

# **TESTICULAR DEVELOPMENT IN BULLS**

A thesis submitted to the college of graduate studies and research, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the

Department of Veterinary Biomedical Sciences,

University of Saskatchewan,

Saskatoon, Saskatchewan, Canada

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## ABSTRACT

In the present study our objectives were (1) to follow the temporal patterns of testicular LH and FSH receptor (LH-R and FSH-R) concentrations and affinity ( $K_a$ ) during sexual maturation in bulls, to see if such patterns could explain the control of rapid testicular growth that occurs after 25 weeks of age, when serum gonadotropin concentrations are low; (2) to see if transformation growth factors (TGF- alpha and beta 1, 2 and 3) and interleukins (IL-1 and IL-6) are produced in the developing bovine testis and if their concentrations change during development; (3) to see if the onset of puberty could be hastened by treating bull calves subcutaneously (sc) with 3 mg of bLH (n=6) or 4 mg of bFSH (n=6) once every 2 days, from 4 to 8 weeks after birth.

Mean LH-R concentrations decreased from 13 to 25 weeks of age and increased to 56 weeks of age ( $P<0.05$ ). LH-R $K_a$  decreased from 9 to 17 weeks of age, increased to 29 weeks and declined to 33 weeks of age ( $P<0.05$ ). FSH-R concentrations declined from 17 to 25 weeks of age then increased to 56 weeks of age ( $P<0.05$ ). FSH-R $K_a$  increased from 17 to 25 weeks of age ( $P<0.05$ ). Testicular TGF-alpha concentrations increased from 13 to 17 weeks of age, decreased to 21 weeks and from 33 to 56 weeks of age ( $P<0.05$ ). Testicular TGF-beta 1 concentrations decreased from 17 to 21 weeks of age, increased to 25 weeks and decreased from 25 to 29 weeks of age ( $P<0.05$ ). Testicular TGF-beta 2 concentrations increased from 5 to 17 weeks of age, decreased to 21 weeks, increased to 25 weeks and decreased at 29 weeks of age ( $P<0.05$ ). Testicular TGF-beta 3 concentrations increased from 13 to 17 weeks of age, decreased to 21 weeks of age and from 25 to 29 weeks of age ( $P<0.05$ ). Mean testicular IL-1 alpha concentrations decreased from 5 to 9 weeks of age and 13 to 21 weeks of age ( $P<0.01$ ) while mean testicular IL-1 beta concentrations decreased from 13 to 17 weeks and 29 to 33 weeks of age ( $P<0.01$ ). Mean IL-1 bioactivity increased from 13 to 17 weeks of age, decreased to 21 weeks, increased to 25 weeks, decreased to 29 weeks and decreased from 33 to 56 weeks of age ( $P<0.05$ ). Mean testicular IL-6 concentrations decreased ( $P<0.05$ ) from 9 to 13 weeks of age, increased ( $P<0.05$ ) to 21 weeks, decreased ( $P<0.05$ ) to 25 weeks of age, increased ( $P<0.05$ ) to 29 weeks and decreased ( $P<0.01$ ) to 56 weeks of age.

We concluded that high concentrations of gonadotropin receptors might be critical to initiate postnatal testis growth and support it after 25 weeks of age in the face of low serum gonadotropin concentrations. Testicular TGF-alpha concentrations were higher in calves than adults while concentrations of TGF-beta and IL-1 were higher in the early postnatal period than the peripubertal period. The changes in testicular concentrations of TGFs and ILs led us to suggest a possible local regulatory role in development. Testicular IL-6 concentrations were higher in prepubertal calves than adults. Treatment of bull calves with bFSH from 4 to 8 weeks of age increased testicular growth (SC), hastened the onset of puberty (SC  $\geq$  28 cm), and enhanced spermatogenesis.

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## *Dedicated to...*

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*For their support through out my education and inculcating the sense of responsibility  
and independence*

### **My Brothers and sisters...**

*For their love and support*

### **My Teachers and mentors...**

*For the arduous task of imparting knowledge in an often disillusioned student*

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## LIST OF COMMON ABBREVIATIONS

AMH	Anti mulerian hormone
T3	Triiodothyronine
ANOVA	Analysis of variance
bINH	Bovine inhibin
CV	Coefficient of variation
FSH	Follicle stimulating hormone
eCG	Equine chorionic gonadotropin
FSH-R	Follicle stimulating hormone receptor
FSH-RKa	Follicle stimulating hormone receptor Affinity
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
IGF	Insulin like growth factor
IL	Interleukin
Kg	Kilograms
LH	Luteinising hormone
LH-R	Luteinising hormone receptor
LHRH	Luteinising hormone releasing hormone
LH-RKa	Luteinising hormone receptor affinity
ng	Nano grams
P	Probability
pg	Pico grams
SC	Scrotal circumference
SEM	Standard error of mean
TGF	Transformation growth factor

## Chapter 1: GENERAL INTRODUCTION

Reproductive development is controlled by the interactions between the testes, pituitary, hypothalamus and higher centers (Macmillan & Hafs 1969; Swanson *et al.* 1971; Pelletier *et al.* 1981; Wilson *et al.* 1981; Garner & Hafez 2000). The higher brain centres release neurochemicals that signal the hypothalamus to release GnRH (Barraclough & Wise 1982; Barraclough *et al.* 1984). GnRH is then transported via portal vessels to the pituitary where it acts on gonadotrophs to stimulate the release of the gonadotropins, luteinising hormone (LH) and Follicle Stimulating Hormone (FSH) (Lacroix & Pelletier 1979a; Thibault *et al.* 1993). In males, LH acts on the Leydig cells (interstitial cells) leading to the production of testosterone (Amann & Ganjam 1976; Amann *et al.* 1978; Schams *et al.* 1978; Sharpe 1990) while FSH acts on Sertoli cells regulating spermatogenesis and the production of proteins such as androgen binding protein (ABP) and inhibin (Chemes *et al.* 1979a, b; Steinberger 1981; Amann & Schanbacher 1983; Miyamoto *et al.* 1989) and carbohydrates such as lactate (Mullaney *et al.* 1994). Testosterone, produced in interstitial cells, crosses into the seminiferous tubule and binds to ABP and within the seminiferous tubules it hastens the development of Sertoli cells (Amann 1983) and enhances spermiogenesis (Wilson *et al.* 1981; Sharpe 1990). Recent reports in mice, rats and pigs indicate gonadotropins and testosterone act on the Leydig and Sertoli cells initiating and/or regulating the production of intratesticular factors such as interleukins (Khan *et al.* 1987; Syed *et al.* 1993) and



growth factors (Avallet *et al.* 1987; Morera *et al.* 1988; Sordoillet *et al.* 1992). The intratesticular factors released then modify the fine details of testicular somatic and germ cell development. In this way, endocrine stimuli in conjunction with cytokines and growth factors are responsible for the development of the testis; hence, the achievement of puberty.

Puberty is defined as the age at which the bull is capable of producing a natural fertile ejaculate. Puberty is often assessed by non-invasive techniques that involve monitoring testicular development such as measuring scrotal circumference (Car & Land 1975; Coulter & Foote 1977; Lunstra *et al.* 1978; Braun *et al.* 1980) and ultrasonographic imaging of the testis (Cartee *et al.* 1986, 1989; Chandolia *et al.* 1997a; Aravindakshan *et al.* 2000b). The invasive techniques of evaluating reproductive development include qualitative and quantitative evaluation of secretory products from reproductive organs, such as semen and tissue biopsies collected from reproductive organs, (for example testicular tissue for histological examination; Chowdhury & Steinberger 1975; Berndtson 1977; Curtis & Amann 1981; Thibault *et al.* 1993). The latter involves the qualitative and quantitative evaluation of changes in somatic and germ cells. Puberty in bulls has been defined as the age at which an ejaculate contains at least 50 million spermatozoa with a 10% linear motility (Wolf *et al.* 1965). Lunstra *et al.* (1978) showed that in bull from six different breeds such an ejaculate was attained at a scrotal circumference (SC) of  $27.9 \pm 0.2$  cm. Age at puberty varies with breed and in Hereford x Charolais bull calves, puberty is attained at about 45 week of age (Evans *et al.* 1993,

1995; Rawlings & Evans 1995; Chandolia *et al.* 1997a,c; Aravindakshan *et al.* 2000a,b).

In bull calves, puberty is preceded by a transient increase in mean serum concentrations of LH and FSH, referred to as the early rise in LH and FSH secretion (Rawlings *et al.* 1978; Amann *et al.* 1986; Wise *et al.* 1987; Rodriguez & Wise 1989; Evans *et al.* 1993, 1995; Rawlings & Evans 1995). This early rise occurs between 5 and 25 weeks of age and is followed by a period when mean serum concentrations of LH and FSH are low but variable (Evans *et al.* 1993,1995,1996; Rawlings & Evans 1995; Chandolia *et al.* 1997a, c). The period between 25 weeks of age and puberty (SC  $\geq$  28 cm; Lunstra *et al.* 1978) is characterized by rapid testicular growth (Evans *et al.* 1993, 1995; Rawlings & Evans 1995; Chandolia *et al.* 1997c). Rapid testicular growth is a reflection of intratesticular changes in somatic and germ cells (Hooker 1970; McCarthy *et al.* 1979a, b; Curtis & Amann 1981; Amann 1983; Chandolia *et al.* 1997a; Aravindakshan *et al.* 2000b). Gonadotrophic hormones (LH and FSH) are purported to be critical for testicular development; however, it is ironic that rapid testicular growth occurs during the period when the mean circulating concentrations of gonadotropins are low (Evans *et al.* 1993, 1996; Rawlings & Evans 1995). It is unclear whether the transient early postnatal increase in mean serum gonadotropin concentrations is sufficient to trigger rapid testicular growth or how important the subsequent low concentrations of gonadotropins are for this rapid growth. Perhaps other testicular regulators are involved.

In beef cattle the rate of genetic progress is dependent on the the time it takes for the male to reach sexual maturity and the fertility of the male. Shortening the generation interval is therefore dependent on the selection of animals that will reach puberty at an early age or hastening the onset of puberty. Selecting the early sexually maturing bulls in addition to culling the slow maturing animals, at a young age optimizes economic benefits to farmers. In beef cattle the delay in attainment of puberty results in to the loss in critical breeding time. The period lost could be close to a year since cattle are bred during a fixed period of the year. It is therefore desirable to identify superior sires early in life and obtain semen from them at the earliest possible age. In beef bulls, often the faster sexually maturing bulls are more fertile than their slow maturing ones.

The literature review for this Ph.D thesis will focus on reproductive development in bull calves with particular emphasis on the following: the ontogeny of serum gonadotropin and testosterone concentrations; the control of the early rise in gonadotropin secretion; testicular somatic and germ cell development; endocrine and paracrine regulation of testicular development; and the evaluation of sexual maturity in bulls using the measurement of scrotal circumference and testicular histological examination. Review papers and the most important research papers are referred to.

## Chapter 2: LITERATURE REVIEW

### 2.1. PUBERTY

Sexual maturation is a slow process that is largely regulated by gonadotropin and steroid secretion (Schams *et al.* 1981; Ojeda 1991). In human males, secondary sex characteristics develop around the time of puberty, and these changes can be staged (Donavan & Vander Werfften Bosch 1965). However, in male farm animals, puberty is assessed on the basis of testicular growth and the beginning of sperm production (Wolf *et al.* 1965; Skinner 1968a, b; Lunstra *et al.* 1978). As a result, in bulls, puberty has been defined as the age at which an ejaculate contains at least 50 million spermatozoa with at least a 10% linear motility (Wolf *et al.* 1965). After the onset of puberty, the number of sperm cells per ejaculate and sperm motility continues to increase greatly for 2 to 3 years to values greater than those above (Almquist & Cunningham 1967; Curtis & Amann 1981; Amann & Schanbacher 1983; Evans *et al.* 1995; Barth 2000). In a study of 6 breeds of beef bulls, Lunstra *et al.* (1978) showed that there was a slight but non-significant difference between breeds in the scrotal circumference (SC) at puberty, with  $27.9 \pm 0.2$  cm as the average SC at puberty. In young bulls, SC is positively correlated with testicular weight (Boyd & VanDemark 1957; Willett & Ohms 1957), spermatozoa output (Boyd & VanDemark 1957; Willett & Ohms 1957; Hahn *et al.* 1969) and fertility (Hahn *et al.* 1969; Foote *et al.* 1970). Age at puberty varies with breed: in Hereford bulls it occurs between 39 and 52 weeks of age; in Charolais bulls

between 33 and 53 weeks of age; and in Holstein bulls between 39 and 41 weeks of age (Wolf *et al.* 1965; Killian & Amann 1972; Lunstra *et al.* 1978). It was suggested that the timing of puberty was dependent on the maturation of the hypothalamus, because the pituitary gland, gonads and steroid dependant target tissues were responsive to their respective tropic hormones prior to puberty (Davidson 1974). In Hereford x Charolais bull calves, the timing of puberty varied with the characteristics of the early rise in gonadotropin secretion; puberty occurred earlier in bull calves that had an earlier and higher early rise in gonadotropin secretion (Amann 1983; McCarthy *et al.* 1979a; Evans *et al.* 1995). Puberty was also hastened by the advancement of the early rise in gonadotropins following administration of exogenous pulses of GnRH (Chandolia 1997c). However, it is unclear whether the transient early postnatal increase in serum gonadotropin concentrations is sufficient to trigger rapid testicular development or how important the subsequent low concentrations of LH and FSH are. In bull calves there could also be important regulatory changes in the testis itself from 25 weeks of age to puberty that are critical for rapid testicular growth (Amann *et al.* 1986; Wise *et al.* 1987; Evans *et al.* 1993,1996; Rawlings & Evans 1995).

## 2.2. ENDOCRINOLOGY OF PUBERTY IN BULL CALVES

Puberty is the culmination of a series of endocrine changes, which begin shortly after birth. Although the temporal changes in serum concentration of various hormones in developing bulls have been fairly well described (Figure 2.1.1 and 2.1.2; Swanson *et al.* 1971; Rawlings *et al.* 1972, 1978; McCarthy 1979a, b; Pelletier *et al.* 1981; Schams *et al.* 1981; Schanbacher 1981; Amann & Schanbacher 1983; Rawlings & Evans 1995;

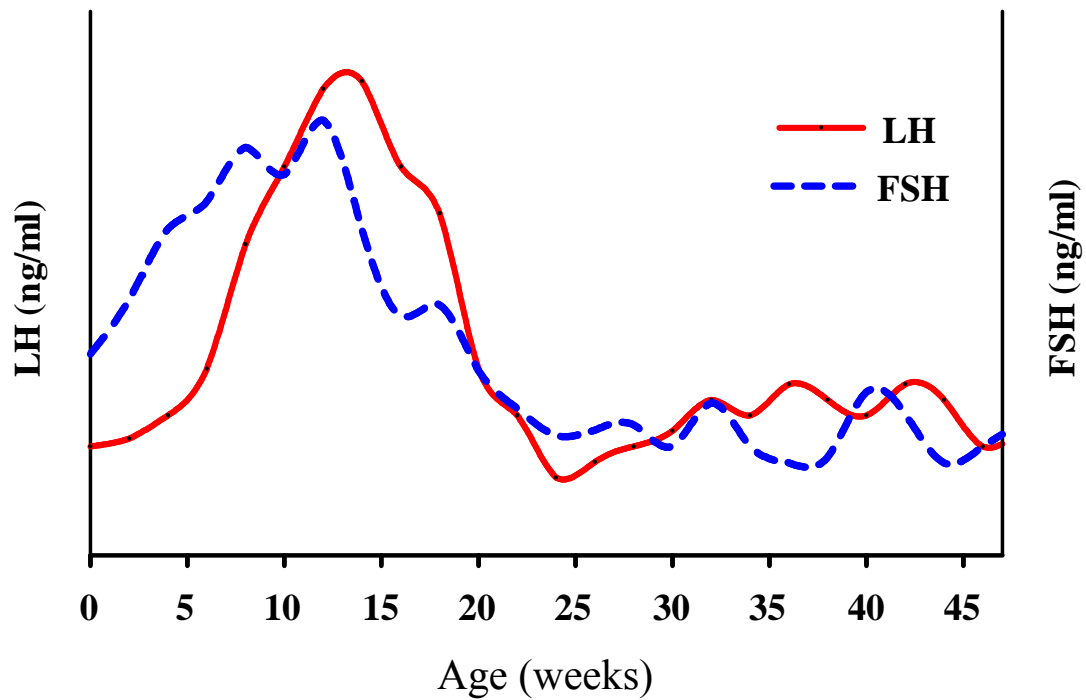
Evans *et al.* 1995; Chandolia *et al.* 1997c) the functional significance of these changes are not yet fully understood.

### **2.2.1. Luteinising Hormone (LH)**

In bull calves, during the very early postnatal period, between birth and 6 weeks of age, mean serum LH concentrations are low (Figure 2.1.1; Rawlings *et al.* 1978; McCarthy *et al.* 1979a, b; Amann & Walker 1983; Amann *et al.* 1986; Rodriguez & Wise 1989; Evans *et al.* 1993, 1995; Chandolia *et al.* 1997c; Aravinsdakshan 2000). The low mean serum LH concentrations have been attributed to infrequent LH discharge [1 to 2 pulses per 10 hours] from the pituitary gland (Rawlings *et al.* 1978; McCarthy *et al.* 1979a, b; Amann & Walker 1983; Amann *et al.* 1986; Rodriguez & Wise 1989; Evans *et al.* 1993, 1995; Chandolia *et al.* 1997c). It was suggested that the infrequent discharge of LH was due to a deficiency in hypothalamic GnRH secretion (Lacroix & Pelletier 1979a, b; Rodriguez & Wise 1989; Chandolia *et al.* 1997c) but not pituitary LH content (Macmillan & Hafs 1968). This deficiency in hypothalamic GnRH secretion is independent of steroid negative feed back (Amann 1983; Wise *et al.* 1987). Serum concentrations of LH are not affected by castration of bull calves during early postnatal life, suggesting that testicular steroids do not regulate LH secretion prior to the early postnatal increase in LH secretion (Wise *et al.* 1987).

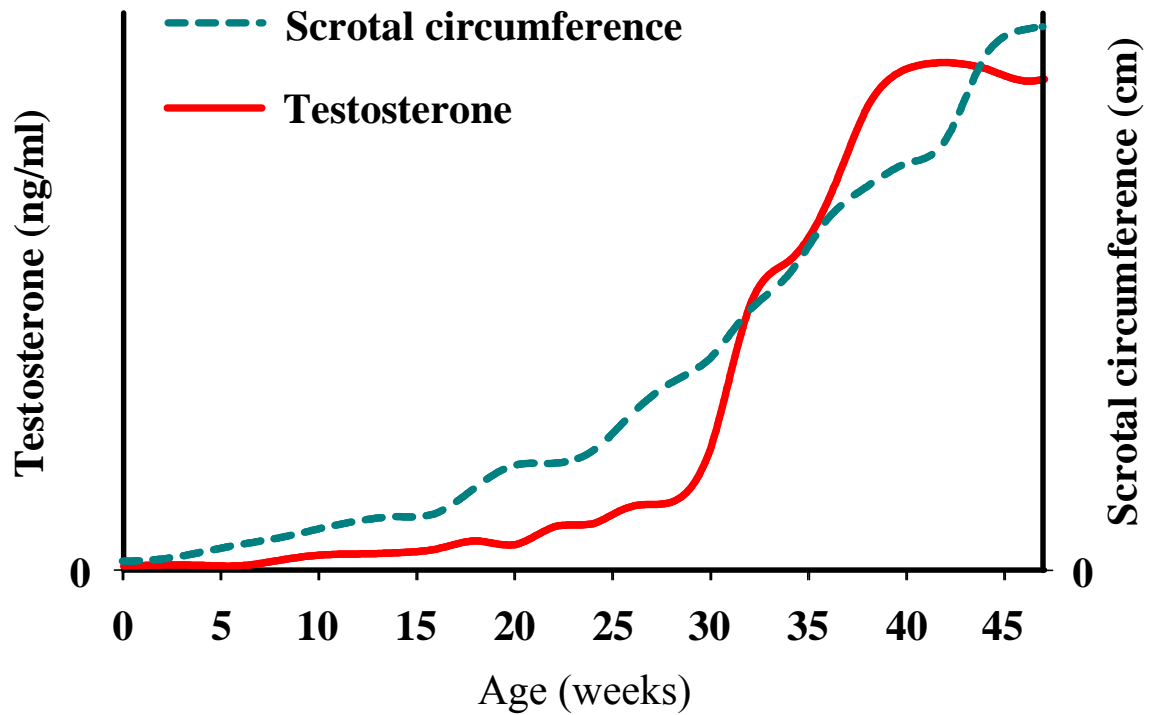
In bull calves, during the early postnatal period between 6 and 16 weeks of age, there is a conspicuous early increase in mean serum LH concentrations (Figure 2.1.1; Rawlings *et al.* 1978; Amann *et al.* 1986; Evans, 1993, 1995; Rawlings & Evans 1995; Chandolia

*et al.* 1997c). It was suggested that the increase in frequency of GnRH pulses that occurred during this early prepubertal period enhanced the pulsatile discharge of LH from the pituitary, which subsequently increased mean serum LH concentrations (Amann & Walker 1983; Amann *et al.* 1986; Pechman & Eilts 1987; Rodriguez & Wise 1989; Evans *et al.* 1993, 1995; Rawlings & Evans 1995; Chandolia *et al.* 1997c). Reports on the timing of the early rise in serum LH concentrations are variable; however, the general consensus is, it starts after 6 weeks of age, reaches peak concentrations between 8 and 16 weeks of age, declines between 20 and 24 weeks of age and reaches a nadir between 25 and 35 weeks of age (Figure 2.1.1; Amann *et al.* 1986; Rodriguez & Wise 1989; Evans *et al.* 1993; 1995; Rawlings & Evans 1995; Evans *et al.* 1995). Although earlier reports had indicated that there was a subsequent increase in mean serum LH concentrations after 35 weeks of age, responsible for the final drive towards puberty (Rawlings *et al.* 1978; McCarthy *et al.* 1979a, b; Schams *et al.* 1981; Evans *et al.* 1993), recent reports have shown that mean serum LH concentration after 25 weeks of age were variable but low (Figure 2.1.1; Rawlings & Evans 1995; Chandolia *et al.* 1997c; Aravindakshan *et al.* 2000a, b). In bull calves, the period from 25 weeks of age until puberty (SC  $\geq$  28cm; Lunstra *et al.* 1978) is characterized by rapid testicular growth and an increase in mean testosterone concentrations (Evans *et al.* 1993, 1995; Rawlings & Evans 1995; Chandolia *et al.* 1997a). Negative feedback effects, from the increasing mean serum testosterone concentrations, are probably involved in the termination of the early rise in LH secretion in bull calves (Rawlings & Evans 1995).



**Figure 2.1. 1** A schematic representation of the temporal changes in serum concentrations of LH and FSH in Hereford x Charolais bull calves from birth (0 weeks) to puberty (45 weeks of age). The x-axis represents age in weeks while the left and right y-axes represent relative hormonal concentrations of LH and FSH respectively. In Hereford x Charolais bull calves puberty is attained at about 45 week of age at a scrotal circumference of  $\geq 28$  cm (Evans et al. 1993, 1996; Rawlings & Evans 1995; Chandolia et al. 1997a, b, c; Aravindakshan et al. 2000a, b).





**Figure 2.1. 2** A schematic presentation of the temporal changes in serum testosterone concentrations and testicular growth profiles of Hereford x Charolais bull calves from birth (0 weeks) to puberty (45 weeks of age). The x-axis represents age in weeks while the left and right y-axes represent relative serum testosterone concentrations and the changes in scrotal circumference (cm), respectively. In Hereford x Charolais bull calves puberty is attained at about 45 week of age at a scrotal circumference of  $\geq 28$  cm (Evans et al. 1993, 1996; Rawlings & Evans 1995; Chandolia et al. 1997a, c; Aravindakshan *et al.* 2000a, b).

### **2.2.2. Follicle Stimulating Hormone (FSH)**

The pattern of FSH secretion in developing bull calves (Figure 2.1.1) is not as consistent as for LH (McCarthy *et al.* 1979a; Rawlings & Evans 1995). In some studies, FSH secretion did not vary significantly throughout the first year of life (Karg *et al.* 1976; McCarthy *et al.* 1979a). However, in other studies, FSH secretory patterns were similar to LH, with an elevated secretion between 4 and 20 weeks of age, and a nadir between 20 and 30 weeks of age (Figure 2.1.1; Evans *et al.* 1993, 1995; Rawlings & Evans 1995; Chandolia *et al.* 1997a, b, c; Aravindakshan *et al.* 2000a, b). Schams *et al.* (1981) reported a longer duration of the early rise in FSH than LH, with mean FSH concentrations in blood increasing from birth up to 28 weeks of age. Amann & Walker (1983) reported that the patterns of FSH secretion seen in young bull calves differed with the frequency of blood sampling employed. When blood samples were collected at weekly intervals, mean serum FSH concentrations were shown to increase by 30% from 4 to 32 weeks of age; however, when the frequency of blood sampling was every 30 minutes for 5 hours no increase was seen nor was there any evidence of pulsatile FSH discharge. Stumpf *et al.* (1993) also argued that the FSH concentrations in jugular blood are not pulsatile; however, in some studies pulsatile FSH secretion has been reported (Evans *et al.* 1993; Rawlings & Evans 1995). The differences above in the patterns of mean serum concentrations of FSH compared to LH could be explained by the longer half life of FSH in circulation compared to LH (Akbar *et al.* 1974); and the fact that testicular inhibin inhibits FSH but not LH secretion (Kaneko *et al.* 1993).

### 2.2.3. Testosterone

During the early rise in serum LH concentrations, mean testosterone concentrations are low, increasing markedly after 28 weeks of age and reaching adult concentrations by 40 weeks of age (Figure 2.1.2; Rawlings *et al.* 1972, 1978; Lunstra *et al.* 1978; Secchiari *et al.* 1976; Amann & Walker 1983; Evans *et al.* 1995). Amann (1983) suggested that the high frequency of LH pulses that occurred in bull calves after 4 weeks of age, initiated differentiation and maturation of adult Leydig cells and a switch from androstenedione to testosterone secretion around 12 to 16 weeks of age. In young bull calves, the testis produces androgens such as androstenedione and 5 $\alpha$ -reduced androgens as well as testosterone (Rawlings *et al.* 1972; Bedair & Thibier 1979; McCarthy *et al.* 1979b; Rawlings & Cook 1986). During the prepubertal period, androstenedione and some 5  $\alpha$ -reduced androgens are secreted in greater amounts than testosterone; however, in the adult, testosterone is the major product (Rawlings *et al.* 1972; Bedair & Thibier 1979; McCarthy *et al.* 1979b; Rawlings & Cook 1986). The highest concentrations of androstenedione are seen between 12 and 16 weeks of age (Rawlings *et al.* 1972). Androgens are synthesized in the smooth endoplasmic reticulum of Leydig cells by enzymatic transformation of pregnenolone; the latter is formed from cholesterol inside the mitochondria of the Leydig cells, and then transported to the smooth endoplasmic reticulum (Gower 1988). It has been suggested that androgens stimulate the functional and anatomical development of the testes, accessory sex glands, the epididymis, ductus deferens and its ampulla, the vesicular gland, the prostate and Cowper's gland (Curtis & Amann 1981; Amann 1983).

#### **2.2.4. Estradiol**

Testosterone produced by Leydig cells that crosses into the seminiferous tubules is converted to estradiol by the Sertoli cells (Purvis et al. 1981). The pattern and role of estrogens in the prepubertal bull calf is not clear. In Holstein bull calves the circulating concentrations of estradiol decreased prior to the early rise in LH secretion (Amann et al. 1986), but in Hereford bull calves, serum concentrations of estradiol were not high during early prepubertal life and significant increases in serum estradiol concentrations occurred after 40 weeks of age (Evans et al. 1993). In bulls, serum concentrations of LH and testosterone were increased by administration of exogenous estradiol 17 $\beta$  (Juniewicz & Johnson 1980), while in another study, secretion was inhibited (Schanbacher et al. 1987; Deaver & Peters 1988). Estradiol may play a role in the feedback control of gonadotropin secretion in bull calves (Schanbacher 1981; Godfrey et al. 1992). It was suggested that estrogens may be important for the development of GnRH neurons in the hypothalamus of bull calves just after birth (Kordon 1994). Treatment of bull calves with estrogens during the early postnatal period was shown to increase in GnRH receptors (Amann et al. 1986; Deaver & Peters 1988). Based on changes in serum concentrations of estradiol around the time of the early rise in LH secretion in dairy bull calves it has been suggested that a decrease in estradiol negative feedback may be involved in the onset of the early increase in LH secretion (Amann et al. 1986; Rodriguez & Wise 1989; Wise et al. 1987). Spermatogenesis was disrupted in dairy bull calves by estradiol treatment (Deaver & Peters 1988). In prepubertal beef bulls, chronic treatment with estradiol inhibited spermatogenesis and production of testosterone by Leydig cells, apparently due to inhibition of LH and FSH secretion from

the pituitary (Weston et al. 1988). The increase in LH secretion near sexual maturation is considered a result of decreases sensitivity of the hypothalamo-pituitary to negative feed back of steroids (Levasseur 1977).

### **2.2.5. Growth Hormone (GH)**

Growth hormone (GH) also referred to as somatotropin, is a protein hormone made up of about 190 amino acids that is synthesized and secreted by *somatotrophs* cells in the anterior pituitary (Kievits et al. 1988). Growth hormone (GH) is involved in the control of several physiological processes such as growth and metabolism (Kievits *et al.* 1988). In female dairy cattle, it was suggested that some of the biological actions of bovine GH are mediated by IGF-I, which stimulates follicular growth and increases the ovulation rate since it hinders apoptosis of granulosa cells (Billig *et al.* 1996; Santos *et al.* 1998). There is some evidence that GH plays an important role in prepubertal testicular development in rats (Ohyama *et al.* 1995) and enhances seminiferous tubule maturity in boars (Swanlund et al. 1995). In bull calves, GH was shown to have no affect on testicular development (MacDonald & Deaver 1993).

McAndrews *et al.* 1993 reported that mean GH concentrations in plasma to decreases with age. This was attributed to a decrease in the GH baseline concentrations between birth and 42 weeks of age and a marked decrease in GH pulse amplitude between 12 and 42 weeks of age. In developing bull calves, the GH pulse frequency increases between birth and 12-weeks of age and then remained constant till the onset of puberty (McAndrews *et al.* 1993).

### **2.2.6. Insulin like growth factor (IGF)**

Insulin like growth factors (IGFs) are single-chain polypeptides expressed ubiquitously in the body tissues (Cohick & Clemmons 1993; Jones & Clemmons 1995; LeRoith *et al.* 1995). There are two forms of IGFs, IGF-I and IGF-II, which share a 62% sequence homology and bind to two known receptors, IGF-I receptor and IGF-II receptor (Cohick & Clemmons 1993; Jones & Clemmons 1995; LeRoith *et al.* 1995). They can also bind to the insulin receptor with low affinity (Cohick & Clemmons 1993; Jones & Clemmons 1995; LeRoith *et al.* 1995). The IGF-1 receptor is the primary mediator of IGF action (Cohick & Clemmons 1993; Jones & Clemmons 1995; LeRoith *et al.* 1995). Biological actions of the IGFs are modulated by six IGF-binding proteins (IGFBPs) that are found in circulation and in the extracellular compartments. The IGFBPs inhibit or enhance IGF effects and may also have ligand-independent effects (Cohick & Clemmons 1993; Jones & Clemmons 1995; LeRoith *et al.* 1995). Insulin like growth factors, their receptors and IGFBPs are cellular modulators of growth and development (Cohick & Clemmons 1993; Jones & Clemmons 1995; LeRoith *et al.* 1995).

In developing bulls, the temporal changes in mean serum IGF-1 concentrations are influenced by age and nutrition (Brito *et al.* 2006a, b). In bull calves, mean serum IGF concentrations increased from 14 to 50 weeks of age and remained at a plateau up to 74 weeks of age (Brito *et al.* 2006a, b). In calves on a high plane of nutrition, mean serum concentrations were greater during the prepubertal (26 to 30 weeks of age) and peripubertal periods (42 to 50 weeks of age) as compared to calves on a lower plane of

nutrition (Brito *et al.* 2006a, b). The temporal changes in mean serum IGF-1 concentrations in bulls during development are similar to those reported in the rhesus monkey (Styne 1991).

### 2.3. CONTROL OF GONADOTROPIN SECRETION

Gonadotropin-releasing hormone is also referred to as Luteinising Hormone Releasing Hormone (LHRH) because of its robust regulatory relationship with LH as compared to FSH (Lacroix & Pelletier 1979a, b; Rodriguez & Wise 1989; Chandolia *et al.* 1997c). In bull calves, it was suggested that the early postnatal increase in pulsatile GnRH secretion resulted from the removal of inhibitory and/or the development of stimulatory mechanisms affecting the LHRH neurons in the hypothalamus (Amann *et al.* 1986; Deaver *et al.* 1988; Rodriguez *et al.* 1993). This concept was referred to as the “central drive” and it was postulated to explain the onset of puberty in rats and primates (Ojeda 1991; Ojeda & Bilger 1999). Changes in concentrations of excitatory amino acids (aspartate and glutamate) and inhibitory amino acids ( $\gamma$ -amino butyric acid [GABA] and taurine) and GnRH in brain perfusates of immature rats, following administration of GABA receptor antagonists and agonists, indicated that amino acid neurotransmitter systems participate in GnRH release in the prepubertal rat (Feleder *et al.* 1996). Gama-amino butyric acid is synthesised from an excitatory amino acid, glutamate, by Glutamic Acid Dehydrogenase (GAD; Ojeda & Bilger 1999). Gama-amino butyric acid acts through two receptors: GABA<sub>A</sub> and GABA<sub>B</sub> (Barnard *et al.* 1998). Mitsushima *et al.* (1994) showed that in monkeys GABA inhibited GnRH secretion and the inhibitory intensity decreased with the onset of puberty. It was suggested that the

decrease in the inhibitory intensity was due to reduction in GABA transmission resulting partly from an increase in excitatory amino acid inputs to the hypothalamus (Ojeda & Bilger 1999).

Glutamate, a major excitatory amino acid in the brain, acts through three types of receptors named after their congeners: kainite/quisqualate;  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA); and NMDA. In juvenile rats and primates, excitatory amino acids control GnRH secretion at the nerve terminals of the median eminence predominately through the kainate receptor (McCann *et al.* 1993), and at the cell bodies in the preoptic and mediobasal hypothalamus preferentially through NMDA receptors (Ondo *et al.* 1988). Activation of the kainate receptors inhibits GABA release (Ojeda 1991). Pulsatile administration of N-methyl-D-aspartic Acid (NMDA, an analog of glutamate) increased GnRH/LH secretion in juvenile rats (Ojeda & Bilger 1999), monkeys (Plant 1994) and heifers (Honaramooz *et al.* 1999). Unlike heifers (Honaramooz *et al.* 1999), in juvenile rats (Ojeda & Bilger 1999) and monkeys (Plant 1994), NMDA advanced the onset of puberty.

The “central drive” hypothesis postulates that immature GnRH neurons have a basal low level of activity that increases at the end of the juvenile period, culminating in the activation of the pituitary and subsequently the gonads (Ojeda 1991). The juvenile period in primates is comparable to the early postnatal period, between 6 to 10 weeks of age in bull calves. It was suggested that GnRH neurons may therefore require a change in external inputs for their activation or may be endowed with the endogenous capacity



to do so (Ojeda 1991; Ojeda & Bilger 1999). In bull calves, prior to the early increase in LH secretion, estrogens were reported to cause the early maturation of GnRH neurons in the hypothalamus (Merten & Stocker-Buschina 1995) and increase GnRH receptors in the pituitary (Amann *et al.* 1986). An increase in the density of GnRH fibres and beads in the median eminence and pituitary GnRH receptors was reported following treatment of neonatal bull calves with estradiol (Deaver *et al.* 1988). In early postnatal bull calves, prior to the increase in mean serum LH concentrations, there is a concomitant increase in GnRH secretion from the hypothalamus, GnRH receptors in the pituitary and serum concentrations of estradiol (Amann *et al.* 1986; Rodriguez & Wise 1989). It was suggested that steroids controlled LH pulse frequency through opioids (Ojeda 1991). Treatment of bull calves with Naloxone, an opioid receptor antagonist, increased LH pulse frequency at 4 and 24 weeks of age but not at 12, 18 or 32 weeks of age (Evans *et al.* 1993). Castration of bull calves during the early postnatal period resulting in the effective removal of steroids did not seem to affect the early increase in LH secretion (Wise *et al.* 1987). It is therefore questionable whether a decrease in opioidergic inhibition of LH pulse frequency is responsible for the initiation of the early rise in LH secretion in bull calves between 8 and 12 weeks of age (MacDonald *et al.* 1990; Evans *et al.* 1993; Rawlings & Evans 1995; Chandolia *et al.* 1997a).

