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Reproductive and metabolic endocrinology in rams selected for high or low plasma IGF-1 concentrations

A thesis presented in partial fulfilment of the requirements of the degree of Master of
Animal Science at Massey University

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Abstract

The objectives of this study were to define reproductive and metabolic endocrinology in Romney rams selected at the time of weaning for high or low peripheral IGF-1 concentrations with particular relevance to the annual changes in the relationships between GH, IGF-I and insulin and the relationship between that system and the activity of the reproductive endocrine axis. Parameters examined in detail included testis function, the response of IGF-I and insulin to GRF, and expression of IGF-I and its binding proteins in the testis. A preliminary experiment (December 2005 and March 2006) examined semen sample high, low and unselected IGF-I rams. In the main experiment, blood and semen samples were collected in July, September and November 2006 and March 2007 from yearling rams (high or low IGF-I concentrations). Scrotal circumference and inguinal skin were recorded. Blood samples were collected via jugular venipuncture. After a collection of the control samples, rams were given 50 ug hGRF and 1,000 IU hCG. Second blood samples were collected 40 min (testosterone) and 10 h later (insulin and IGF-I). Seasonal differences from July to March were evident in the percentage of abnormal sperm, scrotal circumference and sperm motility, and concentrations of testosterone, IGF-I and insulin. IGF-I line differences were also found in scrotal circumference and IGF-I and insulin concentrations. Testosterone concentrations did not differ between lines, but, when actual IGF-I concentrations were taken into consideration, exhibited different secretion patterns between lines. In the experiment 3, samples of liver and testis tissue were collected from four animals for expression of mRNA for IGF-I, Type I IGF receptor and IGF binding proteins. It was found that genes for IGF-I, Types I IGF receptor and IGF binding proteins -2, -3, -4, -5 and -6 were expressed in the testis. Significant high expression of Type I IGF receptor and IGFBP-3 in the testis was found in the low line, with different levels of high and low expression of IGF-I in the liver of the two lines. These studies suggested that circulating concentrations IGF-I are associated with gonadotropin-stimulated steroidogenesis that affects scrotal circumference, but differences in IGF-I status between lines are modulated by the different expression of mRNA for IGF-1R and IGFBPs and by negative feedback between lines.

Chapter 1: General introduction

Insulin-like growth factors (IGFs) are peptide hormones secreted from many different cells. They were previously called somatomedins, because of their growth promoting activity. The IGFs have a similar sequence amino acid to insulin (Rinderknecht and Humbel, 1978), and they are complex systems that allow cells to communicate with their physiologic environment. IGFs play an important role in regulating normal physiology and a number of pathological states, including reproduction. Concentrations of IGFs in the circulation can be influenced by an individual's genetic make-up, photoperiod, age, sex, exercise status, stress levels, nutritional status and levels of sex steroids (Holly, 2004).

Most domestic animals are influenced by seasonal fluctuations of environmental factors, such as temperature, day-length, nutrition and social relationships. Changes in daily photoperiod and annual cycles in environmental temperature play an important role in the regulation of reproductive activity in sheep. In addition, insulin-like growth factors can affect the activity of the reproductive endocrine axis. Changes in seasonal photoperiod and IGF-I concentration throughout the year can alter testis function (Barkawi et al., 2006), and therefore semen production and male reproductive behaviour through the alteration of endocrine and physiological systems (Lincoln et al., 2001).

Massey University has lines that have been genetically selected for high and low IGF-I that differ in terms of their circulating IGF-I concentration. The low selection line displays poorer reproductive performance than does the high and control lines; and low line ewes have with lower ovulation, conception and pregnancy rates (Unpublished observation). The lower conception and pregnancy rates may result from either a poorer uterine environment or poorer ovarian function/ oocyte quality/ follicle growth in low line ewes or poor semen quality in low line rams. The present study investigated the relationship between plasma IGF-I concentration and ram semen and testicular characteristics by examining the reproductive and metabolic endocrine axes of rams from the high and low IGF-I selection lines. Parameters examined in detail included

testis function, responsiveness to GRF, testicular steroid production and expression of IGF-I and its binding proteins in the testis.

1.1 Biology of insulin-like growth factors

The insulin-like growth factors are a family of peptides that are GH-independent and which mediate many of the anabolic and mitogenic actions of growth hormone. The insulin-like growth factors were discovered in 1957, on the basis of studies indicating that growth hormone (GH) did not directly stimulate the incorporation of sulphate into cartilage, but rather acted through a serum factor (Salmon and Daughaday, 1957; Rinderknecht and Humbel, 1978). This factor was initially termed the *sulfation factor*; concurrent studies indicated that only a component of the insulin-like activity of normal serum could be blocked by the addition of anti-insulin antibodies. The remaining activity, termed *nonsuppressible insulin-like growth activity* (NSILA), was subsequently demonstrated to contain two soluble, named NSILA-I and NSILA-II. In 1972, the restrictive labels of *sulfation factor* and NAILA were replaced by the termed *somatomedin*. This study established the criteria for a somatomedin; 1) the concentration in serum must be GH-dependent, 2) the factor must possess insulin-like activity in extraskeletal tissues, 3) the factor must promote the incorporation of sulphate into cartilage and 4) the factor must stimulate DNA synthesis and cell multiplication. Rinderknecht and Humbel (1978) isolated two active somatomedins from human plasma and finally, renamed them *insulin-like growth factor* IGF. The name '*insulin-like growth factor*' was given because of its ability to stimulate glucose uptake into fat cells and muscle. It was subsequently discovered both IGF-I and IGF-II show approximately 50% similarity with the amino acid sequence of insulin (Rinderknecht and Humbel, 1978; Blundell et al., 1983).

THE INSULIN-LIKE GROWTH FACTOR (IGF) SYSTEM

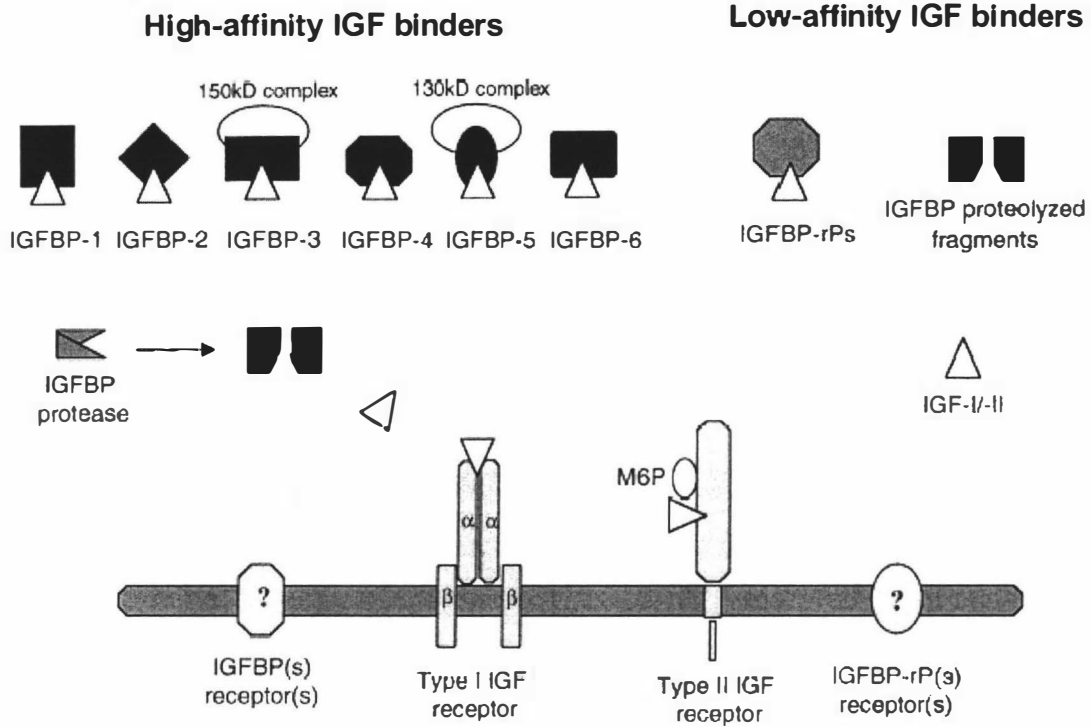


Figure 1.1 The components of IGF system include IGFs (IGF-I and IGF- II), IGF binding proteins-1 to -6, IGFBP proteases, and type I and type II IGF receptors (Hwa et al., 1999).

The IGF system (see Figure 1.1) profound effects on growth, differentiation and the development of many tissues (Hwa et al., 1999). The components of this axis include IGF-I, IGF-II, IGFBP proteases, IGF receptors (Type I and Type II) and the six IGF binding proteins (IGFBPs). The Type I receptor, which is structurally and functionally related to the insulin receptor, mediates most of the insulin-like actions of both IGF-I and IGF-II. The affinity of this receptor for IGF-I is slightly higher than for IGF-II, and much higher than for insulin. However, the Type II receptor binds IGF- I and IGF-II with low affinity, and does not bind insulin. It is therefore highly unlikely that IGF-II receptor mediates some IGF-I actions *in vivo*. Six IGF binding proteins (IGFBPs), which bind IGF-I and IGF-II with high affinity, are present in all biological fluids.

Many studies have demonstrated that GH initiates a signalling cascade which stimulates regulation of IGF-I and related genes, and that systemic growth is promoted by the action of GH on the liver and stimulation of IGF-I production (Rinderknecht and Humbel, 1978). The insulin-like growth factors are mitogenic peptides that potently stimulate proliferation, differentiation of many diverse cell types and inhibition of cell death (apoptosis). These peptides act as endocrine and paracrine/autocrine hormones (Jones and Clemmons, 1995). It is now evident that GH action can be independent of IGF production in the liver, since synthesis of IGF-I and IGF-II occur in most, if not all, tissues under the control of a variety of local and circulating factors that may or may not include GH (Dercole et al., 1980; Roberts et al., 1987; Holly, 2004; Frago and Chowen, 2005). In addition, such local IGF-I production may be directly responsible for the growth promoting effects of GH (Isaksson et al., 1982). It has been shown that multiple tissues in the mouse fetus are capable of IGF-I production, suggesting that the primary biological action of IGF-I are exerted locally at its sites of origin (Dercole et al., 1980). Furthermore, human fetal tissues that synthesise IGF-I include intestine, muscle, kidney, placenta, stomach, heart, skin, pancreas, hypothalamus, brain stem and adrenal tissues (Han et al., 1988). It has been shown that various adult rat tissues such as kidney, liver, lung, heart and testes had higher IGF-I concentrations than could be explained by the

contribution from the blood, and that IGF-I concentrations in many tissues were regulated by GH (Dercole et al., 1984).

Many actions of the IGFs have been demonstrated in the ovary, including an enhancement of cell proliferation, aromatase activity, and progesterone biosynthesis (Yoshimura, 2000). In particular, a growing body of evidence indicates that the IGF system plays a key role in follicular development and atresia in the woman, rodents and domestic animal species (Eckery et al., 1997; Scaramuzzi et al., 1999; Lucy, 2000; Giudice, 2001; Hayashi et al., 2005; Walters et al., 2007). Moreover, IGF-I stimulates the meiotic maturation of follicle-enclosed oocytes *in vitro* via the IGF-I receptor (Yoshimura, 2000).

1.2 Basic physiology of somatotrophic axis

Somatic growth is regulated an interplay of hormonal, genetic, nutritional, developmental and metabolic factors (Daughaday, 1985). The significant role of GH in the regulation of reproductive development through the action of IGF-I was first shown by the original formulation of the somatomedin hypothesis (Salmon and Daughaday, 1957). The somatotrophic axis plays an important role in numerous physiological and pathological processes and it is one of the major hormonal systems regulating somatic growth and cellular proliferation as well as reproduction in mammals.

1.2.1 Regulation of the somatotrophic axis

Growth hormone (GH) is a 191-amino acid, single-chain, polypeptide hormone that is synthesised, stored, and secreted by somatotrophic cells, located in the lateral wing of the anterior pituitary gland. GH is released from the anterior pituitary gland into the bloodstream in a pulsatile manner. The secretion of GH is under the primarily regulatory control of two hypothalamic peptides; somatostatin which inhibits its release and GH-releasing hormone (GRF) which stimulates its secretion (Frohman et al., 1992). The secretion of GRF and

somatostatin is further modified by many neuropeptides, catecholamines, steroid hormones, and metabolites acting on the hypothalamus (Daughaday, 2000). GRF selectively induces GH gene transcription and hormone release, while somatostatin suppresses both basal and GRF-stimulated GH pulse amplitude and frequency, but does not affect GH biosynthesis (Rosenbloom, 1999). In addition, ghrelin can also promote GH secretion (Lengyel, 2006). Serum GH is bound to GH-binding protein, and IGF-I and IGF-II are bound to IGF-binding proteins that play important role in modulating IGF action at target tissue. Upon binding to specific GH receptors in the liver, including other tissues, GH stimulates the production of IGFs and IGFBPs (Chandrashekar et al., 2004). The actions of GH include stimulation of skeletal and muscle growth, regulation of lipolysis, and promotion of cellular uptake of amino acids. The growth-promoting influence of GH is primarily extended by stimulating the liver to secrete IGF-I. In addition, the effects of GH on growth are mostly mediated by the production of IGF-I and IGF-II at local tissue sites (Salomon et al., 1989).

GH regulates its own secretion through a negative feedback mechanism mediated by hypothalamic GRF and somatostatin (Frohman et al., 1992). GH can also inhibit its own secretion by a short feedback loop acting on the hypothalamus. To make the regulation of GH secretion more complicated, both GH and IGF-I are synthesised in the hypothalamus and probably contribute to the regulation of GH secretion. GH stimulates IGF-I release from the liver and IGF-I stimulates hypothalamic somatostatin release and inhibits pituitary GH gene transcription and secretion through a long loop negative feedback (Plotsky and Vale, 1985; Sato and Frohman, 1993; Frago and Chowen, 2005). The functional activities, target sites and regulation of the somatotropic axis are depicted in Figure 1.2.

The actions of GH are mediated by binding to the GH receptor on the cell membrane of target tissues (Clark, 1997). These actions affect a variety of metabolic pathways including those involved in lipid metabolism (Kelder et al., 2007). In mammals and fish, GH receptors are most prevalent in liver but they present in other tissues, including muscle, adipose tissue, bone and kidney

(Mertani and Morel, 1995; Wood et al., 2005). There is evidence that GH receptor signalling in the liver is more relevant than Type I IGF receptor signalling in terms of growth of tissues. Behringer et al. (1990) indicated that GH, and not IGF-I, is the major regulator of liver growth, whereas IGF-I plays an important role for growth of most other tissues. For example, lower levels of GH receptor in the liver compared with that in other tissues may result in more efficient GH receptor signalling in the liver to maintain the liver growth (Iida et al., 2004). However, GH has been shown to reduce adipocyte mass in human (Bengtsson et al., 1993; Burman et al., 1995). In addition, GH receptors are expressed in human subcutaneous and intra-abdominal fat (Blüher et al., 2005). A study has shown that the disruption of the GH receptor is obese with specific accumulation of the excess fat mass in the subcutaneous region.

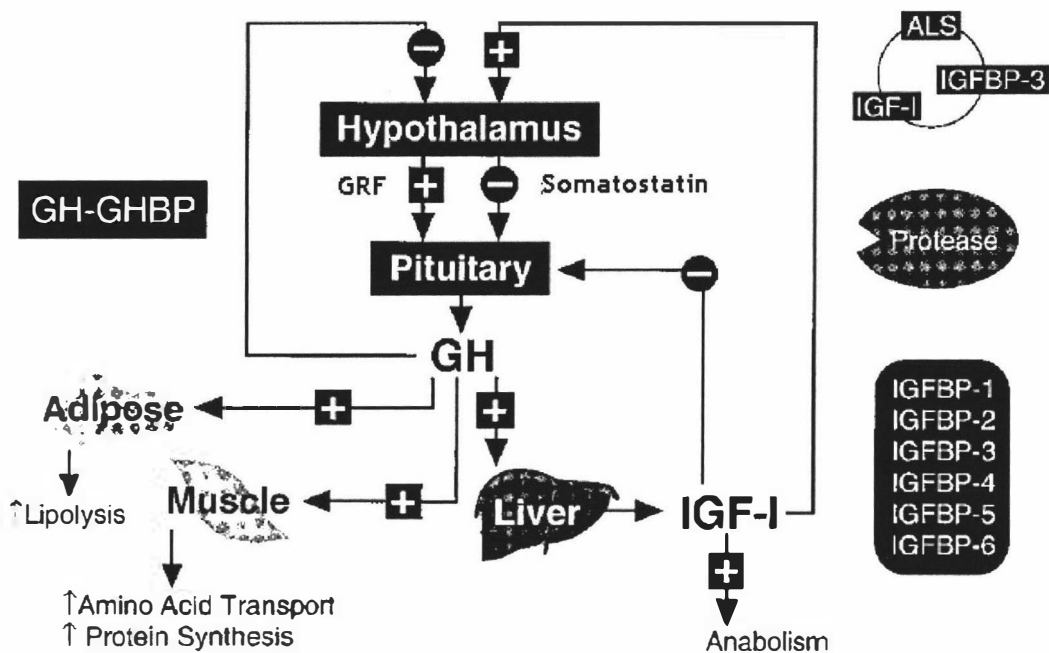


Figure 1.2 Simplified diagram of the somatotrophic axis involving the sites of production of GH and IGF-I, the feedback loops and their secretion, six binding proteins, and various metabolic actions. The hypothalamic hormones GRF and somatostatin control GH secretion from the pituitary. GH, bound to a GH-binding protein, circulates in the blood, inhibits its own secretion and stimulates IGF-I production in liver. IGF-I circulates in the blood bound primarily to IGF-binding protein-3 (IGFBP-3), which is in turn complexed to a third protein, the acid-labile subunit (ALS), but a total of six IGFBPs are known. IGFBP-IGF complexes are subject to protease attack, which assists the dissociation of IGF-I. The action of GH promotes either directly or indirectly the production of IGF-I (Clark, 1997).

1.2.2 Insulin-like growth factors signals

The somatotrophic axis is involved in the combination of a multitude of signals that regulate systemic growth and metabolism including maintenance of metabolic balance, cellular integrity and involvement in normal tissue regeneration. However, IGFs are produced by most tissues, with both the circulating and locally produced factors having physiological functions (Bondy et al., 1992; Flyvbjerg et al., 1995). Thus, both IGFs and GH have important widespread effects on the development and physiology of the organism.

1.2.3 Growth hormone

GH was discovered by Evans and Long (1921) and was isolated from bovine pituitary by Li and Evans (1944). In addition, the first evidence for central nervous system control of GH secretion was introduced by Heatherington and Ranson (1940). The GH secretory pattern is influenced by both age and sex, and is correlated with its ability to promote growth and stimulate the production of growth factors and liver enzymes (Choi and Waxman, 2000).

The actions of GH are well-known in regulating the growth and metabolism that occur multiple tissues, either directly or via IGF-I. These actions involve stimulation of protein synthesis and carbohydrate and lipid metabolism (Frago and Chowen, 2005). GH has both insulin-like and anti-insulin-like effects on cells and tissues. The insulin-like actions include stimulation of transient increases in glucose and amino acid transport, lipogenesis and protein synthesis. This probably occurs as GH and insulin may have similar signalling pathways (Frago and Chowen, 2005). GH appears to inhibit adipocyte differentiation (Hansen et al., 1998). In addition, GH signalling pathways promote protein synthesis that specifically requires tyrosine kinase activity in the cytoplasmic domain of the GH receptor (Frago and Chowen, 2005). The anti-insulin-like actions of GH promote insulin resistance and possibly Type 2 diabetes in humans (Wang et al., 1994). GH action is thought to interfere with the ability of insulin to stimulate carbohydrate metabolism. On the other hand, fetal GH has no role in controlling fetal growth because expression of the GH receptor is

relatively low in fetal tissue. However, GH deficiency can affect lower birth weight (Cottam et al., 1992).

1.2.4 IGFs and IGF receptors

IGF-I and IGF-II are peptides of 70 and 67 amino acids, which have similar actions to that of insulin (Frago and Chowen, 2005) and similar structures to that of proinsulin (Dercole et al., 1984). In adult rodents, IGF-II concentrations in blood are very low, although both IGF-I and IGF-II are present in large amounts in the blood of fetal rodents (Daughaday and Rotwein, 1989). IGF-I concentrations in blood are mainly regulated by GH status, while IGF-II concentrations are relatively independent of GH (Daughaday and Rotwein, 1989). Although it is well-known that IGF-II plays an important role in the control of ovarian function in domestic animals and humans, the situation is not clear in rodents (Dombrowicz et al., 1992).

The IGFs bind to cell surface receptors such as Type I and Type II IGF receptors. The mitogenic effects of IGF are mainly regulated by the Type I IGF receptor (IGF-1R), which is a receptor with tyrosine kinase activity. IGF-1R processes many of the differentiating and mitotic effects found for the receptor complexes of other tyrosine kinase receptors such as *c-kit*/KL and *c-fms*/colony-stimulating factor-I (Hwa et al., 1999). These receptors are important regulators of the differentiation of hematopoietic cells (Sigounas et al., 1997). This study suggested that the IGFs and the Type I IGF receptor may have differentiating and mitogenic activities in hematopoietic cells. The role of Type II IGF receptor, which primarily binds IGF-II, is much less clearly understood.

1.2.5 IGF binding proteins

Insulin-like growth factors (IGFs) circulate in the bloodstream bound to specific high affinity insulin-like growth factor binding proteins (IGFBPs). The free or unbound IGF fraction mediates its response through specific binding to membrane bound IGF receptors. Thus, the rate of IGF production, clearance

and degree of binding to the IGFbps modulate the concentrations of free IGFs. IGFbps not only regulate IGF bioavailability but may also have IGF-independent actions (Hwa et al., 1999; Hayashi et al., 2005).

Insulin-like growth factor-binding proteins control the biological functions of IGFs and may affect cell growth through IGF-independent actions (Yi et al., 2001). IGFs are bound to six different IGFbps that have higher affinities for IGFs than do the Type I IGF receptors. Thus, IGFbps act as carrier proteins to transport IGFs to their target tissues, thereby increasing the half-lives of the IGFs by protecting them from proteolysis, and acting as modulators of IGF availability and activity (Jones and Clemmons, 1995). IGFs must be released from their binding proteins in order to exert their mitogenic and metabolic effects. In consequence that IGFbps influence important biological processes, including bone cell proliferation (Mohan et al., 1995), Leydig cell (Matsui and Takahashi, 2001) and Sertoli cell (Rappaport and Smith, 1995) function, growth arrest of breast, prostate and lung cancer cells (Oh et al., 1993; Djavan et al., 2001; Sato et al., 2006), and developing antral follicle (Walters et al., 2007) and testicular somatic-germ cell (Besset et al., 1996; Bardi et al., 1999), granulosa cells (Alexiadis et al., 2006). In addition, IGFbps play an important role in brain functioning, especially, in regard to hypothalamic control, and in reproductive physiology (Gadd et al., 2000; Bienvenu et al., 2004). Numerous data, *in vitro* and *in vivo*, suggest the importance of IGFbps for cell growth by both IGF-dependent and IGF-independent mechanisms.

1.3 Basic physiology of reproduction in sheep

Most domestic animals are influenced by seasonal fluctuations of environmental conditions such as temperature, daylength, nutrition and social relationships. Animals may respond to seasonal changes in environmental conditions by developing a series of different strategies such as changing feeding habits, laying down energy reserves in the form of fat tissue, lowering basal metabolism, moulting, fur or plumage, hibernating, and migrating (Rosa and Bryant, 2003). Changes in daily photoperiod and annual cycles in environmental temperature

play an important role in regulating reproductive activity in both females and males. Sheep from temperate latitudes are well known as animals with marked seasonality of breeding activity that is primarily controlled by annual photoperiodic changes. Short days stimulate sexual activity in sheep while exposure to long days results in inhibition and thus cessation of reproductive activity (Gundogan et al., 2003; Rosa and Bryant, 2003).

1.3.1 Reproductive seasonality in sheep

The sheep is a seasonal breeder. The domestic ewe is seasonally polyoestrus with interoestrus intervals averaging 16 to 17 days during the breeding season. The ewes become sexually active in response to decreasing day length in the late summer to early autumn. Although some breeds are sexually active throughout the year, the breeding season starts in most ovine breeds during summer or early autumn (Nett and Niswender, 1982). Breeding season length varies markedly among breeds, but in general the season ends during the spring or summer (Hafez, 1952).

Various hormones, such as melatonin, exogenous progestogens and prostaglandins, have been successfully used to control reproductive activities in sheep. The pineal gland, which releases melatonin, plays an important role in regulating the timing of cyclical activity in sheep. The hypothalamus-pituitary-gonadal (HPG) axis changes its activity throughout the year. The activation of physiological functional regulation of the gonadal axis leads to the onset of reproductive function in sheep (Hammond et al., 1991). The HPG axis is activated by gonadotropin-releasing hormone (GnRH), which is released regularly in short bursts from the hypothalamus. GnRH stimulates the release of FSH and LH from the anterior pituitary (Thiery et al., 2002). The gland is influenced by the photoperiod, and pattern of melatonin indirectly regulates the pulsatile secretion of GnRH from the hypothalamus (Lincoln et al., 2001). However, changes in reproductive status are mainly regulated by modifications in the activity of the gonatotropic axis via variations in secretion of pulsatile LH and FSH, which control gonadal activity (Thiery and Malpaux, 2003). In ewes,

LH and FSH secretion begin to increase 2-4 weeks after the photoperiod decreases from the summer solstice. Since these hormones depend on pulsatile stimulation by hypothalamic GnRH, the photoperiodic control of sexual activity in the sheep results from the control of the neurons that release this neuropeptide. However, gonadotropin secretion in rams is usually stimulated at least 1 month earlier than in ewes, indicating that the sensitivity of rams to photoperiod differs from ewes. The increase in LH and FSH secretions is followed by an increase in testosterone, accompanied by growth of the testis (Lincoln and Davidson, 1977). This is because the rams need about 45 days to complete spermatogenesis, while anoestrous ewes can ovulate within a few days of hormonal stimulation, if large-sized follicles are present (Rosa and Bryant, 2003).

There is evidence that GH and IGF-I can exert stimulatory synergistic or permissive effects at each levels of the hypothalamic-pituitary-gonadal (HPG) axis, in the reproductive tract, external genitalia and mammary gland (Chandrashekar et al., 2004). However, overexpression of GH resulting in chronic elevation of GH and IGF-I concentrations can interfere with many aspects of reproduction (Laron, 1999). The somatotropic axis can influence the HPG axis, including gonadotropin release (Colao et al., 2002) and both gametogenic and steroidogenic functions of gonads (Scaramuzzi et al., 1999), and can affect the selection of the dominant follicle and ovulation (Eckery et al., 1997; Gong, 2002), oocyte maturation (Walters et al., 2007) and sperm motility (Vickers et al., 1999).

