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PUBERTAL DEVELOPMENT IN THE MERINO RAM LAMBS AND IMMUNIZATION AGAINST OESTROGENS.

by

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A thesis submitted to the University of Adelaide
in fulfilment of the requirements for the
degree of Doctor of Philosophy

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This thesis is dedicated to the memory of my mother,

Edith Pelletier-Auclair

(1925-1990)

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DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where the due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

10 May 1993

SUMMARY

Several lines of evidence now support the hypothesis that oestrogens regulate many processes of the male reproductive system under physiological conditions.

This thesis presents investigations on pubertal development in South Australian Merino ram lambs and examines the effect of active and passive immunizations against oestradiol-17ß or oestrone on testicular maturation.

All ram lambs were weaned two weeks before the start of the experiment. They were kept indoors, in large pens, under constant lighting (12L:12D) and were fed with commercial sheep pellets and lucerne hay. Each experiment on pubertal development started at 10 or 14 weeks of age and lasted sixteen weeks.

A progressive increase in body weight and testicular volume occurred in all ram lambs studied. Mean LH level tended to decrease as the animals pass through puberty while no distinctive variations in FSH and PRL secretions were noticed. Pituitary responsivess to a GnRH challenge decreased between 14 and 30 weeks of age and probably reflected increasing feedback by gonadal steroids as the steroidogenic activity of the testis increased.

Indeed, mean plasma testosterone level as well as testosterone secretion following a hCG challenge increased markedly as the animals matured. Total testicular blood plasma flow (TTBPF) when expressed per unit weight of testis (µl/g/min) was shown to decrease as the testis grows, although total blood flow per testis (ml/min) is increasing. Subcutaneous scrotal temperature seemed to decrease as testicular size increases. Most of the ram lambs had achieved puberty between 22 and 26 weeks of age as confirmed by the presence of spermatozoa in the seminiferous tubules.

Early active immunization against circulating oestradiol-17ß led to an important increase in gonadotropin concentrations (LH and FSH) and tended to improve the rate of testicular growth until 27 weeks of age, however, testicular volume and weight were comparable. between control and E_2-immunized ram lambs at 30 weeks of age. Detrimental effects have been observed in some E_2-immunized ram lambs. For instance, we have observed a steep decline in testicular size towards the end of the experiment, presence of large vacuoles within the seminiferous

epithelium and, in one lamb, nearly complete absence of germ cells at 30 weeks of age. TTBPF per testis (ml/min) or per unit weight of testis (µl/g/min)] was more elevated in control than in E_2-immunized lambs. The steroidogenic function of the testis was remarkably enhanced in the E_2-immunized lambs as reflected by the high plasma testosterone concentrations, however, body weights were not improved in these lambs (no anabolic effect).

Testicular biopsies have been taken at 22 and 26 weeks of age in some of these ram lambs. This procedure did not seem to affect significantly subsequent testicular development, however, testosterone secretion measured at the end of the experiment (at 30 weeks of age) was significantly reduced in these ram lambs.

In other experiments, some well-characterized antibodies directed against oestradiol-17ß or oestrone (purified IgG or complete antiserum, showing high specificity and high affinity *in vitro*) have been injected to intact ram lambs during pubertal development (10 or 14 week-duration) or to oestradiol-implanted castrated ram lambs for a short-term period (1 or 2 weeks). Unfortunately, no difference was found between the control and the passively immunized intact ram lambs, and only a slight reduction of the oestradiol effect on gonadotropin secretion have been observed in the wethers passively immunized against oestradiol. This lack of effect may have been due to an insufficient amount of antibodies injected into the animals (low titre) and/or incomplete neutralization of the hormone (e.g. relatively low affinity *in vivo*).

In conjunction with this work on puberty, this thesis includes a one year duration experiment using adult Merino rams actively immunized against testosterone. We have demonstrated that the persistent increment in gonadotropin and testosterone secretions observed in T-immunized rams are not sufficient to maintain improved spermatogenic function throughout the year. Furthermore, we have shown that this treatment could lead to a reduction in epididymis weight after twelve months of immunization as well as a reduction in the number of mount culminating in ejaculation performed during a ten-minutes libido trial done on various occasions during the experiment.

PUBLICATIONS

Aspects of the work presented in this thesis have been reported elsewhere:

Abstracts

Auclair, D., Sowerbutts, S.F. and Setchell, B.P. (1993). Active immunization against oestradiol-17ß in developing ram lambs can have a marked effects on the structures and functions of the testis. Proc. Aust. Soc. Reprod. Biol. 25: abstract (submitted), Aug. 23-25 1993, University of Otago, N.Z.

Auclair, D., Sowerbutts, S.F. and Setchell, B.P. (1992). Active immunisation against testosterone in adult Merino rams: A one year-duration study. Proc. Aust. Soc. Reprod. Biol. 24: abstract 83, Sept. 28-30, 1992, University of Adelaide, S.A.

Auclair, D., Sowerbutts, S.F. and Setchell, B.P. (1991). Total testicular blood flow decrease during pubertal development in Merino lambs. Proc. of the 11th North American Testis Workshop, abstract 85, April 24-27, 1991, Montréal, Québec.

Auclair, D., Sowerbutts, S.F. and Setchell, B.P. (1990). Passive immunisation against oestradiol-17ß; its effects on pubertal development in the ram lambs. Proc. Aust. Soc. Reprod. Biol. 22: abstract 8, Sept. 24-26, 1990, University of Western Australia, W.A.

Auclair, D. and Setchell, B.P. (1989). Effects of active immunisation against oestradiol-17ß on testicular growth and testosterone secretion in prepubertal ram lambs. Proc. Aust. Soc. Reprod. Biol. 21: abstract 8, Sept. 25-27, 1989, Monash University, Victoria, Australia.

Paper

Setchell, B.P., Auclair, D., Maddocks, S., Mieusset, R. (1991). Perméabilité vasculaire et débit sanguin testiculaire. Contraception, Fertilité, Sexualité, 19, 725-732.

L. 9: One must keep in mind that various explanations could be given in relation to the primary event necessary for the initiation of puberty depending of which species is studied. For example, the validity of the hypothesis involving a steroid-dependent mechanism has been questioned in the primate in light of increase in gonadotropin secretion in gonadectomized males that have not been given steroid replacement (Plant, 1988). Thus, the exact 'signal' and 'mechanism' for the initiation of puberty may well differ for different species.

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GENERAL INTRODUCTION

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A definition for puberty in males is the period of accelerated reproductive development that culminates in potential fertility (Bronson and Rissman, 1986; Adams and Steiner, 1988). As for any other developmental process, sexual maturation is a genetically programmed and yet environmentally susceptible process. Breed, nutrition and photoperiod are among the best known factors influencing reproductive maturation and timing of the onset of fertility in sheep (Foster et al., 1986; Colas et al., 1987).

An increase in the activity of the GnRH pulse generator is believed to be the primary event responsible for the initiation of puberty and seems to be governed either by steroid-dependent and/or steroid-independent mechanisms (Adams and Steiner, 1988). In the male sheep, the greater frequency of GnRH release is translated into increased pulsatile release of LH and FSH (Crim and Geschwind, 1972b; Foster et al., 1978; Pelletier et al., 1981; Courot and Kilgour, 1984; Yarney and Sanford, 1989). Each of these hormones influence the growth and maturation of the testis by enhancing testicular steroidogenesis and gametogenesis (Courot and Kilgour, 1984).

The increased output of testosterone produces the physical alterations that are typically associated with puberty (Mainwaring et al., 1988). Furthermore, testosterone acts as negative-feedback agent on the hypothalamic-pituitary axis to attenuate gonadotropin secretion (Schanbacher, 1980a, b; 1984b). The effects of testosterone are likely to be partly mediated by its conversion into 5α-dihydrotestosterone and 17β-oestradiol (Bardin et al., 1988; Coffey, 1988). In developing ram lambs, a gradual decrease in responsiveness to the inhibitory actions of gonadal steroids, resulting in increased tonic gonadotropin secretion, seems sufficient to initiate reproductive activity (Olster and Foster 1986; 1988).

Testicular growth is mainly attributable to an increased seminiferous tubule volume resulting from a thickening of the tubular lining (proliferation of germ cells), the formation of a lumen, and the differentiation and growth of Sertoli and Leydig cells (Courot and Kilgour, 1984). Blood flow and vascular permeability in the testis are important aspects of testicular physiology

because they are crucial for the transport of nutrients and secretory products to and from the testis (Setchell and Brooks, 1988). The vascular organization of the testis is also very important to maintain a "special" homeothermia in this organ. In fact, in the ram as in most eutherians, spermatogenesis will not proceed to completion unless the temperature of the testis is a few degrees lower than that of all other internal organs of the body (Setchell, 1978).

A considerable body of evidence now supports the hypothesis that oestrogens regulate many processes of the male reproductive system under physiological conditions. Various tissues are able to aromatize androgens into oestrogens including the hypothalamus (Naftolin et al., 1975), the Sertoli cells (Bardin et al., 1988) and the Leydig cells (Dorrington et al., 1978; Moger, 1980; van der Molen, 1981; Tsai-Morris et al., 1985a, b).

In developing male lambs and in adult rams, picomolar concentrations of oestrogens have been measured in the jugular and in testicular venous plasma (Schanbacher and Ford, 1976; Sanford et al., 1982b; Olster and Foster, 1986; Watts et al , 1989; Pope et al.,1990; Sanford and Robaire, 1990). Besides, oestradiol receptors have been found within the hypothalamic-pituitary axis in rams (Wise et al., 1975; Thieulant and Pelletier 1979; Pelletier and Caraty 1981; Glass et al., 1984).

In rats, high amounts of oestradiol receptors are also found in purified Leydig cells but not in germinal cells (Brinkmann et al., 1972; Van Beurden-Lamers et al., 1974; Mulder et al., 1974; de Boer et al., 1976) and could possibly be produced by some Sertoli cells (Bardin, 1988). In that species, some observations indicate that oestradiol could have a direct inhibitory effect on testicular steroidogenic function (Dorrington et al., 1978; Moger, 1980; van der Molen, 1981).

Immunization against steroid can be used to demonstrate specific hormone dependency of the reproductive system since information accumulated thus far indicates that the binding of gonadal steroid (such as oestradiol-17ß, oestrone or testosterone) to high affinity homologous antibodies can reduce the amount of hormone available to its receptors within the hypothalamic-pituitary-testicular axis and within other target tissues (Nieschlag and Wickings, 1978; Haynes and Southee, 1984).

In adult rams, immunoneutralization of oestradiol-17ß can markedly increase gonadotropin and testosterone secretion (Sanford, 1985; 1987a, b; Schanbacher; 1979; 1984a; Schanbacher et al., 1987; Monet-Kuntz et al., 1988). The results obtained in ram lambs (Land et al., 1981) immunized against oestradiol-17ß or oestrone also indicate that these hormones are likely to be involved in some mechanisms regulating the hypothalamic-pituitary-testicular axis during sexual maturation. Indeed, the rate of growth of the testis was shown to increase in Merino ram lambs passively immunized against oestradiol-17ß and/or oestrone from 14 to 26 weeks of age (Land et al., 1981), however, this effect was not observed in younger crossbred lambs passively immunized against oestradiol-17ß between 2 and 16 weeks of age (Jenkins et al., 1986). Testicular weight was also increased following six or twelve months of active immunization against oestradiol-17ß in adult rams (Schanbacher et al., 1987; Monet-Kuntz et al., 1988). Thompson and Honey (1984) also reported that active immunization against oestrone in prepubertal stallion has led to an increase in testicular weight and daily sperm production (DSP) at 27 months of age (DSP/g testis and DSP/stallion). However, active immunization against oestr , in young bulls (D'Occhio et al., 1987) and in young boars (Wise et al. 1991), did not lead to an improvement of the spermatogenic function.
Immunity against testosterone seems to confer some reproductive advantages (e.g. increase testicular growth and sperm production) in the young bulls (Walker et al., 1984; D'Occhio et al., 1987). However, in ram lambs actively immunized against testosterone, decreased anabolic effect with no improvement in testicular weight have been reported (Schanbacher, 1982). Immunity against testosterone appears not to diminish sexual behaviour in young rams (Haynes and Southee, 1984) and in young boars (Thompson et al., 1985).

The investigations on pubertal development presented in this thesis involve, in most cases, early weaned South Australian Merino lambs aged between 10 and 30 weeks of age, reared under similar well defined experimental conditions (restriction in large pens in a room with controlled light "12L:12D", hand-feeding with commercial sheep pellets and lucerne chaff).

The whole project was designed to obtain relevant information in the following areas:

(1) the endocrinological events occurring, during the period of transition into puberty, within the hypothalamic-pituitary-testicular axis such as changes in LH, FSH, prolactin and testosterone secretion, change in pituitary responsiveness to a single GnRH injection, change in testicular responsiveness to a single hCG injection.

(2) the major maturational changes occurring at the testicular level such as changes in testicular size, changes in histological appearance of testicular tissues, changes in total testicular blood flow, changes in subcutaneous scrotal temperature.

(3) the effects of active immunization against oestradiol-17ß on most of the parameters mentioned above (see 1 and 2).

(4) the effects of passive immunization against oestradiol-17ß on most of the parameters mentioned above (see 1 and 2).

(5) the effects of passive immunization against oestrone on most of the parameters mentioned above (see 1 and 2).

Furthermore, using 30 to 34 weeks old South Australian Merino wethers implanted with a Silactic (polydimethylsiloxane) capsule packed with crystalline oestradiol-17ß or implanted with an empty capsule, we have also obtained some information on:

(6) the effects of active immunization against oestradiol-17ß on LH and FSH secretion.

(7) the effects of passive immunization against oestradiol-17ß on LH and FSH secretion.

Finally, in conjunction with the present work on pubertal development and immunization against oestrogens in ram lambs, we have also carried out an experiment of one year duration with postpubertal Merino rams actively immunized against testosterone since no long-term study using this specific approach has been yet reported in adult rams.

In this additional experiment, our main objective was to gain more information on:

(8) the endocrinological events occurring, over one year, within the hypothalamic-pituitary-testicular axis such as changes in LH, FSH, prolactin and testosterone secretion, change in pituitary responsiveness to a single GnRH injection, change in testicular responsiveness to a single hCG injection.

(9) the effects on the spermatogenic function including possible changes in testicular size, in sperm production, in histological appearance of the testes, in total testicular blood flow.

(10) the effects on sexual behaviour tested in well defined libido trials repeated on various occasions during the study.

The different studies presented in this thesis were also designed to provide answers to these specific interrogations:

(1) if the experimental conditions adopted for the prepubertal ram lambs will allow a satisfactory rate of maturation and achievement of puberty by 30 weeks of age.

(2) if testicular blood plasma flow will increase as testis size increases.

(3) if the subcutaneous scrotal temperature will decrease as testis size increases.

(4) if the immunoneutralization of circulating oestrogens (oestradiol-17ß or oestrone) in prepubertal Merino ram lambs will lead to an increase in gonadotropin secretion along with concomitant activation of the steroidogenic and spermatogenic functions of the testes and consequently accelerate testicular maturation.

(5) if the purified immunoglobulin preparation (purified IgG) is as effective as the complete antiserum for the neutralization of oestradiol-17ß using the passive immunization approach.

(6) if passive and active immunization approaches will lead to comparable results in developing ram lambs immunized against oestradiol-17ß.

(7) if passive and active immunization approaches will lead to comparable results in wethers supplemented with oestradiol-17ß and immunized against oestradiol-17ß.

(8) if testicular biopsies taken at 22 and 26 weeks of age will affect subsequent testicular growth.

(9) if immunoneutralization of circulating testosterone in the adult Merino rams will lead to an increase in gonadotropin secretion, increased stimulation of the testicular functions and consequently improvement of sperm production.

(10) if the response of the adult rams actively immunized against testosterone could be compared to some extent to the response of the ram lambs actively immunized against oestrogens.

(11) if active immunization against testosterone in adult rams or active immunization against oestradiol-17ß in ram lambs can affect total testicular blood flow.

(12) if these immunization approaches have deleterious effects on the reproductive system in the long-term.

In summary, by investigating the period of transition into puberty of the Merino ram lambs, the project aims to gain an insight into the physiological mechanisms involved in the establishment of fertility and aims to determine what is the exact role of oestrogens in that process.

Our central hypothesis is that the negative effects of oestrogens on the hypothalamic-pituitary-testicular axis (e.g. negative feedback) can be efficiently neutralized by antibodies (produced actively or received passively) and therefore that immunization against oestradiol-17ß or oestrone during the prepubertal period should accelerate testicular maturation and consequently advance the time of achievement of puberty.

In addition, we also hypothesize that the feedback effects of testosterone on the hypothalamic-pituitary axis of the adult Merino rams could be partly neutralized by the circulating antibodies produced by T-immunized rams (not completely because of the very high level of testosterone produced by an adult ram) and that this could result in an improvement of the steroidogenic and spermatogenic functions.

CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

The purpose of this chapter is to provide a comprehensive review of the current information regarding the development processes occurring during maturation of the reproductive system in the male sheep with special emphasis on the period of transition into puberty. Along with this approach, we intend to review and place into perspective the contributions of endogenous oestrogens to the regulation of the function of the male reproductive system. Finally, this chapter includes a review of the application of steroid immunization as a tool in reproductive research.

1.2. The ram as a model

The ram is a convenient model to use for a study on sexual development because it grows rapidly, attaining puberty in less than 7 months of age, and shows readily detectable external signs of sexual maturity. Because the ram has a relatively long and well-defined impubertal period which lasts for about ten weeks (Lee et al., 1976), experimental evidence of the endocrine control of testicular ontogenesis can be obtained by many different means including the use of an immunological approach. Its gentle temperament and its large body size make it practical for intensive blood samplings which are necessary for endocrinological studies that take a particular interest in the pulsatile secretion of hormones.

Thus, in covering the topics of this literature review, we will concentrate mainly on the ram for the sake of brevity and because it is the animal model used in all experiments presented in this thesis. We must keep in mind, however, that there is no such thing as a typical mammal or a typical pubertal strategy (Bronson and Rissman, 1986). Although, one may assume that most of the basic mechanisms underlying the process of sexual maturation are conserved across species, results obtained in the ram may not necessarily be extrapolated to other species in all aspects. Therefore, to allow for a better understanding of the reproductive physiology of the male, this review will in some cases cover the information obtained from other farm animals

L. 9: Pulse of GnRH (or LH) are regularly observed in only a few experimental models, amongst which the sheep has been the most commonly used (Thiéry and Martin, 1991). The one-to-one relationship between pulses of GnRH and LH, suspected for many years, was finally demonstrated in conscious, ovariectomized ewes by Clarke and Cummins (1982).

More recently, utilizing a modified technique of hypophyseal portal blood sampling, patterns of GnRH and subsequent patterns of LH were also measured during the juvenile (birth to 6 weeks of age) and prepubertal periods of development in the bull calf (Rodriguez and Wise, 1989). This study provided the first direct measurements of GnRH release in the bovine species and during these stages of sexual development. Patterns of secretion for GnRH and LH were episodic in nature with all detected pulses of LH being temporally associated with pulses of GnRH.

Changes in the pulse profile, particularly the frequency of the pulses, have now become an essential component of our understanding of the control of reproductive activity (Thiéry and Martin, 1991)

(bulls, stallions, bucks, boars), laboratory rodents (mice, rats) and primates (monkeys and humans).

1.3. The components of the reproductive axis and mechanisms of hormonal action

1.3.1. The components of the reproductive axis

The reproductive axis of the male encompasses the following fundamental components:

•The hypothalamus and its transducer neurosecretory neurons. These translate neural signals into a periodic, oscillatory chemical signal, gonadotropin-releasing hormone (GnRH).

•The pituitary gonadotropes (in the anterior pituitary) that, in response to the GnRH rhythmic signal, release luteinizing hormone (LH) and follicle stimulating hormone (FSH) in a pulsatile manner at periodic intervals.

•The various compartments of the testis. The testis produce sex hormones as well as spermatozoa capable of fertilization when the animal has reached puberty.

•The various non-neural targets of the testicular hormones.

It is at each of these loci that modulating factors exercise their effects (Reiter and Grumbach, 1982).

1.3.2. Mechanisms of hormonal action

The fundamental mechanism used by cells to respond to various stimuli have attracted enormous attention in the last decade. The nature of the stimuli and the responses vary greatly from one cell type to another, but the underlying mechanisms are similar.

Cells which respond to peptide hormones (e.g. GnRH, LH, FSH) are distinguished from unresponsive cells by the presence of receptors at the cell surface. Similarly steroid-responsive cells possess intracellular (probably intranuclear) receptors (Gorski et al., 1986). In the particular case of steroid hormones, formation of the steroid-receptor complex facilitates recognition of specific DNA sequences by the receptor and subsequent activation of the

transcription of hormone-regulated genes (see sections 1.6.3.5.iv and 1.9.1.v). For peptide hormones coupling of receptor occupancy and biological response is achieved indirectly by a spectrum of mechanisms, often involving the formation of second messengers as the first step in a complex cascade of events. These messengers, and the changes they produce in intracellular proteins, modify the functions of the various components of the cell which, in turn, provide the response to the original stimulus (see sections 1.6.2.iii and 1.6.3.4.iv; Wakeling, 1988).

Therefore, the arrival at target cells of adequate amounts of a given hormone (and in some cases its conversion in the responding cell to more biologically active derivatives), the presence of sufficient numbers of the corresponding functional receptors in appropriate cellular locations and an intact postreceptor biochemical machinery that amplifies the hormone-receptor signal are all equally important for hormonal regulations. If either the receptor or postreceptor mechanisms are ineffective for genetic or other reasons, then cellular responses to the hormone will be compromised (Williams-Asham, 1988).

1.4. Temporal aspects of the development of the reproductive system in the ram

1.4.1. Stages of sexual maturation

It has been proposed that the events characterizing maturation of the reproductive endocrine system can be viewed as part of a continuum extending from sexual differentiation and the ontogeny of the hypothalamic-pituitary-gonadal system in the fetus to the attainment of the full sexual maturation and fertility, and then ultimately to senescence (Reiter and Grumbach, 1982; Courot and Kilgour, 1984; Bronson and Rissman, 1986; Adams and Steiner, 1988).

Sexual development in the male can be divided morphologically and physiologically into different stages (Pelletier et al., 1981; Courot and Kilgour, 1984; Ojeda and Urbanski, 1988). First of all, there is the ‘fetal period’ which starts at the time of sexual differentiation of the fetal gonad (after the establishment of the gamete sex). The reproductive organs mature to some degree during the fetal life but they undergo final maturation and synchronization during

postnatal development. It progresses after birth through the 'perinatal period' (neonatal or infantile period) which is a stage of relative gonadal quiescence and is immediately followed by the 'prepubertal period' where spermatogenesis is initiated (the perinatal and prepubertal periods are sometimes called impubertal stages). The 'pubertal' period ends with the appearance of mature spermatozoa in the lumen of the seminiferous tubules. Completion of testicular development and improvement of spermatogenesis occurs during the 'postpubertal period'. The 'adult stage' is reached when spermatogenesis attains its ultimate level (onset of the full gametic and endocrine functions of the gonads) and is then dependent on seasonal variation. Differentiation of extragonadal reproductive organs occurs also during those stages of testicular development. In the male sheep, as in other species, the organs of the hypothalamic-pituitary-testicular axis, must develop to a functional state and interact in the correct manner to produce mature gametes (Adams and Steiner, 1988).

1.4.2. The fetal period

i) Sex determination and differentiation

Zygotes and very early embryos cannot be distinguished as masculine or feminine except on the basis of their sex chromosomes. Males have a single X chromosome and a Y chromosome; females have two X chromosomes. Sex determination can be defined as the process that results in either testis or ovary formation. The process subsequent to gonad formation can be defined as sex differentiation.

The Y chromosome determines maleness in mammals. A Y chromosome-linked gene diverts the indifferent embryonic gonad from the default ovarian pathway in favour of testis differentiation, initiating male development (McLaren et al., 1988; Goodfellow and Darling, 1988). This Y-encoded gene has been named *testis-determining factor* (TDF) in man. In mice, a gene (SRY) from the sex-determining region of the Y-chromosome which has been recently identified (Berta et al., 1990) fully qualifies to be equated with the TDF of man (for review: Hawking et al., 1991; Sultan et al., 1991). Interestingly, the use of specialized genetic techniques (e.g. "Zoo

'Default ovarian pathway' means 'the gonad would develop into an ovary in the absence of external influence, i.e. this gene onthe Y chromosome

blot" analysis) in many mammalian species has now confirmed the male specificity of the SRY sequence (Sultan et al., 1991). Of course many points remain to be clarified concerning the exact mechanism of sex determination, including: the mechanism of early activation of SRY, the potential regulatory action of SRY on the activation of other genes (it is clear that genes located in the X chromosome and autosomes are also clearly required for differentiation of testes), the potential existence of other genes involved in sex determination (Sultan et al., 1991). However, that SRY (or TDF) induces the development of the testes and that subsequent hormone production by the testes induces the male phenotype is the current working hypothesis.

Much evidence indicates that differentiation of reproductive tracts in fetuses is genetically programmed to proceed in a feminine direction regardless of the genetic sex, unless testicular hormones, Mullerian-inhibiting substance (MIS) and testosterone (T), produced and acting on their target cells at critical and restricted period of development, effectively prevent expression of this female program and irreversibly impress masculinity (Jost et al., 1973). MIS, secreted by the Sertoli cells, causes regression of the female (mullerian) duct system (Josso et al., 1977). The mechanism of action of MIS is still poorly understood. Testosterone, secreted by the Leydig cells, is essential for the differentiation of male reproductive tracts, and secondary sexual tissues, as well as for masculine characteristics of the central nervous system and many other tissues. Deficiency of androgen receptors in the target tissues may cause dissociation between sex determination and differentiation (presence of testis but female external phenotypic appearance). T formation in the testis begins shortly after the onset of differentiation of the spermatogenic cords (Courot and Kilgour, 1984). Many questions concerning the regulation of T synthesis and secretion by the fetal testis are not resolved. The presence of LH and FSH receptors in fetal testis suggest that those two gonadotropins are functional in controlling testicular development at an early stage. T synthesis is without doubt gonadotropin dependent during the latter two-thirds of gestation (Gluckman et al., 1979; Sklar et al., 1980; Mueller et al., 1978, 1981; Gluckman et al., 1983; Clark et al., 1984; George and Wilson, 1988; Albers et al., 1989b).

ii) The testis

Development of the testis needs harmonious division and differentiation of both somatic and germinal cells. The precursors cell types of Sertoli (supporting cells) and Leydig cells appear early in fetal life. Sexual differentiation of the male gonad starts with the formation of the testicular or seminiferous cords which consist of primordial germ cells associated with immature Sertoli cells. The primordial germ cells will give rise to spermatogonia (Waites et al., 1985; de Kretser and Kerr, 1988).

iii) The hypothalamic-pituitary unit

Differentiation of pituitary gonadotropes and the initiation of a hypophysiotropic drive to the testicular cells is established early in fetal development (Mueller et al 1978, 1981, Sklar et al 1980). The presence of gonadotropins can be detected in the fetal pituitary gland some time before morphological differentiation of the male genital tract (Courot and Kilgour, 1984). The pattern of changes of FSH and LH concentrations in both pituitary glands and serum is consistent with a sequence of increased synthesis and secretion followed by a decline after mid-gestation that persists to term (Foster et al., 1972; Alexander et al., 1973; Foster, 1974; Sklar et al., 1980). GnRH receptors seem to be present and functional in the fetal gonadotropes by mid-gestation, in the ovine fetus, as suggested by the pituitary response to synthetic GnRH by 84 days of gestation, the earliest stage examined (Mueller et al. 1981). Studies employing chronically catheterized ovine fetuses have revealed the presence of distinct LH pulses during this phase of development (Clark et al.,1984). Such discharges of LH are presumably occasioned as in the adult, by the episodic release of GnRH by specific neurones which are synchronised by the hypothalamic pulse generator of the fetal brain. Immunoreactive GnRH has been detected in the hypothalamus of the ovine fetus as early as 59 days of gestation (Mueller et al., 1978). Several observations suggest an increase in pulsatile GnRH secretion that reaches a peak by 80% gestation (after 4 months of gestation in sheep), followed by a fall in late gestation (Mueller et al., 1981). Negative feedback by gonadal steroids on the hypothalamic-pituitary unit is operative in the male and female ovine fetus by 105 days but not at 90 days (Gluckman et al., 1979). In fact, in the late gestational ovine fetus the hypothalamic GnRH-gonadotropin unit becomes increasingly sensitive to oestrogens (Gluckman

et al., 1983). The inhibin-FSH feedback mechanism functional towards the end of gestation (Albers et al., 1989).

1.4.3. Major endocrinological changes of the hypothalamic-pituitary-testicular axis from perinatal to pubertal stages.

A complete chronicle of events that unfold as an individual progresses through puberty is not yet possible but the following endocrinological changes have been identified in the ram lamb.

1.4.3.1. The hypothalamic-pituitary unit

i) GnRH secretion

During infantile development the GnRH pulse generator initiates pulsatile secretion of GnRH at relatively infrequent intervals. The frequency of GnRH release increases distinctly in the latter phase of infancy (Rodriguez and Wise, 1989). This change in the activity of the pulse generator is believed to be the primary event responsible for the initiation of puberty and is governed either by steroid-dependent and/or steroid-independent mechanisms (see section 1.5.1). The greater frequency of GnRH release is translated into pulsatile release of LH, as available anterior pituitary stores of LH and pituitary sensitivity to hypothalamic stimuli increase (Amann et al., 1986). The increase in pulsatile release of LH in turn initiates the pubertal process by enhancing testicular steroidogenesis and gametogenesis.

ii) LH secretion

The serum LH profile in developing ram lambs can therefore be described in general terms by low circulating concentrations during the first few weeks of life, a transient rise between 6 and 10 weeks of age, followed by relatively low concentrations until achievement of fertility (Crim and Geschwind, 1972b; Foster et al., 1978; Pelletier et al., 1981; Courot and Kilgour, 1984; Yarney and Sanford, 1989).

iii) Steroid negative feedback

In the male lamb, a rapid enhancement in LH secretion following removal of gonadal inhibition at a very young age, similar to the response observed in older animals, does not suggest that the steroid negative feedback is weak in the period shortly after birth (Olster and Foster, 1986). However, the magnitude and the time of apparition of this postcastration rise in LH levels may vary greatly between species and with age (Pelletier, 1981; Adams and Steiner, 1988).

The transient increase in LH secretion observed at the onset of puberty could be partly due to a decrease in responsiveness to the inhibitory actions of gonadal steroids (see section 1.5.2.). Then, the decrease in the number of LH pulses observed towards the end of puberty would presumably be due to the increasing level of steroid and to greater feedback inhibition by those steroids on the hypothalamic-pituitary axis (Crim and Geschwind, 1972a; Foster and Ryan, 1981) and to further maturation of the feedback mechanism (D'Occhio et al., 1986b). Shortly before the achievement of puberty, the LH-testosterone feedback mechanism is fully operational (Sanford et al., 1982a).

iv) FSH secretion

In contrast to LH, plasma FSH does not exhibit major variations throughout puberty in the ram lamb (Pelletier et al., 1981; Jenkins and Waites, 1983) although an increase shortly after birth is frequently noticed (Foster, 1974; Lee et al., 1976; Blanc and Terqui, 1976). Recently, Yarney and Sanford (1989) reported an increase in mean FSH concentration just prior to the period of rapid testicular growth in crossbred rams.

v) PRL secretion

In one study, prolactin (PRL) levels remained fairly constant until the completion of puberty, with the exception of a pronounced peak between the tenth and twelfth weeks of life (Ravault and Courot, 1975; Ravault, 1976). In other studies, PRL has been shown to increase briefly during pubertal development (Klindt et al., 1985; Yarney and Sanford, 1989). Nevertheless,

compared with the adult, plasma levels of PRL are relatively low during infancy and are related to the photoperiod soon after birth (Ravault and Courot, 1975; Ravault ,1976; Wilson and Lapwood, 1979).