Increased secretion of catecholamines such as dopamine and epinephrine in the hypothalamus, have been implicated in the early rise in LH secretion (Rodriguez *et al.* 1993). In a review on farm animals, Haynes *et al.* (1989) suggested that opioids acted in conjunction with, or by way of catecholaminergic neuronal pathways to the

hypothalamus. In young bull calves, a dopaminergic drive for LH and FSH secretion has been seen towards the end of the early rise of LH secretion around 24 weeks of age (Chandolia *et al.* 1997b). Inhibition of a dopaminergic drive for GnRH/LH secretion by opioids at the end of the early rise in LH secretion may be important in terminating the early rise in LH secretion (Chandolia *et al.* 1997b).

Although GnRH was reported to stimulate FSH release, in even smaller amounts than LH (McCann *et al.* 1998), there is evidence to suggest the FSH and LH are differentially regulated. In castrated male rats, few of the pulses of both gonadotropins are coincident; however, about half of the FSH pulses occur in the absence of LH pulses (McCann *et al.* 1998). Treatments with GnRH antisera or antagonists suppress the release of LH but not FSH (McNeilly *et al.* 1986; McCann *et al.* 1993). Luteinising Hormone but not FSH pulses can be suppressed by alcohol (Dees *et al.* 1985), delta-9-tetrahydrocannabinol, and interleukin-1 alpha (IL-1 alpha; Rettori *et al.* 1991). When GnRH was given to ewes (Clarke *et al.* 1984) and bull calves (Chandolia *et al.* 1997c), it induced pulses of LH without a consistent acute increase in mean serum FSH concentrations. Studies in the ewe have led to the suggestion that GnRH has an acute regulatory effect on LH secretion but a more chronic/insidious trophic role on FSH secretion (Goodman 1994). However, other authors postulated that there exists a specific FSH-releasing factor (FSH-RF) that could account for the differential secretion rates and patterns of FSH and LH (Bowers *et al.* 1973; McCann *et al.* 1998).

In adult female rats, stimulation of the dorsal anterior hypothalamic area causes selective FSH release, whereas lesions in this area selectively suppress the pulses of FSH but not LH (Lumpkin *et al.* 1989). Conversely, stimulation in the medial preoptic region releases only LH, whereas lesions in this area inhibit LH release but not FSH (McCann *et al.* 1998). The medial preoptic area contains the perikarya of GnRH neurons that project from the preoptic region to the anterior and mid portions of the median eminence (McCann *et al.* 1998). In castrated male rats, lesions confined to the rostral and mid-median eminence selectively inhibit pulsatile LH release without altering FSH pulsations, whereas lesions that destroy the caudal and mid-median eminence selectively block FSH pulses (Lumpkin *et al.* 1989; McCann *et al.* 1993; Rage *et al.* 1997). Therefore, it appears that the putative FSH-RF is produced in neurons with perikarya in the dorsal anterior hypothalamic area with axons that project to the mid and caudal median eminence to control FSH release selectively. In rats, FSH-releasing activity has been shown in the stalk-median eminence, by the demonstration of increased plasma FSH concentrations (Lumpkin *et al.* 1987; McCann *et al.* 1998). Although isolation of FSH-RF is yet to be achieved, an analog of LHRH, gonadotropin-releasing hormone associated peptide (GAP) was shown to selectively release FSH over a 50-fold dose range in vivo in ovariectomized, estrogen-progesterone blocked rats (Yu *et al.* 1990). The selective FSH-releasing activity of GAP was augmented by blocking peptidic digestion by inserting D-trp in position 9 of the molecule. This preparation, rat D-trp-9 GAP produced a sustained increase in plasma FSH without altering LH (Yu *et al.* 1990) and was also active in in vitro assays using hemi pituitaries (McCann *et al.* 1998). Preliminary immunocytochemistry indicates that this peptide is present in the rat

hypothalamus in the region that had previously been shown to control FSH release. Therefore, it is either FSH-RF or a very closely related peptide (Yu *et al.* 1997).

In humans, rats and bulls it has been suggested that the secretion of FSH from the pituitary gland is also under the inhibitory and stimulatory control of inhibin and activin, respectively (Schanbacher 1991; Martin *et al.* 1991; Kaneko *et al.* 1993; Bame *et al.* 1999). Inhibin is found in testicular extracts, rete testis fluid, testicular lymph, seminal plasma, in media from cultures of seminiferous tubules and Sertoli cells (Baker *et al.* 1982; Yu & McCann 1988). Inhibin variants, a 32kDa  $\alpha,\beta$ -dimer and  $\alpha$ -inhibin-92 act on the pituitary gland suppressing FSH release but not LH (Kaneko *et al.* 1993; McCann & Ojeda 1996; McCann *et al.* 1998). In male rats, the actions of variant,  $\alpha$ -inhibin-92 in vivo occur within 10 minutes of treatment and are dose-response related (McCann *et al.* 1998). Whereas in bulls and rats, the actions of variant a 32kDa  $\alpha,\beta$ -dimer are seen after a fairly long latency period of over 1 hour (Kaneko *et al.* 1993; McCann & Ojeda 1996). In humans, the increase in number and maturation of Sertoli cells is accompanied by an increase in blood concentrations of inhibin B (Jensen *et al.* 1997). Concentrations of inhibin B were shown to be higher in semen samples collected from men with high sperm counts as compared to low (Anderson *et al.* 1998). Reports on the relationship between circulating FSH and inhibin in developing bulls are variable: two studies showed a negative correlation between plasma concentrations of inhibin and FSH from 10 to 36 weeks of age (MacDonald *et al.* 1991) or from 2.5 to 108 months of age (Matsuzaki *et al.* 2000); whereas, Miyamoto *et al.* (1989) showed a positive correlation between 1 and 8 months. However, in bull calves active immunization

against inhibin resulted in higher serum FSH concentration than in non-immunized bull calves (Schanbacher 1991; Martin *et al.* 1991; Bame *et al.* 1999). These observations confirm the presence of a strong regulatory interaction between inhibin and FSH in bulls.

FSH and LH themselves have intrahypothalamic actions that alter gonadotropin secretion, which are probably of physiological significance (Yu & McCann 1991). In immature female rats, transformation growth factor alpha (TGF-alpha) hastened maturation of the LHRH neurons, increased LHRH release and caused precocious puberty (Rage *et al.* 1997). Gonadal steroids play an important role in control of LHRH release and pituitary responsiveness to the peptide (McCann *et al.* 1993; McCann *et al.* 1998). In a review by McCann *et al.* (1998) they suggested that in males the influence of androgens at both the hypothalamic and pituitary level is inhibitory, whereas in females, there is a biphasic effect of estrogens. In bull calves, the negative effects of gonadal steroids on LH secretion appear to increase after 2-3 months of age (Evans & Rawlings 1995). Androgens suppress the pulse frequency and amplitude of pulses of LH and this terminates the early rise in LH and FSH (Evans & Rawlings 1995). In turn, pulsatile release of LH and FSH is under the control of a host of classic transmitters and peptides.

#### 2.4. HORMONAL INFLUENCES ON SEXUAL MATURATION

It was suggested that in bulls, age at puberty could be reduced by manipulation of the neuroendocrine system (Amann & Schanbacher 1983). This suggestion was drawn from

observations in bull calves that the timing of puberty in bulls was influenced by a conspicuous increase in serum LH pulse frequency and mean serum LH concentrations that occurred between 6 and 20 weeks of age (Curtis & Amann 1981; Schanbacher 1981; Amann 1983; Amann & Schanbacher 1983). This increase in serum concentrations of LH and FSH; also referred to as the early rise in serum gonadotropins, occurs just prior to tubule lumenation, differentiation of Leydig, Sertoli and germ cells (Curtis & Amann 1981, Amann 1983).

#### **2.4.1. The early rise in serum LH and FSH concentrations**

Several authors have demonstrated the significance of the early rise in serum LH concentrations in the timing of puberty in bull calves (Schanbacher 1981; Amann *et al.* 1986; Rodriguez & Wise 1989; Evans *et al.* 1995). In Hereford x Charolais bull calves, early sexually maturing bulls were shown to have a higher early rise in LH than late maturing bulls (Evans *et al.* 1995). In a study by Chandolia *et al.* (1997c) in which bull calves were treated with 200 ng of LHRH, every 2 hours from 4 to 6 weeks of age, the amplitude and frequency of peaks in LH during the early rise in LH secretion was increased; the treatment accelerated testicular growth and enhanced spermatogenesis. In the study by Chandolia *et al.* (1997c), treatment with GnRH did not affect the serum FSH profiles. The authors suggested that the early rise in serum LH was critical for the initiation and regulation of testicular development (Chandolia *et al.* 1997c). In rats, the early rise of LH secretion was also shown to be important for normal fertility (Pinilla *et al.* 1994) and its inhibition decreased testicular growth and spermatogenesis (Van den Dungen *et al.* 1989, 1990).

In bull calves, treatment with estradiol releasing implants from 7.5 weeks of age to 24 weeks of age decreased mean serum FSH concentrations (MacDonald *et al.* 1991), eliminated LH pulses and reduced testis size (Deaver *et al.* 1988). In that study the decrease in mean serum concentrations of LH and FSH during the early postnatal period hindered growth of the testes and sexual maturation (MacDonald *et al.* 1991). Active immunization against inhibin at 2, 4, 8, 10, 20, 32 and 42 weeks of age (Schanbacher 1991) or 14, 28, 30, and 34 weeks of age (Martin *et al.* 1991) or 60, 90, 104, 124, 270, and 395 days of age (Bame *et al.* 1999) was shown to increase serum concentrations of FSH in prepubertal bull calves. The latter was associated with an increased daily sperm production per gram of testicular parenchyma (Schanbacher 1991; Martin *et al.* 1991; Bame *et al.* 1999). Schanbacher (1991) reported that immunization against bINH had no effect on serum LH and testosterone concentrations or testes and epididymides weight. However, Martin *et al.* (1991) reported an increase in serum testosterone concentrations. Reports of the changes in mean serum LH concentrations were contradictory; Martin *et al.* (1991) showed an increase while Bame *et al.* (1999) a decrease following immunization against bINH.

In the studies above, the role of the individual gonadotropins, LH and FSH in the maturation of bull calves was ambiguous. However, it is clear that the serum concentrations of gonadotropins achieved in bull calves prior to 25 weeks of age are critical for reproductive development. It was suggested that gonadotropins provide a critical early cue or signal for the onset of spermatogenesis and rapid testicular

development (Amann 1983; Evans *et al.* 1995; Chandolia *et al.* 1997c). Thus the early rise in LH and/or FSH secretion may be important for the initiation of rapid testicular growth and development which occurs largely after 20 weeks of age (Amann & Walker 1983; McCarthy *et al.* 1979a, b). However, it is also unclear whether the transient early postnatal increase in serum LH concentrations is sufficient to trigger rapid testicular development or how important the subsequent low concentrations of LH and FSH are. There could also be important regulatory changes in the testis itself from 25 weeks of age to puberty that are critical for rapid testicular growth (Amann *et al.* 1986; Wise *et al.* 1987; Evans *et al.* 1993,1996; Rawlings & Evans 1995).

## 2.5. PARACRINE AND AUTOCRINE REGULATORS

It was suggested that, in addition to the role of gonadotropins and testosterone, functional and anatomical development of the testes in ruminants is regulated by paracrine and autocrine factors (Maddocks *et al.* 1995). However, most of the evidence presented by Maddocks *et al.* (1995) was drawn from in vivo and in vitro studies in rats, pigs and humans (Skinner *et al.* 1991; Verhoeven 1992; Maddocks *et al.* 1995). Studies in rats, mice and humans show that changes in several intratesticular regulators such as interleukins and growth factors do affect testicular somatic and germ cell functional and anatomical development (Gnessi *et al.* 1997; Huleihel & Lunenfeld 2004).

### 2.5.1. Transformation growth factors (TGF)



#### 2.5.1.1. *TGF-alpha*

TGF-alpha is a polypeptide made up of 50 amino acids, and has a 40% amino acid sequence homology with epithelial growth factor (EGF; Gnessi *et al.* 1997). TGF alpha and EGF are amphiregulins and they belong to the family of EGF (Gnessi *et al.* 1997). EGF is the prototype polypeptide, made up of 53 amino acids, that was first isolated from the male rat submandibular salivary glands (Salido *et al.* 1990; Fisher & Lakshmanan 1990; Massague & Pandiella 1993). A variety of basement membrane proteins, extracellular matrix proteins, plasminogen activators and mammalian clotting factors, contain EGF-like domains (Gnessi *et al.* 1997). Transformation growth factor alpha and EGF are produced as integral membrane protein prohormones with hydrophobic transmembrane domains near the carboxyl terminal (Massague & Pandiella 1993). The mature EGF protein is synthesized by proteolytic break down of the prohormone that exhibits eight EGF-like repeating units and a mature EGF precursor (Massague & Pandiella 1993). Transformation growth factor alpha and EGF are the main EGF family peptides involved in mammalian development (Fisher & Lakshmanan 1990; Carpenter & Cohen 1990). They bind to EGF receptor (EGF-R; Fisher & Lakshmanan 1990; Carpenter & Cohen 1990), eliciting similar physiological responses (Verhoeven 1992). In a review on rats, mice and humans, TGF-alpha was reported to have stimulatory effects on testicular somatic cell proliferation and differentiation (Gnessi *et al.* 1997). However, the role of TGF-alpha in testicular development in bulls is unknown.

#### 2.5.1.2. *TGF-beta*

Transformation growth factor beta (TGF-beta) is a polypeptide that consists of two monomers linked by a disulfide bond, synthesized as separate large precursors (Gnessi *et al.* 1997). There are five isoforms of TGF-beta, isoforms 1, 2, 3, 4, and 5 (Fuse *et al.* 1990). TGF-beta belongs to a group of polypeptides that include activin (ACT), inhibin (INH), mullerian inhibiting hormone (MIH) and the bone morphogenic proteins (Kingsley 1994). The TGF-beta receptors are comprised of multiple components; the main TGF-beta binding components are membrane proteins called receptor types I, II, and III; types I and II are involved in signalling, whereas the type III receptor, a betaglycan, regulates access of the TGF-beta to the signalling receptors (Massague' *et al.* 1994; Gnessi *et al.* 1997).

Reports on the effects of TGF-beta on testicular cells in rats and pigs are contradictory (Avallet *et al.* 1987; Morera *et al.* 1988; Sordoillet *et al.* 1992). Morera *et al.* (1988) suggested that the effects of TGF-beta on cell proliferation, differentiation, and organization in pigs were biphasic. Transformation growth factor-beta was either stimulatory or inhibitory depending on whether its concentrations were low or high, respectively (Morera *et al.* 1988). It was later suggested that the stimulatory or inhibitory effects also depended on intratesticular or media conditions (Massague' *et al.* 1994; Gnessi *et al.* 1997). In rats, mice, pigs and humans, TGF-beta has been shown to play a paracrine/autocrine role in testicular somatic and germ cells development (Maddocks *et al.* 1995; Gnessi *et al.* 1997; Huleihel & Nunenfeld 2004). In bulls, there

has not been any report of the presence and/or involvement of TGF-beta in testicular development.

## **2.5.2. Interleukin (IL)**

### **2.5.2.1. *Interleukin -1 (IL-1)***

Interleukins are pro-inflammatory cytokines synthesized by monocytes, macrophage cells, fibroblasts, epithelial cells and keratinocytes (Dinarello & Savage 1989; Arend 1993; Kishimoto *et al.* 1995). In the IL-1 family, there are 2 agonist proteins, IL-1 alpha and IL-1 beta (Dinarello 1996), and one IL-1 receptor antagonist (IL-1ra; Hannum *et al.* 1990; Arend 1993). Although there are two agonist IL-1 isoforms, IL-1 alpha and IL-1 beta, that recognize the same cell surface receptors and have similar biological effects, the cytokines are products of two different distinct genes (Gnessi *et al.* 1997). Interleukin-1 is the prototype of the proinflammatory cytokines; it induces the expression of a variety of genes and the synthesis of several proteins that, in turn, induce acute and chronic inflammatory changes (Gnessi *et al.* 1997). Dinarello (1996) suggested that IL-1 alpha acts mainly intracellularly while IL-1 beta extracellularly. The naturally occurring IL-1 receptor antagonist (IL-ra) has a generic gene structure homologous to that of IL-1 alpha and beta (Eisenberg *et al.* 1990) and binds to the same receptor without transmitting any signal, thereby regulating IL-1 alpha and IL-1 beta agonist action (Hannum *et al.* 1990; Arend 1993). There are two IL-1 receptor (IL-1R) subtypes, IL-1R type I and II (Dinarello 1991; Gnessi *et al.* 1997). The major difference between the IL-1R types I and II is the truncated cytoplasmatic portion of the type II receptor (Dinarello 1991).

#### 2.5.2.2. *Interleukin -6 (IL-6)*

Interleukin-6 (IL-6) is a multifunctional cytokine that causes the differentiation and proliferation of B cells, T cells, hepatocytes, and hematopoietic progenitor cells (Kishimoto *et al.* 1995). The IL-6 family is made up of a group of proteins that bind to the IL-6 receptor; a 130-kDa signal-transduction molecule, glycoprotein 130 (Kishimoto *et al.* 1995).

#### 2.5.2.3. *Interleukins summary*

In rats, mice, pigs and humans, interleukins (IL-1 and IL-6) have been reported to play a paracrine/autocrine role in controlling spermatogenesis (Verhoeven 1992; Maddocks *et al.* 1995; Gnessi *et al.* 1997; Huleihel & Nunenfeld 2004). However, Maddocks *et al.* (1995) reported that several groups using an IL-1 dependant thymocyte proliferation assay failed to demonstrate the presence of IL-1 in testicular lymph collected from rams, bulls and pigs.

### 2.6. TESTICULAR DEVELOPMENT

In bull calves, during the early-postnatal period, from birth up to about 20 weeks of age, testicular growth is slow (Curtis & Amann 1981; Amann & Walker 1983; Evans *et al.* 1993, 1996; Rawlings & Evans 1995; Chandolia *et al.* 1997a, b, c). However, after 25 weeks of age, testicular growth is rapid (Curtis & Amann 1981; Amann & Walker 1983; Evans *et al.* 1993,1996; Rawlings & Evans 1995; Chandolia *et al.* 1997a, b, c). Testicular weight was reported to increase in a curvilinear manner from birth to 9 months of age (Macmillan & Hafs 1969). The rapid increase of testicular weight,

between 25 and 38 weeks of age, results from an increase in the proportion of the parenchyma occupied by seminiferous tubules as well as an increase in tubular diameter and total length of seminiferous tubules per testis (Curtis & Amann 1981; Evans *et al.* 1996). During the period from 12 to 32 weeks of age the proportion of seminiferous tubules within the testicular parenchyma increases from 44 to 81% (Curtis & Amann 1981). The temporal changes in the rate of testicular growth have been associated with serum testosterone concentrations. The initial slow phase of growth is associated with low serum testosterone concentrations and high androstenedione concentrations, while the rapid growth, with high serum testosterone concentrations (Amann & Walker 1983). On the basis of testicular contents, development of the testis in bull calves can be divided into 5 stages (Abdel-Raouf 1960). In stage one (from birth to 8 weeks of age) the testis contains solid sex cords. In stage two (8 to 20 weeks of age), spermatogonial cells develop. In stage three (20 to 32 weeks of age) lumen formation and development of spermatocytes occur. In stage four (32 to 44 weeks of age), spermatids and sperm can be seen. During the fifth stage, testicular cells increase in number.

### **2.6.1. Leydig cell development**

In males, there are two distinct populations of Leydig cells: foetal and adult Leydig cells, which differentiate from mesenchymal cells prenatally and postnatally respectively (Mendis-Handagama & Ariyaratne 2001). The adult Leydig cell lineage is made up of five main cell types, namely the mesenchymal precursor cells, progenitor cells, newly formed adult Leydig cells, immature Leydig cells, and mature Leydig cells (Mendis-Handagama & Ariyaratne 2001). In bull calves, it was suggested that the

demise of foetal Leydig cells occurred during the early postnatal period (Hooker 1970). The differentiation of adult Leydig cells in bull calves starts at around 12 weeks of age (McCarthy *et al.* 1979b; Amann 1983), thereafter, their number and size increases (Hooker 1970). In rats, Leydig cell progenitors are morphologically indistinguishable from precursor cells (mesenchymal cell), but as a result of their commitment to the Leydig cell lineage, they express steroidogenic enzymes (Haider *et al.* 1986; Haider & Servos 1998; Ariyaratne *et al.* 2000). In a review by Mendis-Handagama & Ariyaratne (2001), they reported that in most species, progenitor Leydig cells do express LH-receptors (LH-R). In rats, it was reported that progenitor Leydig cells were capable of producing androgens (Hardy *et al.* 1990; Chemes *et al.* 1992; Shan & Hardy 1992; Risbridger & Davies 1994) and as they matured their ability to synthesize steroids increased and they became large polygonal cells (Mendis-Handagama & Ariyaratne 2001). A transient appearance of intracytoplasmic lipid droplets (Mendis-Handagama & Ariyaratne 2001) and an increase in the number of LH-R in rats (Purvis *et al.* 1977; Hardy *et al.* 1990; Shan & Hardy 1992) and dogs (Inaba *et al.* 1994) was also reported. In the latter, the increase coincides with rapid testicular growth. In rams, testicular LH-R concentrations increase from 90 to 150 days of age (Yarney & Sanford 1989). In bull calves, LH binding sites were demonstrated in testicular parenchyma of neonates (Schanbacher 1979). In the study in bulls in which the changes in LH-R was studied, testes were collected from one separate individual Norwegian red calf every 4 weeks from 12 to 32 weeks of age, and at 40, 52, and 68 weeks of age (Sundby *et al.* 1984). Testicular LH-R concentrations were shown to decrease with age, while binding affinities remained the same (Sundby *et al.* 1984).

#### 2.6.1.1. *Endocrine regulation of Leydig cell development*

Amann (1983) suggested that the high frequency of LH pulses that occurred in bull calves after 4 weeks of age initiated the differentiation and maturation of adult Leydig cells from progenitor cells. In a review on Leydig cell development in rats, pigs, and humans by Mendis-Handagama & Ariyaratne (2001), they suggested that the differentiation of mesenchymal cells into progenitor cells in these species was independent of LH. They suggested that LH was essential for development of later stages in the Leydig cell lineage, such as the induction of cell proliferation, hypertrophy, and the establishment of the full organelle complement required for the steroidogenic function.

Reports on the role of FSH in the development of Leydig cells are variable; in rats, it was suggested that FSH acted via Sertoli cells to hasten Leydig cell maturation (Tabone *et al.* 1984; Kerr & Sharpe 1985). When porcine Leydig and Sertoli cells were co-cultured in vitro, FSH increased Leydig cell LH/human chorionic gonadotropin (hCG) binding sites and the capacity of Leydig cells to secrete testosterone (Tabone *et al.* 1984). Teerds *et al.* (1989) later reported that FSH had no effect on the development of Leydig cells in rats. He suggested that the effect of FSH on Leydig cell development in the earlier reports above was probably due to contamination of FSH with LH.

The effect of androgens on Leydig cell differentiation appears to be direct because all cellular stages in the Leydig cell lineage express androgen receptors (Shan & Hardy 1992). In rats, testosterone was shown to inhibit the differentiation of mesenchymal cells to progenitor Leydig cells but stimulate the latter stages of Leydig cell

differentiation (Mendis-Handagama & Ariyaratne 2001). In bull calves, the switch from androstenedione synthesis to testosterone is associated with maturation of the Leydig cells (Rawlings *et al.* 1972, 1978; Secchiari *et al.* 1976; Amann 1983; Amann & Walker 1983; Rawlings & Cook 1986). Treatment of early postnatal rats with estrogens was shown to inhibit Leydig cell development (Sharpe *et al.* 2003); while in bull calves it inhibited testicular development (Deaver & Peters 1988). The observations above, in rats and bulls, indicate that maturation of the Leydig cell may be enhanced in part by testosterone and inhibited by estrogens. In a review by Mendis-Handagama & Ariyaratne (2001), they suggested that testosterone and estrogens produced by the mature Leydig cells in the adult testis might be of importance in the inhibition of further differentiation of mesenchymal precursor cells to Leydig cells in rats, mice, pigs and humans. The steroid inhibition thereby maintains a fixed number of adult Leydig cells. In bulls, rats, mice, pigs, and humans, it was suggested that Leydig cells that undergo atresia are replaced by new cells to maintain a fixed number (Hooker 1970; Mendis-Handagama & Ariyaratne 2001). However, the mechanisms involved in the maintenance of the Leydig cell population in the adult testes (whether they are derived from the mesenchymal cell pool or by mitosis of newly formed adult Leydig cells) are not yet well established.

#### 2.6.1.2. *Paracrine regulation of Leydig cell development*

In bulls, we do not know the role of transformation growth factors [TGF] and insulin like growth factor-1 [IGF-1] in Leydig cell development, nor has their presence in the testes been demonstrated. In rats, mice and pigs TGF- (alpha and beta) and IGF-1 have



been identified as regulators of Leydig cell development (Skinner *et al.* 1991; Maddocks *et al.* 1995; Huleihel & Lunenfeld 2004). In rats, IGF-1 receptors were identified in Leydig cells, and their numbers increased with mesenchymal cell proliferation and differentiation into Leydig cells (Spiteri-Grech & Nieschlag 1992; Lin 1995). It was suggested that IGF-I induced proliferation of immature Leydig cells and promoted the maturation of the immature Leydig cells into mature adult Leydig cells in rats (Spiteri-Grech & Nieschlag 1992) and mice (Wang & Hardy 2004). This was supported by observations in IGF-1 null mice where Leydig cell numbers were decreased (Wang & Hardy 2004). In mice, the additive effect of LH and IGF-I treatment on testicular cell numbers was reported; it was suggested that the two hormones acted through separate signalling pathways (Wang *et al.* 2003). Interestingly, in *in vitro* cultures of Leydig cells isolated from rats and mice, IGF-1 up-regulated LH-R and testosterone secretion (Spiteri-Grech & Nieschlag 1992; Lin 1995), whereas testosterone in turn up-regulated IGF-I receptors and IGF-I production by Leydig cells (Cailleau *et al.* 1990).

In rats, TGF-alpha interacts with LH to promote DNA synthesis in immature Leydig cells (Khan *et al.* 1992). This indicates that TGF-alpha may be involved in the proliferation of Leydig cells. In pigs, TGF-beta was reported to exert a biphasic effect on hCG-stimulated testosterone production: low concentrations of TGF-beta enhanced hCG-stimulated testosterone production (Morera *et al.* 1988; Sordoillet *et al.* 1992) while high concentrations inhibited it (Morera *et al.* 1988). Low concentrations of TGF-beta increased cholesterol substrate availability in the mitochondria and  $3\beta$ -

HSD/isomerase activity in porcine Leydig cells (Sordoillet *et al.* 1992). The stimulatory effect of TGF-beta increased on addition of TGF-alpha (Sordoillet *et al.* 1992). In rats, TGF-beta inhibited testosterone production by reducing the number of hCG receptors and hCG induced cAMP (Avallet *et al.* 1987; Lin *et al.* 1987; Fauser & Hsueh 1988).

In bulls, the role of interleukins (IL-1 and IL-6) in Leydig cell development is not known nor have they been demonstrated in the testis. In rats, pigs and humans, IL-1 and IL-6 have been shown to interfere with Leydig cell function and development (Calkins *et al.* 1990; Warren *et al.* 1990; Mauduit *et al.* 1992). In cultures of Leydig cells isolated from immature rats, IL-1 stimulated basal and hCG induced steroidogenesis (Verhoeven *et al.* 1988; Svechnikov *et al.* 2001). However, it inhibited steroidogenesis in cultures of Leydig cells isolated from mature rats (Verhoeven *et al.* 1988). The effects of IL-6 are not clear although Leydig cells isolated from the rat were shown to produce it (Boockfor *et al.* 1994).

### **2.6.2. Sertoli cell development**

In the adult bull testis the number of mature Sertoli cells determines both testis size and daily sperm production (Berndtson & Igboeli 1989; Curtis & Amann 1981) because each Sertoli cell has a fixed capacity for the number of germ cells it can support (Sharpe 1994). This capacity varies between species (Sharpe 1994). Immature Sertoli cells have been described as basal or central indifferent cells based on their spatial distribution in cross sections of round seminiferous tubules (Curtis & Amann 1981). The basal indifferent cells constitute up to 95% of the immature Sertoli cells (Curtis & Amann

1981). The timing of Sertoli cell differentiation and proliferation varies with species (Sharpe *et al.* 2003). In bulls, differentiation of basal indifferent supporting cells into mature Sertoli cells begins at about 16 weeks of age and is completed by 33 weeks of age (McCarthy *et al.* 1979a, b; Curtis & Amann 1981; Amann 1983). De Franca *et al.* (2000) suggested that in bulls and pigs, there are two periods during which Sertoli cells proliferate, the neonatal and prepubertal periods, which are separated by a short gap of weeks. There is about a 5 fold increase in the number of Sertoli cells between birth and puberty, but in the adult, once the Sertoli cells are mature, their number is fixed; further aging does not affect their numbers (Hochereau-de-Reviere 1976; Hochereau-de-Reviere *et al.* 1984,1987). Only immature Sertoli cells are capable of proliferating, so the final number of Sertoli cells is determined prior to puberty (Hochereau-de-Reviere *et al.* 1987; Sharpe *et al.* 2003). The number of Sertoli cells can be influenced in prepubertal animals by nutrition, age, endocrine changes and genetics of the animal (Steinberger 1981; Waites *et al.* 1985). With exception of the fragile X gene, FMR-1, the relevant genes are yet to be identified (Slegtenhorst-Eegdeman *et al.* 1998).

Indifferent supporting cells and Sertoli cells resident in the seminiferous tubules are the target cells for FSH action in bull calves (Schanbacher 1979; Amann 1983; Amann & Schanbacher 1983; Miyamoto 1989) and rats (Sharpe 1994). In Holstein bull calves, FSH receptor (FSH-R) concentrations were reported to decrease with age, while binding affinities remained the same (Dias & Reeves 1982). In the study by Dias and Reeves (1982) testicular samples were collected from Holstein calves at 1, 7, 14, and 56 days of age (n = 5); 12 weeks of age (n = 4) and 24 weeks of age (n = 3); however, no samples

were collected during the period of rapid testicular growth or just prior to puberty. Sample size and timing precluded a careful examination of FSH-R changes around the time of the early postnatal increase in serum gonadotropin concentrations or the period of rapid testicular growth.

#### 2.6.2.1. *Endocrine regulation of Sertoli cell development*

In rats and mice, the development of Sertoli cells has been reported to be under the influence of FSH and thyroid hormone (T3), and to some degree growth hormone (GH; Sharpe 1994, 2003). In mice, rats and bulls, it was suggested that FSH acted on Sertoli cells by influencing cellular functions such as mitosis, differentiation, the synthesis of estradiol and androgen-binding protein, and the secretion of tubular fluid (Chemes 1979a, b; Steinberger 1981; Amann & Schanbacher 1983; Miyamoto *et al.* 1989; Bardin *et al.* 1994). In rats, immature Sertoli cells were also shown to produce lactate and growth factors following stimulation with FSH, typical of functionally mature Sertoli cells (Esposito *et al.* 1991; Sharpe 1994, 2003).

In rats, induction of neonatal hypothyroidism was shown to considerably increase the final number of Sertoli cells by 82–157% and delay the age at which cessation of proliferation and/or maturation of Sertoli cells occurs (De Franca *et al.* 1995). However, neonatal hyperthyroidism reduced the final number of Sertoli cells by about 50%, by advancing the age at which cessation of proliferation and/or maturation of Sertoli cells occurred in rats (van Haaster *et al.* 1993). It was suggested that reduced T3 production prolonged the period in which proliferation could occur by regulating maturation of

Sertoli cells (Sharpe *et al.* 2003). It is uncertain whether T3 plays a similar role in all species. Untreated, early postnatal hypothyroidism in humans is associated with precocious and permanent testicular enlargement (Jannini *et al.* 1995). It is unclear as to how thyroid hormones exert their effect directly on Sertoli cells. Sharpe (2003) suggested that T3 decreased Sertoli cell size and therefore their density in the epithelium.

In vitro studies in rats have shown that FSH and T3 both induce androgen receptor (AR) expression in immature Sertoli cells (Arambepola *et al.* 1998a) and suppress the expression of anti mullerian hormone (AMH; Arambepola *et al.* 1998b). The effects of FSH and T3 are additive. In rats it was suggested that rising concentrations of FSH trigger proliferation of Sertoli cells and induce a progressively increased expression of AR. The increased expression of AR was then upregulated by the increase in T3, leading to functional maturation of Sertoli cells and consequently the loss of AMH expression (Sharpe *et al.* 2003). Changing sensitivity of Sertoli cells to T3 during their proliferative phase, due to altered expression of T3 receptors (Buzzard *et al.* 2000), may be another contributing factor to the loss of AMH expression and maturation of Sertoli cells. However, this scenario may differ among species. It is speculated that absence of sufficient T3, or more likely the absence of T3 receptors in the Sertoli cell, prevents induction of AR during the neonatal period in marmosets and possibly in humans (Jannini *et al.* 2000).

It was suggested that LH and/or testosterone play a role in Sertoli development in bull calves (Amann 1983; Chandolia *et al.* 1997c) and rhesus monkeys (Ramaswamy *et al.* 2000). Amann (1983) suggested that the high frequency of LH pulses occurring in bull calves after 4 weeks of age initiated differentiation and maturation of adult Leydig cells from progenitor cells around 12 to 16 weeks of age, and a switch from androstenedione to testosterone secretion. The elevated intratesticular testosterone concentrations, following the onset of Leydig cell differentiation, were suggested to be responsible for causing the differentiation of indifferent supporting cells to Sertoli cells; differentiation is restricted to the period of 16 to 28 weeks of age (McCarthy *et al.* 1979a, b; Curtis & Amann 1981; Amann 1983). In light of observations that human patients with complete androgen insensitivity syndrome (AIS) exhibited Sertoli cells with features of immaturity, such as persistence of AMH expression (Rey *et al.* 1994; Rajpert-de Meys *et al.* 1999), it likely that androgens in conjunction with FSH, initiate maturation of Sertoli cells.

#### 2.6.2.2. *Paracrine regulation of Sertoli cell development*

In bulls, the role of growth factors (TGF-alpha and TGF-beta isoforms 1, 2 and 3) and interleukins (IL-1 alpha, IL-1 beta and IL-6) in the regulation of testicular development is unknown neither has their presence been reported. In rats, pigs and humans various growth factors (TGF-alpha and TGF-beta isoforms 1, 2 and 3) and interleukins (IL-1 and IL-6) have been reported to regulate Sertoli cell development and function (Takao *et al.* 1990; Calkins *et al.* 1990; Mauduit *et al.* 1992; Hoeben *et al.* 1997; Soder *et al.* 1991). In immature rat Sertoli cells TGF-alpha interacts with FSH to promote DNA

synthesis (Petersen *et al.* 2001); while in immature porcine Sertoli cells, TGF-beta 1 was shown to stimulate lactate production and glucose uptake (Esposito *et al.* 1991). Addition of IL-1 alpha to in vitro cultures of Sertoli cells, isolated from immature rats at the age of 15 days, was shown to cause Sertoli cell proliferation, the production of transferrin and to increase Sertoli cell aromatase activity (Hoeben *et al.* 1997). In immature rats, IL-6 was shown to induce the transcription of “early genes” (Jenab & Morris 1997) and basal production of transferrin in Sertoli cells (Boockfor & Schwaz 1991; Hoeben *et al.* 1997).