1.3.2 Male reproductive system in rams

Rams exhibit seasonal fluctuations in sexual behaviour, hormonal activity, spermatogenesis and testis volume and weight during the breeding season (Ortavant et al., 1988). The testis is central to the male reproductive system. The male reproductive system serves two main functions; 1) the continuous production, nourishment and storage of male gametes (spermatozoa), 2) the synthesis and secretion of androgenic hormones necessary for male sexual

differentiation. The testis receives information from the hypothalamus-pituitary-gonadal (HPG) axis, including, the paracrine and autocrine signals to complete its functions.

The male reproductive system is initiated by the pulsatile hypothalamic release of GnRH (gonadotropin-releasing hormone). Pituitary LH (luteinizing hormone) and FSH (follicle-stimulating hormone) secretion are regulated by GnRH pulses from the hypothalamus. FSH secretion produced by Sertoli cells and other components of the testis is influenced by inhibin and activin families. The principal male sex steroid is testosterone, which is synthesised by the Leydig cells of the testes. Secretion of testosterone increases steeply around the time of puberty and is responsible for the development of secondary sexual characteristics. Testosterone and FSH are key regulators of testicular cells that are essential for the production of sperm (spermatogenesis). The production of testosterone is controlled by the release of LH from the anterior lobe of the pituitary gland. The HPG axis is regulated through various factors. For example, testosterone is part of a negative feedback mechanism, in which rising testosterone concentrations suppress the release of GnRH from the hypothalamus. Testosterone is responsible for the development of male characteristics, including muscle mass and strength, fat distribution, bone mass, facial hair growth, voice change and sex drive. Moreover, the Sertoli cells secrete inhibin, which suppresses FSH release from the pituitary. Finally, the Leydig cells and the Sertoli cells stimulate the release of activin, which stimulates FSH secretion.

Both gametogenic and steroidogenic testicular functions are regulated by gonadotropin, which are secreted from the adenohypophyseal cells in a pulsatile fashion. It is well-known that the pituitary gland is essential to the function of seminiferous tubules (Zhou and Bondy, 1993). Experimental evidence suggests that LH stimulates both steroidogenic and gametogenic testicular function through the action of testosterone secreted by the Leydig cells, and FSH is also involved in spermatogenesis (Dickson and Sanford, 2005). Moreover, IGF-I is another important product of Sertoli cells. IGF-I produced by the Sertoli cells

is made available to the spermatocytes undergoing meiosis in the adluminal compartment of the seminiferous epithelium (Soder et al., 1992).

Seasonal changes in the concentrations of LH, FSH and testosterone are influenced by photoperiod (Thiery et al., 2002). Consequently, rams show a seasonal variation in testicular function that affects the quality and fertility of ejaculates. In addition, rams' testicular size, sperm production and mating capacity depend on the season of the year, and are highest during the breeding season, with temperature being another important factor that affects fertility in the ram (Barkawi et al., 2006).

1.4 Insulin-like growth factors and reproduction system

The insulin-like growth factors play an important role in numerous physiological and pathological processes and are one of the major hormonal systems regulating somatic growth and cellular proliferation as well as reproduction in mammals. IGF-I plays an important role in the control of growth and reproduction and influences metabolism, cell division, ovarian folliculogenesis, oogenesis and ovarian secretory activity (Tres et al., 1986; Soder et al., 1992; Schams et al., 1999; Lucy, 2000; Machnik and Lechniak, 2000; Giudice, 2001; Hull and Harvey, 2001; Gong, 2002; Neuvians et al., 2005) in females. In males, it affects germ cell development and the maturation of spermatozoa (Tres et al., 1986; Soder et al., 1992; Hoeflich et al., 1999; Machnik and Lechniak, 2000; Neuvians et al., 2005).

1.4.1 The roles of GH and IGF-I in reproduction

Insulin-like growth factor-I (IGF-I) signalling is necessary for sexual development and full functioning of the reproductive system, whereas GH signalling normally has little direct effect upon male or female fertility (Bartke et al., 2002). However, whilst reproductive processes are controlled by IGF-I rather than by GH, GH may be involved in the stimulation of prenatal and

postnatal growth (Sirotkin et al., 2003). Actions of GH and sufficient concentrations of IGF-I in peripheral circulation are essential for maturation at a normal age and the development of full reproductive potential (Chandrashekar et al., 2004). An increase in plasma IGF-I concentration stimulates granulosa cell proliferation, steroidogenesis (Monget et al., 2002) and LH secretion (Adams et al., 1997). IGF-I is also secreted by granulosa cells, irrespective of their progestogenic status, as a result of the amplification of FSH action at the level of the granulosa cell (Khalid et al., 2000). This result suggested that the IGF system may be involved in the selection of the dominant follicle by way of the amplification of the response to FSH, thereby distinguishing the follicles/testis destined to ovulate. A reduction of mRNA for the GH receptor in the granulosa of follicles from hypophysectomised ewes suggests that GH may play an important role in early stage of folliculogenesis (Eckery et al., 1997).

Clearly, IGFs and GH play a key role in the regulation of follicular development. Furthermore, the consequences of lower IGF-I or resistance to GH in animals and humans include delayed puberty, disrupted spermatogenesis and attenuated gonadal function (Chandrashekar et al., 2004). Many studies suggested that GH and IGF-I influence pituitary and gonadal functions in animals and humans (Chatelain et al., 1991; Zhou and Bondy, 1993; Adam et al., 1998; Machnik and Lechniak, 2000). However, GH overexpression in transgenic animals indicates that an increase in GH and IGF-I concentrations above the normal range can also interfere with normal reproductive function (Bartke et al., 2002). This result reveals that GH deficiency, GH resistance and GH or IGF-I administration can all influence the HPG axis, and that GH and IGF-I are crucial for the regulation of the activity of reproductive hormones.

1.4.2 IGFs and IGFBPs in the testis

1.4.2.1 IGF-I and IGF- II in the testis

Although the role of IGFs in many reproductive processes is not clear, the IGF system plays an important role in the development and functions of reproductive organs. In some activities of the testis, the IGF system functions independently of GH, such as testosterone synthesis by Leydig cells (Lin et al., 1986) and DNA

synthesis in spermatogonia (Soder et al., 1992). The local production of IGF-I by testicular tissues has been confirmed by expression of IGF-I mRNA in whole testis in rats (Casella et al., 1987), production of IGF-I from Sertoli cells in culture in rats (Cailleau et al., 1990) and its localization by *in situ* hybridization in rats and immunohistochemistry in humans (Vannelli et al., 1988; Hansson et al., 1989). IGF-I receptors have been found in Sertoli cells (Borland et al., 1984), Leydig cells (Handelsman et al., 1985), secondary spermatocytes and spermatids, suggesting a role in the coordination of growth, spermatogenesis and steroidogenesis (Tres et al., 1986; Chandrashekar et al., 2004) (see Figure 1.3). It has been shown that IGF-I treatment increases the motility of spermatozoa in rats (Vickers et al., 1999) and testicular activity in Zaraibi goats (Barkawi et al., 2006). The effects of IGF-I on androgen secretion by the testis *in vivo* would be due to direct action or to an its influence on hypothalamic-pituitary function (Chandrashekar et al., 2004).

IGF- II has been detected in the Leydig cells in rat testes from Days 1-11 after birth (Koike and Noumura, 1995), while Sertoli cells secrete IGF-II in immature mice (Tsuruta et al., 2000); indicating a possible role of IGF-II in spermatogenesis. However, IGF-II mRNA has not been identified in ovaries and testes of adult rats, and maturation of fertility is not affected in IGF-II gene-disrupted male mice (Dechiara et al., 1990). Thus, IGF-II may have little effect on female and male reproduction in adult mice and rats.

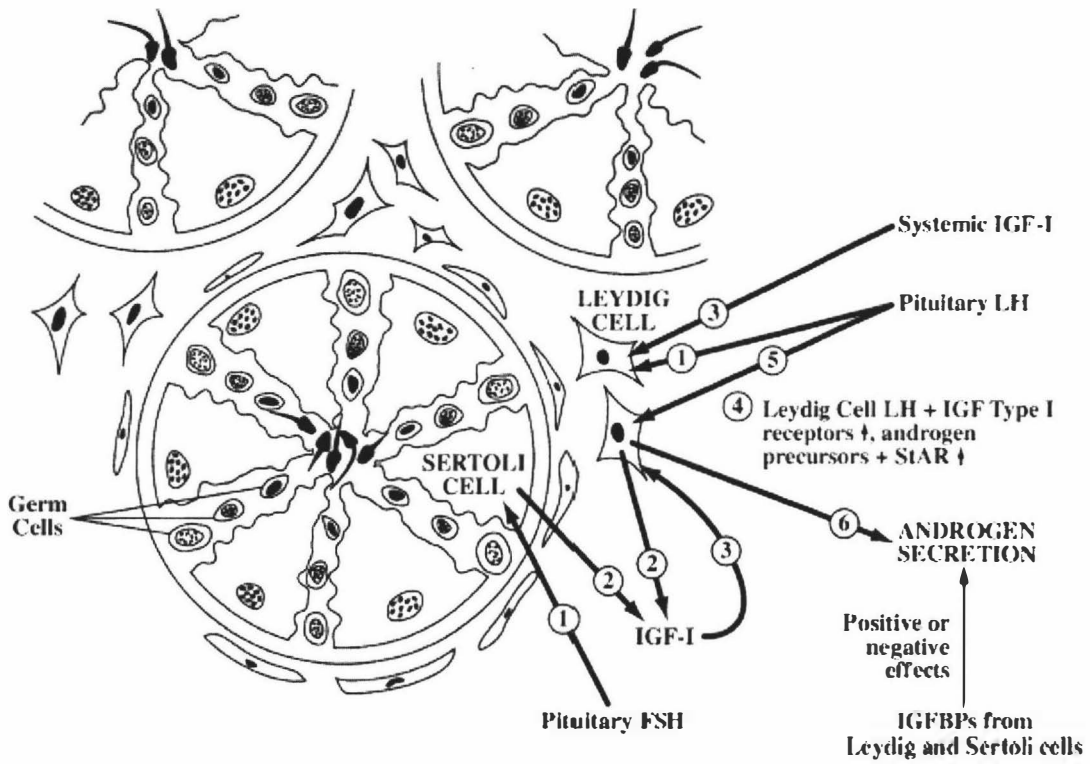


Figure 1.3 The role of the IGF-I system in testicular function (Chandrashekar et al., 2004).

1.4.2.2 IGF binding proteins

The peritubular, Sertoli and Leydig cells in the testis produce IGFBPs (Cailleau et al., 1990). IGFBP-2, -3, and -4 are present in the rat testis but IGFBP-1 is not (Lin et al., 1993). IGFBP-3 is present only in the endothelium of the testicular blood vessels, whereas IGFBP-4 and -5 are present in the Leydig cells and the IGFBP-6 was expressed in peritubular cells (Zhou and Bondy, 1993).

The IGFBPs are known to act as the major modulators of the IGF system by either promoting or inhibiting IGF availability. IGFBP-1 has been found in the endometrium, placenta, liver, and various cell lines. However, IGFBP-1 is not expressed in the rat testis, suggesting that IGFBP-1 probably is not involved in the regulation of testicular function (Lin et al., 1993). High levels of IGFBP-2 mRNA have been found in reproductive tissues, such as Leydig cells in the testis, and thecal interstitial cells and secondary interstitial cells in the ovary (Nakatani et al., 1991; Lin et al., 1993). A study has demonstrated that mural granulosa cells, rather than the oocyte, are the major source of proteases capable of cleaving IGFBP-2 in the developing bovine antral follicle (Walters et al., 2007). In addition, IGFBP-2 shows proteolytic activity in follicular fluid from bovine antral follicles and, furthermore, this proteolysis was negatively correlated with follicular fluid oestradiol and positively correlated with follicular fluid IGFBP-2 concentrations (Spicer, 2004). The effects of hCG and IGF-I on Leydig cell function are partly regulated by the level of IGFBP-2 production (Wang et al., 1994). However, IGFBP-2 protein production in rat Leydig cells is negatively regulated by hCG *in vitro*, and at a concentration of 10 ng/ml hCG, the expression of IGFBP-2 mRNA was reduced by 50% (Lin et al., 1995). This study suggested that IGFBP-2 plays an important modulating role in Leydig cell function.

In general, IGFBP-3 is the most abundant IGFBP in the adult circulation system. In the rat testis, IGFBP-3 mRNA is mainly expressed in Leydig cells and interstitial cells (Smith et al., 1990; Skalli et al., 1992). A study investigated the administration of recombinant human non-glycosylated rIGFBP-3 in rats, and found it inhibited IGF-I stimulated lactate production and that

FSH-stimulated lactate was altered by the addition of rIGFBP-3 (Rappaport and Smith, 1995). This study demonstrated that IGFBP-3 inhibits IGF-I action in the Sertoli cells and that FSH actions on lactate are independent of changes in the autocrine expression of IGFBP-3 in the cultured Sertoli cell environment. Therefore, the potent inhibition of IGFBP-3 by FSH suggests that IGFBP-3 may modulate IGF-I in the rat testis.

The physiological role of IGFBP-4 in the regulation of testis function is not clear. IGFBP-4 appears to exclusively inhibit IGF mitogenic actions under most experimental conditions, because it does not associate with extracellular matrix components or the cell surface (Bardi et al., 1999). This suggests that it may be a negative regulator of cellular proliferation.

In nonpregnant ewes, the expression of IGFBP-5 mRNA shows high concentrations in the maternal caruncles and luminal epithelium, and moderate levels in myometrium (Gadd et al., 2000). Although IGFBP-5 mRNA showed cyclic variations in reproductive organs, IGFBP-5 concentrations reach their peak around ovulation, whereas low expression in the endometrial stroma remained constant in the ovine ovary throughout the oestrous cycle (Hastie and Haresign, 2006a). This study concluded that IGFBP-5 mRNA is expressed in the ovine reproductive tract, with both the concentration and localization differentially regulated during the ovarian cycle and pregnancy.

Although IGFBP-6 was originally thought to be not expressed in follicles (Elroei et al., 1994), recent studies have indicated that IGFBP-6 mRNA is expressed in follicles of anoestrous sheep (Hastie et al., 2004), and in primates (Arraztoa et al., 2002) and mice (Hammond and Wandji, 1999). The IGF-II and IGFBP-6 complex appears to play an important role in the central nervous system, because IGF-II and IGFBP-6 production persists into adulthood and both are normal constituents of cerebrospinal fluid (Roghani et al., 1991). IGFBP-6 binds to IGF-II with an affinity that is stronger than that of the Type I IGF receptor (Roghani et al., 1991). IGFBP-6 has been shown to inhibit proliferation in many cell types, and it is synthesized in the central nervous system. However, transgenic mice with strong expression of IGFBP-6 between

birth and 1 month of age had reduced plasma IGF-I, showed reduction in body weight was significantly reduced and in the number of corpora lutea, had altered ovulation patterns, and showed a reduction of 50% in circulating LH concentration (Bienvenu et al., 2004). The results indicate that IGFBP-6 may play a role in regard to hypothalamic control, and in reproductive physiology.

In summary, IGFBP-2, -3, -4, -5, and -6 have a substantial effect on male reproduction (Zhou and Bondy, 1993). Therefore, as IGFBPs may influence IGF-I action, the ratio of the different IGFBPs to IGF-I may play an important role in action of IGF-I on reproductive system in males.

1.4.3 The effect of IGF-I on female reproduction

Reproductive function in both females and males is primarily regulated by LH and FSH, other factors including IGF-I is also important. Most importantly, it has been demonstrated that IGF-I stimulates GnRH release from rat hypothalamic explants *in vitro*, resulting in greater production of FSH and LH (Dickson and Sanford, 2005). LH and FSH are main hormones regulating the seasonal cycle and spermatogenesis in testicular size and function in males.

It has been recognized that pituitary-derived LH is the major luteotrophic hormone in ruminants (Niswender et al., 2000). The components of the IGF system are also thought to play a role in luteal function. IGF-I appears to stimulate key components in the steroidogenic pathway, leading to increased progesterone secretion in ruminants (Khandawood et al., 1994; Liebermann et al., 1996), humans (Villavicencio et al., 2002) and pigs (Miller et al., 2003).

IGF-I stimulates LH release from rat pituitary cells in culture (Eckery et al., 1997). In addition, a study examined effects of IGF-I on luteinizing hormone (LH) secretion (Adams et al., 1997), and the administration of IGF-I via the carotid stimulated LH output in sheep. However, acute systemic IGF-I administration suppressed GnRH secretion in male rats (Bourguignon et al.,

1993). These studies suggested that IGF-I may be a key luteotrophic factor in sheep.

It has been shown that IGF-I plays an important role in amplifying the effect of FSH and GH in rat (Adashi et al., 1991), pig (Hammond et al., 1991) and sheep (Monniaux and Pisselet, 1992) granulosa cells. In addition, the production of IGF-I by ovine granulosa cells is mainly regulated by GH and FSH (Skalli et al., 1992; Khalid and Haresign, 1996; Adams et al., 1997; Dickson and Sanford, 2005). The results indicated that GH and FSH treatment increase estradiol and IGF-I production by ovine granulosa cells, suggesting a role for GH and FSH in the regulation of ovarian function.

A recent study investigated the effects of LH on IGF gene expression in ovine corpora lutea using a GnRH antagonist model system (Hastie and Haresign, 2006b). The abolition of LH action following GnRH antagonist administration resulted in a significant increase in expression of IGF-I, Type II IGF receptor and IGFBP-5 after 12 h, while the expressions of IGFBP-2, -3, -4 and -6 mRNA were not affected by GnRH antagonist treatment.

1.4.4 The effect of IGF-I on male reproduction

The gonadotropin FSH plays a key role in the control of Sertoli cell function. A recent study presented evidence that FSH can stimulate the phosphatidylinositol 3kinase/protein kinase B (PI3K/PKB) pathway in Sertoli cells and the effects of FSH on phosphorylated PKB levels can be mediated by a combination of increased secretion of endogenous IGF-I, decreased IGFBP-3 production (Meroni et al., 2004). IGF-I increases FSH receptor number and responsiveness to FSH *in vitro* porcine Sertoli cells through increasing cAMP response to FSH during rat spermatogenesis (Soder et al., 1992). Therefore, FSH action on Sertoli cell metabolism may be mediated by regulation of an IGF-I.

Acute treatment of immature pigs with IGF-I increased mRNA for hCG receptor and increased the number of hCG receptors in Leydig cells. IGF-I treatment in male mice with GH insufficiency significantly increased both the testosterone response to hCG and the number of hCG receptors (Chatelain et al., 1994). These results show that IGF-I may induce or maintain the differentiated function of Leydig cells.

It is essential to realize that the IGF-BPs affect the IGF actions in the male reproduction system. One study evaluated the effects of exogenous IGF-I on gonadal function in GH receptor gene knockout (GHR-KO) mice (Chandrashekar et al., 2002). Treatment with IGF-I to GHR-KO mice suppressed the LH-induced testosterone in GHR gene-disrupted mice. Incubation of testes of normal mice with IGF-I increased the total testosterone release, whereas the testes from GHR-KO mice did not respond to IGF-I treatment (Chandrashekar et al., 2002). The different actions between the *in vivo* and *in vitro* effects of exogenous IGF-I suggest that an appropriate ratio of IGF-I and IGF-BPs may be essential for the normal testosterone secretion in GHR-KO mice. These results indicate that IGF-BPs may play an important role in the action of IGF-I and male reproduction.

1.5 Low and high IGF-I selection lines of animals

IGF-I plays an important role in the control of growth and reproduction and influences metabolism, cell division, ovarian folliculogenesis, oogenesis and ovarian secretory activity in females (Hull and Harvey, 2001), and germ cell development and maturation of spermatozoa in males (Hoeflich et al., 1999). Thus, IGF-I might be a candidate for selection to improve reproductive performance in both females and males. A study showed that IGF-I increased the secretion of oestradiol during the follicular phase resulting in an increase in ovulation rates in sheep (Spicer and Zavy, 1992). Additionally, IGF-I is a potent stimulator of follicular steroidogenesis and granulosa cell proliferation in sheep (Scaramuzzi et al., 1999). Likewise IGF-I plays a crucial role in the

initiation, development and maintenance of pregnancy (Simmen et al., 1993), suggesting that IGF-I may be important in the regulation of uterine function.

On the other hand, other results have been less clear. There was no difference in conception rate between mice with high and low IGF-I concentrations, although high line mice produced bigger litters (Kroonsberg et al., 1989). A more recent study of pregnancy rate and age of heifers at puberty and at first calving in Angus beef cattle selected for blood serum IGF-I concentrations showed that the pregnancy rate and age at puberty in cows did not differ between cows with low and high IGF-I concentration (Yimaz et al., 2006).

However, there has been no research on the relationships between IGF-I concentration and male reproductive traits in sheep. IGF-I mRNA has been detected in the testis in rats (Dombrowicz et al., 1992), and IGF-I may play a role in the differentiation of sperm cells in the epididymis (Leheup and Grignon, 1993). IGF-I increases testosterone production in the rat testis (Kasson and Hsueh, 1987). In addition, increased IGF-I concentrations in the GH-deficient rat can improve the motility and morphology of immature spermatozoa (Vickers et al., 1999).

Previous studies have found that infertile men who have a problem with abnormal sperm and low sperm counts generally have reduced IGF-I concentrations in their seminal plasma, compared to normal men (Colombo and Naz, 1999). When the concentrations of IGF-I were increased, there were increased total sperm counts, suggesting that IGF-I may have a role in spermatogenesis and steroidogenesis and that change in IGF-I concentration may be involved in male infertility (Colombo and Naz, 1999). Season also influences scrotal circumference in Damascus bucks (Al-Ghalban et al., 2004), goat (Barkawi et al., 2006), in Brahman and Hereford bulls (Godfrey et al., 1990) and rams (Nowakowski and Cwikla, 1994; Gastel et al., 1995; Bielli et al., 1999). Seasonal changes in scrotal circumference are related to sperm motility and the percentage of normal sperm cells. A study has examined differences in scrotal circumference, sperm motility, and percentage of normal sperm cells between two lines of Angus beef cattle selected for high vs. low blood serum

IGF-I concentration (Yilmaz et al., 1997). The results showed that scrotal circumference, percent sperm motility, and percentage of normal sperm cells are related to blood serum IGF-I concentration in Angus bulls. More recently, Yilmaz et al (2006) have estimated covariance components for reproductive traits and IGF-I concentration in Angus beef cattle, suggesting that selection for increased serum IGF-I concentration should result in increased scrotal circumference, sperm motility and calving rate.

1.6 Aims of the study

The aim of this study was to characterise reproductive and metabolic endocrine parameters in rams selected for high or low plasma IGF-I concentrations. Massey University has high and low IGF-I sheep selection lines that show different concentration of plasma IGF-I. The low selection line displays poorer reproductive performance when compared to the high and control lines, and the low line ewes have been shown to be less likely to ovulate, with lower ovulation, conception and pregnancy rates (Unpublished data). The lower conception and pregnancy rates may result from either a poorer uterine environment in low line ewes or poor semen quality in low line rams. The present study investigated the relationship between plasma IGF-I concentrations, and ram semen and testicular characteristics by characterising in detail the reproductive and metabolic endocrine axes of rams. Longitudinal changes in testis function in rams that have been genetically selected for high or low IGF-I concentrations were also examined.

1.7 Summary of experiments

The IGF-I sheep lines were established in 1986 from a commercial population of Romney sheep. Three IGF-I sheep lines were generated by Massey University on the basis of selection according to plasma IGF-I concentrations at 4-5 months of age, approximately 4 weeks after weaning. These lines consist of high (selection of ram replacements based on high IGF-I concentration (c. ≥ 150 ng/ml) within line), low (selection of ram based on low IGF-I concentration (c.

≤ 80 ng/ml) within line) and unselected line (selection of ram based on normal IGF-I concentration (c. 80-100 ng/ml) within line) (Blair et al., 2002).

This study examined effects of selection for high and low serum insulin-like growth factor I (IGF-I) on reproductive performance in rams. For preliminary experiment, animals were sampled in December 2005 and March 2006. Fifty seven Romney rams (17 from low IGF-I line, 21 rams from high IGF-I line and 19 from unselected group) were used in this study. For the main experiment, animals were examined and samples collected in July, September and November 2006 and March 2007 with two selection lines (13 high and 19 low). Scrotal circumference in the inguinal skin was recorded. Semen was collected by electroejaculation on 6 occasions over a 16 month period. Semen was evaluated according to standard procedures (volume, motility, density and morphology).

This study observed annual changes in the relationships between GH, IGF-I and insulin and to define the relationship between plasma IGF-I concentration and the activity of the male reproductive endocrine axis in two lines of Romney sheep selected for high vs. low blood serum IGF-I concentrations. For the hormone experiment, each ram had 4 blood samples collected in July, September and November 2006 and March 2007. Yearling rams (total 32 rams: 19 from low IGF-I line and 13 rams from high IGF-I) were used in this study. Blood samples were collected in, via jugular venipuncture and two different types of blood samples (serum and plasma) were obtained. After a collection of the first blood samples, rams were given intravenously 50 ug hGRF (growth-hormone-releasing factor) in July 2006 and March 2007, and 1,000 IU hCG (Human chorionic gonadotropin) in all months, because GRF stimulates the GH/IGF and hCG stimulates reproductive endocrine axes. Blood samples were collected 40 min and 10 h later for measurement of testosterone, IGF-I and insulin concentrations.

This study examined expression of genes for IGF-I, Type I IGF receptor and IGF-BPs (-1, -2, -3, -4, -5 and -6) between two lines of Romney rams selected for

high vs. low blood serum IGF-I concentration. RT-PCR was carried out with four animals from each line. Samples of liver and testis tissue were collected for total RNA isolation. Expression of mRNA for IGF-I in both liver and testis were measured. mRNA for IGF Type I receptor and IGFBPs (-1, -2, -3, -4, -5 and -6) in testis was measured.

		2005		2006			2007
Date		Dec	Mar	July	Sept	Nov	Mar
Semen collection							
Challenge experiment	GRF						
	hCG						
Blood collection	Serum						
	Plasma						
RT-PCR experiment							
Experiment 1				Experiment 2			

Figure 1.4 Overall timeline for experiments in this study.