1.4.3.2. The testis

i) Testosterone secretion

LH stimulates the interstitial tissue resulting in a progressive increase in T in blood plasma throughout the impubertal period which in turn acts in feedback fashion to reduce LH secretion to the level seen in the postpubertal and adult ram (Lee et al., 1976; Foster et al., 1978; Pelletier et al., 1981; Klindt et al., 1985; Yarney and Sanford, 1989). The sustained rise in circulating T in the absence of any apparent increases in the secretion of LH in older immature males reflects a progressive increase in Leydig cells numbers and/or their steroidogenic activity (Barenton et al 1983). During this latter period of sexual maturation, patterns of circulating LH and T in the lamb resemble those of the mature adult (Sanford et al., 1974; Schanbacher and Ford, 1976a) in which repeated pulsatile discharges of LH occur followed by marked rises in circulating T. Testicular development of ram lambs is accompanied by increases in the number of LH and FSH receptors within the interstitial tissue and seminiferous tubules respectively (Barenton et al., 1983; Yarney and Sanford, 1989).

ii) Spermatogenesis

In the developing male, spermatogenesis proceeds as a continuum from azoospermia to oligospermia to full fertility (Courot and Kilgour, 1984; see section 1.6.3.3.i and iv).

1.4.4. Achievement of puberty in the ram

i) Definition of puberty

A definition for puberty in males is the period of accelerated reproductive development that culminates in 'potential' fertility. It is convenient to define the first release of spermatozoa from

the seminiferous tubules as the achievement of puberty. Within a larger view (and more practically), the presence of mature spermatozoa in the reproductive tract may be considered to be the criterion that marks the onset of fertility (Bronson and Rissman, 1986; Adams and Steiner, 1988).

ii) Timing of the onset of fertility

It has been recognized for some time now that body weight is a better predictor of the onset of fertility than is chronological age, however, both measurements show extreme irregularity in the ram (Dun, 1955). Thus, based upon various criteria such as changes in testicular weight and morphology and, appearance of spermatozoa in the seminiferous tubules, epididymis, and ejaculate, there is considerable variability among breeds for timing the achievement of pubertal maturation in sheep. Motile spermatozoa are hardly ever produced before 12 weeks of age but are more frequently present between 18 and 25 weeks of age (Watson et al., 1956; Skinner and Rowson, 1968; Dyrmundsson and Lees 1972; Schanbacher et al., 1974; Lee et al., 1976; Olster and Foster, 1986). According to Courot (1979), puberty is attained when ram lambs reach 40-45 % of the adult body weight. The actual time of fertility onset usually will be a reflection of multiple interactions between different environmental influences which will be ultimately limited and interpreted by each individual's unique set of genes i.e. its genetic potential. (see section 1.4.8.).

1.4.5. The postpubertal period

When the total pattern of sexual drives is present in an animal who has reached puberty, we talk of sexual maturity (functional fertility) and this generally occurs during the postpubertal period. Thus the postpubertal rams (e.g. yearling rams) will become sexually mature but because they are still growing, they may not be considered full adults (Olster and Foster, 1988). The production of spermatozoa has not yet reached its maximum level at the end of puberty, and progressively its efficiency increases until its ultimate level is reached. Higher T levels (Schanbacher et al., 1974; Williams et al., 1976) and more LH peaks with higher amplitude

(Sanford et al., 1982a) have been observed in postpubertal rams (6-7 months, yearling) compared with adult rams (approaching 5 years of age).

These observations indicate that further maturational and regulatory adjustments within the hypothalmic-pituitary-testicular axis take place well after achievement of puberty.

1.4.6. The adult ram

In mature rams (seasonal breeders), the production of spermatozoa reaches its ultimate level and then shows periodic fluctuations. In fact, the testes of adult rams of various breeds are smaller during the non-breeding season ("long day" season) and progressively redevelop (decreasing day length) in preparation for the fall breeding season ("short day" season). Spermatogenesis is considerably reduced but never completely arrested during the non-breeding season (Olster and Foster, 1988) while more efficient spermatogenesis (Schanbacher and Ford, 1979; Lincoln 1981) occurs during the breeding season. Although similarities exist between the seasonal transition in the adult rams and the pubertal transition in ram lambs, fundamental differences also exist in the regulation of gonadotropin secretion (Olster and Foster, 1988).

1.4.7. Senescence

To our knowledge, detailed longitudinal studies of changes in the pituitary-gonadal axis and in spermatogenesis with advancing age have not been made for domestic animals. Data that have accumulated in man and in some laboratory species are not discussed here as this topic is considered to be beyond the scope of this review.

1.4.8. Sources of variation in maturational development of the reproductive system

As for any other developmental process, sexual maturation is a genetically programmed and yet environmentally susceptible process (Bronson and Rissman, 1986; Foster et al., 1986).

1.4.8.1. Variations due to genes

It seems reasonable to assume that the component parts of the reproductive axis are assembled sequentially in the developing mammal according to a relatively rigid genetic blueprint. This

blueprint undoubtedly varies from one population to another, but for each individual there should be a genetically fixed, earliest possible time when all the components are mature (Bronson and Rissman, 1986).

1.4.8.2. Variations due to environment

Most individuals will not achieve their fastest possible rate of reproductive maturation as varying degrees of delay will occur because of environmental interventions. The ambient factors known to influence the reproductive maturation of a mammal emanate from the dietary, physical and social dimensions of its environment. These factors could exert their impact either pre-or post-natally and relate somehow to body growth or non-reproductive development (Bronson and Rissman, 1986; Foster et al., 1986; section 1.6.1.viii).

i) Dietary factors

Reproductive development being an energy-consuming process is dependent ultimately upon available calories and nutrients (Bronson and Rissman, 1986). Some metabolic factors may strongly influence the development and maintenance of the reproductive system, however, the nature of the "ultimate" regulatory metabolic signal(s) and the exact site at which it acts have not been identified yet (Adams and Steiner, 1988).

ii) Physical factors

Of the many environmental cues used by seasonal breeders, it is clear that day length or photoperiod is one of the most powerful, constituting a signal which profoundly influences the activity of the reproductive axis in sheep. Photoperiod synchronizes a host of seasonal adjustments in addition to those related directly to reproduction. Many of these affect energy processing and thus puberty could be regulated indirectly as well as directly by photoperiod (Bronson and Rissman, 1986; Foster et al., 1986).

The prepubertal male lamb does not respond to photoperiod as the mature ram does. The question of when the developing hypothalamic-pituitary-testicular system begins to respond to

L. 6: The following questions still need to be answered:

•what is the nature of the photoperiodic signals that can affect testicular growth ?

•what are the mechanisms of action of these photoperiodic signals ?

•what are the roles played by melatonin and PRL during pubertal development ?

•what kind of lightning regimen is required for optimal maturation ?

L. 16: 'Reawakening' is a word borrowed from T.M. Plant (1988) who has written an excellent review on pubertal development in primates and who has also mentioned the important similarities that exist between primates and sheep.

> ''Studies employing chronically catheterized ovine fetus, a species in which the ontogeny of gonadotropin secretion during fetal development appears to resemble that in higher primates, have revealed the presence of distinct LH pulses during this phase of development (Clark et al.,1984)'' (Plant, 1988).
> ''Puberty in primates (and also in the sheep) is separated in a most definite fashion from perinatal ontogeny, a phase of development that is comprised of both growth and differentiation'' (Plant, 1988).

Nevertheless, we agree that, contrary to the situation observed in sheep, the steroid-dependent mechanism has not been sufficient to explain the pubertal changes occurring in primates.

> ''Thus, the continued application of the gonadostat hypothesis to account for the initiation of puberty in primates seems to be unwarranted. A much more attractive explanation for the onset of primate puberty, in light of data presently available, is the hypothesis that the open-loop capacity, or drive of the hypothalamic-pituitary unit to produce gonadotropin, increases at this phase of development'' (Plant, 1988).

Thus, to avoid misinterpretation, we suggest to use the word 'reactivation' instead of the word 'reawakening'. 'Reactivation' suggests a transient increase in the activity of the hypothalamic-pituitary unit but does not imply that this unit was inactive before the "prepubertal" rise.

photoperiod has not been answered with certainty (Olster and Foster, 1988). Colas and co-workers.(1987), have shown that exposure of ram lambs (born in autumn) to long days and then to short days accelerate testis growth. It was also shown that photoperiodic treatment can only influence ram lambs during the phase of rapid growth of the testis (Alberio, 1976; Alberio and Colas, 1976). Several important gaps remain in our knowledge of the photoperiodic regulation of puberty (Lindsay et al., 1984; Olster and Foster, 1988).

iii) Social factors

Many mammals have evolved direct and specific neural and hormonal pathways via which the final stages of reproductive development can be modulated for adaptative purposes (e.g. pheromonal cues: Bronson and Macmillan, 1983; Vandenbergh, 1983). Likewise it is also well established that non-specific, aversive, emotional states can have potent effects on pubertal development, and that the social environment is a rich source of situations that yield such neurogenic stimulation (Bronson and Rissman, 1986; see section 1.6.1.viii). The role of those factors in the regulation of puberty is a poorly studied subject in farm animals.

1.5. Hormonal signals for puberty

1.5.1. Reawakening of the hypothalamic GnRH pulse generator

The hypothalamic-pituitary-gonadal system differentiates and functions during fetal life and early infancy, is then suppressed to a low level of activity for a variable amount of time and is reactivated during the prepubertal period. In this light, the initiation of puberty is set in motion by the reawakening of a dormant, but apparently, fully mature, hypothalamic GnRH pulse generator. However, the question of what holds the GnRH pulse generator in check during the impubertal period and what causes its reactivation later on has not been answered with certainty, despite receiving considerable attention for many years (Adams and Steiner, 1988).

Thus, an increase in hypothalamic pulsatile GnRH discharge is generally accepted to be a key event that underlies the changes in the secretory patterns of the gonadotropins (LH and FSH)

that occur during prepubertal maturation. Each of these humoral factors influence the growth and maturation of the testis.

Both steroid-dependent and steroid-independent mechanisms have been invoked to explain the prepubertal change in the activity of the pulse generator (Adams and Steiner, 1988) and will be discussed in the next sections.

1.5.2 Sex-steroid dependent mechanism

The basic premise of the "gonadostat" hypothesis (Ramirez and McCann, 1963; Ramirez and McCann, 1965; Ramirez, 1973) is that before puberty, the hypothalamic-pituitary axis responsible for gonadotropin secretion is extremely sensitive to gonadal steroid inhibition, resulting in low tonic LH and FSH secretion. As prepubertal maturation proceeds, there is a decrease in responsiveness to the inhibitory actions of gonadal steroids, resulting in increased tonic gonadotropin secretion sufficient to initiate reproductive activity. What precisely accounts for the change in steroid feedback sensitivity is unclear and certainly has not been neurochemically defined. This hypothesis has received strong support as a developmental concept in many species (for rat (Ramirez, 1973), for golden hamster (Sisk and Turek,) 1983, for pig (Elsaesser et al., 1978), for cattle (Pelletier et al., 1981) for human (Reiter and Grumbach, 1982)). In the male sheep, two studies from Olster and Foster (1986 and 1988) strongly support this hypothesis. However, there is no convincing evidence to support the notion that the initiation of puberty is triggered solely by a decrease in sensitivity to gonadal steroid feedback.

That GnRH pulse generator in the brain can be activated independently of gonadal steroids is evident from studies performed in gonadectomized rats, sheep and primates. For example, the fact that the augmentation of pulse generator activity occurs at about the same time in gonadectomized and gonad intact monkeys suggests that the time course of pubertalmaturation is to some extent a gonad-independent event (Adams and Steiner, 1988; Plant, 1988).

1.5.3 Sex-steroid independent mechanism

Another explanation is that the onset of puberty is merely a consequence of further maturation of central nervous system (CNS) components which finally override the suppressive effects which

gonadal steroids had exerted. This steroid independent mechanism would perhaps play the primary, if not the most decisive, role in the initiation of puberty in the male, as can be observed in gonadectomized animals. Two theoretical physiological systems have been developed to explain this maturational change in the activity of the GnRH pulse generator. One proposes that it is the consequence of the loss of an intrinsic CNS inhibitory system independent of steroid negative feedback control. The other model involves the concept of a mechanism that might depend on the activation of excitatory inputs to the CNS .

i) The loss of a central restraint

The nature of the postulated intrinsic CNS inhibitory system during impubertal life is not known (Ojeda and Urbanski, 1988).

In the rats, the opiate system appears to play an active role in inhibiting gonadotropin release well before puberty. However, an important link between hypothalamic opiate peptides and the inhibitory effects of gonadal steroids on LH secretion has been established (Cicero et al., 1980) and it has been interpreted as being indicative of an involvement of the opiate system in mediating the pubertal "resetting of the gonadostat" (Bhanot and Wilkinson. 1983). Therefore, although the opiate system may be a crucial component in the mechanism(s) leading to sexual maturation it does not appear to be a "steroid-independent" mechanism. Furthermore, there is no evidence that the actual initiation of puberty is determined only by the removal of opiate-restraining influences. The importance of opiate involvement during sexual maturation in ram lambs has not been demonstrated yet (Bhanot and Wilkinson. 1983).

Dopamine has also been implicated in a possible neurochemical inhibitory mechanism. Whether the inhibitory effects of dopamine on gonadotropin secretion can be translated to the pubertal period remains to be established (see section 1.6.1.1.xi).

ii) The activation of excitatory inputs

Some data suggest that in the impubertal animal, GnRH neurons are relatively quiescent only because they lack sufficient excitatory stimulation. Therefore, a more plausible mechanism for

the onset of puberty is that the initiation of pulsatile GnRH release is primarily dependent on the activation of excitatory inputs (Ruf, 1982). The nature of those excitatory inputs is also unknown. Possible candidates are the cathecholaminergic and the serotoninergic systems (Ojeda and Urbanski, 1988). Establishment of these excitatory mechanisms is suggested, at the morphological level, by the increased synaptogenesis that occurs during prepubertal development, as well as by the histological changes observed in normally maturing GnRH neurons (Silverman, 1988). At the functional level, the establishment of excitatory mechanisms is suggested by the changes in neurotransmitter metabolism and in GnRH responses to secretagogues during juvenile-peripubertal development (Ojeda and Urbanski, 1988).

1.5.4. Summary

To summarize, it may now be suggested that the GnRH-releasing system develops under the influence of both excitatory and inhibitory inputs. The steroid independent initiation of pulsatile GnRH secretion probably constitutes the fundamental activation of the system, but the output of the system is adjusted or fine-tuned by the magnitude of feedback effects exerted by gonadal steroids (Adams and Steiner, 1988; Ojeda and Urbanski, 1988).

It remains that experimental separation of changes in negative feedback sensitivity from changes in the steroid-independent mechanism has proven difficult (as indicated in section 1.5.3. i). As might be expected, the different steps in the development of the reproductive axis probably vary from mammal to mammal. For example, changing patterns of gonadatropin secretion in prepubertal bulls seem to involve both feedback dependent and feedback independent shifts in activity of the hypothalamic-pituitary axis (D'Occhio et al., 1986a). In contrast, the gonadostat hypothesis alone seems to offer an attractive explanation for sexual maturation of the male lamb (Olster and Foster 1986; 1988).

1.6. Organization of the hypothalamic-pituitary-testicular axis and oestrogen within this axis

1.6.1. Central nervous system (CNS) and hypothalamus

i) Anatomy

The GnRH neurosecretory network is very diffuse: it contains individual neurons, located in vastly different regions of the brain, presumably integrating different kinds of information (Silverman, 1988). Thiéry and Martin (1991) had recently reviewed what is actually known about this neural pathway in the sheep brain. The dispersion of functional GnRH neurons and a complex organization (connection with various afferent systems) permit the integration of endocrine, autonomic, and higher cerebral function and permit appropriate regulation of the reproductive system in response to various stimuli (Page, 1988).

ii) Pulse generator

It is well established now that at the core of the process of reproduction in the sheep, as well as in other mammals, is a hypothalamic pulse generator that synchronizes the activity of GnRH-secreting neurons. The GnRH pulse generator is thought to comprise the basic neuroendocrine unit responsible for LH and FSH secretion. The origin and nature of the hypothalamic pulse generator are unknown (Wakeling, 1988; Thiéry and Martin , 1991).

iii) GnRH

GnRH is produced as a small portion (10 amino acids) of a large precursor molecule (92 amino acids) (Jennes and Conn, 1988). The development of techniques for sampling pituitary portal blood has led to a good understanding of the way in which GnRH controls the anterior pituitary gland, including the importance of its mode of secretion. GnRH is released into the portal system in a pulsatile manner, reaches the anterior pituitary and stimulates the pulsatile discharge of LH and a more regular discharge of FSH. Both the frequency and amplitude of GnRH

pulses vary according to the physiological state of the animal. In the absence of feedback by gonadal hormones, episodic secretion of GnRH occurs at intervals of 20-60 minutes (Wakeling, 1988).

iv) Regulation of GnRH release by steroids

The release of a GnRH pulse involves both inhibitory and excitatory inputs (see section 1.5.). Androgens, oestrogens and progestagens are capable of reducing the activity of the GnRH pulse generator in intact and castrated rams at various ages (see section 1.6.2.v). It is unlikely that the steroids alter pulse frequency and/or amplitude by directly affecting the GnRH cells but more likely by acting via interneurons because steroid receptors have not been found in the GnRH neurons (Karsch and Lehman 1988; Silverman, 1988). Oestrogen and androgen (T and DHT) concentrating neurons have been identified in different regions of the rodent brain by autoradiographic means (Sachs and Meisel, 1988). The anatomy of the GnRH nerve cells and fibres in the rat and their physical connections with steroids-concentrating neurons and other neural systems have been reviewed by Kalra (1986).

v) Evidence for oestrogen participation within the CNS

Evidence for the presence of aromatase enzymes in the central neuroendocrine tissues of male animals (human, rhesus monkey, rat, mouse, and rabbit) has been provided by Naftolin et al. (1975). The areas where oestrogens are formed are those long associated with brain differentiation, feedback control of gonadotropins, control of sexual maturation, and initiation of sexual behaviour. Perfusion of living isolated brain from rhesus monkey showed that oestrogen was produced *in vivo* in that species (Naftolin et al., 1975). Interestingly, in experiments with either hypothalamic or limbic tissues, homogenates from male subjects (human, rat, mouse, rabbit) have had more aromatase activity than those from females (Naftolin et al., 1975). Many regions within the brain of males have cells that contain oestrogen receptors, with the distribution of such regions broader than the distribution of regions in which aromatization of T can occur. This would imply that not all of the actions of oestrogens on the brain are the result

of intracellular conversion of T to oestradiol (Sacks and Meisel, 1988). In the ram, the demonstration of specific oestrogen receptor sites in the hypothalamus (Pelletier and Caraty, 1981; Glass et al., 1984) also supports a role for oestrogen within the CNS.

vi) Pubertal development

The morphology of the GnRH neurons has been observed to change both during development and with changes in reproductive status (Silverman, 1988). The exact nature of the changes in the pulsatile pattern of GnRH release that must underlie the pubertal increase in hypophysiotropic drive to the gonadotropes remains to be fully defined (see section 1.5.). The key role of GnRH in the control of gonadotropins and their importance for the ontogeny of testicular function has been experimentally demonstrated by active immunization against GnRH in ram lambs and boars (Schanbacher, 1982; Falvo et al., 1986) and by GnRH pulsatile treatment of impubertal bulls implanted with oestradiol-17ß (Schanbacher et al., 1982).

vii) The control of GnRH by neurotransmitters

Studies have been initiated in several laboratories to examine the nature of the neurotransmitter systems that innervate the GnRH neurons and their processes. In this section we will present only the major findings that could help us to understand the relationships between gonadal steroids and the GnRH secretory pathways. An enormous amount of work has been done in this relatively "new" field of research, however this area is still characterized by controversy rather than clarity (Thiéry and Martin, 1991).

<u>Catecholamines</u>

The catecholamines, noradrenaline (NA) and dopamine (DO), play an important role in regulating the reproductive system and perhaps in timing the onset of puberty as well (Adams and Steiner, 1988). Because catecholaminergic cells are steroid-sensitive and have terminals on the GnRH cells, they can be viewed as a necessary mediator of the inhibitory effects of steroid such as oestrogen. However, opinions differ concerning the exact effects of NA and DO (which may relate to differences in experimental methodology and the animal model used) and no final

conclusion can yet be drawn from these studies. The question of how oestrogen could affect the activity of catecholaminergic cells remains to be answered. The different possibilities have been reviewed by Thiéry and Martin (1991).

Endogenous opioids

Neurons using opioid peptides as neurotransmitters generally inhibit GnRH and gonadotropin release and this effect appears to require the presence of gonadal steroids. Their anatomical distribution in the diencephalon is consistent with their participation in the control of gonadotropin secretion. In sheep, the inhibitory effect is evident from the responses to drugs, such as naloxone, that block opioid receptors and evoke a very rapid increase in the frequency of GnRH and LH pulses (Schanbacher, 1985; Caraty et al 1987). Various other results suggest that the primary role of the opioidergic neurons is to mediate responses to negative feedback (Ebling et al., 1987; Lincoln et al., 1987). There is also ample evidence for steroid-independent effects of the opioid neurons on the activity of GnRH cells (Thiéry and Martin 1991). Thus, opioids could be important neurotransmitters participating in the timing of the onset of puberty in mammals (section 1.5.3.1.).

Pineal indolamines

At the present time there is no doubt that the pineal gland of mammals functions as a true endocrine organ that transduces neuronal information about day length into endocrine secretion. The pineal gland produces, among other compounds, the indolamines: melatonin and serotonin. The importance of melatonin in the control of reproduction in seasonal breeders has been recognized for many years (Adams and Steiner, 1988). Synthesis and release of melatonin are tightly coupled to light-dark cycle, such that plasma and pineal levels of melatonin are high at night and low during the day. At this point in time, it is unclear exactly where the action of melatonin is exerted. A growing body of evidence indicates that the neuroendocrine centres of the brain are the most likely primary target, although a few *in vitro* studies have suggested that melatonin may also affect the pituitary gland and even the gonads directly. The ultimate effect of inhibitory photoperiods ("long days" in sheep) appears to be to inhibit tonic LH secretion by maintaining a high degree of responsiveness to oestradiol negative feedback (Kennaway et al., 1992). Serotonin is also believed to play a role

in the control of reproduction, although its exact functions have not yet been fully characterized (Adams and Steiner, 1988).

viii) Other factors that influence the GnRH neurons

Bearing in mind the central role played by the hypothalamus in regulating the tempo of sexual maturation, it is logical to consider that several kinds of internal and external factors might well become integrated simultaneously to influence the activity of the GnRH pulse generator. Among those factors known to affect the activity of the GnRH neurons are nutrition, pheromones, social cues (see section 1.4.8.2.). The hormonal pathways used by pheromonal cues have been reviewed recently (Bronson and Macmillan, 1983). Obviously many of the effects of social cues on puberty involve the CRF/ACTH (corticotrophin/adrenocorticotrophic hormone)/adrenal axis, at least at some level. Indeed, there is evidence that increased activity of this axis is felt at many levels in the reproductive axis: by enhancing the activity of inhibitory opiate pathways in the brain, by decreasing pituitary responsiveness to GnRH, and by decreasing steroidogenesis in the gonad (River and Vale, 1984; Rivier and Rivest, 1991). Aversive emotional states also can interfere with food intake and then, act via energetic restriction as well (Bronson and Rissman, 1986).

ix) Other roles for GnRH

That GnRH neurons could also influence the reproductive system by a mechanism not involving the regulation of gonadotropin secretion has been suggested. For example, it has been shown that some GnRH cells project to cerebral and brain-stem regions that could promote sexual behaviour (Page, 1988). This finding is consistent with studies suggesting that GnRH may facilitate or provoke certain reproductive behaviours (Page, 1988; Jennes and Conn, 1988; Thiery and Martin, 1991).

1.6.2. Pituitary

i) GnRH transport to pituitary gonadotropes

The median eminence, being the final point of converging GnRH neural system, forms the humoral relay of information from the brain to the anterior pituitary gland. The termination of a secretory neuron near a blood vessel without an interposed blood-brain barrier constitutes the fundamental organizational pattern of the neurohypophysis. GnRH is then carried via a blood portal system to the anterior pituitary. GnRH leaves the blood to interact with receptors at the cell's surface of gonadotropes and to regulate the release of LH and FSH that, in turn, enter the capillaries and are carried away from the gland (Page, 1988).

This review includes photos and diagrams picturing the various interactions between the glands and the general organization of the hypothalaic-pituitary unit.

ii) Pituitary gonadotropes and gonadotropins (LH, FSH)

The gonadotropes are regulated by GnRH but also by the interaction with the hormonal secretions of target organs that have reached their appropriate receptors through the systemic circulation (e.g. steroid hormones, inhibin). The ultrastructural elements necessary for protein synthesis, storage and release into the extracellular space (rough endoplasmic reticulum, vesicles, Golgi apparatus, and secretory granules) are present in the endocrine cells of the adenohypophysis (Page, 1988). The majority of gonadotropes contain both LH and FSH which are glycoproteins that contain an alpha chain (common to LH, FSH and TSH) and a beta chain (specific for each gonadotropin) (Page, 1988).

The one-to-one relationship between pulses of GnRH and LH, that was suspected for many years, was finally demonstrated in conscious, ovariectomized ewes by Clarke and Cummins (1982). Until this time, we had relied on indirect arguments to show that the LH pulses in the peripheral circulation corresponded with GnRH pulses secreted by the hypothalamus . Changes in LH pulse profile, particularly the frequency of the pulses, have now become an essential component of our understanding of the finer aspects of hypothalamic-pituitary activity. Variations in the frequency and amplitude of LH pulses are primarily a reflection of the intensity of the action of the sex steroids (via feedback mechanisms) (Thiéry and Martin, 1991).

No specific FSH releaser has been clearly demonstrated *in vivo* [although activin appears to be a potent and selective stimulator of pituitary FSH release *in vitro* (Tsonis and Sharpe, 1986; see section 1.6.3.3. v)] and consequently it is generally accepted that GnRH promotes secretion of the two gonadotropins. Because of the longer circulating half-life and the less acute rate of rise of FSH to an GnRH stimulus, distinct pulsatile patterns of plasma FSH are not obvious. Differences in the factors that modulate the action of GnRH on FSH and LH release have been considered to explain the two distinct secretory patterns (e.g. inhibin regulation of FSH) (Reiter and Grumbach, 1982; Wu et al., 1989).

It is now clear that different types of gonadotropins exist and that their biological activity and the immunoreactivity are not always correlated (Jeffcoate, 1988). Further studies are required to characterize the different gonadotropin isoforms (e.g. isoelectric focusing pattern of FSH) and to prove directly that they have distinct physiologic functions (e.g. pleimorphism of FSH vs biological activity) (Beitins and Padmanabhan, 1991).

In brief, FSH is the principal hormone binding to seminiferous tubules and allowing spermatogenesis while LH binds to Leydig cells and regulates steroidogenesis (Courot, 1967; 71; Courot et al.,79; Williams-Ashman, 1988).

iii) GnRH receptors and mechanism of action

The binding of GnRH to the pituitary membrane receptors initiates a cascade of events leading eventually to new synthesis and release of LH and FSH. Calcium mobilized from the extracellular space fulfils the requirement of a second messenger or mediator of GnRH action on gonadotropin release. Calmodulin, GTP binding protein ('G-protein') and phospholipase C also appear to participate in some way in the response to GnRH (Jennes and Conn, 1988).

The rate of hypothalamic secretion of GnRH seems to be a primary regulator of the number of its own pituitary receptors (this is referred to as a "priming effect") (Jennes and Conn, 1988). The inhibition of gonadotropin secretion by the continuous infusion of GnRH is termed "desensitization". Early desensitization appears to result from a loss of receptors (down-regulation), but the desensitized state is maintained by loss of functional Ca^{2+} ion channels even

at a time when receptor numbers return to normal. Discontinuous or pulsatile stimulation by exogenous GnRH infusion reverses or prevents refractoriness to GnRH and restores pulsatile release of gonadotropin (Jennes and Conn, 1988). Thus these studies provide evidence that the pulsatile mode of secretion of GnRH is necessary for normal pituitary function.

iv) Steroid negative feedback

Aromatizable androgen (Pelletier, 1970; Bolt, 1971; Crim and Geschwind, 1972b; Garnier et al., 1977; Foster et al., 1978; Parrott and Davies, 1979; Schanbacher, 1980b; D'Occhio et al., 1982; 1983a; 1983b; 1985), non-aromatizable androgen (Parrott and Davies, 1979; D'Occhio et al., 1982; 1983b), oestrogen (Bolt, 1971; Riggs and Malven, 1974; Edgerton and Baile, 1977; Schanbacher and Ford 1977; Parrott and Davies, 1979; Schanbacher 1980a; Jenkins and Waites, 1983; D'Occhio et al., 1983b; Olster and Foster, 1986; 1988; Sanford and Robaire, 1990) and progestagen (Bolt, 1971; Echternkamp and Lunstra, 1984) have been recognized as steroids capable of controlling gonadotropin secretion in the male sheep (negative feedback mechanism). In rams, support for the hypothesis that picomolar concentrations of oestrogen normally contribute to the regulation of gonadotropins is provided by the observation that the administration of antioestrogen such as tamoxifen (Sanford, 1985; Sanford, 1987a) or immunization against oestrogens results in a rise in the secretion of LH and/or FSH (Schanbacher, 1979; 1984a; Schanbacher et al., 1987; Monet-Kuntz et al., 1988; Sanford, 1985; 1987a; 1987b). The presence of androgenic and oestrogenic receptors in the ram pituitary support the finding that both steroids can have direct effects on the pituitary gland. Interestingly, pituitary androgen and oestrogen receptors increase with age in the ram during pubertal development (see section 1.9.1.iv). Both the pituitary and the hypothalamus contain the 5 α-reductase (converting T into DHT), but the pituitary seems to lack the aromatase enzyme (Naftolin et al., 1975). In a passive immunization study, Land et al (1981) have shown that the concentration of FSH was correlated with the oestrone antibody titre suggesting that oestrone may have a particular part to play in the feedback control of FSH release in the ram lamb.

v) Non-steroidal feedback (inhibin)

Information on the feedback control inhibin in males derived largely from *in vitro* studies involving isolated pituitary, Sertoli and Leydig cells; there are few studies on the interaction of inhibin with these cells based on *in vivo* results (de Kretser et al., 1987). The participation of inhibin in modulating anterior pituitary responses to GnRH assumes increasing significance for the differential regulation of FSH and LH synthesis and discharge in the adult ram (Schanbacher, 1980a). In maturing sheep, some evidences indicate a role for inhibin in the negative feedback control of FSH secretion (Blanc and Terqui, 1976; Walton et al., 1978; 1980; Clarke et al., 1991). Merino ram lambs immunized against partly purified inhibin at 3 weeks of age showed an increase in FSH level but had similar LH concentration during their postpubertal development (Al-Obaidi et al., 1987). More recently, Voglmayr and co-workers (1990) have shown that active immunization against human recombinant α-unit could amplify the season-related increase in both serum gonadotropin levels in the adult Suffolk rams. That inhibin system could also be involved in the regulation of LH release in the male lamb (Schanbacher and Ford, 1976b) and in the adult ram (Blanc et al., 1978) has been considered.

vi) Pubertal development and gonadotropin secretion

There is no doubt as to the necessity of gonadotropins for the process of sexual maturation. In the absence of LH and FSH, as in the hypophysectomized male lamb, neither testicular development nor spermatogenesis proceeds; replacement with gonadotropins restores testicular function and the initiation of spermatogenesis to a certain extent (Courot, 1967; 71; Courot et al.,79). The evolving pattern of LH and FSH secretion (see section 1.4.3.) during pubertal development could be explained by the combined effects of changing hypothalamic GnRH pulse frequency and gonadal feedback so that a new equilibrium for sustaining adult function is achieved (see section 1.5.). Interestingly it has been suggested that the increase in the amount of gonadotropins secreted during pubertal development is accompanied by a shift in the of gonadotropins such that the LH and FSH molecules have greater biological activity (Adams and Steiner, 1988). The pituitary exhibits striking variations in its responsiveness to GnRH

L. 1: The magnitude of LH response to GnRH injection increases during the first weeks following birth. Thereafter (>60-80 days of age), modifying factors (e.g. steroid feedback) appear to be acting, and the total response may continue to increase slightly (Galloway and Pelletier, 1974) or decrease (Pelletier et al, 1981).

L. 12: In postpubertal rams, treatment with 2-bromo-alpha-cryptine (CB154), a drug that suppresses PRL secretion, was associated with decreased testicular size and lower testosterone levels (Sanford and Dickson, 1980). These authors concluded that PRL secretion is a prerequisite for normal testicular development in preparation for the fall ovine breeding season.