### **2.6.3 Germ cell development (spermatogenesis)**

During early embryonic development, primordial germ cells migrate from the embryonic yolk sac into the undifferentiated foetal gonad where they undergo several divisions prior to formation of gonocytes (Garner & Hafez 2000). In bull calves, during the early postnatal period from birth to 12 weeks of age, gonocytes and prespermatogonia are the predominant germ cells (Curtis & Amann 1981; Evans *et al.* 1995). Spermatogenesis is a complex process that involves the production of A-spermatogonia from gonocytes and the conversion of the A-spermatogonia into mature spermatozoa (Hochereau-de Reviers 1976; Curtis & Amann 1981; Amann 1983; Garner & Hafez 2000). Spermatogenesis starts with spermatocytogenesis and culminates with spermiogenesis (Garner & Hafez 2000).

During the course of spermatocytogenesis, immature germ cells undergo a series of divisions, proliferation and then finally a meiotic division in order to halve the

chromosome number (Garner & Hafez 2000). In bulls, during the course of spermatocytogenesis, type A1 spermatogonia undergo six progressive divisions (type A1, type A2, type A3, intermediate spermatogonia [type In], type B1 and type B2) prior to formation of primary spermatocytes (Berdtsen & Desjardins 1974; Hochereau-de Reviere *et al.* 1976; Hochereau-de Reviere 1981; Amann & Schanbacher 1983; Garner & Hafez 2000). Type A2 cells divide not only to produce many germ cells that eventually form sperm but also undergo a specific division to replace the stem cell population of type A1 spermatogonia (Amann & Schanbacher 1983; Garner & Hafez 2000). In adult bulls, the number of A1 spermatogonia is highly correlated with Sertoli cell numbers (Setchell 1978). The primary spermatocytes duplicate their DNA and undergo meiosis before dividing to form secondary spermatocytes (Amann & Schanbacher 1983; Garner & Hafez 2000). In bull calves, between 16 and 28 weeks of age the number of seminiferous tubules with primary and secondary spermatocytes as the most mature germ cell begins to increase (Curtis & Amann 1981).

During spermiogenesis round spermatids undergo morphological changes in order to transform into elongated spermatozoa (Garner & Hafez 2000). In bull calves, between 28 and 32 weeks of age, spermatocytes are gradually replaced by round and elongated spermatids as the most mature germ cells (Curtis & Amann 1981; Amann 1983; Evans *et al.* 1996). Amann (1983) suggested that elongated spermatids are present in semen samples collected from in bulls at the 38 weeks of age.



#### 2.6.3.1. *Endocrine regulation of germ cell development*

In bull calves, the differentiation of Leydig cells and Sertoli cells coincides with increased production of gonadotropins from the pituitary and production of testosterone from the Leydig cells (Amann 1983; McCarthy *et al.* 1979b). Spermatogenesis is initiated just after the early transient increase in serum gonadotropin concentrations and continues as serum concentrations of testosterone increase towards adult concentrations (Curtis & Amann 1981). In bull calves, indifferent supporting cells and Sertoli cells resident in the seminiferous tubules are the target cells for FSH action (Schanbacher 1979). It is therefore likely that FSH exerts its influence on germ cells via Sertoli cells. In ram lambs the proliferation of A-spermatogonia has been correlated with increased FSH secretion in prepubertal ram lambs (Courot 1967; Courot *et al.* 1979). In the rat, FSH plays an important role in the onset of spermatogenesis and is required from the initiation of spermatogonial division through the formation of the secondary spermatocytes; thereafter, testosterone takes responsibility for sperm cell formation (Steinberger & Duckett 1967; Chowdhury 1979; Steinberger 1981). Follicle stimulating hormone also induces Sertoli cell “binding capacity” to spermatids, which is needed for their maturation into spermatozoa (Thibault *et al.* 1993). Inhibition of LH and FSH secretion from the pituitary in developing rats disrupted spermatogenesis (Kolho *et al.* 1988) and disruption of Leydig cell function also affected spermatogenesis (Molenaar *et al.* 1985), indicating the importance of FSH, LH, testosterone and other Leydig cell products for spermatogenesis (Sharpe 1990).

Maturation of spermatids in bull calves takes place in stage VI of spermatogenesis, which corresponds to stage VII in rats (Clermont & Leblond 1955; Berdtson & Desjardins 1974). In rats, withdrawal of testosterone causes increased apoptosis of germ cells in these stages leading to atrophy of the seminiferous epithelium (Sharpe 1990). Estradiol treatment in dairy bull calves disrupts spermatogenesis (Deaver *et al.* 1988), and in prepubertal beef bull calves, chronic treatment with estradiol inhibits spermatogenesis and the production of testosterone by Leydig cells, apparently due to the inhibition of LH and FSH secretion from the pituitary (Weston *et al.* 1988).

#### 2.6.3.2. *Paracrine regulation of germ cell development*

The influences of intratesticular factors on spermatogenesis are exerted directly on the germ cells or indirectly via the somatic testicular cells. The expression of Epithelial Growth Factor Receptor (*EGF-R*) gene in spermatogenic cells suggests TGF-alpha may be involved in Sertoli-germ cell interactions in rats (Mullaney & Skinner 1992). TGF-beta 1 was reported to enhance the expression of a 50 KDa protein in human spermatozoa (Naz & Kumar 1991). In rats, mice and humans and IL-1 and IL-6 have been shown to have effects in the regulation of spermatogenesis and spermiogenesis (Takao *et al.* 1990; Calkins *et al.* 1990; Mauduit *et al.* 1992; Hoeben *et al.* 1997; Soder *et al.* 1991). In rats, IL-1 alpha stimulates DNA replication in stage I spermatogonia and in preleptotene spermatocytes (Parvinen *et al.* 1991; Soder *et al.* 1991) whereas IL-6 inhibits meiotic DNA synthesis in preleptotene spermatocytes and to a lesser extent in spermatogonia (Hakovirta *et al.* 1995).

#### **2.6.4. Testicular development summary**

In summary, the early transient increase in LH and FSH concentrations is followed by altered testicular steroidogenesis, increased circulating concentrations of testosterone and differentiation of the Leydig and Sertoli cells and the onset of spermatogenesis (Curtis & Amann 1981; Amann 1983). Differentiation of Leydig cells in bull calves occurs between 12 and 16 weeks of age while differentiation of Sertoli cells is restricted to the period between 16 and 28 weeks of age (Abdel-Raouf 1961; Curtis & Amann 1981; Amann 1983). Spermatogenesis in bull calves is initiated around 16 to 20 weeks of age and sperm are produced by 32 weeks of age (Macmillan & Hafts 1969; Curtis & Amann 1981). In beef bulls, puberty is attained between 38 and 46 weeks of age (Lunstra *et al.* 1978; Evans *et al.* 1995). The onset of spermatogenesis is dependent on the pituitary gonadotropins (LH and FSH) and testicular androgens (Gondos 1980; Guraya 1980; Hochereau-de Reviers *et al.* 1980; Courot & Ortavant 1981; Amann 1983). In rats, mice pigs and human, the presence of growth factors (TGF-alpha and beta) and interleukins (IL-1 and IL-6) in the testis has been established but their role as the modulators of its development is yet to be established. The presence of these factors in the bovine testis has not been studied.

## 2.7. EVALUATION OF PUBERTY IN BULL CALVES

### 2.7.1. Semen

Electroejaculation has been widely accepted for use in bull calves (Roberts 1986; Barth 2000). However, semen collected by electroejaculation will vary in volume and concentration of spermatozoa. In bull calves rectal massage over the urethra and accessory sex glands, prior to electroejaculation, improves semen collection (Garner & Hafez 2000). Proper cleanliness should be maintained to avoid contamination of the semen from the bull; a rectal probe with two or three electrodes is usually used (Roberts 1986). Electric stimulation should begin with the lowest voltage and stimuli should be given for 2 or 3 seconds; 4 to 5 stimuli should be given followed by a short rest interval of about 1 to 2 minutes (Barth 2000). The number of stimuli required to obtain semen varies with individual bulls (Roberts 1986; Barth 2000). In bull calves, semen evaluation involves the qualitative and quantitative evaluation of the ejaculate (Barth 2000). Qualitative evaluation requires establishing the density and volume, gross and individual motility, and sperm morphology of the semen collected. Quantitative evaluation involves establishing the number of sperm cells per unit volume of semen, total number of spermatozoa per ejaculate and the percentage distribution of abnormal and normal sperm morphology (Barth 2000). There is a decrease in the incidence of the abnormal morphological features of sperm cells as bulls mature; the greatest decreases are in the incidence of proximal droplets and knobbed acrosomes (Evans *et al.* 1995). The removal of proximal droplets and other changes to the acrosome occur in the epididymis and are testosterone dependent (Martig & Almquist 1969). The decrease in these abnormalities could be attributed to increasing testosterone concentrations in bulls

as they mature (Evans *et al.* 1995). Significant changes in semen characteristics, such as the decrease in abnormal sperm and the increase in individual sperm motility and numbers per ejaculate, are noted just prior to puberty (Evans *et al.* 1995). These changes continue up to 12 weeks postpuberty (Evans *et al.* 1995). In bulls, puberty is defined as the time when the semen collected contains at least 50 million spermatozoa with at least 10% linear motility (Wolf *et al.* 1965).

### **2.7.2 Age and body weight**

Body weight and age are also considered indicators of approaching puberty but should be used in association with testicular parameters and semen characteristics (Macmillan & Hafts 1969; Lunstra *et al.* 1978). The peripubertal period is not associated with marked change in the rate of body growth, but there is a good correlation between body weight and testicular weight (Macmillan & Hafts 1969; Coulter & Foote 1977). Body weight and age at puberty differ according to breed and nutritional management. In well-fed Charolais bulls, puberty occurred at a mean age of  $41 \pm 1$  weeks, and mean body weight of  $396 \pm 13$  kg (Barber & Almquist 1975). In Hereford bull calves mean age at puberty was found to be  $44 \pm 1$  weeks, ranging from 39 to 52 weeks of age and a mean body weight of  $321 \pm 12$  kg, with a wide range from 214 to 407 kg (Almquist & Cunningham 1967). In another study, in Hereford bull calves, age at puberty was reported as  $326 \pm 9$  days at a body weight of  $261 \pm 6$  kg (Lunstra *et al.* 1978).

### 2.7.3. Scrotal circumference

Measurement of scrotal circumference is a non-invasive easily obtainable method used in the assessment of puberty in maturing bulls and the evaluation of fertility in breeding soundness examinations of bulls (Willett & Ohms 1957; Hahn *et al.* 1969; Foote *et al.* 1970; Lunstra *et al.* 1978; Coulter & Foote 1979; Coulter & Keller 1982; Barth 2000). Measurements of scrotal circumference should be taken from the widest circumference of the testis with moderate tightness on the measuring tape and repeated to give accurate measurements (Coulter & Foote 1979; Barth 2000). In bulls the paired testicular weight can be estimated by measuring scrotal circumference (Coulter & Foote 1979). In sexually maturing bulls, the temporal relationship between scrotal circumference and body weight was curvilinear with a correlation coefficient of  $R = 0.81$  ( $P < 0.01$ ; Coulter & Foote 1977). In young bulls, scrotal circumference is correlated with paired testis weight ( $r = 0.95$ ; Coulter & Keller 1982), spermatozoa output (Boyd & VanDemark 1957; Willett & Ohms 1957; Hahn *et al.* 1969) and fertility (Willett & Ohms 1957; Hahn *et al.* 1969). Despite reports of a linear relationship between changes in scrotal circumference and body weight there is considerable variation in scrotal circumference among bulls of the same body weight (Coulter & Foote 1977). Correlations between SC measurements and birth weights were negative to low ( $r = -0.04$  to  $0.18$ ) (Bourdon & Brinks 1985; Kriese 1991; Smith 1989). Both weaning weights ( $r = 0.00$  to  $0.86$ ) and yearling weights ( $r = 0.10$  to  $0.68$ ) were poorly to highly correlated with SC measurements (Coulter & Foote 1979; Coulter & Keller 1982).

#### **2.7.4. Testicular histology**

Testicular development, and indirectly puberty have been evaluated by histological examination of spermatogenesis in the testicular tissue (Toppari *et al.* 1989). Unfortunately, histological evaluation is an invasive method of qualitative or quantitative evaluation of puberty or spermatogenesis, thus limiting its usefulness (Berdtson 1977; Curtis & Amann 1981; Evans *et al.* 1996). Needle biopsy of the testis can also be useful in assessing the progress of spermatogenesis in some species (Arvindan *et al.* 1990).

##### *2.7.4.1. Qualitative histological evaluation of the testis*

In bull calves, the diameter of a round seminiferous tubule and its lumen has been reported to increase with age (Curtis & Amann 1981; Evans *et al.* 1996). The rate of increase is greater after 25 weeks of age and formation of the seminiferous tubular lumen occurs around the same period (Evans *et al.* 1996). During the course of testicular development there are somatic and germ cell changes that constitute qualitative changes in the testicular parenchyma (Curtis & Amann 1981; Evans *et al.* 1996). Due to the cyclic nature of spermatogenesis any given area within the seminiferous tubule has a well defined series of events that occur over time and which follow each other in a precise orderly sequence (Berndtson 1977). These events occur at extremely well timed intervals with respect to one another and give rise to a precise number of distinct cellular associations, each consisting of one or two generations of spermatogonia, spermatocytes and spermatids (Clermont 1972; Berndtson 1977; Curtis & Amann 1981; Berndtson & Igboeli 1989). The sequence of events occurring from the

disappearance of a given cellular association to its reappearance constitutes one cycle of the seminiferous epithelium (Clermont 1972). As a result, in a cross section of any randomly selected round seminiferous tubule, the epithelium holds a precise set of germ cell associations at different stages of development which could include any of the following cell types; type A or B spermatogonia; primary spermatocytes; secondary spermatocytes; rounded spermatids and/or elongated spermatids (Berndtson & Desjardins 1974; Berndtson 1977; Curtis & Amann 1981; Berndtson & Igboeli 1989). In bulls, there are 12 such germ cell associations or stages of the cycle of the seminiferous epithelium (Berndtson & Desjardins 1974; Berndtson 1977; Curtis & Amann 1981). In prepubertal calves and early pubertal bulls complete germ cell associations as reported by Berndtson & Desjardins (1974) are not seen and some germ cells are present with cell types different from those they are associated with in the adult (Curtis & Amann 1981; Evans *et al.* 1996). In order to circumvent this dilemma, during qualitative evaluation of the developing testis, each randomly selected round tubular cross section is classified on the basis of the most advanced germ cell type present (Curtis & Amann 1981; Evans *et al.* 1996). The relative differences between treatments or developing bull calves can then be compared

#### 2.7.4.2. *Quantitative histological evaluation of testis*

Clermont and Morgentaler (1955) proposed a method for measuring relative spermatozoa production rates, in which one stage of the cycle was selected to be representative of spermatogenesis as a whole (Clermont & Harvey 1967). The determination of relative spermatozoa production rates by testicular histology has been



reported in bulls (Berndtson 1977; Curtis & Amann 1981; Berndtson & Igboelli 1989). Germ cell and Sertoli cell nuclei or their identifiable fragments were enumerated in round seminiferous tubular cross sections of both experimental and control animals. The number of nuclei/cells counted was designated the crude count [CC], this count required adjustment for two factors: nuclei (since nuclei of larger diameters were more likely to be counted in sections of any thickness); and thickness of the section (since variation in thickness can introduce errors). Abercrombie (1946) developed a procedure by which crude counts can be adjusted to permit their direct comparison. This correction is only valid for a spherical structure in a histological section. Abercrombie's formula for the true count [TC] is as follows:

$$TC = CC \times [\text{Section thickness } (\mu) / \text{Section thickness} + \text{nuclear diameter } (\mu)]$$

Correction can be made for changes in histological processing such as shrinkage, by expressing the germ cells in terms of Sertoli cell ratios also referred to as a Sertoli cell correction factor (Clermont & Morgentaler 1955) This correction is based on the fact that Sertoli cells rarely divide in adult animals and are extremely resistant to other factors that affect germ cells (Berndtson *et al.* 1977). The number of Sertoli nuclei should be corrected for true counts.

Calculation of daily spermatozoa production has been done from the different cell types (Berdtson 1977). This involves the determination of the total testes weight at castration and the number of sperm per gram of testicular parenchyma. In the latter, a fixed weight

of testicular tissue is homogenized in a known volume of saline, then the total number of elongated sperm in the homogenate determined. The later is then divided by the known homogenized testicular weight in order to establish the number of elongated sperm per gram of testicular tissue. In order to establish the total number of elongated sperm per bull, the testes weight determined at castration is multiplied by the number of elongated spermatids per gram of testis. The value obtained on a whole testes weight is then converted to daily sperm production by use of a time divisor of 5.2 days, this division is equivalent to the number of days it takes for a spermatid to form a spermatozoa in bulls (Amann & Almquist 1961). Later several authors reported methods in which the daily spermatozoa production was estimated from different germ cell stages such as round spermatids in stage VIII of the seminiferous epithelium cycle (Amann & Almquist 1962; Swierstra 1966).

## 2.8. HYPOTHESES AND OBJECTIVES

### 2.8.1. Hypotheses

The hypotheses tested in this thesis were:

1. Changes in testicular gonadotropin receptor numbers and their affinity to serum gonadotropins, during development would enhance the role of the early postnatal increase in serum concentrations of LH and FSH and support rapid testicular growth, when serum LH and FSH concentrations are low.
2. Transformation growth factors (TGF-alpha, TGF-beta isoforms 1, 2, and 3) are produced in an age dependent pattern by the developing bovine testis

3. The temporal changes in concentrations of serum IGF-1, testicular TGF-alpha and TGF-beta isoforms 1, 2, and 3 occur at significant developmental points during testicular development.
4. Interleukins IL-1 (alpha and beta) and IL-6 are produced in an age dependent pattern by the developing bovine testis
5. The temporal changes in testicular concentrations of interleukins IL-1 (alpha and beta) and IL-6 occur at significant developmental points during testicular development.
6. Treatment of bull calves subcutaneously with gonadotropins (LH and FSH) from 4 to 8 weeks of age, prior to the early rise in endogenous serum gonadotropin concentrations, hastens their attainment of puberty.

### **2.8.2. Objectives**

The objectives of the studies conducted in this thesis were to determine if:

#### Experiment 1 (Chapter 3)

Changes in gonadotropin receptor concentrations and affinity occur in a pattern that could be related to the early postnatal increase in serum LH and FSH concentrations, differentiation of Sertoli cells, the onset and progression of spermatogenesis, and the period of rapid testicular growth, which occurs while serum LH and FSH concentrations are low.

#### Experiment 2 (Chapter 4)

(1) TGF-alpha, TGF-beta isoforms 1, 2, and 3 are produced in the developing bovine testis; (2) there are temporal changes in concentrations of serum IGF-1 and testicular TGF-alpha, TGF-beta isoforms 1, 2, and 3 during postnatal reproductive development; (3) shifts in concentrations occur at significant developmental time points in testicular maturation.

#### Experiment 3 (Chapter 5)

(1) IL-1 and IL-6 are produced in the developing bovine testis; (2) there are temporal changes in testicular concentrations during postnatal reproductive development; (3) shifts in concentrations occur at significant developmental time points in testicular maturation.

#### Experiment 4 (Chapter 6)

Treatment of early postnatal bull calves, prior to the early increase in gonadotropin concentrations, with supra-physiological doses of gonadotropins (LH or FSH), in a more practical but longer treatment regime as compared to the GnRH regime used by Chandolia *et al.* (1997c) (1) would hasten the onset of puberty (2) have long term effects on spermatogenesis and fertility.

**Chapter 3: POSTNATAL CHANGES IN TESTICULAR GONADOTROPIN  
RECEPTORS, SERUM GONADOTROPIN, AND TESTOSTERONE  
CONCENTRATIONS AND FUNCTIONAL DEVELOPMENT OF THE TESTES  
IN BULLS**

Bagu E T, Cook S, Gratton CL and Rawlings NC

**3.1. Abstract**

The objectives of this study were to follow the temporal patterns of testicular LH and FSH receptor (LH-R and FSH-R) concentrations and affinity ( $K_a$ ) during sexual maturation in bulls and to see if such patterns could help explain the control of rapid testicular growth that occurs after 25 weeks of age, when serum gonadotropin concentrations are low. Separate groups of Hereford x Charolais calves ( $n = 6$ ) were castrated every 4 weeks from 5 to 33 weeks of age and at 56 weeks of age. A week prior to castrations, from 5 to 33 weeks of age, blood was collected every 15 minutes for 10 h. The transition from indifferent supporting cells to Sertoli cells in seminiferous tubules was rapid between 13 and 25 weeks and rapid testis growth occurred after 25 weeks of age. Serum LH and FSH concentrations were transiently elevated at 12 weeks of age ( $P < 0.05$ ). LH-R concentrations decreased from 13 to 25 weeks of age and increased to 56 weeks of age ( $P < 0.05$ ). LH-R $K_a$  decreased from 9 to 17 weeks of age, increased to 29 weeks of age and declined to 33 weeks of age ( $P < 0.05$ ). FSH-R

concentrations declined from 17 to 25 weeks of age then increased to 56 weeks of age ( $P < 0.05$ ). FSH-RK $\alpha$  increased from 17 to 25 weeks of age ( $P < 0.05$ ). High concentrations of gonadotropins and their receptors may be critical to initiate testis growth postnatally and support it after 25 weeks of age in the face of low serum gonadotropin concentrations.

### **3.2. Introduction**

In bulls, puberty has been defined as the time when an ejaculate has at least  $5.0 \times 10^7$  sperm and a 10% linear motility (Wolf *et al.* 1965). Age at puberty varies with breed: in Hereford bulls it occurs between 39 and 52 weeks of age, in Charolais bulls between 33 and 53 weeks of age, and in Holstein bulls between 39 and 41 weeks of age (Wolf *et al.* 1965; Killian & Amann 1972; Lunstra *et al.* 1978). In bull calves, serum Luteinising Hormone (LH) concentrations are transiently increased between 4 and 25 weeks of age, with minimal or no subsequent increase prior to puberty (McCarthy *et al.* 1979a, b; Amann *et al.* 1986; Wise *et al.* 1987; Evans *et al.* 1993, 1996). In developing bulls, the patterns of serum FSH concentrations are variable; with reports of no change with time (McCarthy *et al.* 1979a, b), a slight increase in the early postnatal period (Rawlings & Evans 1995; Aravindakshan *et al.* 2000a, b), and an increase from 4 to 32 weeks of age (Amann & Walker 1983). During the early rise in serum LH concentrations, serum testosterone concentrations are low, increasing markedly after 28 weeks of age and reaching adult concentrations by 40 weeks of age (Rawlings *et al.* 1972, 1978; Secchiari *et al.* 1976; Amann & Walker 1983).

In males, there are two distinct populations of Leydig cells: foetal and adult Leydig cells, which differentiate from mesenchymal cells prenatally and postnatally respectively (Mendis-Handagama & Ariyaratne 2001). In bull calves, it was suggested that the demise of foetal Leydig cells occurred during the early postnatal period (Hooker 1970). Amann (1983) suggested that the high frequency of LH pulses that occurred in bull calves after 4 weeks of age, initiated differentiation and maturation of adult Leydig cells from progenitor cells around 12–16 weeks of age, and a switch from androstenedione to testosterone secretion. The elevated intratesticular testosterone concentrations, following the onset of Leydig cell differentiation, may initiate differentiation of indifferent supporting cells to Sertoli cells; differentiation is restricted to the period of 16 to 28 weeks of age (McCarthy *et al.* 1979a, b; Curtis & Amann 1981; Amann 1983). Subsequent increases in serum testosterone concentrations after 28 weeks of age probably reflect enhanced production by Leydig cells or increased Leydig cell numbers (Amann 1983). In bull calves, spermatogenesis is initiated as early as 16 weeks of age and the appearance of elongated spermatids in the seminiferous tubules at 32 weeks of age marks the initial achievement of complete spermatogenesis (Curtis & Amann 1981). Rapid testicular growth that occurs after 25 weeks of age is, therefore, a reflection of the above changes. It is unclear whether the transient early postnatal increase in serum LH concentrations is sufficient to trigger rapid testicular development or how important the subsequent low concentrations of LH and FSH are. There could also be important regulatory changes in the testis itself from 25 weeks of age to puberty that are critical for rapid testicular growth (Amann *et al.* 1986; Wise *et al.* 1987; Evans *et al.* 1993, 1996; Rawlings & Evans 1995).

During sexual development, an increase in testicular Luteinising Hormone Receptor (LH-R) concentrations was reported in rats (Purvis *et al.* 1977; Hardy *et al.* 1990; Shan & Hardy 1992) and dogs (Inaba *et al.* 1994). In the latter, the increase coincided with rapid testicular growth. In rams, testicular LH-R concentrations increase from 90 to 150 days of age (Yarney & Sanford 1989). In the two studies with bulls, testes were either collected from one separate individual Norwegian red calf every 4 weeks from 12 to 32 weeks of age, and at 40, 52, and 68 weeks of age (Sundby *et al.* 1984) or from Holstein calves at 1, 7, 14, and 56 days of age (n = 5); 12 weeks of age (n = 4) and 24 weeks of age (n = 3); however, no samples were collected during the period of rapid testicular growth or just prior to puberty (Dias & Reeves 1982). In these studies, gonadotropin receptor concentrations decreased with age, while binding affinities remained the same. Sample size and timing precluded a careful examination of gonadotropin receptor changes around the time of the early postnatal increase in serum gonadotropin concentrations or the period of rapid testicular growth.

The objectives of the present study were to determine if changes in gonadotropin receptor concentrations and affinity were related to the early postnatal increase in serum LH and FSH concentrations, differentiation of Sertoli cells, the onset and progression of spermatogenesis, and the period of rapid testicular growth, which occurs while serum LH and FSH concentrations are low.



### **3.3. Materials and methods**

#### *3.3.1. Animals and experimental procedures*

Fifty-four spring-born, age matched ( $\pm 3$  days) bull calves (Hereford x Charolais) were divided into 9 groups of 6 calves each. Calves were suckled at pasture until they were weaned at 26 weeks of age. After weaning, calves were kept in corrals and provided with water and a standard feed ration ad libitum (Evans *et al.* 1995). Calf body weights were measured bi-weekly until castration. All experimental procedures were done in accordance with the regulations of the Canadian Council for Animal Care.

#### *3.3.2. Blood and testes collection*

Separate groups of calves ( $n = 6$ ) were castrated every 4 weeks from 5 to 33 weeks of age, and at 56 weeks of age. A week prior to castration, from 5 to 33 weeks of age, blood samples (5 ml) were collected every 15 minutes for 10 hours from the jugular vein by an intravenous catheter, starting at 08:00 hours (Evans *et al.* 1995). Blood samples were left to clot for at least 12 hours at room temperature and then serum was harvested and frozen at  $-20^{\circ}\text{C}$  until assayed. At castration, the testes were weighed and a tissue sample from the mid section of the right testis of each bull was fixed in Helly's reagent [ $\text{HgCl}_2$  (70 g/L) and  $\text{K}_2\text{Cr}_2\text{O}_7$  (25 g/L) (VWR international Ltd, Edmonton, Alberta, Canada) were dissolved in warm distilled water and formaldehyde (40%) was then added immediately prior to fixing the testis (VWR international Ltd, Edmonton, Alberta, Canada)]. The rest of the right and left testes were plunged into liquid nitrogen for approximately 20 minutes and stored at  $-70^{\circ}\text{C}$  until homogenisation.

### 3.3.3. *Radioimmunoassays*

Serum LH and FSH concentrations were determined using previously validated double-antibody radioimmunoassays (Rawlings & Evans 1995). The LH concentrations are expressed in terms of NIDDK-bLH4. Sensitivity of the LH assay, defined as the lowest concentration of LH capable of significantly displacing labelled LH from the antibody (t-test), was 0.1 ng/ml. Intra or inter assay coefficients of variation (CVs) for the LH assay were 7.1% and 8.6% or 12.7% and 11.9% for reference sera with LH concentrations of 0.4 ng/ml or 0.8 ng/ml, respectively. The sensitivity of the FSH assay was 0.1 ng/ml and concentrations are expressed in terms of USDA-bFSH-I1. Intra or inter assay CVs for the FSH assay were 6.4% and 5.7% or 17.5% and 18.8% for reference sera with FSH concentrations of 1.9 ng/ml or 6.8 ng/ml, respectively. Serum testosterone concentrations were determined in a single pool of samples collected from each intensive bleed using a commercial, double-antibody radioimmunoassay kit (Coat A-Count total testosterone, Diagnostics Product Corporation, Los Angeles, CA). The testosterone assay sensitivity was 0.04 ng/ml. The intra assay CVs were 19.7%, 12.7% and 6.6% for reference sera with testosterone concentrations of 1.2, 2.7 and 7.0 ng/ml respectively.

### 3.3.4. *Bovine testicular LH and FSH receptor membrane fractions*

A frozen section (6 g) from the right testis of each of the bull calves castrated at 4 week intervals from 5 to 33 weeks of age and at 56 weeks of age (n = 6 per age group), was homogenized to obtain membrane fractions using a published procedure (Sairam 1978; Yarney and Sairam 1989). The testicular parenchyma was weighed, diced, and

homogenized at maximum speed for 2.5 minutes, using a Brinkmann polytron® (PT 10203500, Steinhofhalde 22 Switzerland) in 25 mM-Tris-HCL buffer, containing 100 mM sucrose at pH 7.5. Five ml of buffer were used per gram of tissue. All procedures, unless otherwise indicated, were carried out at 4 °C. The homogenate was centrifuged at 500 x g for 30 minutes and the supernatant decanted into a fresh polystyrene tube and re-centrifuged at 39,000 x g for 1 hour. The pellet was re-suspended in 25 mM-Tris-HCL buffer at pH 7.5, containing 10 mM MgCl<sub>2</sub>, at a concentration of 1 g of original tissue per ml. Aliquots of these fractions were stored at -70 °C. Prior to assay, membrane fractions were gently pulsed to obtain a uniform suspension. The protein concentration in the testicular membrane fractions were determined by a Bio Rad Assay (Bio Rad, Richmond, CA, USA) using bovine serum albumin (BSA) as a standard.

### 3.3.5. Radio-receptor assay

The radio-receptor assay was a modification of published methods (Sairam 1978; Yarney *et al.* 1988; Yarney & Sairam 1991). Bovine LH (USDA-bLH-I1) or FSH (NIDDK-AFP5332B) was iodinated (<sup>125</sup>I) (Amersham Biosciences, Baie d'Urfe' Quebec, Canada) using published methods (Sairam 1978; Yarney & Stanford 1989). When 730 ng of <sup>125</sup>I-bLH and 491 ng of <sup>125</sup>I bFSH were incubated with an excess of bovine testicular membrane preparations total binding ranged from 12% to 13.7% and 17% to 19%, respectively. The specific activity of <sup>125</sup>I-bLH or <sup>125</sup>I-bFSH was 0.162 mCi/μg or 0.192 mCi/μg, respectively. To determine the affinity constant (K<sub>a</sub>) and receptor concentrations by Scatchard analysis, 3 serial dilutions of 120 ± 2, 243 ± 3 and 364 ± 4 CPM/μl of <sup>125</sup>I-bLH (USDA-bLH-I1) and 99 ± 2, 194 ± 1 and 291 ± 2 CPM/μl

of  $^{125}\text{I}$ -bFSH (NIDDK-AFP5332B) were used in the assay with each testicular homogenate. All incubations for the radio receptor assays were done in triplicate in borosilicate glass test tubes (12 x 75 mm; VWR West Chester, PA 19380). To determine total binding, 300  $\mu\text{l}$  of  $^{125}\text{I}$  bLH or  $^{125}\text{I}$  bFSH was added to 100  $\mu\text{l}$  of testicular membrane fractions, the volume was made-up to 500  $\mu\text{l}$  with 100  $\mu\text{l}$  of buffer (25 mM-Tris-HCL buffer at a pH 7.5, containing 10 mM  $\text{MgCl}_2$  and 0.1% BSA). In order to determine the non-specific binding (NSB) for each of the testicular membrane fractions, 100  $\mu\text{l}$  of buffer containing 1500 ng of non radiolabelled bLH or bFSH was added. Specific binding was defined as the difference between the total binding and non-specific binding and was approximately  $37 \pm 3\%$  and  $61 \pm 1\%$  of total binding for LH and FSH, respectively. The assays were incubated 24 hours at 4 °C. Incubation was terminated by adding 2.5 ml of 25 mM-Tris-HCL buffer (pH 7.5, containing 10 mM  $\text{MgCl}_2$ , 0.1% BSA and 8% Polyethylene Glycol) and test tubes were immediately centrifuged at 4000 x g for 25 minutes in a Beckman JS-5.2 Rotor (Beckman instruments Inc. Palo Alto, CA, USA). The supernatant was decanted and the radioactivity in the pellet determined using a gamma counter (efficiency 80%; Apex gamma counter, Titertek instruments Inc. Huntsville, AL, USA). Affinity constants ( $K_a$ ) and binding capacities were determined by analyzing the binding data using a nonlinear regression analysis or Scatchard analysis (Sigma Stat for Windows®, version 1.0; Jadel Corporation, San Rafael, CA, USA) similar to Munson *et al.* (1980). After the ratio of receptor bound radiolabelled hormone to free radiolabelled hormone (B/F) was plotted against the receptor bound radiolabelled hormone (B), a nonlinear curve was fitted to determine the slope or the binding affinity ( $K_a$ ) and x-intercept (binding capacity). The

receptor concentrations were then expressed as moles per mg of protein (specific binding capacity). The inter assay CV for the binding capacity and  $K_a$  were determined by replicating pooled testicular membrane fractions collected at 56 weeks of age, in all assays, as an internal control. The inter assay CV for LH-R with a mean specific binding capacity and  $K_a$  of  $4.8 \pm 0.5$  pM/mg of protein and  $1.82 \pm 0.1 \times 10^{-10} \text{ M}^{-1}$ ; were 16.2% and 13.5%, respectively. The inter assay CV for FSH-R with a mean specific binding capacity and  $K_a$  of  $9.8 \pm 0.7$  pM/mg of protein and  $2.0 \pm 0.1 \times 10^{-10} \text{ M}^{-1}$ , were 12.8% and 7.4%, respectively.

In the bovine LH-R assay 21 or 45% displacement of  $^{125}\text{I}$ -bLH was caused by 10 or 100 ng of bLH/tube (USDA-bLH-I1) respectively. No displacement was seen with 100 ng of either bovine prolactin (AFP 4835b) or bovine growth hormone (bGH AFP 11182B); however, 100 ng/tube of bovine FSH (USDA-bFSH-I-1) and bovine TSH (NIADDK bTSH I3) caused 14 and 16% displacement, respectively. In the bovine FSH-R assay 30 or 51% displacement of  $^{125}\text{I}$  bFSH was caused by 10 or 100 ng of bFSH/tube respectively, (USDA-bFSH-I-1). No displacement was seen with 100 ng of either bovine prolactin (AFP 4835b) or bovine growth hormone (bGH AFP 11182B); however, 100 ng/tube of bovine LH (USDA-bLH-I1) and bovine TSH (NIADDK bTSH I3) caused 24 and 23% displacement, respectively.

### 3.3.6. *Histology*

Sections of testicular parenchyma were stored in Helly's reagent for 24 hour, rinsed in water for 48 hour, washed in 70% ethanol, then dehydrated in alcohol and embedded in

paraffin wax. A 5  $\mu\text{m}$  thick section from each of the bull calves castrated at 4 week intervals from 5 to 33 weeks of age and at 56 weeks of age ( $n = 6$  per age group) was stained with haematoxylin and periodic acid Schiff's (H-PAS) and evaluated for development of spermatogenesis. A systematic, uniform, random sampling technique as described by Curtis and Amann (1981) and Evans *et al.* (1995), was used to select 20 round seminiferous tubules in the section from each bull. In each seminiferous tubule, at 1000 x magnification, Sertoli cells and their progenitor indifferent supporting cells were enumerated and the most mature germ cell identified; the tubule was classified according to the most mature germ cell in it. This classification was based on a scheme of 7 groups of germ cell types: 1) gonocytes; 2) pre-spermatogonia; 3) A- and B-spermatogonia; 4) primary spermatocytes; 5) secondary spermatocytes; 6) round spermatids; 7) elongating spermatids. The percentage of tubules with a particular germ cell type as the most mature germ cell was determined by multiplying the number of seminiferous tubules with a particular germ cell type as the most mature germ cell by 100% and then dividing the product by 20 (the total number of seminiferous tubules evaluated from the section of each bull). Sertoli cells and their precursor cells were only evaluated in sections collected from at 5, 13, 21, 25, 33 and 56 wk of age. Testicular germ cells and somatic cells were identified according to published descriptions (Berndston & Desjardins 1974; Curtis & Amann 1981). All the histological evaluations were done by a single operator in a random order without knowledge of the age groups.