Chapter 2: Effects of selection for high and low serum insulin-like growth factor I (IGF-1) on reproductive performance in rams

2.1 Introduction

The insulin-like growth factors (IGFs) are single chain polypeptides that function as endocrine and paracrine/autocrine hormones (Jones and Clemmons, 1995). In addition to its well recognised as a metabolic regulator, the IGF system is also required for sexual development and full functioning of the testis and ovary (Bartke, et al., 2002). IGF-I has numerous functions in the testis, including testosterone synthesis in Leydig cells (Lin et al., 1986) and DNA synthesis in spermatogonia (Soder et al., 1992). Furthermore, IGF-I produced by Sertoli cells stimulates lactate synthesis (Oonk and Grootegoed, 1988), glucose transport (Oonk et al., 1989) and cell proliferation (Borland et al., 1984). The local production of IGF-I by testicular tissues has been confirmed by the expression of IGF-I mRNA in whole rat testis (Casella et al., 1987), production of IGF-I from Sertoli cells in culture (Cailleau et al., 1990) and its localization by *in situ* hybridization and immunohistochemistry (Vannelli et al., 1988; Hansson et al., 1989). In addition, IGF-I receptors, including Type 1 and Type 2 IGF receptors, have been found in Sertoli (Borland et al., 1984) and Leydig cells (Handelsman et al., 1985), and in secondary spermatocytes and spermatids (Tres et al., 1986). Although IGF-I production is mainly regulated by growth hormone (GH) secretion, it has also been shown to be locally stimulated by follicle stimulating hormone (FSH) which plays a key role in the control of gonadal function (Cailleau et al., 1990). Receptors for IGF-I have been detected in the rat epididymis, suggesting that IGF-I may play a role in regulating sperm modulation in the epididymis (Spiterigrech and Nieschlag, 1992).

IGF-I action on sperm cells has been proven by the discovery of active receptors in porcine testis and in bovine spermatozoa (Machnik and Lechniak, 2000). Increases in IGF-I production from Sertoli and Leydig cells stimulate maturation of spermatozoa (Colombo & Naz, 1999) and improve sperm motility (Ovesen et al., 1998). In addition, IGF-I and GH interact with pituitary hormones to

regulate reproductive and sexual function (Spiterigrech and Nieschlag, 1992). IGF-I and GH increase both basal and LH-stimulated testosterone production in rat Leydig cells (Kasson and Hsueh, 1987) and stimulate the actions of gonadotropins on the Leydig cells *in vivo* by changing their sensitivity to LH and influencing the process of spermatogenesis (Chatelain et al.,1991).

Colombo and Naz (1999) found that men with abnormal sperm and low sperm counts generally have lower IGF-I concentrations in their seminal plasma than men with normal sperm production. When IGF-I concentrations were increased, there were increased total sperm counts. Thus, IGF-I appears to have a significant biological role in spermatogenesis and steroidogenesis in the testis and IGF-I system might be involved in male infertility (Colombo and Naz, 1999).

Sexual behaviour and semen quality are the main factors that regulate male reproductive performance during the year in rams. These factors are influenced by the breed, geographical location, season of the year (Nett and Niswender, 1982; Ortavant et al., 1988; Gundogan et al., 2003; Al-Ghalban et al., 2004), testicular size (Nowakowski and Cwikla, 1994a; Barkawi et al., 2006) and annual changes in gonadotropins secretion (Thiery et al., 2002; Filicori et al., 2002; Dickson and Sanford, 2005; Barkawi et al., 2006). The current study examined the relationship between IGF-I concentrations, semen quality and scrotal circumference over the breeding and non-breeding seasons between lines of New Zealand Romney rams that had been selected for low and high blood serum IGF-I concentration. In this study, a breeding soundness exam (BES) was conducted to identify rams with obvious problems that may impair their subsequent reproductive performance. This process included evaluation of their physical condition as well as an evaluation of the semen itself. It aims to clarify the possible relationships between IGF-I concentrations and breeding soundness examination

2.2 Materials and methods

2.2.1 Animals

Three IGF-I sheep lines were generated by Massey University on the basis of selection according to plasma IGF-I concentrations in 1986. These lines consist of three lines; high (selection of ram replacements based on high IGF-I concentration (c. ≥ 150 ng/ml) within line), low (selection of ram based on low IGF-I concentration (c. ≥ 80 ng/ml) within line) and unselected (selection of ram based on normal IGF-I concentration (c. 80-100 ng/ml) within line) (Blair et al., 2002).

Two experiments (preliminary and main experiments) were conducted.

Experiment-1 (Preliminary experiment)

Animals were sampled in December 2005 and March 2006. Fifty seven Romney rams with three selection lines (17 from low IGF-I line, 21 rams from high IGF-I line and 19 from unselected group) were randomly selected and were used in this study.

Experiment-2 (Main experiment)

Yearling rams were examined and samples collected in July, September and November 2006 and March 2007, with two selection lines (13 from the high line and 19 from the low line).

Seasonal changes in scrotal circumference between lines of rams selected for low and high blood serum IGF-concentration were recorded. Semen was collected by electroejaculation and sperm motility and density were recorded. Semen was evaluated according to standard procedures (volume, motility, density and morphology).

2.1.1 Physical examination

Physical examination involved measurement of scrotal circumference and palpation of the penis and prepuce, scrotum and its contents and accessory sex glands. Scrotal circumference was measured by grasping the scrotum above the testis and pressing the testis down into the base of the scrotum. Circumference was measured across the fullest part of the scrotum, pulling the tape measure just tight enough to rest on the skin (side-by side). After inspection of the scrotal skin, testes were palpated in two positions; while hanging freely with one hand pushing the testes down into the base of the scrotum.

2.1.2 Semen examination

Semen was collected by electroejaculation because this method is particularly useful to collect semen from untrained rams for soundness of breeding examination (Soderquist and Hulten, 2006). The ram's head was held down by one person and the hind limbs were held by another person. The penis was extruded and held in a gauze bandage. The electroejaculation probe was inserted into the rectum and a series of five electronic stimulations, each of 4 seconds duration, was applied. The probe used in these experiments had ring electrodes and delivered a pulse wave of about 60 Hz with a maximum of 10 V. Semen was collected into a warmed 30 ml universal bottle. Semen analyses were performed immediately after collection of sample and included measurements of the volume of the ejaculate, sperm colour, temporary assessment of sperm morphology and the percentage of sperm motility. At the laboratory, semen smears were stained with a 5% aqueous solution of eosin-B (Casarett, 1953) for permanent assessment of sperm morphology, and semen were diluted to check sperm density. For field use, a microscope with x 100 and x 400 magnification, a low power lens and x 1000 oil immersion lens were used for examination of sperm morphology.

Semen temperature was kept between 30°C and 37°C while motility was measured and while morphology smears were prepared. When the smears dried, the slides were allowed to cool down, immediately after sperm collection.

Semen motility was measured by subjective an assessment of wave activity on a 10 point scale using a microscope at low power with a defocused condenser. After assessment of motility, the semen was diluted 1:1 with 0.1% formal saline at body temperature and kept in Eppendorf tubes for sperm density measurement. For individual sperm morphology in the field, two slides of sperm smears were stained with Eosin-Nigrosin. A drop of semen was added to stain at a dilution factor of 1: 3 to 1: 4. Small amounts of stained semen were placed on warmed slides and smeared in order to avoid sperm cell overlap. The slides were left for a few seconds on the heated stage to dry and then were quickly checked at x100 or x400 magnification to ensure that the sperm morphology was appropriate.

For individual sperm morphology in the laboratory, two slides of semen smears were stained for permanent assessment of sperm morphology. Sperm morphology classified, according to the strict criteria of Blom (1983). Two slides from the farm and two from the laboratory were assessed and the mean value of abnormal sperm from each slide was calculated. 100 sperm were analyzed per slide.

2.1.3 Sperm density

Spermatozoa density was assessed using a haemocytometer with an improved double Neubauer ruling counting chamber. Firstly, all semen samples were diluted 20-fold (10 μ l semen + 200 μ l 0.1% formal saline) followed by a second dilution dependent upon sperm concentration. Spermatozoa were counted under x400 magnification.

2.1.4 Sperm morphology

A total of 100 spermatozoa were recorded per slide using bright field illumination and an oil immersion objective with a total magnification of x1000. At least ten different areas of the slide at random from different locations were examined. Sperm abnormalities were classified by site (head, tail, midpiece,

cytoplasmic droplets) or site of origin (testis, epididymis, accessory glands/post ejaculation) and were assigned, according to the criteria of Blom (1983), into major and minor defects. Major defects include many abnormalities, such as the head and midpiece, proximal cytoplasmic droplets of abnormal sperm and single abnormalities that are present at a high percentage. Minor defects include looped tails, detached sperm heads and distal cytoplasmic droplets.

2.1.5 Statistical analyses

Six rams (2 from low IGF-I line and 4 from high IGF-I line) were excluded from statistical analyses as they were removed for the flock before the completion of the study. The total percentage of sperm that were abnormal was calculated for the lines of sheep each month from the mean percentages of sperm classified with major and minor abnormalities. Data were log transformed before analysis, with 0.1 added to values for the total percentage of sperm that were abnormal, percentages of sperm with major or minor abnormalities, percentage of sperm with abnormalities in the head region, and motility scores. Levene's tests were conducted for all variables to check for equality of variances before parametric analyses were performed. Repeated measures two-way ANOVA followed by Bonferroni comparisons were used to compare the lines of rams between two months for total percentage of sperm that were abnormal, percentages of sperm with major, minor or head abnormalities, scrotal circumference and motility score. Kruskal-Wallis nonparametric ANOVAs were used for comparisons of density and proportions of sperm with cytoplasmic droplets between lines for each month, with pairwise comparisons between months for each line performed with Mann-Whitney U tests. Statistical analyses were performed using Systat (Systat Software). Data are presented as mean \pm S.E.

2.2 Results: scrotal circumference and semen quality

2.2.1 Effects of IGF-I selection line and season on scrotal circumference

Experiment 1

In the preliminary experiment, there were significant differences between months ($p < 0.001$) but not lines ($p = 0.837$) in scrotal circumference (Figure 2.1, see Table 2.1 for statistics). Scrotal circumference was less in March than December in all three lines ($F_{1,30} = 11.895$, $p = 0.002$; $F_{1,30} = 21.418$, $p < 0.001$; $F_{1,30} = 20.855$, $p < 0.001$ for low, high and control lines).

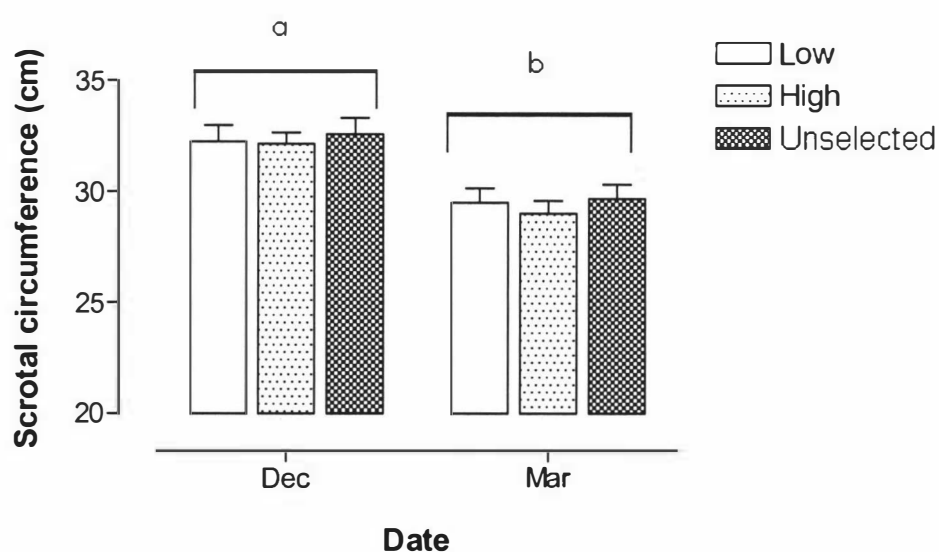
Table 2.1 Statistical analysis of Experiment 1 for comparisons of scrotal circumference between lines for each month.

Scrotal circumference

	F	df	P
Line	0.179	2,30	0.837
Month	51.407	1,30	<0.001*
Interaction of line and month	0.060	2,30	0.942

Asterisks indicate significant differences ($*P < 0.05$) between the values.

Figure 2.1 Scrotal circumference in rams of three IGF-I selection lines in December 2005 and March 2006. Groups of means labelled with different letters are significantly different from each other ($p < 0.05$).



Experiment 2

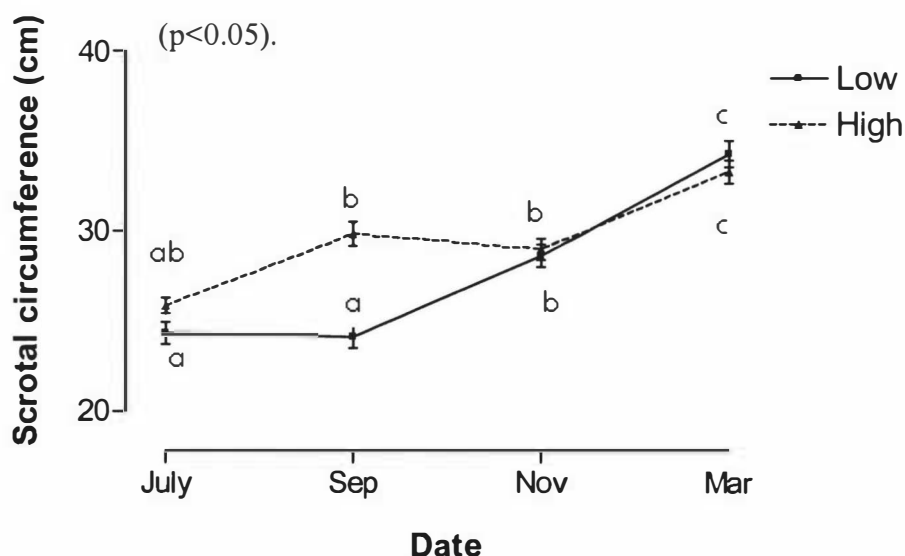
In the main experiment, there were significant differences between months ($p < 0.001$) and lines ($p = 0.042$) in scrotal circumference (Figure 2.2, see Table 2.2 for statistics). Scrotal circumference was greater in the high than the low line in September ($F_{1, 24} = 25.253$, $p < 0.001$), but not in other months. The circumference did not change from July to September in the low line, then increased in successive months thereafter ($F_{1, 24} = 1.393$, $p = 0.252$; $F_{1, 24} = 16.842$, $p = 0.001$; $F_{1, 24} = 48.986$, $p < 0.001$). The circumference increased from July to September in the low line, did not change between September and November then increased from November to March ($F_{1, 24} = 13.269$, $p = 0.002$; $F_{1, 24} = 0.000$, $p = 1.000$; $F_{1, 24} = 8.116$, $p = 0.010$).

Table 2.2 Statistical analysis of Experiment 2 for comparisons of scrotal circumference between lines for each month.

Scrotal circumference	F	df	P
Line	4.715	1,24	0.042*
Month	44.872	3,72	<0.001*
Interaction of line and month	4.678	3,72	0.005*

Asterisks indicate significant differences ($*P < 0.05$) between the values.

Figure 2.2 Scrotal circumference in rams of two IGF-I selection lines in July, September and November 2006 and March 2007. Points labelled with different letters are significantly different from each other ($p < 0.05$).



2.2.2 Effects of IGF-I selection line and season on sperm motility and sperm density

Experiment 1

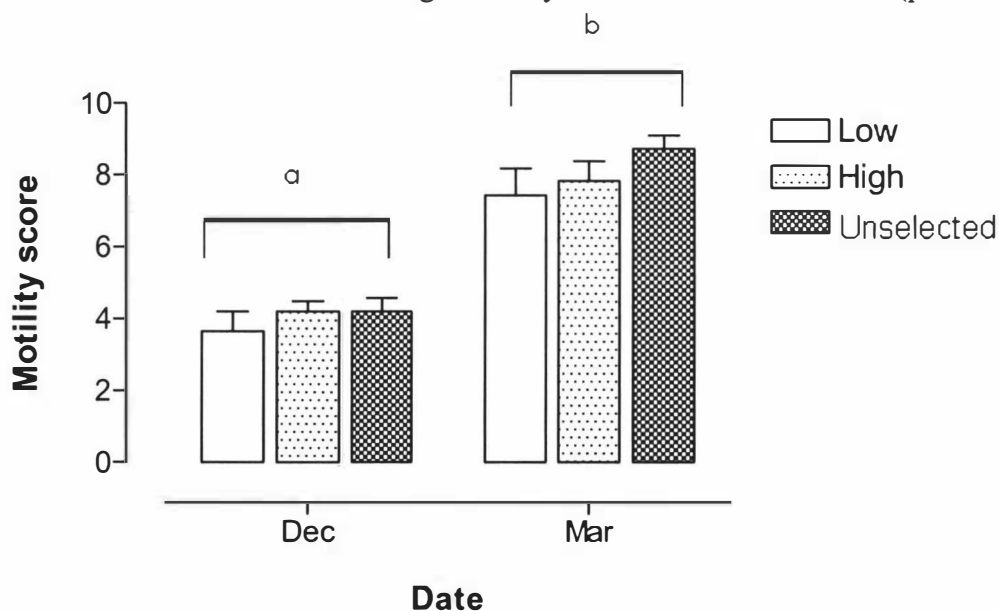
In the preliminary experiment, sperm motility scores were higher in March than December in the high and control but not low lines ($F_{1,30} = 6.150$, $p=0.019$; $F_{1,30}=7.192$, $p=0.009$; $F_{1,30} = 1.532$, $p=0.225$ for high, unselected and low lines, Figure 2.3, see Table 2.3 for statistics).

Table 2.3 Statistical analysis of Experiment 1 for comparisons of sperm motility between lines for each month.

Sperm motility score	F	df	P
Line	2.995	2,30	0.065
Month	13.158	1,30	0.001*
Interaction of line and month	0.339	2,30	0.715

Asterisks indicate significant differences ($*P < 0.05$) between the values.

Figure 2.3 Sperm motility score in rams of three IGF-I selection lines in December 2005 and March 2006. Groups of means labelled with different letters are significantly different from each other ($p < 0.05$).



In sperm density, there were no differences in December, compared to March, either in overall mean values or between lines. In December, there were no differences between lines but in March, values (Kruskal-Wallis one way ANOVA; $K_2 = 1.814$ $p=0.404$; $K_2 = 8.191$, $p=0.017$; Figure 2.3, see Table 2.3 for statistics) for the low line were significantly lower than high line. A similar trend was present in December, but the difference was not significant ($p=0.404$).

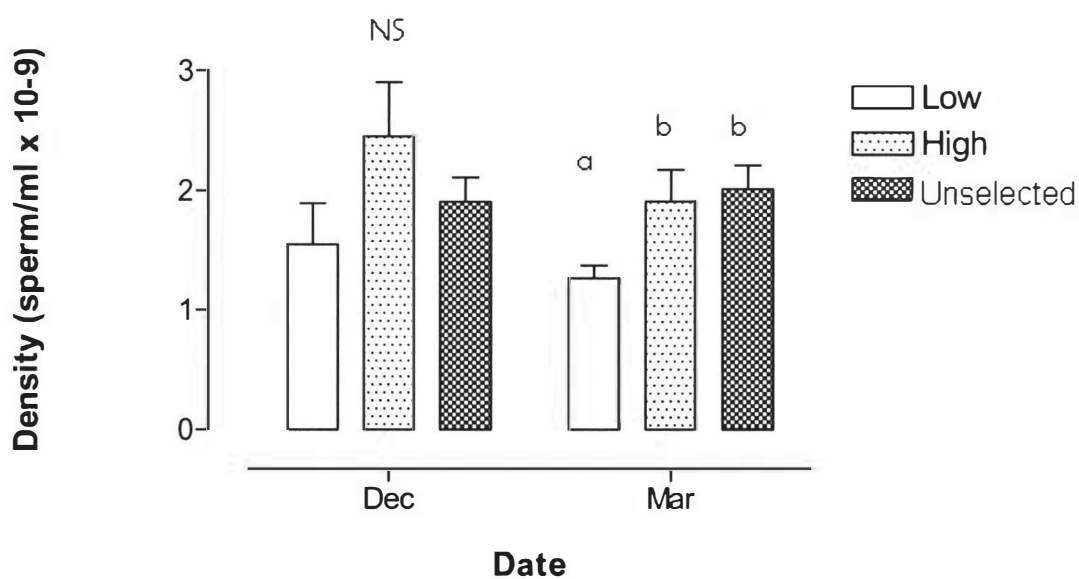
Table 2.4 Statistical analysis of Experiment 1 for comparisons of sperm motility between months.

Sperm density

	Kruskal Wallis K	P
December	1.814	0.404
March	8.191	0.017*

Asterisks indicate significant differences ($*P < 0.05$) between the values.

Figure 2.4 Sperm density in rams of three IGF-I selection lines in December 2005 and March 2006.



Experiment 2

Sperm motility scores and the density of sperm did not differ between lines or months (Figure 2.5 and Table 2.5 for statistics).

Table 2.5 Statistical analysis of Experiment 2 for comparisons of sperm motility and density between lines for each month.

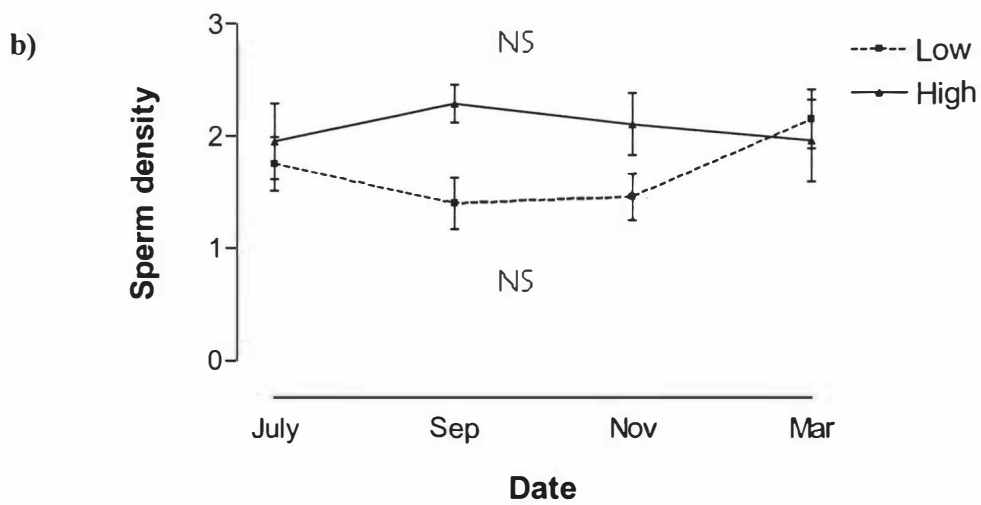
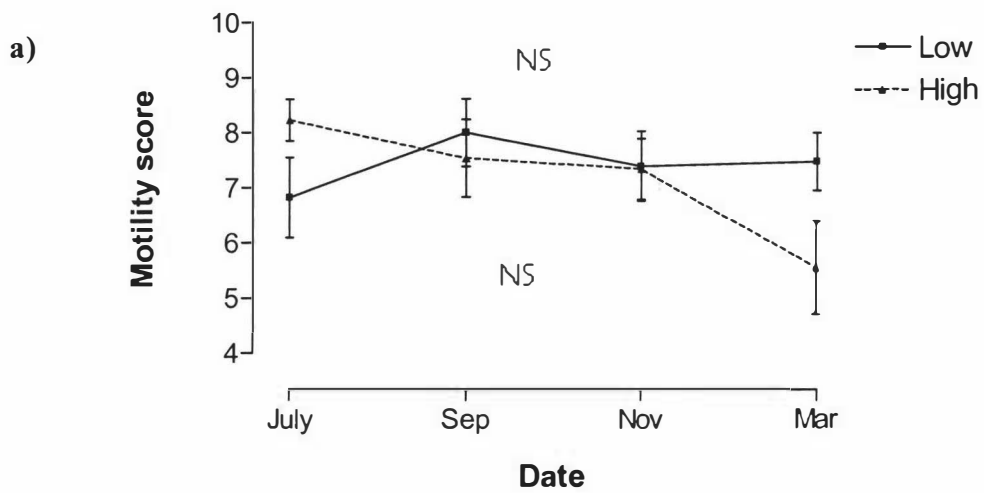
Sperm motility score

	F	df	P
Line	0.498	1,24	0.487
Month	0.822	3,72	0.486
Interaction of line and month	2.304	3,72	0.084

Sperm density

	F	df	P
Line	0.276	1,24	0.604
Month	0.977	3,72	0.409
Interaction of line and month	1.265	3,72	0.293

Figure 2.5 Sperm motility (a) and sperm density (b) in rams of two IGF-I selection lines in July, September and November 2006 and March 2007. No significant differences were present in sperm motility score or sperm density between months and lines.



