in bovine and rat ^{125}I-HCG binding with age is not understood.

Although these results do not clearly define changes in receptor numbers per Leydig cell with advancing age, they do suggest that absence of LH receptors is probably not the cause of low testicular response to endogenous LH episodes in vivo in prepubertal bulls.

^{125}I-FSH binding to testicular homogenates was greater in 1- and 3-month-old bulls than 4-, 5-, 7- and 9-month-old bulls ($P<.05$) when expressed on a per μg DNA or protein basis (Figure 12). FSH binds specifically to Sertoli cells (Castro et al., 1972; Means and Vaitukaitis, 1972; Steinberger et al., 1974), but loss of binding per μg of DNA or protein during puberty did not result from a loss of Sertoli cells or binding sites per Sertoli cell, but an increase in the other testicular cellular components during puberty. Total binding per testis x 2 was constant for months 1, 3 and 4, and then increased to 9 months ($P<.05$). Since Sertoli cell populations remain stable and do not undergo mitotic divisions after 2 weeks of age in rats (Steinberger and Steinberger, 1977), I suspect that increased ^{125}I-FSH binding per testis is due to increased binding per Sertoli cell. The relatively high concentration of FSH binding sites in testicular homogenates from bulls before puberty suggests that the ability of testicular tissue to bind FSH is not a limiting factor to the initiation of spermatogenesis.

Affinity constants for binding of ^{125}I-HCG and ^{125}I-FSH to bovine testicular tissue are in agreement with those found previously using rat tissue (Table 9). Catt et al. (1972) and Means and Vaitukaitis (1972) found affinities of $2.4 \times 10^{10} M^{-1}$ and $.14 \times 10^{9} M^{-1}$ for HCG and FSH, respectively. I found no instances where affinities have been shown to change with age or physiological state. However, there is a trend in these data for 7-month-old bull testes to have a higher affinity for HCG and 1-month-old bulls to have a lower affinity for FSH. A sufficient number of animals was not examined to estimate variability within an age group. This question merits further investigation

Table 9. Affinity constants (K_A) for ^{125}I-HCG and ^{125}I-FSH binding to bovine testicular homogenates.

Age (Mo)	Animal (ID)	$K_A \times 10^9 M^{-1}$	
		HCG	FSH
1	6091	9.9	1.1
	6092	--	0.7
	6104	--	0.4
3	6046	8.3	3.3
4	6048	10.3	2.4
5	783	11.0	2.7
7	766	36.6	3.2
	762	21.1	--

because this could be a mechanism whereby equivalent serum hormone concentrations in bulls of different ages could elicit different responses.

GENERAL DISCUSSION

The major hormonal change observed in these studies in bulls during maturation was the pronounced increase in basal serum testosterone concentrations and in peak testosterone concentrations following endogenous LH peaks. Increased androgen production is unquestionably the most important factor in sexual maturation, and it is involved in spermatogenesis, sex accessory gland function and secondary sex characteristics (Steinberger, 1971). Episodic LH peaks were present in 1-month-old bulls, but they did not elicit increases in testosterone secretion. By 4 months episodic LH peaks were followed by androstenedione peaks, but by 5 months the number and size of androstenedione peaks was reduced. Testosterone peaks followed LH episodes at 5 months and these peaks increased in size through 10 months of age. The hormonal requirements for increased androgen production are not known, but increased frequency of LH peaks at 4 months could stimulate differentiation of precursor cells into Leydig cells (Chemes et al., 1976) and maturation of the steroidogenic capacity of Leydig cells (Dufau et al., 1976). Increased frequency of LH peaks before puberty could be the major trigger to the onset of puberty.

LH is released in distinct episodes in bulls but the physiological importance of this release pattern is unknown. Episodic release may be biologically the most efficient way to deliver serum hormone to a target organ. Temporarily elevated hormone level would load testicular LH

receptors and prolonged half-life of LH on receptors (Mendelson et al., 1975) would provide constant hormonal stimulation long after serum hormone levels had declined. In addition, episodic release is possibly the best method to overcome "testicular desensitization" (Hsueh et al., 1977) in which testicular response to LH is reduced immediately following LH exposure.