### 3.37. *Data Analysis*

The PC-pulsar program was used to determine serum LH and FSH secretory characteristics in blood samples collected every 15 minutes for 10 hours (J. Gitzen and V Ramirez, University of Illinois, Urbana, IL). Serum LH pulse frequency and amplitude as well as basal and mean serum concentrations are presented. The secretory patterns of FSH were judged to be non-pulsatile, therefore mean concentrations are presented. The LH pulses were defined using standard deviation criteria of height (G Values) and duration (Merriam & Wachter 1982). Data for LH pulse amplitude, LH pulse frequency, basal and mean LH concentrations, mean FSH concentrations, mean testosterone concentrations, testicular weight, testicular gonadotropin receptor concentration per mg of protein, gonadotropin receptor  $K_a$ , the percentage distribution of the most mature germ cell in each of the 20 seminiferous tubules, indifferent supporting cell counts per seminiferous tubule, Sertoli cell counts per seminiferous tubule and body weight were analysed for effects of age by one-way analysis of variance (one-ANOVA, Sigma Stat for Windows®, version 1.0; Jadel Corporation, San Rafael, CA, USA). If main effects were significant, paired comparisons were made using the Fisher LSD method for post ANOVA multiple comparisons ( $P < 0.05$ ). Pearson correlations were used to examine the relationships between mean serum LH concentrations; serum LH pulse frequency; mean serum FSH concentrations; mean serum testosterone concentrations; testicular LH-R concentrations; LH-R affinity constant; testicular FSH-R concentrations; testicular FSH-R affinity constant; testicular weight; indifferent supporting cell counts per seminiferous tubule and Sertoli cell counts per seminiferous tubule.

### **3.4. Results**

#### *3.4.1. Body weights and testicular growth*

Mean body weight increased from 5 to 21 weeks of age and from 25 to 29 weeks of age then subsequently from 33 to 56 weeks of age ( $P<0.05$ ; Figure 3.1). Mean testicular weight increased from 9 to 21 weeks of age and then increased from 25 to 56 weeks of age ( $P<0.01$ ; Figure 3.1).

#### *3.4.2. Hormonal patterns*

Mean serum LH concentrations increased from 4 to 12 weeks of age then declined to 20 weeks of age ( $P<0.05$ ; Figure 3.2a). Basal serum LH concentrations increased from 8 to 12 weeks of age then declined to 16 weeks of age ( $P<0.05$ ; Figure 3.2b). The LH pulse frequency increased from 4 to 12 weeks of age and then declined to 16 weeks of age ( $P<0.05$ ; Figure 3.2c). The LH pulse amplitude declined from 24 to 28 weeks of age ( $P<0.05$ ; Figure 3.2d). Mean serum FSH concentrations declined from 12 to 20 weeks of age ( $P<0.05$ ; Figure 3.3). Mean serum testosterone concentrations increased from 8 to 20 weeks of age, declined to 28 weeks of age and then subsequently increased from 28 to 32 weeks of age ( $P<0.01$ ; Figure 3.4).

#### *3.4.3. Testicular gonadotropin receptor patterns*

The mean testicular LH-R concentrations (pM/mg of protein) decreased from 13 to 25 weeks of age and then increased to 56 weeks of age ( $P<0.05$ ; Figure 3.5a). Mean testicular LH-R concentrations (pM/mg of protein) were positively correlated with LH



pulse frequency and mean serum concentrations of LH and FSH ( $P<0.05$ ; r-values of 0.47, 0.51 and 0.51, respectively). Testicular LH-RK<sub>a</sub> decreased from 9 to 17 weeks of age, increased to 29 weeks of age and then declined to 33 weeks of age ( $P<0.05$ ; Figure 3.5b). Testicular LH-RK<sub>a</sub> was negatively correlated with mean serum LH concentrations, LH pulse amplitude and mean testicular LH-R concentrations (pM/mg of protein) ( $P<0.05$ ; r-values - 0.32, - 0.32 and - 0.31, respectively). The mean testicular FSH-R concentrations (pM/mg of protein) declined from 17 to 25 weeks of age then increased to 56 weeks of age ( $P<0.05$ ; Figure 3.6a). Mean testicular FSH-R concentrations (pM/mg of protein) were positively correlated with mean testicular LH-R concentrations (pM/mg of protein) and mean testicular weight ( $P<0.05$ ; r-values of 0.53 and 0.63, respectively). The testicular FSH-RK<sub>a</sub> increased from 17 to 25 weeks of age ( $P<0.05$ ; Figure 3.6b). Testicular FSH-RK<sub>a</sub> was positively correlated with mean testicular weight ( $P<0.05$ ; r-value 0.4); however, it was negatively correlated with mean testicular FSH-R concentrations (pM/mg of protein), from 5 to 33 weeks of age ( $P<0.01$ ; r-value -0.43).

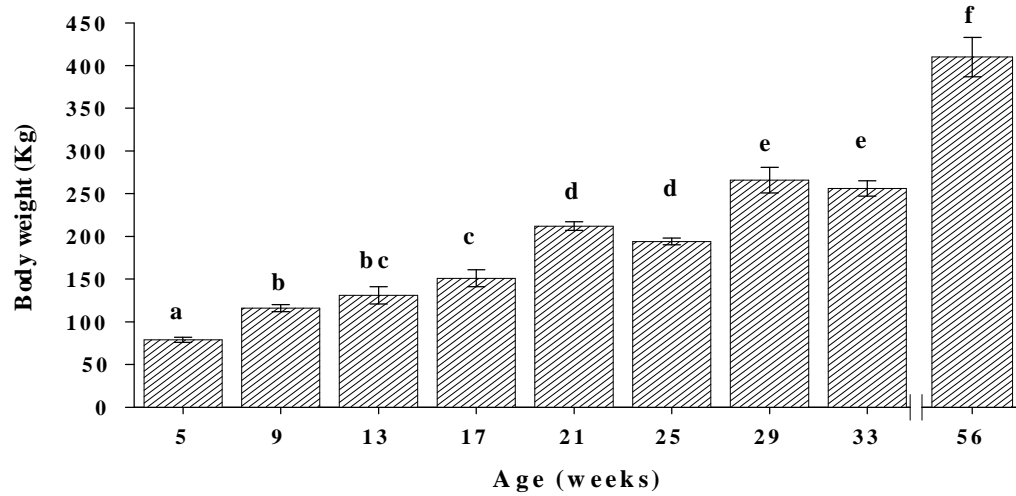
#### 3.4.4. Sertoli cell development

The mean indifferent supporting cell (immature Sertoli cell) count per seminiferous tubule decreased from 13 to 33 weeks of age ( $P<0.05$ ; Figure 3.7). As the indifferent supporting cells differentiated into mature Sertoli cells, the mean Sertoli cell count per seminiferous tubule increased from 13 to 33 weeks of age ( $P<0.05$ ; Figure 3.7).

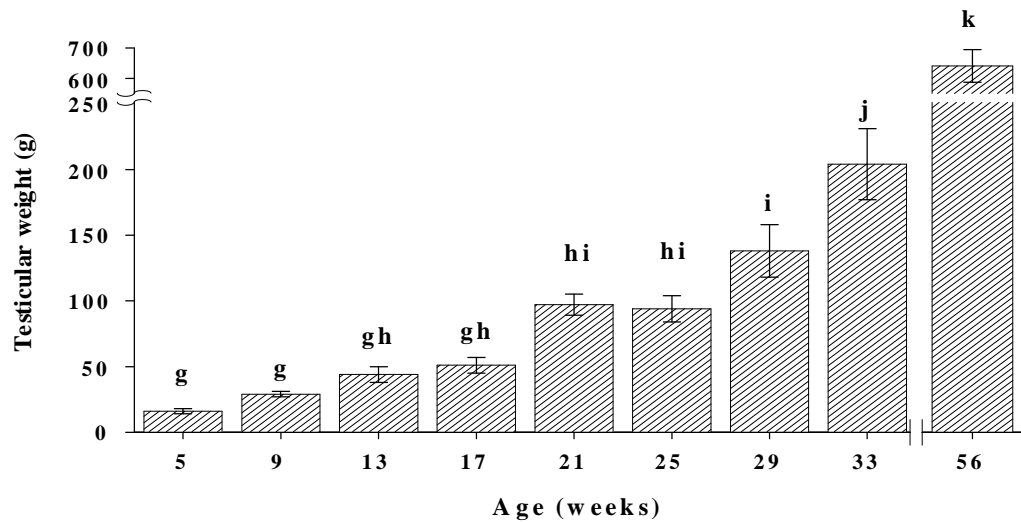
#### 3.4.5. *Germ cell development*

The percentage of seminiferous tubules with gonocytes as the most mature germ cell type decreased from 5 to 13 weeks of age and from 17 to 21 weeks of age ( $P < 0.01$ ; Table 3.1). The percentage of seminiferous tubules with pre-spermatogonia as the most mature germ cell type decreased from 5 to 9 weeks of age and from 17 to 21 weeks of age ( $P < 0.01$ ). The percentage of seminiferous tubules with A- and B-spermatogonia as the most mature germ cell type increased from 5 to 13 weeks of age and decreased from 13 to 25 weeks of age ( $P < 0.01$ ; Table 3.1). The percentage of seminiferous tubules with primary spermatocytes as the most mature germ cell type increased from 17 to 25 weeks of age and then decreased from 33 to 56 weeks of age ( $P < 0.01$ ; Table 3.1). The percentage of seminiferous tubules with secondary spermatocytes as the most mature germ cell type increased from 21 to 25 weeks of age ( $P < 0.01$ ; Table 3.1). The percentage of seminiferous tubules with round spermatids as the most mature germ cell type increased from 21 to 25 weeks of age and from 29 to 33 weeks of age, then subsequently decreased from 33 to 56 weeks of age ( $P < 0.01$ ; Table 3.1). The percentage of seminiferous tubules with elongated spermatids as the most mature germ cell type increased from 21 to 25 weeks of age and from 33 to 56 weeks of age ( $P < 0.01$ ; Table 3.1).

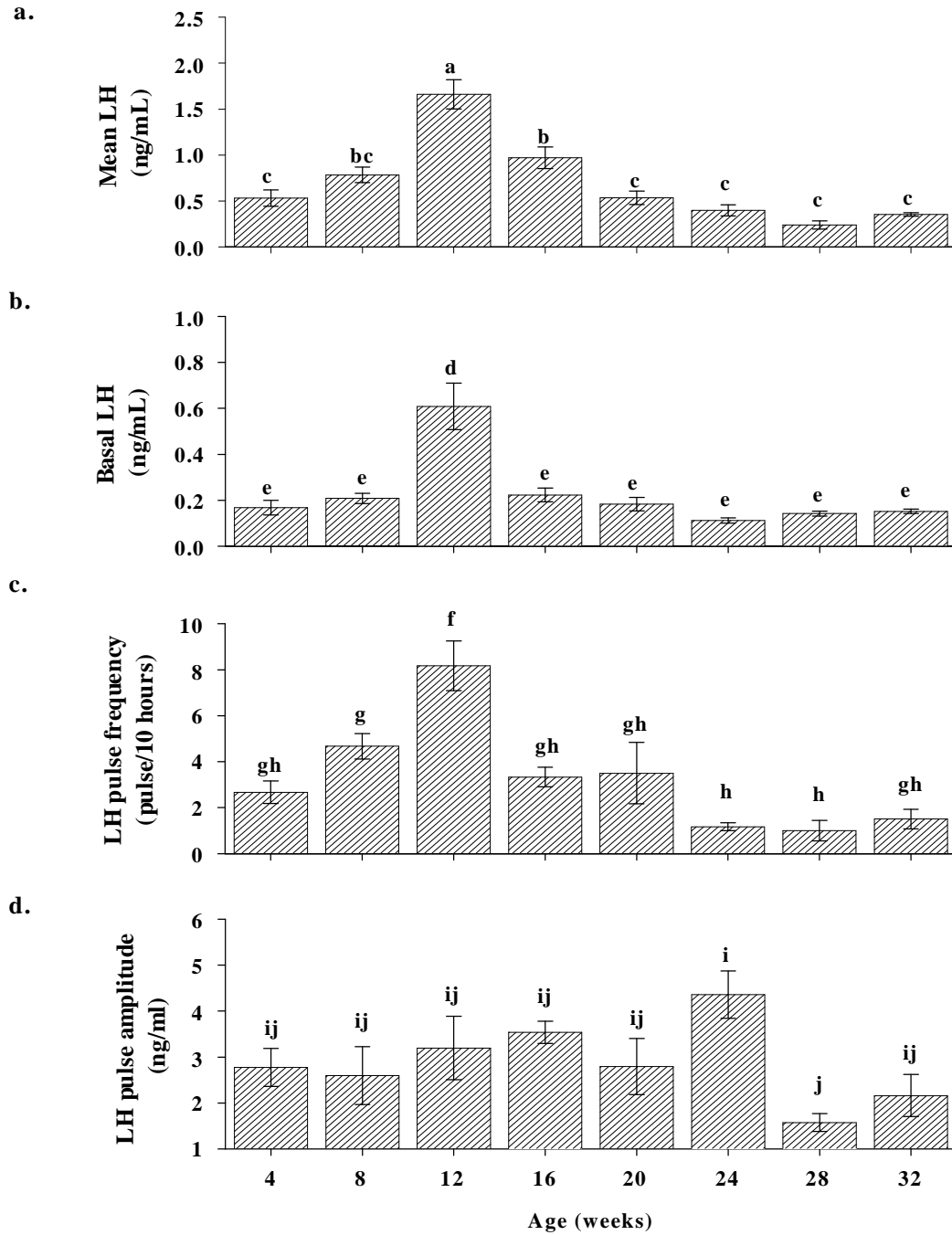
(a)



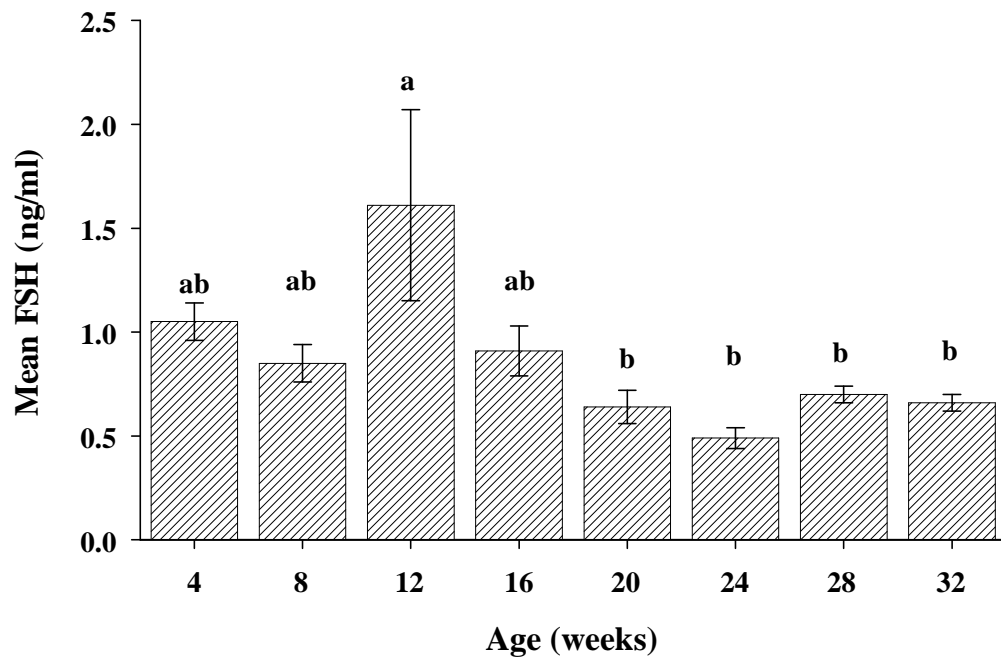
(b)



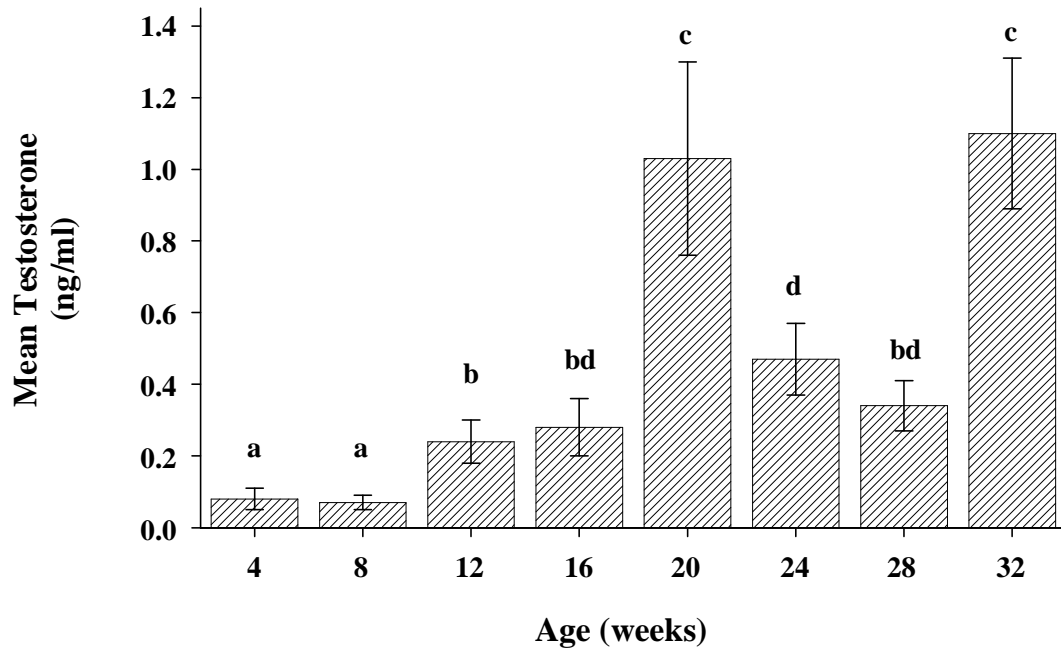
**Figure 3. 1** Mean ( $\pm$  SEM) (a) body weight and (b) testicular weight of bull calves. Separate groups of bull calves ( $n = 6$ ) were weighed and then castrated at 4 week intervals from 5 to 33 weeks of age and at 56 weeks of age. The testes (right and left) were weighed at castration. The differences between ages within variables are indicated by different superscripts ( $P < 0.05$ ).



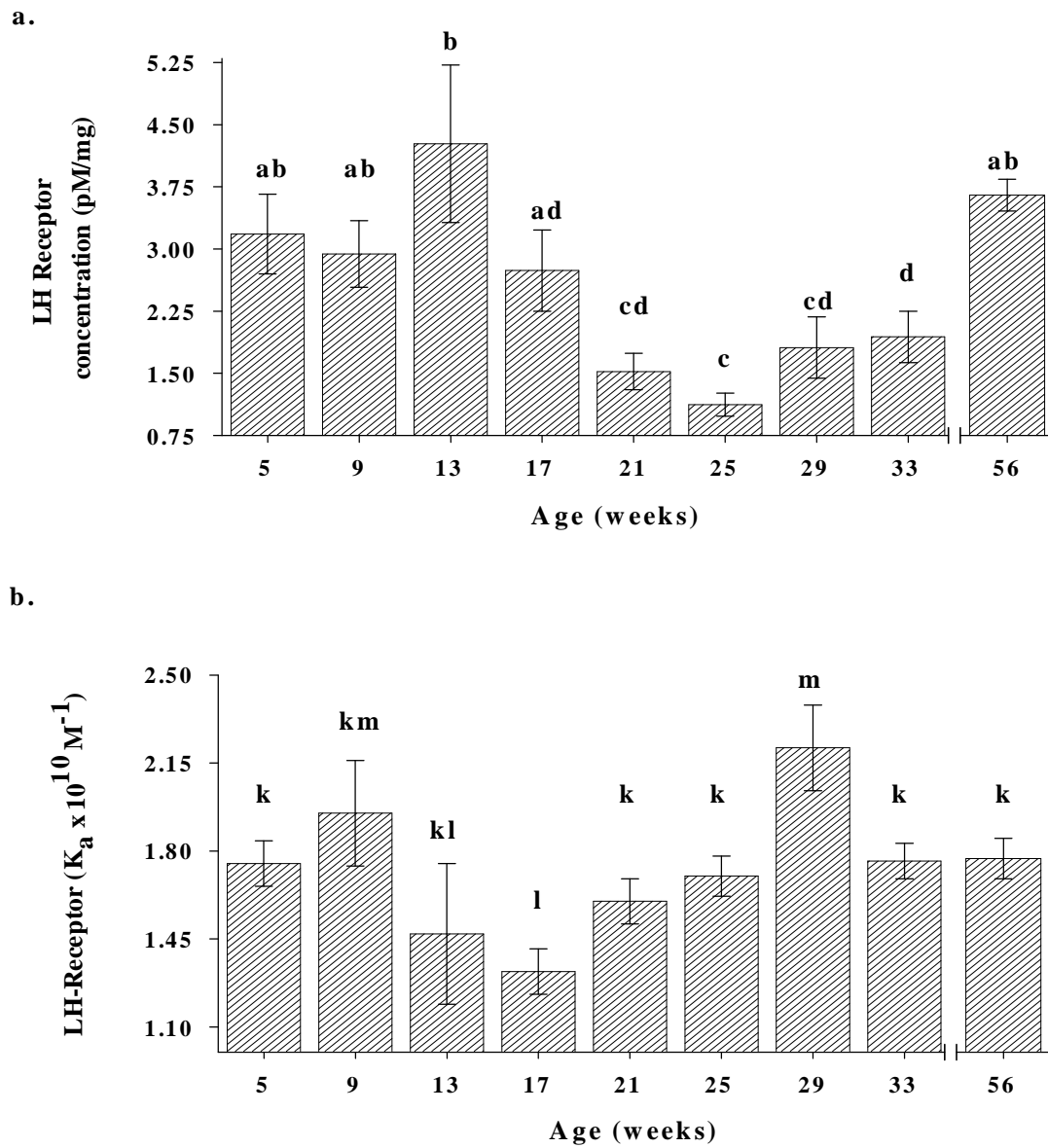
**Figure 3.2** Mean ( $\pm$  SEM) (a) serum LH concentrations, (b) basal LH concentrations, (c) LH pulse frequency and (d) LH pulse amplitude in blood samples collected every 15 minutes for 10 hours, at 4 week intervals from 4 to 32 weeks of age, from separate groups of calves ( $n = 6$ ). The differences between ages within variables are indicated by different superscripts ( $P < 0.05$ ).



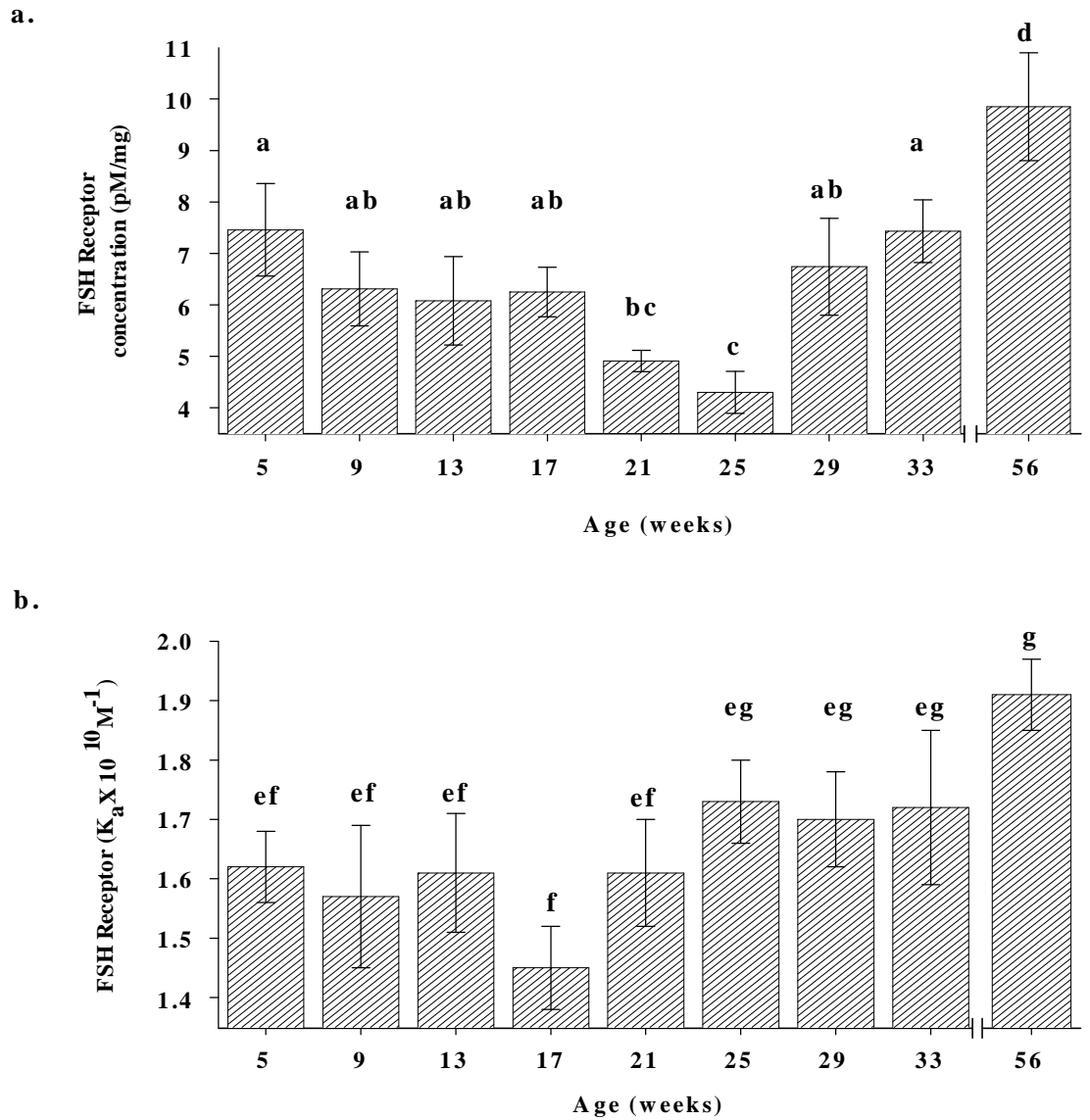
**Figure 3.3** Mean ( $\pm$  SEM) serum FSH concentrations in blood samples collected every 15 minutes for 10 hours, at 4 week intervals from 4 to 32 weeks of age from separate groups of calves ( $n = 6$ ). The differences between ages are indicated by different superscripts ( $P < 0.05$ ).



**Figure 3.4** Mean ( $\pm$  SEM) serum testosterone concentrations in pooled blood samples collected every 15 minutes for 10 hours, at 4 week intervals from 4 to 32 weeks of age from separate groups of calves ( $n = 6$ ). The differences between ages are indicated by different superscripts ( $P < 0.05$ ).

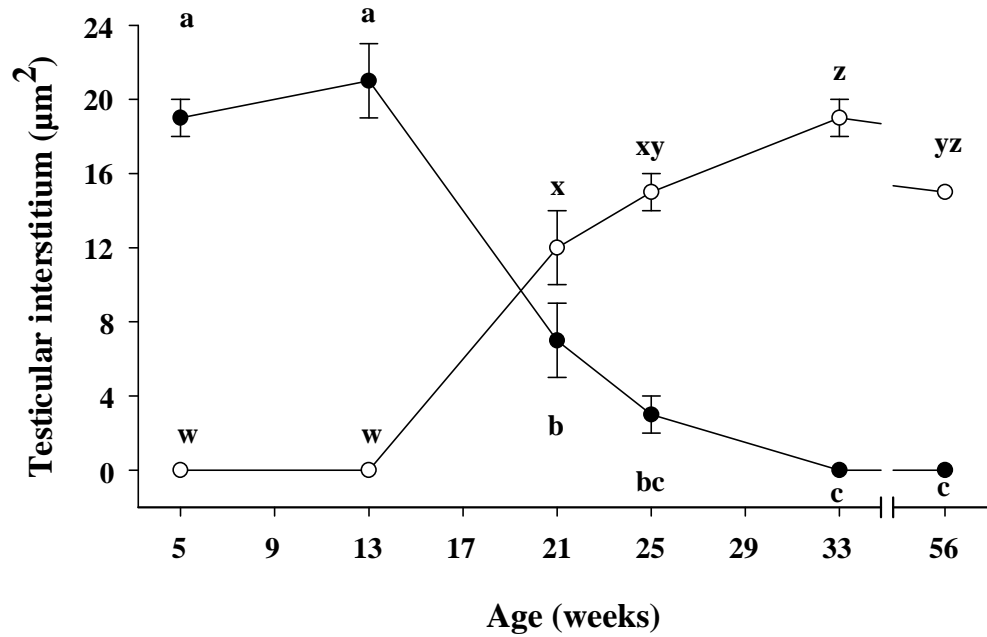


**Figure 3.5** Mean ( $\pm$  SEM) (a) testicular Luteinising Hormone Receptor (LH-R) concentrations per mg of protein; (b) testicular LH-R affinity ( $K_a$ ) for samples collected from separate groups of bull calves ( $n = 6$ ) castrated at 4 week intervals from 5 to 33 weeks of age and at 56 weeks of age. The differences between ages within variables are indicated by different superscripts ( $P < 0.05$ ).



**Figure 3.6** Mean ( $\pm$  SEM) (a) testicular Follicle Stimulating Hormone Receptor (FSH-R) concentration per mg of protein; (b) testicular FSH-R affinity ( $K_a$ ) for samples collected from separate groups of bull calves ( $n = 6$ ) castrated at 4 week intervals from 5 to 33 weeks of age and at 56 weeks of age. The differences between ages within variables are indicated by different superscripts ( $P < 0.05$ ).





**Figure 3.7** Mean ( $\pm$  SEM) Sertoli cell numbers (open circles) and their progenitor indifferent supporting cells (closed circles) per section of seminiferous tubule. Twenty randomly selected tubules were examined from one testis per bull at 1,000 x magnification. Counts were done for separate groups of bull calves ( $n = 6$ ) castrated at 5, 13, 21, 25, 33 and 56 weeks of age. The differences between ages within variables are indicated by different superscripts ( $P < 0.05$ ).

Table 3. 1 Mean ( $\pm$  SEM) percentage of seminiferous tubules with a particular germ cell type as the most mature germ cell (gonocytes; pre-spermatogonia; A- and B- spermatogonia; primary spermatocytes; secondary spermatocytes; round spermatids; elongating spermatids) in 20 randomly selected round seminiferous tubules per histological section of testis samples collected from separate groups of bull calves (n = 6) castrated at 4 week intervals from 5 to 33 weeks of age and at 56 weeks of age.

Age (weeks)	Gonocytes	Pre - Spermatogonia	A- and B- Spermatogonia	Primary spermatocytes	Secondary - Spermatocytes	Rounded - Spermatids	Elongated - Spermatids
5	41 $\pm$ 5 <sup>a</sup>	49 $\pm$ 4 <sup>a</sup>	10 $\pm$ 3 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>
9	31 $\pm$ 2 <sup>b</sup>	29 $\pm$ 5 <sup>b</sup>	36 $\pm$ 4 <sup>b</sup>	2 $\pm$ 1 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>
13	13 $\pm$ 4 <sup>cd</sup>	32 $\pm$ 4 <sup>b</sup>	51 $\pm$ 4 <sup>c</sup>	3 $\pm$ 3 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>
17	19 $\pm$ 6 <sup>d</sup>	27 $\pm$ 6 <sup>b</sup>	38 $\pm$ 9 <sup>bc</sup>	15 $\pm$ 6 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>
21	5 $\pm$ 3 <sup>ce</sup>	6 $\pm$ 2 <sup>c</sup>	35 $\pm$ 13 <sup>bcd</sup>	37 $\pm$ 15 <sup>ab</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>
25	2 $\pm$ 1 <sup>e</sup>	1 $\pm$ 1 <sup>c</sup>	18 $\pm$ 12 <sup>ad</sup>	40 $\pm$ 11 <sup>b</sup>	8 $\pm$ 4 <sup>bc</sup>	18 $\pm$ 8 <sup>b</sup>	14 $\pm$ 7 <sup>b</sup>
29	0 $\pm$ 0 <sup>e</sup>	0 $\pm$ 0 <sup>c</sup>	13 $\pm$ 6 <sup>a</sup>	50 $\pm$ 8 <sup>b</sup>	9 $\pm$ 3 <sup>bc</sup>	18 $\pm$ 7 <sup>b</sup>	9 $\pm$ 5 <sup>b</sup>
33	0 $\pm$ 0 <sup>e</sup>	0 $\pm$ 0 <sup>c</sup>	6 $\pm$ 6 <sup>a</sup>	44 $\pm$ 9 <sup>b</sup>	3 $\pm$ 2 <sup>ab</sup>	31 $\pm$ 7 <sup>c</sup>	17 $\pm$ 9 <sup>b</sup>
56	0 $\pm$ 0 <sup>e</sup>	0 $\pm$ 0 <sup>c</sup>	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0 <sup>a</sup>	3 $\pm$ 3 <sup>ab</sup>	18 $\pm$ 3 <sup>b</sup>	76 $\pm$ 6 <sup>c</sup>

The differences between ages within columns are indicated by different superscripts a, b, c ( $P < 0.05$ ).

### 3.5. Discussion

In the present study, between 4 and 25 weeks of age, serum concentrations of LH and FSH were transiently elevated confirming previous reports (Wise *et al.* 1987; Evans *et al.* 1993; Evans *et al.* 1996; Aravindakshan *et al.* 2000a, b). As was previously suggested, the early postnatal increase in LH secretion, noted in the present study, was caused by an increase in LH pulse frequency (McCarthy *et al.* 1979a; Amann & Walker 1983; Evans *et al.* 1993, 1996). It is probable that the high frequency discharge of LH between 4 and 25 weeks of age influenced Leydig cell differentiation and maturation, and led to increased secretion of testosterone after 12 weeks of age (Rawlings *et al.* 1972, 1978; Secchiari *et al.* 1976; Amann 1983; Amann & Walker 1983; Rawlings & Cook 1986; Mendis-Handagama & Ariyaratne 2001). The marked increase in mean serum testosterone concentrations from 16 to 20 weeks of age, noted in the present study, probably terminated the early postnatal increase in serum concentrations of LH and FSH (Rawlings *et al.* 1978; Evans *et al.* 1995).

In males, there are two distinct populations of Leydig cells; foetal and adult Leydig cells, which differentiate from mesenchymal cells prenatally and postnatally, respectively (Mendis-Handagama & Ariyaratne 2001). In bull calves, it was suggested that the demise of foetal Leydig cells occurred during the early postnatal period (Hooker 1970). Differentiation and maturation of adult Leydig cells in bulls is initiated around weeks 12 to 16 and continues actively up to 28 weeks of age (Amann 1983). This phase of differentiation and maturation would appear to be reflected in the increased testosterone production from 8 to 20 weeks of age in the present study.

Amann (1983) suggested that in bull calves the enhanced testosterone production after 28 weeks of age involved increased cellular synthesis or increased Leydig cell numbers. The peaks in testosterone production seen at 20 and 32 weeks of age in the present study, with the intervening trough, have been noted previously (Rawlings *et al.* 1972, 1978; Secchiari *et al.* 1976). Progenitor Leydig cells and Leydig cells resident in the testicular interstitium are the target cells for LH action (Schanbacher 1979; Mendis-Handagama & Ariyaratne 2001). In the present study, the high concentrations of testicular LH-R in the early postnatal calves and the decline in concentrations from 13 to 21 weeks of age probably reflected high numbers of foetal Leydig cells and undifferentiated Leydig progenitor cells followed by a decline in numbers of both. It is interesting that as adult Leydig cells differentiated and matured, as reflected in enhanced testosterone production from 8 to 20 weeks of age, testicular LH-R concentrations declined but LH-RK<sub>a</sub> was maintained. However, the early postnatal increase in mean serum LH concentrations rising to a peak at 12 weeks of age is clearly positioned to initiate the differentiation and maturation of adult Leydig cells. At least LH drives the latter stages of Leydig cell differentiation in rats, mice and humans (Mendis-Handagama & Ariyaratne 2001). It is likely that the increases seen in LH-R concentration in the testis beyond 25 weeks of age, in the present study, especially at 56 weeks of age, reflected further Leydig cell maturation or increased numbers of Leydig cells as suggested by Amann (1983). In the present study, the positive correlation of testicular LH-R concentrations with testicular FSH-R concentrations suggested FSH affected Leydig cell development, acting via Sertoli cells. Administration of FSH to *in vitro* co-cultured purified pig Leydig and Sertoli cells increased Leydig cell LH/human

Chorionic Gonadotropin binding sites and their capacity to secrete testosterone (Tabone *et al.* 1984). In the present study, LH-R concentrations increased from 25 to 56 weeks of age along with the Leydig cell testosterone production.