2.2.3 Effects of IGF-I selection line and season on sperm abnormalities

Experiment 1

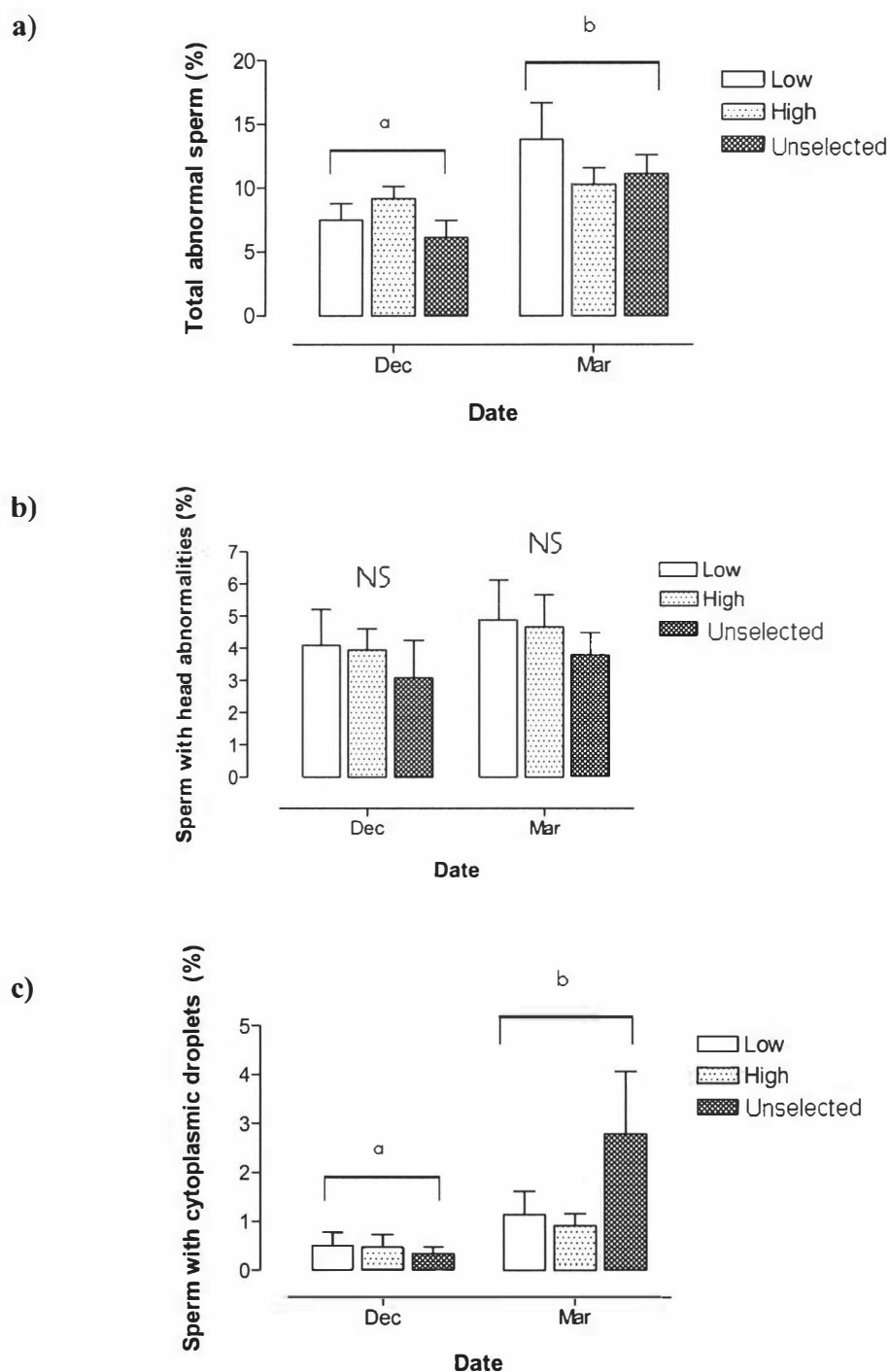
In the preliminary experiment, the total percentage of abnormal sperm differed between months ($p=0.008$) but not lines ($p=0.390$), and there was no interaction between months and lines ($p=0.100$; Figure. 2.6, see Table 2.6a for statistics). The total percentage of abnormal sperm was greater in March than December in the low and unselected but not high line ($F_{1,30} = 5.021, p=0.030$; $F_{1,30} = 6.521, p=0.016$; $F_{1,30} = 0.010, p = 0.919$; see Figure 2.6a). There were no effects of line or month on the percentage of sperm with major abnormalities (see Table 2.6b), whereas the percentage of sperm with minor abnormalities differed between months ($p=0.002$) but not lines ($p=0.294$; see Table 2.6c). The percentage of sperm with minor abnormalities tended to be higher in March than December in the low and unselected but not high lines ($F_{1,30} = 5.009, p=0.033$; $F_{1,30} = 10.260, p=0.003$; $F_{1,30} = 0.049, p=0.827$; data not shown). There were no differences between months in the percentage of abnormal sperm in the head region (Figure. 2.6b, see Table 2.6d for statistics). There were no differences between lines in the percentage of sperm with cytoplasmic droplets (Kruskal-Wallis one way ANOVA; $K_2 = 2.699, p=0.259$; $K_2 = 0.187, p=0.911$ for December and March; Figure 2.6c see Table 2.6e for statistics). The percentage of sperm with cytoplasmic droplets was higher in March than December in the high but not the low or unselected lines ($U = 47.0, p=0.018$; $U=25.5, p=0.450$; $U = 39.5, p=0.117$; Figure 2.6c).

Table 2.6 Statistical analysis of Experiment 1 for comparisons of sperm with abnormalities between lines for each month.

a) % Total abnormal sperm			
	F	df	P
Line	0.972	2,30	0.390
Month	8.047	1,30	0.008*
Interaction of line and month	2.490	2,30	0.100
b) % sperm with major abnormalities			
	F	df	P
Line	0.193	2,30	0.825
Month	0.656	1,30	0.424
Interaction of line and month	0.082	2,30	0.836
c) % sperm with minor abnormalities			
	F	df	P
Line	1.277	2,30	0.294
Month	11.070	1,30	0.002*
Interaction of line and month	2.629	2,30	0.089
d) % sperm with abnormalities in the head region			
	F	df	P
Line	0.353	2,30	0.706
Month	0.243	1,30	0.625
Interaction of line and month	0.137	2,30	0.720
e) % sperm with cytoplasmic droplets			
	Mann-Whitney U	P	
Low	25.5	0.450	
High	47.0	0.018*	
Unselected	39.5	0.11	

Asterisks indicate significant differences ($*P < 0.05$) between the values.

Figure 2.6 Percentages of sperm with (a) total abnormalities, with (b) abnormalities of the head region, and with (c) cytoplasmic droplets in rams of three IGF-I selection lines in December 2005 and March 2006. Points labelled with different letters are significantly different from each other ($p < 0.05$).



Experiment 2

In the main experiment, the total percentage of abnormal sperm differed between months ($p < 0.001$) but not lines ($p = 0.221$; Figure 2.7a, see Table 2.7a for statistics). The percentage increased from July to March in both lines (comparisons between July and March in each line; $F_{1,24} = 7.672$, $p = 0.011$; $F_{1,24} = 13.791$, $p = 0.001$).

The percentage of sperm with major abnormalities differed between months in the high but not the low line (Figure 2.7b, see Table 2.7b for statistics), with a decline in this percentage from November to March in the high line (Wilcoxon $Z = -2.650$, $p = 0.040$).

The percentage of sperm with minor abnormalities had the same pattern as the total percentage of abnormal sperm, with differences between months but not lines (Figure 2.7c and Table 2.7c) and an increase from July to March in both lines (comparisons between July and March in each line; $F_{1,24} = 15.682$, $p = 0.001$; $F_{1,24} = 21.711$, $p < 0.001$).

There were no differences between months in the percentage of sperm with abnormalities in the head region, and differences between months in the low but not the high line in the percentage of sperm with cytoplasmic droplets (Figure 2.7e, see Table 2.7e for statistics). The percentage increased from July to September, then decreased and increased again from November to March (Wilcoxon $Z = 2.634$, $p = 0.008$; $Z = 2.043$, $p = 0.041$; $Z = 2.807$, $p = 0.005$).

Table 2.7 Statistical analysis Experiment 2 for comparisons of sperm with abnormalities between lines for each month.

a) Total % abnormal sperm

	F	df	P
Line	1.581	1,24	0.221
Month	7.549	3,72	<0.001*
Interaction of line and month	1.412	3,72	0.246

b) % sperm with major abnormalities

	Friedmans F	P
Low line	1.021	0.796
High line	10.010	0.018*

c) % sperm with minor abnormalities

	F	df	P
Line	0.025	1,24	0.876
Month	17.707	3,72	<0.001*
Interaction of line and month	0.393	3,72	0.759

d) % sperm with abnormalities in the head region

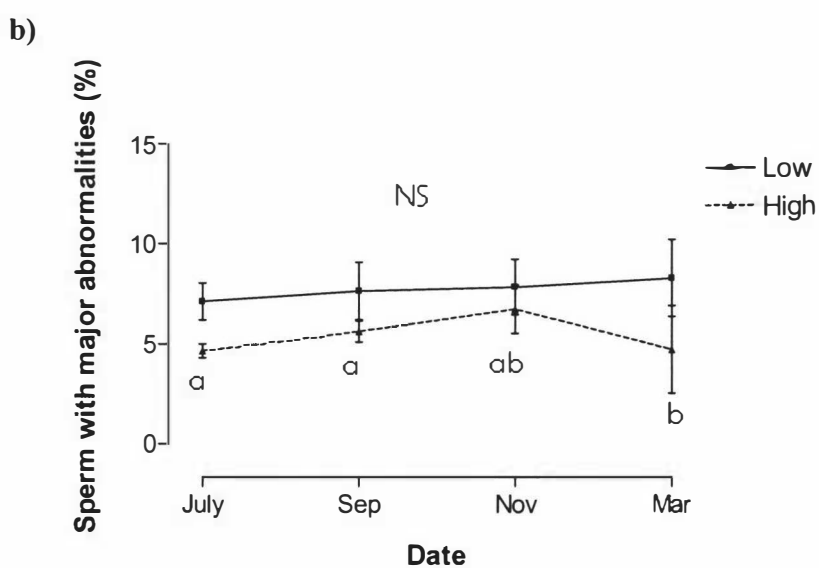
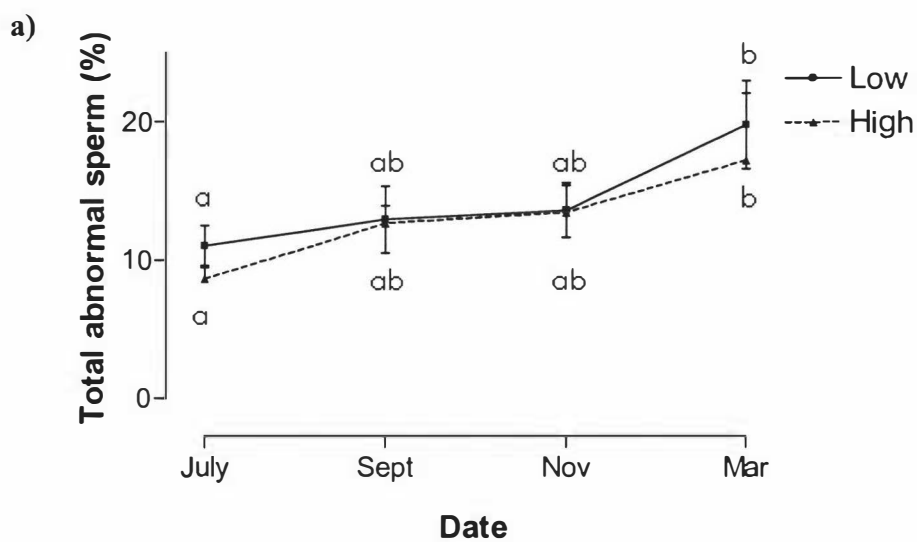
	Friedmans F	P
Low line	5.511	0.138
High line	3.932	0.269

e) % sperm with abnormalities in cytoplasmic droplets

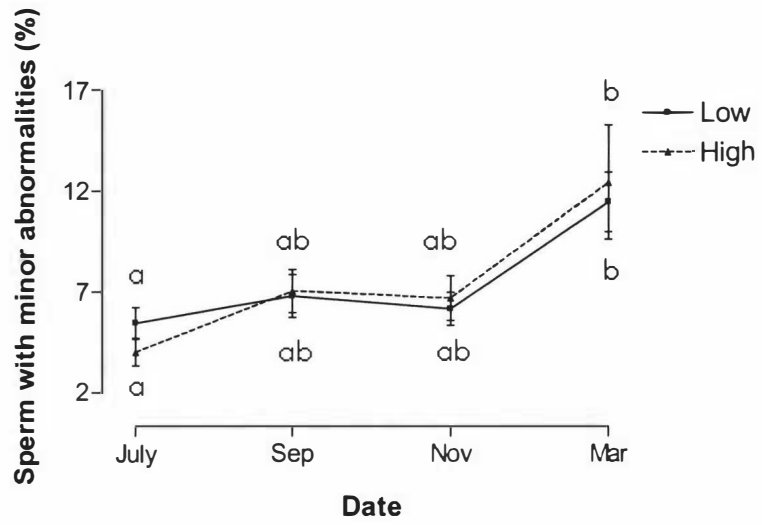
	Friedmans F	P
Low line	14.973	0.002*
High line	2.806	0.422

Asterisks indicate significant differences ($*P < 0.05$) between the values.

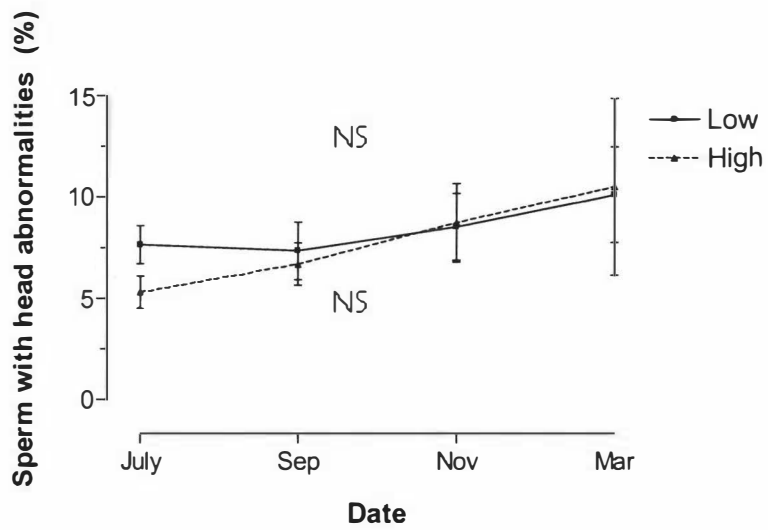
Figure 2.7 Percentages of sperm with (a) total abnormalities, with (b) major abnormalities, with (c) minor abnormalities, with abnormalities of the (d) head region and of (e) cytoplasmic droplets, in rams of two IGF-I selection lines throughout the year. Points labelled with different letters are significantly different from each other ($p < 0.05$).



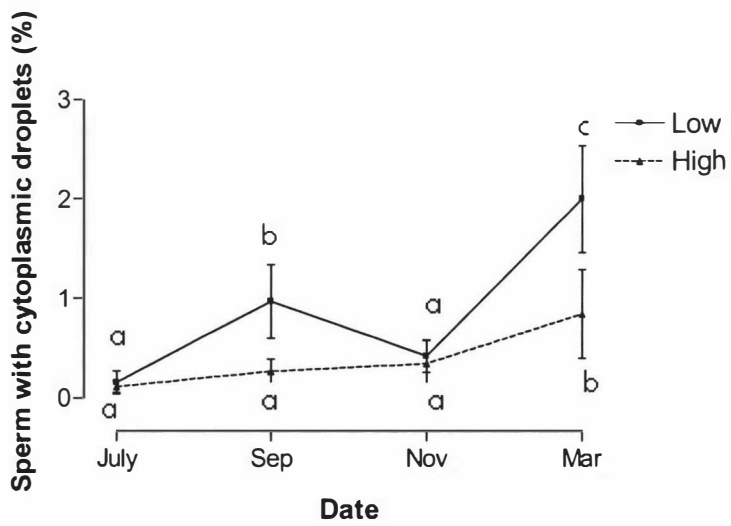
c)



d)



e)



2.3 Discussion

This study examined seasonal differences and reproductive parameters between rams in selected for high and low serum insulin-like growth factor-I (IGF-I). Seasonal changes were found in the percentage of abnormal sperm, scrotal circumference, sperm motility and sperm density, but there were no differences between lines in any reproductive parameters.

Scrotal circumference is a good indicator of sperm output, because testis size is highly correlated with sperm production, and the testis is the target of both LH and FSH (Pelletier and Ravault, 1988). In addition, scrotal circumference is related to semen quality parameters such as numbers of sperm, percentage of motile sperm and percentage of morphologically normal sperm (Brito et al., 2002; Martins et al., 2003; Kafı et al., 2004; Parkinson, 2004). Thus, sperm output is primarily determined by size of the testis in rams (Nowakowski and Cwikla, 1994; Soderquist and Hulten, 2006). Subsequently, many studies have demonstrated that scrotal circumference can be positively related to conception and pregnancy rate (Hamada et al., 2005; Bormann et al., 2006; Pereira et al., 2006). However, there are different growth patterns of testes during the peak breeding season in adult rams (Nowakowski and Cwikla, 1994b). Scrotal circumference of rams in Experiment 1 was significantly larger in December than in March (see Figure 2.1). On the other hand, scrotal circumference of rams in Experiment 2 was significant larger in breeding season (see Figure 2.2), in agreement with other studies (Nowakowski and Cwikla, 1994b; Al-Ghalban et al., 2004). A decrease in scrotal circumference results in an increase in the occurrence of morphologically abnormal sperm (Mickelsen et al., 1982), and rams with low scrotal circumference are less likely to have normal semen quality or fertility than rams with a normal circumference (Mickelsen et al., 1982; Nowakowski and Cwikla, 1994; Al-Ghalban et al., 2004; Kafı et al., 2004). In contrast, the present study found that there were no correlation between the scrotal circumference and the percentage of morphologically abnormal sperm. Scrotal circumference in Experiment 1 did not differ between lines, in agreement with Yilmaz et al. (1999), who reported that there was no relationship between IGF-I concentrations and scrotal circumference. However, scrotal

circumference in Experiment 2 was a significant difference between lines. In addition, there was a significant effect of interaction of line and month. These results suggest that differences in IGF-I concentration may be associated with scrotal circumference.

Sperm motility and the percentage of abnormal sperm also show seasonal variation (Al-Ghalban et al., 2004). Sperm motility in their study was low during winter and autumn with a minimum in summer, in agreement with Boland et al. (1985), who reported motility to increase steadily during breeding season. In Experiment 1, motility scores were significantly higher in March than in December (see Figure 2.3), as found by Hulet et al (1964), but did not differ in Experiment 2 (see Figure 2.5). In the present results, this pattern of motility scores corresponded with the pattern of scrotal circumference. As the scrotal circumference decreases, there is an increase in the motility score. Sperm motility scores in both Experiments 1 and 2 did not differ between the selection lines (see Figure 2.3 and 2.5), suggesting that the large differences in serum IGF-I concentrations between the lines did not affect in sperm motility, in agreement with Yilmaz et al. (1999). In contrast, Breier et al. (1996) reported that increased IGF-I concentrations in seminal plasma were significantly correlated with increased sperm motility without changes in morphology of sperm cells in GH-deficient dwarf rats during GH treatment.

Sperm concentration is considered in most studies of artificial insemination. However, no significant differences in sperm density were observed in either Experiment 1 or 2. These findings is, as has been shown by Ollero et al. (1996) and Lezama et al. (2003), who reported no significant difference in semen concentration between months and between selection lines (see Figure 2.4 and 2.5).

Male reproductive performance in rams is high at the end of the summer and in autumn and low at the end of winter and in spring (Gundogan et al., 2003; Rosa and Bryant, 2003). A similar autumnal peak of seminal fructose levels was noted for ejaculates from Romney and Merino rams (Barrel and Lapwood. 1978). Hence it is surprising that, in Experiment 1, the percentage of total abnormal

sperm (including in abnormalities in the head, midpiece, tail and cytoplasmic droplets abnormal sperm) was greater in March than in December (see Figure 2.6). In Experiment 2, the total percentage of abnormal sperm differed between months (see Figure 2.7). Although many studies have reported that percentage of morphologically abnormal sperm are related to conception rate (Suttiyotin et al., 1992; Singh et al., 1995; El-Darawany, 1999; Naqvi et al., 2002; O'Brien and Robeck, 2006), the literature on the relationship between semen quality and fertility is contradictory. The percentage of abnormal sperm did not differ between lines. A similar study reported that percentage of normal sperm cells did not differ between high and low line in bulls (Yilmaz et al., 1999), whereas there was a correlation between seminal IGF-I concentrations and percentage of morphologically normal sperm cells in humans (Glander et al., 1996).

In conclusion, current and previous data suggest that concentration of blood serum IGF-I is involved in growth and reproductive performance in rams. Rams have a distinct seasonal sexual activity. There was seasonal variation in the scrotal circumference, sperm morphology and sperm motility in Romney rams, but no seasonal variation in sperm density. In this experiment, the incidence of morphologically abnormal sperm cells, the scrotal circumference and sperm motility were higher in breeding season than in non-breeding season. There were no differences between lines in any of the reproductive parameters, with the exception of scrotal circumference in Experiment 1. Selection for high IGF-I may not be associated with improved reproduction, but seasonal variation does influence the male reproduction.

Chapter 3: Effects of selection for high vs. low blood serum IGF-I concentrations on plasma concentrations of testosterone and insulin and on reproductive performance in rams

3.1 Introduction

Insulin-like growth factor-I is a mitotic polypeptide (Svoboda et al., 1980) that stimulates sulphate (Salmon and Daughaday, 1957), lactate synthesis (Oonk and Grootegoed, 1988), glucose transport (Oonk et al., 1989) and cell proliferation (Borland et al., 1984). IGF-I plays an important role in the control of growth and reproduction and influences metabolism, cell division, ovarian folliculogenesis, oogenesis and ovarian secretory activity (Tres et al., 1986; Soder et al., 1992; Schams et al., 1999; Lucy, 2000; Machnik and Lechniak, 2000; Giudice, 2001; Hull and Harvey, 2001; Gong, 2002; Neuvians et al., 2005) in females, and germ cell development and maturation of spermatozoa (Tres et al., 1986; Soder et al., 1992; Hoeflich et al., 1999; Machnik and Lechniak, 2000; Neuvians et al., 2005) in males. The actions of IGF-I involve not only the actions of endocrine, GH-dependent, IGF-I, produced in the liver and its target cells, but also those of paracrine IGF-I, regulated independently of GH (Frohman et al., 1992).

IGF-I in the testis has various functions, such as testosterone synthesis by Leydig cells (Lin et al., 1986) and DNA synthesis in spermatogonia (Soder et al., 1992). IGF-I production in testicular tissues has been confirmed by the expression of IGF-I mRNA in whole testis (Casella et al., 1987), production of IGF-I from Sertoli cells in culture (Cailleau et al., 1990) and its localization by *in situ* hybridization and immunohistochemistry (Vannelli et al., 1988; Hansson et al., 1989). In addition, IGF-I receptors have been also found in Sertoli (Borland et al., 1984) and Leydig cells (Handelsman et al., 1985), and in secondary spermatocytes and spermatids (Tres et al., 1986). IGF-I has also been shown to be locally stimulated by follicle stimulating hormone (FSH) which plays a key role in the control of gonadal function (Cailleau et al., 1990). Experiments with cultures of Leydig cells and the testicular cells in seminiferous tubules have shown that IGF-I might influence the process of spermatogenesis (Spiterigrech

and Nieschlag, 1992). For example, increased IGF-I production from Sertoli and Leydig cells stimulates maturation of spermatozoa (Colombo & Naz, 1999), and affects the actions of gonadotropins on the Leydig cells *in vivo* by changing their sensitivity to LH (Chatelain et al., 1991). Circulating IGF-I can increase sperm motility (Ovesen et al., 1998), and increase both basal and LH-stimulated testosterone production in rat Leydig cells (Kasson and Hsueh, 1987).

Annual changes in daylength have been widely demonstrated to modulate reproduction in sheep (Nett and Niswender, 1982; Ortavant et al., 1988; Thiery et al., 2002). The breeding season begins at the end of summer and finishes in late winter or early spring. Rams show seasonal fluctuations in sexual behaviour and semen quality which affect male reproductive performance during the year (Gundogan et al., 2003; Rosa and Bryant, 2003).

IGF-I can stimulate circulating gonadotropins as a putative metabolic signal, and photoperiod is the possible factor that alters the pituitary secretion of FSH and LH, and consequently testosterone production from the testis. The objectives of this study were to define annual changes in the relationships between testosterone, IGF-I and insulin and to define the relationship between plasma IGF-I concentration and the activity of the male reproductive endocrine axis in two lines of Romney sheep selected for high vs. low blood serum IGF-I concentrations.

3.2 Materials and methods

3.2.1 Animals

Yearling rams (total 32 rams: 19 from low IGF-I line and 13 rams from high IGF-I) were used in this study. Each ram had blood samples collected at 4 occasions; in July, September and November 2006 and March 2007. After collection of blood samples, rams were given intravenously 50 ug hGRF (growth-hormone-releasing factor) in 2 ml 0.9% saline in July 2006 and March 2007, and 1,000 IU hCG (human chorionic gonadotropin) in 2 ml 0.9% saline in all months, to determine the extend to which GRF would stimulate the GH/IGF and hCG stimulate reproductive endocrine axes. Blood samples were collected 40 min and 10 h later for measurement of testosterone, IGF-I concentrations and insulin.

3.2.2 Collection of blood samples and administration of hormones

Pre-challenge (control) blood samples were collected via jugular venipuncture before the injections of hGRF and hCG and two different types of blood samples (serum and plasma) were obtained. Electric shears were used to shave part of the neck in order to allow for easier viewing of the vein and to minimize contamination from dirt and bacteria into the vein. Blood sampling was done with assistance to turn the head of animal to allow for easy access to the vein. The animal's body was restrained in a corner or against a wall to help control its hindquarters. After the area had been cleaned and the vein had been located, the control sample was taken by using a 20-gauge vacutainer needle and vacutainer. No anticoagulants vacutainers were used for serum collection and heparinised vacutainers were used for plasma collection. Blood samples (approximately 10 ml) were held on ice until they were centrifuged. After a collection of control samples, rams were given intravenously 50 µg hGRF (growth-hormone-releasing factor; Bachem) in July 2006 and March 2007, and 1,000 iu hCG (human chorionic gonadotropin; Chorulon, Intervet, Auckland, New Zealand) in July, September and November 2006 and March 2007.

Second blood samples were collected 40 min and 10 h later for measurement of testosterone, IGF-I concentrations and insulin. Blood samples were allowed to clot at room temperature for 2 h and serum or plasma were then separated by centrifugation. The serum and plasma were stored at -20°C for further assay.

3.2.3 Radioimmunoassay of testosterone

Testosterone concentrations in plasma were measured by radioimmunoassay. Samples were assayed in duplicate. Plasma samples 10 µl were mixed with 20 µl of sex binding globulin inhibitor solution (SGBI, MP Biomedicals, USA), then incubated with 100 µl of iodinated testosterone and 100 µl of antiserum (¹²⁵I- testosterone and antiserum ImmuChem™ Double Antibody Testosterone ¹²⁵I RIA kit for *in vitro* diagnostic use, MP Biomedicals, USA) for 2 h at 37°C. Second antibody 20 µl (MP Biomedicals, USA) was added and each sample was vortexed thoroughly, incubated for 1 hour at 37°C, then centrifuged for 15 min at 2 000 g at 4°C. 20 µl starch (50 g/l starch (Sigma) plus 0.1 g/l neutral red (BDH) in 10 ml of PBSG (pregnant mare serum gonadotropin) was added to increase adhesion of the pellet to the tube, then the samples were centrifuged for a further 15 min at 2000 g at 4°C and the supernatant aspirated. The pellets were counted on a PerkinElmer Wallac 1470 Automatic gamma counter for 5 min.