L. 20: GH (i.e. somatotropin) is the primary growth-promotting hormone in postnatal vertebrates, and IGF-1 has been shown to mediate many of the somatogenic effects of GH (Galbraith and Topps, 1981; Gluckman, et al., 1987; Isaksson et al., 1988). GH can act synergistically with LH, FSH and testosterone (Setchell, 1978). Moreover, it has been recently shown that the positive effect of exogenous GH and oestradiol on growth may be additive in steers (Wagner et al., 1988; Enright et al., 1990).

treatment over the course of sexual maturation in ram lambs (Galloway and Pelletier, 1974; Lee et al., 1976) (see INSERT 1). Change in the number of pituitary GnRH receptors (pituitary sensitivity) is probably an important component of the maturational changes that occur during pubertal development (Amann et al., 1986).

vii) Other pituitary hormones involve in reproductive function

Prolactin (PRL)

The role played by PRL in the male reproductive system seems to vary greatly between species. That PRL may affect the development of the testes (by synergism with LH and testosterone) in the ram has been suggested by Ravault et al. (1977). Whether it is important in the establishment and maintenance of testicular receptors, as it is in the rat, remains to be determined. Interestingly, Yarney and Sanford (1989) found an increase in PRL secretion in crossbred rams between 90 and 150 days of age which occurred at the same time that LH-receptor numbers and T secretion were increasing. (see INSERT 12) PRL seems to be regulated the most by direct-seasonal influences. The endocrine products of the testis do not appear to alter the normal secretion rate of PRL in the different seasons (Sanford and Robaire, 1990). Active immunization of prepubertal rams against PRL did not affect pubertal development (Spicer, 1979) however this treatment in yearling rams tended to reduce body and testes weights (Ohlson et al., 1981).

Growth Hormone (GH)

The pulsatile nature of the secretion of growth hormone ensuring normal growth is an essential component of sexual maturation (Short, 1980; Klindt et al., 1985). (see INSERT 20)

Thyroid stimulating hormone (TSH)

TSH is the hormone that regulates the function of the thyroid gland. To our knowledge, no experiment has demonstrated that TSH can affect the hypothalamic-pituitary-testicular axis directly but it has been shown that both hypothyroidism and hyperthyroidism are associated with disruptions in reproductive processes in adult rams (Chandrasekhar et al., 1985b) and prepubertal ram lambs (Chandrasekhar et al., 1985a). It seems that thyroxin which is produced

by the thyroid gland plays a permissive role in reproductive function in male sheep (Chandrasekhar et al., 1986). The role of thyroid hormones in animal growth and the relationship between thyroid hormones, GH and IGF-1 in the control of growth have received increased attention (Cabello and Wrutniak, 1989)

1.6.3. The testis

1.6.3.1. Testicular growth

The general development of the gonads is closely associated with body growth. In the fetal sheep, testicular weight increases slowly from sexual differentiation to birth. After birth the pattern of testicular growth is sigmoidal, with firstly a period of slow development at a rate similar to that in the fetus. This is then followed by a period of marked, rapid increase in weight (starting with the initiation of spermatogenesis) which finally reaches a plateau (Courot, 1971; Courot and Kilgour, 1984).

1.6.3.2. Testicular development: general points

As the animal goes through puberty, important changes occur within the gonad. The testes become more sensitive to the stimulatory actions of the gonadotropins, primarily because of elevated LH secretion, and so the level of T production becomes enhanced. Meanwhile, both T and the gonadotropins provide the basic stimulus for initiating spermatogenesis. The increased output of T also produces the physical alterations that are typically associated with puberty, namely, the development and maintenance of the accessory sex organs, and further facilitates the maturation of the seminiferous tubules (Mainwaring et al., 1988). In addition, T and other gonadal hormones (e.g. oestradiol, inhibin) act as negative-feedback agents to attenuate gonadotropin secretion. The developing testis undergoes active differentiation and cellular multiplication at the same time as the central neuroendocrine system is developing. Testicular growth is mainly attributable to an increased seminiferous tubule volume resulting from a thickening of the tubular lining, the formation of a lumen, and the differentiation and growth of Sertoli and Leydig cells (Courot and Kilgour, 1984).

1.6.3.3. Seminiferous tubules

i) Histological appearance and testicular development

During the fetal and impubertal phase, the sex cords (seminiferous tubules) contain supporting cells (which are the precursors of the Sertoli cells of the adult), and gonocytes (primordial germ cells). Spermatogenesis has not yet begun, and throughout this period, the histological appearance of the sex cords does not change (Courot and Kilgour, 1984). The nuclei of the supporting cells lie at the periphery of the cords, while their cytoplasm fills the central part of the cords, there is no lumen. The germ cells are found squeezed in between the supporting cells and surrounded by their cytoplasm. Mitoses are seen in the two populations of cells, and thus their numbers increase. Thereafter, the proliferation progressively declines to reach zero during the first few steps of spermatogenesis. The testicle reaches the prepubertal phase, where the supporting cells differentiate to become the highly specialized Sertoli cells (Courot and Kilgour, 1984). Sertoli cells have a well developed Golgi apparatus and morphologically appear to be well equipped for protein synthesis. At some stage during the prepubertal phase, the gonocytes begin rapidly dividing and differentiating, their rate of division resulting in a drastic increase in testicular size. As this process of differentiation progresses, the different types of germ cells progressively appear. At first, just a few primary spermatocytes are seen, distributed at random throughout the tubules. Later, their numbers increase progressively, and the various stages of meiotic prophase appear so that the cellular associations seen in adult spermatogenesis become established. The cellular associations become gradually completed as spermatids and spermatozoa are formed (de Kretser and Kerr, 1988). Very few spermatozoa are produced at first, but eventually production reaches adult levels and the testis begins to show normal spermatogenic cycles (Courot and Kilgour, 1984).

Electron microscopic studies demonstrated that adjacent Sertoli cells formed specialized "tight" junctions, thereby forming the blood-testis barrier which effectively divides the tubule into basal and abluminal compartments. This formation is necessary to create a specific intratubular environment needed for spermatogenesis. The morphological differentiation of the blood-testis

barrier appears after cessation of Sertoli cell mitosis and commencement of the meiotic maturation of germ cells. The factors controlling the formation of the blood-testis barrier are not known (de Kretser and Kerr, 1988).

Closer examination of the ultrastructural features of the seminiferous epithelium revealed the presence of ectoplasmic specializations as well as various types of tight junctions or desmosomes like structures (desmosomes, hemidesmosomes, gap junctions) associated with the plasma membrane of the Sertoli cell. The possible functions of these structures have been recently discussed by de Kretser and Kerr (1988).

ii) Hormonal control of seminiferous tubules

After hypophysectomy of lambs at 50 days there was no evidence of spermatogenesis in the seminiferous tubules and there was an immediate and irreversible regression of the population of supporting cells. While there was no quantitative modification of the primordial germ cells, there was a total failure of the initiation of spermatogenesis. It was suggested that this effect of hypophysectomy on lambs germ cells might be primarily due to modifications of supporting cells (Courot, 1971; Courot and Kilgour, 1984).

Supplementation of impubertal hypophysectomized lambs with LH and FSH induced initiation of spermatogenesis (synergistic effect) (Courot, 1971). The FSH hormonal dependence in the initiation of spermatogenesis in lamb has been further demonstrated in an immunization study (Courot et al., 1984a,b; Kilgour et al., 1984). Passive immunization with an anti-ovine-FSH commencing several weeks before the initiation of spermatogenesis induced an early inhibition of testicular growth with severe reduction in Sertoli cell numbers. There was also an impairment of spermatogenesis at the level of the last spermatogonial divisions . This was truly an effect of the antibodies against FSH, since LH and T remained normally active as shown by the absence of an effect on the accessory glands (Courot et al., 1984a,b; Kilgour et al., 1984). It seems that the effect of LH on spermatogenesis is indirect because the Sertoli cells lack LH-receptors (Steinberger and Steinberger, 1977; Tsai-Morris et al., 1985b; Williams-Ashman, 1988). The main function of LH in the males is to stimulate *de novo* synthesis by Leydig cells of T, high

concentrations of which must be present in Sertoli cells for sperm production to occur. However, Courot (1971) has also shown that T treatment alone in hypophysectomized ram lambs was not able to initiate spermatogenesis. Similarly, T alone does not quantitatively support spermatogenesis in the adult ram (Courot et al., 1979; Courot and Ortavant, 1981).

In the rat, FSH and testosterone are required for both the initiation of spermatogenesis in immature animals and its reinitiation after prolonged regression such as occurs in adults after hypophysectomy (Sanborn et al., 1986). Recently, Awoniyi et al (1990) reported that qualitative restoration of spermatogenesis can be achieved with T alone in hypophysectomized adult rats but that this treatment failed to produce the quantitative restoration of spermatogenesis. Interestingly, in adult rats actively immunized against GnRH or LH, quantitative restoration of spermatogenesis with T alone has been demonstrated (Awoniyi et al., 1989; 1990).

In summary, although the exact hormonal control of the seminiferous tubules is still unknown and might vary from one species to another, for the male sheep, it is generally considered that the two hormones that are directly required by Sertoli cells to support spermatogenesis in all its phases are FSH and T. The mechanism whereby the combination of FSH and T maintains spermatogenesis remains to be clarified. Androgen and FSH receptors are present in the Sertoli cells but are apparently lacking in all differentiating male germ cells, which are thus not directly affected by these hormones (Steinberger and Steinberger, 1977; Bardin et al., 1988). LH plays a crucial role as it stimulates T secretion by a direct action on the Leydig cells (Williams-Ashman, 1988).

Besides the direct effect of these hormones on the Sertoli cells, their function is regulated by a complex interplay of signals from the local environment (e.g. paracrine regulation from Leydig, peritubular and germ cells). The complex interactions between the different cells suggest that intercellular communication between the components of the testis is important in maintaining the proper milieu for the spermatogenic process (Sanborn et al., 1986).

iii) Oestrogen receptors in seminiferous tubules

The observation that Sertoli cell lines have oestrogen receptors (ER) and are growth inhibited by low levels of oestradiol suggests that these cells might respond to this hormone under physiological situation (Bardin et al., 1988). Interestingly, ER are present at or below the level of detection (i.e. undetectable level) in newly isolated Sertoli cells. However, following 15 days of culture, ER rise to a detectable level (Nakla et al., 1984; see section 1.9.1.iv). The appearance of ER in primary Sertoli cells with increasing time in culture suggests that these cells are capable of producing this class of receptors even though they are undetectable in freshly isolated cells. The reasons why ER may not be measurable in newly isolated Sertoli cells have been recently discussed by Bardin et al. (1988).

e.g. ER could be present in only a limited number of Sertoli cells along the length of the tubule or ER could be uniformly suppressed by some testicular products (Bardin et al., 1988).

iv) Spermatogenesis and germ cells

The Sertoli cells clearly provide mechanical support for the developing germ cells at all stages of differentiation and contribute to a localized environment conducive to germ cell development (de Kretser and Kerr, 1988). The sequence of cytological events that result in the formation of mature spermatozoa from precursor cells is known as spermatogenesis. The whole spermatogenic process in the ram lasts 49 days (Ortavant, 1959).

There are three major elements that together constitute spermatogenesis: stem cell renewal by the process of mitosis, reduction of chromosomal number by meiosis and the transformation of a conventional cell into the complex structure of the spermatozoa by a series of changes involving no further cell division, but representing a metamorphic process termed spermiogenesis (de Kretser and Kerr, 1988).

The stem cells for the spermatogenic process are termed spermatogonia, which undergo mitotic division. Morphologically, the spermatogonia can be classified into three types: A, Intermediate (IN) and B spermatogonia. The spermatogonia not only must provide the precursors for the meiotic process but also must renew themselves. The cells in the spermatogenic process that are involved in meiosis are the primary and secondary spermatocytes. The process of meiosis actually involves two cell divisions. In the first, which involves the primary spermatocytes, the

chromosomes appear each as pairs of chromatids, subsequent to which homologous chromosomes pair by synapsis to form bivalents. Each member of the bivalent pair subsequently moves to the daughter cells, termed secondary spermatocytes which contain half the number of chromosomes (haploid number), but because each chromosome is composed of a pair of daughter chromatids, the actual total DNA content is equivalent to that of somatic cells. The second division occurs after a relatively short duration, and during this, the chromatids of each chromosome separate into daughter cells by mechanisms similar to those of mitotic divisions. The daughter cells, termed spermatids, contain an haploid number of chromosomes and half the DNA content of somatic cells. The transformation of spermatids to spermatozoa involves a fascinating but complex sequence of events that constitute the process of spermiogenesis. No cell division is involved, but the process is in essence a metamorphosis in which a regular cell is converted into a highly organized motile structure. Spermiogenesis differs greatly in detail among eutherian species, as does the morphology of testicular, epididymal and ejaculated spermatozoa (de Kretser and Kerr, 1988).

Meiosis does not proceed completely before puberty in most species including the sheep. Because pachytene primary spermatocytes are formed prior to the effective establishment of the blood-testis barrier, it seems that the meiotic maturation process taking place during the initiation of spermatogenesis is not dependent on the formation of an adluminal epithelial compartment (de Kretser and Kerr, 1988). There are suggestions that a specific meiosis-preventing substance is present in the testis in juvenile animals, but the nature and physiological importance of this material is uncertain (Williams-Ashman, 1988).

v) Peptides and proteins produced by Sertoli cells

It is important to realize that a broad spectrum of biochemical activities have been ascribed, directly or indirectly, to Sertoli cells. They include general metabolic properties, specific enzymatic and metabolic activities and secretory products (review: Sanborn et al., 1986). Among these secretory products, various peptides and proteins have been identified.

Inhibin and members of the inhibin family

Current knowledge on inhibin has been reviewed extensively during the past years (Findlay, 1986; de Jong, 1987, 1988; Ying 1988; Lincoln et al., 1989; Risbridger et al., 1989; de Kretser and Robertson, 1989; de Kretser, 1990; Risbridger et al., 1990; Knight, 1991).

Inhibin is a heterodimer molecule comprising α and ß subunits linked with disulfide bonds. There are two different subunits, termed ßA and ßB, which share approximately 70% amino acid sequence homology. Consequently, two mature forms of inhibin have been isolated (α-ßA and α-ßB), both of which demonstrate the same biological action of suppressing FSH release. Even though Sertoli cells are considered to be the major intratesticular sources of inhibin, mRNA for the α, ßA and ßB subunits are expressed in a variety of tissues, including the placenta, pituitary and brain. Recent findings suggest that the Leydig cells can themselves secrete inhibin-α (Maddocks and Sharpe, 1989b).

The production of inhibin appears to increase with age and to be stimulated by FSH (Lee et al., 1984; Lee et al., 1986). Furthermore, the distribution of inhibin also appears to change from a predominantly extratubular to an intratubular pattern as the testes mature (Maddocks and Sharpe, 1989a; 1990). That steroids and gonadotropic stimulation (hCG) also affect production of inhibin-α by the testis and perhaps its route of secretion as been suggested by these last authors.

Inhibin has been implicated in the control of FSH secretion in adult and prepubertal mammals (see section 1.6.2 v). Inhibin may have a (negative) paracrine effect on some aspect of spermatogenesis. Leydig cells and spermatogonia have been suggested as intratesticular targets for inhibin (Risbridger et al., 1990).

Active immunization against partly purified inhibin from 3 weeks of age increased sperm production and testis diameter in Merino lambs at 23 months of age but did not significantly affect those parameters in crossbred ram lambs. If immunization was started at 9 weeks of age, no effect was observed in those lambs (Al-Obaidi et al., 1987).

Other members of the inhibin family are produced by Sertoli cells. Activin (also termed FSH-releasing peptide) are composed of the two ß subunits of inhibin ($ß_A ß_A$ or $ß_A ß_B$). This molecule exhibits the opposite biological activity of inhibin and stimulates FSH secretion from

pituitary cells. Two other products that belong to this same family of peptides are transforming growth factor-ß (TGF-ß) and Mullerian-inhibiting substance (MIS) (see section 1.4.2.i.).

Other peptides and proteins produced by Sertoli cells

A number of proteins have been reported to be secretory products of Sertoli cells. Among them, we find: plasminogen activator, transferrin, testibumin, GnRH-like substance (Sanborn et al., 1986). One important protein secreted by the maturing testis (in rat, rabbit, ram, bull, man) is androgen binding protein (ABP) which specifically binds T and DHT and is thought to be under the direct control of FSH (French and Ritzen, 1973; Jegou et al., 1978; Bardin et al., 1981; Lobl, 1981). This protein may influence androgen action or metabolism particularly in the caput epididymis where it is present in high concentrations (Bardin et al., 1981). Certain growth factors have been localized to and are known to be produced by Sertoli cells, including seminiferous growth factor (SGF), somatomedin C / insulin-like growth factor (IGF-1), Sertoli cell-secreted growth factor (SCSGF), transforming growth factor α and ß (TGFα, TGFß) and interleukin-1α (1L-1α) (Bellvé and Zheng, 1989). These trophic peptides act through autocrine and paracrine pathways. Other postulated Sertoli cell secretory factors may be involved in the regulation of Leydig cell function and their future characterization and identification should provide a better understanding of the paracrine control of testicular cells (Sharpe 1984; Sanborn et al., 1986; Saez et al., 1989). Interestingly, protein secretion is increased at the time when spermatogenesis is starting in ram lambs (Waites et al., 1985).

vi) Steroids produced by Sertoli cells

Some steroidogenic enzymes are found within the seminiferous tubule, which can convert progesterone to T. Immature rat Sertoli cells are capable of aromatization under FSH stimulation (see section 1.9.1.iii). It seems unlikely that Sertoli cells contribute significantly to the androgen pool in the blood of adult animals. There is some in vitro evidence however that the seminiferous tubules could make a physiologically detectable contribution to steroid production (e.g. maintaining the function of the seminiferous tubule, rete testis, or epididymis), but there is no

evidence that the seminiferous tubules can maintain spermatogenesis by their own production of androgens (Bardin et al., 1988).

1.6.3.4. Interstitial tissue

The organization of the intertubular tissue varies dramatically between species, but contains the blood vessels, lymphatics, and nerve fibres. The Leydig cells (interstitial cells) are scattered in groups in the intertubular tissue in relation to vasculature and lamina propria of the seminiferous tubules, the outer layers of which consist of modified smooth-muscle cells termed myoid cells (de Kretser and Kerr, 1988). Some of the secreted proteins in the rat were shown to arise from those peritubular myoid cells. Such cells in co-culture can influence the efficiency of ABP secretion by Sertoli cells and act cooperatively with Sertoli cells to synthesize the various components of the extracellular matrix of the basal lamina (Waites et al., 1985). Furthermore, they could play an important role as intermediate between Sertoli cells and the interstitial environment by their capacity to produce important regulatory factors (Skinner 1990; 1991).

i) Leydig cells

The Leydig cells arise through metamorphosis of mesenchymal cells. This is generally accepted as the sole means of formation of new cells since mitosis are rarely seen (Courot and Kilgour, 1984). In general, the numbers of these cells increase during fetal development, then after a certain time decrease, often to reach a very low level at birth. In ram lambs, the number of Leydig cells per testis increase about 7 fold between 25 and 100 days of age (Monet-Kuntz et al., 1984). Once full sexual maturity has been reached, the number of Leydig cells remains fairly constant. The adult population of Leydig cells appears to be functionally different from the population found in the fetal testis (Courot and Kilgour, 1984).

The most conspicuous feature of the Leydig cell is an extensive endoplasmic reticulum, most of which is smooth (Hall, 1988). The smooth endoplasmic reticulum (SER) is generally believed to be related to the synthesis of cholesterol, (which is consistent with the idea that the Leydig cell makes much of its own steroidogenic cholesterol) and steroid hormones. In addition, the

Leydig cell contains rough endoplasmic reticulum (RER) with ribosomes. The RER interconnects with the SER and must be responsible for the synthesis of the proteins required for the response of Leydig cells to LH (Hall, 1988).

The Leydig cells are the most significant source of androgen, and it is generally accepted that they maintain a high local concentration of androgen in and around the seminiferous tubules. Leydig cells are also capable of aromatization (formation of oestrogen) (Hall, 1988; Williams-Asham, 1988). It is also important to remember that Leydig cells are not only active in producing androgens but that they can also produce prostaglandins, opiates and angiotensins (Rommerts et al., 1987).

ii) Hormonal control of steroidogenesis

In hypophysectomized animals, the synthesis of androgens by the testis proceeds at a greatly reduced rate. The pituitary owes its ability to stimulate the synthesis of androgens to the synthesis and secretion of LH, the hormone providing the most important physiological regulation of the production of androgens by the testes (Hall, 1988). The membrane of the Leydig cell contains the LH-receptors coupled to adenylate cyclase. The receptors can bind both LH and hCG, so it is known as the LH/hCG receptors. The Leydig cell is said to show spare receptors, because maximal steroidogenesis can be obtained at concentrations of LH that are sufficient to occupy only a fraction of the total number of available LH receptors. Recently, with a pure preparation of FSH, it has been shown that injection of this hormone to hypophysectomized rats induces a dose-dependent increase in testicular LH receptor number (Closset and Hennen, 1989) and Leydig cell steroidogenic capacity (Teerds et al., 1989). A direct action of FSH on Leydig cells seems unlikely since FSH binding in isolated Leydig cells is negligible (Dorrington et al., 1978). FSH has also been implicated in the control of maturation of Leydig cells. It is possible that immature Sertoli cells, in response to FSH, secrete a factor which induces increased numbers of LH-binding sites of the testicular tissue (Dorrington et al., 1978). It is also possible that oestrogen may be important in the control of Leydig cell function (see section 1.9.2.3.).

The list of other agents known to influence response of Leydig cells to gonadotropins and to alter the rate of production of the gonadal steroids, includes PRL, GH, GnRH, insulin and catecholamines (Sharpe 1984; Sharpe et al., 1986; Rommerts et al., 1987).

iii) Steroidogenic pathway

The synthesis of steroid hormones require a substrate (that is, a source of the steroid ring system or depots of cholesterol), a series of enzymes and cofactors that together constitute the biosynthetic pathway and a source of energy (Hall, 1988). The pathway begins with the conversion of cholesterol to pregnenolone (C27 side-chain cleavage P-450) which takes place in mitochondria. The pregnenolone so formed moves to the microsomal compartment, where it is attacked by membrane-bound enzymes including the C21 side-chain cleavage P-450 which converts the 21-carbon steroid to a 19-carbon androgen, androstenedione, or dehydroepiandrosterone (DHEA), depending on whether the $\Delta 4$ or $\Delta 5$ pathway is used. Androstenedione is converted to the principal androgen, testosterone, by 17ß-hydroxysteroid dehydrogenase. The processes of transport to and within the mitochondria appear to be points at which LH accelerates steroid synthesis. There may be, in addition, stimulation of the side-chain cleavage reaction itself (Hall, 1988).

iv) LH-receptors and mechanism of action

Down-regulation (loss of LH receptors) and desensitization (blockage beyond c-AMP) of Leydig cells have been observed following repeated injections of LH (see GnRH-receptors, section 1.6.2.iii). Down-regulation should be seen as part of the normal cellular processing of receptors. Various explanations have been given to the phenomenon of desensitization. It is not clear if cholesterol depletion occurs *in vivo* under physiological circumstances. When the individual steps in the biosynthetic pathway to T are examined in desensitized Leydig cells, C21 side-chain cleavage is seen to be depressed, and, with higher doses of LH at first injection, C27 side-chain cleavage of cholesterol may also be inhibited (Hall, 1988). One possible factor

involved in the mechanism of desensitization appears to be the synthesis of oestrogens by the Leydig cell (see section 1.9.2.3).

1.6.3.5. Major testicular steroids

i) Androgens

The principal steroids secreted by the testis of most adult mammals are androgens, and T is by far the most important androgen (with the exception of the pig testis which secretes as much of 5 α-androsterone as T and almost as much dehydroepiandrosterone sulphate and oestrone sulphate; see Setchell and Brooks, 1988). Other androgens also leave the testes by the spermatic vein, and these include androstenediol, androstenedione, dehydroepiandrosterone (DHEA), and dihydrotestosterone (DHT) but the concentration of these androgens is much lower than T, with all being generally less than 15 % of that of T (Coffey, 1988). The complete biologic importance of many of these steroids circulating in the plasma has not been determined. The predominance of T production throughout prepubertal development distinguishes male sheep from other mammals, in which androstenedione (e.g. bulls) and other 5 α-reduced (e.g. rats) products are preferentially secreted during certain periods of juvenile life (Setchell and Brooks, 1988). DHT is a considerably more potent androgen than T in several bioassays (Coffey, 1988). Thus, it is considered that in those tissues with a capacity to reduce T to DHT, the latter derivative serves to amplify the androgenic potency of T (intracellular mediator). Several observations indicate that DHT is the major active androgen in the accessory sex organs (see section 1.7.), whereas in the mature testis it is T (Bardin et al., 1988; Coffey, 1988). Steroid 5 α-reductases are also present in the hypothalamus and the pituitary. In the testis, spermatocytes and Sertoli cells have a greater 5 α-reductase activity than do spermatids, but there is very little of this enzyme in Leydig cells. This enzyme is higher in testes from younger animals (Bardin et al., 1988). The concentration of DHT in blood is mainly derived by peripheral conversion from plasma T since the testes secrete relatively little DHT. The steroid 5 α-reductase reaction is essentially irreversible and DHT is not a substrate for the aromatase system that converts T, DHEA or

androstenedione to oestrogens. Thus, DHT once formed from T cannot be transformed into oestrogens. T and DHT can be enzymatically converted into several other derivatives with diminished or altered types of biological activities in many tissues. T and DHT act via a common receptor. An important role for DHEA and androstenedione, two weaker androgens, may be their peripheral conversion to oestrogens through the aromatase reaction (Coffey, 1988). Androgens play a paramount role in the development and maintenance of masculine characteristics in male mammals. These hormones play a pivotal role in several aspects of sexual maturation including fetal differentiation of the extragonadal organs of the male genitourinary tract, and the complete development of the male tract and secondary sexual tissues that begins at puberty. T also exerts a tight control over gonadotropin secretion by negative-feedback loop (see section 1.6.2.iv). The stimulation of spermatogenesis by T via its action on Sertoli cells is probably the most fundamental function of this hormone. Androgens are essential for the establishment of male courtship, coital behaviour patterns, and the maintenance of libido. In addition, androgens stimulate the development of the musculoskeletal system and a variety of other tissues including the skin and the kidneys (Mainwaring et al., 1988).

ii) Oestrogens

The testis also produces small amounts of oestrogens (Hall, 1988). The testis of the stallion and of the boar are remarkable because of their important oestrogen secretion (Setchell and Cox, 1982; Setchell et al., 1983). In the rat, indirect evidence points to the Leydig cells as the major testicular source of such oestrogens, although the Sertoli cells (more probably in immature animals) also contribute (Tsai-Morris et al., 1985a; section 1.9.1. iii). Since oestrogen is the major subject of interest of this thesis, a complete section of this literature review will be devoted to this steroid (see section 1.9.).

iii) Progestagens

Progesterone, 17 α-hydroxyprogesterone, pregnenolone, 17 α-hydroxypregnenolone are also produced by the testis but at very low concentration. They are intermediate metabolites in the

steroidogenic pathways (Hall, 1988). Progesterone is capable of controlling gonadotropin secretion in rams (see section 1.6.2.iv). Although progesterone is weakly androgenic, it has been shown that it does not exert a significant effect on the prostate at the low concentrations present in normal male plasma (Coffey, 1988).

iv) Sulphated steroids

Sulphated steroids (e.g. oestrone-sulphate, DHEA-sulphate) are secreted by the testis of many species including pig, human, horse (Setchell and Cox, 1982; Setchell et al., 1983; Setchell and Brooks, 1988). The significance of these conjugated steroids is not known at present but may be important in testicular development in some species since, an approximate 5-fold increase in the sulphated steroids occurs during pubertal development in the boar (Setchell et al., 1983).

v) Mechanism of action of steroids

Steroids (free form) are small lipophilic molecules that can readily diffuse into cells where they are retained if that cell is a responsive or target cell (Gorski et al., 1986). The separate receptors for androgens (AR) and oestrogens (ER) are detectable in a wide variety of cell types formed in the reproductive tract of both sexes and in many other locations in mammals. The expression of the AR and ER genes, and hence the tissue contents of these receptors, are regulated differentially in various tissues by a large number of factors, including developmental status, age, and many sorts of homologous and heterologous hormones (Williams-Ashman, 1988). AR and ER are often present together in responsive cells. These receptors contain hormone-binding sites that exhibit extremely high affinities for their steroid ligands. DHT binds to the AR of most tissues in most species with a greater affinity than does T. The net consequence is that DHT formation generally amplifies the androgenic signal (Williams-Ashman, 1988). The intracellular localization of steroid receptors has been a much debated issue in recent years (Gorski et al., 1986). Immunocytochemical (King and Greene, 1984) and enucleation studies (Welshons et al., 1984) have demonstrated that ER was exclusively nuclear regardless of the

hormone status. The presence of ER in cytosol fractions is now generally regarded as an homogenization artefact resulting from dissociation of loosely bound nuclear ER.

The hormone-receptor complex formed bind to DNA and other anionic substances and lead to differential changes in the transcription of subsets of genes. Thus, the majority of the cellular effects of these hormones can ultimately be accounted for by alterations in the production and accumulation of intracellular or secretory enzymes and structural proteins (Williams-Ashman, 1988; Hall, 1988). However, other possible mechanisms of action that also may be important will be further discussed for oestrogens in section 1.9.1.v.

vi) Steroid binding proteins

Under physiological conditions, gonadal steroids are bound with a low-affinity to albumin (relatively unspecific binding) and with higher affinity to the more specific sex hormone-binding globulin (SHBG). In relation to DHT and T binding to SHBG, oestradiol binds to a lesser degree with the relative order being DHT 3 times that of T which is, in turn, approximately 2 times that of oestradiol (Mawhinney and Neubauer, 1979). Free levels of plasma oestradiol and T range from 1.7 to 2 % of the total hormone (this percentage in higher for rat and pig which lack SHBG). Gorski and co-workers (1986) believe that this estimate is directly proportional to the available intracellular pool of free steroid (biologically active form), if care is taken to accurately measure the free (non-protein bound) extracellular concentration of steroid at equilibrium. However, other authors have reported that plasma protein-bound steroids are not necessarily biologically inactive (Siiteri et al., 1982; Siiteri and Simberg, 1986 ; Pardridge, 1985, 1986, 1987). However, because this hypothesis is still very much debated (Edwards and Ekins, 1988) and for the sake of brevity, we will assume in the remainder of this review that the protein-bound steroids (including antibody-bound steroids) are biologically inactive (see section 1.10.5.i).

Using ammonium sulphate precipitation, Chandrasekhar et al. (1986) estimated that the level of SHBG found in one litre of plasma of mature Merino rams could bind approximately 5 nM of radiolabelled DHT (~ 1.5 ng DHT/ml). In man, the plasma concentration of SHBG exceeds that

of T (bound and free forms) and is far more than the normal peripheral oestradiol concentration. Steroid-protein binding does not appear to vary during prepubertal development, however, it has been shown to increase after oestrogen administration and to decrease after T treatment (van der Molen et al., 1981). Physiological functions of SHBG include protection of steroids from rapid degradation and the dampening of the fluctuations of the free steroid concentration (van der Molen et al., 1981).

Other plasma proteins that bind steroids include corticosteroid-binding globulin (CBG, also called transcortin), progesterone-binding globulin (PBG), and, to a lesser extent, the α-acid glycoprotein (AAG). The total amount of steroid bound to PBG and AAG is not large and is usually ignored (Coffey, 1988). As T levels increase in the plasma, the order of increasing saturation of the plasma proteins proceeds from SHBG to CBG to albumin. *In vivo* , complex binding equilibria occur between free steroid, serum proteins, non-specific tissue binding, and the steroid receptors. Thus in all cases, one deals with complex binding equilibria. Generally the disappearance of steroids from circulation is inversely related to the extent of their protein binding. In fact, only free steroids are available for metabolism by the liver and intestines, primarily to 17-ketosteroids, which are then secreted into the urine as final water-soluble conjugates with sulfuric acid or glucuronic acid (Coffey, 1988).

vii) Steroids dynamics and puberty

Further studies are required to elucidate the role of steroid dynamics in the endocrine processes of puberty in different species, e.g. movements and exchanges within and between various tissues + fluids. In the domestic pig, changes in steroid production and clearance rates are thought to contribute to the endocrine mechanisms of puberty (Elsaesser et al., 1982).

viii) Extragonadal source of steroids: The adrenal gland

The adrenal gland secretes many steroidal hormones, including glucocorticoids, mineralocorticoids, progestagens, oestrogens and aromatizable androgens (Coffey, 1988; Hall, 1988). In a number of species (e.g. human, rat) it has been shown that some of these hormones

are secreted in amounts sufficient to influence the reproductive system. There have been few studies on the role of adrenal steroids in reproduction in sheep.