In the present study, the high mean testicular FSH-R concentrations from 5 to 13 weeks of age were probably a result of the high number of indifferent supporting cells per seminiferous tubule (Curtis & Amann 1981). In bull calves, indifferent supporting cells and Sertoli cells resident in the seminiferous tubules are the target cells for FSH action (Schanbacher 1979). The drop in mean testicular FSH-R concentrations from 17 to 25 weeks of age accompanied the precipitous decline in numbers of indifferent supporting cells as they differentiated into mature Sertoli cells and may also have been influenced by the rapid onset of spermatogenesis, particularly increased spermatocytes. The latter would have effectively decreased FSH-R concentrations per mg of protein. The increase in testicular FSH-R concentrations from 25 to 56 weeks of age was probably due to maturation of the immature Sertoli cells. In bull calves, it was suggested that testosterone stimulates the differentiation of indifferent supporting cells (immature Sertoli cells) to mature Sertoli cells (Amann 1983).

In the present study, mean serum FSH concentrations and FSH-R concentrations were high when the seminiferous tubules were occupied primarily by immature germ cells (gonocytes, prespermatogonia). This may have facilitated the FSH dependent proliferation and differentiation of the immature germ cells to primary spermatocytes (Means *et al.* 1976; Amann 1983; Jegou *et al.* 1983). In the present study, the

percentage of seminiferous tubules with secondary spermatocytes as the most mature germ cell increased from 21 to 25 weeks of age and coincided with the period of low serum LH concentrations and testicular LH-R concentrations, from 20 to 33 weeks of age. In the present study, the LH dependent release of testosterone, essential for the progression of primary spermatocytes to secondary spermatocytes, could have been facilitated by the increased LH-RK<sub>a</sub> noted from 21 to 29 weeks of age (Purvis *et al.* 1977). In the present study, elongated and rounded spermatids were the most mature germ cells in the seminiferous tubules between 25 and 33 weeks of age, during the period of high serum testosterone concentrations. A similar association between stages VII and VIII of spermatogenesis and elevated serum testosterone was seen in rats (Pearson & Tubbes 1967). In the present study, during the period of increase in the percentage of seminiferous tubules with elongated spermatids as the most mature germ cell type, from 29 to 56 weeks of age, mean serum FSH concentrations were low but testicular FSH-RK<sub>a</sub> and FSH-R concentrations were high. This implied that, the increased Sertoli cell sensitivity to FSH ensured the progression and sustenance of spermatogenesis during low serum FSH concentrations (Means *et al.* 1976; Amann 1983; Jegou *et al.* 1983; Orth 1984).

In the present study, a rapid increase in testicular weight occurred after 25 weeks of age and coincided with increased serum testosterone concentrations, gonadotropin receptor concentrations and affinity. It is likely that testicular growth was initiated by the high postnatal serum gonadotropin concentrations and testicular gonadotropin receptor concentrations and was maintained by the increased sensitivity of the Sertoli and Leydig

cells to low serum FSH and LH concentrations, respectively, as reported for the ram (Yarney & Sanford 1989) and rat (Dufau & Catt 1978). The increase in gonadotropin receptor concentrations from 25 to 56 weeks of age differs from previous reports in bull calves (Sundby *et al.* 1984; Dias & Reeves 1982); however, low sample number and lack of sampling during the period of rapid testicular growth and just prior to puberty could have confounded trends in those studies.

We concluded that high concentrations of serum gonadotropins and testicular gonadotropin receptors during the early postnatal period in bull calves may be critical to the initiation of rapid testis growth; however, increased gonadotropins receptor concentrations and affinity in the face of low circulating gonadotropin concentration and high testosterone concentrations may support rapid testicular growth after 25 weeks of age. The high testicular gonadotropin receptor concentration and affinity during low serum gonadotropin concentrations suggested a high Leydig and Sertoli cell sensitivity to low serum LH and FSH concentrations, respectively.

**Chapter 4: POSTNATAL CHANGES IN SERUM CONCENTRATIONS OF  
INSULIN LIKE GROWTH FACTOR 1 AND TESTICULAR  
CONCENTRATIONS OF TRANSFORMATION GROWTH FACTORS -ALPHA  
AND -BETA 1, 2 AND 3 IN BULLS**

Bagu ET, Gordon JR and Rawlings NC

**4.1. Abstract**

Our objectives were to see if transformation growth factors (TGF- alpha and beta 1, 2 and 3) are produced in the developing bull testis and to establish the temporal relationship amongst serum insulin like growth factor 1 (IGF-1), testicular TGF-alpha and beta 1, 2 and 3. Separate groups of Hereford x Charolais calves (n=6) were castrated every 4 weeks from 5 to 33 weeks of age and at 56 weeks of age. A week prior to castrations, from 5 to 33 weeks of age, blood was collected every 15 minutes for 10 hours. Serum IGF-1 concentrations increased from 8 to 12 weeks of age, decreased from 24 to 28 weeks of age and increased to 32 weeks of age ( $P<0.05$ ). Testicular TGF-alpha concentrations increased from 13 to 17 weeks of age, decreased to 21 weeks of age and from 33 to 56 weeks of age ( $P<0.05$ ). Testicular TGF-beta 1 concentrations decreased from 17 to 21 weeks of age, increased to 25 weeks of age and decreased from 25 to 29 weeks of age ( $P<0.05$ ). Testicular TGF-beta 2 concentrations increased from 5



to 17 weeks of age, decreased to 21 weeks of age, increased to 25 weeks of age and decreased at 29 weeks of age ( $P < 0.05$ ). Testicular TGF-beta 3 concentrations increased from 13 to 17 weeks of age, decreased to 21 weeks of age and from 25 to 29 weeks of age ( $P < 0.05$ ). In conclusion, testicular TGF-alpha concentrations were greater in calves than adults while concentrations of TGF-beta 1, 2 and 3 were greater in the early postnatal than peripubertal period.

#### **4.2. Introduction**

In bulls, puberty has been defined as the time when an ejaculate has at least  $5.0 \times 10^7$  sperm and a 10% linear motility (Wolf *et al.* 1965). In Hereford x Charolais bulls, puberty occurs at about 45 weeks of age (Evans *et al.* 1993; Rawlings & Evans 1995; Evans *et al.* 1996; Aravindakshan *et al.* 2000a, b). In bull calves, there is an early transient increase in mean serum LH concentrations between 4 and 25 weeks of age, with minimal or no subsequent increase prior to the onset of puberty (Evans *et al.* 1993; Rawlings & Evans 1995; Evans *et al.* 1995, 1996; Aravindakshan *et al.* 2000a, b). Mean serum FSH concentrations are often slightly elevated in the early postnatal period, decreasing from 24 to 32 weeks of age (Evans *et al.* 1996; Rawlings & Evans 1995; Aravindakshan *et al.* 2000). In the early postnatal period, mean serum testosterone concentrations are low, increasing minimally from 18 to 24 weeks of age and markedly after 28 weeks of age, reaching adult concentrations by 40 weeks of age (Rawlings *et al.* 1972, 1978; Secchiari *et al.* 1976; Amann & Walker 1983; Rawlings & Cook 1986).

In males, there are two distinct populations of Leydig cells; foetal and adult cells, which differentiate from mesenchymal cells prenatally and postnatally, respectively (Mendis-Handagama & Ariyaratne 2001). In bull calves, it was suggested that the demise of foetal Leydig cells occurred during the early postnatal period (Hooker 1970). Amann (1983) suggested that the high frequency of LH pulses that occurred in bull calves after 4 weeks of age, initiated differentiation and maturation of adult Leydig cells and a switch from androstenedione to testosterone secretion around 12 to 16 weeks of age. It was also suggested that the increased intratesticular testosterone concentrations initiated the differentiation of indifferent supporting cells to Sertoli cells; differentiation is restricted to the period of 16 to 28 weeks of age (McCarthy *et al.* 1979a; Curtis & Amann 1981; Amann 1983). In bull calves at 12 weeks of age, Gonocytes are the predominant germ cells in the seminiferous tubule later replaced by spermatocytes between 16 to 28 weeks of age (Curtis & Amann 1981). The appearance of elongated spermatids in the seminiferous tubules at 32 weeks of age marks the onset of the final maturation stages of spermatogenesis (Curtis & Amann 1981). Rapid testicular growth that occurs after 25 weeks of age is therefore a reflection of the changes above (Evans *et al.* 1993, 1996; Rawlings & Evans 1995).

It is unclear whether the transient early postnatal increase in serum LH concentrations is sufficient to trigger rapid testicular growth or how important the subsequent low concentrations of LH and FSH are for the process. In rats, mice and pigs transformation growth factors [TGF] and insulin like growth factors [IGF] have been identified as regulators of testicular development (Skinner *et al.* 1991; Maddocks *et al.* 1995;

Huleihel & Lunenfeld 2004). TGF-alpha enhanced gonadotropin induced proliferation of Leydig, Sertoli and germ cells isolated from rats (Khan *et al.* 1992; Tajima *et al.* 1995); whereas IGF-1 enhanced maturation and increased steroidogenesis of Leydig cells isolated from immature rats (Wang *et al.* 2003, 2004). However, there are many contradicting findings surrounding the role of TGF-beta in rats and pigs (Avallet *et al.* 1987; Morera *et al.* 1988, Sordoillet *et al.* 1992) and in bulls there is a paucity of information for the role of these factors in testicular development.

In bull calves, elevated serum IGF-1 concentrations following treatment with GH had no effect on testicular development (Santos *et al.* 1999; Sauerwein *et al.* 2000). The role of IGF-1 in testicular development in bulls is not clear and the importance of TGF-alpha and TGF-beta 1, 2 and 3 in this process has not been studied. In the present study, our objectives were to see if: (1) TGF-alpha, TGF-beta isoforms 1, 2, and 3 are produced in the developing bovine testis; (2) there are temporal changes in concentrations of serum IGF-1 and testicular TGF-alpha, TGF-beta isoforms 1, 2, and 3 during postnatal reproductive development and (3) shifts in concentrations occur at significant points in during testicular development.

### **4.3. Materials and methods**

#### *4.3.1. Animals*

Fifty-four spring-born, age matched ( $\pm 3$  days) bull calves (Hereford x Charolais) were divided into 9 groups of 6 each. Bull calves were suckled at pasture until they were weaned at 26 weeks of age. After weaning, calves were kept in corrals and provided

with water and a standard feed ration ad libitum (Evans *et al.* 1995). All experimental procedures were done in accordance with the regulations of the Canadian Council for Animal Care.

#### *4.3.2. Blood and testes collection*

Separate groups of calves were castrated every 4 weeks from 5 to 33 weeks of age, and at 56 weeks of age. A week prior to castration, from 5 to 33 weeks of age, blood samples (5 ml) were collected every 15 minutes for 10 hour (intensive bleeds) from the jugular vein by an intravenous catheter starting at 08:00 hours (Evans *et al.* 1995). Blood samples were left to clot for at least 12 hours at room temperature and serum harvested and frozen at -20 °C until assayed. At castration, the testes were weighed and cut into sections, plunged into liquid nitrogen for approximately 20 minutes and stored at -70 °C until homogenisation.

#### *4.3.3. Serum IGF-1 concentrations*

The serum IGF-1 concentrations for each calf were determined from pooled blood samples from each of the intensive bleeds. The pooled serum was first subjected to acid ethanol extraction and centrifugation. The supernatant was decanted, neutralized with 0.86M Tris buffer and IGF-1 concentrations determined using a previously validated, double antibody radioimmunoassay (Kerr *et al.* 1990). All samples were analyzed in one assay and the intra assay coefficient of variation (CV) was 16.5% for a reference serum with 252 ng/ml of IGF-1.

#### 4.3.4. Testicular homogenates

Frozen sections (2 – 3 g) of testicular parenchyma were weighed, diced and homogenized at maximum speed for 2.5 minutes, using a Brinkman polytron® (PT 10203500, Steinhofhalde 22, Switzerland) in phosphate buffer saline (PBS) (1.71 mM NaH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub> 154 mM NaCl) pH 7.4 at 2 ml of buffer per gram of tissue. All procedures unless otherwise indicated, were carried out at 4 °C. The testicular homogenates were centrifuged at 17,500 X g for 45 minutes and the supernatant decanted and into polystyrene tube. Aliquots of the supernatant fractions were then stored at -70 °C. The protein concentrations in the testicular supernatant fractions were determined using a Bio Rad Assay (Bio Rad, Richmond, CA, USA) with bovine serum albumin (BSA) as a standard.

#### 4.3.5. Mean testicular concentrations of TGF- alpha and TGF-beta isoforms 1, 2 and 3

Testicular supernatants were analyzed for concentrations of TGF-alpha and TGF-beta 1, 2 and 3 by a sandwich enzyme immunosorbent assay [ELISA] (Theoret *et al.* 2001). ELISA plates (Nunc maxisorp surface; Nalge Nunc International Rochester, NY USA) were coated overnight at 4°C with a primary antibody (R & D Systems Inc. Minneapolis, MN USA) a goat anti-human TGF-alpha (AF-239-NA), which cross reacts with bovine TGF-alpha; or mouse anti-human TGF-beta 1 (MAB 240), which cross reacts with bovine TGF-beta 1; or mouse anti-porcine TGF-beta 2 (MAB612), which cross reacts with bovine TGF-beta 2; or mouse anti-human TGF-beta 3 (MAB 6430), which cross reacts with bovine TGF-beta 3.

The standards (R & D Systems Inc.) used were: recombinant human TGF-alpha (rhTGF-alpha (DY239); rhTGF- beta 1 (100-B); rhTGF-beta 2 (302-B2) and rhTGF-beta 3 (243-B3). Bound TGF-alpha and TGF-beta isoforms 1, 2 and 3 were detected using biotinylated secondary antibodies as follows; goat anti-human TGF-alpha (DY239), chicken anti-human TGF-beta 1 (BAF240), goat anti-human TGF- beta 2 (BAF302) and goat anti-human TGF-beta 3 (BAF243), respectively with streptavidin-horse radish peroxidase (A-2004 Vector Laboratories Inc. Burlingame, CA USA). For the TGF-alpha ELISA 3,3',5,5'-tetramethylbenzidine [TMB] (R & D Systems Inc.) was used for colour development whereas in the TGF-beta isoforms 1, 2 and 3 assay 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) [ABTS] (Vector Laboratories Inc.). The colour changes were measured with an ELISA plate reader (model 3550, Bio-Rad Laboratories, Inc. Hercules, CA USA) at an OD of 450 nm for the TGF-alpha assay and 405 nm for the assays for TGF-beta isoforms 1, 2 and 3 assays. The TGF-alpha concentrations in the testicular supernatant samples were determined from a calibration curve established by standard concentrations of 0, 6.25, 12.5, 25, 50, 100, 200, 400 pg/ml of rhTGF alpha. TGF-beta isoforms 1, 2 and 3 concentrations in testicular supernatant samples were determined from calibration curves established by standard concentrations of 0, 31.25, 62.5, 125, 250, 500, 1000, 2000 pg/ml of rhTGF-beta 1, rhTGF-beta 2 and rhTGF-beta 3 respectively. The TGF-alpha intra assay CV was 2.8% for reference testicular supernatant concentrations of 200 pg/ml. The intra assay CV for the TGF-beta 1 assay was 3.4%, for reference testicular supernatant concentrations of 542 pg/ml. The intra assay CV for the TGF-beta 2 was 10.8%, for reference testicular

supernatant concentrations of 742 pg/ml. The intra CV for the TGF-beta 3 assay was 10.2% for reference testicular supernatant concentrations of 1613 pg/ml.

#### 4.3.6. Data analysis

Serum IGF-1 concentrations, testicular concentrations of TGF-alpha and TGF-beta isoforms 1, 2 and 3 were analysed for effects of age by a one-way analysis of variance (one-ANOVA, Sigma Stat for Windows®, version 1.0; Jadel Corporation, San Rafael, CA, USA). If the main effects were significant multiple comparisons were done using the Fisher least significant difference method for post ANOVA multiple comparisons ( $P < 0.05$ ). Pearson correlations were used to examine the relationships between serum IGF-1 concentration and testicular TGF concentrations (TGF-alpha and TGF-beta isoforms 1, 2 and 3) at different ages (Sigma Stat for Windows®, version 1.0; Jadel Corporation, San Rafael, CA, USA). Data are presented as Mean  $\pm$  SEM

## 4.4. Results

### 4.4.1. Serum IGF-1

Mean serum IGF-1 concentrations increased from 8 to 12 weeks of age, decreased from 24 to 28 weeks of age and subsequently increased to 32 weeks of age ( $P < 0.01$ ; Figure 4.1).

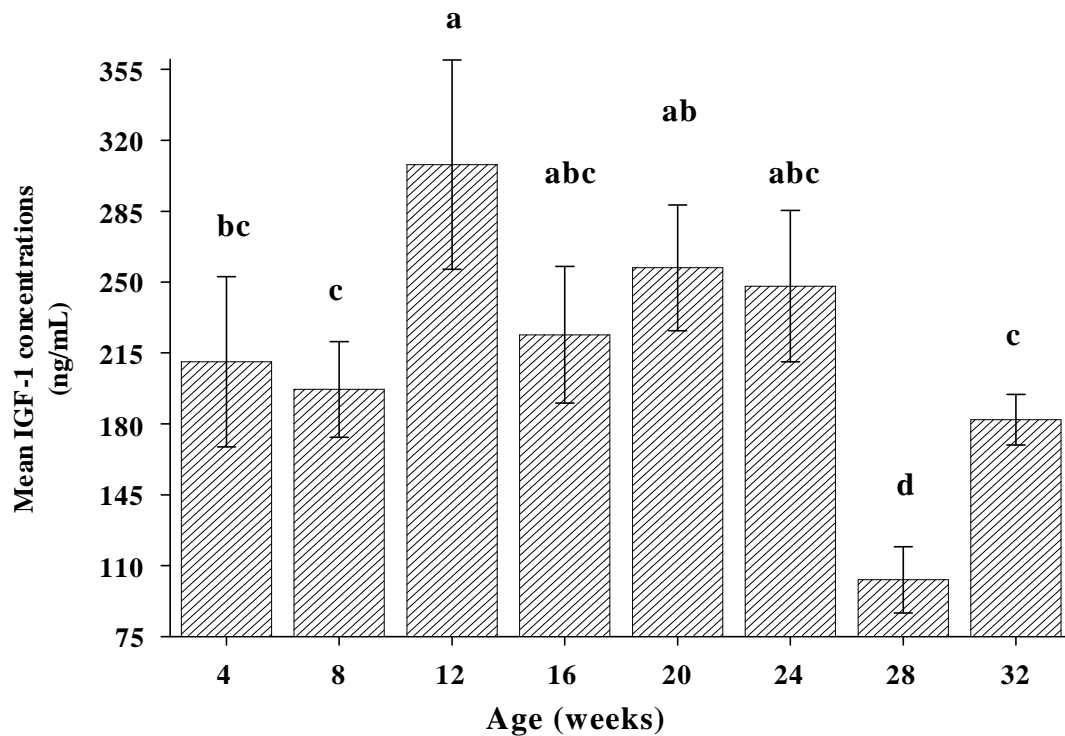
#### 4.4.2. Mean concentration of testicular TGF- $\alpha$ and TGF- $\beta$ isoforms 1, 2 and 3

Mean testicular TGF-  $\alpha$  concentrations (pg/g of testis) increased from 13 to 17 weeks of age, decreased to 21 weeks of age and subsequently from 33 to 56 weeks of age ( $P < 0.05$ ; Figure 4.2). Mean testicular TGF-  $\beta$  1 concentrations (ng/g of testis) decreased from 17 to 21 weeks of age, increased to 25 weeks of age and subsequently decreased from 25 to 29 weeks of age ( $P < 0.01$ ; Figure 4.3).

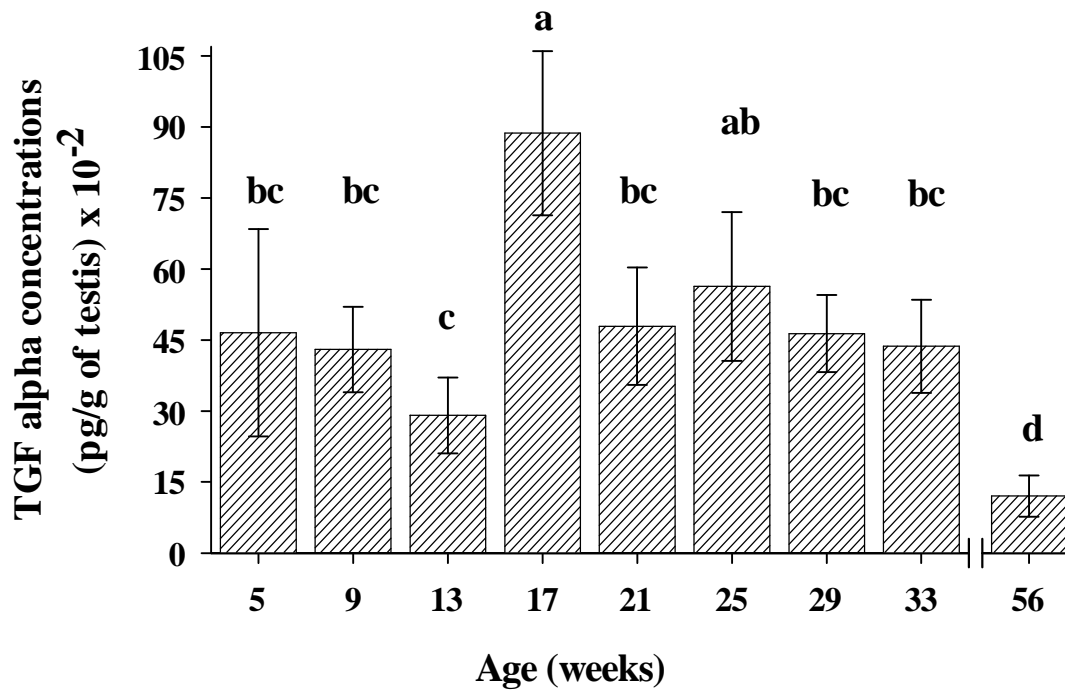
Mean testicular TGF-  $\beta$  2 concentrations (ng/g of testis) increased from 5 to 17 weeks of age, decreased to 21 weeks of age, increased to 25 weeks of age and subsequently decreased to a nadir at 29 weeks of age ( $P < 0.05$ ; Figure 4.4). Mean testicular TGF- $\beta$  2 concentrations were positively correlated with mean testicular TGF- $\beta$  1 concentrations ( $P < 0.01$ ; r-value 0.87).

Mean testicular TGF-  $\beta$  3 concentrations (ng/g of testis) increased from 13 to 17 weeks of age, decreased to 21 weeks of age, and subsequently decreased from 25 to 29 weeks of age ( $P < 0.05$ ; Figure 4.5c). Mean testicular TGF- $\beta$  3 concentrations were positively correlated with mean testicular TGF- $\beta$  1 and TGF- $\beta$  2 concentrations ( $P < 0.01$ ; r values of 0.74 and 0.69, respectively).

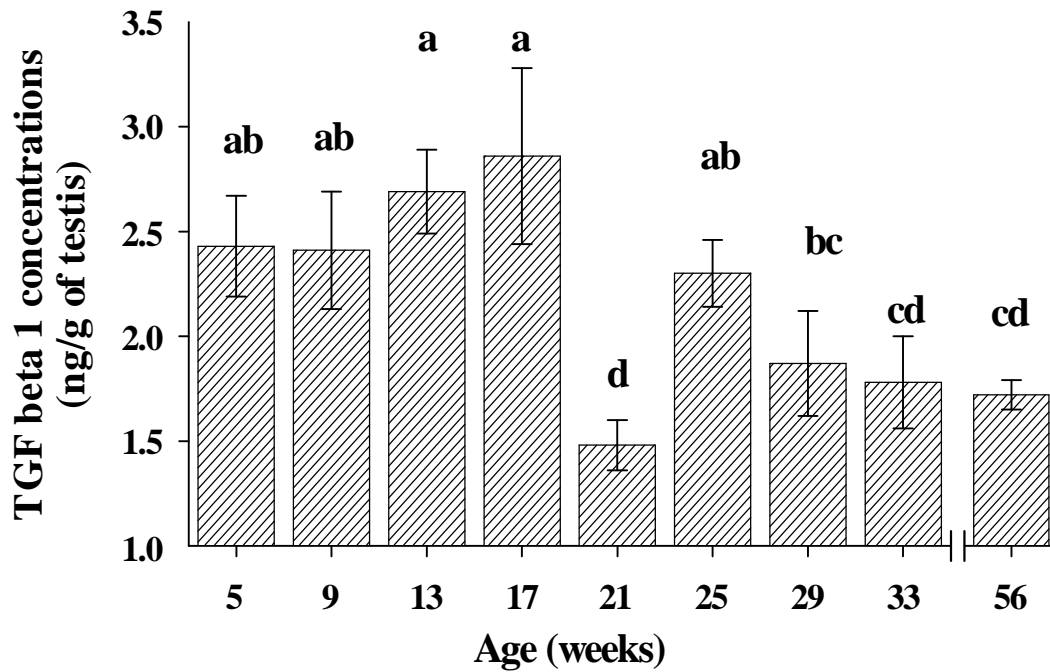




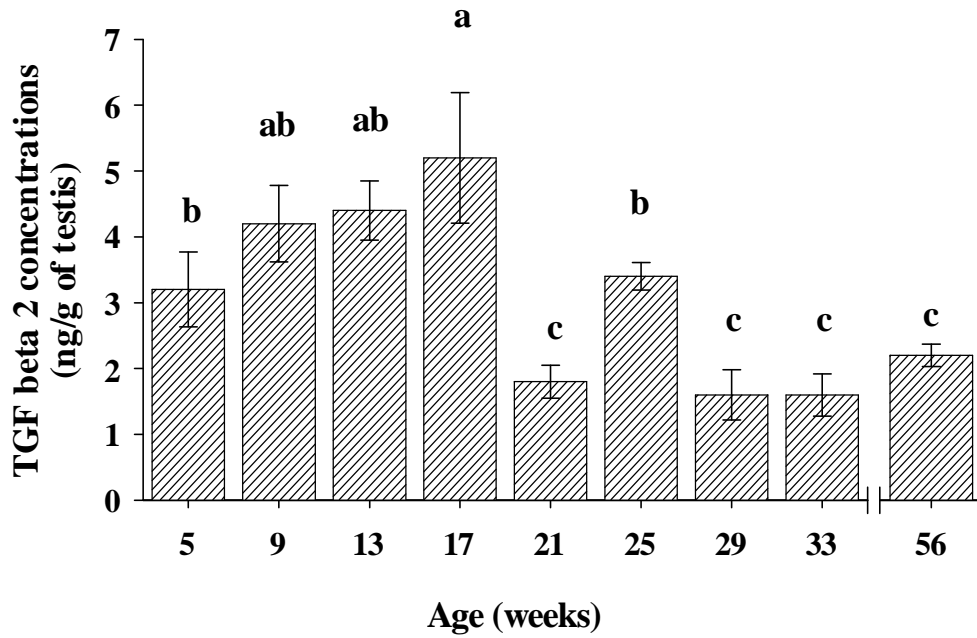
**Figure 4. 1** Mean ( $\pm$  SEM) serum IGF-1 concentrations in pooled blood samples collected every 15 minutes for 10 hours, at 4 week intervals from 4 to 32 weeks of age from separate groups of calves ( $n = 6$ ). The differences between ages are indicated by different superscripts a, b, c ( $P < 0.05$ ).



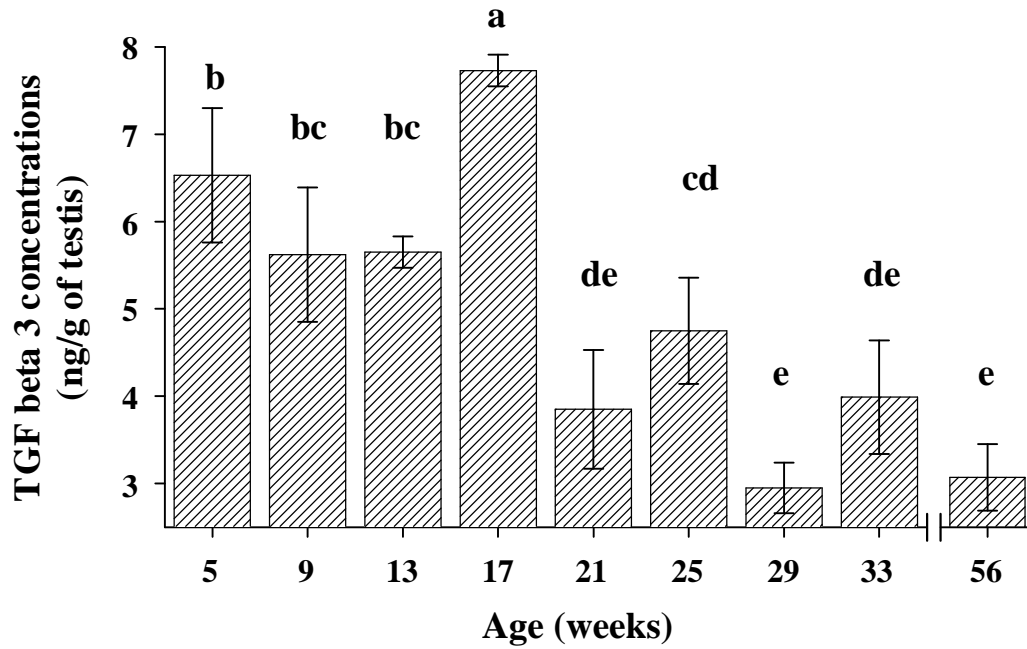
**Figure 4. 2** Mean ( $\pm$  SEM) testicular TGF-alpha concentrations in testes collected from separate groups of bull calves ( $n = 6$ ) castrated at 4 week intervals from 5 to 33 weeks of age and at 56 wk of age. The differences between ages within variables are indicated by different superscripts a, b, c ( $P < 0.05$ ).



**Figure 4. 3** Mean ( $\pm$  SEM) testicular TGF-beta 1 concentrations in testes collected from separate groups of bull calves ( $n = 6$ ) castrated at 4 week intervals from 5 to 33 wk of age and at 56 weeks of age. The differences between ages within variables are indicated by different superscripts a, b, c ( $P < 0.05$ ).



**Figure 4.4** Mean ( $\pm$  SEM) testicular TGF-beta 2 concentrations in testes collected from separate groups of bull calves ( $n = 6$ ) castrated at 4 week intervals from 5 to 33 wk of age and at 56 weeks of age. The differences between ages within variables are indicated by different superscripts a, b, c ( $P < 0.05$ ).



**Figure 4.5** Mean ( $\pm$  SEM) testicular TGF-beta 3 concentrations in testes collected from separate groups of bull calves ( $n = 6$ ) castrated at 4 week intervals from 5 to 33 wk of age and at 56 weeks of age. The differences between ages within variables are indicated by different superscripts a, b, c ( $P < 0.05$ ).

#### 4.5. Discussion

In the present study, TGF-alpha and TGF-beta isoforms 1, 2 and 3 were detected in the developing testis of bull calves of all ages. Mean testicular concentrations of TGF-alpha were higher in calves than in adults. Testicular concentrations of TGF-beta isoforms 1, 2 and 3 were higher in bull calves during the early postnatal period (5 to 17 weeks of age) than in those in the pre- and peri- pubertal period (21 to 56 weeks of age). The temporal changes in testicular concentrations of TGF-alpha and TGF-beta isoforms 1, 2 and 3 in the developing bull testis were similar to those seen for TGF-alpha (Mullaney & Skinner 1992) and TGF-beta isoforms 1, 2 and 3 (Mullaney & Skinner 1993) mRNA expression patterns in the developing rat testis. Mean testicular concentrations of TGF-beta 3 were higher than TGF-beta isoforms 1 or 2 at all ages in agreement with data from the rat (Mullaney & Skinner 1992, 1993).

We were unable to determine testicular IGF-1 concentrations in the present study as testicular concentrations were below the assay sensitivity; however, IGF-1 was detected in serum samples. With exception of calves at 28 weeks of age, mean serum IGF-1 concentrations in calves between 4 and 32 weeks of age were high. In a study by Brito *et al.* (2006), mean serum IGF-1 concentrations were shown to increase with age, reaching maximum concentrations in bull calves between 34 and 51 weeks of age. When mean serum concentrations of IGF-1 in the present study were compared with the previously published (Bagu *et al.* 2006) mean serum LH and testicular LH receptor concentrations (LH-R) for the present bull calves, serum IGF-1 concentrations were found to be positively correlated with mean serum LH and testicular LH receptor

concentrations ( $P < 0.01$ ;  $r$  values of 0.45 and 0.39, respectively). In rats, IGF-1 receptors have been identified in Leydig cells and have been shown to increase mesenchymal cell proliferation and differentiation into Leydig cells (Spiteri-Grech & Nieschlag 1992; Lin 1995). Leydig cell numbers are decreased in IGF-1-null mice (Wang & Hardy 2004) and the additive effect of LH and IGF-I treatment on testicular cell numbers has been reported, indicating that the two hormones use separate signalling pathways (Wang *et al.* 2003). Interestingly, in in vitro cell cultures, IGF-1 has been reported to up-regulate LH-R and testosterone secretion (Spiteri-Grech & Nieschlag 1992; Lin 1995), whereas testosterone in turn up-regulates IGF-I receptors and IGF-I production by Leydig cells (Cailleau *et al.* 1990). In the present study there was no relationship between serum concentrations of IGF-1 and testosterone.

When testicular concentrations of TGF-beta isoforms 1, 2 and 3 in the present study, from bull calves aged between 5 and 33 weeks, were compared with mean serum LH and testosterone concentrations (Bagu *et al.* 2006), mean serum LH concentrations were shown to be positively correlated with mean testicular concentrations of TGF-beta isoforms 1, 2, and 3 ( $P < 0.01$ ;  $r$  values of 0.36, 0.53 and 0.30, respectively); however, mean serum testosterone concentrations were negatively correlated with TGF-beta isoforms 1, 2, and 3 ( $P < 0.01$ ;  $r$  values of -0.45, -0.41 and -0.40, respectively). No interaction in vivo between LH and TGF-beta isoforms has been previously demonstrated in any species although human chorionic gonadotropin [hCG] was shown to cause a dose and time dependent increase in TGF-beta receptors in Leydig cells isolated from pigs (Goddard *et al.* 2000). Testosterone was shown to inhibit the

synthesis of TGF-beta 1 mRNA in Sertoli cells isolated from immature pigs (Avallet *et al.* 1994); while TGF-beta inhibited hCG/LH induced cAMP and testosterone release and reduced the number of LH-R in Leydig cells isolated from immature rats (Lin *et al.* 1987; Fauser & Hsueh 1988) and pigs (Avallet *et al.* 1987). When the testicular concentrations of TGF-beta isoforms 1, 2 and 3 from the present study were compared with testicular gonadotropin receptor concentrations (Bagu *et al.* 2006); mean testicular TGF-beta 2 concentrations were positively correlated with mean testicular LH-R concentrations ( $P < 0.01$ ;  $r = 0.34$ ); whereas mean testicular concentrations of TGF-beta isoforms 1 and 3 were positively correlated with mean testicular FSH-R concentrations ( $P < 0.05$ ;  $r$  values of 0.32 and 0.29, respectively). This implied that TGF-beta 2 might have stimulated the testicular LH-R concentrations while TGF-beta isoforms 1 and 3 the testicular FSH-R concentrations. An interaction between LH-R concentrations and TGF-beta has not been previously demonstrated. Although treatment of Sertoli cells isolated from immature pigs with TGF-beta 1 did not affect FSH-R numbers (Morera *et al.* 1992), it increased the expression of FSH-R messenger RNA in granulosa cells isolated from female rats (Inoue *et al.* 2003) and birds (Woods & Johnson 2005). Granulosa cells are FSH sensitive cells comparable to Sertoli cells in males (Yding & Andersen 2000).