The mechanism responsible for increased frequency of LH peaks is not known. It has been suggested that the increase in serum gonadotropin concentrations that occurs in male rats before puberty is due to maturation of the hypothalamus and decreased sensitivity of the hypothalamus to circulating steroids (Ramirez and McCann, 1965). Increased frequency of LH peaks before puberty in bulls could result from a similar mechanism, but the fact that bulls, steers and SS bulls all had increased LH peak frequency at 4 months irrespective of circulating steroid concentrations suggests maturation of the hypothalamus and increased LH production is independent of alterations in steroid negative feedback.

The limited responsiveness of the testis to LH in vivo before puberty seems not due to a lack of LH binding sites. Although older bulls had a greater LH binding capacity/testis; 1-, 3- and 4-month-old bulls had a higher concentration of binding sites. Also, testes from 1-month-old bulls responded in vitro to LH with elevated testosterone production, indicating that adequate numbers of functional LH binding sites and some mature Leydig cells were present.

By contrast to the in vitro testicular response to LH, in vivo endogenous LH peaks are followed by testosterone release in bulls only after 4 months of age. In vitro, testes responded to LH at all ages.

Reasons for this discrepancy are unknown. Testicular vasculature early in life in bulls may limit access of gonadotropins and other substances to the interstitium, or Leydig cell products may not have free access to peripheral circulation. In addition, testicular substances (e.g., estrogens) may inhibit production of testosterone in response to LH in vivo, but these substances may be diluted in vitro to allow testosterone secretion. A greater volume of testicular tissue is present relative to body size in older bulls. More steriodogenic tissue is available to respond to LH and could result in greater elevation of peripheral testosterone concentrations. In vitro results indicate that so far as testosterone secretion is concerned, a mature population of Leydig cells is present as early as 1 month of age in bulls.

Serum FSH concentrations did not differ between bulls of different ages. If FSH is involved in the induction of spermatogenesis as in other species, constant serum concentrations of FSH probably perform this function in bulls. Possibly a change in the production of other compounds (i.e., steroids or pituitary hormones) with age causes constant serum FSH concentrations to become stimulatory to Sertoli cells and spermatogenesis. Unlike LH, FSH was not released episodically in bulls, steers or SS bulls. Different negative feedback on FSH than LH secretion may partially account for this difference. Also, a longer half-life for FSH in serum (Van Hall et al., 1971) might make FSH episodes more difficult to detect.

Serum FSH concentrations were greater in steers than in bulls or SS bulls. Lack of elevated FSH concentrations in SS bulls was surprising, especially since histological preparations indicated that spermatogenesis was halted. The only cell type abundant in the

seminiferous tubules was the Sertoli cell. This suggests that Sertoli cells are a major source of inhibitory compounds that influence FSH secretion. This agrees with work by Steinberger and Steinberger (1977) who demonstrated that media from Sertoli cell cultures inhibited FSH release when placed in pituitary cell cultures.

To establish the function of pituitary hormones other than LH and FSH in the onset of puberty will require further study. Growth hormones and prolactin concentrations change during puberty in bulls and roles for these hormones in testicular function have been demonstrated in other species. A complex of pituitary hormones acting concurrently or sequentially may be required to initiate full steroidogenesis and spermatogenesis.

SUMMARY AND CONCLUSIONS

The objective of these experiments were to describe serum hormone changes in bulls from birth until after puberty; to examine negative feedback relationships between the testis and hypothalamus/pituitary using bulls, steers and bulls in which the scrotum had been shortened (SS bulls); and to examine testicular changes during the peripubertal period.