Recently, Sanford (1987b) found an increase in LH pulse frequency in castrated rams after prolonged immunization against oestradiol-17ß. This result suggested that there was still some extragonadal oestrogens acting on the hypothalamic-pituitary axis in these animals (in which the gonadal source of steroids has been completely eliminated) and that the antibodies could prevent the hormone from reducing LH secretion. There is no evidence indicating whether the adrenal glands act by secreting oestrogens directly, or by secreting androgenic steroids which are aromatized to oestrogens at other sites in the body (Adams et al., 1990).

1.6.3.6. Other important factors to consider when testicular function is concerned

i) Testicular Blood flow

Blood flow and vascular permeability in the testis are important aspects of testicular physiology because they are crucial for the transport of nutrients and secretory products to and from the testis. The vascular organization of the testis is also very important to maintain a "special" homeothermia in this organ (see section 1.6.3.6.ii). Arterio-venous transfer of blood takes place in the spermatic cord of the ram (Noordhuizen-Stassen et al., 1985). In the rat, there are important changes in the organization and permeability of the vasculature of the testis during testicular development (Kormano 1967a; Setchell et al., 1988; Damber et al., 1990) including a tendency for arterio-venous transfer of blood to increase with age (Maddocks and Sharpe, 1990). During pubertal development, testicular capillary blood flow (TCBF: the fraction of the total blood flow that exchanges with tissue via the capillaries) increased in the fox (Joffre, 1973). No modification of TCBF has been observed between impubertal, prepubertal and adult ram in the breeding season and, lower TCBF was found in the adult ram in the non-breeding season (Courot and Joffre, 1977). No relationship between total testicular blood flow (TBF) and testicular growth has been reported yet for the male sheep. Factors involved in the control

of testicular blood flow have been recently reviewed (Setchell and Brooks, 1988; Setchell et al., 1991). It is not known whether or not the reproductive hormones (e.g. hCG, steroids) directly influence the blood flow to the testis in the ram lamb (see section 1.9.2.4.).

ii) Thermoregulation

In the ram, as in the majority of eutherians, spermatogenesis will not proceed to completion unless the temperature of the testis is a few degrees lower than that of all other internal organs of the body. Two structures are responsible for maintenance of the testis at this lower temperature: the scrotum and the pampiniform plexus of testicular veins (Waites, 1970; Setchell, 1978). The cooling effect of the scrotum on the testis is in large part due to evaporative heat loss effected by the luxuriant population of sweat glands in the scrotal skin; this occurs even in species such as sheep in which sweating from other parts of the body is not important for maintenance of homeothermia (Waites and Voglmayr, 1963). The arterial blood is pre-cooled as it passes through the spermatic cord by counter-current heat exchange with cooler venous blood. This system ensures that this cooling is rapidly spread through the testis (Setchell, 1978; see recent reviews in Zorgniotti, 1991). It must be noted that T secretion by Leydig cells is much less deleteriously affected by exposure of the testis to body temperatures than is spermatogenesis, so that cryptorchid or short-scrotum animals may develop and maintain many androgen-dependent masculine characteristics other than the capacity of the seminiferous tubules to produce spermatozoa (Setchell, 1978).

The descent and growth of the testis occurring during testicular maturation must certainly drive important adjustments in the thermoregulatory mechanisms and to our knowledge this subject (including change in testicular vasculature, blood flow and temperature) has not been studied in ram lambs.

1.7. Morphological changes in other accessory male organs during pubertal development in ram lambs.

The differentiation and maintenance of scrotal structure is highly dependent on androgenic hormones (Setchell, 1978). The time of descent of the testes into the scrotum is species dependent but generally, takes place during later stages of fetal development in the sheep. Dependence of the process of testicular descent on androgens and/or gonadotropins is suggested however one must keep in mind that this process is both complex and still incompletely understood (Wensing and Colenbrander, 1986). In addition to the requirement for androgens there is a significant contribution of pituitary growth hormone to penile growth. Because T synthesis is gonadotropin dependent during the latter two-thirds of gestation, it follows that growth of the penis and testicular descent which takes place during this time are probably gonadotropin-dependent as well (Georges and Wilson, 1988). A feature of immature rams is the presence of adhesions between penis and prepuce. With maturity the penis gradually becomes freed (urethral process first, then gland). In well grown rams this change is completed at approximately five months (Dun, 1955).

All male mammals develop a series of specialized secretory organs from the fetal urogenital sinus (the prostate and bulbourethral glands) and in some species the wolffian duct develops into seminal vesicles and a secretory ampulla of the vas deferens (present in the ram). The differentiation of these structures in the fetus, as well as their extensive growth and secretory activity initiated at puberty, is utterly dependent on androgens. Although T is the primary plasma androgen that induces growth of the prostate gland and other sex accessory tissues, it appears to function as a prohormone in that the active form of the androgen in the organs is not T but DHT (Bardin et al., 1988; Coffey, 1988). From the fact that the epididymis and the prostate contain oestrogen receptors (see section 1.9.1.iv), it has been suggested that this hormone plays a role in the functioning of these reproductive structures. Theories have been advanced that prolactin stimulates growth of the accessory sex organs by a direct effect on the tissues themselves or by synergism with LH and T (Ravault et al., 1977; Klindt et al., 1985; Yarney and Sanford, 1989).

Of the many different types of mammalian organs, the greatest variation between species is observed in the anatomy, biology, and function of the sex accessory tissues (Coffey, 1988). Even, the ejaculate volume varies dramatically between species for no known reason (only 1 ml in the ram vs 250 ml in the boar). This variation is also reflected in the biochemical composition of the glandular secretions and combined ejaculate. Although it might be argued that these variations have no biological meaning, this is difficult to accept and it might be suggested that the great diversity of environments and reproductive habits of mammals regulates different sex accessory functions to protect their genitourinary tracts from invasion by pathogens or external insult (Coffey, 1988).

The only important function of the prostate and bulbourethral glands together with those of the seminal vesicles is to produce the bulk of seminal plasma, which also contains components of rete testis and epididymal fluids. Seminal fructose is the principal sugar in the seminal plasma of most mammals and serves as the major exogenous source of substrate for lactate production by sperm glycolytic enzymes (Mann, 1964; 1975; Mann and Lutwak-Mann, 1981). The principal substance in seminal plasma is water. There can be no doubt that the overriding function of seminal plasma is a hydrodynamic one, to ensure that the semen is effectively ejaculated into the female genital canal (Williams-Ashman, 1988). Antibacterial properties of seminal plasma are also recognized (Foster et al., 1988).

Epididymal development and function are strictly regulated by androgens. Sperm maturation itself is dependent upon androgen-controlled secretory products of the epididymal epithelium.(Orgebin-Crist, 1986; Amann, 1987; Brooks; 1987; Fournier-Delpech and Courot, 1987). In fact, mammalian spermatozoa leave the testis as immature cells unable to fertilize until they have traversed the epididymis. Sperm maturation is an orchestrated series of sequential events occurring at different levels of the epididymis: sperm-zona pellucida binding ability in the proximal epididymis, progressive motility and the ability to penetrate and fertilize the oocyte in the middle epididymis and the ability to initiate normal embryonic development in the distal epididymis (Orgebin-Crist, 1986). When ejaculation is not exceptionally frequent, virtually all

the sperm in semen originate from the cauda of the epididymis and the vas deferens (Amann, 1981; Amann and Schanbacher, 1983; Amann, 1987).

Pubertal changes in accessory sex organs weights have been reported by Skinner et al. (1968) for the Suffolk rams and more recently by Yarney and Sanford (1989) for crossbred rams. The weights of the bulbourethral and vesicular glands approximately doubled between 1 and 3 months of age while epididymal weight increased 4-fold during this period. Between 3 and 5 months of age, the weights of all three accessory sex organs increased approximately 6-fold. Thereafter, the accessory sex organs continued to grow constantly to reach adult size.

In some breeds of sheep, horn development is one of the most apparent physical changes that occurs during pubertal development in the male.

1.8. Behaviour: from male infancy to adulthood

The various aspects of the mating behaviour of rams have been fully described by Banks (1964). Generally, full mating is not achieved until the age of 6 months (Bryant, 1975). However, physiological age is again more important than chronological age in the determination of mating behaviour (Dyrmundsson and Lees, 1972). Young postpubertal rams often seem to find themselves in situations where their reproductive axis is functional, but where they are inhibited from using it, or where they can use it only inefficiently, because of behavioural immaturity (Al-Nakib et al., 1986). The degree to which emotional stress and its endocrine ramifications contribute to this problem is unknown, but it seems probable that this is a contributing factor. Repeated libido tests during the growing phase of ram lambs do not initiate sexual interest in the inactive animals, but enhance it in those lambs which already possess it. This observation demonstrates the importance of sexual experience (Al-Nakid et al., 1986).

Although social and environmental factors play a role in sexual behaviour, a large body of evidence supports a cause-and-effect relationship between steroid hormones and sexual behaviour. Castration of the postpubertal rams cause a reduction in libido and the capacity for sexual intercourse but its effects are gradual and often incomplete. In general, T (but not DHT) replacement to castrated rams can restore normal sexual behaviour. The possibility exists,

however, that oestrogens also contribute to the regulation of normal sexual behaviour (see section 1.9.2.8; Mawhinney and Neubauer, 1979; D'Occhio and Brooks, 1980; Parrott and Baldwin, 1984).

1.9. Oestrogen participation in the male reproductive system

1.9.1. Oestrogens in the male: general points

i) Estimation of oestrogens in body fluids

Estimation of oestrogens was for a long time performed with bioassay methods and more precise biochemical detection techniques (spectrophotometry, fluorimetry, double isotope dilution). However, these techniques are usually not very sensitive and relatively unreliable (van der Molen et al., 1981). The introduction of radioimmunoassay techniques for oestrogens (Abraham et al., 1970) made it possible to perform oestrogen estimations in male plasma and tissue samples on a routine basis. The result of such estimations have provided useful data on the origin and functions of oestrogens in males. In fact, the demonstration of the presence of oestrogens in male tissues, destroyed the myth that these steroids were specific to the female.

ii) Oestrogen level in the ram

Fetal circulating oestrogen concentrations are reported to increase after 100 days of gestation (Findlay and Seamark, 1973; Mueller et al., 1981). Olster and Foster (1986) reported that oestradiol levels range between 0.25 pg/ml and 1.0 pg/ml in prepubertal ram lambs up to 20 weeks of age. By 32 weeks of age, however, oestradiol concentrations had increased to 2.0 pg/ml. Concentrations of oestradiol-17ß ranging up to 4.2 pg/ml have also been reported in pubertal ram lambs (Pope et al.,1990). In castrated ram lambs (4 and 10 weeks of age), detectable concentrations of oestradiol-17ß was also found (<1 pg/ml). Furthermore, sub-capsular testis venous plasma, pooled from intact and hemicastrated prepubertal lambs (~14 weeks old), contained concentrations of oestradiol-17ß in the range of 80-120 pg/ml (Pope et

al.,1990). In testis venous plasma of intact ram lambs (8-12 weeks old), Watts et al (1989) reported concentrations of oestradiol ranging between 10.6 pg/ml and 33.5 pg/ml.

In adult rams, oestradiol levels in the peripheral blood circulation are less than 0.5% of T levels (Schanbacher and Ford, 1976a; Sanford et al., 1982b, Sanford and Robaire, 1990). A definite seasonal increase (approximately 40%) in mean circulating oestrogen levels (~ 12 pg/ml) during the fall breeding season which was highly correlated with the concurrent increase in T levels (r=0.88) have been reported (Sanford et al., 1982b). Oestradiol levels decrease only 50 % (from ~12 to 6.0 pg/ml; Sanford and Robaire, 1990) following castration of adult rams.

Note: These are relatively high oestradiol levels for rams and may reflect the performance of the assay rather than 'true' levels for rams.

iii) Sites of aromatization and regulation

Plasma oestradiol and oestrone originate from gonadal and extragonadal tissues (Longcope et al., 1969; 1978; Dorrington et al., 1978; van der Molen et al., 1981). The androgenic C19 steroids are converted to the oestrogenic C18 steroids by removing the 19-methyl group and by subsequently forming an aromatic or phenolic steroid A ring (aromatase reaction), present in both oestradiol and oestrone. Oestradiol is formed from T, and oestrone is formed from androstenedione; these two oestrogens are then interconvertible (Naftolin et al., 1975; Mawhinney and Neubauer, 1979; Coffey, 1988).

With regard to the reproductive system, aromatization of androgen occurs in crucial tissues including the hypothalamus, the Sertoli cells (at least in the immature testes) and the Leydig cells (Naftolin et al., 1975, Tsai-Morris et al., 1985a). In the pituitary, only low level of aromatization has been found (Naftolin et al., 1975).

In the adult human, on whom the most extensive studies have been done, radioisotope infusion has identified adipose tissue, skeletal muscle, skin and prostate (connective tissue fibroblasts or stromal cells) as sites of peripheral oestrogen synthesis (Longcope et al., 1978; Hodgins, 1989). The dynamics of the synthesis of oestrogens in the ram have not been quantitated yet.

Observations in the rat suggest that during development the predominant site of the testicular aromatase activity shifts from the Sertoli cells to the Leydig cells and that both gonadotropins are able to stimulate aromatization of androgens, FSH acting on Sertoli cells and LH on Leydig cells

(Dorrington et al., 1978; Valladares and Payne, 1979; Moger, 1980; van der Molen et al., 1981; Tsai-Morris et al., 1985a,b). It has been recently suggested that testicular androgens and a paracrine factor produced by testicular peritubular cells (PModS) contribute to the natural age-dependent decline in Sertoli cell aromatase activity (Verhoeven and Cailleau, 1988a, b; Rosselli and Skinner, 1992). Interestingly, inhibition of rat Sertoli cell aromatase by a high molecular weight factor secreted specifically at spermatogenesis stages VII-VIII has been demonstrated (Boitani et al., 1981). The germinal cells at these stages are the most sensitive germ cells to T deprivation (Saez et al., 1987).

That some differences exist between species concerning the testicular cells responsible for the aromatization of androgens is likely. In the pig, Leydig cells seem to be responsible for all testicular oestrogen production (Saez et al., 1989). In the ram, it is still unknown which testicular cells produce oestrogen and if such a shift occurs during testicular maturation.

iv) Localization of oestrogen receptors (ER) in the male

The hypothalamus contains significant amounts of ER (Pelletier and Caraty, 1981; Glass et al., 1984). However, the concentration of ER and the localization of injected ^{3}H-oestradiol have been shown to be highest in the pituitary (Wise et al., 1975; Thieulant and Pelletier 1979; Pelletier and Caraty 1981; Glass et al., 1984). Unlike androgen receptors (Schanbacher et al., 1984), ER in the hypothalamic-pituitary axis do not decrease in number following castration (Thieulant and Pelletier 1979) which support the idea that there may be still some biologically active oestrogen in those rams. Pituitary DHT and oestradiol-17ß receptors tend to increase between 20 and 80 days of age in Préalpes du Sud rams. Values at 80 days were only 50-75% of the adult level (Pelletier et al., 1981). In bulls, during the transition into prepubertal development, levels of ER within hypothalamic tissue are considerably elevated (Amann et al., 1986).

Stumpf (1969) found radioactivity concentrated in the nuclei of the Leydig cells of immature rat testes after the injection of ^{3}H-oestradiol. High amounts of ER are present in purified rat Leydig cells but not in germinal cells (Brinkmann et al., 1972; Van Beurden-Lamers et al., 1974;

Mulder et al., 1974; de Boer et al., 1976). ER become detectable in primary Sertoli cells with increasing time in culture (see section 1.6.3.3.iii). Unlike the androgen receptors that have been well studied in ram testis (Monet-Kuntz et al., 1984), intratesticular ER have not yet been identified and characterised specifically in that species.

ER have been located also in the epididymis and the prostate gland of many species (e.g. mice, rats, rabbits, dogs, men) (Robaire and Hermo, 1988).

v) Mechanism of action of oestrogens

Oestrogen-receptor complexes bind to DNA and cause increased rates of transcription of a variety of genes (see section 1.6.3.5. iv), depending upon the respective target cell. For example, in the pituitary, oestrogen increases lactotroph transcription of prolactin gene, but decreases FSH and LH production in gonadotrophs. The transformed ER complexes bind with high affinity to many other nuclear components (such as RNA, histones, nuclear matrix, etc) and these could be the actual site of action of oestrogen (Gorski et al., 1986; Sutherland et al., 1988). Oestrogen may also be implicated in post-transcriptional regulation (Sutherland et al., 1988).

Recent studies on oestrogen-mediated cell growth indicate that oestradiol induces the secretion of specific growth factors. These growth factors may, in turn, affect cell growth by autocrine or paracrine mechanisms and form an obligate link between activation of transcription and oestrogen-induced cellular replication (Bellvé and Zheng, 1989). Proliferation may also be mediated directly through ER-mediated changes in the transcription of specific genes critical to cell cycle progression and DNA synthesis, or indirectly by the release from inhibition by serum-borne growth inhibitory factors. The direct effects of oestrogens on cell replication still remain speculative and controversial (Sutherland et al., 1988).

1.9.2. Oestrogen actions in the male

Relatively high circulating levels of oestrogens are known to exert marked demasculinizing and feminizing effects in males (Mawhinney and Neubauer, 1979). However, many experimental

evidences now support the hypothesis that, even under physiological conditions, oestrogens regulate many processes of the male reproductive system, including differentiation (see section 1.9.2.1), development (see 1.9.2.3 - 5) and maintenance (see 1.9.2.2 & 3, 1.9.2.6 - 8) of its various components.

1.9.2.1. Sexual differentiation of the hypothalamus

At present, most evidence supports the idea that the inability of the male rat to emit gonadotropins in a cyclic fashion is attributable, at least in part, to the action of the oestrogenic metabolites of T on the hypothalamus shortly after birth (Mawhinney and Neubauer, 1979). DHT did not sterilize neonatally treated female rats, but T, androstenedione, and oestradiol did; oestradiol was by far the most potent of the three agents in this action (Naftolin et al., 1975). However, sexual differentiation of the central nervous system may vary depending on the species studied (e.g. T does not cause defeminization in primates) (Mawhinney and Neubauer, 1979).

1.9.2.2. Oestrogens regulating the hypothalamic-pituitary axis

A [illegible] role for oestradiol in the steroidal regulation of the hypothalamic-pituitary axis is suggested by the presence of oestradiol receptors in the hypothalamus and pituitary (see section 1.9.1.iv) and by the extensive data demonstrating the effects of immunoneutralization as well as oestradiol administration on gonadotropin secretion (see section 1.6.2.iv). In ovariectomized female rhesus monkeys, oestrogen injected into the arcuate nucleus decreased the rate of GnRH secretion, as reflected in lowered gonadotropin levels and decreased frequency of LH pulses (Chappel et al. 1981). Moreover, recently developed techniques have allowed the direct measurement of GnRH pulse amplitude in sheep and revealed inhibitory effects of oestradiol, but it is not known whether this effect is due to a reduction in the amount of GnRH released by each neurone or a reduction in the number of neurones releasing a pulse (Thiéry and Martin, 1991). Interactions between oestrogen and catecholaminergic, opioidergic and other neurotransmittory systems could be of crucial importance in the control of gonadotropin secretion (see section 1.6.1.vii).

The possibility that oestradiol is more important than T in providing the homeostatic balance between the gonads and gonadotropin secretion in the male was suggested by Ciaccio et al.

L. 2: Subcutaneous implant containing oestradiol (E_2) were often used to maintain serum E_2 levels in castrated rams at concentrations similar to that found in intact rams and to suppress gonadotropin secretion (Riggs and Malvern, 1974; Schanbacher, 1979; 1980a; D'Occhio et al., 1983b; 1985; Sanford and Robaire, 1990). In hemicastrated and castrated ram lambs, E_2 implantation has also been shown to be very effective in reducing gonadotropins (Jenkins and Waites, 1983; Olster and Foster, 1986; 1988).

Whereas testosterone (T) and E_2 had qualitatively similar effects on LH pulse frequency (i.e. suppression), the amplitudes of LH pulses in E_2-replaced castrated ram lambs were usually lower than those observed in castrated and T treated-castrated ram lambs (Olster and Foster, 1986; 1988). Other researchers have also found that E_2-replacement therapy was more efficient than T treatment in reducing LH and FSH secretion in castrated rams (Edgerton and Baile, 1977; Schanbacher and Ford 1977; Parrott and Davies, 1979; Schanbacher 1979; D'Occhio et al., 1985). Evidence supports the theory that castration of males, in general, leads to either a loss or decrease in sensitivity to T negative feedback (but not to E_2 or DHT negative feedback) (D'Occhio et al., 1985). Overall, the suppression of gonadotropin secretion by DHT and E_2 is consistent with the theory that T acts as a prohormone and is converted to active metabolites at target tissues.

(1979). More recently, the significance of oestradiol feedback regulation was further illustrated by the fact that oestradiol replacement in rams is more effective than T in suppressing gonadotropin secretion in the non-breeding season when the hypothalamus-pituitary axis is most responsive to steroidal feedback (Olster and Foster, 1988; see INSERT)

Aromatization does not seems to be involved in the feedback action of T at the level of the pituitary where aromatase is lacking but is very important within the CNS (Naftolin et al., 1975). Peripheral and testicular oestrogens may directly affect the pituitary and the hypothalamus (Mawhinney and Neubauer, 1979).

Oestradiol and/or oestrone immunization did not affect LH concentrations in some experiments in rats (Hillier et al., 1975b), in rams (Land et al., 1981; Jenkins et al., 1986; Sanford, 1989; Sanford et al., 1991), in bulls (D'Occhio et al., 1987) or in stallions (Thomson and Honey, 1984). However, these findings do not necessarily exclude a role for oestrogens in the control of gonadotropins since feedback could presumably be maintained in those animals by increased circulating concentrations of T and consequently more central aromatization (beyond the blood-brain barrier where the oestradiol antibodies have no access and therefore could have no effect). However, in other studies, simultaneous increases in LH and T secretion have been observed in oestradiol-immunized animals (Nishihara and Takahashi, 1983; Sanford, 1985; 1987a; Schanbacher, 1979; 1984a; Schanbacher et al., 1987; Monet-Kuntz et al., 1988) and therefore, it is generally assumed that testicular and/or peripheral oestradiol production (within the reach of antibodies) is of greater physiologic significance to the ram than oestradiol production derived from central aromatization.

Antioestrogens, tamoxifen and clomiphene, also raise plasma LH levels however, it had been difficult to determine the physiological role of circulating oestradiol using those two antioestrogens because they were known to possess not only antioestrogenic but also oestrogenic properties in several experimental conditions (Hsueh et al., 1978; Sanford, 1985; 1987a). Administration of aminoglutethimide (AG: aromatase inhibitor), to testosterone-treated wethers was associated with a decrease in circulating oestradiol concentrations and a rise in LH secretion (Schanbacher, 1984a). Again it was concluded that peripheral aromatization (inhibited

by AG) contributes to the central pool of oestrogen which participates in the regulation of LH secretion in testosterone-implanted castrated rams. However, since many unspecific effects can be associated with the use of aromatase inhibitor, the interpretation of this result also remains difficult (Jordan, 1990; see section 1.9.3.).

The susceptibility of FSH secretion to oestradiol feedback has also been demonstrated in the ram. In fact, immunoneutralization of circulating oestradiol resulted in elevated plasma FSH in adult rams (Schanbacher, 1979; Sanford, 1987a; Schanbacher et al., 1987; Monet-Kuntz et al., 1988), suggesting that endogenous oestradiol does exert tonic inhibitory control over FSH secretion in the adult male sheep. Moreover, treatment of intact or castrated adult rams with this steroid results in a suppression of FSH secretion (Schanbacher and Ford, 1977; Alexander and Miller, 1982; Schanbacher, 1979). Schanbacher and Ford (1977) have shown that neither T nor DHT (in physiological amounts) could suppress the elevated concentrations of gonadotropins in cryptorchid rams. Oestradiol, on the other hand, reduced serum LH to concentrations observed in normal intact rams and serum FSH to intermediate levels. In this experiment, the inhibition of LH secretion by oestradiol lasted less than 12 hours, although the negative effects of oestradiol on FSH secretion lasted 72 to 144 hours. In another study, while oestradiol in the treated castrated rams suppressed LH-pulse frequency more effectively in the non-breeding season, its feedback effect on FSH secretion was constant throughout the year (Sanford and Robaire, 1990). Goodman and Karsch (1981) have also suggested that oestradiol plays a significant role in the control of gonadotropin secretion in the male and that it is a major mediator of seasonal breeding in sheep.

In ram lambs, no significant changes in either plasma FSH or LH values were observed during passive immunization against oestrogen (Land et al., 1981; Jenkins et al., 1986). However, it has been shown that oestradiol implants induced a suppression of the secretion of FSH in intact male lambs (Jenkins and Waites, 1983). Moreover, following hemicastration or unilateral cryptorchidism, administration of oestradiol can prevent compensatory testicular growth (Schanbacher and Ford, 1977; Jenkins and Waites, 1983).

Perhaps low levels of oestradiol synergize with testicular inhibin to regulate FSH (Price, 1991).

Increases in blood T to supraphysiological levels followed either active, chronic (Schanbacher 1984a; Schanbacher et al., 1987) or passive, acute (Sanford 1985; 1987a; Sanford et al., 1991) immunization against oestradiol. Oestrogen immunization does not alter the metabolic clearance rate (MCR) of T and therefore changes in serum T concentrations can be interpreted as reflecting changes in the rate of T production (Nishihara and Takahashi, 1983; Schanbacher et al., 1987).

1.9.2.3. Oestrogen effects on the testis

i) Introduction

Peripheral oestrogens can affect Leydig cell function indirectly via their feedback actions on the hypothalamic-pituitary axis but considerable evidence points to oestrogens also having a direct effect on testicular steroidogenic function. In fact, the oestrogen concentration in the testis could be high enough to exert important local effects (Moger, 1980). The presence of oestrogen receptors (ER) and the observed aromatase activity in testicular cells (see section 1.9.1.iii and iv) strongly suggest that oestrogen may act as an intracellular regulator of these cells and would further suggest that it could play a physiological role in controlling testicular function.

It is known that the secretion of oestrogen, as well as testicular content of ER, changes during testicular development in the rat (Pomerantz, 1984). In the rams, Barenton and Pelletier (1983) and Barenton et al. (1983) have shown that oestradiol production by the testis paralleled the onset of spermatogenesis and T secretion. Such findings suggest an intratesticular role for oestrogens in some of the endocrine changes that occur during testicular development.

ii) Oestrogen effects on steroidogenesis

Many *in vivo* experiments with intact and hypophysectomized animals have demonstrated that oestrogens can inhibit T secretion by acting directly on the testis (Moger, 1980). It seems also that immunity against oestradiol can alter possible direct negative actions of this steroid within the testis, allowing increased steroidogenic responses to gonadotropin input. In oestradiol-immunized rams, this is suggested by the facts that marked increases in serum T level can occur

in the absence of appreciable changes in LH level and that the T response to a standardized dose of exogenous LH is enhanced relative to that of nonimmunized rams (Sanford 1985; 1987a; Sanford et al., 1991). Similarly, bulls immunized against oestradiol also secreted more T compared with controls in response to GnRH and hCG treatment (D'Occhio et al., 1987).

In laboratory rodents, several studies have shown that oestrogens can influence directly testicular androgen production *in vivo* (Samuels et al., 1964; Hsueh et al., 1978; Kalla et al., 1980, Moger 1980; Nozu et al., 1981a,b; van der Molen et al., 1981; Grotjan and Steinberger, 1982). Interestingly, the data presented by Dufau et al. (1979) appear to show a 50% increase in basal and hCG-stimulated T production by isolated Leydig cells from Tamoxifen-treated rats; this suggests that the T production may be normally restrained by testicular oestrogens.

Attempts to demonstrate a direct action of oestradiol *in vitro* have been much less successful than *in vivo* studies (Moger, 1980). In fact, in *in vitro* experiments using rat decapsulated testes or dispersed Leydig cells incubated with oestradiol and LH, high concentrations of oestradiol were required to obtain a significant effect (Sholiton et al., 1975; Bartke et al., 1977; Hunt et al., 1979; Sairam and Berman, 1979a,b; Brinkmann et al., 1980). This suggests that the incubation conditions commonly employed may not adequately maintain all aspects of Leydig cell function or that the short-term incubation of testicular tissue may not be a suitable technique to study the effects of oestradiol on androgen secretion (Moger, 1980).

The direct effect of oestrogens on Leydig cells has been unequivocally demonstrated in the rat although Daehling et al (1985) had also reported some direct inhibitory effects of natural and synthetic oestrogens in T release from human testicular tissue *in vitro*.

iii) Oestrogen effects on Leydig cells: mechanism of action

Oestradiol dampening of Leydig cell responsiveness to LH could be achieved in a number of ways. Regulation may be mediated by reductions in the number of LH receptors (Moger 1980; Navickis et al., 1981) and/or in the ability of these receptors to bind LH (Sairam and Berman 1979a,b). Further, the activity of steroidogenic enzymes involved in metabolizing progesterone to androgen could be impaired by oestradiol (Huhtaniemi et al., 1980; Moger 1980).

As the complete mechanism by which LH stimulates steroidogenesis is not known, it is not yet possible to determine with certainty the site(s) at which oestrogens antagonize the action of LH (Moger, 1980). It appears that the inhibitory effect of oestrogens on LH-stimulated androgen secretion is due to an effect beyond the production of cAMP; the most likely sites are reduced activity of the 17 α-hydroxylase and C17-20 lyase, although other sites are not excluded (Moger, 1980; van der Molen et al., 1981). A decrease in testicular microsomal cytochrome P450 in rats treated with oestradiol has been demonstrated (Kalla et al., 1980; Brinkmann et al., 1980; van der Molen et al., 1981). It is possible that oestradiol directly inhibits the enzymes but it is more likely that it induces the production of a protein that can inhibit both C21 and C27 cleavages. The nature of this protein and the mechanism of inhibition are likely to be important. An oestradiol-mediated RNA polymerase activation has been demonstrated which would result from the nuclear actions of oestradiol in gonadotropin-treated Leydig cells (Aquilano and Dufau, 1983). Interestingly, it has been recently shown that oestradiol *in vitro* can also induce a marked dose-dependent inhibition of RNA synthesis by purified Leydig cells of mature rats as well as impairment of T synthesis after hCG stimulation (Ronco et al., 1988).

The physiologic importance of all these observations still remains to be demonstrated.

iv) "Short" feedback loop

In the rat, it has been suggested that testicular oestrogens may function as part of a "short" feedback loop to regulate T secretion (Moger, 1980; Nozu et al., 1981c). LH stimulation of the Leydig cell stimulates steroidogenesis and leads to increased T. An increase in substrate would result in increased oestradiol synthesis either within the Leydig cell or after diffusion to the Sertoli cell. Activation and (or) synthesis of the aromatase system by LH in Leydig cells or by FSH in Sertoli cells would also favour increased oestradiol synthesis. The increase in testicular oestradiol concentration, acting via the oestradiol receptor mechanism, would reduce T synthesis by inhibiting 17 α-hydroxylase and/or C17-C20 lyase activity (Moger, 1980).

Whether gonadotropin-stimulated testicular oestrogen synthesis is part of a physiologically relevant "short" feedback loop that participates in the regulation of T synthesis remains unclear.