In bull calves, Amann (1983) suggested that the high frequency of LH pulses that occurred after 4 weeks of age, initiated differentiation and maturation of adult Leydig cells from progenitor cells around 12 to 16 weeks of age. Differentiation of basal indifferent supporting cells into Sertoli cells is restricted to the period between 16 to 28



wk of age (Curtis & Amann 1981; Bagu *et al.* 2006). In the present study, high testicular concentrations of TGF-beta isoforms in the early postnatal bull calves during the period of Leydig and Sertoli cell differentiation indicated a possible paracrine regulatory role for growth factors in Leydig cell and Sertoli cell development. This was further supported by the positive correlation between testicular concentrations of TGF-beta isoforms with gonadotropin receptors. Increased gonadotropin receptor number could have been a consequence of cell proliferation or maturation. In immature rats, TGF-beta was shown to increase LH or FSH induced incorporation of [ $H^3$ ] thymidine into DNA by Leydig (Khan *et al.* 1992) and Sertoli cells (Dorrington *et al.* 1993) respectively. In the present study increased testicular concentrations of TGF beta isoforms 1, 2 and 3 at 25 weeks of age, coincident with the period of initiation of rapid testicular growth (Evans *et al.* 1993, 1996; Rawlings & Evans 1995) indicated a possible involvement of TGF-beta isoforms in the initiation of spermatogenesis. In rats, increased TGF-beta 3 mRNA expression prior to puberty was coincident with the initiation of spermatogenesis (Mullaney & Skinner 1993) and the formation of spermatocytes was shown to be accompanied by the release of the highest concentrations of bioactive TGF-beta isolate (Haagmans *et al.* 2003).

In conclusion, the production of TGF-alpha and TGF-beta isoforms 1, 2, and 3 in the testis of the bull calf was age dependent. Testicular TGF-alpha concentrations were greater in calves than adults and the testicular concentrations of TGF-beta isoforms 1, 2 and 3 were generally greater in the early postnatal than peripubertal period.

**Chapter 5: POSTNATAL CHANGES IN TESTICULAR CONCENTRATIONS OF INTERLEUKINS (IL-1 ALPHA, IL-1 BETA AND IL-6) DURING SEXUAL MATURATION IN BULLS**

Bagu E T, Gordon JR and Rawlings NC

**5.1. Abstract**

Serum gonadotropin concentrations are high during the early postnatal period but low during the prepubertal period of rapid testicular development. It is likely that other factors may regulate testicular development. The objectives of this study were to see if IL-1 and IL-6 are produced in the developing bovine testis and to establish the temporal relationship amongst testicular interleukins (IL-1 and IL-6) during reproductive development. Separate groups of 6 bull calves were castrated every 4 weeks from 5 to 33 weeks of age, and at 56 weeks of age. Testicular IL-1 alpha concentrations decreased ( $P < 0.01$ ) from 5 to 9 weeks of age and 13 to 21 weeks of age. Testicular IL-1 beta concentrations decreased ( $P < 0.01$ ) from 13 to 17 weeks of age and from 29 to 33 weeks of age. Mean IL-1 bioactivity increased from 13 to 17 weeks of age, decreased to 21 weeks of age, increased to 25 weeks of age, decreased to 29 weeks of age and from 33 to 56 weeks of age ( $P < 0.05$ ). Testicular IL-6 concentrations decreased ( $P < 0.05$ ) from 9 to 13 weeks of age, increased ( $P < 0.05$ ) to 21 weeks of age, decreased ( $P < 0.05$ ) to 25 weeks of age, increased ( $P < 0.05$ ) to 29 weeks of age and then decreased ( $P < 0.01$ ) to 56

weeks of age. In conclusion, testicular IL-1 concentrations were highest in the early postnatal period but lowest in the peripubertal period; however, IL-1 bioactivity, although variable, was low in the early postnatal period and greater in the immediate prepubertal period. Testicular IL-6 concentrations were higher in prepubertal calves than adults.

## **5.2. Introduction**

In bulls, puberty has been defined as the time when an ejaculate has at least  $5.0 \times 10^7$  sperm and a 10% linear motility (Wolf *et al.* 1965). Age at puberty varies with breed: in Hereford bulls it occurs between 39 and 52 weeks of age while in Charolais bulls between 33 and 53 weeks of age (Wolf *et al.* 1965; Killian & Amann 1972; Lunstra *et al.* 1978). In bull calves, during the early postnatal period there is an early transient increase in mean serum LH concentrations between 4 and 25 weeks of age, with minimal or no subsequent increase prior to puberty (Evans *et al.* 1993, 1996; Rawlings & Evans 1995; Aravindakshan *et al.* 2000a, b). Mean serum FSH concentrations are often slightly elevated in the early postnatal period, decreasing from 25 to 32 week of age (Evans *et al.* 1996; Rawlings & Evans 1995; Aravindakshan *et al.* 2000a, b). In the early postnatal period, mean serum testosterone concentrations are low, increasing minimally from 18 to 24 weeks of age and markedly after 28 weeks of age; adult concentrations are attained by 40 weeks of age (Secchiari *et al.* 1976; Rawlings *et al.* 1972, 1978; Amann & Walker 1983; Rawlings & Cook 1986).

In males, there are two distinct populations of Leydig cells; foetal and adult, which differentiate from mesenchymal cells prenatally and postnatally, respectively (Mendis-Handagama & Ariyaratne 2001). In bull calves, it was suggested that the demise of foetal Leydig cells occurred during the early postnatal period (Hooker 1970). Amann (1983) suggested that the high frequency of LH pulses that occurred after 4 weeks of age, initiated differentiation and maturation of adult Leydig cells and the switch from androstenedione to testosterone secretion; both events occurring around 12 to 16 weeks of age. The elevated intratesticular testosterone concentrations that followed maturation of the Leydig cells then initiated differentiation of indifferent supporting cells to Sertoli cells; such differentiation is restricted to the period of 16 to 28 weeks of age (McCarthy et al 1979; Curtis & Amann 1981; Amann 1983). In bull calves spermatogenesis is initiated as early as 16 weeks of age with the appearance of preleptotene and diplotene spermatocytes in the seminiferous tubules (Curtis & Amann 1981). The first appearance of elongated spermatids in the seminiferous tubules, at 32 weeks of age, marks the final phase of spermatogenesis (Curtis & Amann 1981). Rapid testicular growth that occurs during the prepubertal period after 25 weeks of age is therefore a reflection of the changes above. It is unclear whether the transient early postnatal increase in serum LH concentrations is sufficient to trigger rapid testicular development or how important the subsequent low concentrations of LH and FSH are.

It is likely that there are regulatory changes in the testis itself that are critical for rapid testicular growth (Amann *et al.* 1986; Wise *et al.* 1987; Evans *et al.* 1993; Rawlings & Evans 1995; Evans *et al.* 1996). In rats, interleukin (IL) 1 bioactivity was detected in

testicular extracts from prepubertal and pubertal rats but not early postnatal rats (Khan *et al.* 1987). Syed *et al.* (1993) later demonstrated IL-6 bioactivity in Sertoli cells isolated from early postnatal and pubertal rats. In the IL-1 family, there are 2 agonist proteins, IL-1 alpha and IL-1 beta (Dinarello 1996), and one IL-1 receptor antagonist (IL-1ra) (Hannum *et al.* 1990, Arend 1993). The IL-6 family is a group of proteins with a common agonistic action (Kishimoto *et al.* 1995). Concentrations of immuno reactive IL-1 alpha, IL-1 beta, IL-1ra (Huleihel *et al.* 2003) and IL-6 (Potashnik *et al.* 2005) were shown to be higher in testicular homogenates of mice during the early postnatal as compared to the peripubertal period.

In early postnatal rats, IL-1 was shown to stimulate Leydig cell steroidogenesis (Svechnikov *et al.* 2001; Verhoeven *et al.* 1988), Sertoli cell DNA synthesis and proliferation (Petersen *et al.* 2002) and immature germ cell DNA synthesis (Khan *et al.* 1987; Soder *et al.* 1988, 1991; Parvinen *et al.* 1991); while IL-6 stimulated the expression of “early” genes and production of transferin in Sertoli cells (Boockfor & Schwaz 1991; Hoeben *et al.* 1997). In bull calves, the role of interleukins in testicular development has not been studied. In the present study, our objectives were to see if: (1) IL-1 and IL-6 are produced in the developing bovine testis; (2) there are temporal changes in testicular concentrations during postnatal reproductive development and (3) shifts in concentrations occur at significant points during testicular development.

### **5.3. Materials and methods**

#### *5.3.1. Animals*

Fifty-four spring-born, age matched ( $\pm 3$  d) bull calves (Hereford x Charolais) were divided into 9 groups of 6 each. Bull calves were suckled at pasture until they were weaned at 26 weeks of age. After weaning, calves were kept in corrals and provided with water and a standard feed ration ad libitum (Evans *et al.* 1995). All experimental procedures were done in accordance with the regulations of the Canadian Council for Animal Care.

#### *5.3.2. Bovine testes collection and testicular supernatant preparation:*

Separate groups of calves (n=6) were castrated every 4 weeks from 5 to 33 weeks of age, and at 56 weeks of age. The testes were weighed, sectioned, wrapped in aluminium foil and plunged into liquid nitrogen for approximately 20 minutes and stored at  $-80^{\circ}\text{C}$  until homogenisation.

Frozen sections (2 - 3 g) of testicular parenchyma were weighed, diced and homogenized at maximum speed for 2.5 minutes, in 2 ml of phosphate buffered saline (PBS, 1.71 mM  $\text{NaH}_2\text{PO}_4$ , 8.1 mM  $\text{Na}_2\text{HPO}_4$  154 mM  $\text{NaCl}$ ; pH 7.4) per gram of tissue, using a Brinkman polytron® (PT 10203500, Kinematica, GmbH). All procedures, unless otherwise indicated, were carried out at  $4^{\circ}\text{C}$ . The homogenates were centrifuged at  $17,500 \times g$  for 45 minutes and the supernatants decanted into polystyrene tubes. Aliquots of the supernatants were then stored at  $-80^{\circ}\text{C}$ . The protein

concentrations in the testicular supernatants were determined with a Bio Rad Assay (Bio Rad, Richmond, CA, USA) using bovine serum albumin (BSA) as a standard.

### 5.3.3. *Measurement of IL-1 and IL-6 by Enzyme Linked immunosorbent Assay (ELISA)*

Testicular supernatants were analyzed for concentrations of IL-1 alpha, IL-1 beta and IL-6 by a sandwich ELISA (Egan *et al.* 1994; Shoda *et al.* 2000). ELISA plates with 96 flat bottom wells (Nunc maxisorp surface; Nalge Nunc International Rochester, NY USA) were coated overnight at 4°C with primary antibody: mouse anti-ovine IL-1 alpha (OvIL-1 alpha; monoclonal antibody (mAb) 10.82; University of Melbourne, Australia), which cross reacts with bovine IL-1 alpha; or mouse anti-OvIL-1 beta (mAb 1658; Serotec, Cedarlane laboratories LTD, Ontario Canada), which cross reacts with bovine IL-1 beta; or mouse anti-OvIL-6 (mAb 1004; Chemicon, Temecula, CA USA) which cross reacts with bovine IL-6. The standards used were: recombinant OvIL-1 alpha (recOvIL-1 alpha; University of Melbourne, Australia); recOvIL-1 beta and recOvIL-6 (recOvIL-1 beta and recOvIL6 were a gift from Dr Peter McWaters, Commonwealth Scientific Industrial and Research Organization, Australia). Bound IL-1 alpha, IL-1 beta and IL-6 was detected using rabbit polyclonal sera raised against OvIL-1 alpha (University of Melbourne, Australia), or secondary antibodies (polyclonal antibody raised in rabbits), anti-OvIL-1 beta (AHP423; Serotec) and anti-OvIL6 (AB1839; Chemicon), respectively. An anti rabbit IgG antibody conjugated to horse radish peroxidase (IgG-HRP; Chemicon) and 3,3',5,5'-tetramethylbenzidine (TMB; Chemicon) were used for colour development. The colour change was measured at an OD of 450 nm with an ELISA plate reader (model 3550, Bio-Rad Laboratories, Inc. Hercules, CA

USA). The concentration of IL-1 alpha, IL-1 beta and IL-6 in the sample were determined from a calibration curve established by standard concentrations of 0, 31.25, 62.5, 125, 250, 500, 1000, 2000 pg/mL of recombinant ovine IL-1 alpha, IL-1 beta or IL-6. The intra assay coefficients of variation (CV) for mean concentrations of 250 and 1000 pg/mL of IL-1 alpha, IL-1 beta or IL-6 were 12.3% and 4.2% or 1.7 % and 1.6% or 7.1% and 5.6%, respectively.

#### 5.3.4. Measurement of IL-1 bioactivity using a LM-1 cell bioassay

The IL-1 bioactivity in the testicular homogenates was determined using LM-1 cell proliferation assays, as was reported by Billingham *et al.* (1995). Testicular homogenates were randomly selected from four of the six bulls at each age and the IL-1 bioactivity evaluated. LM-1 cells (Vaccine and Infectious Disease Organization, University of Saskatchewan, SK Canada) cells are a subclone of the IL-1 responsive cell line D10.G4.1 (American Type Culture Collection (ATCC) Rockville, Maryland USA) and proliferate in the presence of IL-1 with no other mitogens. LM-1 cells ( $4 \times 10^5$  cells /mL) were suspended in Rosewell Park Memorial Institute Medium (RPMI 1640; GIBCO invitrogen Corporation Grand Island NY USA) with 10 % heat-inactivated foetal calf serum (FCS; GIBCO Invitrogen Inc. Burlington, Ontario Canada) and 1% antibiotic/antimycotic solution (10,000 units/mL Penicilline G sodium, 100,000 µg/mL streptomycin sulphate, 25 µg/mL Amphotericin B in 0.85% saline; GIBCO invitrogen Corporation) and dispensed into 96 well plates, at 100 µL /well. Twenty µL of serial dilutions (0, 0.5, 5, 50, 500 and 1000 pg/mL) of recombinant mouse IL-1 beta (R & D Systems Inc. Minneapolis, MN USA) or testicular homogenate samples were



added in triplicate to wells and the volume of each well was made up to 200  $\mu$ L/well with RPMI-10% FCS. The plates were incubated for 3 days in a humidified CO<sub>2</sub> incubator at 37°C. Twenty  $\mu$ L of 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT 5 mg/mL of PBS; Sigma-Aldrich Canada Ltd. Oakville, Ontario) was added to each well, incubated for 1 h, and then 150  $\mu$ L was aspirated from each well. MTT is converted to Formazan in the mitochondria of the LM-1 cells. The Formazan was dissolved in acidified isopropanol (100  $\mu$ L) and the resulting dark-blue color change was measured at an OD of 595 nm using an ELISA plate reader (model 3550, Bio-Rad Laboratories). The bioactivity of IL-1 in testicular supernatants was expressed in terms of recombinant mouse IL-1 beta (R & D Systems Inc.). The intra and inter assay CV's for mean IL-1 beta (R & D Systems Inc.) concentrations of 50 pg/mL or 500 pg/mL were 4.5% and 23% or 6.8 % and 18%, respectively.

#### 5.3.5. *Data analysis*

Testicular IL-1 alpha, IL-1 beta and IL-6 concentrations (pg/mg of protein, pg/g of testis) and the IL-1 bioactivity of the testicular supernatants were analysed for effects of age by a one-way analysis of variance (ANOVA, Sigma Stat for Windows®, version 1.0; Jadel Corporation, San Rafael, CA, USA). If the main effects were significant multiple comparisons were made using the Fisher LSD method for post ANOVA multiple comparisons ( $P < 0.05$ ). Pearson correlations were used to examine the relationships between the temporal patterns of mean testicular IL-1 alpha, IL-1 beta and IL-6 concentrations. All data are expressed as mean  $\pm$  SEM.

## 5.4. Results

### 5.4.1. Testicular concentrations of IL-1 alpha and IL-1 beta

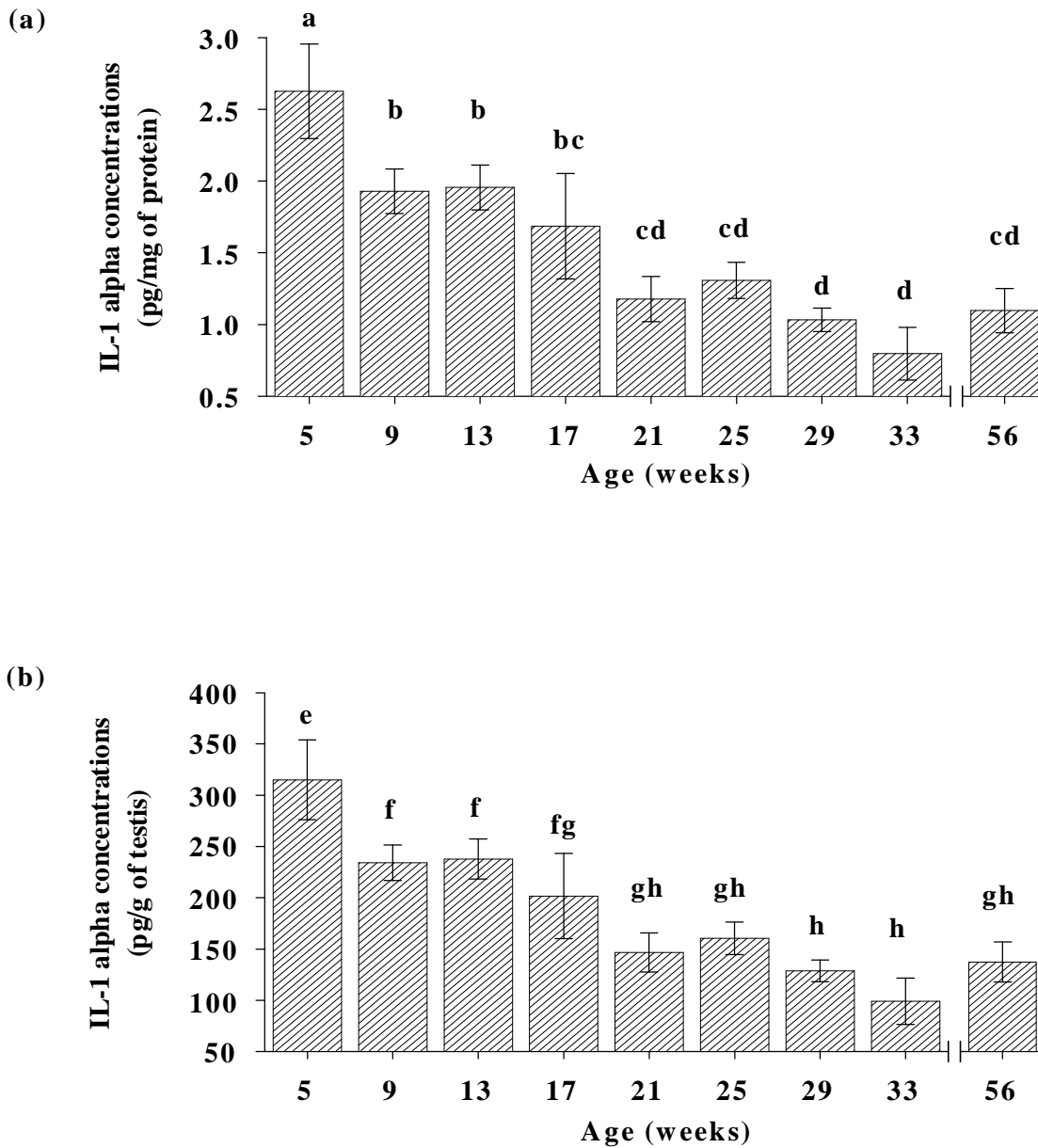
Mean testicular IL-1 alpha concentrations (pg/mg of protein or pg/g of testis) decreased from 5 to 9 weeks of age and subsequently from 13 to 21 weeks of age ( $P < 0.01$ ; Figure 5.1a, b). Mean testicular IL-1 beta concentrations (pg/mg of protein or pg/g of testis) decreased from 13 to 17 weeks of age and subsequently from 29 to a nadir at 33 weeks of age ( $P < 0.05$ ; Figure 5.2a, b). Mean testicular concentrations of IL-1 alpha (pg/mg of protein or pg/g of testis) were positively correlated with mean testicular concentrations of IL-1 beta (pg/mg of protein or pg/g of testis) ( $P < 0.01$ ;  $r = 0.55$  and  $0.58$ ; respectively).

### 5.4.2. Testicular supernatant IL-1 bioactivity

Mean IL-1 bioactivity (pg/mg of protein or pg/g of testis) increased from 13 to 17 weeks of age, decreased to 21 weeks of age, increased to 25 weeks of age, decreased to 29 weeks of age and subsequently from 33 to 56 weeks of age ( $P < 0.05$ ; Figure 5.3a, b).

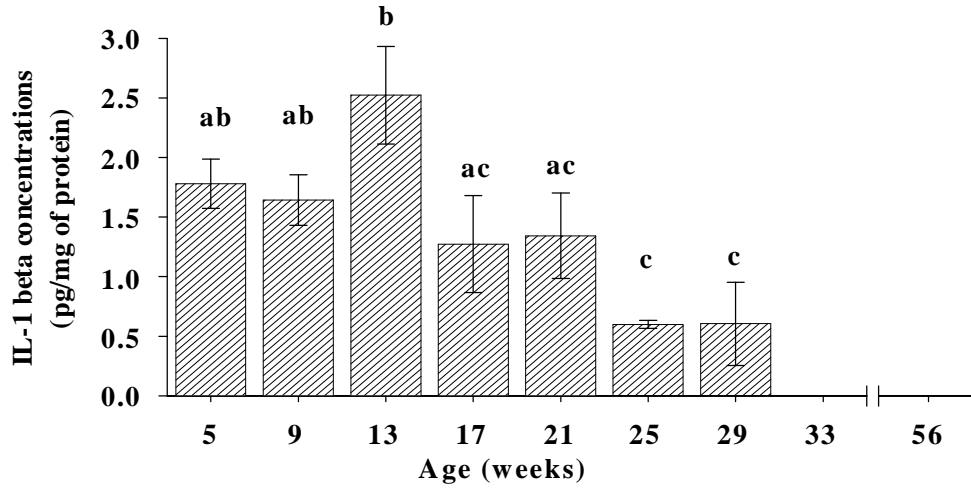
### 5.4.3. Mean testicular concentrations of IL-6

Mean testicular IL-6 concentrations (pg/mg of protein or pg/mg of testis) decreased from 9 to 13 weeks of age, increased from 13 to 21 weeks of age, decreased from 21 to 25 weeks of age, increased from 25 to 29 weeks of age and then subsequently decreased from 29 weeks of age to a nadir at 56 weeks of age ( $P < 0.05$ ; Figure 5.4a, b). Mean testicular IL-6 concentrations (pg/g of testis) were negatively correlated with mean IL-1 alpha concentrations (pg/g of testis) ( $P < 0.05$ ;  $r = -0.37$ ).

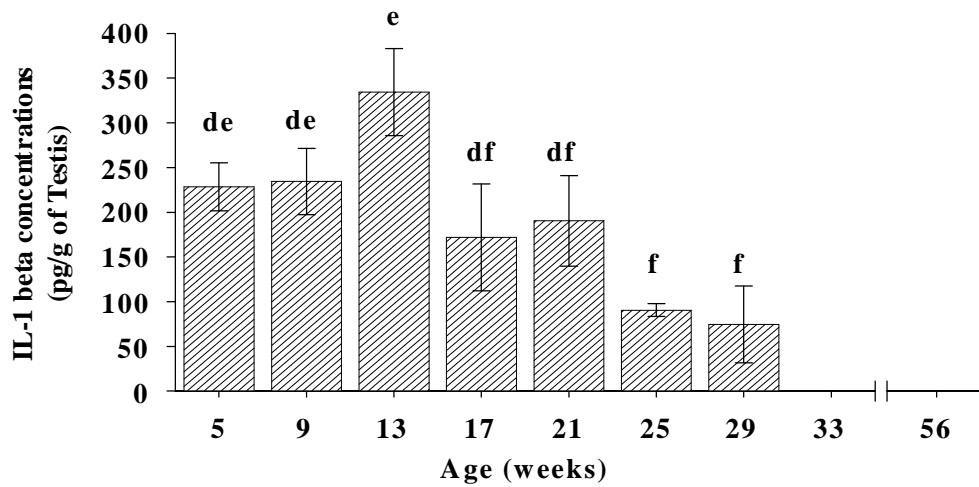


**Figure 5 1** Mean ( $\pm$  SEM) concentrations of IL-1 alpha ([a] pg/mg of protein and [b] pg/g of testis) in testes collected from separate groups of bull calves ( $n = 6$ ), castrated at 4 week intervals, from 5 to 33 weeks of age and at 56 weeks of age. The differences between ages within variables are indicated by different superscripts a, b, c ( $P < 0.05$ ).

(a)

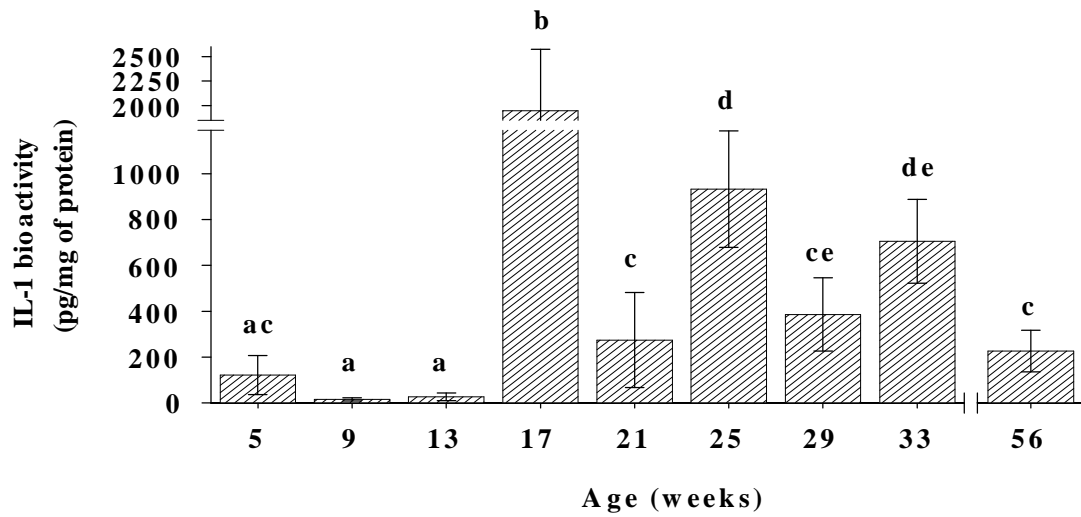


(b)

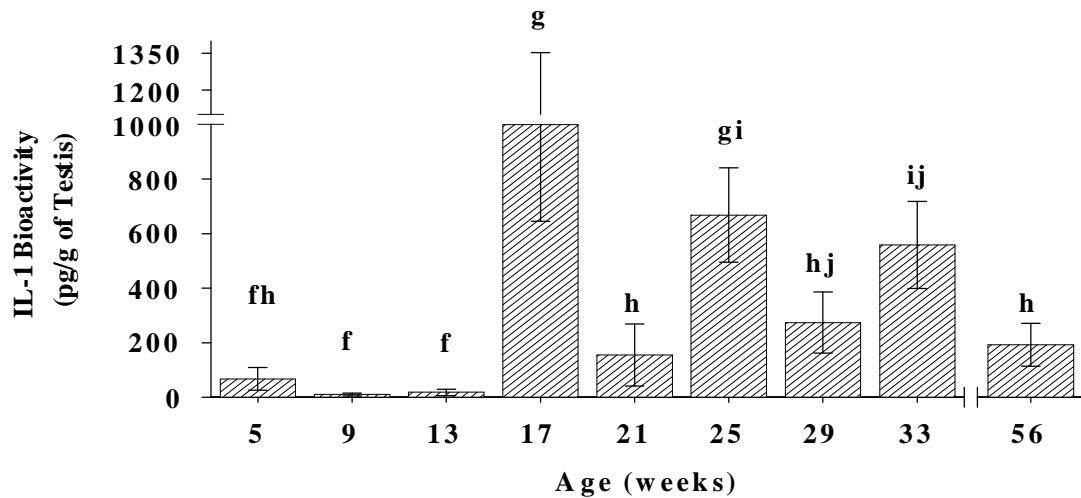


**Figure 5 2** Mean ( $\pm$  SEM) concentrations of IL-1 beta ([a] pg/mg of protein and [b] pg/g of testis), in testes collected from separate groups of bull calves ( $n = 6$ ), castrated at 4 week intervals, from 5 to 33 weeks of age and at 56 weeks of age. The differences between ages within variables are indicated by different superscripts a, b, c ( $P < 0.05$ ).

(a)

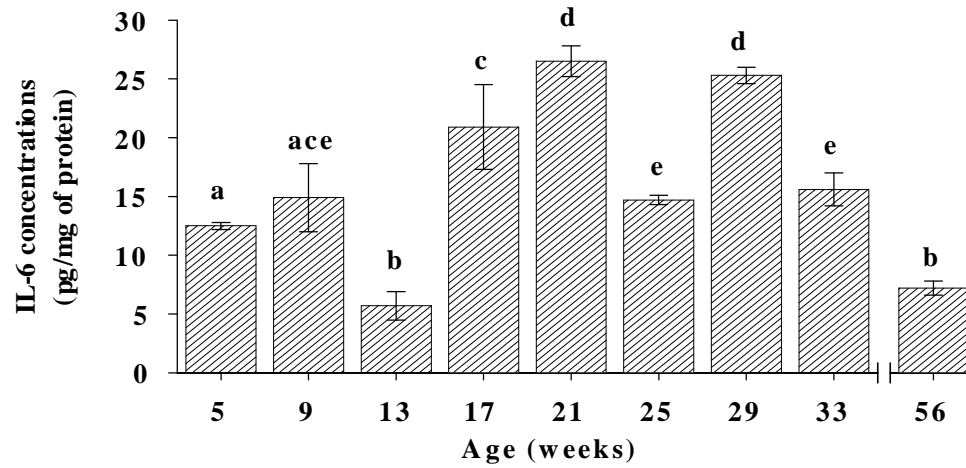


(b)

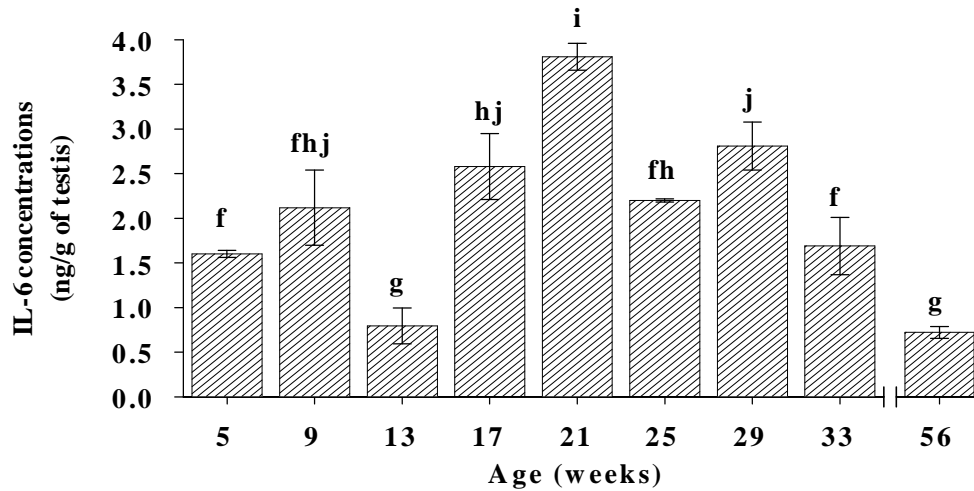


**Figure 5 3** Mean ( $\pm$  SEM) IL-1 bioactivity (a) pg/mg of protein and (b) pg/g of testis in testes collected from separate groups of bull calves ( $n = 6$ ), castrated at 4 week intervals, from 5 to 33 weeks of age and at 56 weeks of age. The differences between ages within variables are indicated by different superscripts a, b, c ( $P < 0.05$ ).

(a)



(b)



**Figure 5 4** Mean ( $\pm$  SEM) of IL -6 concentrations ([a] pg/mg of protein and [b] pg/g of testis, in testes were collected from separate groups of bull calves ( $n = 6$ ), castrated at 4 week intervals, from 5 to 33 weeks of age and at 56 weeks of age. The differences between ages within variables are indicated by different superscripts a, b, c ( $P < 0.05$ ).

## 5.5. Discussion

In the present study, IL-1 alpha and IL-6 were detected in the testis of bull calves of all ages; however, IL-1 beta was only detected in the testis of calves aged between 5 and 29 weeks. Testicular concentrations of IL-1 alpha and IL-1 beta were high during the early postnatal period and then decreased with age to a nadir in the peripubertal period. In Hereford X Charolais bull calves, puberty is attained around 45 week of age (Evans *et al.* 1993; Rawlings & Evans 1995; Evans *et al.* 1996; Aravindakshan *et al.* 2000a, b). With the exception of calves at 13 weeks of age, mean testicular concentrations of IL-6 were higher in immature calves during the early postnatal and prepubertal period as compared to post pubertal bulls. The temporal patterns of testicular concentrations of IL-1 alpha, IL-1 beta and IL-6 seen during development in bull calves, in the present study, agree with observations in mice (Huleihel & Lunenfeld 2002; Potashnik *et al.* 2005). In the present study, the positive correlation of mean testicular concentrations of IL-1 alpha with IL-1 beta indicated a possible regulatory interaction between the two cytokines. In previous reports, treatment of Sertoli cells isolated from early postnatal mice (Zeyse *et al.* 2000) or Leydig cells from pubertal rats (Wang *et al.* 1991), with increasing concentrations of recombinant IL-1 beta caused a dose dependent increase in IL-1 alpha concentrations. In the present study, the negative correlation of mean testicular concentrations of IL-1 alpha with IL-6 indicated a possible antagonistic regulatory mechanism. However, previous reports in rats showed that residual bodies shed during the late stages of spermiation triggered Sertoli cell production of IL-1, which in turn activated the production of leukotrienes, the latter subsequently stimulating IL-6 secretion (Gerad *et al.* 1992; Syed *et al.* 1995).

In the present study, IL-1 bioactivity was lowest in testicular supernatants from bull calves aged between 5 and 13 weeks during the early postnatal period. The IL-1 bioactivity was higher in testicular supernatants collected from bull calves aged between 17 and 56 weeks during the pre and post pubertal period. These observations are in agreement with earlier reports in rats in which IL-1 bioactivity was only detected in pre and post pubertal but not early postnatal rat testes (Khan *et al.* 1987; Syed *et al.* 1988). In the present study, the disparity between changes in testicular IL-1 bioactivity with age and concentrations of IL-1 alpha and beta determined by ELISA was probably due to the presence of high concentrations of endogenous IL-receptor antagonist (IL-1 ra) in the early postnatal calves blocking the biological IL-1 agonist activity at that time. The ability of IL-1 ra to inhibit the IL-1 bioactivity has been demonstrated in several species (Hannum *et al.* 1990; Arend 1993). In mice, FSH was shown to induce an acute increase in production of IL-1 ra but had no effect on the production of IL-1 alpha by Sertoli cells (Huleihel & Lunenfeld 2002). The relatively high serum FSH concentrations reported in bull calves during the early postnatal period (Evans *et al.* 1993; Rawlings & Evans 1995, Evans *et al.* 1996; Aravindakshan *et al.* 2000a, b; Bagu *et al.* 2006) might have induced IL-1 ra production in addition to the other IL-1 isoforms. We were unable to determine IL-1 ra concentrations in the present study due to lack of an appropriate bovine IL-1 ra assay.