The first experiment was designed to determine serum hormone concentrations and the importance of negative feedback in prepubertal and pubertal bulls. Eight prepubertal and eight pubertal bulls were assigned four each to be left intact and four to be castrated 2 weeks before the experiment. Serum growth hormone, prolactin, testosterone and luteinizing hormone concentrations were determined from a 9-hr sampling period. Steers had higher mean serum LH concentrations and a greater frequency of LH peaks per 9 hr ($P<.05$) than bulls, but serum LH did not differ between ages. The frequency of testosterone peaks and mean testosterone concentrations were greater in pubertal than prepubertal bulls ($P\simeq.10$). Prolactin concentrations did not differ between ages or castrational state, but prolactin in older animals tended to be higher and more variable. Prepubertal bulls had greater mean serum growth hormone concentrations and a greater frequency of growth hormone peaks ($P<.07$) than pubertal bulls, steers and prepubertal steers.

The second experiment was designed to describe hormone patterns during development in normal bulls, bulls with altered negative feedback (SS bulls) and bulls with no negative feedback (steers). Two-week-old bulls were assigned four each to be bulls, steers or SS bulls. Serum samples were collected during a 24-hr sampling period, monthly for 10 months, and were assayed for LH, FSH, testosterone and androstenedione. Bulls, steers and SS bulls grew at similar rates. Bull reached puberty by 10 months; indicated by the presence of spermatozoa in epididymidal homogenates and active spermatogenesis apparent histologically in the seminiferous tubules. SS bulls had no spermatozoa in the epididymides, and only Sertoli cells and scattered spermatogonia were seen in the seminiferous tubules.

Mean LH concentrations were greater in steers than bulls or SS bulls from 3 to 10 months, while SS bulls had greater LH concentrations than bulls at 5 and 6 months ($P<.05$). In steers and SS bulls, average LH concentrations increased from 1 to 3 months ($P<.05$) and then remained constant. In bulls, LH was greater at 4 months than at 1 month, but not different among other months. Frequency of LH peaks increased from 1 month to 4 months in bulls, steers and SS bulls and then declined in bulls and SS bulls. Steers had a greater frequency of LH peaks than bulls from 2 to 10 months, while SS bulls had a greater frequency of LH episodic peaks than bulls at 3 and 4 months.

Serum testosterone concentrations were less than 1 ng/ml in bulls from 1 to 4 months and increased ($P<.05$) abruptly at 5 months. In SS bulls, testosterone increased from 5 to 6 months, later than in bulls. Increased frequency and height of testosterone peaks and increased baseline concentrations each contributed to the increase in mean

testosterone concentrations. Small testosterone peaks (<1 ng/ml) followed episodic LH peaks in bulls from 1 to 3 months and SS bulls from 1 to 5 months, but after this age the amplitude of testosterone peaks increased to 2 ng/ml and continued to increase to 5 ng/ml by 10 months. Average androstenedione concentrations did not differ between bulls and SS bulls; it increased transiently at 4 months in both treatments.

Serum FSH concentrations increased from 1 to 2 months in steers, did not change through 10 months and were greater in steers than in bulls or SS bulls from 2 to 10 months. Serum FSH did not change with age in normal of SS bulls, and did not differ between these two treatments at any month.

I conclude from Experiment 2 that increased mean LH concentrations and frequency of LH episodic peaks at 4 months probably stimulated the increase in testosterone secretion that occurred by 5 months. Slightly greater LH concentrations in SS bulls during puberty indicates reduced negative feedback from SS bull testes. In addition, comparable FSH concentrations in bulls and SS bulls suggests that Sertoli cells are the major source of compounds that feedback on FSH secretion.

The major objective of Experiment 3 was to examine serum hormone changes due to age, independent of season and photoperiod. A second objective was to examine in vitro changes in testicular response to LH and in vitro gonadotropin binding in bulls of different ages. Blood samples were collected from five bulls each at 1, 3, 4, 5, 7 and 9 months of age for a 24-hr period on a single day. On the following day testes were removed for in vitro studies.