Currently there is evidence that this proposed feedback loop indeed functions in the case of gonadotropin-induced desensitization of Leydig cells (Moger, 1980; van der Molen et al., 1981; see section 1.6.3.4.iv).

v) Oestrogen effects on spermatogenesis and testicular growth

It is unclear whether oestrogens exert a direct effect on the seminiferous tubules since there is still controversy concerning the presence of ER in Sertoli cells and no ER have been identified in the germinal cells so far (see section. 1.9.1.iv). However, the fact that the rat Sertoli cell aromatase, as for many other functional activities of Sertoli cell, seems to be modulated by the stage of the spermatogenic cycle, suggests that the formation of oestrogens needs to be closely monitored in order to obtain the special requirements of each stage of the spermatogenic cycle (Boitani et al., 1981; Saez et al., 1987). Whether oestrogen can act on other testicular cells such as the myoid cells surrounding the seminiferous tubules remains to be determined.

Chronic oestradiol-17ß treatment can delay the onset of normal testicular growth and development in prepubertal bulls ('delayed puberty'). In fact, young bulls implanted with oestradiol-17ß had smaller testes and did not show signs of spermatogenesis at 42 weeks of age (Schanbacher et al., 1982). However, chronic treatment with oestradiol during the pubertal period appears not to be detrimental to the subsequent development of the bovine testis. For example, testicular growth was accompanied by active spermatogenesis in each of the calves implanted with oestradiol-17ß and infused with GnRH suggesting that the negative influence of oestradiol-17ß on the testes was mediated primarily by a central action of the oestrogen, possibly in the hypothalamus (Schanbacher et al., 1982).

The rate of growth of the testis was greater in oestrogen-immunized than control Merino ram lambs (14-26 wks old; Land et al., 1981) however this effect was not observed in the other passive immunization study done in younger crossbred lambs (2-16 wks old; Jenkins et al., 1986). Unfortunately, the assessment of spermatogenic function was not reported in these two experiments. Thompson and Honey (1984) reported that active immunization against oestrone in prepubertal stallion led to an increase in testicular weight and daily sperm

production (DSP) at 27 months of age (DSP/g testis and DSP/stallion). Because sperm production rates were assessed only at one age in this experiment, it was not possible to determine whether immunization against oestrogens in this species caused a permanent increase in DSP, a hastening of puberty or a change in the seasonality of spermatogenesis. Further experiments are needed to determine if the effects of active immunization against oestrogens observed would persist beyond the final maturation of the testes. The spermatogenic function was not affected following active immunization against oestradiol in prepubertal bulls (D'Occhio et al., 1987) or following active immunization against oestrone in young boars (Wise et al., 1991)

Therefore, in two species in which the testes secrete significant oestrone (stallion and boar) the findings have been inconsistent.

In crossbred adult rams passively immunized against oestradiol, no change in testicular weight was observed and the author suggested that the immunoneutralization period (2-8 weeks) may not have been sufficient for increments in spermatogenic activity to be manifest as increased testis weight (Sanford, 1985, 1987a, 1989). In a longer study (6 months), testis weight was increased following active oestrogen-immunization in Ile de France rams (Schanbacher et al., 1987), however, no improvement in sperm production have been reported in those rams (Monet-Kuntz et al., 1988). More recently, Sanford et al (1991) reported that testicular regression was partially prevented in crossbred adult rams passively immunized against oestradiol and in which the testicular cycle was entrained to alternating 4 month periods of long-inhibitory and short-stimulatory day lengths. No change in daily sperm output (based on sperm voided in urine) early in the redevelopment stage has been found in those E_2-immunized rams even though the size of their testes was found to be larger during most of the regression stage. Thus important age, season, and species differences may exist with regard to the physiological role played by oestrogens in the regulation of spermatogenesis.

Furthermore, oestradiol immunization appears to influence testis size; however a clear effect on spermatogenic function has not been demonstrated yet.

vi) Oestrogen effects on testicular blood flow and vasculature

Not much is known in the ram, on the effect of oestrogen (oestradiol or oestrone) with regard to the regulation of testicular blood flow. Within the interstitium in oestradiol-immunized rams, blood and lymphatic vessels had a larger volume than in controls (Monet-Kuntz et al., 1988). Similarly,

Nieschlag et al. (1975c) observed a higher percentage volume of blood vessels in the testes of oestradiol-immunized rabbits. These effects are probably due to higher LH levels, since hCG injections have been shown to cause increased testicular lymph flow and similar changes in blood flow and the volume of blood within the testis (Setchell and Sharpe, 1981). Such an effect of LH could be mediated by vasoactive secretory products of the Leydig cells, since selective elimination of Leydig cells from the testis abolishes the action of hCG on the permeability of testicular blood vessels (Setchell and Rommerts, 1985; Veijola and Rajaniemi, 1985).

1.9.2.4. Oestrogen role in pubertal development: summary

Oestrogens can have significant inhibitory effects on the hypothalamic-pituitary axis in the male lambs (Jenkins and Waite, 1983; Schanbacher, 1984a, b; see section 1.9.2.2.). Indeed, manipulation of circulating oestradiol concentrations by steroid implantation (Jenkins and Waites, 1983) or by immunization (Land et al., 1981; Jenkins et al., 1986) has provided evidence for a role in the prepubertal rams. Escape from the "feedback action" of oestradiol during prepuberty may result in the establishment of an episodic secretory pattern for LH with concomitant activation of the steroidogenic and spermatogenic potential of the testes (Olster and Foster, 1986, 1988; see section 1.5).

Land et al. (1981) have shown that although no significant changes in either plasma FSH or LH values were observed at the time of measurement, the rate of growth of the testis was greater in oestradiol-immunized than control Merino ram lambs between 14 and 26 weeks of age. Jenkins et al (1986) have shown that passive immunization of crossbred lambs against oestradiol-17ß from 2 to 16 weeks of age significantly elevated androgen concentrations in plasma and depressed the median eminence content of dopamine. Jenkins and Waites (1983) have shown that oestradiol implants induced a suppression of the secretion of FSH in intact male lambs and prevented compensatory testicular growth in hemicastrated ram lambs.

As mentioned previously (see section 1.9.2.3. i), oestrogens are likely to be playing an important role within the seminiferous tubules as it has been shown that the rat Sertoli cell

L 6-18: Growth usually is defined as production of new cells. But because growth typically is measured as an increase in mass, growth includes not only cell multiplication (hyperplasia) but also cell enlargement (hypertrophy) and incorporation of specific components from the environment (e.g. appetite deposition). Most mammals are born with nearly their full complement of skeletal muscle fibers. Muscle hyperplasia occurs primarily prenatally, and muscle fiber numbers increase only slightly postnatally (Owens et al., 1993). For the lamb, cumulative weight plotted against age follows a sigmoid curve; this curve is composed of the prepubertal, self-accelerating phase and the post-pubertal, self-limiting phase. Puberty is the point at which self-accelerating growth changes to self-inhibiting growth and that weight at puberty occurs at approximately 60 % of mature weight in all species (Owens et al., 1993). Growth rate at later fetal stages and after birth but before maturity can be influenced greatly by factors such as plane of nutrition, hormonal status, and environment. Endogenous (e.g. insulin, GH, IGF-I, IGF-II) and exogenous hormones promote translation, transcription, and amino acid uptake. Administration of GH has improved growth rate and gain:feed ratio in pigs, lambs, and cattle and decreased fat content of pig and lamb carcasses. GH effects seem to be indirect and dependent on locally produced somatomedins (IGF-I and IGF-II) (see section 1.6.2. vii.).

The ability of steroid implants to improve efficiency of growth, feed conversion, and carcass composition of beef steers is well established (Galbraith and Topps, 1981), but the mechanism(s) responsible for these improvements remains unknown. Some studies suggest that steroid implant function indirectly through increased GH concentrations, which should increase mature size (Beitz, 1986; Gluckman et al., 1987). Oestrogen administration consistently has increased protein and decreased fat deposition for growing cattle. Effects of T or the synthetic anabolic agent, trenbolone acetate, are synergistic with oestrogen for enhancing deposition of lean tissue, again potentially through increasing mature body size.

Many researchers have studied the use of steroid implant to control animal growth (Davies et al. 1977; Buttery et al., 1984; Roche et al, 1986; O'Callaghan et al., 1986; Field et al., 1990; Dayton and Hathaway, 1991; Goldspink, 1991).

aromatase is precisely modulated by the stage of the spermatogenic cycle (Boitani et al., 1981; see section 1.9.1, iv). Furthermore, the fact that oestradiol can influence the metabolism of the Leydig cells (section 1.9.2.3. iii) also suggest that this steroid has a role to play within the interstitial tissue (Moger, 1980). However, the direct intratesticular roles for oestrogens have not been demonstrated yet in the developing testis of the ram lambs.

1.9.2.5. Anabolic effects of oestrogens

In many mammalian species, steroids hormones are responsible for sex-related differences in mature body size (Short, 1980; Bronson and Rissman, 1986). In man, it is probable that these hormones are responsible for the growth spurt at puberty (Stanhope and Brook, 1988). In ruminants, there is no clear understanding of the role of sex steroids in the control of skeletal growth and there is no evidence of pubertal growth spurt (Foster et al., 1986). Recently, Mahgoub et al. (1988) have shown that oestrogens accelerate the natural process of skeletal maturation in lambs. Exogenous oestrogens are commonly used as growth promotants for farm animals (Schanbacher, 1984c). There is increasing evidence that steroid hormone (especially oestrogen) modulation of growth factor synthesis provides another important link in physiological growth control (see section 1.9.1.v). Growth factors are thought to function as autocrine or paracrine regulators of both normal and abnormal growth and differentiation (Sutherland et al., 1988). (see facing page)

1.9.2.6. Oestrogen effects on the epididymis and the accessory male organs

Oestrogens produce a number of effects in the male accessory organs (Mawhinney and Neubauer, 1979; Robaire and Hermo, 1988). First, small amounts of oestrogen can enhance the action of T on epithelial cells. For example, low doses of oestradiol enhances the action of T on the secretory activity of the canine prostate in dogs and seminal vesicles in bulls. On the other hand, oestrogens have the capability in some systems to inhibit the action of T (Mawhinney and Neubauer, 1979). Furthermore, oestrogens can induce squamous epithelial metaplasia and can promote growth of the fibromuscular organs (Mawhinney + Neubauer, 1979). Oestrogen administration to

young dogs or rabbits resulted in increases in weight of the epididymis that were almost entirely due to a hyperplasia of the stroma (lamina propria) of this tissue. This effect on stroma is caused partly by an increased number of cells (Robaire and Hermo, 1988). Similar effect of oestrogens on the adult epididymis has not yet been reported. It will be important to determine if any of these actions are expressed by normal endogenous levels of oestrogens in the male.

That endogenous circulating, or potentially locally synthesized, oestradiol may serve a specific function(s) in the epididymis (e.g. modulation of clear cell function) has been suggested by Schleider et al. (1984). The difficulty in identifying oestradiol receptors and aromatase activity in the epididymis of adult mammals may be due to the localization of activity to cells that are not abundant in this tissue (clear cells). Oestradiol administration to adult male mice has been shown to result in an increase in the rate of transport of spermatozoa through the epididymis. Whether this effect of oestradiol on epididymal function was direct or indirect was not addressed experimentally, but the effect was clearly opposite to the effect observed with T (Meistrich et al., 1975).

1.9.2.7. Oestrogen effects on male behaviour

Several lines of evidence indicate that the CNS converts T to oestradiol which, in concert with androgens (T or DHT), causes expression of male sexual behaviour (Naftolin et al., 1975; Mawhinney and Neubauer, 1979). The suppression of female sexual behaviour (defeminization) during the neonatal period (in the rats but not in primates) is thought to occur by the same process (see section 1.9.2.1.; Mawhinney and Neubauer, 1979). In support of the role of oestrogens in sexual behaviour, data obtained from rams indicate a strong and positive relationship between circulating oestrogen and mating frequency early in the breeding season (Sanford et al., 1982b). D'Occhio and Brooks (1980) have shown that oestrone, oestradiol-17ß and the synthetic oestrogen, diethylstilbestrol, were the most effective oestrogens in eliciting mounting behaviour in adult castrated rams. Nonaromatizable androgens such as DHT were relatively incapable of inducing male sexual behaviour (D'Occhio and Brooks, 1980). Microgram quantities of oestrogenic hormones are sufficient to elicit mounting behaviour in

castrated sheep. T, on the other hand, must be given in milligram quantities for it to elicit an equivalent response. Oestrogen administration alone can partially activate mounting behaviour and combination therapy with DHT and oestradiol produced full restoration of male copulatory behaviour. It was concluded that induction and/or maintenance of male sexual behaviour in adult rams by T depend in part upon T aromatization to oestradiol (D'Occhio and Brooks, 1980). Parrott (1978) has also suggested from his results that oestradiol-17ß and DHT act synergistically in the brain to elicit mating behaviour in male sheep. There is no evidence that sexual behaviour can be in any way suppressed by active immunization against testosterone hormones in male sheep (Haynes and Southee, 1984). Furthermore, no change in sexual behaviour has been reported in any of the experiments done in rams using antioestrogens or aromatase inhibitors (Sanford 1985, 1987a). However, in castrated rats, compounds which block the aromatization of androgens to oestrogens inhibit testosterone-induced mating behaviour whilst this inhibition can be overcome by the concurrent administration of oestrogens (Morali et al., 1977).

1.9.2.8. Other oestrogen effects

Relatively high circulating levels of oestrogens are known to exert marked demasculinizing and feminizing effects in males (Mawhinney and Neubauer, 1979). In Australia, enlargement of the teats and lactation, and enlargement of the accessory male glands were found to be common among the wethers grazing oestrogenic pastures ("clover disease" syndrome) (see review: Moule et al., 1963). Because the sheep is a ruminant and is also the farm animal most widely affected by oestrogenic pastures, methods of bioassay determining increase in teat length in wethers have been developed. Indeed, the simplicity of the method made it very useful for rough quantitative indications of the oestrogenic activity of pastures as grazed by sheep (Moule et al., 1963). More recently, Mahgoub et al. (1988), have reported that teat length increases linearly with size of oestradiol implants in castrated lambs.

1.9.3. Tools for studying oestrogen action

The first antioestrogens to be described were MER 25 [1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenylethanol], clomiphene and tamoxifen. The action of these compounds is made extremely complex by the expression of partial or full agonist as well as antagonist properties (Sutherland et al., 1988). At the molecular level, antioestrogens act as competitive inhibitors of oestrogen binding to the ER with consequent formation of antioestrogen-ER complexes that no longer possess proper receptor function. A number of the diverse biological effects of antioestrogens are apparently not mediated by ER and may result instead from the binding of these compounds to a variety of other cellular components. The activity expressed by those antioestrogens varies depending on the species, tissue or oestrogen-responsive parameter studied (Sutherland et al, 1988) (for example, in the rat, tamoxifen is classified as an antagonist with partial agonist activity whereas, in the mouse, tamoxifen is classified as an agonist (Sutherland et al, 1988); in the ram, tamoxifen has been shown to possess oestrogenic activity). (Sanford 1985, 1987a)

The recent discovery of the first pure antagonist (e.g ICI 164,384) promises to resolve the problem of partial agonist activity found with other antioestrogens. The trophic actions of exogenous and endogenous oestradiol, and of partial agonist antioestrogens, are blocked completely by ICI 164, 384 whilst the compound itself is devoid of stimulatory activity (Wakeling, 1988).

Aromatase inhibitors have also been used with some success on the principle that "the only true antioestrogen is no oestrogen". Unfortunately increased side effects, and the fact that endogenous oestrogenic steroids are not all controlled by inhibitors of aromatization, rather limit their usefulness (Jordan, 1990).

1.10. Studying the steroid participation in the reproductive system using immunization techniques.

1.10.1. Introduction

Immunization against steroids is not a special field of research, but rather an investigation tool applying immunological principles to endocrinology. Steroid antisera can be used to characterize biological effects of hormones, to investigate feedback control mechanisms, and to demonstrate hormone dependency of reproductive functions (Nieschlag and Wickings, 1977; 1978). Although the main value of immunological methods is an aid to research, practical applications have also been developed (e.g. Fecundin™: immunization of ewes against androstenedione to improve fecundity, see Cox, 1984; "immunological castration" using active immunization against GnRH, see Robertson et al., 1982 ; Hoskinson et al., 1990) Antibodies can provide a long-term and specific withdrawal technique. It allows the particular steroid-producing gland to remain *in situ* to pursue any other functions it might have (Nieschlag and Wickings, 1978; Haynes and Southee, 1984). Thus this approach overcomes many of the obstacles produced by more radical technique such as ablation of the gland. Since antibodies should have no biological activity other than by their capacity to bind the homologous hormone in the peripheral circulation (Ferin et al., 1973), they have unique advantages over other methods such as the use of antihormones (e.g. antioestrogen) which have a complex activities (see section 1.9.3.). It is assumed that the antibodies directed against a steroid neutralize the hormone in the peripheral circulation, therefore leaving the hormonal receptor sites intact. Both passive and active immunization against steroid hormones have contributed significantly to our present understanding of the functions of the steroid hormones in male and female mammals (Haynes and Southee, 1984). The lack of widespread usage may be attributed to the difficulties encountered in determining precisely the effectiveness (uncertainty concerning the degree to which steroid hormones are actually neutralized) and specificity of antibodies *in vivo* (Nieschlag and Wickings, 1978; Haynes and Southee, 1984).

1.10.2. General characteristics of the immune system

The immune system can be divided into the non-specific (innate) and the specific (acquired) immune system (Grossman, 1984). The non-specific immune system encompasses all reactions which are nonantigen dependent, while the specific immune system involves reactions of lymphocytes (T and B cells) and antibodies (immunoglobulin, Ig), all of which are antigen-dependent. Also, the specific immune system is composed of the two distinct but interrelated immunological responses, humoral and cell-mediated reactions. The first response has been shown to be activated by the host exposure to foreign substance called antigen and the consequent production of antibodies by B lymphocytes (B cells). Such molecules circulate in both the blood and lymph and are able to recognize and specifically bind to antigens. The formation of an antibody-antigen complex then triggers a series of events (including phagocytosis by polymorphonuclear granulocytes and macrophages and activation of the complement system) which then leads to the elimination of the antigens from the systems. The macrophages play an important role in immune response because they phagocytize foreign material or particles and digest them. After digestion the macrophages will present antigenic determinants (removed from these particles) to the B cells and T cells (Grossman, 1984).

The second type of specific immune response is dependent on reactions which are commonly defined as those immunological responses in which the lymphoid tissues develop a specific cell mediated defense to foreign antigen. The process depends on a specific type of lymphocytes termed a T cell which is able to recognize and bind to the antigen that is presented by a macrophage (or any other cell capable of presenting the antigen). Such binding will then lead to elimination of the foreign material from the system. In fact, after activation, the T cells will secrete some regulatory molecules ("lymphokines" such as: interleukin-2, interferon γ,) that will stimulate B and T cell clone formation and activate the immune response against the antigen-containing structures (Roitt et al., 1989).

While the humoral and cell mediated reactions function separately and require specific types of lymphocytes, they are dependent upon each other because certain subclasses of T cells (helper

and suppressor cells) have been shown to modulate B cell production of antibody (Grossman, 1984).

In general, when an antigen is injected into an animal for the first time, antibodies are produced after a time lag of 10-12 days. In this primary response antibodies are usually of the high-molecular weight IgM class; they reach a maximum titre at 15-18 days, then disappear from the serum. If the same animal is now re-injected with the same antigen, a more vigorous response follows after a time-lag of only 3-4 days (Szelke, 1983). In addition to IgM antibodies seen in the primary response, much higher levels of IgG antibodies are also produced which persist for long periods (Szelke, 1983). Several reports can be found in the literature, where specificity increases during immunization. A change from IgM immunoglobulins to IgG during the course of immunization may account for the change in cross-reactivity (Wickings et al., 1977; Nieschlag and Wickings, 1978; Roitt et al., 1989).

1.10.3. Immunogen and immunization procedure

Steroid hormones are relatively small compounds and are normally present to some extent in most animals; they therefore have no
inherent immunogenic properties. However, when covalently coupled to large molecule carriers they become immunogenic acting as haptens (Ferin et al., 1973). The most commonly used carrier proteins have been: human or bovine serum albumin (HSA, BSA), although other proteins such as keyhole limpet hemacyonin (KEL), ovalbumin, thyroglobulin have also been used. Injection of such conjugates into experimental animals under the appropriate conditions results in the formation of antibodies which recognized and combined with the steroid moiety. Such antibodies can be reasonably specific to the particular steroid used as hapten. In addition we can further stimulate antibody response and prolong the release of such immunogenic conjugates into the animal's body by the use of suitable adjuvants (the most widely used are Freund's complete adjuvant and Freund's incomplete adjuvant) (Ferin et al., 1973; Nieschlag and Wickings, 1978).

Immunization protocols employed in the production of antisera to steroids vary widely between laboratories and there is no single route to success (Scaramuzzi et al., 1975). Each antiserum

developed has individual properties which vary with the site of hapten-coupling, route and schedule of immunization, and length of immunization (Wickings et al., 1977). In order to obtain antibodies of high titre and avidity, it is almost always necessary to elicit a secondary immune response by booster immunization which must be given when the antibody titre produced by the primary response begins to fall (Szelke, 1983). It is also important to use a small dose of antigen that will favour the selection of clones of lymphocytes B that can produce high affinity antibodies (Roitt et al., 1989). Each experimental animal reacts differently to immunization (Wickings et al., 1977).

Active immunization against steroid-protein conjugates produces antibodies directed not only towards the steroidal hapten but also towards the antigenic determinants of the carrier protein and adjuvant, indeed, antibodies capable of binding steroids may represent only a small proportion of the whole immune response (Nieschlag and Wickings, 1978). Possible interference by these non-specific antibodies may be accounted for by appropriate control measures such as immunization of a control group of animals with adjuvant and carrier protein or by the removal of non-specific antibodies by absorption prior to passive immunization.

1.10.4. Passive vs active immunization against steroid hormone

Immunization against steroids may be achieved in two ways, either actively, when the animal produces its own antibodies in response to immunization with steroid-protein conjugates, or passively, when antibodies (produced in another actively-immunized animal) are administered exogenously to the recipients (Nieschlag and Wickings, 1978). It is important to bear in mind the differences between active and passive immunization.

i) Active immunization

The advantages of active immunization are that a high concentration (titre) of antibodies can be obtained and there is continual production of the antibodies so they are always likely to be in excess of endogenous antigen production (Nieschlag and Wickings, 1978; Martin, 1984). Obviously, active immunization has the advantage of producing longer-lasting effects. Active

immunization is likely to be more practical than passive immunization provided that a reproducible and appropriate antibody response could be achieved. Usually, a reproducible immune response can be attained with careful standardization of immunogens, adjuvants and treatment protocols. However, this immunological approach may present a number of complicating factors (Nieschlag and Wickings, 1978). The reactions in actively immunized animals cannot be easily controlled, since the amount and type of antibodies produced is largely out of the hands of the investigator (with considerable between animals variation). Since the time required to attain an adequate titre is considerable, only chronic effects of neutralizing the hormone can be observed (Martin, 1984). Furthermore, the specificity of antibody during active immunization is not constant and can change significantly with time. The high concentration of circulating antibodies (predominantly low affinity antibodies) may result in the formation of immune complexes which are most likely to be deposited in the renal glomeruli (Roitt et al., 1989). However, Nieschlag and Wickings, (1978) have reported no signs of ill health or no higher mortality rate in the steroid immunized animals that they have examined precisely in this regard. One unpleasant side effect of active immunization is the local reaction at the site of immunization (ulcerations, enlargement of the local lymph nodes). This effect is due to the use of Freund's complete adjuvant and can be reduced by using Freund's incomplete adjuvant in the booster injections (Nieschlag and Wickings, 1978).

ii) Passive immunization

In comparison with active immunization, the passive immunization approach allows more control of antisera specificity and titre; the later advantage is important because the magnitude of the endocrine change(s) subsequent to neutralization of a given hormone may relate to the titre of the antiserum in the circulation (Sharpe and Fraser, 1983). Passive immunization provides a reasonably fast method of neutralizing circulating levels of steroid hormones and consequently, it is possible to study the acute effects of such neutralization (Scaramuzzi, 1976; Rawlings et al., 1979). Passive immunization suffers from a relatively short duration of action related to the half-life of the immunoglobulins administered (half-life of about 4 weeks) (Nieschlag and

Wickings, 1978; Haynes and Southee, 1984). Also, this approach requires large quantities of antisera which depend on the antibody capacity to bind the steroid concerned and the quantity of this steroid present in the circulation (Martin, 1984). Repeated application of antisera during the course of passive immunization introduces large amounts of foreign proteins into the experimental animal and may result in serum sickness or anaphylactic reactions and therefore limits the extent to which passive immunization can be used in experimental animals (Nieschlag and Wickings, 1978). In fact, the experimental animal that received several injections of immunoglobulins (antiserum) produced in a different animal, has the potential to build an immune reaction against the foreign proteins introduced in its system (e.g. immunoglobulins with foreign allotype or idiotype). In that respect, it would appear that the main factor determining the duration of the antiserum's effects is the ability of the recipient to mount an effective antibody-mediated clearance of the exogenous immunoglobulins (Kurtenbach et al., 1989; Madon et al., 1991).

1.10.5. Mechanism of action of circulating antibodies

i) Bioavailability of steroids after immunization

In an immunized animal, the total mass of steroid in the blood has two components: free steroid which is biologically active and protein-bound steroid (mainly antibody-bound) which is presumed to be inactive. These two components are in stoichiometric equilibrium and the proportion of free, active hormone is presumably a major determinant of the efficacy of the immunization (Nieschlag and Wickings, 1978; Martin, 1984; see section 1.6.3.5.vi).

Binding affinities for populations of steroid antibodies are of the order of 10^9 litres/moles and are thus about 100-fold higher than the affinities of the specific binding proteins, such as SHBG, but are in the same range as or slightly higher than the binding affinities of the cellular steroid receptors (Nieschlag and Wickings, 1978).

ii) General effects of circulating antibodies

Antibodies, resulting from active or passive immunization, are able to bind selected circulating hormones and create an artificial hormonal deficiency at the target tissue level (preventing the hormone from getting to the receptor sites, leaving these sites free) (Ferin et al., 1973). Such neutralization of hormones can lead to physiological changes which can be directly associated with the binding of hormone to antibody or indirectly from disturbance to the endocrine networks and feedbacks (Nieschlag and Wickings, 1978; Haynes and Southee, 1984).

Thus, two aspects of steroid metabolism will be affected by the immunization procedure. Firstly, the steroid that is bound to the antibody is cleared from the blood more slowly than the free steroid, presumably because the antibody prevents uptake by the liver (increased half-life) (Martin, 1984). Second, the rates of gonadal steroid secretion could be increased due to reduced feedback effect and increased gonadotropin stimulation, and could contribute to the increase in the total mass of steroids in the blood (Nieschlag and Wickings, 1978; Haynes and Southee, 1984).

iii) Importance of the characteristics of the antibodies (specificity, affinity, and titre)

The value of immunization as an experimental technique lies in the high affinity and degree of specificity with which antibodies can bind their antigens. The circulating antibodies will compete with the receptors for the steroid hormones in the sensitive tissues. The degree to which the receptors can compete with the antibodies will depend on the relative affinities of these two agents for the steroid (see section 1.10.5.i), and in turn this will determine the degree to which the steroid is biologically neutralized. High affinity binding in blood and extracellular fluids would tend to draw steroid out of target tissues however, if the affinity of the antibody is similar to the affinity of the receptor, then the antibody could virtually act as a carrier protein (Martin, 1984). However, in an active immune response, the antigen-antibody complex formed will be eliminated by the activated macrophages (stimulated by T cells) that possess

some F_c receptors capable of binding the antibody. Therefore, these macrophages can eventually eliminate the antigen-antibody complexes from the circulation by phagocytosis.

The specificity of the antibodies produced is one of the most important parameters for the interpretation of the results of physiological studies (Nieschlag and Wickings, 1978). Specificity is usually established *in vitro* by displacement studies and will, in most cases, indicate that the antisera react not only with the homologous steroid, but also cross-react with structurally related steroids (Ferin et al., 1973). Whilst this procedure is widely used to test antibody specificity in radioimmunoassay, its application in biological studies is questionable since it is usually performed with a high dilution of antiserum and therefore cannot account for low titre, non-specific antibodies which may be of importance in the physiological situation (Martin, 1984).

It has been demonstrated that specific antisera (with high affinity antibodies) can effectively prevent the entry of labelled oestradiol into uteri, ovaries, and pituitary gland in immature rats (Ferin et al., 1973). The latter findings provide good evidence for the hypothesis that the antibodies prevent the steroids from reaching receptors in target tissues.

Finally, the antibody must be present in sufficient quantity (appropriate titre) to be able to bind the mass of steroid present in the circulation and constantly renewed.

1.10.6. Immunization against steroids in the ram and other species

Active immunization of mature rams against oestradiol-6-CMO-BSA resulted in the production of circulating antibodies to oestradiol (Schanbacher, 1979; 1984a; Schanbacher et al., 1987; Monet-Kuntz et al., 1988). This method of neutralizing the biological activity of a steroid as well as passive transfer of antibodies (Sanford, 1985; 1987a; 1989; Sanford et al., 1991) have proven themselves useful in determining the biological actions of endogenous circulating oestradiol in adult rams. Prepubertal ram lambs have also been passively immunized against oestrogens (Land et al., 1981; Jenkins et al., 1986) however, no investigation has been reported yet using active immunization against oestrogens in the young male sheep (see summary in appendices).

Immunity against testosterone in ram lambs seems to confer no reproductive advantages. In fact, decreased anabolic effect with no improvement in testicular weight have been reported by Schanbacher (1982). Interestingly, Haynes and Southee (1984) reported that immunity against testosterone appears not to diminish sexual behaviour in young rams. In adult rams, Schanbacher (1979) reported an increase in LH and FSH secretion following active immunization against T. However, no long-term study using actively T-immunized adult rams have been reported yet (see summary in appendices).

In males of other species, immunization against oestrogens or testosterone have been also used successfully at various ages (for summary see tables in appendices). Because of the dynamic aspects of the response that follows passive or active immunization against steroids, variable and sometimes contradictory observations have been reported in these various species. It must be noted that very few immunization studies have included a complete estimation of the steroidogenic and spermatogenic function of the testis and, no one has reported a complete evaluation of sexual behaviour in the immunized animals.

1.10.7. Special considerations concerning immunization against oestrogens

Oestrogen antibodies in blood and extracellular fluids would bind circulating oestrogens and would also tend to draw oestrogens out of the target cells and the cells capable of aromatization. Because oestrogens would not be able to reach its receptors within responsive cells, its actions will be neutralized. It is generally assumed that the oestrogen antibodies have access to circulating oestrogens of testicular and peripheral origin including oestrogens formed at the level of the pituitary (which has low aromatase activity) and at the level of the median eminence (outside the blood-brain barrier). The oestrogens formed beyond the blood-brain barrier (within the CNS, in the hypothalamus) are probably protected to a certain extent from antibody binding because the immunoglobulins do not cross this barrier. The same is also true for the oestrogens that are found beyond the blood-testis barrier (e.g. inside the seminiferous tubules). This, of course, does not exclude the possibility that free oestrogens produced outside the blood-brain

barrier and the blood-testis barrier could diffuse down concentration gradient and therefore become within the reach of the neutralizing antibodies.

Briefly, immunoneutralization of oestrogens should lead to an increase in gonadotropin secretion (LH and FSH) because of the "partial" reduction of an important component of the steroid negative feedback system that regulates the hypothalamic-pituitary axis. An increase in LH concentration and possibly the neutralization of the intratesticular negative effects of oestrogens should lead to enhancement of T production by Leydig cells. More T will in turn bind to the androgen receptors within the Sertoli cells which simultaneously received more FSH.

Although, most oestradiol antibodies have been shown to cross-react slightly with T and DHT, the overall binding of T to circulating antibodies in oestrogen-immunized animals appears not to influence its metabolic clearance rate (Nishihara and Takahashi, 1983; Schanbacher et al., 1987). Therefore an increase in serum T concentrations in rams immunized against oestrogens can be interpreted as reflecting increase in the rate of T production.

Overall, these endocrinological changes due to immunity to oestrogens should eventually lead to an improvement of spermatogenesis which is, in fact, the central hypothesis of the work presented in this thesis (see General Introduction, pp. 1-6).