The temporal changes in serum concentrations of LH, FSH and testosterone of the bull calves in the present study have been published (Bagu *et al.* 2006). However, when the mean testicular concentrations of IL-1 alpha, IL-1 beta and IL-6 of bull calves aged



between 5 and 33 weeks were correlated with mean serum concentrations of FSH and testosterone in blood samples collected a week prior to each castration, the following correlations were found: mean testicular concentrations of IL-1 alpha and IL-1 beta, were positively correlated with mean serum FSH concentrations ( $r = 0.47$  and  $0.46$ , respectively) while mean testicular concentrations of IL-6 were positively correlated with mean serum testosterone concentrations ( $r = 0.45$ ). The correlation of mean serum FSH concentrations with mean testicular concentrations of IL-1 alpha and IL-1 beta indicated a possible regulatory interaction of FSH with IL-1 alpha and IL-1 beta. In previous reports, addition of FSH to cell cultures of human Sertoli cells, exposed to hypotonic shock, stimulated the production of IL-1 alpha (Cudicini *et al.* 1997). In bull calves, mean serum FSH concentrations are slightly elevated in the early postnatal period, decreasing from 24 to 32 week of age (Evans *et al.* 1996; Rawlings & Evans 1995; Aravindakshan *et al.* 2000a, b; Bagu *et al.* 2006). The reported temporal changes of mean serum FSH concentrations in bull calves are similar to the trend of mean testicular concentrations of IL-1 alpha and IL-1 beta seen in the present study. A possible regulatory interaction between testosterone and IL-6 was indicated by the positive correlation between them in the present study. Physiological concentrations of testosterone have been shown to stimulate the production of IL-6 in mouse Sertoli cells (Stephan *et al.* 1997). In bull calves, mean serum testosterone concentrations are low in the early postnatal period increasing minimally from 18 to 24 weeks of age and markedly after 28 weeks of age (Rawlings *et al.* 1972; 1978, Secchiari *et al.* 1976; Amann & Walker 1983; Rawlings & Cook 1986; Rawlings & Evans 1995). In the present study, the increase in mean testicular concentrations of IL-6 coincided with the

period in bull calves when the mean serum testosterone concentrations are reported to be on the increase.

In bull calves, the demise of foetal Leydig cells during the early postnatal period (Hooker 1970) is followed by a phase of rapid differentiation and maturation of adult Leydig cells from progenitor cells around 12 to 16 weeks of age. Amann (1983) suggested that the high frequency of LH pulses that occurred after 4 weeks of age initiated the process of rapid Leydig cell differentiation. In the present study, the coincidence of high mean testicular concentrations of IL-1 isoforms with the period when foetal Leydig cells are actively being replaced by adult Leydig cells indicated a possible paracrine regulatory role for IL-1 in Leydig cell development. In immature rats, IL-1 was shown to stimulate basal and gonadotropin induced production of testosterone in Leydig cells (Verhoeven *et al.* 1988; Svechnikov *et al.* 2001) as well as DNA synthesis and proliferation in Sertoli cells (Petersen *et al.* 2002) and immature germ cells [spermatogonia and preleptotene spermatocytes] (Parvinen *et al.* 1991; Soder *et al.* 1991). In bull calves, initiation of spermatogenesis occurs between 16 and 28 weeks of age (Curtis & Amann 1981) and around the same period, indifferent supporting cells were shown to differentiate into Sertoli cells (McCarthy *et al.* 1979a, b; Curtis & Amann 1981; Amann 1983). In the present study, high mean testicular concentrations of IL-1 alpha and IL-1 beta during the early postnatal period, prior to the time in bull calves when spermatocytes (preleptotene and diplotene) and mature Sertoli cells appear in the seminiferous tubules, indicated a possible paracrine involvement of IL-1 in the initiation of germ cell and Sertoli cell development. In the present study, the

presence of testicular IL-1 alpha but not IL-1 beta in bull calves during the peripubertal period indicated IL-1 alpha might have been involved in the maintenance of spermatogenesis. The coincidence of increased mean testicular concentrations of IL-6 with the period of Sertoli cell differentiation in bull calves (17 to 29 weeks of age) indicated a possible paracrine role of IL-6 in Sertoli cell development. In immature rats, IL-6 was shown to induce the transcription of “early genes” (Jenab & Morris 1997) and basal production of transferin in Sertoli cells (Boockfor & Schwaz 1991; Hoeben *et al.* 1997). In the present study the presence of IL-6 during the peripubertal period provided support that IL-6 could have been involved in the regulation of spermiogenesis and spermatogenesis. In rats, testicular IL-6 concentrations were shown to be highest during stages II-VI and lowest during stages VII-VIII, when the elongated spermatids and rounded spermatids respectively are the most mature germ cells (Syed *et al.* 1993). In bulls elongated spermatids appear in seminiferous tubules as the most mature germ cell at 32 weeks of age (Curtis & Amann 1981).

In conclusion, the production of testicular IL-1 and IL-6 concentrations was age dependent. Testicular IL-1 concentrations were greatest in the early postnatal period but lowest in the peripubertal period; however, IL-1 bioactivity, although variable, was low in the early postnatal period and greater in the immediate prepubertal period. Testicular IL-6 concentrations were greatest in prepubertal calves as compared to adults.

**Chapter 6: EFFECTS OF TREATMENT WITH LH OR FSH FROM 4 TO 8 WEEKS OF AGE ON THE ATTAINMENT OF PUBERTY IN BULL CALVES**

Bagu ET, Madgwick S, Duggavathi R, Bartlewski PM, Barrett DMW, Huchkowsky S, Cook SJ and Rawlings NC.

**6.1. Abstract**

A transient increase in gonadotropin secretion between 6 and 20 weeks of age is critical for the onset of puberty in bull calves. To try and hasten the onset of puberty, bull calves were treated (sc) with 3 mg of bLH (n = 6) or 4 mg of bFSH (n = 6) once every 2 d, from 4 to 8 wk after birth; control calves received saline (n = 6). At 4 and 8 weeks of age, mean LH concentrations were greater ( $P < 0.05$ ) in bLH-treated ( $2.3 \pm 0.04$  ng/ml and  $1.20 \pm 0.04$  ng/ml) than control calves ( $0.50 \pm 0.1$  ng/ml and  $0.70 \pm 0.10$  ng/ml). Mean serum FSH concentrations at 4 and 8 weeks of age, were greater ( $P < 0.05$ ) in bFSH-treated ( $1.60 \pm 0.20$  ng/ml and  $1.10 \pm 0.2$  ng/ml) than saline-treated calves ( $0.38 \pm 0.07$  ng/ml and  $0.35 \pm 0.07$  ng/ml). A scrotal circumference (SC)  $\geq 28$  cm was attained earlier in bFSH- than saline-treated calves ( $P < 0.05$ ;  $39.3 \pm 1.3$  and  $44.8 \pm 1.3$  weeks of age, respectively). Based on testicular histology at 56 weeks of age, treatment with bFSH resulted in greater ( $P < 0.05$ ) numbers of Sertoli cells ( $5 \pm 0.2$ ,  $6 \pm 0.3$  and  $5 \pm 0.3$  in bLH-, bFSH- and saline- treated calves, respectively); elongated spermatids ( $42 \pm 2$ ,  $57 \pm 8$  and  $38 \pm 5$  in bLH-, bFSH- and saline- treated calves, respectively) and spermatocytes ( $31 \pm 3$ ,  $38 \pm 3$  and  $29 \pm 2$  in bLH-, bFSH- and saline- treated calves, respectively) per

seminiferous tubule. We concluded that treatment of bull calves with bFSH from 4 to 8 weeks of age increased testicular growth (SC); hastened onset of puberty (SC  $\geq$  28 cm); and enhanced spermatogenesis.

## **6.2. Introduction**

In cattle puberty is evaluated on the basis of testicular growth and the beginning of sperm production (Wolf *et al.* 1965; Skinner *et al.* 1968a, b; Linstra *et al.* 1978). In the bull puberty has been defined as the time when an ejaculate has at least  $5.0 \times 10^7$  sperm and a 10% linear motility (Wolf *et al.* 1965); this occurs at a scrotal circumference (SC) of  $27.9 \pm 0.2$  cm (Linstra *et al.* 1978). The age at puberty varies with the breed; in Hereford bulls it ranges from 39 to 52 weeks of age while in Charolais from 33 to 53 weeks of age (Wolf *et al.* 1965; Almquist & Cunningham 1967; Killian & Amann 1972; Evans *et al.* 1996).

In bull calves there is an early postnatal increase in mean serum LH concentrations occurring over the period from 4 to 25 weeks of age; this has been attributed to an increase in LH pulse frequency (Rawlings *et al.* 1978; McCathy *et al.* 1979; Wise *et al.* 1987; Evans *et al.* 1993, 1996). The mean serum LH concentrations between 12 to 17 weeks of age are greater in early compared to late pubertal bulls (Evans *et al.* 1995). The pattern of FSH secretion is variable; mean serum FSH concentrations are often slightly elevated in the early postnatal period then decreasing between 24 to 32 weeks of age (Rawlings & Evans 1995; Evans *et al.* 1996; Aravindakshan *et al.* 2000a, b). McCarthy *et al.* (1979a), showed that mean serum FSH concentrations did not change

with age; however, according to Amann & Walker (1983), mean serum FSH concentrations increase by 30% between 4 to 32 weeks of age with no evidence of pulsatile discharge. Stumpf *et al.* (1993) also argued that the FSH concentrations in jugular blood are not pulsatile; pulsatile secretion is perhaps masked by the long half-life of FSH (Akbar *et al.* 1974).

The early postnatal rise in gonadotropin secretion precedes rapid testicular growth occurring after 25 weeks of age (Curtis & Amann 1981; Evans *et al.* 1996). During rapid testicular development, mean serum LH concentrations are low (Evans *et al.* 1996); while mean serum FSH concentrations are quite variable with an increase in concentration reported in some studies (Mac Donald *et al.* 1990; Rawlings & Evans 1995; Moura & Erickson 1997). Moura and Erickson (1997) found basal FSH concentrations from 8 to 12 and 8 to 48 weeks of age to be negatively correlated with the number of Sertoli cells per testis and testicular diameter respectively, at 1 year of age.

In a review on sexual development in bull calves by Amann (1983), he suggested that many attempts to hasten the onset of puberty with exogenous GnRH have not been successful because calves were treated too late in development. Chandolia *et al.* (1997) gave Hereford bull calves pulses of GnRH every 2 h from 4 to 6 weeks of age; this increased the LH pulse frequency prior to the normal endogenous postnatal increase in LH and at 6 weeks of age caused a numerical but non significant increase in basal and mean serum FSH concentrations. Treatment with GnRH hastened the onset of puberty,

increased Sertoli cell counts, sperm output and testicular weight although the exact role of LH and FSH on hastening the onset of puberty was unclear (Chandolia *et al.* 1997c).

We designed the present experiment to see the effects of LH or FSH individually, prior to the early postnatal increase in LH secretion, on the onset of puberty. Our objective was also to examine the effects of supra-physiological doses of gonadotropins, in a more practical but longer treatment regime as compared to the GnRH regime used by Chandolia *et al.* (1997c).

### **6.3. Materials and methods**

#### *6.3.1. Animals and experimental procedures*

Eighteen spring-born, age matched ( $\pm 3$  days) bull calves (Hereford x Charolais) were divided into three groups of six calves. Calves were suckled at pasture until they were weaned, at 26 weeks of age. After weaning, calves were kept in corrals and provided ad libitum with water and a standard feed ration as described by Evans *et al.* (1995). All experimental procedures were done in accordance with the regulations of the Canadian Council for Animal Care.

From 4 to 8 weeks of age, bull calves were treated subcutaneously in the neck region, once every 2 days with either 4 mg of bFSH (equivalent to 10 mg NIH-FSH-SI; LH contamination  $<0.05\%$ ; NOBL, Laboratories Inc., Sioux Centre, IA, USA), 3 mg of bLH (equivalent to 5 mg NIH-LH-S1; bFSH contamination,  $<0.05\%$ ; NOBL, Laboratories Inc., Sioux Centre, IA, USA) in saline (1 mg/mL) or saline alone. All

injections were done between 08:00 and 10:00. The doses of bLH and bFSH were derived from preliminary studies and the treatments were designed to create supra-physiological gonadotropin concentrations in 6 weeks old bull calves.

### 6.3.2 *Blood sampling*

Blood samples were taken from the jugular vein by an intravenous catheter as described by Evans *et al.* (1995). During the “intensive bleed”, blood (5 ml) was collected every 15 minutes for 10 hours, at 4 and 8 weeks of age and then every 6 weeks, from 8 to 38 weeks of age. At 4 and 8 weeks of age saline, bFSH or bLH treatments were administered 1 h after commencement of blood sampling. Blood samples were also taken every 6 hours for 36 hours post treatment. Blood samples were left to clot for  $\geq 12$  h at room temperature then serum was harvested and frozen at  $-20^{\circ}\text{C}$  until assayed (Evans *et al.* 1995).

### 6.3.3. *Body weight, testicular growth and sperm production*

Body weight and scrotal circumference (SC) were measured every other week; body weights were recorded from birth to 54 weeks of age and SC from 2 to 48 weeks of age. In the present study age of puberty was regarded the age at which a SC of  $\geq 28$  cm was attainment. To confirm the achievement of puberty, calves were electroejaculated once every 2 wk from the time they attained a SC  $\geq 26.5$  cm until an ejaculate of  $\geq 5 \times 10^7$  sperm per ml with progressive linear motility of  $\geq 10\%$  was collected [based on Wolf *et al.*, (1965) but expressed as per ml of ejaculate].



#### *6.3.4. Castration and histology*

At 56 weeks of age, bulls were castrated, the epididymis was dissected from each testis, testes and epididymides were weighed. Samples were taken from the dorsal and ventral poles of the left testicular parenchyma of each bull and fixed in Helly's reagent for 24 h, rinsed in water for 48 h and then washed in 70% ethanol. The tissue was dehydrated in alcohol and then embedded in paraffin wax. Five  $\mu\text{m}$  thick sections were prepared and stained with haematoxylin and periodic acid Schiff's (H-PAS). Tissue sections were evaluated for stage of development of spermatogenesis. A systematic, uniform, random sampling technique as described by Curtis & Amann (1981) was used to select five round seminiferous tubules between Stages I to VI of the spermatogenic wave per cross section (10 round seminiferous tubules per bull). Sertoli and germ cells (elongated spermatids, round spermatids, spermatocytes and spermatogonia) counts per seminiferous tubule selected were determined at a magnification of X 1000. In order to get the true counts, the crude counts were corrected for section thickness and cell nuclear diameter (Abercrombie 1946). All histological evaluations were done by a single operator, in a random order with out knowledge of the treatments.

#### *6.3.5. Radioimmunoassays*

Serum LH and FSH concentrations in all samples collected were determined using previously validated, double-antibody, radioimmunoassays (Rawlings & Evans 1995). The assay coefficients of variation (CV) were determined by replicating low and high reference sera in each assay (0.2 ng/ml or 0.9 ng/ml for LH and 1.9 ng/ml or 6.1 ng/ml

for FSH respectively). Serum LH concentrations are expressed in terms of NIDDK-bLH4. The intra- and inter-assay CVs were 9.7 and 12.8 % or 7.1 and 9.2 % respectively. Concentrations of FSH were expressed in terms of USDA-bFSH-I1. The sensitivity of the FSH assay was 0.1 ng/ml. The intra- and inter- assay CVs were 6.9 % and 12 % or 5.8 % and 9.9 %, respectively.

#### *6.3.6. Data analysis*

The characteristics of secretory patterns of serum LH and FSH in blood samples collected every 15 minutes for 10 h, were determined by the PC-Pulsar program (J. Gitzen and V Ramirez, University of Illinois, Urbana, IL, US). The secretory patterns of LH were pulsatile and pulse frequency and amplitude are presented in addition to basal and mean serum concentrations. The secretory patterns of FSH were judged to be nonpulsatile and mean concentrations are given. LH pulses were defined using standard deviation criteria of height (G Values) and duration (Merriam & Wachter 1982). The PC-Pulsar program computes basal concentrations of LH by subtraction of all data points that make up pulses from the 10 hours LH profile.

Data for LH pulse amplitude, LH pulse frequency, basal and mean LH and mean FSH concentrations, scrotal circumference and body weight, were analysed for effects of treatment, age and interactions by two-way repeated measures analysis of variance (RM-ANOVA, Sigma Stat for Windows®, Version 1.0; Jadel Corporation, San Rafael, CA, USA). If the main effects were significant, multiple comparisons were made using a Student's t test for post-ANOVA multiple comparisons ( $P < 0.05$ ). All data collected

for SC were analysed in two ways, with and without a control bull that had abnormal spermatogenesis (oligospermia). This abnormal calf did not attain desired semen characteristics ( $\geq 5 \times 10^7$  sperm per mL with progressive linear motility  $>10\%$ ) until 12 wk after reaching a  $SC \geq 28$  cm (puberty); however, other control calves met the criteria after  $4 \pm 2$  weeks. Histological evaluation at 56 weeks of age revealed that spermatogenesis in the one abnormal control calf was at par with other control calves but not ahead, as the SC had suggested. A one-way ANOVA was used to evaluate effects of treatment on testicular and cauda epididymal weights at castration, Sertoli and germ cell numbers per seminiferous tubule, and age at puberty based on SC measurements and confirmed by semen characteristics. Pearson correlations were used to examine the relationships between age at puberty; testicular weight; cauda epididymal weight; Sertoli and germ numbers per seminiferous tubule and mean serum LH and FSH concentrations at 4 and 8 weeks of age.

## **6.4. Results**

### *6.4.1 Hormone concentrations*

In bull calves given bLH at 4 weeks of age, mean serum LH concentrations were elevated within 15 minutes after injection, with a peak at 75 minutes from injection ( $P < 0.05$ ; control calves:  $0.9 \pm 0.3$  ng/ml; bLH-treated calves:  $6.0 \pm 0.8$  ng/ml). Mean serum LH concentrations returned to control values by 375 minutes after injection ( $P > 0.05$ ; control calves  $0.7 \pm 0.4$  ng/ml; bLH-treated calves:  $1.2 \pm 0.1$  ng/ml). Over the 9 h after injection, mean serum LH concentrations did not differ between control calves and those given bFSH ( $P > 0.05$ ; Figure 6.1). At 4 week of age, injection of bFSH increased

mean serum FSH concentrations within 15 minutes, with a peak at 105 minutes from injection ( $P < 0.05$ ; control calves  $0.5 \pm 0.1$  ng/ml; bFSH-treated calves:  $3.01 \pm 0.47$  ng/ml). Mean serum FSH concentrations returned to control values by 24 h after injection ( $P > 0.05$ ; control calves:  $0.5 \pm 0.1$  ng/ml; bFSH-treated calves:  $0.6 \pm 0.1$  ng/ml). Over the 9 h from injection, mean serum FSH concentrations did not differ between control calves and calves given bLH ( $P > 0.05$ , Figure. 6.2).

At 8 weeks of age, injection of bLH resulted in increased mean serum LH concentrations over the period of 9 h after injection, compared to the controls calves ( $P < 0.05$ , Figure 6.1). However, there were no differences between the bLH-treated and control calves at individual time points. Mean serum LH concentrations in bFSH-treated calves did not differ from control bull calves ( $P > 0.05$ , Figure 6.1). Treatment of calves with bFSH at 8 weeks of age in increased mean serum FSH concentrations within 30 minutes of injection, with a peak 120 minutes after injection ( $P < 0.05$ ; control calves:  $0.4 \pm 0.1$  ng/ml; bFSH-treated calves  $1.5 \pm 0.3$  ng/ml). Mean serum FSH concentrations returned to control values by 495 minutes after injection ( $P > 0.05$ ; control calves  $0.6 \pm 0.2$  ng/ml; bFSH treated calves  $1.0 \pm 0.2$  ng/ml). Over the 9 h from injection, mean serum FSH concentrations were slightly greater in bLH-treated calves compared to control calves ( $P < 0.05$ ; Figure 6.2).

In control calves, mean serum LH concentrations were greatest at 14 weeks, reached a nadir at 20 weeks of age and subsequently remained low until 38 weeks of age ( $P < 0.05$ ; Figure 6.1). Mean serum concentrations of FSH in control calves decreased from 14

weeks of age to a nadir at 26 weeks of age, then increased significantly at 32 weeks of age ( $P < 0.05$ ; Figure. 2). In control calves, the highest basal serum LH concentrations were noted at 8 wk of age; there was a subsequent decline to 20 weeks of age ( $P < 0.05$ ; Figure 6.3). Calves treated with bLH or bFSH from 4 to 8 weeks of age had a significantly lower LH pulse frequency at 14 weeks of age as compared to control calves ( $P > 0.05$ ; Figure 6.3). In control calves, LH pulse frequency increased from 4 to 14 weeks of age, decreased at 26 weeks and subsequently remained low until 38 weeks of age ( $P > 0.05$ ; Figure 6.3).

#### *6.4.2. Body weight, testicular growth and sperm production*

Treatment had no effect on the rate of body weight gain, determined from weights measured every 2 weeks ( $P > 0.05$ ). At birth, mean weights in bLH-treated, bFSH-treated, and control calves were  $51 \pm 2$ ,  $50 \pm 3$  and  $48 \pm 3$  kg, respectively. At 54 weeks of age, mean body weight increased to  $406 \pm 51$ ,  $408 \pm 55$  and  $410 \pm 60$  kg in bLH-treated, bFSH-treated and control calves, respectively ( $P > 0.05$ ).

When analysis of scrotal circumference included data from the one abnormal control calf with oligospermia, there was no significant treatment effect ( $P > 0.05$ ; Figure 6.4a).

When data from the calf with oligospermia was excluded, bFSH-treated calves had significantly greater SC than control calves from 38 to 44 weeks of age ( $P < 0.05$ ; Figure 6.4b). Puberty based on age at which an SC of  $\geq 28$  cm occurred earlier in bFSH-treated calves as compared to control calves ( $P < 0.05$ ;  $42.7 \pm 2.2$ ,  $39.3 \pm 1.3$  and  $44.8 \pm 1.3$  weeks of age for LH, FSH and saline treated calves respectively), when data of a control calf

that was diagnosed with oligospermia was eliminated. Treatment had no significant ( $P>0.05$ ) effect on age at which puberty was confirmed by semen characteristics, with or without the oligospermic control calf. Mean age at puberty for bLH, bFSH and saline treated calves, including all calves were  $47\pm 1.8$ ,  $44.3\pm 1.1$  and  $47.6\pm 2.1$  weeks of age, respectively ( $P>0.05$ ).

#### 6.4.3 *Castration and histology*

At castration, the mean testicular and cauda epididymal weights did not differ among the three groups of bull calves ( $P>0.05$ ). The paired testes weights in bLH, bFSH and saline treated calves were  $671\pm 51$ ,  $657\pm 29$  and  $630\pm 53$  g, respectively; while paired epididymal weights in bLH-treated, bFSH-treated and control calves were  $11.1\pm 1.2$ ,  $11.9\pm 0.8$ , and  $10.59\pm 1.1$  g, respectively. Based on histological evaluation of testicular tissue collected from all bulls at 56 weeks of age, bFSH treatment, unlike bLH, resulted in significantly greater numbers of Sertoli cells, elongated spermatids and spermatocytes per seminiferous tubule compared to control calves ( $P<0.05$ ; Table 6.1).

Age at puberty ( $SC\geq 28$  cm) was negatively correlated with paired testicular weight ( $r = 0.7$ ;  $P<0.05$ ); however, it was positively correlated with age at semen characteristics ( $\geq 5 \times 10^7$  sperm per mL with progressive linear motility  $\geq 10\%$ ) were attained ( $r = 0.7$ ;  $P<0.05$ ). The correlations of age at puberty ( $SC\geq 28$  cm) with mean serum FSH concentrations at 8 wk of age and number of Sertoli cells were:  $r = -0.47$  and  $-0.45$  respectively; and  $P = 0.05$  and  $0.06$  respectively. Mean serum FSH concentrations at 8 wk of age had a correlation value of  $r = -0.46$  with age at which semen characteristics ( $\geq 5 \times 10^7$  sperm per mL with progressive linear motility  $\geq 10\%$ ) was attained ( $P=0.06$ ).

Mean serum FSH concentrations at 4 and 8 wk of age were positively correlated with Sertoli and germ cell counts (Table 6.2). The age at which the desired semen characteristics was reached, was negatively correlated with the number of Sertoli cells, elongated spermatids and round spermatids (Table 6.2).

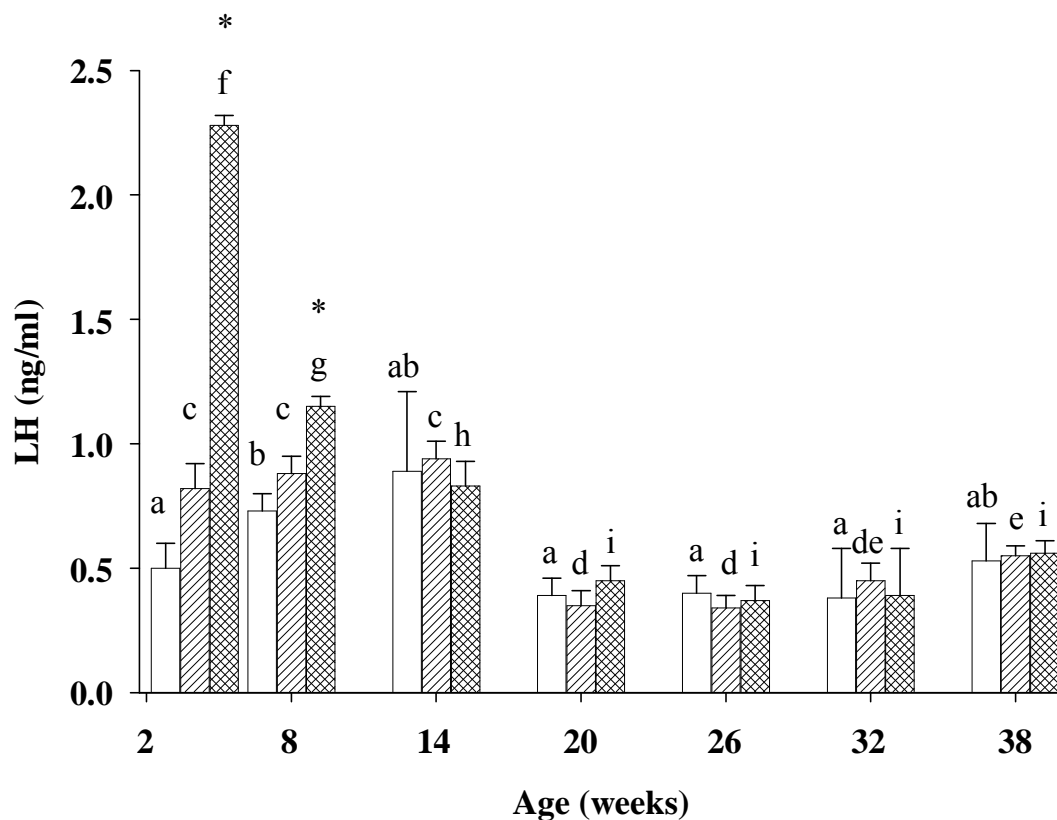
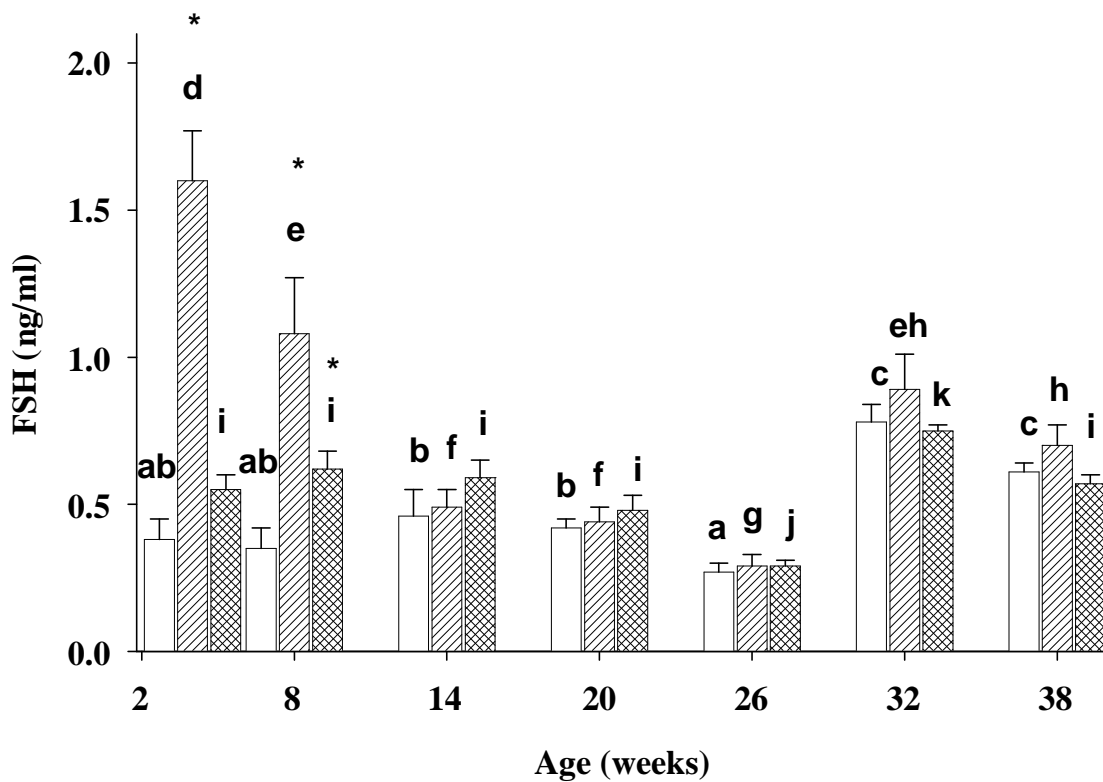
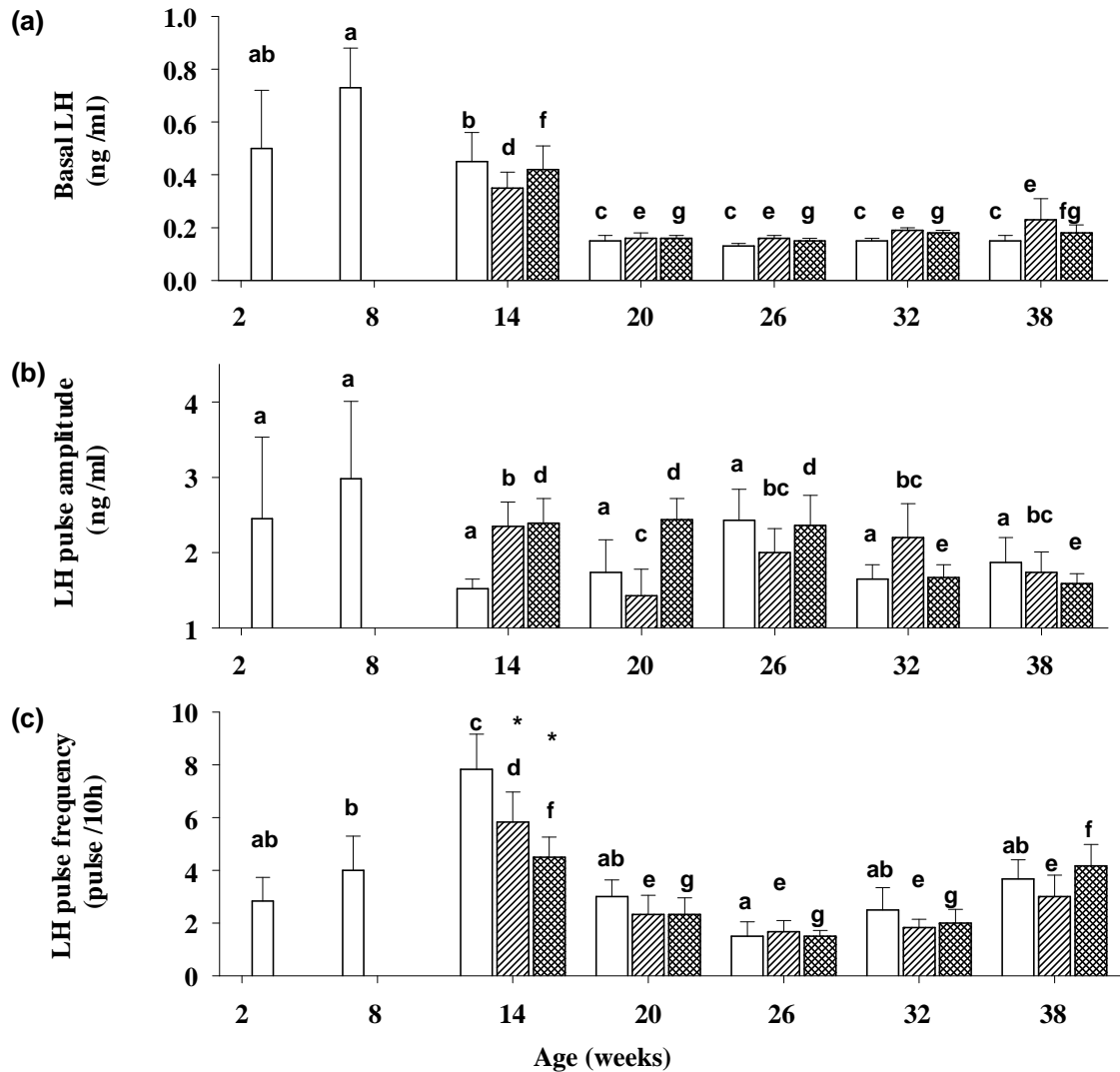


Figure 6.1 Mean ( $\pm$  SEM) serum LH concentrations at 4, 8, 14, 20, 26, 32, and 38 wk of age, in control calves (open bars,  $n = 6$ ) and calves treated once every 2 d with a subcutaneous injection of either 4 mg of bFSH (left hatched bars,  $n = 6$ ) or 3 mg of bLH (crisscrossed bars,  $n = 6$ ), from 4 to 8 wk of age. Blood samples were collected every 15 minutes for 10 h, and injections were given 1 h after commencement of the intensive bleed. Differences between ages within treatment are indicated by different letters ( $P < 0.05$ ), \* values are different from control bull calves at the same age.

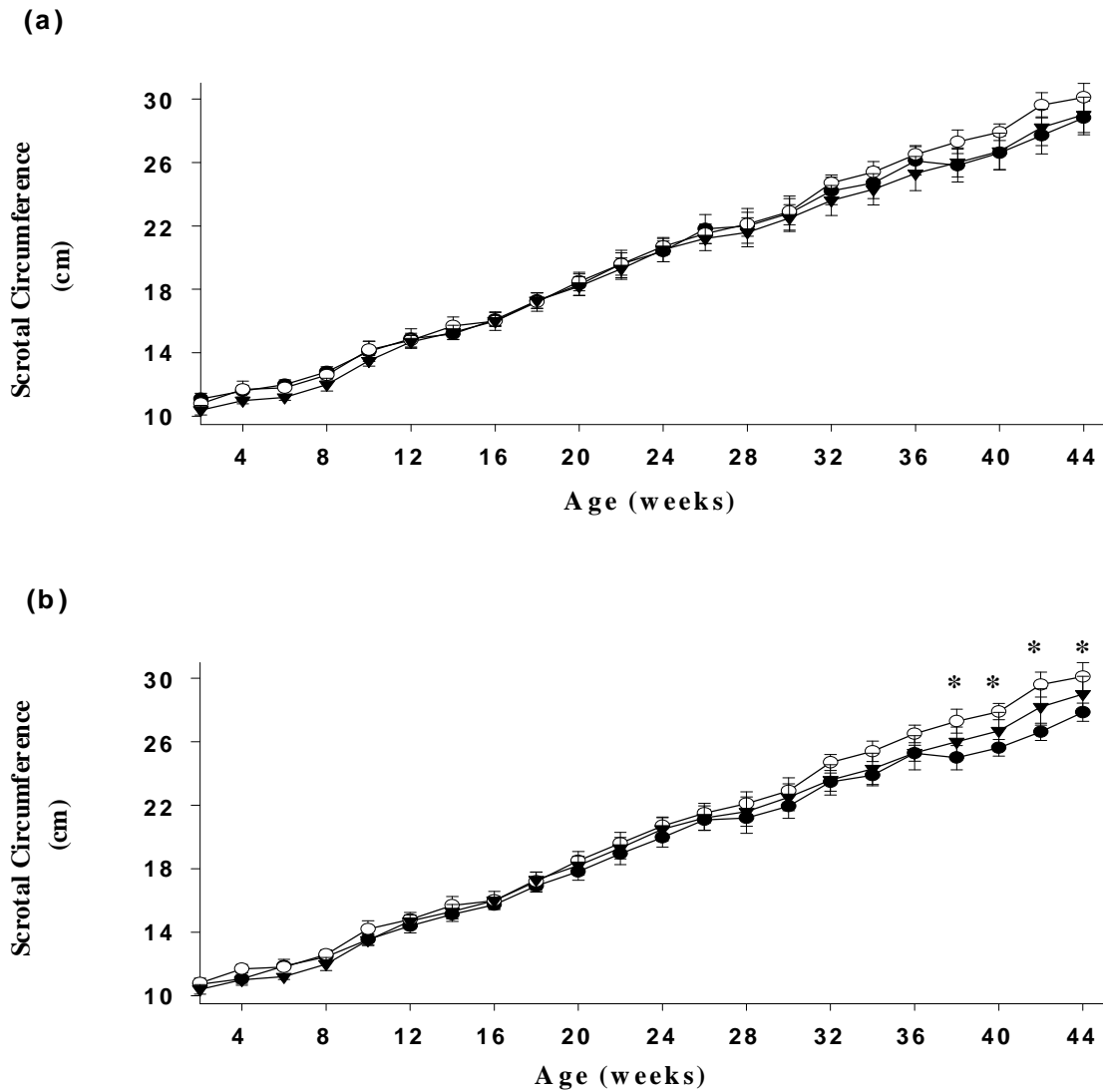




**Figure 6.1** Mean ( $\pm$  SEM) serum FSH concentration, at 4, 8, 14, 20, 26, 32, and 38 wk of age, in control calves (open bars,  $n = 6$ ), and calves treated once every 2 d with a subcutaneous injection of either 4 mg of bFSH (left hatched bars,  $n = 6$ ), or 3 mg of bLH (crisscrossed bars,  $n = 6$ ), from 4 to 8 wk of age. Blood samples were collected every 15 minutes for 10 h, and injections were given 1 h after commencement of the intensive bleed. Differences between ages within treatment are indicated by different letters ( $P < 0.05$ ), \* values are different from control bull calves at the same age.