The cross-reactivity of the testosterone antibody with other steroids was provided by MP Biomedicals. Cross-reactions are as follows: 5α-dihydrotestosterone (3.40%), 5α-androstane-3, 17-diol (2.20%), 11-oxotestosterone (2.00%), 6-hydroxytestosterone (0.95%), 5-androstane-3, 17-diol (0.71%), 5-dihydrotestosterone (0.63%), androstenedione (0.56%), epiandrosterone (0.20%) and 11-hydroxyandrostenedione, 11-hydroxytestosterone, androsterone, 5α-androstane-3, 17-dione, 5-androstane-3, 17-dione, 5α-androstane-3α, 17-diol, dehydroepiandrosterone, oestrone, oestradiol-17β, oestriol, progesterone, corticosterone and desoxycorticosterone (<0.01%).

A serial dilution of plasma in PBSG was parallel to the testosterone standard curve. The quantitative recovery of testosterone was measured by adding

different amounts of standard testosterone to three plasma samples. The recoveries of added testosterone were $100.0 \pm 3.3\%$, $94.5 \pm 2.4\%$ and $101.1 \pm 4.9\%$.

The sensitivity of the testosterone assay was the minimum hormone level that could be consistently distinguished from zero. It was determined as the hormone concentration at the mean minus 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity was 0.006 ng/ml.

Solutions of testosterone in PBSG at concentrations that gave approximately 80, 50 and 20% binding on the standard curve were used as low, medium and high quality controls in every assay. The mean concentrations of testosterone in these solutions were 122.7 ± 8.5 , 502.5 ± 20.5 and 1472 ± 171 pg/ml respectively. The intra-assay coefficient of variation for each solution was determined by conducting an assay with twenty duplicates of each solution. The intra-assay coefficients of variation for testosterone were 8.5%, 4.1% and 9.8% for low, medium and high solutions respectively. Inter-assay coefficients of variation were calculated from duplicates of the solutions included at the beginning and end of each assay. The inter-assay coefficients of variation for twenty assays were 15.2%, 7.3% and 8.5% for low, medium and high solutions respectively.

3.2.4 Radioimmunoassay of insulin

Insulin concentrations in serum were measured by radioimmunoassay. Samples were assayed in duplicate. Serum 50 μ l was incubated with 50 μ l of iodinated insulin and 50 μ l of antiserum (125 I- insulin and antiserum, DSL-1600 kit, Diagnostic Systems Laboratories, USA) for 16 h at 4°C. Precipitating reagent (500 μ l; Diagnostic Systems Laboratories, USA) was added and each sample was vortexed thoroughly, incubated for 20 min at room temperature, 20°C, then centrifuged for 20 min at 2 000 g at 4°C. The pellets were counted on a PerkinElmer Wallac 1470 Automatic gamma counter for 2 min.

The cross-reactivity of the insulin antibody with other steroids was tested by Diagnostic Systems Laboratories. Cross-reactions are as follows: human insulin (100%), porcine insulin (99%), des 64, 65 human proinsulin (68%), human proinsulin (55%), des 31, 32 human proinsulin (50%) and glucagon, somatostatin, pancreatic polypeptide and insulin-like growth factor 1 (<0.01%).

A serial dilution of serum in kit zero standard was parallel to the insulin standard curve. The quantitative recovery of insulin was measured by adding different amounts of standard insulin to two serum samples. The recoveries of added insulin were $102.4 \pm 3.7\%$, and $90.8 \pm 1.3\%$.

The sensitivity of the insulin assay was the minimum hormone level that could be consistently distinguished from zero. It was determined as the hormone concentration at the mean - 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity was 1.6μ insulin/ml serum.

Solutions of insulin in a protein-based buffer (Diagnostic Systems Laboratories, USA) at concentrations that gave approximately 50 and 20% binding on the standard curve were used as medium and high quality controls in every assay. The mean concentrations of insulin in these solutions were 18.7 ± 1.6 and 63.0 ± 2.4 IU/ml respectively. Inter-assay coefficients of variation were calculated from duplicates of the solutions included at the beginning and end of each assay. The inter-assay coefficients of variation for two assays were 8.4 % and 3.8% for medium and high solutions respectively. The intra-assay coefficient of variation was determined by the manufacturer (Diagnostic Systems Laboratories, USA) with ten replicates of three samples. The intra-assay coefficients of variation for insulin were 8.3%, 4.5% and 6.4% for low, medium and high samples respectively.

3.2.5 Two-site immunoradiometric assay of IGF-I

Serum samples (50 μ l) were extracted by adding 200 μ l extraction solution (Diagnostic Systems Laboratories, USA), vortexing then incubating at room

temperature (~25°C) for 30 minutes. For samples with high levels of endogenous IGF-I, 25 µl serum and 225 µl extraction solution were used. The extracts were centrifuged at 10 600 g for 3 minutes, then 500 µl of neutralising solution (Diagnostic Systems Laboratories, USA) was added to 100 µl of supernatant and vortexed. The extracts were refrigerated (4°C) overnight and assayed the next day.

IGF-I concentrations in serum were measured by two-site immunoradiometric assay. Samples were assayed in duplicate. Serum extracts (50 µl) were incubated with 200 µl of iodinated anti-IGF-I in anti-IGF-I coated tubes (anti-IGF-I [¹²⁵I] and coated tubes, DSL-5600 kit, Diagnostic Systems Laboratories, USA) for 3 h at 25°C on an orbital shaker (Chiltern Scientific SS70) set at 180 rpm. The tubes were decanted then washed three times with 3 ml double-distilled water. The tubes were counted on a PerkinElmer Wallac 1470 Automatic Gamma Counter for 1 min each.

The cross-reactivity of the IGF-I antibody with other steroids was tested by Diagnostic Systems Laboratories. Cross-reactions are as follows: human IGF-I (100%) and human IGF-II, insulin, pro-insulin and growth hormone (non-detectable at 1 µg/tube).

A serial dilution of serum extract in kit zero standard was parallel to the IGF-I standard curve. The quantitative recovery of IGF-I was measured by adding different amounts of standard IGF-I to a serum extract. The recovery of added IGF-I was $87.0 \pm 3.4\%$.

The sensitivity of the IGF-I assay was the minimum hormone level that could be consistently distinguished from zero. It was determined as the hormone concentration at the mean - 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity was 20 IGF-I/ml.

Solutions of IGF-I in buffer (Diagnostic Systems Laboratories, USA) at concentrations that gave approximately 5 and 10% binding on the standard curve

were used as medium and high quality controls in every assay. The mean concentrations of IGF-I in these solutions were 75.0 ± 13.6 and 239.0 ± 46.1 ng/ml respectively. Inter-assay coefficients of variation were calculated from duplicates of the solutions included at the beginning and end of each assay. The inter-assay coefficients of variation for two assays were 18.4 % and 19.3% for medium and high solutions respectively. The intra-assay coefficient of variation was determined by the manufacturer (Diagnostic Systems Laboratories, USA) with eight replicates of three samples. The intra-assay coefficients of variation for IGF-I were 3.4%, 3.0% and 1.5% for low, medium and high samples respectively.

3.2.6 Statistical analyses

Three rams (2 from high line and 1 from low line) without complete sets of blood samples were excluded from statistical analyses as they were sick or died during the study. All hormone data were log e transformed before analysis, and Levene's tests were conducted for all variables to check for equality of variances before parametric analyses were performed. Changes in plasma testosterone concentrations were compared between lines and before and after the hCG challenge using repeated measures ANOVA with month, lines and strains and before and after the hCG challenge as the grouping factors. Post hoc comparisons were made using univariate F tests. Friedmans nonparametric repeated measures ANOVAs were used for each line to compare IGF-I concentrations between months, with Wilcoxon Z tests used to compare pairs of months. Mann-Whitney U tests were used for pairwise comparisons between lines, and for comparisons of IGF-I concentrations before and after hGRF challenges. Changes in plasma insulin concentrations were compared between lines and months using repeated measures ANOVA with month and lines as the grouping factors. Post hoc comparisons were made using univariate F tests. T-tests were used to compare insulin concentrations before and after hGRF challenges. Statistical analyses were performed using Systat (Systat Software). Data are presented as mean \pm S.E.

Testosterone data were subjected to analysis of variance, with respect to line and month, using a repeated-measures model, in which individual rams were nested within lines. Initially, time relative to hCG challenge (i.e. pre- and post-challenge) were included in the model, but as the magnitude of the differences between the pre- and post-challenge concentrations were so great, pre-challenge and post-challenge data were separately analysed. Basal IGF and insulin data were similarly analysed with respect to line and month, also using a repeated-measures model in which rams were nested within lines. Data for the two months in which GRF challenges were undertaken were analysed with respect to line, month and time (i.e. pre- and post- challenge), also using a repeated-measures model.

Initial inspection of the testosterone data suggested that it might be correlated with concentrations of IGF-I and insulin. This was examined by correlation and regression analysis. Thereafter, pre- and post-hCG challenge testosterone concentrations were re-analysed using either basal IGF-I or insulin concentrations, or both IGF-I and insulin concentrations, as covariates, in the same repeated measures analysis that had been undertaken previously.

Where statistical significance was established, post-hoc comparisons of individual pairs of means were undertaken using the method of least significant differences.

3.3 Results

3.3.1 Effects of IGF-I selection line and season on plasma testosterone and on testosterone responses to an hCG challenge

There were differences between months in basal testosterone concentrations ($p < 0.012$), but not between lines ($p = 0.407$; see Figure 3.1 and Table 3.1 for statistics). However, there were significant differences between months ($p < 0.001$) in testosterone concentration after hCG challenge in both lines ($p = 0.028$; see Figure 3.1a and 3.1b, and Table 3.1 for statistics). Results of the statistical analysis are shown in Table 3.1. Basal testosterone concentrations were higher in the low than the high line in July ($p = 0.043$) but not in other months. Basal testosterone concentrations in the low line did not change significantly from July to September, decreased from September to November then did not change significantly. Testosterone concentrations in the high line did not change from July to November then significant increased in March. Testosterone concentrations increased markedly after hCG challenge in all months and in both lines.

Table 3.1 Statistical analysis for testosterone comparisons of lines and before/after challenge for each month.

Basal			
Effect	F	df	P
Line	0.71	1,30	0.407
Month	3.84	3,84	0.012*
Interaction of line and month	0.19	3,84	0.908
Post hCG			
Effect	F	df	P
Line	5.36	1,30	0.028*
Month	5.93	3,84	<0.001*
Interaction of line and month	0.55	3,84	0.647
Comparisons before and after challenge for each month			
Low line			
July	78.966	1,50	<0.000*
September	133.526	1,50	<0.000*
November	109.761	1,50	<0.000*
March	38.375	1,50	<0.000*
High line			
July	32.377	1,50	<0.000*
September	108.140	1,50	<0.000*
November	111.201	1,50	<0.000*
March	14.544	1,50	<0.000*
Comparisons between months for initial testosterone			
Low line			
July c.f. September	0.059	1,50	0.810
July c.f. November	4.162	1,50	0.047*
July c.f. March	7.486	1,50	0.009*
September c.f. November	4.564	1,50	0.038*
September c.f. March	9.039	1,50	0.004*
November c.f. March	1.842	1,50	0.181
High line			
July c.f. September	1.240	1,50	0.271
July c.f. November	0.457	1,50	0.502
July c.f. March	2.347	1,50	0.132
September c.f. November	0.129	1,50	0.721
September c.f. March	6.909	1,50	0.011*
November c.f. March	4.960	1,50	0.030*

Asterisks indicate significant differences (* $P < 0.05$) between the values.

Figure 3.1a Plasma testosterone concentrations before and 40 min after hCG challenges in rams of two IGF-I selection lines. Pairs of means labelled with different letters are significantly different from each other ($p < 0.05$).

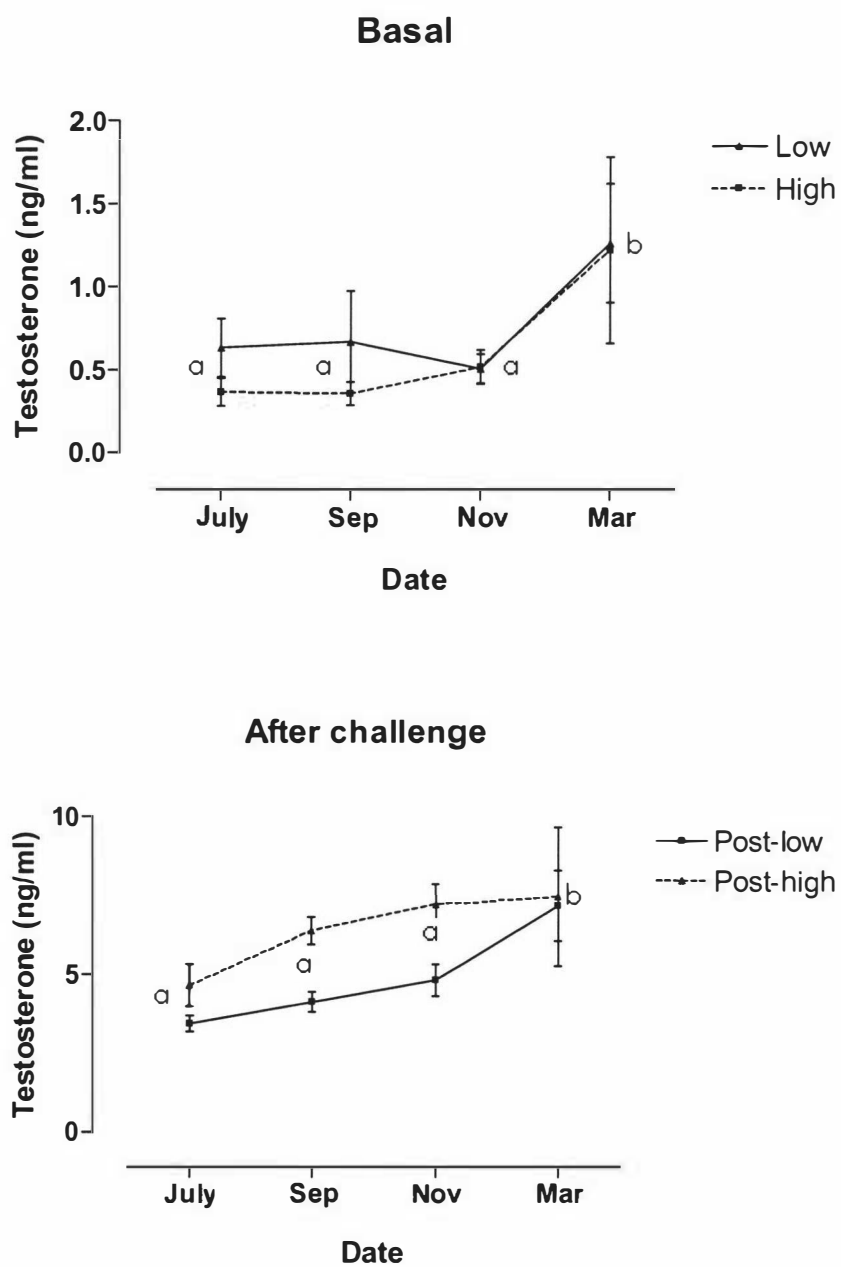
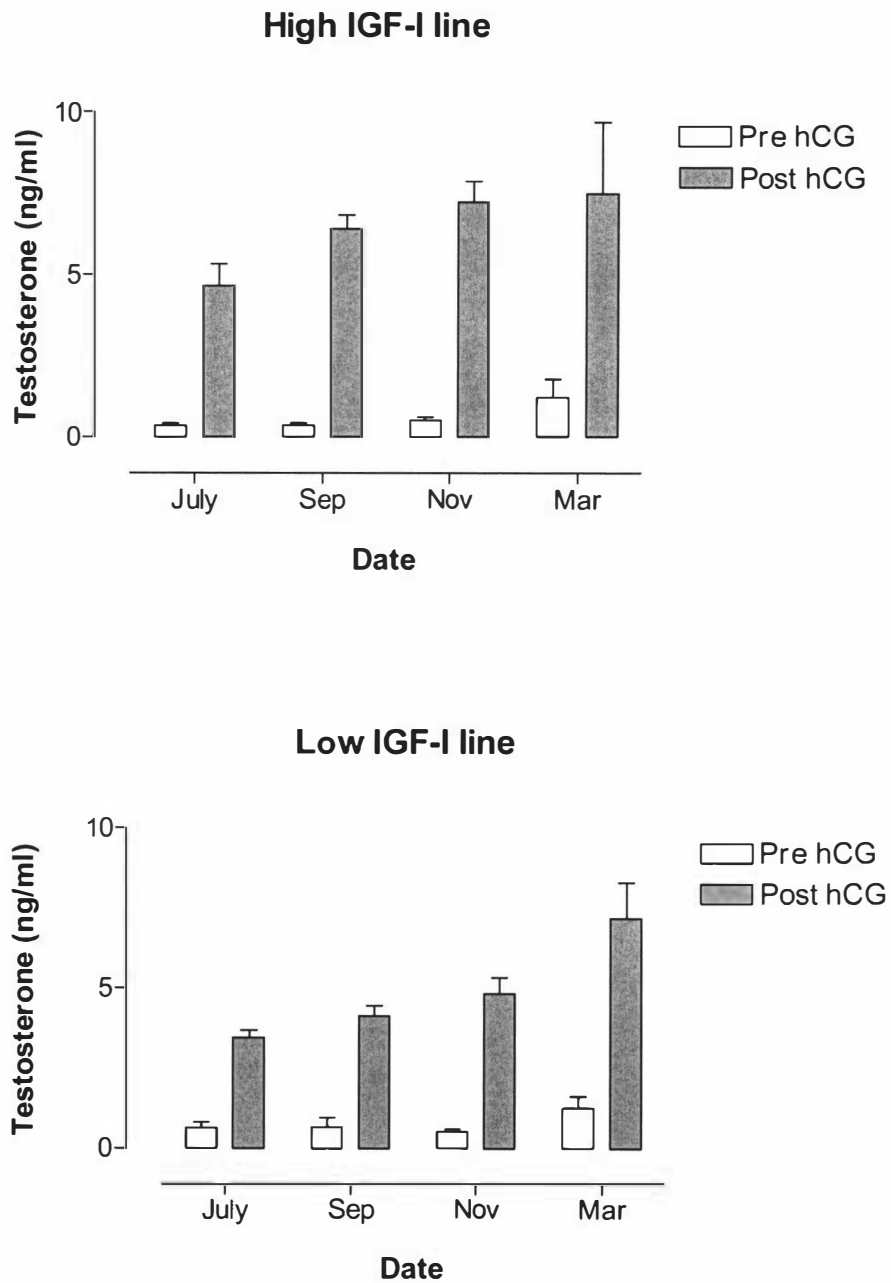


Figure 3.1b Plasma testosterone concentrations in rams of two IGF-I selection lines before and 40 min after hCG challenges (see Table 3.1 for statistics).



3.3.2 Effects of IGF-I selection line and season on plasma IGF-I and on IGF-I responses to an hGRF challenge

There were significant effects of IGF-I selection lines and seasonal changes on plasma IGF-I concentrations before and after hGRF challenge (see Figure 3.2, see Table 3.2a for statistics). IGF-I decreased significantly from July to September in the low line, increased from September to November then decreased again in March (Wilcoxon $Z = -2.803$, $p=0.005$; $Z = 2.803$, $p=0.005$; $Z = -2.803$, $p=0.005$, see Table 3.2b for statistics). IGF-I in the high line decreased from July to September, did not change in November and decreased in March (Wilcoxon $Z = -2.803$, $p=0.005$; $Z = -0.153$, $p=0.878$; $Z=-2.803$, $p=0.005$). IGF-I was greater in the high than the low line in all months ($U = 64.50$, $p<0.001$; $U = 0.00$, $p<0.001$; $U = 6.00$, $p<0.001$; $U = 19.50$, $p<0.001$, see Table 3.2c for statistics). There was no change in IGF-I after the hGRF challenge in the low line in July or March ($U = 382.50$, $p=0.784$; $U = 382.50$, $p=0.784$, see Table 3.2c for statistics). IGF-I decreased markedly after the hGRF challenge in July and increased significantly after the challenge in March ($U = 612.00$, $p=0.001$; $U = 612.00$, $p=0.001$) in high line.

Table 3.2 Statistical analysis for comparisons of IGF concentrations between months for each line.

a) Basal

Effect	F	df	P
Line	46.77	1,18	<0.001*
Month	12.47	3,54	<0.001*
Interaction of line and month	10.31	3,54	<0.001*

Post GRF

Effect	F	df	P
Line	8.82	1,10	0.008*
Month	10.34	3,54	<0.001*
Interaction of line and month	2.95	3,54	0.041*

b) Comparisons between months for each line

	Wilcoxon Z	P
Low line		
July vs September	-2.803	0.005*
July vs November	0.968	0.333
July vs March	-2.803	0.005*
September vs November	2.803	0.005*
September vs March	-2.803	0.005*
November vs March	-2.803	0.005*
High line		
July vs September	-2.803	0.005*
July vs November	-2.497	0.013*
July vs March	-2.803	0.005*
September vs November	-0.153	0.878
September vs March	-2.803	0.005*
November vs March	-2.803	0.005*

c) Basal

Mann Whitney U tests

July	U = 64.50, p<0.001*
September	U = 0.00, p<0.001*
November	U = 6.00, p=0.001*
March	U = 19.50, p<0.001*

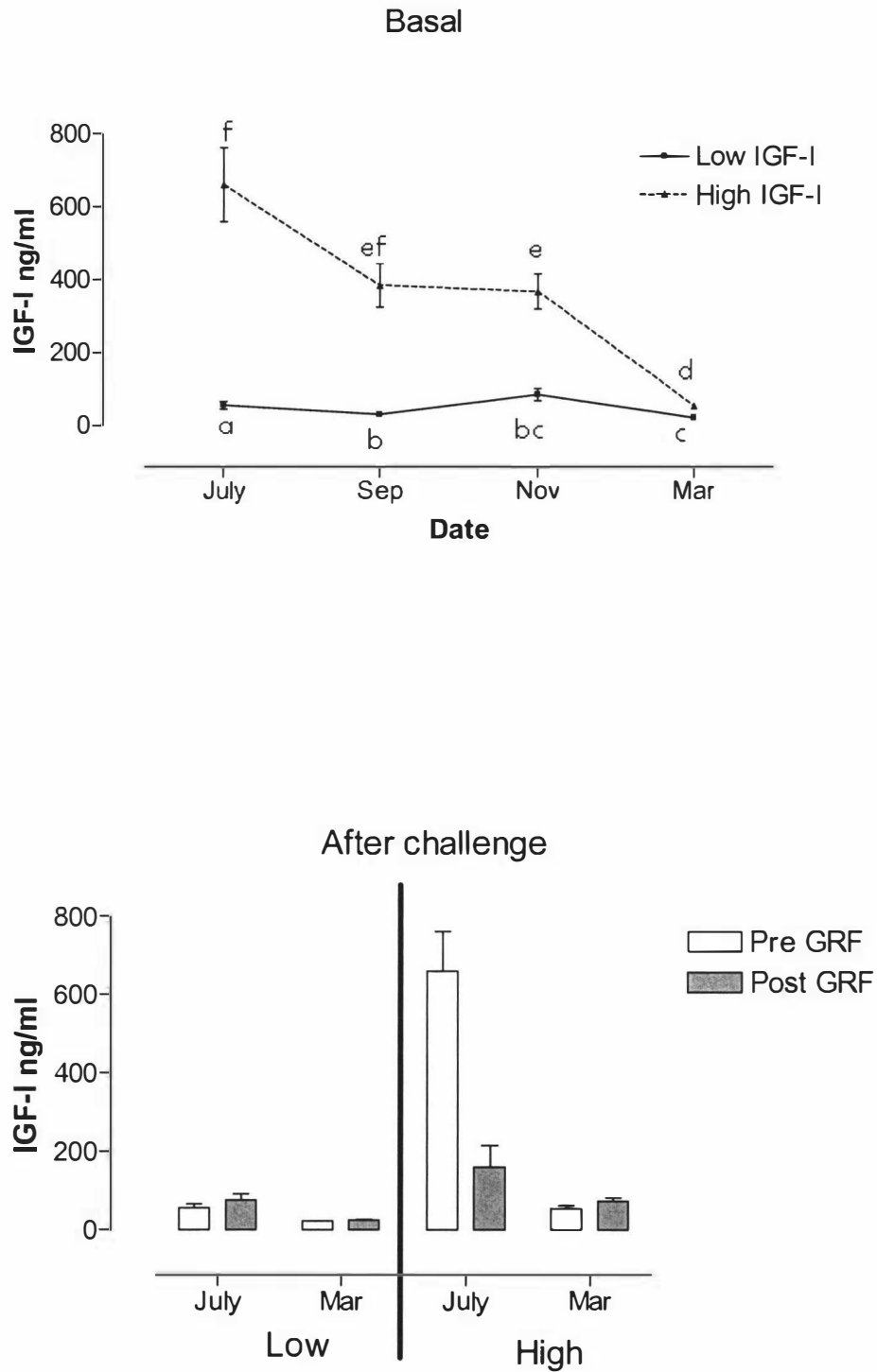
Post GRF

Mann Whitney U tests

July low line	U = 382.50, p=0.784
July high line	U = 612.00, p=0.001*
March low line	U = 382.50, p=0.784
March high line	U = 612.00, p=0.001*

Asterisks indicate significant differences (* $P < 0.05$) between the values.

Figure 3.2 Plasma IGF-I concentrations before and 10 h after hGRF challenges in rams of two IGF-I selection lines. Points labelled with different letters are significantly different from each other ($p < 0.05$).