CHAPTER 2. MATERIALS AND METHODS

2.1. Reagents

Gonadotropin releasing hormone (GnRH), human chorionic gonadotropin (hCG) and steroids were obtained from Sigma Chemical Co., St-Louis, Missouri, USA. Radiolabelled ^{3}H-steroids and sodium iodide (^{125}I) were purchased from Amersham Australia Pty, Ltd, Sydney, N.S.W. Other materials were obtained as follows: Heparin from Commonwealth Serum Laboratories, Melbourne, Victoria; Sodium pentobarbitone (Nembutal, Abbott) from Ceva Chemicals Australia Pty, Ltd, Hornsby, N.S.W.; Xylazine 2% (Rompum) from Bayer Australia Ltd, Botany, N.S.W.; Lidocaine hydrochloride (Lignocaine 2%) from Australian Analytical Laboratories Pty, Ltd, Thornleigh, N.S.W.; Benzylpenicillin injection (sodium salt) from Commonwealth Serum Laboratories, Melbourne, Victoria; Polyethylene cannulae tubing from Dural Plastics and Engineering, Dural, N.S.W.; Dialysis membrane from Selbys Scientific Ltd, Adelaide, S.A. All solvents used were analytical grade.

The following immunogens were used:

- 17ß-Oestradiol-6(o-carboxymethyl)oxime-bovine serum albumin conjugate were purchased from Steraloids, Inc., Wilton, New Hampshire, U.S.A.
- 17ß-Oestradiol-3(O-carboxymethyl)oxime-human serum albumin conjugate was a gift from Dr. R.M. Hoskinson, CSIRO, Division of Animal Production, Prospect, N.S.W.
- Oestrone-3-O-carboxymethylether-bovine serum albumin conjugate (#429) was a gift from Dr. R.I. Cox, Hormone Assay Development Group, CSIRO, Division of Animal Production, Prospect, N.S.W.
- Testosterone-3(O-carboxymethyl)oxime-bovine serum albumin conjugate (#422z) was a gift from Dr. R.I. Cox, Hormone Assay Development Group, CSIRO, Division of Animal Production, Prospect, N.S.W.
- Bovine serum albumin (BSA) was purchased from Sigma Chemical Co.,St-Louis, Missouri, USA.

2.2. Experimental animals

Sheep used were of the South Australian Merino strain, bred on the Field Experiments Station, Glenthorne, O'Hallaran Hill (which is located approximately 20 km South of Adelaide) or at the Waite Agricultural Research Institute, Glen Osmond, S.A.

2.2.1. Ram lambs

The lambs used were part of a behavioural study on maternal recognition at birth therefore the flocks were kept under continuous observation during peak lambing periods. Lambing periods were of short duration because oestrus has been synchronized in the ewes by intravaginal application of polyurethane sponges impregnated with progesterone (Repromap, 60 mg, Upjohn) for 14 days. The lambs were eartagged at birth and the date of birth recorded. The lambs were kept outdoors with their mother until weaning (between 8 and 12 weeks of age) then they were transferred to a room with controlled light (12 hours light: 12 hours dark) and kept there until the end of the experiment.

[The absence of photoperiodic changes ('constant cycle') do not appear to have a major influence on pubertal development. Furthermore, based on PRL data, it seems that the rams interpret the 12L:12D cycle as short day (Klindt et al., 1985; Kennaway & Gilmore, 1985).]

The controlled light room contained 4 large pens (6 metre square), each pen receiving 4 to 5 lambs. The lambs were grouped according to body weight at their arrival in this environment to allow better feeding management and to minimize competition between larger and smaller animals. The lambs were initially fed twice a day with commercial sheep pellets (approximately 400 g /day) and lucerne chaff (50 g /day). The amount of pellets was increased throughout the trial to achieve an average liveweight gain of 120 g per day.

2.2.2. Adult rams and wethers

All adult rams and wethers used came from the Waite Agricultural Research Institute farm. The rams were kept on pasture throughout the year and received cereal hay and sheep commercial pellets as supplement during the dry season. The wethers used for the production of antisera were kept indoors in individual pens and were fed with commercial sheep pellets and oat straw according to their maintenance requirements.

2.3. Immunization procedure

2.3.1. Active immunization

One mg of immunogen was dissolved in 1 ml of sterile physiological saline and 1 ml of Freund's complete adjuvant (Commonweath Serum Laboratories, Melbourne, Victoria). This preparation was emulsified by vigorous shaking using a vortex mixer for approximately 10 minutes (or until a stable emulsion was obtained) and immediately injected into sheep to be immunized at 8 subcutaneous or intradermal sites (0.25 ml x 2 sites under each fore and hind limbs). A booster injection was given one month after the primary injection. The booster consisted of 1 mg immunogen dissolved in 1 ml of saline and emulsified with an equal volume of Freund's incomplete adjuvant (Commonweath Serum Laboratories, Melbourne, Victoria).

2.3.2. Passive immunization

2.3.2.1. Method of purification of Immunoglobulin G (IgG) by caprylic acid precipitation

Antisera obtained from wethers immunized against oestradiol-3-HSA, oestrone-3-BSA and BSA (see chapter 5, section 5.2.) were purified according to the method described by Steinbuch and Audran (1969). Briefly, 500 ml of antisera was purified at a time. Caprylic acid (25 g) (n-octanoic acid, BDH Chemicals Ltd, Adelaide, SA) was added very slowly to the antisera diluted in 1 litre of acetic acid 0.1 M. (pH 4) This mixture was agitated vigorously for 30 minutes until

precipitation was complete. Centrifugation was used to separate precipitate from supernatant. The precipitate was discarded and the supernatant (which contains IgG at ~90% purity) was dialysed against 0.015 M acetate buffer (pH 5.7) at room temperature for approximately 48 hours. The supernatant was then lyophilized and kept in a dessicator at 4 °C until needed.

The degree of purity of the IgG fraction was tested by electrophoresis (see sections 6.2.1.6. and 6.2.2.5).

2.3.2.2. Treatment

The passive immunization procedure involved injecting a known amount of antibodies (undiluted antiserum or purified IgG diluted in saline) into the jugular vein of ram lambs every 14 days.

Before the injection to the experimental lambs, purified IgG (1.66 g.) was diluted in 25 ml saline and centrifuged at high speed (20Kdpm) to remove possible aggregates that could have formed in the solution. The supernatant (purified IgG) and the undiluted antiserum were both sterilized by filtration (pore size: 0.22 µm).

With only approximate information regarding the ultimate titre desired, the dose of anti-oestrogen serum or purified IgG diluted in saline was a compromise between that considered effective on the basis of earlier experiments (Land et al., 1981; Jenkins et al., 1986; Sanford, 1985; 1987a, 1989) and the number of animals available for treatment (e.g. dose giving a titre ranging between 1:100 and 1:200) (more details will be given in chapter 6, sections 6.3.1.3. and 6.4.1.3., and in chapter 7, section 7.3.1.2.).

2.4. Body weight

Body weight was recorded weekly between 10 and 11 AM, approximately 2 hours after the beginning of ingestion of food in the morning.

The words 'body weight' have been used throughout the thesis and should be consider equivalent to the word 'liveweight'.

2.5. Estimation of testicular volume

2.5.1. Measurement of testicular diameter

Left and right testicular diameter was measured weekly with calipers by the same person in all cases. The diameter was measured at the widest level of the testis and including two thicknesses of scrotal skin. The scrotum was kept well clipped at all times, to facilitate measurement.

2.5.2. Measurement of testicular length

At the same time, by the same person and in a similar way as for testicular diameter, left and right testicular length was measured.

2.5.3. Calculation of testicular volume

The volume of the testis was estimated from caliper measurements of its diameter and length by assuming that it is a prolate spheroid (Setchell and Waites, 1964). It was calculated according to the formula $1/6 \pi a^2b$ (a= largest width; b=length, of the testis).

2.6. Blood sampling

2.6.1. Intensive bleeding

2.6.1.1. Blood collection.

Peripheral blood samples were taken in conscious animals from the jugular vein. The jugular was cannulated with an indwelling polyethylene cannulae (1.5 mm O.D., 1.0 mm I.D.) on the day prior to sampling. After drawing a 5 ml blood sample the cannulae were flushed with 0.9% sterile saline containing heparin (50 I.U./ml). Blood samples were collected into 10 ml heparinized centrifuge tubes and usually centrifuged immediately or after a short waiting period at 4 °C. The plasma separated after centrifugation were stored at -20 °C, until required for assay.

2.6.1.2. Pool

In most cases, intensive bleedings involved taking a blood sample every 20 minutes for 6 hours, a total of 19 blood samples per animal Before storing these blood samples, equal aliquots of plasma taken from each of the 19 samples were pooled. Mean hormonal levels were determined by assaying plasma pool representing the 6-hour sampling periods.

2.6.2. Pituitary responsiveness to GnRH

Pituitary function in sheep was assessed by monitoring the response of the pituitary to intravenous of GnRH (5 ng/kg body weight). The GnRH was administered immediately after an intensive bleeding. Blood samples were taken at 10, 20, 30, 40, 60, 90 and 120 minutes after the injection.

2.6.3. Testicular responsiveness to hCG

2.6.3.1. Blood collection and hCG challenge.

Human chorionic gonadotropin (hCG) was administered to anaesthetized sheep as a single intravenous injection (20 I.U./kg body weight) at the time of testicular blood flow measurement (see section 2.7.2). Blood samples from the jugular (JUG) and the internal spermatic veins (ISV) were taken at 10 minute interval, starting 40 minutes before the hCG challenge and for another 100 minutes thereafter.

2.6.3.2. Pool

The "pre-hCG" pool for each site of blood collection (JUG, left-ISV, right-ISV) consists of equal aliquots of plasma taken from the 4 serially collected blood samples. Similarly, the "post-hCG" pool for each site of blood collection consists of equal aliquots of plasma taken from the 10 serially collected blood samples. Testosterone mean levels were determined by assaying these plasma pools representing the "pre-hCG" and the "post-hCG" periods.

2.7. Surgery

2.7.1. Oestradiol-17ß implantation

2.7.1.1. Silastic capsules

All implants were prepared according to the method of Klincl and Rudel (1971) using Silastic (polydimethylsiloxane) medical tubing (3.35 mm I.D, 4.65 mm O.D.) and Silastic medical adhesive (Silicone Type A) which were obtained from Dow Corning Corporation, Missouri, U.S.A. Silastic tubing was cut into 24 cm length and sealed at one end with Silastic adhesive. The tubes were packed with crystalline oestradiol-17ß the following day. The open ends were sealed with adhesive and stored in a desiccator at 4 °C until needed. The day before utilization, the implants were soaked in absolute ethanol followed by a surgical disinfectant (Zephiran™, Winthrop Laboratories, Sydney, N.S.W.). For the control wethers, empty Silastic implant of the same length were used. A single oestradiol implant of this length was shown to suppress LH secretion in wethers to the level found in intact rams (D'Occhio et al, 1985).

2.7.1.2. Implantation

At the time of implantation the lambs were tranquilized with Xylazine 2% (Rompum). Lambs were held firmly but gently on a table, laying on their left side. The right side of the thorax was well shaved and cleaned. The sites of incision were infiltrated with 5 ml Lignocaine 2% (Lidocaine HCl). Two small incisions situated at 30 cm apart, along the last rib, were made through the skin with the help of a sterile scalpel blade. A long and thin surgical plier was gently inserted by the top incision under the skin. The implant was then inserted by the bottom incision and was positioned subcutaneously along the last rib with the help of the surgical plier. Catgut (no. 0 Chromic gut) was used to suture the cut made at the point of insertion.

2.7.2. Testicular blood flow: cannulation, PAH infusion and blood collection

At the end of the experiment, the ram lambs were anaesthetized using sodium pentobarbitone (Nembutal, Abbott), given intravenously and anaesthesia was maintained with further doses of

the same anaesthetic. The jugular (JUG) was cannulated with an indwelling polyethylene cannulae (1.5 mm O.D., 1.0 mm I.D.). Ram lambs were placed in a supine position on a surgical table. The scrotum and testis were drawn through an opening in a surgical drape to lie on a sterile surgical field. A longitudinal incision was made through the scrotum over the cranial aspect of each testis. Another incision was made through the tunica vaginalis to expose the testis and the spermatic cord. The distal part of the head of the epididymis (caput epididymis) was separated from the testis by careful blunt dissection or with sharp small scissors. The head was reflected dorsally and a cannulae (tapered catheter: 0.5 mm O.D., 0.2 mm I.D. at its finer end) was positioned in one of the exposed testicular vein. Another cannulae (1.2 mm O.D., 0.8 mm, I.D.) was positioned in the ipsilateral internal spermatic vein (ISV) approximately 2 cm above the pampiniform plexus. The same procedure was repeated on the other side. A 2% solution of para-amino-hippuric acid (PAH) was then infused at the rate of 100 μl/min into the testicular vein using a peristaltic infusion pump (Minipulse 2 Gibson, Villiers, L.E.B.E.L., France). Beginning 10 minutes after the commencement of the infusion, blood samples were withdrawn from the ISV and JUG at 10 minute intervals for 140 minutes. A dose of 20 I.U. hCG/kg body weight was injected into the JUG 40 minutes after the start of the PAH infusion (see section 2.6.3).

2.7.3. Testicular biopsy

Testicular biopsy was performed unilaterally on ram lambs tranquilized with Xylazine 2% (Rompum). Ram lambs were placed in a supine position on a surgical table. The subcutaneous tissues around the spermatic cord were infiltrated with 5 ml Lignocaine 2% (lidocaine HCl). The scrotum (well shaved and cleaned) and testis were drawn through an opening in a surgical drape to lie on a sterile surgical field. A 2 cm incision was made through the scrotum over the testis at its widest diameter. An avascular area of the testicular capsule was located and a 1 cm opening in the tunica vaginalis was made with a scalpel. A small triangular incision (5 mm x 5 mm x 5 mm) was made into the tunica albuginea (extreme care was taken to avoid severing blood vessels in the tunica vasculosa layer of the capsule) with a new sharp scalpel blade and the

protruding testicular tissue with the tunica albuginea were removed and transferred to the fixative (Bouin's solution). A small amount of antibiotic powder (Benzylpenicillin, sodium salt) was placed directly on the biopsy site and spread between both tunica. The tunica albuginea was then closed with fine catgut (no. 000 Chromic gut), followed by the tunica vaginalis and finally the skin, using catgut (no. 0 Chromic gut). Following surgery, rams were given 3 ml of the penicillin preparation (Vetspen injection, Glaxovet, Glaxo Animal Health, Ltd) via i.m. injection as a precautionary measure against infection.

2.7.4. Castration

At the end of the experiment, (generally after testicular blood flow measurement and/or response to hCG challenge) the testes were removed, separated from tunica vaginalis and epididymis, then weighed (with tunica albuginea). Testicular diameter and length were again measured with calipers and testicular volume recalculated (see section 2.5.3.). The spermatic cord was ligated with strong non-absorbable suture (Dacron polyester fiber 2, Davies and Geck American Cyanamid Company). The skin was sutured with catgut (no. 0 Chromic gut).

2.8. Laboratory analysis

2.8.1. Hormone assay

2.8.1.1. Phosphate buffer and solutions

(A). Phosphate buffer 0.05 M (PBS); pH 7.5

1.42 g. Na_2HPO_4 (anhydrous)

0.37 g. EDTA

1.00 g. NaN_3

8.18 g. NaCL

pH adjusted to 7.5

made up to 1 litre using double distilled water

(B). Phosphate buffer 0.05 M, 0.2% gelatin (gel PBS); pH 7.5

Gelatin added to PBS to get 0.2% solution (2 g. in 1 litre of PBS)

pH adjusted to 7.5

2.8.1.2. Radioimmunoassays (RIA) for gonadotropin hormones

Principle of radioimmunoassay:

In the assay, a limited amount of specific antibody (Ab) is reacted with the homologous hormone labelled with a radioisotope (*H). Upon addition of an increasing amount of the hormone (H), a correspondingly decreasing fraction of *H is bound to the antibody. After separation of the bound from the free *H by various means, the amount of radioactivity in one or both of these two fractions is evaluated and used to construct a standard curve against which the unknown samples are measured. (Chard, 1982)

2.8.1.2.1. Iodination of gonadotropin hormones

Gonadotropin hormones were iodinated using Chloramine-T method of Greenwood et al. (1963).

i) Preparation of Bio-Gel P-60 columns for gel filtration of iodinated hormones.

A. Washing columns

1. 10 ml disposable glass pipettes were used with the top 2 cm. of the pipette cut-off.
2. Columns were thoroughly washed in Biochlor (Gibson Chemical, Ltd), rinsed and dried, siliconized with Cotasil (Ajax Chemical, Sydney, N.S.W.), and washed in double distilled water and dried again.

B. Preparation of Bio-Gel

1. Approximately 3 g Bio-Gel (Bio-Rad Laboratories, California, USA) was placed into 500 ml beaker and 300 ml PBS (at room temperature) added and mixed with continued stirring for 2-3 hours.

2. The solution was allowed to settle and the excess buffer removed from above the gel. A new volume of PBS was added to the gel and stirring recommenced for 15 minutes.
3. Step 2 was repeated 3 times to wash the gel solution.

C. Preparation of columns

1. A medium size glass bead was introduced into a 10 ml pipette which was placed in a column-holding device. A small piece of silastic tubing (4.7 mm OD., 3.4 mm ID.) was attached to the bottom of the pipette, and a screw clamp placed around the tube to control column flow.
2. Column was rinsed with PBS and filled with Bio-Gel slurry.
3. Bio-Gel was allowed to settle to the 10 ml mark.
4. Clamp released and Bio-Gel allowed to settle further.
5. Column was kept wet all times with PBS.
6. Column was washed with 20 ml PBS.
7. 1.5 ml 5% bovine serum albumin in PBS was gently pipetted onto column-bed, below buffer, for better recovery of iodinated protein.
8. Column was washed with another 20 ml PBS.
9. Columns were usually prepared 24 hours prior to use and kept at room temperature.

ii) Reagents

1. Purified hormone

   Highly purified hormone preparations of LH + FSH were used. Details have been described separately in the procedures under each hormone.
2. Radioactive iodine

   Sodium iodide (^{125}I) was obtained from Amersham, Australia. For each iodination 0.5 mCi (5 μl) was used.

3. Chloramine-T

   A stock solution of Chloramine-T was prepared by dissolving 6 mg Chloramine-T in 15 ml PBS, immediately prior to each iodination.

4. Sodium metabisulphite ($Na_2S_2O_5$):

   Sodium metabisulphite (10 mg) was dissolved in 10 ml PBS prior to each iodination.

5. Transfer solution

   50 mg of Potassium iodide (KI) was dissolved in 5 ml of 16 % sucrose in PBS prior to each iodination.

6. Rinse solution

   50 mg of Potassium iodide (KI) was dissolved in 5 ml of 8 % sucrose in PBS prior to each iodination.

iii) Iodination procedures

1. Five µg of protein to be iodinated was dissolved in 25 µl PBS, in an Eppendorf tube.
2. Sodium iodide (^{125}I) (5 µl = 0.5 mCi) was added to the hormone and mixed well by tapping on the tube.
3. Chloramine-T stock solution (10 µl = 4 µg) was added and mixed by gentle tapping on the tube for one minute.
4. Sodium metabisulphite (50 µl = 50 µg) was added to stop the reaction.
5. One minute later, transfer solution (100 µl) was added, and the mixture was transferred into the PBS layer above the surface of the Bio-Gel column. The Eppendorf tube was then rinsed with rinse solution (100 µl) and transferred on to the column.
6. Approximately 20 fractions (~ 0.5 ml/fraction) were collected in tubes (12 x 75 mm) containing 5% BSA solution (100 µl). The ^{125}I labelled hormone was eluted first, followed by the free ^{125}I.
7. A sample (10 µl) of each fraction was counted for radioactivity.
8. The fraction with the highest radioactivity in the hormone peak was retained and diluted in PBS to give approximately 20000 cpm per 100 µl.

2.8.1.2.2. Luteinizing hormone (LH) RIA.

i) General Method

200 µl of standard hormone or plasma samples (unknown) were placed in 12 x 75 mm glass vials using a reagent dispensor (Micromedic Systems, Horsham, PA, USA), along with 400 µl of antibody diluted in Gel PBS to give appropriate binding and displacement (dilution that could bind approximately 40 % of the amount of radiolabelled hormone added in tubes containing only 200 µl of Gel PBS buffer - 'blank samples'). Iodinated hormone (100 µl) was then added with another dispenser, and tubes shaken to ensure mixing of contents. The samples were assayed in duplicate and the standards in triplicate. Assay tubes were held at room temperature (~22 °C) for 24 hours.

ii) Separation of antibody -bound from free hormone

100 µl of Antirabbit gamma glogulin (ARGG) antiserum raised in wether (W297, 5 % dilution) was then added to the tubes containing free and antibody - bound hormone; the tubes were mixed well and held at room temperature for another 24 hours and then at 4 °C for a final 24 hour period. ARGG formed a precipitate with the antibody along with the bound fraction of the hormone. The free hormone was washed with 1.7 ml of PBS (at 4 °C) and decanted ('vacuum system') after centrifugation (2000 rpm for 15 minutes at 4 °C). The pellet containing the bound fraction in the precipitate was counted in the auto radioimmunoassay gamma counter. (see section 2.8.1.2.2.v)

iii) Hormones

A highly purified form of ovine LH (NIDDK-oLH-I-3) was used for iodinations. Standards of 0, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10 ng/200 µl oLH were prepared in gel PBS, using ovine LH (NIADDK-oLH-25). Both hormone preparations were obtained from the National Institute of Arthritis, Diabetes & Digestive & Kidney Diseases (NIADDK), Bethesda, Maryland, U.S.A.

iv) LH Antiserum

The anti-ovine LH serum (NIADDK-anti-oLH-I) was also obtained from the National Institute of Arthritis, Diabetes & Digestive & Kidney Diseases (NIADDK), Bethesda, Maryland, U.S.A. The antiserum was used at a dilution of 1 in 2000000. The antiserum exhibited low cross-reactions with TSH (0.12 %), PRL (< 0.01 %), FSH (5.4 %) and GH (0.6 %) (Information supplied by NIADDK). The sensitivity of the assay using this antisera was 0.195 ng/ml and the inter and intra-assay coefficients of variation were 13.4 % and 5.5 % respectively. The sensitivity of the assay was defined as the least concentration of unlabelled hormone which can be distinguished from a sample containing no unlabelled hormone (zero standard), the distinction being based on the confidence limits (mean ± 1.96 SD) of the estimate of the zero standard on the one hand and the standard on the other (Chard, 1982). Intra-assay coefficients of variation were calculated from six to eight replicates of a sample run at the beginning and at the end of the same assay. One sample of low and one sample of high concentration of the hormone, each run in duplicate in all assays, were used to calculate inter-assay coefficients of variation.

v) Calculation of results

A. Non-specific binding component (NSB)

An estimate of the percentage of counts added which contribute to the bound fraction, in the absence of antibody, was required so that a correction could be applied to the calculation of actual counts bound. To assess this within each assay, 3 tubes containing 200 µl of gel PBS, 100 µl of ^{125}I LH and 400 µl of gel PBS + 0.002% RGG were included.

B. Calculation of % bound

The percentage of total counts bound to antibody, including correction factor for NSB % was calculated as follow:

$$\% \text{ Bound} = \frac{\text{Total counts bound to antibody}}{\text{Total counts added to the tube}} \times 100$$

Standard curves were constructed by plotting % bound against LH concentration. The % bound data was fitted in a spline curve plot of :

$$\frac{B^{o}\text{ (\% bound in the absence of LH)}}{B^{x}\text{ (\% bound at each concentration of LH)}}$$

against LH concentration, from which test samples were quantitated. The counting and calculations were carried out in a LKB-Wallac automatic microcomputer-controlled two channel gamma counter dedicated to RIA applications.

C. Results

The hormonal concentration was finally expressed in ng/ml. If the values in samples were below assay sensitivity, then these samples were given the value of assay sensitivity.

2.8.1.2.3. Follicle stimulating hormone (FSH) RIA

General methods, separation of antibody bound from free hormone and calculation of the results are similar to those used for oLH.

i) Hormones

A highly purified form of ovine FSH (NIDDK-oFSH-I-1) was used for iodinations. Standards of 0, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10 ng/200 µl oFSH were prepared in gel PBS, using ovine FSH (NIDDK-oFSH-RP-1). Both hormone prêparations were obtained from the National Institute of Arthritis, Diabetes & Digestive & Kidney Diseases (NIADDK), Bethesda, Maryland, U.S.A.

ii) FSH antiserum

The anti-ovine FSH serum raised in rabbits (NIDDK-anti-oFSH-1) was obtained from the National Institute of Arthritis, Diabetes & Digestive & Kidney Diseases (NIADDK), Bethesda, Maryland, U.S.A. The FSH antiserum was used at a dilution of 1 in 80000. The antiserum

exhibited low cross-reactions with PRL (< 0.01%), LH (0.17 %) and GH (< 0.01%). (Information supplied by NIADDK) The sensitivity of the assay using this antisera was 0.200 ng/ml and the inter and intra-assay coefficients of variation were 13.9 % and 8.4 % respectively.

2.8.1.2.4. Prolactin (PRL) RIA

General methods (except that we have used 50 µl of standard, or 50 µl plasma samples diluted at 1:10, instead of 200 µl), separation of antibody bound from free hormone and calculation of the results are similar to those used for oLH and o FSH.

i) Hormones

A highly purified form of ovine PRL (NIDDK-oPRL-I-2) was used for iodinations. Standards of 0, 0.098, 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 ng/50 µl oPRL were prepared in gel PBS, using ovine PRL (NIDDK-oPRL-I-2). Both hormone preparations were obtained from the National Institute of Arthritis, Diabetes & Digestive & Kidney Diseases (NIADDK), Bethesda, Maryland, U.S.A.

ii) PRL antiserum

The anti-ovine PRL serum raised in rabbits (NIDDK-anti-oPRL-2) was obtained from the National Institute of Arthritis, Diabetes & Digestive & Kidney Diseases (NIADDK), Bethesda, Maryland, U.S.A. The PRL antiserum was used at a dilution of 1 in 600000. The antiserum exhibited low cross-reactions (< 0.06 %) with TSH, FSH, LH and GH. (Information supplied by NIADDK) The sensitivity of the assay using this antisera was 1.0 ng/ml and the inter and intra-assay coefficients of variation were 15.0 % and 9.3 % respectively.

2.8.1.3. RIA for steroid hormones

2.8.1.3.1. Liquid scintillation system

(A). Scintillation fluid

5.0 g. PPO (2,5-Diphenyloxazole)

dissolved in 1 litre of toluene

(B). Counting of aqueous systems

After adding the aqueous sample and scintillation fluid (1 ml/vial), the polyethylene vials were shaken vigorously for approximately 20 minutes on a mechanical shaker to facilitate the transfer of radioactivity from the aqueous to the organic phase. Radioactivity was determined in a LKB-Wallac, Rack Beta II liquid scintillation counter, Model no. 1215.

2.8.1.3.2. Testosterone RIA

i) Testosterone antiserum

The testosterone antiserum (no. 457) was raised in sheep against testosterone-3-carboxymethyloxime-BSA conjugate and was a gift from Dr. R.I. Cox, Hormone Assay Development Group, CSIRO, Division of Animal Production, Prospect, Blacktown, N.S.W.. The cross reactivity of this antiserum is 98 % with 5 α-dihydrotestosterone, 47 % with 4-androsten-3ß, 17ß-diol, 4.7 % with androstenedione, 3.6 % with 4-androsten-17ß, 19-diol-3-one and less than 1 % with other steroids (Information supplied by Dr. R. I. Cox). The sensitivity of the assay was 0.06 ng per ml and the inter and intra assay coefficients of variation were 17.1 % and 5.2 %.

ii) Assay method

One ml of toluene:hexane (2:1) was added to 100 µl of sample or standard (0, 6.25, 12.5, 25, 50, 100, 200, 400, 800, 1600 pg testosterone in 100 µl gel PBS) in disposable glass tubes (12 x 75 mm) and extracted for 30 seconds on a vortex mixer. The aqueous phase was then frozen in a liquid nitrogen and ethanol bath, the solvent extract was decanted into clean tubes and dried at 43 °C in a stream of air. 200 µl of [1,2,6,7-^{3}H] testosterone (~10000 cpm/200µl) together

with 200 µl of antiserum (1:80000) was added to the dried samples and standards and assay tubes were incubated at 4 °C overnight. Free and bound testosterone were separated with 200 µl dextran coated charcoal (62.5 mg dextran T70 and 625 mg Norit A charcoal in 100 ml PBS) for 15 minutes at 4 °C. The tubes were centrifuged at 2000 rpm for 20 minutes, the supernatant that contained the bound fraction of the hormone was transferred to polyethylene scintillation vials and the radioactivity was counted as described in section 2.8.1.3.1.

iii) Results

The hormonal concentration was finally expressed in ng/ml. If the values in samples were below assay sensitivity, then these samples were given the value of assay sensitivity (0.06 ng/ml).

2.8.2. Titre check and specificity

2.8.2.1. Titre check assay

i) Plasma dilution

All samples were diluted with PBS. Dilutions 1:100 and 1:200 were made for the samples coming from passively immunized lambs. For the active immunization samples, the following dilutions were prepared: 1:500, 1:1000, 1:5000 and 1:10000. For all control animals, straight plasma, dilution 1:10 or dilution 1:100 were used for titre check.

ii) Labelled steroids

- (2,4,6,7,16,17-^{3}H[N])oestradiol used for titre determination during immunization against oestradiol-17ß.
- (2,4,6,7-^{3}H[N])oestrone used for titre determination during immunization against oestrone.
- (1,2,6,7-^{3}H[N])testosterone used for titre determination during immunization against testosterone.

Specific activites: 158, 96 and 80 curies/mmol (558, 347 and 272 Ci/ml) respectively.

iii) Assay method

Circulating antibody titres were determined using the instructions outlined in detail by Abraham (1974).

The titre was determined by incubating 100 µl of diluted serum with 100 µl of labelled steroid (~10000 cpm/100µl) and 100 µl of gel-PBS overnight at 4 °C (final volume: 300 µl). Free and bound hormone were separated with 500 µl dextran coated charcoal (25 mg dextran T70 and 250 mg Norit A charcoal in 100 ml PBS) for 15 minutes at 4 °C. The tubes were then centrifuged at 2000 rpm for 15 minutes, the supernatant that contained the bound fraction was transferred to polyethylene scintillation vials and the radioactivity was counted as described in section 2.8.1.3.1.

Antiserum titre was defined as that dilution binding 50% of the radioactive steroid.

The dilution was calculated for the assay tube before any other solutions were added.

2.8.2.2. Specificity

Cross-reactivity with common steroids were determined by incubating increasing amounts of 'cold' (unlabelled) steroid in the presence of homologous labelled steroid [*S] and the corresponding antiserum to be tested.(dilution that bind 50 % of added *S).

Briefly, the assay consists of incubating 100 µl of diluted antiserum with 100 µl of homologous labelled steroid [*S] (~10000 cpm/100µl) and 100 µl of increasing amounts (0 pg/ml to 1000 pg/ml in gel pBS) of different unlabelled steroids (e.g. [S] or [X] = [E_2], [E_1], [E_1S], [T], [DHT], [A_4], etc.). The tubes were left overnight at 4 °C. Bound and free hormone were separated by charcoal stripping as described in section 2.8.1.3.2. ii.

The relative inhibitory activity of each steroid for the antiserum was calculated from the ratio of the mass of homologous steroid [S] required to displace 50% of the radiolabelled homologous steroid [*S] to the màss of the cross reacting steroid [X] required to displace the same fraction of the labelled steroid.

2.8.3. Total testicular blood plasma flow (TTBPF)

2.8.3.1. Solutions

10 % Trichloroacetic acid (10 g in 100 ml double distilled water, 'ddH_2O')

0.1 % Sodium nitrite ($NaNO_2$) (100 mg in 100 ml ddH_2O),

this solution should be freshly made; do not keep more than 24 hours.

0.5 % Ammonium sulphamate (500 mg in 100 ml ddH_2O)

0.1 % N-1-Naphthylethylene diamine dihydrochloride (100 mg in 100 ml ddH_2O)

2.8.3.2. Para-amino-hippuric acid (PAH) determination by spectrophotometry

The method of Kalant and MacArthur (1950) was used to determine plasma PAH concentration.

i) Precipitation

1. Pipet 100 µl of each plasma sample in 12 x 75 mm glass vials.
2. Add 1.9 ml of 10% trichloroacetic acid.
3. Mix well (vortex).
4. Centrifuge at 2000 rpm for 10 minutes.
5. Transfer 1 ml of supernatant in a new clean 12 x 75 mm glass vial.

ii) Chemical reaction

6. Add 100 µl of 0.1% sodium nitrite:
   - to each sample (each vial containing 1 ml of supernatant),
   - to each tube containing standards (tubes containing 0, 5, 10, 20, 40, 50, 100, 200, 400 µg of PAH in 1 ml of 10% trichloroacetic acid)
   - and to tubes containing 1 ml of the infusate preparation

     (the PAH infusate collected during 1 minute before each surgery [~100 µl/min] was diluted in 9.9 ml of ddH_2O; --> 50 µl of this dilution is placed in a glass vial and 950 µl of 10 % trichloroacetic acid is added).
7. Mix well (vortex).
8. Wait 10 minutes.