**Figure 6.2** Serum basal LH concentrations, LH pulse amplitude and LH pulse frequency, at 4, 8, 14, 20, 26, 32, and 38 wk of age, in control calves (open bars, n = 6), and calves treated once every 2 d with a subcutaneous injection of either 4 mg of bFSH (left hatched bars, n = 6) or 3 mg of bLH (crisscrossed, n = 6), from 4 to 8 wk of age. Blood samples were collected every 15 minutes for 10 h, and injections were given 1h after commencement of the intensive bleed. Differences between ages within treatment are indicated by different letters (P<0.05), \*values are different from control bull calves at the same age.



**Figure 6.3** Scrotal circumference measured once every 2 wks from 2 to 48 wk of age in control calves (●; n = 6), and in calves treated once every 2 d with a subcutaneous injection of either 4 mg of bFSH (○, n = 6) or 3 mg of bLH (▼, n = 6), from 4 to 8 wk of age. Data presented from 2 to 44 wk of age since many calves had reached puberty by 44 wk of age with (a) in the presence and (b) in the absence of one abnormal (oligospermic) control calf, \* values are different (P < 0.05) from control bull calves at the same age.

Table 6. 1 Mean ( $\pm$ S.E.M) Sertoli and germ cell counts (elongated spermatids, round spermatids, spermatocytes and spermatogonia) per seminiferous tubule in all calves treated once every 2 days with: 4 mg of bFSH (n = 6), 3 mg of bLH (n = 6), or saline (n = 6) from 4 to 8 weeks of age.

Group	Sertoli Cells	Elongated spermatids	Rounded Spermatids	Spermatocytes	Spermatogonia
Control	5 $\pm$ 0.3 <sup>a</sup>	38 $\pm$ 5 <sup>a</sup>	47 $\pm$ 5	29 $\pm$ 2 <sup>a</sup>	17 $\pm$ 2
bFSH	6 $\pm$ 0.3 <sup>b</sup>	57 $\pm$ 8 <sup>b</sup>	59 $\pm$ 5	38 $\pm$ 3 <sup>b</sup>	18 $\pm$ 1
bLH	5 $\pm$ 0.2 <sup>a</sup>	42 $\pm$ 2 <sup>a</sup>	53 $\pm$ 3	31 $\pm$ 3 <sup>a</sup>	17 $\pm$ 2

Values in columns with different superscripts (a, b, c) are different ( $P < 0.05$ ). Germ cell counts were determined from 10 randomly sampled, round seminiferous tubules from stages I to VI of the spermatogenic cycle, from testes collected at 56 weeks of age.

Table 6. 2 Correlations (Pearson moments correlations coefficient) of Sertoli cells and germ cells (elongated spermatids, round spermatids, spermatocytes and spermatogonia) counts per seminiferous tubule, mean serum FSH concentrations at 4 and 8 weeks of age and age at puberty based on semen characteristics ( $\geq 5.0 \times 10^7$  sperm/ml with progressive linear motility > 10%) in all calves treated once every 2 days with either 4 mg of bFSH (n = 6), 3 mg of bLH (n = 6), or saline (n = 6) from 4 to 8 weeks of age.

	Sertoli cells	Spermatocytes	Rounded spermatids	Elongated spermatids
Mean FSH at 4 weeks of age	0.60 <sup>***</sup>	0.54 <sup>**</sup>	0.47 <sup>*</sup>	0.52 <sup>**</sup>
Mean FSH at 8 weeks of age	0.67 <sup>***</sup>	0.61 <sup>***</sup>	0.55 <sup>**</sup>	0.76 <sup>***</sup>
Age at puberty based on semen characteristics	-0.50 <sup>**</sup>	-0.15 <sup>*</sup>	-0.60 <sup>*</sup>	-0.62 <sup>***</sup>
Elongated spermatids	0.63 <sup>***</sup>	0.66 <sup>***</sup>	0.73 <sup>***</sup>	

\* P=0.05;    \*\*P<0.05;    \*\*\* P<0.01

## 6.5. Discussion

In this study, treatment with either bFSH or bLH at 4 and 8 weeks of age significantly increased mean serum concentrations of FSH and LH respectively. Serum gonadotropin concentrations following bLH and bFSH treatments were within physiological limits (McCarthy *et al.* 1979a, b; Rawlings & Evans 1995; Amann & Walker 1983; Stumpf *et al.* 1993). According to Amann and Walker (1983), there are no clear pulses in serum FSH concentrations in bull calves; as a result, the testis is not exposed to a pulsatile pattern of FSH concentrations. The biological efficacy of a prolonged exposure of the testes to FSH was shown in this study; increases in serum concentrations of FSH over 24 hours post-treatment at 4 weeks of age and over 8.25 hours post-treatment at 8 weeks of age were stimulatory, advanced puberty and enhanced spermatogenesis. Chandolia *et al.* (1997) advanced puberty by giving pulses of GnRH every 2 hours, from 4 to 6 weeks of age; therefore it is likely that the testes in young bull calves are sensitive to increased LH pulse frequency. In the present study, creating a prolonged high-amplitude peak of LH every 2 days appeared to have some stimulatory effects on spermatogenesis, but these effects were not significant. However, this relatively simple regimen of delivery of exogenous FSH to bull calves prior to the early transient postnatal increase in endogenous LH (FSH) secretion accelerated the onset of puberty. The lower efficacy of the present treatment with LH probably reflected the fact that endogenous LH is delivered to the testis in a pulsatile pattern (Rawlings *et al.* 1978; McCarthy *et al.* 1979a, b; Amann *et al.* 1986; Wise *et al.* 1987; Evans *et al.* 1995, 1996).

The preparations of bFSH and bLH used in this study were semi-purified but had <0.05% contamination with bLH and bFSH respectively. When bFSH and bLH were

injected, small increases in mean concentrations of LH and FSH respectively, were noted; this increase was significant only in the case of mean serum FSH concentrations in bLH-treated calves at 8 weeks of age. Treatments with either bFSH or bLH at 4 and 8 weeks of age significantly suppressed LH pulse frequency at 14 weeks of age. The reason for this transient suppression of LH pulse frequency is not clear; however, no other parameters of LH secretion were affected.

When age at puberty ( $SC \geq 28$  cm) was analysed, in the presence of one abnormal control calf, there was a numerical but nonsignificant acceleration of puberty in bFSH-treated calves as compared to control calves. The one abnormal control calf, diagnosed as oligospermic experienced a rapid increase in SC without an immediate and commensurate increase in spermatogenesis. Semen and histological evaluation revealed that sexual development (spermatogenesis) in the one abnormal control calf was at par with other control calves but not ahead, as the SC had suggested. Testicular hypertrophy in association with low sperm counts has been reported in Ayrshire bulls (Anderson & Makinen 1999). The re-analyzed data for age at puberty as determined by  $SC \geq 28$  cm, without the abnormal (oligospermic) control calf, showed that treatment of calves with bFSH from 4 to 8 weeks resulted in early attainment of puberty and a significantly higher mean SC from 38 to 44 weeks of age as compared to the control calves. The mean age, at which puberty was confirmed by semen characteristics, was numerically but not significantly lower in bFSH-treated calves compared to control calves. The age at which the desired semen characteristics were attained, is a reflection of the daily sperm production and is reached earlier in animals with greater daily sperm production.

In adult dairy bulls, the number of Sertoli cells accounted for a significant proportion of the variability in daily sperm production ( $R^2 = 0.68$ ) (Berndtson *et al.* 1987). In the present study, Sertoli cell counts were negatively correlated with the age at which the desired semen characteristics were reached; this suggests that calves with a greater Sertoli cell count (bFSH-treated calves) are likely to have a greater daily sperm production. Age at puberty ( $SC \geq 28$  cm) appeared to be lower in bLH-treated calves as compared to control calves but this difference was not significant. Interestingly, testicular growth as indicated by SC did not show a marked rapid increase after 25 weeks of age (Curtis & Amann 1981; Evans *et al.* 1996). Apart from perhaps a slower phase of growth from birth to 8 weeks of age, SC increased steadily over the period of sexual development. There was no obvious explanation for this.

Based on histological evaluation of the testes at 56 weeks of age, the number of Sertoli cells, elongated spermatids and spermatocytes were greater in the FSH-treated calves as compared to the control calves. The number of round spermatids and spermatogonia appeared to be greater in the bFSH treated calves as compared to control calves, but this was not significant. The positive correlation of mean serum FSH concentrations at 4 and 8 weeks to spermatocyte counts at 56 weeks of age suggests treatment with bFSH had a profound effect on spermatogonia cell division leading to a greater spermatocyte count. It is likely that treatment with bFSH decreased germ cell apoptosis leading to a greater elongated spermatid count as was suggested by the positive correlation of elongated spermatid counts at 56 weeks of age with mean serum FSH concentrations at 4 and 8 weeks of age. The number of germ cells is influenced by mitotic division of



spermatogonia and germ cell degeneration (apoptosis) during meiosis and spermiogenesis (Clermont 1972; Roosen-Runge 1973; Berndtson & Desjardins 1974; Amann 1981). During the period of rapid cell proliferation, Sertoli cells acquire the machinery required to support spermatogenesis in the mature animal and can support only a limited number of germ cells (Bardin *et al.* 1994). In rats and bulls, FSH has been shown to initiate and indirectly affect spermatogenesis through Sertoli cell functions, such as the synthesis and secretion of estradiol and androgen binding protein, tubular fluid and growth factors (Odell *et al.* 1973; Orth 1984; Bardin *et al.* 1994). The increase in germ cell counts in bFSH treated calves is therefore a reflection of more functionally competent Sertoli cells (Berndtson *et al.* 1987). The present study clearly demonstrated that treatment with bFSH increased Sertoli cell counts as shown by the correlation of Sertoli cell count at 56 weeks of age with mean serum FSH concentrations at 4 and 8 weeks of age. This increase was likely to have been a result of a greater Sertoli cell proliferation in bFSH treated calves during the course of sexual maturation (Bardin *et al.* 1994; Orth 1984). These observations in calves treated with bFSH were consistent with those of Bame *et al.* (1999), in which immunization against inhibin at 60 days of age and booster immunizations at 90, 104, 124, 270 and 395 days of age, increased mean serum FSH concentrations and augmented spermatogenesis and sperm output. Calves treated with bLH had numerically greater numbers of advanced stages of spermatogenesis (elongated spermatids and spermatocytes) as compared to control but this was not significant.

In summary, treatment of bull calves with bFSH from 4 to 8 weeks of age, starting before the early postnatal increase in gonadotropin secretion, increased the rate of

testicular growth (SC) from 38 to 44 weeks of age, hastened the onset of sexual maturity as defined by  $SC \geq 28$  cm, and increased spermatogenic potential per unit testicular weight. The enhanced numbers of Sertoli cells per tubular cross section suggested that FSH treatment permanently enhanced spermatogenic potential. Endocrine manipulation of young calves may hold promise to hasten sexual maturation, increase adult fertility and accelerate genetic progress.

## **Chapter 7: GENERAL SUMMARY AND CONCLUSION**

### **7.1. General summary**

In chapters 3 and 6 we established that in bull calves, between 4 and 25 weeks of age, serum concentrations of LH and FSH were transiently elevated as previously reported (Wise *et al.* 1987; Evans *et al.* 1993, 1996; Aravindakshan *et al.* 2000a, b). The early postnatal increase in LH secretion was caused by an increase in LH pulse frequency (McCarthy *et al.* 1979a, b; Amann & Walker 1983; Evans *et al.* 1993, 1996). No clear pulses in serum FSH concentrations are seen in bull calves (Amann & Walker 1983). Mean serum testosterone concentrations are low during the early postnatal period, increasing from 16 to 20 weeks of age, decreasing to 28 weeks of age and then subsequently increasing from 28 to 32 weeks of age (Rawlings *et al.* 1972, 1978; Secchiari *et al.* 1976).

In chapter 3 of the present study, the high concentrations of testicular LH-R in the early postnatal calves and the decline in concentrations from 13 to 21 weeks of age probably reflected high numbers of foetal Leydig cells and undifferentiated Leydig progenitor cells postnatally, followed by a decline in numbers of both (Hooker 1970; Mendis-Handagama & Ariyaratne 2001). In bull calves, it was suggested that the demise of foetal Leydig cells occurred during the early postnatal period (Hooker 1970) and that differentiation and maturation of adult Leydig cells was initiated around 12 to 16 weeks of age continuing actively up to 28 weeks of age (Amann 1983). Testicular Leydig cells

were shown to be the LH positive cell in early postnatal bull calves (Schanbacher 1979). It is likely that the increases seen in LH-R concentrations in the testis beyond 25 weeks of age, especially at 56 weeks of age, reflected further Leydig cell maturation or increased numbers of Leydig cells as suggested by Amann (1983). The positive correlation of testicular LH-R concentrations with testicular FSH-R concentrations (chapter 3) were of interest. Administration of FSH to in vitro co-cultured purified pig Leydig and Sertoli cells increased Leydig cell LH binding sites and their capacity to secrete testosterone (Tabone *et al.* 1984).

The high mean testicular FSH-R concentrations from 5 to 13 weeks of age in chapter 3 were probably a result of the high number of indifferent supporting cells per seminiferous tubule (Curtis & Amann 1981). Testicular indifferent supporting cells were shown to be FSH positive in early postnatal bull calves (Schanbacher 1979). Mean serum FSH concentrations and FSH-R concentrations were high when the seminiferous tubules were occupied primarily by gonocytes and prespermatogonia. This may have facilitated the FSH dependent proliferation and differentiation of the immature germ cells to primary spermatocytes (Means *et al.* 1976; Amann 1983; Jegou *et al.* 1983). The decrease in mean testicular FSH-R concentrations from 17 to 25 weeks of age may have been caused by the precipitous decline in numbers of indifferent supporting cells as they differentiated into mature Sertoli cells and the increase in germ cell numbers. The latter would have effectively decreased FSH-R concentrations per mg of protein. The increase in testicular FSH-R concentrations from 25 to 56 weeks of age was probably due to maturation of immature Sertoli cells as was reported in sheep (Yarney *et al.* 1997). In

chapter 3 of the present study, the LH dependent release of testosterone, essential for the progression of primary spermatocytes to secondary spermatocytes, could have been facilitated by the increased LH-RK<sub>a</sub> noted from 21 to 29 weeks of age (Purvis *et al.* 1977). Elongated and rounded spermatids were the most mature germ cells in the seminiferous tubules between 25 and 33 weeks of age, during the period of high serum testosterone concentrations. From 29 to 56 weeks of age, during the period of increase in the percentage of seminiferous tubules with elongated spermatids as the most mature germ cell type, mean serum FSH concentrations were low but testicular FSH-RK<sub>a</sub> and FSH-R concentrations were high. This implied that the increased Sertoli cell sensitivity to FSH ensured the progression and sustenance of spermatogenesis during low serum FSH concentrations (Means *et al.* 1976; Amann 1983; Jegou *et al.* 1983; Orth 1984).

In chapter 4 of the present study, with exception of calves at 28 weeks of age, mean serum IGF-1 concentrations in calves between 4 and 32 weeks of age were high. In a study by Brito *et al.* (2006), mean serum IGF-1 concentrations were shown to increase with age, reaching maximum concentrations in bull calves between 34 and 51 weeks of age. In chapter 4 of the present study, mean serum IGF-I concentrations were analyzed from pooled blood samples collected every 15 minutes for 10 hours every 4 week from bull calves between 4 and 32 weeks of age. There were no observation during the peripubertal period, unlike in previous reports by Brito *et al.* (2006a,b). The temporal patterns of mean serum IGF-1 concentrations reported in chapter 4 were positively correlated with the mean serum LH and testicular LH-R concentrations during

development reported for the same bull calves in chapter 3. Serum concentrations of IGF-and testosterone concentrations were not correlated.

From experiments in chapter 4 we showed that TGF-alpha and TGF-beta isoforms 1, 2 and 3 were present in the testis of bull calves at all ages. Mean testicular concentrations of TGF-alpha were higher in calves than in adults. Testicular concentrations of TGF-beta isoforms 1, 2 and 3 were higher in bull calves during the early postnatal period (5 to 17 weeks of age) than in the pre- and peri- pubertal periods (21 to 56 weeks of age). Of all the three TGF-beta isoforms, TGF-beta 3 was the most prevalent at all ages in the bull testis as was shown in rats (Mullaney & Skinner 1992, 1993). The positive correlation between the temporal patterns of serum LH concentrations (chapter 3) with testicular concentrations of TGF-beta isoforms 1, 2, and 3 during development (chapter 4) indicated a positive regulatory interaction between LH and TGF-beta isoforms 1, 2, and 3; however, no such interaction has been previously demonstrated in any species. Human chorionic gonadotropin was shown to cause a dose and time dependent increase in TGF-beta receptors in Leydig cells isolated from pigs (Goddard *et al.* 2000). The negative correlation of serum testosterone concentrations (chapter 3) with testicular concentrations of TGF-beta isoforms 1, 2, and 3 (chapter 4) suggested an inhibitory interaction between testosterone and TGF-beta isoforms 1, 2, and 3. Testosterone was shown to inhibit the synthesis of TGF-beta 1 mRNA in Sertoli cells isolated from immature pigs (Avallet *et al.* 1994); while TGF-beta inhibited hCG/LH induced cAMP and testosterone release and reduced the number of LH-R in Leydig cells isolated from immature rats (Lin *et al.* 1987; Fauser & Hsueh 1988) and pigs (Avallet *et al.* 1987).

Mean testicular TGF-beta 2 may have a stimulatory influence on numbers of Leydig cells as was implied by the positive correlation between the two (chapter 4). The positive correlation of TGF-beta isoforms 1 and 3 concentrations (chapter 4) with testicular FSH-R concentrations (chapter 3) suggested a stimulatory relationship between Sertoli cells and TGF-beta isoforms 1 and 3. TGF-beta increased the expression of FSH-R messenger RNA in granulosa cells isolated from female rats (Inoue *et al.* 2003) and birds (Woods & Johnson 2005). Granulosa cells are FSH sensitive cells comparable to Sertoli cells in males (Yding & Andersen 2000).

The high testicular concentrations of TGF-beta isoforms in the early postnatal bull calves (chapter 4), during the period of Leydig and Sertoli cell differentiation, indicated a possible paracrine regulatory role for growth factors in Leydig cell and Sertoli cell development. This was further supported by the positive correlation between testicular concentrations of TGF-beta isoforms with gonadotropin receptor numbers. Increased gonadotropin receptor numbers could have been a consequence of cell proliferation or maturation. In immature rats, TGF-beta was shown to increase LH or FSH induced incorporation of [ $H^3$ ] thymidine into DNA by Leydig (Khan *et al.* 1992) and Sertoli cells (Dorrington *et al.* 1993) respectively. In chapter 4 of the present study, the increased testicular concentrations of TGF beta isoforms 1, 2 and 3, at 25 weeks of age, coincident with the period of initiation of rapid testicular growth (Evans *et al.* 1993, 1996; Rawlings & Evans 1995) indicated a possible involvement of TGF-beta isoforms in the initiation of spermatogenesis as was shown in rats (Mullaney & Skinner 1993; Haagmans *et al.* 2003).

Experiments in chapter 5 showed that IL-1 alpha and IL-6 were present in the testes of bull calves of all ages; but IL-1 beta was only present in the testes of calves aged between 5 and 29 weeks. Testicular concentrations of IL-1 alpha and IL-1 beta were high during the early postnatal period and then decreased with age to a nadir in the peripubertal period. Puberty in Hereford x Charolais bulls was attained at  $45 \pm 1$  weeks of age at a scrotal circumference of  $\geq 28$  cm (Chapter 6; Evans *et al.* 1993, 1996; Rawlings & Evans 1995; Chandolia *et al.* 1997a, b; Aravindakshan *et al.* 2000a, b). In chapter 5, with the exception of calves at 13 weeks of age, mean testicular concentrations of IL-6 were higher in immature calves during the early postnatal and prepubertal period as compared to postpubertal bulls. The positive correlation of mean testicular concentrations of IL-1 alpha with IL-1 beta indicated a possible regulatory interaction between the two cytokines. The negative correlation of mean testicular concentrations of IL-1 alpha with IL-6 indicated a possible antagonistic regulatory mechanism.

In chapter 5, IL-1 bioactivity was lowest in testicular supernatants from bull calves aged between 5 and 13 weeks (early postnatal period). The IL-1 bioactivity was higher in testicular supernatants collected from bull calves aged between 17 and 56 weeks during the pre and postpubertal period. These observations are in agreement with earlier reports in rats in which IL-1 bioactivity was only detected in pre- and postpubertal but not early postnatal rat testes (Khan *et al.* 1987; Syed *et al.* 1988). In the present study, the disparity between changes in testicular IL-1 bioactivity with age and concentrations of IL-1 alpha and beta determined by ELISA was probably due to the presence of high



concentrations of endogenous IL-receptor antagonist (IL-1 ra) in the early postnatal calves blocking the biological IL-1 agonist activity at that time. The ability of IL-1 ra to inhibit the IL-1 bioactivity has been demonstrated in several species (Hannum *et al.* 1990; Arend 1993). In mice, FSH was shown to induce an acute increase in production of IL-1 ra but had no effect on the production of IL-1 alpha by Sertoli cells (Huleihel & Lunenfeld 2002). The relatively high serum FSH concentrations reported in bull calves during the early postnatal period (Evans *et al.* 1993, 1996; Rawlings & Evans 1995; Aravindakshan *et al.* 2000a, b; Bagu *et al.* 2006) might have induced IL-1 ra production in addition to the other IL-1 isoforms. We were unable to determine IL-1 ra concentrations due to lack of an appropriate bovine IL-1 ra assay.

The correlation of the temporal patterns of mean serum FSH concentrations during development reported in chapter 3 with mean testicular concentrations of IL-1 alpha and IL-1 beta in chapter 5 indicated a possible regulatory interaction of FSH with IL-1 alpha and IL-1 beta. A possible regulatory interaction between testosterone and IL-6 was indicated by the positive correlation between them. Physiological concentrations of testosterone have been shown to stimulate the production of IL-6 in mouse Sertoli cells (Stephan *et al.* 1997).

The coincidence of high mean testicular concentrations of IL-1 isoforms (chapter 5) with the period when foetal Leydig cells were actively being replaced by adult Leydig cells indicated a possible paracrine regulatory role for IL-1 in Leydig cell development. In immature rats, IL-1 was shown to stimulate basal and gonadotropin induced production of testosterone in Leydig cells (Verhoeven *et al.* 1988; Svechnikov *et al.*

2001) as well as DNA synthesis and proliferation in Sertoli cells (Petersen *et al.* 2002) spermatogonia and preleptotene spermatocytes (Parvinen *et al.* 1991; Soder *et al.* 1991). High mean testicular concentrations of IL-1 alpha and IL-1 beta during the early postnatal period, prior to the time in bull calves when spermatocytes and mature Sertoli cells appear in the seminiferous tubules (Chapter 3; McCarthy *et al.* 1979a, b; Curtis & Amann 1981; Amann 1983), indicated a possible paracrine involvement of IL-1 in the initiation of germ cell and Sertoli cell development. The presence of testicular IL-1 alpha but not IL-1 beta in bull calves during the peripubertal period indicated IL-1 alpha might have been involved in the maintenance of spermatogenesis. The coincidence of increased mean testicular concentrations of IL-6 with the period of Sertoli cell differentiation and spermatogenesis in bull calves (17 to 29 weeks of age) indicated a possible paracrine role of IL-6 in Sertoli cell development. In the present study the presence of IL-6 during the peripubertal period provided support that IL-6 could have been involved in the regulation of spermiogenesis and spermatogenesis. In rats, testicular IL-6 concentrations were shown to be highest during stages II-VI and lowest during stages VII-VIII, when the elongated spermatids and rounded spermatids, respectively, are the most mature germ cells (Syed *et al.* 1993). In bulls elongated spermatids appear in seminiferous tubules as the most mature germ cell at 32 weeks of age (Curtis & Amann 1981).

In chapter 3 of the present study, the rapid increase in testicular weight occurred after 25 weeks of age and coincided with increased serum testosterone concentrations, gonadotropin receptor concentrations and affinity. It is likely that testicular growth was

initiated by the high postnatal serum gonadotropin concentrations and testicular gonadotropin receptor concentrations and was maintained by the increased sensitivity of the Sertoli and Leydig cells to low serum FSH and LH concentrations, respectively, as reported in rams (Yarney & Sanford 1989) and rats (Dufau & Catt 1978). The increase in gonadotropin receptor concentrations from 25 to 56 weeks of age differs from previous reports in bull calves (Sundby *et al.* 1984; Dias & Reeves 1982). Interestingly, in chapter 6 of the present study, testicular growth as indicated by SC did not show a marked rapid increase after 25 weeks of age (Curtis & Amann 1981; Evans *et al.* 1996). There was no obvious explanation for this.

Treatment of bull calves from 4 to 8 weeks of age, once every 2 days with either bFSH (4 mg) or bLH (3 mg) increased mean serum concentrations of FSH and LH respectively within physiological limits (McCarthy *et al.* 1979a, b; Rawlings & Evans 1995; Amann & Walker 1983; Stumpf *et al.* 1993). On analysing SC data in the absence of a control calf diagnosed with oligospermia, treatment with bFSH from 4 to 8 weeks of age hastened the onset of puberty and resulted in a higher mean SC from 38 to 44 weeks of age. Treatment with bFSH from 4 to 8 weeks of age increased spermatocytes, elongated spermatids and Sertoli cells counts at 56 weeks of age. This suggested treatment with bFSH increased spermatogonia cell division and led to a greater spermatocyte count. Treatment with bFSH might have decreased germ cell apoptosis leading to a greater elongated spermatid counts (Clermont 1972; Roosen-Runge 1973; Berndtson & Desjardins 1974; Amann 1981). In rats and bulls, during sexual maturation, Sertoli cells proliferate and acquire the capacity to support a fixed number

of germ cells (Bardin *et al.* 1994). FSH initiates and indirectly affects spermatogenesis through Sertoli cell functions (Odell *et al.* 1973; Orth 1984; Bardin *et al.* 1994). In the present study, the increase in germ cell counts in bFSH treated calves was therefore a reflection of more functionally competent Sertoli cells (Berndtson *et al.* 1987). The correlation of Sertoli cell count at 56 weeks of age with serum FSH concentrations at 4 and 8 weeks of age supports the notion that treatment with bFSH increased Sertoli cell counts (Bardin *et al.* 1994; Orth 1984). These observations in calves treated with bFSH were consistent with those of Bame *et al.* (1999), in which immunization against inhibin at 60 days of age, with booster immunizations at 90, 104, 124, 270 and 395 days of age, increased serum FSH concentrations and augmented spermatogenesis and sperm output.

Treatment of bull calves, from 4 to 8 weeks of age, once every 2 days with 3 mg of bLH, appeared to lower the age at puberty ( $SC \geq 28$  cm) and stimulate spermatogenesis but these effects were not significant. The lower efficacy of the present treatment with bLH probably reflected the fact that endogenous LH is delivered to the testis in a pulsatile pattern (Rawlings *et al.* 1978; McCarthy *et al.* 1979a, b; Amann *et al.* 1986; Wise *et al.* 1987; Evans *et al.* 1995, 1996; Chandolia *et al.* 1997a, b).

## **7.2. Conclusions**

1. High concentrations of serum gonadotropins and testicular gonadotropin receptors, during the early postnatal period in bull calves, would suggest that they would be critical for the initiation of rapid testis growth. Increased gonadotropin receptor

concentrations and affinity, in the face of low circulating gonadotropin concentration and high testosterone concentrations, support rapid testicular growth after 25 weeks of age. High testicular gonadotropin receptor concentration and affinity during the period of low serum gonadotropin concentrations, indicate a high Leydig and Sertoli cell sensitivity to low serum LH and FSH concentrations, respectively.

2. The production of transformation growth factors (TGF-alpha and TGF-beta isoforms 1, 2, and 3) and interleukins (IL-1 alpha, IL-1beta and IL-6) in the testis of the bull calf is age dependent. Testicular TGF-alpha concentrations are greater in calves than adults and the testicular concentrations of TGF-beta (isoforms 1, 2 and 3) and IL-1 (alpha and beta) are greater in the early postnatal period than the peripubertal period. Testicular IL-6 concentrations are greater in prepubertal calves as compared to early postnatal calves and adults. Transformation growth factors (TGF-alpha and TGF-beta isoforms 1, 2, and 3) and interleukins (IL-1 alpha, IL-1beta and IL-6) may be involved in the paracrine/autocrine regulation of testicular development.
3. Treatment of bull calves with bFSH from 4 to 8 weeks of age, starting before the early postnatal increase in gonadotropin secretion, increases the rate of testicular growth (SC), hastens the onset of puberty as defined by  $SC \geq 28$  cm, and increases the spermatogenic potential per unit testicular weight. Endocrine manipulation of young calves may hold promise to hasten sexual maturation, increase adult fertility and accelerate genetic progress.

## **Chapter 8: FUTURE RESEARCH DIRECTIONS**

In chapter 3 of the present study, we were not able to characterise the functional and histological changes in Leydig cells. In other species such as rats, mice, pigs and hamsters, it has been suggested that foetal Leydig cells, mesenchymal cells, progenitor Leydig cells, newly formed adult Leydig cells and adult Leydig cells belong to the adult Leydig cell lineage (Mendis-Handagama & Ariyaratne 2001). In bull calves the temporal changes in the Leydig cell lineage have not been clearly characterized. These studies would involve the use of immunohistochemistry and/or insitu hybridization. The LH receptors, steroid enzymes and metabolites are expressed at different times in the Leydig cell development. Using antibodies against steroidogenic enzymes, steroid metabolites and LH, we could therefore identify the different cells in the Leydig cell lineage.

In chapter 4 of the present study, we established the changes in serum IGF-1 concentrations in bull calves between 4 and 32 weeks of age. It therefore would be of interest to establish if there is a diurnal variation of IGF-I serum concentrations and how IGF-1 concentrations change during the peripubertal period.

In chapter 4 of the present study, we were unable to determine testicular IGF-1 concentrations as testicular concentrations were below the assay sensitivity. This could have been due to that fact that acidified alcohol extraction was performed on

homogenate preparations rather than diced tissue. It therefore would be of interest to establish testicular concentrations of IGF-1 following extraction from tissue and using a more sensitive assay.

In chapters 4 and 5 of the present study we established the temporal changes in TGF-alpha, TGF-beta isoforms (1, 2, and 3), IL-1 alpha, IL-1 beta and IL-6 during sexual development in bull calves. In chapter 5 of the present study, there was a discrepancy in the bioactivity and immuno reactivity of IL-1 during development. Differences in the secretion of IL-1ra could have partially explained the discrepancy. However, due to lack of antibodies against IL-1ra we were unable to establish the trend in IL-1 ra. It would therefore be imperative to establish the postnatal changes in testicular IL-1ra concentrations during sexual maturation in bulls. In order to verify the temporal changes in testicular TGF-alpha, TGF-beta isoforms (1, 2, and 3), IL-1 alpha, IL-1 beta and IL-6 protein, comparison with their respective mRNA concentrations in testicular tissue could be determined by real time polymerase chain reaction (RT-PCR).

In chapters 4 and 5 of the present study, the cellular origin(s) and target(s) of TGF-alpha, TGF-beta (isoforms 1, 2 and 3), IL-1alpha, IL-1 beta and IL-6 in the bull testis were not established. It would be useful to know what contribution the testicular macrophages make to the testicular cytokine pool. In chapter 3 of the present study, we showed that the cellular composition of the testis changes during sexual maturation in bulls. Analysis of whole tissue homogenates at various time points thus represents different cellular contributions. To get a better understanding of the physiological implications of TGF-alpha, TGF-beta (isoforms 1, 2 and 3), IL-1alpha, IL-1 beta and

IL-6 it is therefore imperative to know their origin(s) and target(s) within the testis. We recommend the identification of the different testicular cell types that synthesize and/or respond to these cytokines during the course of sexual maturation in bulls. The presence of both protein and mRNA of TGF-alpha, TGF-beta, IL-1 alpha, IL-1 beta and IL-6 within a cell would confirm local synthesis. The use of in-situ hybridization with cDNA probes designed to hybridize with the cytokine mRNA would facilitate the establishment of the spatial distribution of the mRNA of interest in testicular cells. The use of immunohistochemistry using antibodies directed against the cytokine proteins of interest would facilitate the establishment of the spatial distribution of the cytokine protein within the bull testis.

The presence of TGF-alpha, TGF-beta, IL-1 alpha, IL-1 beta and IL-6 protein in absence of mRNA within a given testicular cell leads to the suggestion that the cell could be a target of the cytokine. Testicular cells responsive to a TGF-alpha, TGF-beta, IL-1 alpha, IL-1 beta and IL-6 could be identified by the presence of the respective receptor in or on the cell by immunohistochemistry or autoradiography. Immunohistochemistry would involve the use of antibodies directed against the cytokine receptor while autoradiography would involve the use of a radiolabelled cytokines capable of binding to the receptor. The mechanistic and functional characteristics of TGF-alpha, TGF-beta, IL-1 alpha, IL-1 beta and IL-6 on testicular somatic and germ cell development could only be established by the use of in-vitro cell culture studies using testicular cell isolates from bull calves at different ages. Inferences could then be drawn about mechanistic and functional characteristics of these cytokines in vivo.



In chapter 6 of the present study, we demonstrated that treatment of bull calves, from 4 to 8 weeks of age, once every 2 days with either 4 mg of bFSH increased serum concentrations of FSH over 24 h post-treatment at 4 weeks of age and over 8.25 h post-treatment at 8 weeks of age, hastened the onset of puberty and enhanced spermatogenesis. However, it would be of interest to look at the effect of a more practical and higher efficiency treatment regime with FSH. This would require a decrease in the number of FSH injections administered and could be achieved by incorporating FSH in an organic matrix such as polyvinyl pyrrolidone (PVP). An organic matrix such as PVP would create a subcutaneous FSH depot that would control the release of FSH into circulation and increase the availability of FSH and hence decrease the need for frequent FSH injections. An alternative approach would involve the use of a more potent and longer acting FSH-like ligand such as equine chorionic gonadotropin hormone (eCG) also referred to as pregnant mare's serum gonadotropin (PMSG; Hoppen 1994; Garner & Hafez 2000). Equine chorionic gonadotropin is a glycoprotein hormone produced by endometrial cuff cells attached to the uterine wall (chorionic membrane) during early pregnancy (Sobiraj & Bostedt 1985). In most farm species, eCG has been reported to have prolonged FSH- and LH-like activity (Hoppen 1994; Garner & Hafez 2000).

In chapter 6 of the present study, treatment of bull calves with bLH elevated serum LH concentrations but failed to hasten the onset of puberty. In bull calves, unlike FSH, discrete pulsatile LH patterns can be detected in blood. It is therefore likely that the testicular Leydig cells detect and respond to discrete pulsatile LH changes.

In the present study, treatment of bull calves with bLH may have down regulated the Leydig cells LH receptors. It would therefore be interesting to investigate the effect of pulsatile bLH during the early postnatal period on the timing of puberty.

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