3.3.3 Effects of IGF-I selection line and season on plasma insulin and on insulin responses to an hGRF challenge

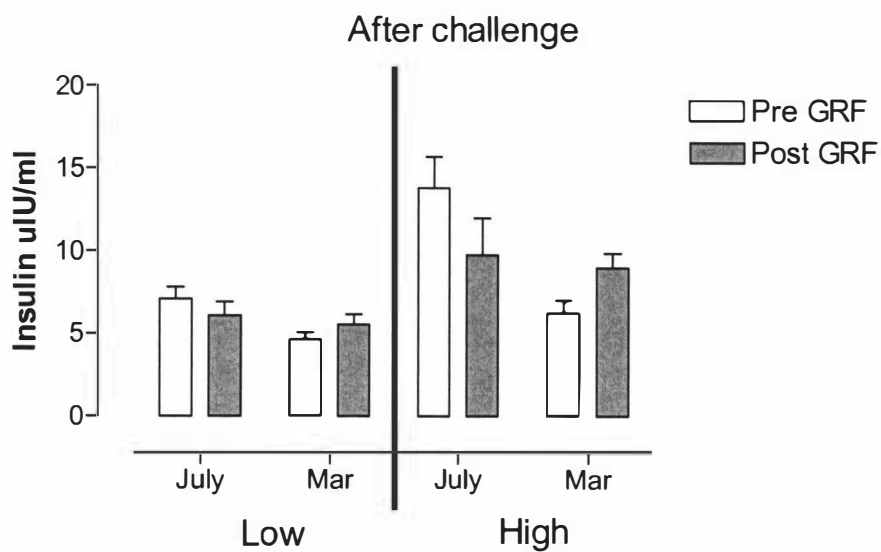
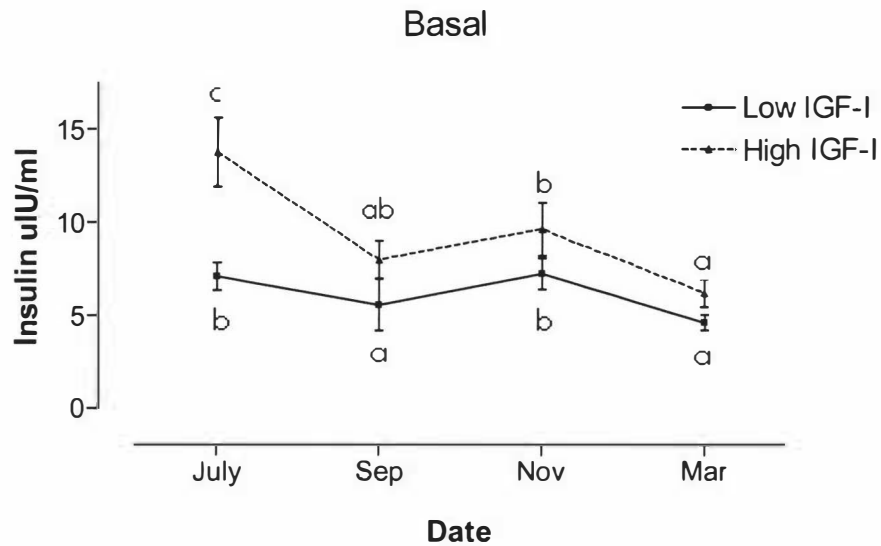
There were differences between months ($p < 0.001$) and lines ($p = 0.008$) in plasma insulin concentrations (see Figure 3.3 and Table 3.3 for statistics). Insulin was higher in the high than the low line in July and September, and did not differ between lines in November and March ($F_{1,18} = 15.852$, $p = 0.001$; $F_{1,18} = 4.317$, $p = 0.052$; $F_{1,18} = 2.472$, $p = 0.133$; $F_{1,18} = 2.563$, $p = 0.127$). Insulin decreased from July to September, increased from September to November then decreased again in March in the low line ($F_{1,18} = 4.571$, $p = 0.046$; $F_{1,18} = 5.174$, $p = 0.035$; $F_{1,18} = 6.163$, $p = 0.023$). Insulin in the high line also decreased from July to September, did not change significantly in November then decreased in March ($F_{1,18} = 6.915$, $p = 0.017$; $F_{1,18} = 0.865$, $p = 0.365$; $F_{1,18} = 7.183$, $p = 0.015$). Insulin did not change after the hGRF challenge in the low line, whereas insulin in the high line decreased after the hGRF challenge in July and increased after the challenge in March (low line $t_9 = 1.620$, $p = 0.140$; $t_9 = -2.021$, $p = 0.074$; high line $t_9 = 3.182$, $p = 0.011$; $t_9 = -2.804$, $p = 0.021$).

Table 3.3 Statistical analysis for insulin for comparisons of before and after challenge for each line in July and March.

Basal				
Effect		F	df	P
Line		8.82	1,10	0.008*
Month		10.34	3,54	<0.001*
Interaction of line and month		2.95	3,54	0.041*
Post GRF				
Effect		F	df	P
Line		3.66	1,18	0.072
Month		18.39	1,54	<0.001*
Interaction of line and month		0.18	1,54	0.671
Paired T tests				
Low line	July	$t_9 = 1.620$, $p = 0.140$		
	March	$t_9 = -2.021$, $p = 0.074$		
High line	July	$t_9 = 3.182$, $p = 0.011*$		
	March	$t_9 = -2.804$, $p = 0.021*$		

Asterisks indicate significant differences ($*P < 0.05$) between the values.

Figure 3.3 Plasma insulin concentrations before and 10 h after hGRF challenges in rams of two IGF-I selection lines. Points labelled with different letters are significantly different from each other ($p < 0.05$).



3.3.4 Covariation for IGF-I and insulin concentrations on testosterone concentrations before/after hCG challenge

Analysis of covariance between concentrations of IGF-I and insulin with those of testosterone (see Table 3.4 for the results of the statistical analysis), showed that both pre- and post-challenge testosterone concentrations were related to those of IGF-I. Pre-challenge testosterone concentrations were dependent upon variation, IGF-I (Figure 3.4), but not with those of insulin. When adjusted for the covariance of IGF-I concentration, there were statistically significant differences in basal testosterone concentrations between lines and months, such that the changes in testosterone concentrations with time varies between lines ($P < 0.05$; Figure 3.4a). The correlations between IGF-I and testosterone in this analysis of covariance was significant ($P < 0.05$). When adjusted for the effects of insulin concentrations there were significant difference in basal testosterone concentrations between months ($P = 0.01$), but not between lines. When both IGF-I and insulin were added into the model as covariates, there were no significant differences in basal testosterone concentrations between either line or month; moreover, the error term (residual mean squares) was significant ($P < 0.05$) greater than when IGF-I alone was used as a covariate. Post-challenge testosterone concentrations were less closely related to those of IGF-I and insulin than were basal testosterone concentrations. However, when adjusted for the effect of IGF-I concentrations, there was a significant difference between months ($P < 0.05$) and line ($p = 0.007$), but not the month and line interaction term ($P = 0.28$), in testosterone concentrations (Figure 3.4b). There was a significant ($p = 0.01$) correlation between the covariate, IGF-I, and post-challenge testosterone concentrations (Figure 3.5). Post-challenge testosterone concentrations were unrelated to those of insulin. When both IGF-I and insulin were added to the model as covariates, the effect of line upon testosterone concentrations was slightly increased (to $P < 0.005$), but that that of month became non-significant ($P = 0.07$).

Table 3.4 Statistical analysis of covariation for IGF-I and insulin concentrations on testosterone concentrations before and 40 min after hCG challenge.

a)	IGF		Insulin		IGF+insulin		
	F	P	F	P	F	P	
Basal testosterone							
Line	0.25	0.62	3.01	0.10	0.23	0.56	
Month	1.93	0.14	4.07	0.012*	2.15	0.10	
Interaction of line and month	2.71	0.05*	2.25	0.09	2.33	0.09	
Covariate	IGF	3.92	0.05*		3.91	0.05*	
	Insulin		0.82	0.37	0.93	0.34	
Testosterone after hCG challenge							
Line	9.34	0.007	3.96	0.06	10.88	0.005*	
Month	3.06	0.037*	1.57	0.21	2.55	0.07	
Interaction of line and month	1.31	0.28	0.04	0.99	1.21	0.05*	
Covariate	IGF	6.52	0.014*		6.38	0.015*	
	Insulin		0.00	0.962	0.00	0.99	
b)				F	P		
Correlation between IGF-I and insulin concentrations				26.74	<0.001*		

Asterisks indicate significant differences (* $P < 0.05$) between the values.

Figure 3.4 Testosterone concentrations before and 40 min after hCG challenge adjusted for IGF-I concentrations.

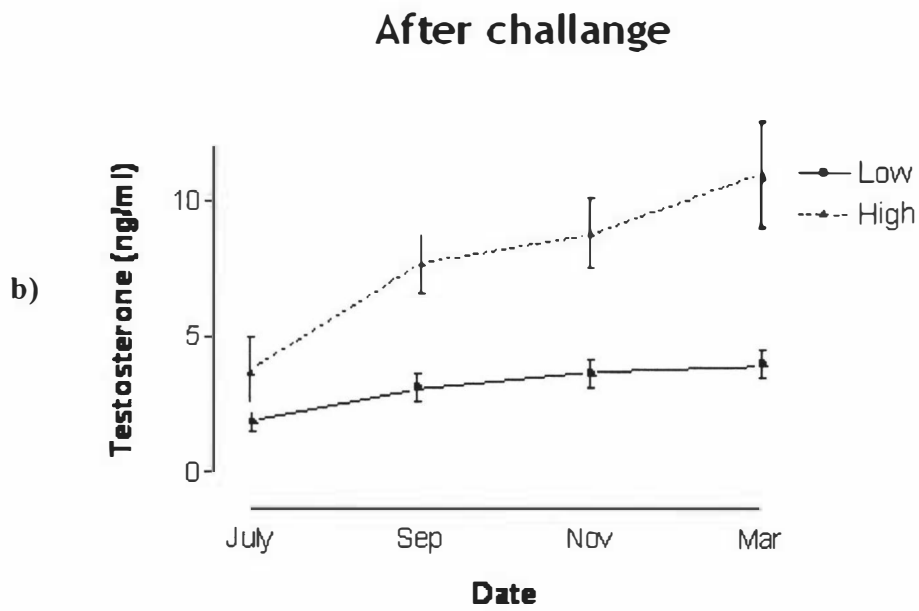
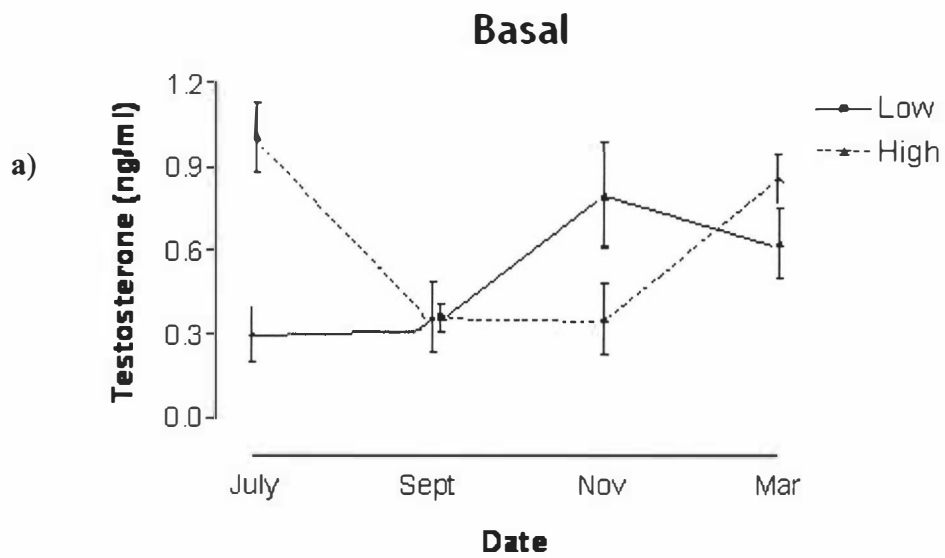
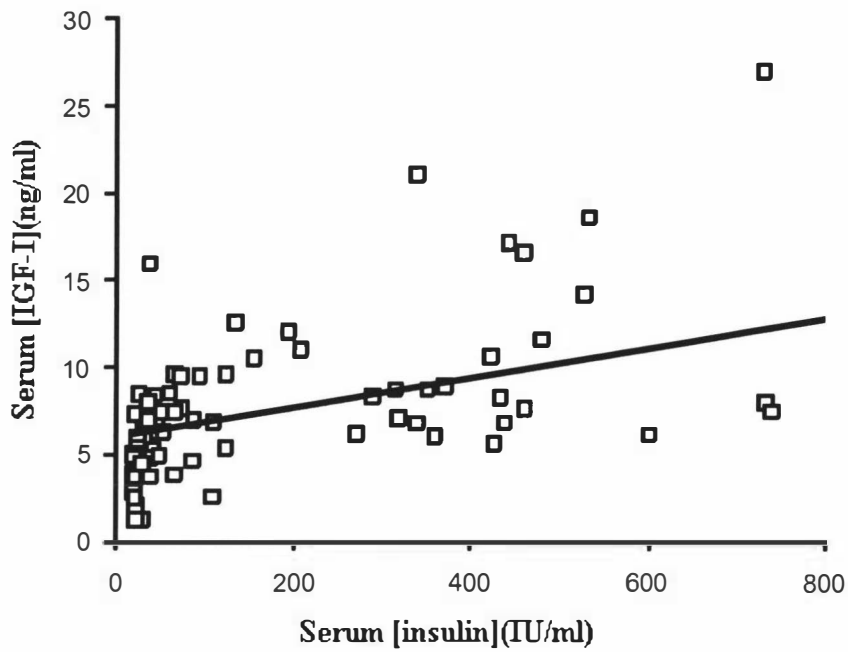


Figure 3.5 Plot of least squares means for testosterone and IGF-I concentration. The equation for the regression line was $Y=0.008x + 6.020$, where variable Y =serum IGF-I concentrations and variable X = serum insulin concentrations.



3.4 Discussion

This study tested the hypothesis that insulin-like growth factor-I (IGF-I) is a putative metabolic signal, which could modulate the photoperiod-driven changes of FSH and LH, and consequently testosterone production from the testis. In this study, there were seasonal changes in plasma testosterone, IGF-I and insulin concentrations, and differences between lines were found in the concentrations of IGF-I and insulin secretion throughout the year, but not in testosterone concentrations.

The sheep is a seasonal breeder, in which short days stimulate sexual activity, whilst exposure to long days results in inhibition and thus cessation of reproductive activity (Gundogan et al., 2003; Rosa and Bryant, 2003). Annual changes in daily photoperiod play an important role in the regulation of reproductive activity in both female and male sheep. The effects of photoperiod on reproductive status are mainly regulated by modifications in the activity of the gonatotropic axis in terms of variations in the secretion of LH and FSH (Thiery et al., 2002; Thiery and Malpaux, 2003). Gonadotropic hormones reach their peak near the onset of the breeding season in sheep (Ortavant et al., 1988; Eckery et al., 1997; Thiery et al., 2002; Gundogan et al., 2003; Rosa and Bryant, 2003; Dickson and Sanford, 2005). In present study, plasma testosterone concentrations were greatest during the breeding season, as has been widely reported from previous studies (Sghiri and Driancourt, 1999; Rosa et al., 2003; Anukulitch et al., 2007). . The role of hCG as an luteinizing hormone-like hormone to stimulate testosterone synthesis in reproductive tissue is likewise well-recognised (Zamaratskaia et al., 2007). In the present study, the changes in the response of plasma testosterone concentrations to stimulation by hCG throughout the year were generally similar to those previously shown in other studies (Bablok et al., 1979; Chandrashekar and Bartke, 1993; Adiyaman et al., 2004).

There is increasing evidence, both from the present study and from the literature, that the response of the testis to hCG is modulated by the GH-IGF axis. For example, acute treatment of immature pigs with IGF-I increased mRNA for the hCG receptor and increased the number of hCG receptors in Leydig cells (Lin et al., 1993). IGF-I treatment of male mice with GH insufficiency significantly increased

both the testosterone response to the hCG and the number of hCG receptors (Chatelain et al., 1994). In addition, IGF-I stimulates LH output in sheep, which, in turn, stimulates testosterone production (Adam et al., 1998; Khalid et al., 2000). The present study showed no differences between lines in basal testosterone concentrations, although basal testosterone concentrations were related to those of IGF-I. Thus, both in simple regression analysis and in the analysis of covariance, basal testosterone concentrations were related to those of IGF-I. Moreover, there was a significant difference between lines in their responses to hCG and, in the analysis of covariance, there was also a relationship between testosterone response to hCG and IGF-I concentrations (Figures 3.4 and 3.5). Conversely, insulin concentrations were largely unrelated to those of testosterone, either before or after hCG-stimulation and, except in the case of the relationship between line and post-hCG testosterone correlations, most of the correlations between IGF-I and testosterone were worsened when insulin was added into the model.

Taken together, these data provide evidence that the response of the testis to stimulation by LH is differentially regulated by IGF-I in the two lines of rams. If so, this infers that the seasonal changes in testosterone secretion may be partly controlled by the IGF-I system, at least at the level of the ability of the testis to respond to LH and, possibly, via the secretion patterns of LH as well.

There is, however, some disagreement on whether the effects of IGF-I in the testis are dependent on GH. In relation to testis functions, one study reported that the IGF system acts independently of GH such as testosterone synthesis, (Lin et al., 1986). The main action of GH in the regulation of reproductive development through the action of IGF-I was firstly shown by the original formulation of the somatomedin hypothesis (Salmon and Daughaday, 1957). The GH-IGF axis plays an important role in numerous physiological and pathological processes and it is one of the major hormonal systems regulating somatic growth and cellular proliferation as well as reproduction in mammals. Growth hormone is released from the anterior pituitary gland into the bloodstream in a pulsatile fashion that is particularly pronounced in males and is present in the plasma both in the free form and bound to GH binding protein (GHBP). The actions of GH are mediated by binding to the GH receptor on the cell membrane of target tissues (Clark, 1997). GHBP is

derived from cleavage of the extracellular portion of the GH receptor or from alternative transcripts of the GH receptor/ GHBP gene (Bartke, 2000). Secretion of GH is controlled primarily by hypothalamic peptides; somatostatin which inhibits its release and GH-releasing hormone (GRF) which stimulates its secretion (Spiterigrech and Nieschlag, 1992; Jones and Clemmons, 1995; Mohan et al., 1995; Hwa et al., 1999; Chandrashekar et al., 2004). GH also regulates its own secretion through a short loop negative feedback mechanism mediated by hypothalamic GRF and somatostatin (Frohman et al., 1992). GH can also inhibit its own secretion by a short feedback loop acting on the hypothalamus. The actions of GH are mediated by binding to the GH receptor on the cell membrane of target tissues (Clark, 1997). GH receptors in the liver, including other tissues, GH stimulates the production of IGFs and IGFbps (Chandrashekar et al., 2004). GH receptors are expressed at many sites, including the hypothalamus, other brain regions (Nyberg and Burman, 1996), and the testis, including Leydig and Sertoli cells (Lobie et al., 1990), suggesting that GH can affect directly on male reproductive function. The best-known response to GH in liver is the increase in the synthesis and secretion of IGF-I.

Insulin-like growth factor-I acts as an endocrine regulator of GH release through a negative feedback pathway (Chandrashekar et al., 2004). Low IGF-I concentrations reduce negative feedback on GH and result in an increase in blood GH concentrations. However, Laron (1999) reported that the over-expression of GH resulting in chronic elevation of GH and IGF-I concentrations can interfere with many aspects of reproduction. Furthermore, Dyck et al. (1999) reported that the testicular over-expression of IGF-I induces increased IGF binding proteins, resulting in inhibition of IGF activity. A study of the effects of IGF-I, insulin and GH, on basal and GRF-induced GH release, in goat anterior pituitary cells *in vitro* (Kato et al., 2004), showed that the secretion of GH was significantly suppressed by the presence of IGF-I. It was considered that this was because the increase in basal GH release increases IGF-I concentration and the increase in IGF-I release reduces GRF release through the negative feedback.

In the present study, IGF-I concentrations after hGRF challenge increased slightly during the breeding season. However, the high basal concentrations of IGF-I that were present in July decreased markedly after hGRF challenge in the high IGF line. It may be that this occurred as the exogenous hGRF increased GH release, which lead to increased IGF-I secretion, which in turn, had a negative feedback effect to reduce endogenous GH. Consequently, the net effect was to reduce IGF-I release, as also suggested by Katoh et al., (2004). Concentrations GH were not measured, due to practical limitations, so the veracity of this suggestion cannot be confirmed.

Both IGF-I and insulin fluctuate according to energy balance. Lucy (2006) showed that cows in negative energy balance and cows selected for increased milk production have lower IGF-I and insulin concentrations. Infertility results in such cows since both insulin and IGF-I facilitate the secretion of GnRH and LH. Thus, both nutrition and energy balance, as well as the genetically-determined activity of the GH/IGF/insulin axis can affect reproductive status. However, the effects of nutrition and energy balance may well primarily depend upon the GH receptor. Lucy (2006) showed that low insulin concentrations lead to low GH receptor expression, decreased blood IGF-I and greater blood GH concentration, and insulin resistance. Berek et al. (1999) reported a stimulatory effect of insulin on liver GH receptors in human and other species, suggesting that the coincident increase in blood insulin and liver GH receptors depends on insulin. Low insulin concentrations have an opposite effect on adipose tissue where GH receptor expression increases in response to a decrease in blood insulin (Butler et al., 2003). Furthermore, insulin resistance may affect IGF-I secretion when manifested at the intracellular level, because insulin and IGF-I share second messenger systems (Lucy, 2006). Growth hormone signals through its receptor in liver and adipose tissue and inhibits the activity of the insulin receptor (Blüher et al., 2005). Thus, an increase in insulin concentrations causes an increases in liver GH receptors which, in turn, leads to an increase in liver IGF-I synthesis (and, hence, an increase in blood IGF-I concentrations).

Lucy (2002) reported that ovarian cells treated with either insulin or IGF-I had a greater response to LH and FSH. In addition, reproductive organs (including testis

and ovary) have the capacity to respond to GH, insulin and IGF-I because they possess receptors for the respective hormones. Likewise, Blüher et al. (2005) suggested that reproductive tissues generally respond positively to insulin and IGF-I, and that these hormones may link nutrition and energy balance to reproductive success. In the present study, insulin concentrations in high and low lines had similar seasonal patterns and insulin concentrations before and after hGRF challenges were higher in the high than the low line throughout the year (Figure 3.3). The present study showed that there was a relationship between IGF-I and insulin concentrations (Figure 3.5), with profiles for plasma insulin in both lines similar to the pattern of high line of IGF-I concentrations across months. Insulin concentrations increased significantly after hGRF challenge during breeding season and high concentrations of insulin decreased considerably after hGRF challenge in July. Thus, the decrease in insulin concentrations coincided with the decrease in IGF-I concentrations, in agreement with Lucy (Lucy, 2006), who reported that low insulin concentrations lead to low GRF expression, decreased IGF-I and greater GH release in humans and cows. However, simultaneous addition of insulin did not have any significant effects on GH release at the pulsatile levels (Kato et al., 2004) and, in the present study, there was no relationship between testosterone and insulin concentrations. These results suggested that secretion of insulin may not be directly related to GRF and testosterone release, but may have a positive effect on IGF-I release.

In summary, the present study showed that there were differences in basal and hCG-stimulated testosterone concentrations that were related to breeding season, and between high and low IGF-I lines in plasma IGF-I and insulin concentrations throughout the year. Testosterone concentrations did not differ significantly between lines in simple analyses but, when IGF-I concentrations were taken into consideration, there was a significant difference in the temporal changes in basal and post-hCG testosterone concentrations between lines. These differences were largely unrelated to changes in insulin concentrations, although differences in insulin concentrations may have affected the responses to hCG. Seasonal changes in IGF concentrations within lines (themselves probably at least partly constrained nutritionally) may contribute to, or modulate, the seasonal changes in the activity of the hypothalamo-pituitary-gonadal axis.

Chapter 4: Expression of mRNA encoding IGF-I, IGF Type I receptor, IGF-binding proteins (IGFBP-1 to -6) between rams selected on basis of blood serum IGF-I concentration

4.1 Introduction

The insulin-like growth factors are single chain polypeptides which are structural analogues of proinsulin. They stimulate proliferation and differentiation of many diverse cell types and function as endocrine and paracrine/autocrine hormones (Jones and Clemmons, 1995). The insulin-like growth factor system consists of two ligands (IGF-I and IGF-II), Type 1 and Type 2 IGF receptors (Froesch et al., 1985), IGF-binding proteins (IGFBPs), and IGFBP proteases. IGF-I is mainly produced by the liver under the control of growth hormone (GH) (Clemmons and Underwood, 1991) and acts on target tissues as a hormone (Borland et al., 1984). The biological actions of IGFs are mediated by cell-surface receptors (insulin receptor (1R) and Type I and Type II IGF receptor (IGF-1R and IGF-2R respectively). IGFs are normally bound to at least six different IGF binding-proteins (IGFBP-1,-2, -3, -4, -5, -,6 ; (Hwa et al., 1999). The six IGFBPs function as regulators of the biological activities of IGF-I and IGF-II (Jones and Clemmons, 1995; Hwa et al., 1999). IGFBPs have higher affinities for IGFs than do the Type 1 IGF receptors, so that IGFBPs not only prolong the half-life of the IGFs as carrier of IGFs, but also modulate IGF availability and activity (Hwa et al., 1999).

Although the role of IGF in many reproductive processes is not clear, the IGF system plays an important role in the development and functions of the testis and ovary. IGF-I acts on numerous functions in the testis, including testosterone synthesis by Leydig cells (Lin et al., 1986) and DNA synthesis in spermatogonia (Soder et al., 1992). Furthermore, IGF-I produced by Sertoli cells stimulates lactate synthesis (Oonk and Grootegoed, 1988), glucose transport (Oonk et al., 1989) and cell proliferation (Borland et al., 1984). The local production of IGF-I by testicular tissues has been confirmed by expression of IGF-I mRNA in the rat testis (Casella et al., 1987), production of IGF-I from Sertoli cells in culture (Cailleau et al., 1990) and its localization by *in situ* hybridization and

immunohistochemistry (Vannelli et al., 1988; Hansson et al., 1989). In addition, IGF-I receptors have been found in Sertoli cells (Borland et al., 1984), Leydig cells (Handelsman et al., 1985) and secondary spermatocytes and spermatids (Tres et al., 1986). Although IGF-I production is mainly regulated by GH secretion, it has also been shown to be locally stimulated by follicle stimulating hormone (FSH) which plays a key role in the control of gonadal function (Cailleau et al., 1990).

The testicular synthesis of IGF-I, its receptor and the IGF binding proteins have not previously been examined in sheep. The aim of this study was to compare the expression of genes for IGF-I, IGF Type I receptor and IGFBPs (-1, -2, -3, -4, -5 and -6) between two lines of Romney rams selected for high vs. low blood serum IGF-I concentration.

4.2 Materials and methods

4.2.1 Animals

Yearling rams (4 from low IGF-I line and 4 rams from high IGF-I) were used in this study. Samples of liver and testis tissue were collected for total RNA isolation. The animals were euthanised immediately before tissue collection. After collection, tissues were serially sectioned using stainless steel blade and allow them to be immediately frozen in dry ice and stored at -80°C. Expression of mRNA for IGF-I in both liver and testis were measured. mRNA for IGF Type I receptor (IGF-1R) and IGFBPs (-1, -2, -3, -4, -5 and -6) in testis was measured.