9. Add 100 μl of 0.5 % ammonium sulphamate to each tube.
10. Mix well (vortex).
11. Wait 10 minutes.
12. Add 100 μl of 0.1% N-1-Naphthylethylene diamine dihydrochloride to each tube.

13. Mix well (vortex).
14. Wait 30 minutes.

iii) Reading on spectrophotometer

15. Read colour on a spectrophotometer adjusted at 540 nm (wavelength).

2.8.3.3. Calculation of Total Testicular Blood Plasma Flow (TTBPF)

TTBPF was calculated by multiplying the PAH concentration of the infusate by the infusion rate and dividing by the difference between PAH concentration in plasma from the ISV and that in the peripheral plasma (Laurie and Setchell, 1978).

The total volume of blood plasma that leaves the testis per minute was expressed in ml/min. The total volume of blood plasma per unit weight of testis was calculated and expressed in μl/g/min.

2.8.4. Daily sperm production (DSP)

2.8.4.1. Fixation

At the end of the experiment, the testis was removed and perfused via the testicular artery with saline followed by 2% glutaraldehyde and 0.1 M. cacodylate buffer, pH 7.2 (Johnson, Petty and Neaves, 1981).

2.8.4.2. Homogenization solution

0.77 g. NaCL (150 mM)

0.50 ml Triton X-100 (0.05% v/v)

0.25 g. NaN_3 (3.8 mM)

made up to 1 litre using ddH_2O

L. 21: For each testis, one small triangular piece of tissue (including tunica albuginea) was taken at the level of the widest diameter and not deeper than 1 cm from the surface of the testis.

2.8.4.3. Method

Determination of DSP rates using homogenization-haemacytometric techniques (Amann, 1970).
Diced testicular parenchyma (0.5 g fixed tissue in 100 ml homogenizing fluid) was homogenized at room temperature for 6 minutes in a Sorval omnimixer. Duplicate homogenates were prepared and evaluated for each testis. Fifteen ml of homogenate was mixed with three drops of eosin-negrosin stain before evaluation under microscope.

The concentration of spermatids in each homogenate (100 ml homog. fluid + 0.5 g fixed tissue) was evaluated by enumeration of the elongated spermatids nuclei in a haematocytometer.

The total number of spermatid nuclei in one large square (0.1 mm^3) x 10000 x (100 ml + 0.5 g) = total number of spermatids in the homogenate.

2.8.4.4. Calculation of daily sperm production (DSP)

DSP per unit weight of testis was calculated by dividing the number of spermatids in the homogenate by the product of the weight of tissue homogenized (0.5 g) and then by the time divisor. The time divisor is the number of days of production represented by these spermatid reserves; for rams, this is 3.56 days (Amann, 1970). This finally gives sperm production in no. of spermatozoa/g/day (DSP/g).

We have also calculated the average DSP for one animal:

$$\frac{[\text{DSP/g (left testis) x left testis weight (g)}] + [\text{DSP/g (right testis.) x right testis weight (g)}]}{2}$$

2.9. Testicular histology

Small blocks of testicular tissue were fixed in Bouin's solution for 24 hours and then transferred to 70% ethanol for storage. The tissues were embedded in paraffin wax and sections of 7 µm thickness were cut with a microtome and stained with haematoxylin and eosin.

Microscopic evaluation was achieved to verify the presence of seminiferous tubules exhibiting complete spermatogenesis at the time of biopsy or castration (main criteria for achievement of

puberty). The occurrence of complete spermatogenesis within each seminiferous tubule was confirmed by the presence in the luminal epithelium of spermatids with condensed elongated nuclei.

2.10. Statistical analysis

All data are presented as mean ± standard error of the mean (sem). Results were analyzed using factorial analysis of variance or using analysis of variance for repeated measures, depending on each experimental design concerned. CLR ANOVA computer program (CLR ANOVA, Clear Lake Research, Morningside, Houston, TX, 77005) was used to to carry out the analysis of variance. Single pairwise comparisons were made using Student's *t* test. Multiple comparisons were made using Duncan's New Multiple-Range test. An appropriate transformation was applied to some hormone data to equalize variances. The data presented in tables and graphs are the actual ones (i.e. non-transformed data). Regression analysis were carried out using Statview computer program (Statview™ 512+, Abacus Concepts, BrainPower Inc., Calabasas, CA) to determine the equation that best fitted the data and the correlation coefficients. The LH profiles were analyzed for pulses using a modified version of the 'Pulsar' algorithm (Merriam & Wachter, 1982) developed for the Apple Mackintosh microcomputer (Munro; Elsevier-BIOSOFT, Cambridge, UK). Statistical methods used for analyzing experimental data are explained in more details in the "Experimental Procedure" sections of chapters 3, 4, 5, 6, 7, 8.

CHAPTER 3.

PUBERTAL DEVELOPMENT IN SOUTH AUSTRALIAN MERINO RAM LAMBS KEPT UNDER SPECIFIC EXPERIMENTAL CONDITIONS.

3.1. INTRODUCTION

A complete chronicle of the events that unfold as an individual progresses through puberty is not yet possible although some characteristic endocrinological changes have been well identified in ram lambs. For instance, increases in LH frequency and mean level have been observed during prepubertal development (Pelletier et al., 1981; Courot and Kilgour, 1984) and seem to reflect an increase in hypothalamic pulsatile GnRH discharge which is generally accepted to be a key event leading to the initiation of puberty (Adams and Steiner, 1988). The increase in the amount of gonadotropins (LH and FSH) secreted at the end of infancy are necessary for the process of sexual maturation and testicular development (Courot, 1967; 71). In ram lambs, testicular volume rises slowly from birth until approximately three months of age then it increases sharply as important cell proliferation and differentiation take place within the seminiferous tubules and lead to the first release of spermatozoa at puberty. The production of steroids by the testis increases also sharply during the period of transition into puberty (Courot and Kilgour, 1984).
The actual time of fertility onset usually will be a reflection of multiple interactions between different environmental influences which will be ultimately limited and interpreted by each individual's unique set of genes (Bronson and Rissman, 1986). Photoperiod is one of the most powerful environmental cues influencing the activity of the reproductive axis in the adult sheep (Lincoln, 1981) and can possibly influence testicular development in ram lambs (Colas et al., 1987).

Although the changes in the spermatogenic and steroidogenic functions of the testis during maturation are well known, no information has been reported concerning the changes in total testicular blood flow occurring during testicular development in the male sheep. The only relevant experiment we have been able to find indicated no difference in relative testicular capillary blood flow (TCBF expressed per gram of testis) between impubertal, prepubertal and

adult ram in the breeding season while lower TCBF was found in the adult ram in the non-breeding season (Courot and Joffre, 1977).

The present experiment was undertaken with the aim of investigating the reproductive endocrinological events which occur during pubertal development in the South Australian Merino ram lambs kept under well defined experimental conditions (early weaning, hand feeding, pen restriction, controlled light environment). It was hypothesized that these experimental conditions will allow a satisfactory rate of maturation and that all lambs will exhibit complete spermatogenesis by 30 weeks of age. Furthermore, we intended to study the changes in total testicular blood plasma flow (TTBPF) occurring during testicular development. Our hypothesis is that an increase in TTBPF should support the increase in testicular size.

3.2. EXPERIMENTAL PROCEDURE

3.2.1. Experimental animals

Sixteen South Australian Merino ram lambs born between 1st and 8th April 1989 were used in this experiment. The lambs were kept outdoors with their mothers until weaning at 12 weeks of age. The lambs were then transferred to a room with controlled light (12 hours light: 12 hours dark). The lambs were grouped in large pens (4 lambs per 6 metre square pen) (as described in section 2.2.1.). After castration, the lambs were returned to their pen and remained there until the completion of the experiment at 30 weeks of age.

3.2.2. Treatment

Ram lambs were randomly assigned to one of the four following groups:

group 14: castration at 14 weeks of age (n=4)

group 22: castration at 22 weeks of age (n=4)

group 26: castration at 26 weeks of age (n=4)

group 30: castration at 30 weeks of age (n=4)

3.2.3. Body weight

Body weight was recorded at the time of castration.

3.2.4. Intensive bleeding and response to GnRH

A few days (~4 days) before castration, the lambs were intensively bled (every 20 minutes for 6 hours as described in section 2.6.1.) then were given a single GnRH injection (5 ng/kg body weight) and additional blood samples were collected as described in section 2.6.2.

3.2.5. Measurement of testicular blood plasma flow and response to hCG

TTBPF was measured on anaesthetized lambs before castration as described in section 2.7.2. Testicular responsiveness to a single hCG injection (20 I.U./kg body weight) was simultaneously evaluated as described in section 2.6.3.1. PAH concentration was assayed in each blood sample collected as described in section 2.8.3. The production of testosterone by each testis (defined as the product of plasma flow per testis and the veno-arterial concentration difference for testosterone, i.e.: (ISV [T] - JUG [T]) x TTBPF) was also calculated.

3.2.6. Hormone assays

Plasma samples were assayed for LH, FSH, PRL and testosterone as described in section 2.8.1.2.2., 2.8.1.2.3., 2.8.1.2.4. and 2.8.1.3.2., respectively. Mean hormone levels were determined by assaying a plasma pool representing the 6-h sampling period (section 2.6.1.2.). Each blood sample collected following a GnRH challenge was assayed for LH. Pool samples collected before and after a hCG challenge (section 2.6.3.2.) were assayed for testosterone.

3.2.7. Castration and testicular weight

All ram lambs were castrated and testicular weight recorded as described in section 2.7.4.

3.2.8. Testicular histology

Blocks of testicular tissue taken at castration were prepared as described in section 2.9.

3.2.9. Statistical analysis

Body weight, testicular weight, gonadotropin and testosterone concentrations in plasma were analyzed using one-factor analysis of variance. When significant age differences were noted, means were tested using Duncan's New Multiple-Range test. Analysis of variance for repeated measures was used to compare LH concentrations preceding and following a GnRH injection, as well as testosterone concentrations, TTBPF and testosterone production preceding and following a hCG injection. CLR ANOVA computer program (Clear Lake Research,

Morningside, Houston, TX, 77005) has been used for these analysis. In addition, regression analysis were carried out to determine the curve which best characterized the changes in TTBPF occurring during testicular development. Statview™ 512+ computer program (Abacus Concepts, BrainPower Inc., Calabasas, CA.) has been used for the regression analysis.

3.3. RESULTS

3.3.1. Body weight and testicular weight at castration

As expected, body and testicular weight (average left and right testis) recorded at the time of castration increased significantly with age (for both parameters: $p < 0.01$) (table 3.1).

3.3.2. Testosterone concentration

Mean testosterone concentration in plasma at different ages is shown in fig. 3.1. As expected, there was a continuous increase in testosterone concentration as the lambs got older. Testosterone level started at 0.095 ± 0.010 ng/ml at 14 weeks of age and reached 4.300 ± 1.777 ng/ml at 30 weeks of age.

3.3.3. LH and FSH concentrations

Mean LH concentrations in plasma varied between 0.271 ± 0.034 ng/ml and 0.737 ± 0.121 ng/ml and were significantly lower ($p < 0.05$) at 30 weeks of age compared with the values found at 14 weeks and 26 weeks of age (fig. 3.2.).

Mean FSH concentrations in plasma varied between 0.196 ng/ml $\pm$ 0.001 and 0.558 ± 0.119 ng/ml and were significantly higher ($p < 0.05$) at 26 weeks of age than at the other ages (fig. 3.3.).

3.3.4. PRL concentration

Mean PRL concentrations in plasma varied between 44.066 ± 14.325 ng/ml and 190.907 ± 21.935 ng/ml and were significantly higher ($p < 0.01$) at 22 weeks of age than at the other ages (fig. 3.4.).

3.3.5. LH concentration after a GnRH challenge

The pituitary response to exogenous GnRH is shown in fig. 3.5. The maximal increase in LH was observed ten minutes after GnRH injection in all lambs. The peak height, defined as the

Table 3.1.

Mean* body and testicular weight at castration in South Australian Merino ram lambs of four age groups.

Age at castration (weeks)	Body weight (kg ± s.e.m.)	Testicular weight ** (g ± s.e.m.)
14	25.38 ± 0.66^{a}	11.14 ± 1.78^{a}
22	30.13 ± 0.72^{b}	46.04 ± 10.59^{b}
26	32.30 ± 0.27^{b}	68.94 ± 7.04^{c}
30	37.50 ± 1.30^{c}	116.27 ± 7.49^{d}

*: mean of 4 lambs per age group

**: mean left and right testes

values with different superscripts are significantly different at 95%

Fig. 3.1. Testosterone Concentration

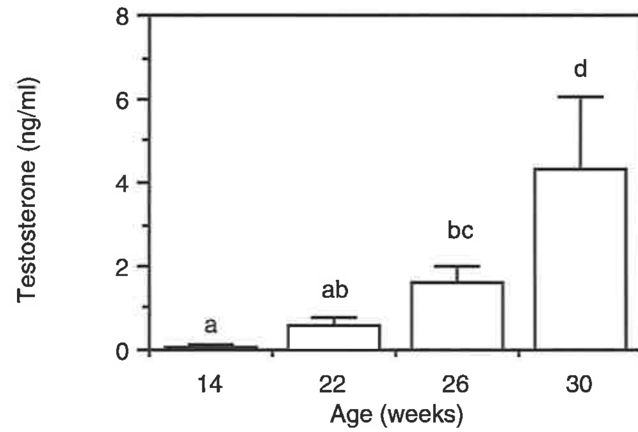


Fig. 3.2. LH Concentration

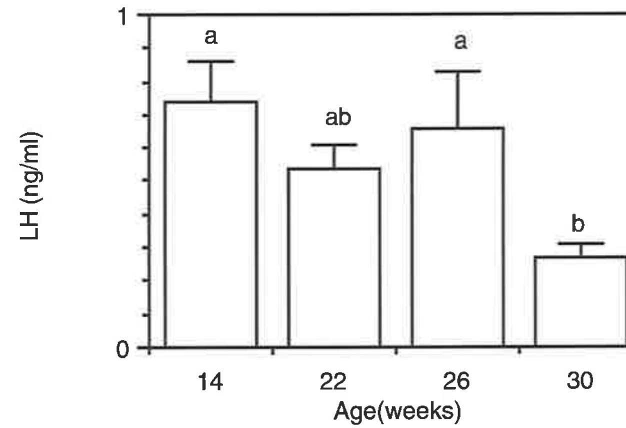


Fig. 3.3. FSH Concentration

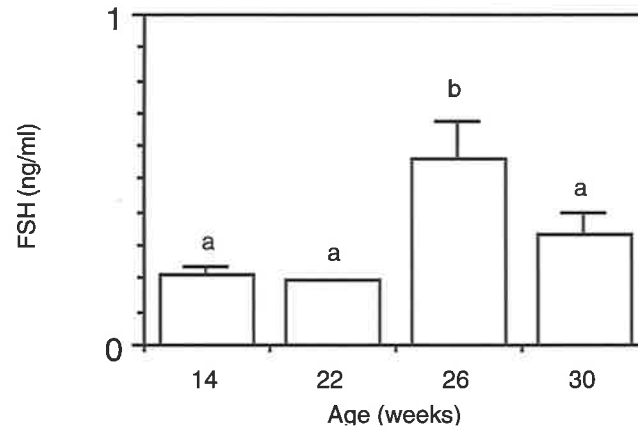
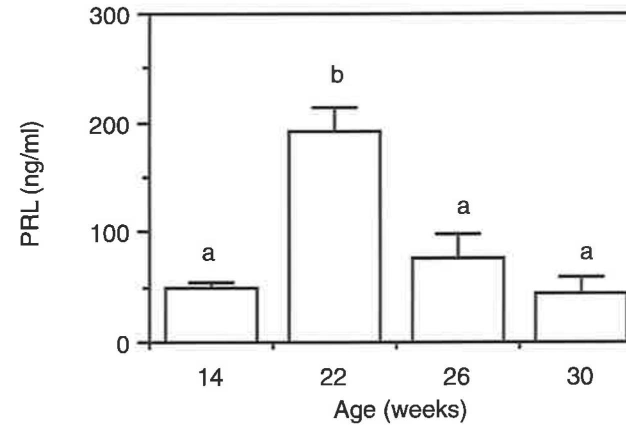
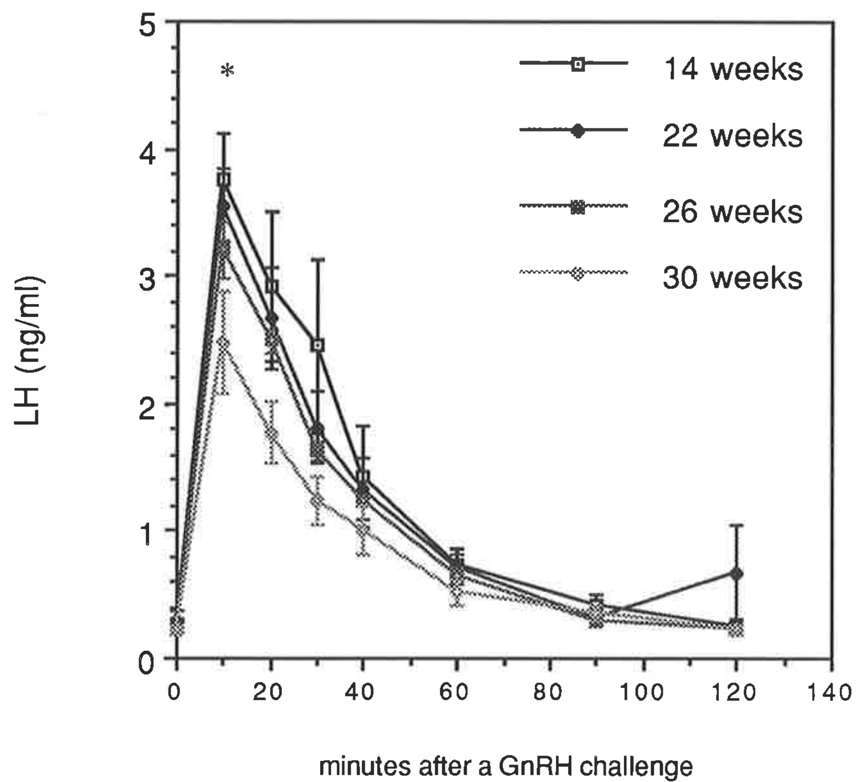


Fig. 3.4. Prolactin Concentration



values with different superscripts are significantly different at 95%

Fig. 3.1, 3.2, 3.3 and 3.4 : Mean Testosterone, LH, FSH and PRL concentrations (ng/ml ± sem) in plasma collected at 14, 22, 26 and 30 weeks of age from South Australian Merino ram lambs (mean of 4 lambs per age group).



* : values found at 14 weeks of age being significantly different from values found at 30 weeks of age ($p < 0.05$)

Fig. 3.5.: Time course of circulating LH (mean ± sem) after a single i.v. injection of GnRH (5 ng/kg BW) to South Australian Merino ram lambs at 14, 22, 26 and 30 weeks of age (mean of 4 lambs per age group).

L. 15: The lack of significance (i.e. the fact that the hCG injection did not significantly affect T concentration in the JUG or in the ISV at 14, 22 and 26 weeks of age) appears to be due to the large "between animal" variation .

highest LH concentration associated with the response curve, decreased with age ($p < 0.07$). Circulating LH concentration was significantly higher ($p < 0.05$) 10 minutes post injection in the 14 week old than in the 30 week old lambs.

3.3.6. Testosterone concentration after a hCG challenge

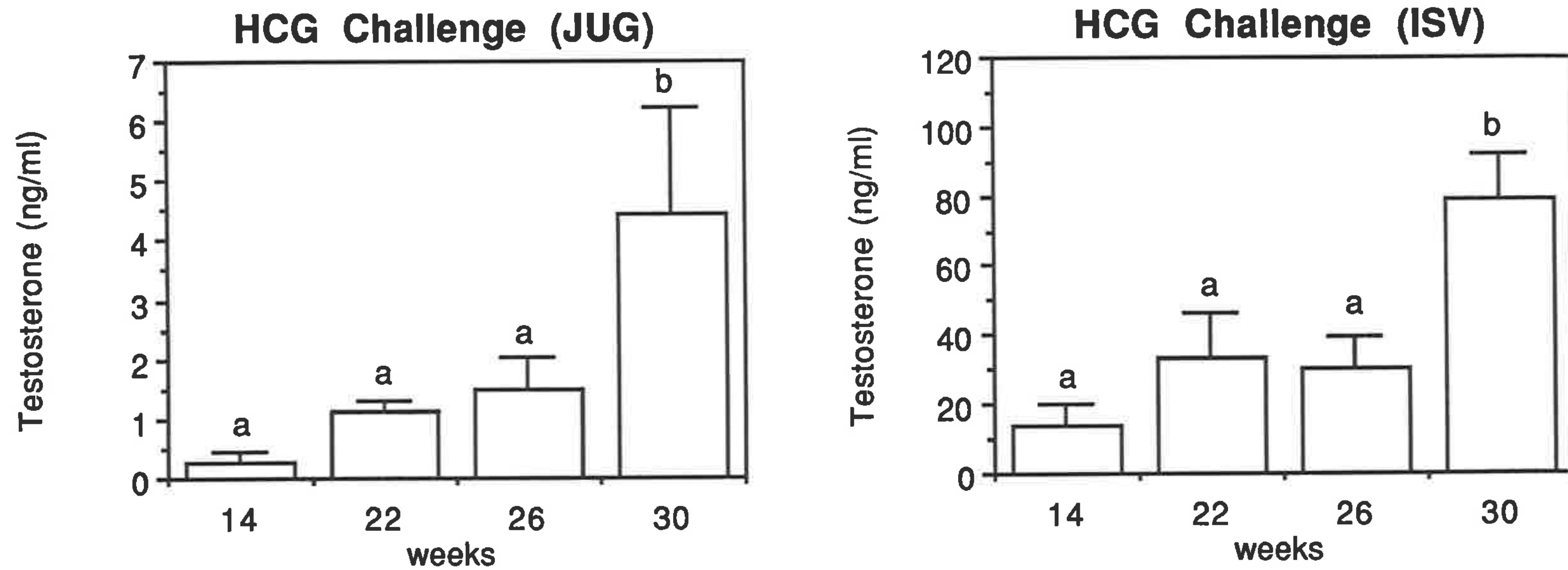
The testicular response to a single dose of hCG is summarized in fig. 3.6. In contrast to the significantly different mean testosterone levels found in the jugular of the four age groups during intensive bleedings (in conscious lambs), no significant differences among the four age groups were found in the testosterone concentration measured in the jugular (JUG) and both internal spermatic veins (ISV) (in anaesthetized lambs) before the hCG injection. However a significant age effect was demonstrated in the level of testosterone measured in the jugular vein ($p < 0.06$) and the left-ISV ($p < 0.05$) after a hCG challenge; the older lambs producing more testosterone. Although testosterone concentration tended to be more elevated in all samples collected after the hCG challenge, a significant difference between the testosterone concentration measured in JUG and in both ISV before and after the hCG injection was demonstrated only in the 30 week old lambs. (see INSERT)

3.3.7. Total testicular blood plasma flow (TTBPF)

The testicular blood flow from 19 testes (one testis from each lamb of the three youngest age groups and from one lamb at 30 weeks and both testes from three of the 30 week old lambs) have been successfully measured. TTBPF per testis (ml/min) and TTBPF per unit weight of testis (µl/g/min) did not change significantly after the hCG injection (data not presented). TTBPF per unit weight of testis was significantly higher ($p < 0.05$) at 14 weeks of age compared with the values found at 26 weeks and 30 weeks of age (table 3.2.). TTBPF per testis was significantly higher ($p < 0.05$) in the 30 week old groups compared with the values found at 14 weeks and 26 weeks of age (table 3.2.).

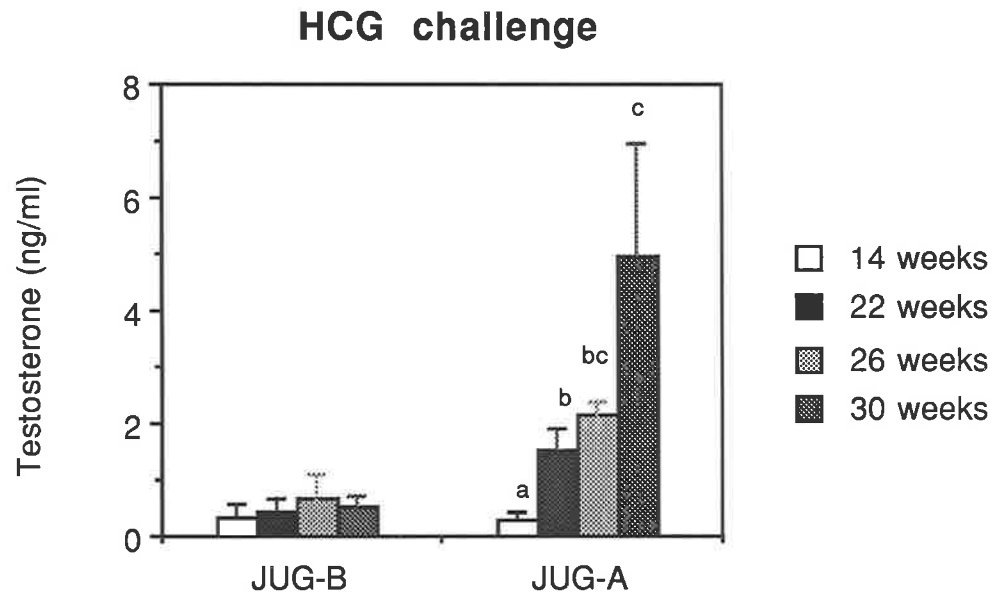
The regression lines that best described the changes in TTBPF in relation to changes in testicular weight (TW) are shown in fig. 3.7.

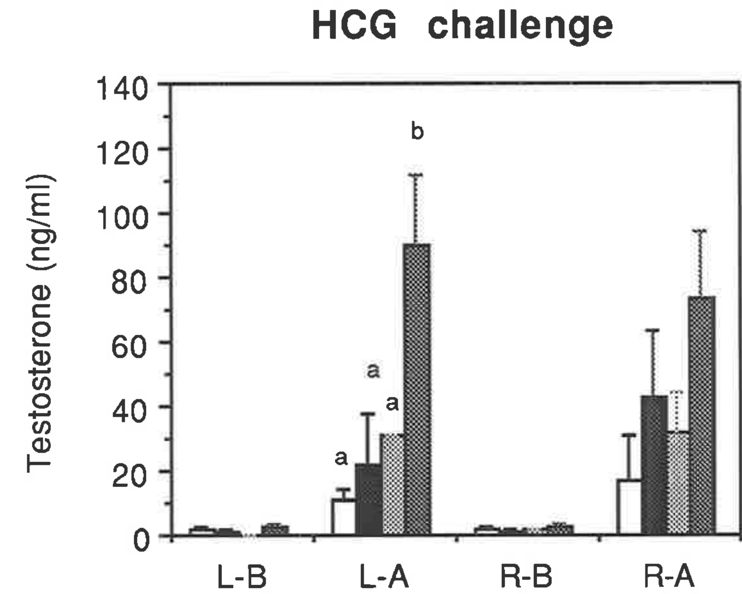
TTBPF per testis (y) increases as TW (x) increases. This relationship can be represented by the following equation: $y = 0.084x + 3.671$ (r: 0.533; $p = 0.0188$).



values with different superscripts are significantly different at 95%

Fig. 3.6.a: Differences in mean plasma testosterone concentrations (ng ± sem), between POST-hCG values and PRE-hCG values measured in the jugular vein (JUG) and in the internal spermatic veins (ISV) of South Australian Merino ram lambs at 14, 22, 26 and 30 weeks of age (mean of 4 lambs per age group). (hCG challenge = a single i.v. injection of hCG; 20 I.U./kg BW).





values with different superscripts are significantly different at 95%

Fig. 3.6.: Mean testosterone concentration (mean ± sem) measured in the jugular (JUG) and in the left (L) and right (R) internal spermatic vein before (B) and after (A) a single i.v. injection of hCG (20 I.U./kg BW) to South Australian Merino ram lambs at 14, 22, 26 and 30 weeks of age (mean of 4 lambs per age group).

Table 3.2.

TTBPF per testis and per unit weight of testis in South Australian Merino ram lambs of four age groups.

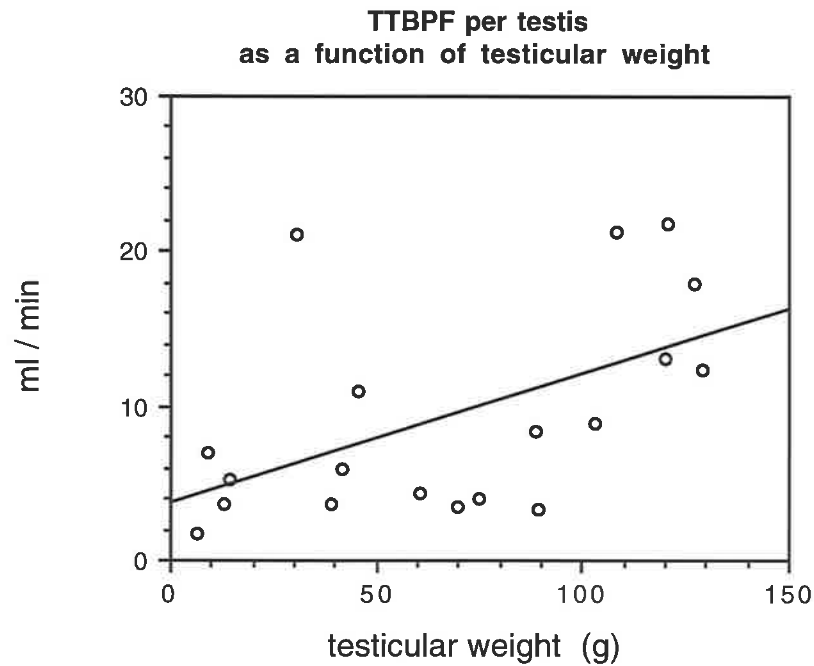
Age at castration (weeks) (number of testes)	**TTBPF per testis (ml/min ± s.e.m.)**	**TTBPF per unit weight of testis (µl/g/min ± s.e.m.)**
14 (n=4 testes)	**4.36 ± 1.10[a]**	**416 ± 117[a]**
22 (n=4 testes)	**8.67 ± 4.16[a b]**	**244 ± 149[a b]**
26 (n=4 testes)	**5.56 ± 1.83[a]**	**99 ± 47[b]**
30 (n=7 testes)	**14.83 ± 2.12[b]**	**129 ± 17[b]**

values with different superscripts are significantly different at 95%

Some results obtained from the 26 week old lambs [i.e. mean FSH (see fig. 3.3), TTBPF (see table 3.2) and T production (see table 3.3)] did not seem to follow the same trend as the results obtained from the other groups of ram lambs. We cannot give an explanation for this ~~disruption~~ discrepancy however it seems that growth rate in these lambs was slightly lower than growth rate in the other groups of lambs (mean live weight of the 26 weeks old lams was not significantly different from that of the 22 weeks old lambs - see table 3.1). One must note that, for the 26 week old lamb, T production and TTBPF follow the same trend since T production by each testis is defined as the product of TTBPF per testis and the veno-arterial concentration difference for testosterone (see other comments presented on P. 118a).

Fig. 3.7:

In both graphs, the two points that seem to distort the equation cannot be deleted, they have to be accepted as such. Measurement of blood flow in a very small testis is quite difficult (because of the size of the blood vessels) and the fact that we noticed a large "between animal" variation for the impubertal testis was not surprising. Thus one or two values that differ from the others is acceptable. Of course, a better estimate of the changes occurring during testicular maturation could have been obtained with several measurements taken on a larger number of small impubertal testes (< 30 g).