Serum LH concentrations increased from 1.4 ng/ml in 1-month-old bulls to <2 ng/ml at 3, 4 and 5 months and then decreased to 1.4 ng/ml

at 7 and 9 months. The frequency of LH peaks increased from 4.4 at 1 month to 11.8 at 4 months and then decreased to 6 months. LH peak height was constant through 5 months, and then decreased to 7 months. Increased LH secretion preceded increased testosterone production by 1 month. Testosterone was <1 ng/ml at 1, 3 and 4 months and then increased markedly at 5, 7 and 9 months. Frequency and peak height (ng/ml) increased linearly from .4 and .9 at 1 month to 3.0 and 13.2 at 9 months, respectively. I suggest that a major factor responsible for turning on testosterone production at 5 months is the increased frequency of LH episodic peaks in bulls at 4 months of age.

Androstenedione tended to be higher in 4-month-old bulls. FSH did not change with age and did not have distinct episodic releases similar to those observed for LH. The absence of elevated serum FSH concentrations before puberty is surprising. Changing FSH concentrations appear not to be a requirement for the onset of puberty in bulls. Mean prolactin and prolactin peak height increased from 1 to 5 months while peak frequency increased from 1 to 4 months. Increased prolactin concentrations around puberty may stimulate steroidogenesis, or could be stimulated by rising androgen concentrations.

Testosterone concentration in testicular tissue increased linearly in bulls from 1 to 9 months. Incubation <u>in vitro</u> caused a significant increase in testosterone production. Over all treatments, 4-, 5- and 7-month-old bulls produced more testosterone than 1- and 3-month-old bulls. LH caused equivalent testosterone production from testicular tissue of bulls of all ages. LH caused androstenedione release into media, but tissue from 1-,3- and 4-month-old bulls produced significantly more androstenedione than tissue from 5-, 7- and 9-month-old bulls.

Similar *in vitro* response to LH in testicular tissue from bulls at all ages was unexpected and differs with *in vivo* findings where only minimal amounts of testosterone are produced in response to endogenous LH episodic peaks before 5 months of age. *In vitro* results suggest however, that mature, responsive Leydig cells are present in bulls as early as 1 month of age. Decreased androstenedione production *in vivo* and *in vitro* between 4 and 5 months suggests maturation of 17β-hydroxysteroid dehydrogenase system.

^{125}I-HCG binding to testes homogenates per µg DNA was greater in tissue from 3- and 4-month-old bulls than from 7- and 9-month-old bulls. Binding per µg protein was greater at 1, 3 and 4 than at 5, 7 and 9 months. Total HCG bound per testis increased from 1 to 9 months. FSH binding was greater in 1- and 3-month-old bulls than in 4-, 5-, 7- and 9-month-old bulls when expressed on a per µg DNA or protein basis. Total FSH binding per testis was constant at 1, 3 and 4 months, and then increased to 9 months.

Gonadotropin binding studies demonstrate increased binding per testis in older bulls, but higher concentration of sites in younger bulls. I conclude that limited testicular responsiveness to gonadotropins *in vivo* is not due to absence of binding sites. Binding studies are difficult to interpret without being able to equate changes in binding with changes in a specific cell population (i.e., Leydig cells).

Results of these experiments prove that the major hormonal change during puberty is increased testosterone production. Increased androstenedione at 4 months and a decrease at 5 months indicates that maturation of testicular steroidogenic enzymes takes place to favor testosterone production. I conclude that increased frequency of LH

peaks at 4 months probably induces testicular maturation and increases testosterone production by 5 months. Other pituitary hormones may participate but the role of FSH is difficult to assess because the concentration in serum does not change during puberty.

FSH and LH concentrations were greater in steers, demonstrating the importance of negative feedback of testicular products on gonadotropin secretion as early as 2 months of age. LH in SS bulls was only slightly greater than that in normal bulls showing a change in negative feedback in SS bulls. FSH concentrations in SS bulls were not different than bulls, suggesting that Sertoli cells may produce inhibin in bulls.

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