4.2.2 Primer design

For this study, primer sequences of IGF-I, IGFBP-1, -2, -3, -4 and -6 taken from the sheep IGF and sequences of IGF-1R and IGFBP-5 taken from bovine IGF were used to design primers to determine their gene expression in the rams' testis. Previous studies of IGF gene expression have been conducted using

human- or rat-or bovine-specific primers. RNA samples from the liver of rams were used as an internal control.

Upstream and downstream primers for the components of the IGF-1R, IGFBP-1,2,3,4,5 and 6 and the abundant ovine glyceraldehydes-3-phosphate dehydrogenase (GAPDH; positive control) were reported in a previous study (Hayashi et al., 2005). Primer sequences for the components of the IGF-I were designed from the coding regions of each gene sequence using a commercial computer programme (Primer 3). The annealing temperatures were optimised and different from those of Hayashi et al. (2005). The sequences of the upstream and downstream primer, the GenBank[®] Accession numbers, the annealing temperatures and the size of PCR product obtained for each component are shown in Table 4.1.

Table 4.1 Summary of upstream and downstream PCR primer sequences, annealing temperatures (AT) used during PCR and Genbank[®] accession NO and size of cDNA.

Primer	Sequences of upstream and downstream primers (5' to 3')	Genbank [®] (Species)	AT	cDNA size (bp)
IGF-I	CCTCGCATCTCTTCTATCTGGC	NM001009774 (Ovine)	60	351
	CACTCCCTCTGCTTGTGTTCTTC			
IGF-1R	GAGATTGCAGATGGCATGG	54980 (Bovine)	61	441
	GCTGATGATCTCCAGGAACG			
IGFBP-1	GATGACCGAGTCCAGTGAGG	AF327650 (Ovine)	61	210
	CCATTCTTGTTGCAGTTTGG			
IGFBP-2	AGCAGGTTGCAGACAATGG	S44612 (Ovine)	61	353
	ACAGTTGGGGATGTGTAGGG			
IGFBP-3	CAGAACTTCTCCTCCGAGTCC	AF327651 (Ovine)	63	205
	CCACACACCAGCAGAAACC			
IGFBP-4	CTTGGCCAAAATTCGAGACC	S77394 (Ovine)	61	286
	AAGCTTCACTCCCGTCTTCC			
IGFBP-5	GCGGCGTCTACACTGAGC	S52657 (Bovine)	63	212
	GAAGATCTTGGGCGAGTAGG			
IGFBP-6	AAGGAGAGTAAGCCCAAGC	AF327653 (Ovine)	63	212
	GGCACGTAGAGTGTATGAGACC			
GAPDH	CTGACGCTCCCATGTTT	AF030943 (Ovine)	61	162
	CGTGGACAGTGGTCATAAG			

4.2.3 Total RNA extraction and RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was carried out with four animals from each line. Total RNA was isolated from frozen testis using Trizol reagent (Life Technologies, Inc.-BRL, Bethesda, MD) according to the manufacturer's instructions. Concentrations of RNA in samples were quantified from their spectrophotometric absorbance at 260 nm. When the RNA sample was contaminated by DNA or the A260:A280 ratio was less than 1.8, DNA digestion with a restriction enzyme was performed.

Reverse transcription PCR was conducted using a commercial kit (Access RT-PCR System; Promega) using Genius thermal cycler (Bio-Rad) following the standard protocol in the manufacturer's instructions. In brief, the total volume of mixture was 25 μ l, including 1 μ g total RNA, 1 μ M upstream primer, 1 μ M downstream primer, 1X AMV/*Tfl* reaction buffer, 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 1 mM MgSO₄, 0.1U AMV-RT and 0.1 U *Tfl* DNA polymerase. The annealing temperatures of each primer were calculated from the percentage of GC content of each set of primers. For the first-strand cDNA synthesis, all samples were placed in tubes in a thermal cycler equilibrated at 45°C and incubated for 45 minutes to allow the RNA reverse transcription to work. The samples were incubated at 94°C for two minutes in order to allow AMV RT inactivation and RNA/cDNA/primer denaturation. For the second-strand cDNA synthesis with the creation of cDNA, all PCR reactions were performed at 94°C for 30 sec, 60-63°C for 1 min and 68°C for 1 min. The annealing temperatures used for each primer pair are given in Table 4.1. The number of cycles was 40 for all primers.

After RT-PCR, electrophoresis was performed for analytical purposes at 135 v for 30 min, 15 μ l of all reaction products were analysed using a 2% agarose gel (Bio-Lab Laboratories Pty Ltd) with ethidium bromide staining (Sigma-Aldrich New Zealand) and TBE buffer (In Vitro Technologies Ltd; Biotek). The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using an AlphaImager

(Alpha Innotech Corporation, San Leandro, CA). The GAPDH values were used to correct for differences in the amounts of cDNA used for each sample.

4.2.4 Statistical analyses

Statistical analyses were performed using Systat (Systat software). Levene's tests were performed on all variables to test for homogeneity of variances. mRNA expressions were compared between lines using T-tests and U-tests. Data are presented as individual points or as mean \pm S.E.

4.3 Results

4.3.1 Expression of mRNA encoding IGF-I and IGF Type I receptor

Expression of mRNA for IGF-I was greater in the high than in the low line in the liver, but not in the testis ($t_6 = 3.260$, $p = 0.017$; $u = 7.00$, $p = 0.773$; see Figure 4.1, see Table 4.1 statistics). mRNA for the Type I IGF receptor showed higher expression in the testis in the high line compared with the low line ($t_6 = 3.084$, $p=0.022$; see Figure 4.1).

Table 4.2 Statistical analysis of mRNA expression of IGF-I and Type I IGF receptor in the testis, and IGF-I in the liver for comparisons of variables between lines.

T tests using pooled variance

IGF-I liver $t_6 = 3.260$, $p=0.017$

IGF-I receptor $t_6 = 3.084$, $p=0.022$

U-test

IGF-I testis $U = 7.00$, $p=0.773$

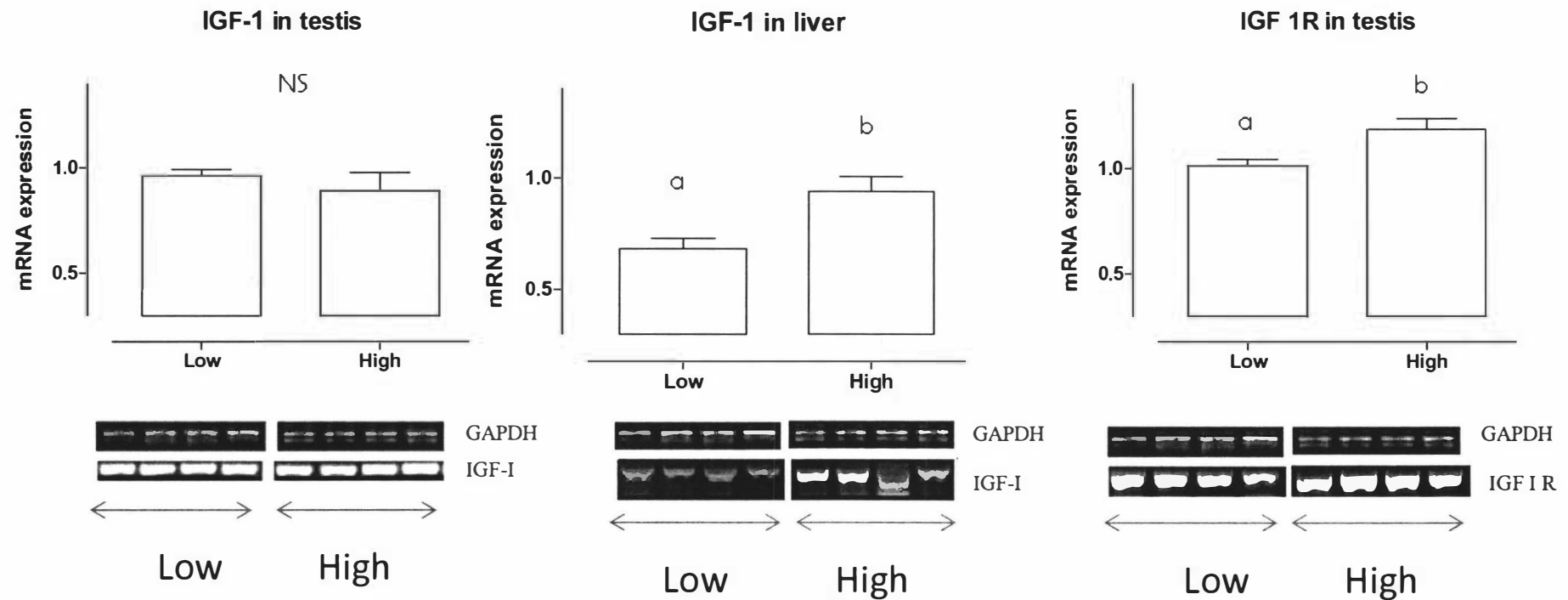


Figure 4.1 Mean IGF-I and Type I IGF receptor mRNA expression in the testis and liver between high and low line. Means labelled with different letters are significantly different from each other ($p < 0.05$).

4.3.2 Expression of mRNA encoding IGF-binding proteins

The expression of mRNA for IGFBP-3 was lower in the high line than in the low line ($t_6 = -4,115$, $p = 0.006$; see Figure 4.2, see Table 4.2 for statistics). Expressions of mRNA for IGFBP -2, -4, -5 and -6 did not differ significantly between lines ($t_6 = 1.651$, $p = 0.334$; $u = 2.00$, $p = 0.083$; $t_6 = -2.054$, $p = 0.086$; $t_6 = 2.168$, $p = 0.073$, respectively; see Figure 4.2 and 4.3).

Table 4.3 Statistical analysis of mRNA expression of IGFBP-2, -3, -4, -5 and -6 in the testis for comparisons of variables between lines.

T tests using pooled variance

IGFBP-2	$t_6 = 1.051$, $p = 0.334$
IGFBP-3	$t_6 = -4.115$, $p = 0.006$
IGFBP-5	$t_6 = -2.054$, $p = 0.086$
IGFBP-6	$t_6 = 2.168$, $p = 0.073$

U-test

IGFBP-4	$U = 2.00$, $p = 0.083$
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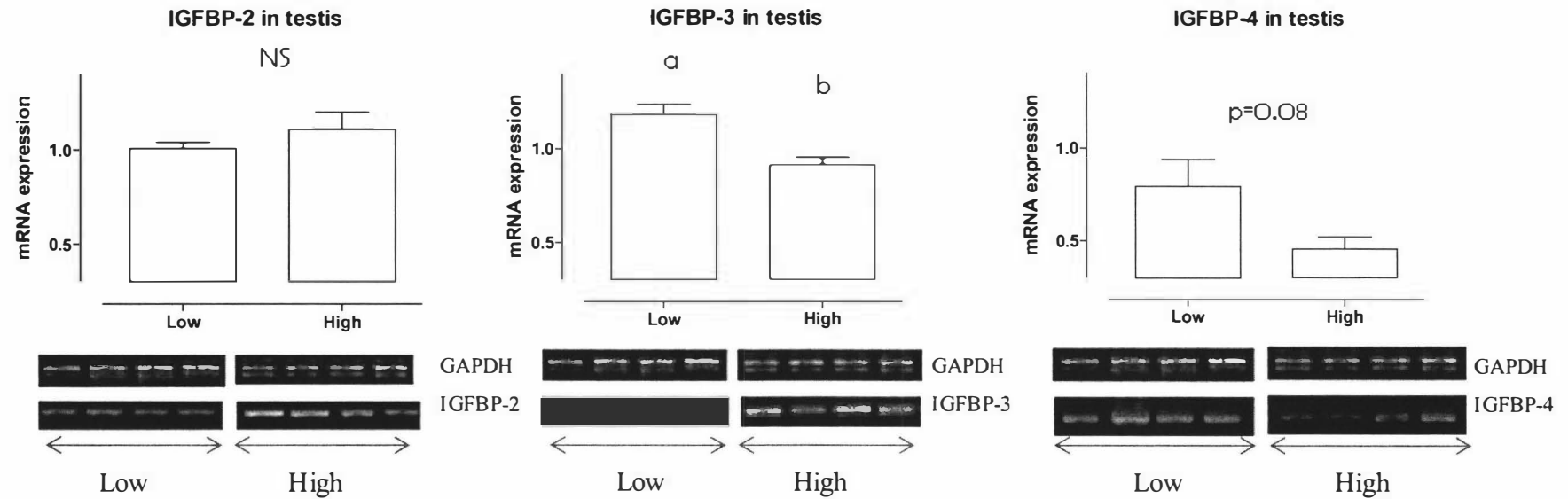


Figure 4.2 Mean IGFBP-2, -3 and -4 mRNA expression in the ram testis. Means labelled with different letters are significantly different from each other ($p < 0.05$).

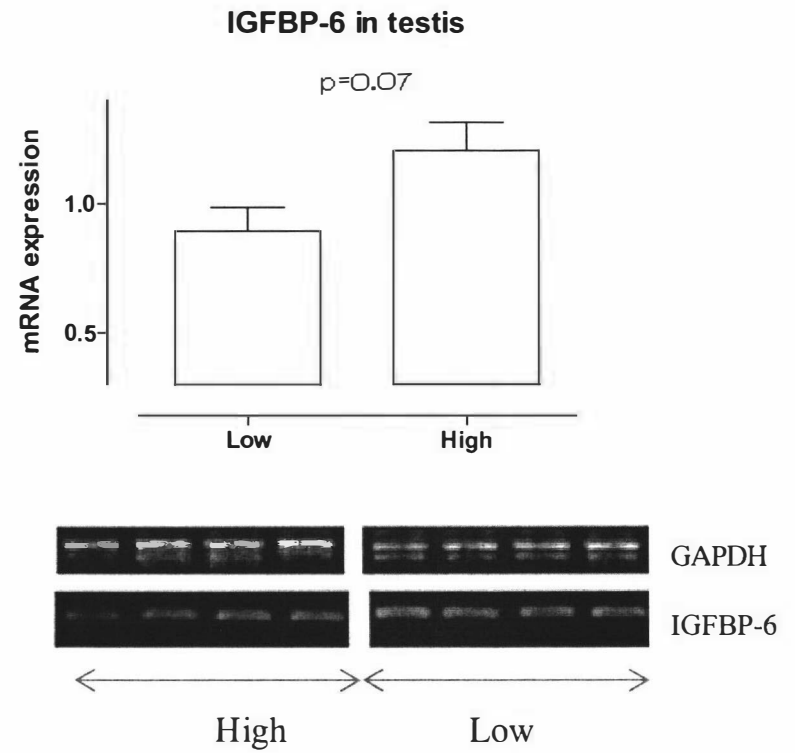
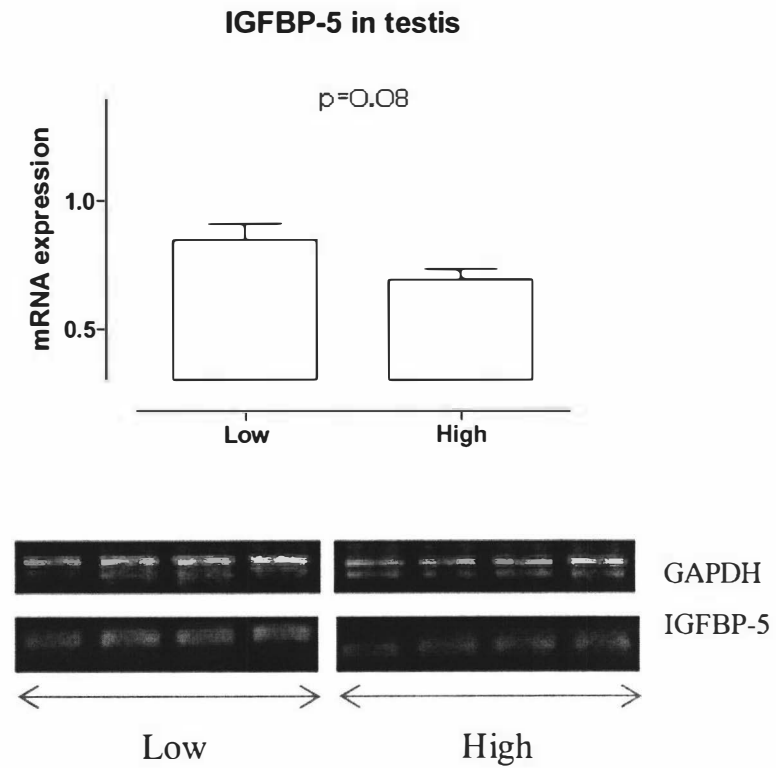


Figure 4.3 Mean IGFBP-5 and -6 mRNA expressions in the ram testis.

4.4 Discussion

This study examined the expression of genes for insulin-like growth factor (IGF)-I, Type I IGF receptor and IGF binding proteins (IGFBPs -1, -2, -3, -4, -5 and -6) between two lines of rams selected for high and low blood serum IGF-I concentration. It was found that genes for all of these, except IGFBP-1, were expressed in the testis. Significant differences between lines of sheep selected for high and low plasma IGF-I concentrations were found in the expression of IGF-I in the liver, Type I IGF receptor in the testis and IGF binding protein-3. Moreover, expressions of mRNA for IGFBP-4, -5 and -6 did tend to differ between IGF-I selection lines, but did not reach statistical significance.

Many studies have demonstrated that the importance of the IGF system in the mammalian testis. Production of IGF-I by rat Sertoli cells has been demonstrated *in vitro* (Cailleau et al., 1990) and its localization in testis demonstrated by *in situ* hybridization in rats and by immunohistochemistry in humans (Vannelli et al., 1988; Hansson et al., 1989). IGF receptors on Leydig cells are regulated by gonadotropins both *in vivo* and *in vitro* (Lin et al., 1995). IGF receptors have been found in Sertoli cells (Borland et al., 1984), Leydig cells (Handelsman et al., 1985), epididymis (Dombrowicz et al., 1992), secondary spermatocytes and spermatids, suggesting a role in the coordination of growth, spermatogenesis and steroidogenesis (Tres et al., 1986; Chandrashekar et al., 2004). In addition, the expression of mRNA for IGF-1R increases when the testis grows rapidly and decreases when the sexual maturation is almost complete (Jones and Clemmons, 1995). IGF-I may be a regulator of Leydig cell function and steroidogenesis in the testis (Lin et al., 1993; Matsui and Takahashi, 2001). Taken together, the concept that the IGF system plays an important role in the development and functions of the male reproductive organs (Machnik and Lechniak, 2000; Sirotkin et al., 2003; Hayashi et al., 2005; Walters et al., 2007). The production of IGF-I in the liver is mainly regulated by GH but, in some activities of the testis, for example in testosterone synthesis by Leydig cells (Lin et al., 1986) and DNA synthesis in spermatogonia (Soder et al., 1992), the IGF system functions independently of GH. Local production of IGF-I by testicular tissues has been confirmed by expression of IGF-I mRNA in

whole testis in rats (Casella et al., 1987) and the present study, IGF-I was expressed in both the liver and as local production in the testis. IGF-I expression in the liver was greater in the high than in the low line, whereas by contrast, the expression of mRNA for IGF-I in testis did not differ between lines. Thus, selection for high and low IGF concentrations at weaning is likely to be on the basis of hepatic IGF synthesis; which is not necessarily related in testicular IGF synthesis, understanding the different concentrations of hepatic and local IGF production in the regulation of testicular activity is of some importance to mediating the effects of selection for IGF upon fertility.

Most of the biological actions of IGF-I are mediated by its high-affinity receptor. The Type I IGF receptor (IGF-1R), which is a receptor with tyrosine kinase activity and which is structurally and functionally related to the insulin receptor, mediates most of the insulin-like actions of both IGF-I and IGF-II. The mitogenic effects of IGF are also regulated by the IGF-1R. The present study found expression of mRNA for IGF-1R in the testis, such that mRNA for the IGF-I receptor showed a higher level of expression in the high line compared with the low line. This was opposite to the trend that was present for the expression of IGF-I in the testis. Different patterns of IGF-I and IGF-1R expressions in different groups of animals, have already been reported in the chick liver (Antich et al., 1995) and in rat tissues (Werner et al., 1989). In consequence, IGF-I mRNA levels increased, whereas IGF-1R mRNA levels decreased in the same tissues. Hence, the present results suggest that selection for high serum IGF-I is associated with greater expression of mRNA for IGF-I in the liver, and the expression of mRNA for IGF-I in testis is associated with changes in the local regulation of IGF-I receptors.

Insulin-like growth factors (IGFs) circulate in the bloodstream bound to specific high affinity IGF binding proteins (IGFBPs). IGFBPs modulate the availability of unbound IGF for interaction with IGF-1R (Wood et al., 2005) and are known to act as major modulators of the IGF system by promoting or inhibiting the biological activity and availability of IGF (Conover, 1992; Hwa et al., 1999). Moreover, IGFBPs have higher affinities for IGFs than do the Type I IGF receptors and IGFs must be released from their binding proteins in order to exert

their mitogenic and metabolic effects. Therefore, IGFBPs act as carrier proteins to transport IGFs to their target tissues, thereby increasing the half-lives of the IGFs by protecting them from proteolysis, and by acting as modulators of IGF availability and activity (Jones and Clemmons, 1995). Growth factors and hormones have been shown to alter IGFBP production by target cells, suggesting that the effects of these factors may be partially mediated by the local production of IGFBPs. In consequence, IGFBPs influence several biological processes, including bone cell proliferation (Mohan et al., 1995), Leydig cell (Matsui and Takahashi, 2001) and Sertoli cell (Rappaport and Smith, 1995) function, can inhibit growth of breast, prostate and lung cancer cells (Oh et al., 1993; Djavan et al., 2001; Sato et al., 2006), and stimulate granulosa cells (Alexiadis et al., 2006) and developing antral follicles (Walters et al., 2007), and testicular somatic-germ cells (Besset et al., 1996; Bardi et al., 1999). In addition, IGFBPs play an important role in brain functioning, especially, in regard to hypothalamic control of reproductive physiology (Gadd et al., 2000; Bienvenu et al., 2004). Numerous *in vitro* and *in vivo* data suggest that IGFBPs may affect cell growth by both IGF-dependent and IGF-independent mechanisms (Mohan et al., 1995; Yi et al., 2001).

IGFBP-1 has been found in the endometrium, placenta, liver and in various cell lines. Other studies reported that mRNA for IGFBP-1 was not expressed in rat testis, as found in the present study, suggesting that IGFBP-1 is probably not involved in the regulation of testicular function. This has also been suggested by other studies (Lin et al., 1993; Zhou and Bondy, 1993).

IGFBP-2 has been shown to either inhibit and or promote IGF actions, depending on cellular context (Feyen et al., 1991; Bourner et al., 1992). High levels of IGFBP-2 mRNA have been found in reproductive tissues, such as Leydig cells, theca interstitial cells and secondary interstitial cells in the ovary (Nakatani et al., 1991; Lin et al., 1993). Another study has demonstrated that granulosa cells, rather than the oocyte, are the major source of proteases capable of cleaving IGFBP-2 in the developing bovine antral follicle (Walters et al., 2007). In addition, IGFBP-2 shows proteolytic activity in follicular fluid from bovine antral follicles and, furthermore, this proteolysis was negatively

correlated with follicular fluid oestradiol and positively correlated with follicular fluid IGFBP-2 concentrations (Spicer, 2004). The effects of hCG and IGF-I on Leydig cell function are negatively regulated by IGFBP-2 (Wang et al., 1994). It has been reported that the level of IGFBP-2 is decreased markedly in rat Leydig cells after hypophysectomy (Lin et al., 1993) and that the expression of IGFBP-2 mRNA *in vitro* is reduced in the presence of 10ng/ml hCG (Lin et al., 1995). However, the addition of recombinant IGF-I had no effect in terms of modulating IGFBP-2 in bovine granulosa cell (Walters et al., 2007). In the present study, IGFBP-2 was expressed in the testis, but the amount of mRNA for IGFBP-2 did not differ between lines, suggesting that selection of IGF concentrations may not influence the local production of IGFBP-2 in testis.

In general, IGFBP-3 is the most abundant of the IGFBPs (Ali et al., 2003). More than 90% of circulating IGF is bound to IGFBP-3. IGFBP-3 is known to impair IGF action and inhibit cell growth, either by blocking free IGF or, possibly, through an IGF-independent mechanism (Oh et al., 1993; Flyvbjerg et al., 1995; Ali et al., 2003). In the rat testis, IGFBP-3 mRNA is mainly expressed in Leydig cells and interstitial cells (Smith et al., 1990; Skalli et al., 1992). Administration of recombinant human non-glycosylated rIGFBP-3 to rats found it inhibited IGF-I stimulated lactate production and that FSH-stimulated lactate was altered by the addition of rIGFBP-3 (Rappaport and Smith, 1995). This study is of significance in as much as it demonstrated that IGFBP-3 inhibits IGF-I action in the Sertoli cells, and that FSH actions on lactate are independent of changes in the autocrine expression of IGFBP-3. Therefore, the potent inhibition of IGFBP-3 by FSH suggests that IGFBP-3 may modulate IGF-I in the rat testis. Conover (1992) reported a mechanism by which IGFBP-3 potentiates IGF-I action in cultured bovine fibroblasts, suggesting that it may involve changes in Type I IGF receptor responsiveness, but not receptor number or affinity. This idea is corroborated by the present study, in which the expression of mRNA for IGFBP-3 was significantly lower in the high line than in the low line. In the other words, high IGF-I in the circulating concentrations are negatively related to IGFBP-3 level in the testis.

The physiological role of IGFBP-4 in the regulation of testis function is not clear. IGFBP-4 mRNA is expressed predominantly in liver, but smaller amounts are also present in Leydig cells and in possible other interstitial cells (Lin et al., 1993). IGFBP-4 appears to exclusively inhibit mitogenic actions of IGF under most experimental conditions, because it does not associate with extracellular matrix components or the cell surface (Bardi et al., 1999). This suggests that it may be a negative regulator of cellular proliferation. In the present study, the expression of mRNA for IGFBP-4 in the low line was greater than in the high line, which is compatible with the idea that high IGF-I concentration may have a negative effect on IGFBP-4 in testis.

Gadd et al. (2000) reported that the high expression of IGFBP-5 mRNA was present in the maternal caruncles and luminal epithelium, with moderate levels in the myometrium in nonpregnant ewes. A similar study revealed that the highest expression for IGFBP-5 was present in uterus and placenta (Hou et al., 2005). These studies suggest a possible relationship between IGFBP-5 and sex steroids in sheep. IGFBP-5 concentrations reach their peak around ovulation, whereas low expression in the endometrial stroma remained constant throughout the oestrous cycle (Hastie and Haresign, 2006a). Their study concluded that IGFBP-5 is expressed in the ovine reproductive tract, with both the concentration and localization differentially regulated during the ovarian cycle and pregnancy. In the present study, the expression of mRNA for IGFBP-5 in the low line was greater than in the high line, again suggesting that high IGF-I concentration may have a negative effect on IGFBP-5 levels in the testis.

Recent studies have indicated that IGFBP-6 mRNA is expressed in follicles of anoestrous sheep (Hastie et al., 2004), as well as in primates (Arraztoa et al., 2002) and mice (Hammond and Wandji, 1999). Furthermore, the IGF-II and IGFBP-6 complex appears to play an important role in the central nervous system, because IGF-II and IGFBP-6 production persists into adulthood and both are normal constituents of cerebrospinal fluid (Roghani et al., 1991). Numerous studies of diverse cell types have shown that IGFBP-6 is involved in the arrest of proliferation, increase in apoptosis and diminished tumorigenic potency (Grellier et al., 1998; Leng et al., 2001). IGFBP-6 binds to IGF-II with

an affinity that is stronger than that to the Type 1 IGF receptor (Roghani et al., 1991). IGFBP-6 has been shown to inhibit proliferation in many cell types, and it is synthesized in the central nervous system (Seurin et al., 2002). However, transgenic mice with strong expression of IGFBP-6 between birth and one month of age had reduced plasma IGF-I concentrations, showed a reduction in body weight, had fewer corpora lutea, had altered ovulation patterns, and showed a reduction of 50% in circulating LH concentrations (Bienvenu et al., 2004). The results indicate that IGFBP-6 may play a role in regard to hypothalamic control, and in reproductive physiology. In the present study, the expression of IGFBP-6 was present in testis; the expression of mRNA for IGFBP-6 was lower in the low line than in the high line. Whether this should be interpreted to mean that high IGF-I in the circulation is positively related to IGFBP-6 levels in the testis is unclear.

In general, IGFBPs control IGF bioactivity by regulating its availability to IGF receptors. In the present study, IGF-I was differently expressed in the liver and testis, and mRNA expressions differed between IGF-I and IGF-1R in the testis, suggesting that IGF-1R not only regulates IGF bioavailability but may also has IGF-independent actions via local production of IGF-I in the testis. The present results suggest that selection for high and low circulating IGF-I is associated with different levels of gene expression for IGF-I, IGF-1R, IGFBP -2, -3, -4, -5, and -6 in the testis, which is in agreement with other studies of the actions of IGF within the reproductive system actions and availability (Hastie et al., 2004; Hayashi et al., 2005; Neuvians et al., 2005; Alexiadis et al., 2006). Also, animals selected for high circulating IGF concentrations had lower expression of testicular IGFBP-3, -4 and -5 and greater expression of testicular IGFBP-6, but did not affect IGFBP-2 expression in the testis. Locally produced IGFBPs may function as autocrine/paracrine agents and may play a significant role in regulating IGF-I actions and availability. However, this study found that IGF-I expression in the liver was significantly higher in the high line, while opposite patterns of testicular mRNA expression for IGF-1R and IGFBP-3 were present, suggesting that the IGF-I in the testis can modulate expression of mRNA for its receptors and binding proteins, and that IGF-1R and IGFBP-3 may be important regulators of IGF-I actions in the testis.

In conclusion, IGF-I, its Type I receptor and IGFBP 2-6 (but not IGFBP-1) are expressed in the testis, with differential expression of IGF-I in the liver, Type I receptor and IGFBP-3 in the testis between the high and low IGF selection lines. Thus, it is like that these affect male reproductive organs, via Sertoli cells-mediated actions on spermatogenesis or via Leydig cells-mediated actions upon steroidogenesis. Moreover, IGF-I may influence IGF-1R and IGFBPs actions in the testis tissue, the ratio of the different IGFBPs to IGF-I may play an important role in actions of IGF-I on reproductive system in males.

Chapter 5: General discussion

This thesis examined the effect of genetic selection of rams for high or low circulating concentrations IGF-I at the time of weaning upon the subsequent activity of the GH/IGF/insulin system and the interrelationship between that system and the hypothalamo-pituitary-testis axis of sexually mature animals. Seasonal differences were found in the percentage of abnormal sperm, scrotal circumference and sperm motility, and in testosterone, IGF-I and insulin concentrations throughout the year, but not in sperm density. Differences between IGF-I selection lines were found in scrotal circumference, IGF-I and insulin, but not in numbers of abnormal sperm, sperm motility scores or sperm density. Testosterone concentrations did not show a simple difference between lines, but, when actual IGF-I concentrations were taken into consideration, exhibited different temporal changes in secretion patterns between lines. Expression of IGF-I, Type I IGF receptor and IGF binding proteins -2, -3, -4, -5 and -6, as determined by RT-PCR analysis of the presence of mRNA, occurred in testis tissue, and there were significant differences between IGF-I selection lines in the expression of IGF-I in the liver, and of Type I IGF receptor and IGFBP -3 in the testis.

The insulin-like growth factors, as endocrine and paracrine/autocrine hormones, are mitogenic peptides that potently stimulate proliferation, differentiation of many diverse cell types, as well as inhibition of cell death. The IGFs play an important role in numerous physiological and pathological processes and are one of the major hormonal systems regulating somatic growth and cellular proliferation in mammals. The IGF system involves IGF-I, IGF-II, IGFBP proteases, IGF receptors (Type I and Type II) and the six IGFBPs. GH initiates a signalling cascade which stimulates regulation of IGF-I and related genes, and systemic growth is promoted by the action of GH on the liver and stimulation of IGF-I production. However, IGFs are also produced by most tissues, with both the circulating and locally produced factors having physiological functions (Bondy et al., 1992; Flyvbjerg et al., 1995). Moreover, local production of IGF-I is influenced by a variety of local and circulating factors, which may or

may not include GH (Dercole et al., 1980; Roberts et al., 1987; Holly, 2004; Frago and Chowen, 2005).

The IGF system also plays an important role in the development and functions of reproductive organs. Many actions of the IGFs have been demonstrated in the female, including the control of growth and reproduction and influences on metabolism, cell division, ovarian folliculogenesis, oogenesis and ovarian secretory activity (Tres et al., 1986; Soder et al., 1992; Schams et al., 1999; Lucy, 2000; Machnik and Lechniak, 2000; Giudice, 2001; Hull and Harvey, 2001; Gong, 2002; Neuvians et al., 2005). Lucy et al (2002) showed that ovarian cells treated with either insulin or IGF-I had an increased response to LH and FSH. It has also been shown that IGF-I plays an important role in amplifying the effect of FSH and LH on rat (Adashi et al., 1991), pig (Hammond et al., 1991) and sheep (Monniaux and Pisselet, 1992), and GH and FSH treatment increase estradiol and IGF-I production by ovine granulosa cells (Eckery et al., 1997). The effects of IGF-I upon the testis could be due to direct actions upon testicular cells or via an influence on hypothalamo-pituitary function (Chandrashekar et al., 2004). There is considerable evidence of a central role of IGF. For example, IGF-I stimulates GnRH release from rat hypothalamic explants *in vitro*, resulting in greater production of FSH and LH (Dickson and Sanford, 2005). Similarly, IGF-I stimulates LH release from rat pituitary cells in culture (Eckery et al., 1997), and the administration of IGF-I via the carotid stimulates LH output in sheep (Adams et al., 1997).

Many studies have demonstrated that the IGF system plays an important role in regulating reproductive function in the testis in mammals (Rappaport and Smith, 1995; LeGac et al., 1996; Drescher et al., 1997; Neuvians et al., 2005). Production of IGF-I by rat Sertoli cells has been demonstrated *in vitro* (Cailleau et al., 1990) and its localization in testis demonstrated by *in situ* hybridization in rats and by immunohistochemistry in humans (Vannelli et al., 1988; Hansson et al., 1989). In the testis, the IGF system can function independently of GH, and influences testosterone synthesis by Leydig cells (Lin et al., 1986) and DNA synthesis in spermatogonia (Soder et al., 1992), suggesting direct effects upon the coordination of growth, spermatogenesis and

steroidogenesis (Tres et al., 1986; Chandrashekar et al., 2004). Furthermore, FSH can stimulate the combination of increased secretion of endogenous IGF-I and decreased IGFBP-3 production in Sertoli cells (Meroni et al., 2004). Interestingly, there may be two-way regulation of testosterone and IGF in the testis, since concentrations of IGF-I are increased in response to the testosterone treatment (Arnold et al., 1996). In the present study, scrotal circumference, IGF-I and insulin showed significant differences between IGF-I selection lines, but the percentage of abnormal sperm, sperm motility scores, the density of sperm and testosterone concentrations did not differ.

Scrotal circumference is a good indicator of sperm output, because it is related to semen quality parameters such as numbers of sperm, percentage of motile sperm and percentage of morphologically normal sperm (Brito et al., 2002; Martins et al., 2003; Kafi et al., 2004; Parkinson, 2004). A decrease in scrotal circumference has been shown to be associated with negative effects upon semen production, including an increase in the occurrence of morphologically abnormal sperm (Mickelsen et al., 1982) and low semen quality or fertility (Mickelsen et al., 1982; Nowakowski and Cwikla, 1994; Al-Ghalban et al., 2004; Kafi et al., 2004). Scrotal circumference is highly correlated to testicular weight and therefore sperm production have been show in rams (Al-Ghalban et al., 2004; Kafi et al., 2004). Previous reports showed that scrotal circumference varies according to age and body weight (Braun et al., 1980). Furthermore, it has been shown that season and breed had more effect on scrotal circumference than did body weight (Mickelsen et al., 1982). It is therefore likely that the greater scrotal circumference of high- than low-line rams in the present study is indicative of higher fertility in the former group of animals, and that, whist there were differences in body weight between the two lines, these were unlikely to have been the main determinant of differences in reproductive activity between lines.

Glander et al. (1996) reported that, in humans, there is a correlation between seminal IGF-I concentrations and the percentage of morphologically normal sperm cells, and that differences in IGF-I concentrations are likely to be of physiological or pathological significance in the determination of the conception

rates achieved by oligospermic men (Suttiyotin et al., 1992; Singh et al., 1995; El-Darawany, 1999; Naqvi et al., 2002; O'Brien and Robeck, 2006). Whether this also applies to animals is less clear, since the percentage of normal sperm cells did not differ between high and low IGF-I line in bulls (Yilmaz et al., 1999). Breier et al. (1996) reported that increased IGF-I concentrations in seminal plasma were significantly correlated with increased sperm motility without changes in morphology of sperm cells in rats during GH treatment. Yilmaz et al. (1999) reported that there was no significant difference in semen concentration between bulls in selected for high and low serum insulin-like growth factor-I (IGF-I). However, in the present study, the percentage of abnormal sperm, sperm motility scores and density of sperm did not differ between the selection lines, suggesting that the large differences in serum IGF-I concentrations between the lines did not affect sperm morphology, sperm motility and density of sperm in rams. On the other hand, single collections of semen, especially if collected by electroejaculation, are not highly correlated with fertility: hence, the difference in scrotal circumference between lines is likely to be more representative of a biological effect of selection for IGF upon reproductive performance than is a lack of a difference in semen-assessment characteristics.

The sheep is a seasonal breeder and changes in daily photoperiod play an important role in the regulation of reproductive activity in sheep. Sheep from temperate latitudes are well known as animals with marked seasonality of breeding activity that is primarily controlled by annual photoperiodic changes. Rams exhibit seasonal fluctuations in sexual behaviour, hormonal activity, spermatogenesis and testis volume and weight during the breeding season (Ortavant et al., 1988). Although seasonal modulation is not an all or-nothing condition, increased quality and quantity of sperm output and scrotal circumference (Mickelsen et al., 1982; Brito et al., 2002; Martins et al., 2003), and increased concentrations of testosterone, FSH and LH (Dickson and Sanford, 2005; Barkawi et al., 2006) occur during breeding season. Changes in seasonal photoperiod and IGF-I concentration throughout the year can alter testis function (Barkawi et al., 2006), and therefore semen production and male reproductive

behaviour through the alteration of endocrine and physiological systems (Lincoln et al., 2001).

The activation of the gonadal axis leads to the onset of reproductive function in sheep (Hammond et al., 1991). Reproductive activity during the breeding season is mainly controlled via the release of FSH and LH which is, in turn, regulated by photoperiod, since the pattern of melatonin indirectly determines the pulsatile secretion of GnRH from the hypothalamus (Lincoln et al., 2001). FSH probably has the central role in regulating seasonal changes in testicular function, whereas LH acts primarily by stimulating testosterone secretion (Dickson and Sanford, 2005). Each hormone plays a unique role in the differentiation and proliferation of spermatogonia, after which testosterone stimulates the formation and differentiation of spermatocytes (Ghosh et al., 1992; Kilgour et al., 1993).

Sexual behaviour and semen quality are the main factors that regulate male reproductive performance during the year in rams. These factors are influenced by breed, geographical location, season (Nett and Niswender, 1982; Ortavant et al., 1988; Gundogan et al., 2003; Al-Ghalban et al., 2004), testicular size (Nowakowski and Cwikla, 1994a; Barkawi et al., 2006) and annual changes in gonadotropins secretion (Thiery et al., 2002; Filicori et al., 2002; Dickson and Sanford, 2005; Barkawi et al., 2006). Many studies have reported that the percentage of morphologically abnormal sperm is related to reproductive performance (Suttiyotin et al., 1992; Singh et al., 1995; El-Darawany, 1999; Naqvi et al., 2002; O'Brien and Robeck, 2006), but the literature on the relationship between semen quality and fertility is contradictory. In the present study, seasonal differences were found in the percentage of abnormal sperm. However, it is surprising that the percentage of total abnormal sperm (including in abnormalities in the head, midpiece, tail and cytoplasmic droplets abnormal sperm) during the breeding season was greater than during the non-breeding season. This is probably a reflection of the relatively low numbers of abnormal sperm that were present, as well as of the technical limitations of electroejaculation as a means of accurately assessing small differences in sperm morphology.

On the other hand, Yilmaz et al. (1999) reported no significant difference in sperm density between months and between high and low IGF-I selection lines in bulls. A similar result was observed in the present study with no seasonal differences found in sperm density. However, motility scores during the breeding season were significantly higher than during the non-breeding season. This pattern of motility scores corresponded with the pattern of scrotal circumference. As the scrotal circumference decreased, there was an increase in the motility score, reflecting the view that increased in scrotal circumference is related to improve sperm motility. Moreover, it has been shown that testosterone is the main hormone in the regulation of testicular size (Dickson and Sanford, 2005), and, as expected, testosterone concentrations reached peak values during the breeding season.

In mammals, the generally accepted model of the hypothalamic regulation of GH release is that GH is primarily controlled by two factors; GH-releasing factor (GRF) which stimulates, and somatostatin (SRIF) which inhibits GH release (Spiterigrech and Nieschlag, 1992; Jones and Clemmons, 1995; Mohan et al., 1995; Hwa et al., 1999; Chandrashekar et al., 2004). Multiple tissues are affected by GH, but coordinated events in the liver and adipose tissue may be important in responses to GH. The interaction of the hypothalamic factors is such that SRIF regulates baseline GH secretion, whereas GRF regulates GH pulses (Giustina et al., 1995). GH regulates its own secretion through a short loop negative feedback mechanism mediated by hypothalamic GRF and somatostatin (Frohman et al., 1992). The actions of GH are mediated by binding to the GH receptor on the cell membrane of target tissues (Clark, 1997). GH receptors in the liver, including other tissues, GH stimulates the production of IGFs and IGF-BPs (Chandrashekar et al., 2004). GH receptor are expressed at many sites, including brain regions (Nyberg and Burman, 1996), and the testis, including Leydig and Sertoli cells (Lobie et al., 1990), suggesting that the GH/IGF axis can directly affect reproductive function in the male. However, the effects of GH on growth are mostly mediated by the production of IGF-I at local tissue sites (Salomon et al., 1989). IGF-II produced by the liver acts as an endocrine hormone that regulates GH release through negative feedback, and

IGF produced locally in various GH target organs acts as paracrine/autocrine regulator of cell function (Chandrashekar et al., 2004). Its actions are modulated by 6 different IGF binding proteins (IGFBPs) which control IGF bioactivity by regulating its availability.

Low IGF-I concentrations stimulate GRF release and promote pituitary GH gene transcription and secretion through a long loop negative feedback (Plotsky and Vale, 1985; Sato and Frohman, 1993; Frago and Chowen, 2005). However, Laron (1999) reported that the overexpression of GH, resulting in chronically elevated GH and IGF-I concentrations, can interfere with many aspects of reproduction. Katoh et al. (2004) showed that the secretion of GH was significantly suppressed by the presence of IGF-I. In the present study, IGF-I concentrations in the high line reached a peak during the non-breeding season and decreased markedly after the hGRF challenge in the high line. In contrast, IGF-I concentrations were lower during the breeding season and increased slightly after hGRF challenge in the high line. These results suggested that high concentrations of IGF-I may have negative effects on GRF release through a negative feedback control on GH secretion, although GRF stimulated IGF-I synthesis.

Regulation of the reproductive organs by that insulin and IGF-I may also be dependent upon energy status, since blood concentrations of IGF-I and insulin play important roles in the control of energy balance. Cows in negative energy balance and cows selected for milk production have lower IGF-I and insulin concentrations (Lucy, 2006). There is evidence of direct effects of energy status upon GnRH and LH secretion, but these may also be affected by IGF and insulin, through their responsiveness to nutrition and energy balance. In the present study, IGF-I and insulin concentrations were higher in the high IGF-I line than in the low line throughout the year. Thus, high IGF-I in the circulation may differentially affect scrotal circumference throughout the year, via effects upon IGF-I and insulin concentrations.

Blood concentrations of IGF-I and insulin are related to positive energy balance. Growth hormone acts through a cell surface receptor (GH receptor) which is

found in most tissues and especially in the liver (Lucy et al., 2001). Bereket et al. (1999) reported the stimulatory effect of insulin on liver GH receptor in the human and other species, suggesting that the coincident increase in liver GH receptor numbers depends on insulin. In a recent study, Lucy (2006) reported that low insulin concentrations lead to low GH receptor expression, decreased blood IGF-I, greater blood GH concentrations, and insulin resistance. Growth hormone signals in liver and adipose tissue through its receptor and inhibits the activity of the insulin receptor (Bluher et al., 2005). The present study showed similar profiles of IGF-I and insulin concentrations, with seasonal changes in plasma insulin concentrations in both lines similar to changes in IGF-I concentrations in the high line. Higher insulin concentrations may lead to increases in liver GH receptor numbers, with a consequent increase in liver IGF-I synthesis and blood IGF-I concentrations. Raised concentration of IGF-I decreases GH secretion through negative feedback. In the present study, insulin concentrations reached a peak during the non-breeding season and were lowest during the breeding season. Insulin concentrations were increased after hGRF challenges. These results suggest that insulin concentrations may vary seasonally and that the administration of hGRF can stimulate insulin concentrations throughout the year.

The production of IGF-I is mainly regulated by GH secretion in liver but, in some activities of the testis, the IGF system functions independently of GH. In the present study, IGF-I expression was found in both liver and testis. IGF-I expression in the liver was greater in the high than in the low line. In contrast, the expression of mRNA for IGF-I in the testis was slightly lower in the high than in the low line.

The biological actions of IGF-I are mainly mediated by the IGF Type I receptor (IGF-1R). IGF-1R is a receptor with tyrosine kinase activity which mediates the mitogenic effects of IGF-I. IGF receptors have been found in Sertoli cells (Borland et al., 1984), Leydig cells (Handelsman et al., 1985), epididymis (Dombrowicz et al., 1992), secondary spermatocytes and spermatids, suggesting a role for IGF-I in the coordination of testicular growth, spermatogenesis and steroidogenesis (Tres et al., 1986; Chandrashekar et al., 2004). The present

study found expression of mRNA for IGF-1R in the testis. mRNA for the IGF-I receptor showed higher expression in the testis in the high line compared with the low line. It is interesting that different patterns of mRNA expression were found for IGF-I and IGF-1R in the testis because the free or unbound IGF fraction mediates its response through specific binding to membrane-bound IGF receptors (Bondy et al., 1992; Antich et al., 1995). These results suggested that selection for high serum IGF-I is associated with the expression of mRNA for IGF-I in the liver, and IGF-I expression in testis is associated with changes in the local regulation of Type I IGF receptors.

Type I IGF receptors process many of the differentiating and mitotic effects of this hormone. IGFs circulate in the bloodstream bound to specific high affinity IGFBPs. These binding proteins modulate the availability of unbound IGF for interaction with IGF-1R (Wood et al., 2005). The IGFBPs are known to act as the major modulators of the IGF system by either promoting or inhibiting the biological activity and availability of IGF (Conover, 1992; Hwa et al., 1999). The various IGFBPs all have higher affinities for IGFs than do the Type I IGF receptors. Therefore, IGFBPs act as carrier proteins to transport IGFs to their target tissues, thereby increasing the half-lives of the IGFs by protecting them from proteolysis, and acting as modulators of IGF availability and activity (Jones and Clemmons, 1995). In the present study, expressions of mRNA for IGF-I, Type I IGF receptor (IGF-1R), IGF binding protein -2, -3, -4, -5 and -6 were present in ram testis, but IGFBP-1 mRNA was not expressed. Significant differences between lines were found in the expression of IGF-I in liver, Type I IGF receptor in testis and IGFBP-3. Also, expressions for mRNA for IGFBP-4, -5 and -6 differed between IGF-I selection lines, but did not reach the level of significance.

IGFBPs play an important role in the control of IGF bioactivity by regulating its availability to IGF receptors. It has been shown that IGFBP-1 is not expressed in the testis (Zhou and Bondy, 1993; Matsui and Takahashi, 2001; Neuvians et al., 2005), suggesting that IGFBP-1 is probably not involved in the regulation of testicular function. IGFBP-2 shows proteolytic activity, which is negatively correlated with follicular fluid oestradiol and positively correlated with follicular

fluid IGFBP-2 concentrations (Spicer, 2004), and the effects of hCG and IGF-I on Leydig cell function are negatively regulated by IGFBP-2 (Wang et al., 1994). Many studies have demonstrated that IGFBP-3, which is the most abundant IGFBP, inhibits IGF-I action in the Sertoli cells (Rappaport and Smith, 1995; Besset et al., 1996; Matsui and Takahashi, 2001). IGFBP-4 appears to exclusively inhibit mitogenic actions of IGF (Bardi et al., 1999). IGFBP-5 mRNA is expressed in the ovine reproductive tract, with both the concentration and localization differentially regulated during the ovarian cycle and pregnancy (Hastie and Haresign, 2006a). IGFBP-6 has been shown to inhibit proliferation in many cell types, and it is synthesized in the central nervous system (Seurin et al., 2002). In the present study, the expression of mRNA for IGFBP-4 and -5 in the low line was greater than in the high line, suggesting that high IGF-I concentration may have a negative effect on IGFBP-4 and -5 in the testis. On the other hand, the expression of mRNA for IGFBP-6 was lower in the low line than in the high line, suggesting that high IGF-I in the circulation is positively related to IGFBP-6 level in the testis.

In the present study, IGF-I expressions in the liver and testis differed which consisted with the local production of IGF-I by testicular tissues functioning independently of GH. mRNA expressions also differed between IGF-I and IGF-1R in the testis, suggesting that the IGF-1R not only regulate IGF bioavailability but may also have IGF-independent actions in local production of testis IGF-I. The present results showed that IGF-I concentrations may modulate gene expression of IGF-I, IGF-1R, IGFBP -2, -3, -4, -5, and -6 in reproductive organs, in agreement with other studies (Hastie et al., 2004; Hayashi et al., 2005; Neuvians et al., 2005; Alexiadis et al., 2006).

High IGF-I in the circulation is negatively related to IGFBP-3, -4 and -5 levels and positively related to IGFBP-6 in testis, and not related to expression of IGFBP-2 in testis. Locally produced IGFBPs may functions as autocrine/paracrine agents and may play an important role in regulating IGF-I synthesis. However, this study found higher IGF-I expression in the liver, and lower expression of IGF-1R and IGFBP-3 in the testis in the high line, suggesting that the action of IGF-I in testis can modulate expressions of mRNA

for its receptors and binding proteins, and IGF-1R and IGFBP-3 may be important regulators of mRNA expression for IGF-I in the testis.

To sum up, the present study concluded:

- 1) IGF-I affects scrotal circumference, but not semen quality.
- 2) IGF-I concentrations were related to testosterone concentrations, with a significant difference between lines when IGF concentrations were taken into consideration. Testosterone concentrations were unrelated to insulin.
- 3) IGF-I gene expression in the liver was higher in the high, compared to the low line.
- 4) It is likely that sensitivity to IGF-I negative feedback was higher in the high line than in the low line.
- 5) Gene expression for IGFBP3 and Type I IGF receptor were higher in the low line than in the high line.

Taken together, the results suggest that IGF-I affects steroidogenesis and spermatogenesis, possibly directly (as there are IGF-1R and IGFbps in the testis) and probably via potentiating the effects of gonadotropins. In addition, selection for plasma IGF-I does not imply selection for local IGF-I production and, indeed, some of the current evidence suggests that there may be opposing effects of local and systemic IGF-I activity. The differences in negative feedback highlight this point. Moreover, there are strain-related differences in IGFbps, suggesting there is a local regulation of IGF-I availability to the testis, thereby damping the effect of elevated systemic IGF-I concentrations.

Finally, it suggests that the infertility that is seen in female low line sheep cannot be simply explained in terms of direct responses to gonadotropins. This is as one would expect, though, since there are many layers of regulation of IGF-I availability and activity in the ovary, and are more likely to require explanation through regulation of mitosis in the granulosa than of simple steroid responses to gonadotropin. Hence, differences in testosterone, whilst giving an important insight into the possible effects of IGF-I upon steroidogenesis in the male (which has a more simple regulation than in the complex system represented by the

ovary), is probably of less biological significance than is the effect of line upon scrotal circumference. It has been shown that Sertoli cell function and mitosis is critically dependent upon IGF-I concentrations presumably also in fetal and pre-pubertal life when Sertoli cell populations are being established. If so, increased scrotal circumference (=increased volume of seminiferous tubules = increased number of Sertoli cells) is an exactly analogous effect in the male to the mitogenic effects of IGF-I upon the granulosa of the female.

It is suggested that gonadotropin-stimulated steroidogenesis is affected by IGF I status, but differences in IGF-I status between lines are modulated by the differences between lines in IGF-1R, IGF-BPs and negative feedback. The general effects of IGF-I as a mitogen are reflected in the differences in testis size, although this was not reflected in semen quality, because of technical limitations of methodology.

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