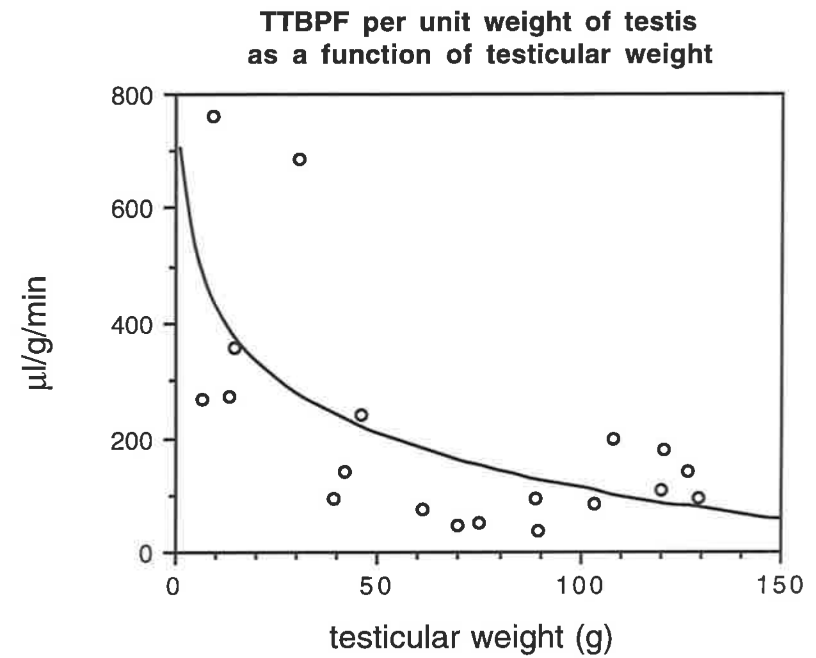


Fig. 3.7.: Total testicular blood plasma flow (TTBPF) per testis (ml/min ± sem; left) and per unit weight of testis (µl/g/min ± sem; right) as a function of testicular weight in 16 South Australian Merino ram lambs aged between 14 and 30 weeks (blood flow measured in 19 testes).

By contrast, TTBPF per unit weight of testis (y) falls as TW (x) increases and the equation that best described this relationship is: $y = 746.44 - 317.72 * \log(x)$ (r: 0.645; $p = 0.0029$).

3.3.8. Testosterone production.

The production of testosterone has been calculated for 19 testes (one testis from each lamb of the three youngest age groups and from one lamb at 30 weeks and both testes from three of the 30 week old lambs). For all groups of lambs, mean testosterone production by one testis (ng/min) increased significantly ($p < 0.01$) after the hCG injection. Before stimulation with hCG, mean testosterone production by one testis was significantly higher ($p < 0.05$) in the 30 week old groups compared with the values found at 14 weeks and 26 weeks of age (table 3.3.). After the hCG injection, mean testosterone production by one testis was significantly higher ($p < 0.01$) in the 30 week old groups compared with the values found at 14, 22 and 26 weeks of age (table 3.3.).

3.3.9. Spermatogenesis

Testicular tissue from all lambs was examined under light microscopy. Spermatozoa were present in the seminiferous tubules in 0 lamb out of 4 lambs at 14 weeks of age, in 1 out of 4 lambs at 22 weeks, in 2 out of 4 lambs at 26 weeks and finally in all lambs (4 out of 4 lambs) at 30 weeks of age. The average body and testicular weight of the 3 rams in which spermatozoa were present in seminiferous tubules at 22 and 26 weeks of age were 30.90 ± 1.19 kg.(range: 28.6 - 32.6 kg.) and 78.45 ± 2.87 g.(range: 69.34 - 89.57 g.). The testes that have not started to release spermatozoa were all below 61 g (< 80 cm^3).

3.4. DISCUSSION

The continuous increase in body weight, circulating testosterone concentration and testicular weight observed as the lambs grew older was comparable to that reported by other investigators for the Merino and Merino/Corriedale rams (Lee et al., 1976; Papachristoforou, 1987) and for other breeds of sheep (Skinner and Rowson, 1968; Dyrmundsson and Lees 1972; Schanbacher et al., 1974; Echternkamp and Lunstra, 1984; Klindt et al., 1985; Al-Nakid et al., 1986; Olster and Foster, 1986; Yarney and Sanford, 1989).

Table 3.3.

Mean testosterone production (ng/min) by one testis in South Australian Merino ram lambs of four age groups before and after a hCG challenge.

Age at the time of measurement (weeks) (number of testes)	T production per testis (ng/min ± s.e.m.) before hCG	T production per testis (ng/min ± s.e.m.) after hCG
14 (n=4 testes)	6.97 ± 2.39^{a}	65.27 ± 30.63^{a}
22 (n=4 testes)	18.00 ± 11.36$^{a\,b}$	267.89 ± 131.37^{a}
26 (n=4 testes)	6.36 ± 5.84^{a}	178.10 ± 69.07^{a}
30 (n=7 testes)	32.26 ± 6.16^{b}	1213.63 ± 313.36^{b}

values with different superscripts are significantly different at 95%

L. 1-5: We do not know if the difference between our results and the results reported by Lee and coworkers (1976) was due to differences in growth rate since we did not directly measured this variable in the present study and since these authors did not report this variable in their paper either. Nevertheless, we assume that growth rate of our Merino ram lambs was better than their Merino/Corriedale ram lambs since the ~~latest~~ latter were reared outdoors. In other studies presented in this thesis (see chapters 5 and 6), repeated measurements of body weight throughout the experiments clearly indicated that growth rate for Merino lambs kept in the same controlled environment was very good if the lambs were weaned at 12 weeks of age (as it was done in the present study).

All the 30 week old and half of the 26 week old ram lambs have started to release spermatozoa in their seminiferous tubules. This timing is earlier than that reported by Lee et al. (1976) who observed that event by 39-42 weeks of age, in Merino/Corriedale lambs born in spring, weaned at 3 months of age and reared outdoors in Victoria, Australia. Therefore, our result indicates that our experimental conditions allowed an advantageous rate of maturation. The reactivation of the hypothalamic pulse generator that is considered to be the key event leading to the initiation of puberty (Adams and Steiner, 1988) and which is reflected by a transient increase in LH level in the prepubertal ram lambs (Crim and Geschwind, 1972b; Foster et al., 1978; Pelletier et al., 1981; Courot and Kilgour, 1984; Yarney and Sanford, 1989) has not been observed in the youngest lambs. Thus, we suspect that this rise in LH secretion must have occurred before the beginning of the study. On the contrary, in the 30 week old lambs, we have observed a slight decrease in mean LH concentration and, a pituitary response to a GnRH challenge that was significantly less pronounced at that age than in the 14 weeks old lambs. Furthermore, the testicular response to a hCG challenge (measured in anaesthetized lambs) was clearly more pronounced in the 30 week old groups than in the younger lambs in agreement with previous report (Foster et al., 1978). Overall, these results support the idea of an increasing negative feedback effect on the pituitary as the steroidogenic function of the testis becomes considerable (Crim and Geschwind, 1972a; Foster and Ryan, 1981).

Although no particular tendency in the secretion of FSH was observed between our groups of lambs, mean FSH level in plasma was more elevated in the 26 week old age group. Therefore, the maturational changes in the secretion of FSH did not follow the pattern of LH secretion and did not appear to be related to circulating testosterone concentration. This observation is also in agreement with other reports (Pelletier et al., 1981; Jenkins and Waites, 1983) and supports the idea that although the two gonadotropins are believed to be regulated by GnRH and released by the same pituitary cells, different factors modulate their releases (Reiter and Grumbach, 1982). Indeed, although the mechanisms of regulation of the gonadotropins are not totally understood, it is now generally accepted that FSH release is regulated by the feedback action of some peptides of testicular origin (e.g. inhibin, activin; de Kretser et al., 1987; Clarke et al., 1991;

L. 11: Although, the exact mechanism of prolactin action was not determined, these authors suggested a possible synergistic effect on the testis with LH and testosterone.

L. 20: As mention previously (see P. 113a), live weight of the 26 week old ram lambs was not significantly different from live weight of the 22 week old ram lambs suggesting that growth rate may have been slower for the 26 week old ram lambs. Nevertheless, the fact that half of the 26 week old ram lambs had achieved puberty at that age clearly indicates that these lambs had reached an appropriate level of maturation (2 out of 4 lambs had achieved puberty at 26 weeks of age).

L. 27: Although the values observed in the 26 week old age group tended to invalidate this hypothesis.

Price, 1991) as well as by some gonadal steroids, while LH release is mainly under the control of gonadal steroids (Schanbacher, 1980). Unfortunately, we have not measured the changes in inhibin secretion occurring during pubertal development in our lambs and thus, we do not know if FSH concentration was somewhat related to this hormone during this period of transition.

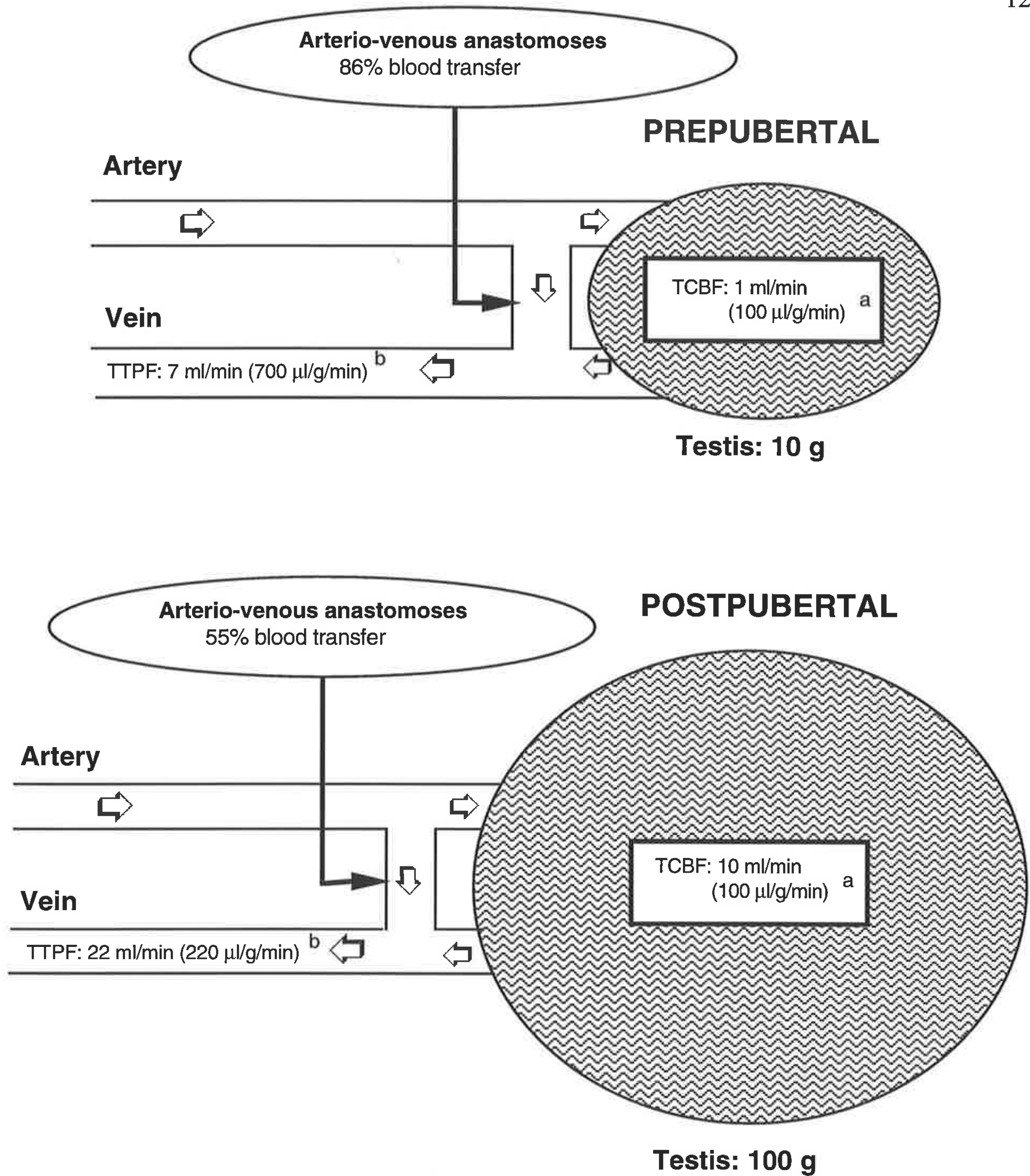
Our lambs were born in April (autumn) and thus grew outdoors with decreasing day length until their arrival to the room with controlled light at 12 weeks of age. PRL levels in plasma were relatively low for all groups of lambs except for the 22 week old age group in which PRL levels were doubled. In prepubertal Ile-de-France ram lambs born in autumn (a faster growing breed than the Merino), low PRL concentrations were also found until a short sharp elevation occurs at 10 to 12 weeks of age (Ravault and Courot, 1975). These authors suggested that this peak in PRL coincided with the onset of spermatogenesis. In our study, the PRL peak observed at 22 weeks of age could also be associated with the time of onset of spermatogenesis for the ram lambs which would have reached puberty by 28-30 weeks of age (in fact, the majority of our lambs) since the complete spermatogenic process in rams lasts 7 weeks. PRL values may vary considerably from one study to another and this could be explained by the fact that PRL secretion is sensitive to photoperiod (Ravault, 1976) and stress (Raud et al., 1971), two factors that can vary greatly between experiments conducted in different countries (e.g. different latitude, different food), under different lighting (e.g. natural or artificial), and in different environment (e.g. field or pen restriction). Since we have obtained a good and homogeneous rate of maturation, we assume that stress or seasonal influences were kept to a minimum in this study and therefore, could not explain the high level of PRL found in the 22 week old lambs. The exact function played by PRL in male reproduction and in pubertal development has not yet been demonstrated. In ram lambs, PRL can possibly be involved in the establishment and maintenance of testicular receptors (Yarney and Sanford, 1989) or may play a role in the secretory activity of the seminal glands (Ravault et al., 1977).

Going with our second hypothesis, we have found that TTBPF per testis increases as testicular weight increases. This finding was expected as more blood is required to feed the increasing mass of tissues present in the developing testis. Besides, it is generally accepted that the amount

of blood flowing through the testis is determined largely by the mass of the tubules (Setchell and Brooks, 1988). Lindner (1961) has also reported that testicular blood flow (per testis) was increasing during testicular development in bulls. However, a surprising observation in the present study is that TTBPF expressed per unit weight of testis falls as testicular weight increases. Indeed, the highest TTBPF values (values ranging between 266-762 µl/g/min) were found in the younger lambs with the smallest testis (< 31 g, n=5). Lower TTBPF values (mean: 114 ± 16 µl/g/min; max.: 238 µl/g/min) were found in the larger testes (> 31 g, n=14) and were more like the values measured in control adult rams studied in the experiment presented in chapter 8 (n=9, mean: 152 ± 26 µl/g/min, max.: 321 µl/g/min). Therefore, it seems that a steep decline in TTBPF per unit weight of testis has occurred before the establishment of spermatogenesis and thus before the rapid increase in testicular weight.

To pursue our discussion on testicular blood flow and to help us explain our rather "opposite" results, we must consider an important observation reported on the fraction of the total blood flow that exchanges with testicular tissue via the capillaries in male sheep. Courot and Joffre (1977) found no modification in testicular capillary blood flow (TCBF expressed per gram of testis) between impubertal, prepubertal and adult ram in the breeding season while lower TCBF was found in the adult ram in the non-breeding season. This suggests that testicular metabolism is considerable during the impubertal period since the TCBF measured during that period was found to be similar to the TCBF of the adult ram at a time of important cellular metabolism. Larson et al. (1974) also reported that there was no difference in TCBF per gram of testis between prepubertal, postpubertal and adult rabbits. Therefore, it seems that, during testicular development in sheep, when expressed per unit weight of testis, TTBPF decreases while TCBF remains constant. However, when expressed per testis, both parameters increase.

A diagram illustrating the relationship that may exist between total testicular blood flow (TTBF) and TCBF in the prepubertal and postpubertal testis is presented in fig. 3.8. Both our data and the ones reported by Courot and Joffre (1977) have been used to demonstrate the next point. The TTBPF values have been transformed by multiplying by a correction factor [1/(1-0.40); 40% being the haematocrit value estimated for the experimental lambs] (see Laurie and Setchell,



a: approximate value based on data from Courot and Joffre (1977)
b: approximate value based on our data
[TTBF=1/(1-haematocrit) x TTBPF]; haematocrit value used 40%
TTBPF: prepubertal testis: 4 ml/min (400 µl/g/min)
postpubertal testis: 13 ml/min (130 µl/g/min)]

Fig. 3.8. A diagram illustrating the relationship between total testicular blood flow (TTBF) and testicular capillary blood flow (TCBF) in the prepubertal and postpubertal testis.

1978) to get an approximate TTBF value for the prepubertal and the postpubertal testes. Assuming that the difference between TCBF and TTBF was due solely to the arterio-venous anastomoses which are present in the spermatic cord of the ram (Noordhuizen-Stassen et al., 1985), we have estimated what could be the percentage of arterial blood transferring to the venous blood in the prepubertal and the postpubertal testes. Therefore, we have estimated that approximately 86% of the arterial blood transfers to the pampiniform plexus in the prepubertal testis (10 g) while this percentage is reduced to 55 % in the larger postpubertal testis (100 g). This model needs further verification using homogeneous animals since the present calculations are based on approximate values and include measurements taken in two different populations of sheep (Merino and Ile-de-France breeds). However, we believe that important vascular adjustments such as the one described in this diagram (fig. 3.8) are likely to occur during pubertal development since the testis enlarged rapidly and required more blood and since an important reduction in testicular temperature seems to take place early during testicular development (see chapter 4). Interestingly, Noordhuizen-Stassen et al., (1988) have recently reported for the pig testes, that the overflow of blood through arterio-venous anastomoses could be seen most clearly in those cases in which there were either less active and/or abnormally located testes. In either less active and/or abnormally located testes, the cooling function of this vascular complex is less efficient. This observation supports our postulate since a more important blood transfer is suspected in the prepubertal testis which is generally considered less active in comparison with the postpubertal testis. Moreover, our estimate of the percentage of blood transfer found for the postpubertal testis (55%) compare well with the values reported by Noordhuizen-Stassen et al. (1985) for adult Texel rams (28-60%) and by Fleet et al. (1982) for Clun Forest rams (~60%). A tendency for an increase in arterio-venous transfer of blood taking place during testicular maturation in the rat has recently been suggested, however, this observation is based on an increase in the amount of dilution of testosterone measured in the internal spermatic vein in comparison with the hormone measured in a testicular vein (Maddocks and Sharpe, 1989c). Nevertheless, it remains possible that the changes in testicular blood flow and in arterio-venous blood transfer occurring during testicular maturation can differ from one species to another. For

instance, using the same method of measurement as for the lambs, TCBF was found to increase in the fox during pubertal development (Joffre, 1973; Courot and Joffre, 1977).

Finally, we have shown that TTBPF does not change following a hCG challenge. This result is in agreement with other observations reported on the "short-term" effect of hCG on testicular blood flow (Setchell, 1990; Setchell et al., 1991).

In conclusion, we have demonstrated that the South Australian Merino ram lambs grew well under specific experimental conditions such as early weaning, hand feeding, pen restriction, and constant light environment (12 L:12D). The ram lambs has achieved puberty at a relatively early age (< 30 weeks of age) under these conditions. Furthermore, we have shown that important changes in testicular blood flow occur during testicular development which probably involve variations in the arterio-venous blood transfer occurring within the spermatic cord.

CHAPTER 4.

SUBCUTANEOUS SCROTAL TEMPERATURE AND TESTICULAR DEVELOPMENT IN RAM LAMBS

4.1. INTRODUCTION

In the ram, as in the majority of eutherian mammals, spermatogenesis will not proceed to completion unless the temperature of the testis is a few degrees lower than that of all other internal organs of the body. The scrotum and the spermatic vasculature produce an efficient thermoregulatory mechanism (Waites, 1970; Setchell, 1978). The cooling effect of the scrotum on the testis is in good part due to evaporative heat lost effected by the luxuriant population of sweat glands in the scrotal skin (Waites and Voglmayr, 1963). Moreover, since the tunica dartos and cremaster muscle can regulate scrotal surface area and the position of the testes with respect to the abdominal wall, both can indirectly regulate heat loss. The arterial blood is pre-cooled as it passes through the spermatic cord by counter-current heat exchange with cooler venous blood. Arterial blood is further cooled as it courses on the surface of the testicle prior to passing into the testis (Setchell, 1978; see recent reviews in Zorgniotti, 1991).

Blood flow can influence testicular homeothermia, since it determines the amount of blood that convey the heat to and out of the testis and since the blood carries the hormonal and nutritional elements affecting testicular metabolism and thus, the amount of heat generated by the testis. The arterio-venous blood transfer occurring at the level of the spermatic cord could indirectly influence the temperature of the testis by modifying the arterial blood flow entering the testis (Noordhuizen-Stassen et al., 1988). Furthermore, an important entry of arterial blood within the venous circulation at the level of the spermatic cord would probably reduce the gradient of temperature between the venous and the arterial blood and thus would consequently reduce the cooling effect of the venous blood by counter-current heat exchange mechanism (Sealfon and Zorgniotti, 1991). This factor may be worth considering during pubertal development since our data presented in the preceding chapter suggests that the percentage of arterial blood transferring to the pampiniform plexus decreases during testicular maturation (see chapter 3, fig. 3.8.).

In the rat, Kormano (1967b) has studied the rectal-testis temperature gradient during pubertal development, but a similar investigation has not been carried out in the male sheep.

The aim of this experiment was to measure the subcutaneous scrotal temperature in ram lambs at different stages of testicular development. The hypothesis is that the subcutaneous scrotal temperature will decrease as testicular volume increases because the scrotal surface area also increases while the position of the developing testes changes with respect to the abdominal wall and, since we suspect that there is a decrease in the percentage of arterial blood transferring to the pampiniform plexus during testicular maturation.

4.2. EXPERIMENTAL PROCEDURE

4.2.1. Experimental animals

Eleven South Australian Merino ram lambs born between March and May 1990 were used in this experiment (group 1). Five additional cross-bred (Merino-Dorset) lambs born in September 1990 were also used (group 2: lambs #252, 280, 257, 263 and 256). The lambs were kept outdoors with their mothers. The following measurements were taken on the 1st November (group 1) and the 5th November (group 2), 1990.

4.2.2. Body weight and testicular volume measurement

Body weight was measured and testicular diameter and length were recorded as described in section 2.5.

4.2.3. Subcutaneous scrotal temperature measurement

Ram lambs were simply held firmly but gently in a sitting position by one person during the whole procedure so that no anaesthesia was required. The scrotum had been well shaved the day before temperature measurement and and a small area was cleaned with 70 % ethanol immediately before the following procedure. A very small incision was made through the scrotum. Approximately 5 cm of a long and thin flexible probe connected to an electronic temperature monitor was gently inserted under the scrotal skin and the tip of the probe was positioned adjacent to the anterior face of testis. The temperature was recorded after one minute of monitoring, when the temperature of the probe had stabilized. After this measurement, the

probe was removed and the skin was sutured with catgut (no. 0 Chromic gut). Following this short procedure, ram lambs were given penicillin (1 ml; i.m.) (Vetspen injection, Glaxovet, Glaxo Animal Health, Ltd) as a precautionary measure against infection.

4.2.4. Rectal temperature measurement

Rectal temperature was recorded after the subcutaneous scrotal temperature measurement while the animal was standing up. This temperature was recorded by inserting into the rectum a different probe connected to the same electronic temperature monitor used for subcutaneous scrotal temperature measurement. The temperature was recorded one minute after the introduction of the probe into the rectum.

4.2.5. Ambient temperature

During subcutaneous scrotal and rectal temperature measurement, the ambient temperatures were 15 °C (1 November, group 1) and 25 °C (5 November, group 2).

4.2.6. Statistical analysis

Regression analysis was carried out to determine the curve which best characterized the changes in subcutaneous scrotal temperature and in rectal-testis temperature gradient occurring during testicular development for each group. Statview™ 512+ computer program (Abacus Concepts, BrainPower Inc., Calabasas, CA.) has been used for this analysis.

4.3. RESULTS.

4.3.1. Body weight

Body weights ranged between 10.3 kg and 15.5 kg for group 2 and between 33.5 kg and 43.5 kg for group 1 (table 4.1.).

4.3.2. Testicular volume

Testicular volumes ranged between 4.58 cm^3 and 11.13 cm^3 for group 2 and between 52.23 cm^3 and 131.46 cm^3 for group 1 (table 4.1.).

Table 4.1.

Summary table indicating body weight, testicular volume, subcutaneous scrotal temperature, rectal temperature and rectal-testis temperature gradient for each lamb.

Lamb (#)	Body Weight (kg)	Testicular Volume[1] (cm^3)	Subc.scrotal Temperature (°C)	Rectal Temperature (°C)	Rectal-testis gradient (°C)
252 [b]	10.3	4.58	37.3	41.2	3.9
280 [b]	10.6	4.92	36.8	40.9	4.1
257 [b]	12.0	10.97	33.7	41.3	7.6
263 [b]	15.0	11.13	35.2	40.1	4.9
256 [b]	15.5	6.49	34.9	41.0	6.1
744 [a]	33.5	89.26	32.9	40.5	7.6
740 [a]	33.6	88.00	30.4	41.6	11.2
750 [a]	34.4	52.23	32.9	41.2	8.3
745 [a]	34.6	55.18	30.9	41.1	10.2
743 [a]	36.6	80.46	33.1	41.3	8.2
748 [a]	38.0	92.94	30.9	41.6	10.7
747 [a]	39.8	71.71	32.9	41.0	8.1
746 [a]	40.9	82.97	31.9	40.8	8.9
751 [a]	41.0	128.29	33.7	41.4	7.7
749 [a]	42.0	114.51	32.6	41.3	8.7
752 [a]	43.5	131.46	30.7	39.9	9.2

[1] Volume and subcutaneous scrotal temperature were measured on the same testis.

[a]: group 1: Merino ram lambs; ambient temp.: 15 °C

[b]: group 2: Dorset-Merino ram lambs; ambient temp.: 25 °C

4.3.3. Subcutaneous scrotal temperature

Subcutaneous scrotal temperatures ranged between 33.7 °C and 37.3 °C for group 2 and between 30.4 °C and 33.7 °C for group 1 (table 4.1.). In group 2, subcutaneous scrotal temperature tended to fall as testicular volume increases (fig. 4.1). The regression line that best described this change in subcutaneous scrotal temperature (y) in relation to change in testicular volume (x) can be represented by the following equation: $y = -0.372 x + 38.415$, (r: 0.816; $p = 0.0917$).
In group 1, however, there was no clear relationship (simple regression, r: 0.011; $p = 0.9733$) between subcutaneous scrotal temperature and testicular volume (fig. 4.1).

4.3.4. Rectal temperature

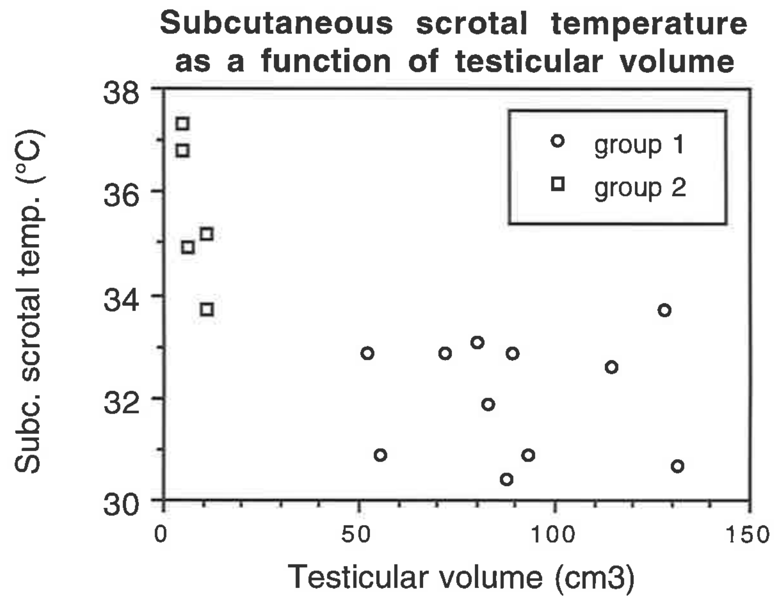
Rectal temperatures ranged between 40.1 °C and 41.3 °C for group 2 and between 39.9 °C and 41.6 °C for group 1 (table 4.1.).

4.3.5. Rectal-testis temperature gradient

Subcutaneous scrotal temperature was subtracted from rectal temperature to obtain the gradient of temperature between the body core and the gonads.
The lowest rectal-testis temperature gradient was 3.9 °C and was found in the lamb with the smallest testes (lamb # 252, testis size: 4.58 cm^3). The highest gradient was 11.2 °C and the testicular volume in that lamb (#740) was 88 cm^3.
In group 2, the gradient of temperature tended to increase as testicular volume increases (fig. 4.1). The regression line that best described this change in gradient of temperature (y) in relation to change in testicular volume (x) can be represented by the following equation: $y = 0.312 x + 2.945$, (r: 0.651; $p = 0.2342$). In group 1, however, there was no clear relationship (simple regression, r: 0.118; $p = 0.7301$) between gradient of temperature and testicular volume (fig. 4.1).

4.4. DISCUSSION

Our data suggest that, in the group of lambs with the small developing testes (group 2), the subcutaneous scrotal temperature decreases as the testis size increases, in another words, that rectal-testis temperature gradient increases as the testis develops. However, in the group of



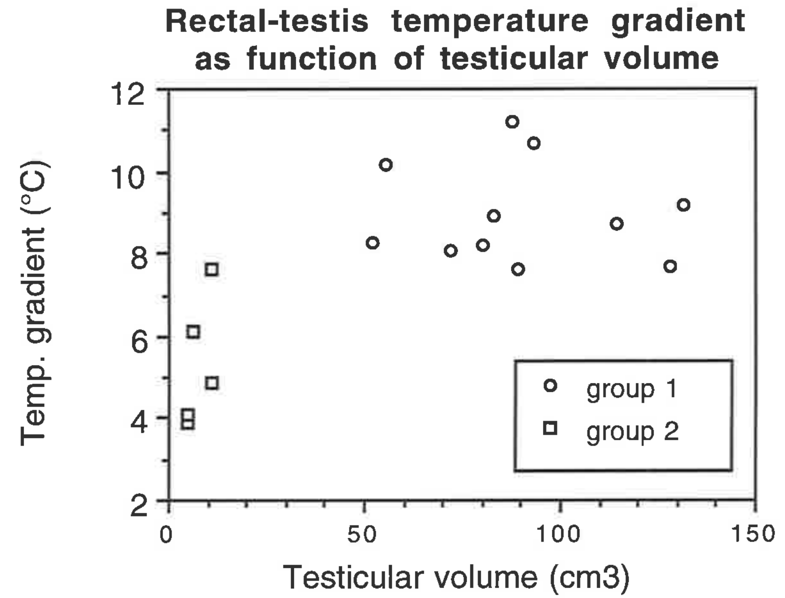


Fig. 4.1.: Subcutaneous scrotal temperature (left) and rectal-testis temperature gradient (right) as a function of testicular volume.

group 1: Merino ram lambs, ambient temp. 15 °C

group 2: Dorset-Merino ram lambs, ambient temp. 25 °C

lambs with the larger testes (group 1), in which spermatogenesis is presumed to be initiated, the subcutaneous scrotal temperature and the rectal-testis temperature gradient do not vary with testis size. It would therefore appear that the major structural and functional adjustments required to reduce the temperature of the testis at a few degrees lower than that of all other internal organs of the body occur early during the prepubertal period in the male sheep. However, one should keep in mind that considerable differences exist between the two groups of lambs used in that experiment, including genetic (e.g. Merino vs Merino/Dorset breed) and environmental variations (e.g. different ambient temperature at the time of measurement) which could account to some extent for the variety in temperature values recorded in these animals.

Interestingly, Kormano (1967b) has also studied the development of the rectal-testis temperature gradient in young rats and reported that the gradient increased from 1.05 °C at 20 days of age to 2.04 °C at 30 days and attained adult levels of about 3.5 °C at 35 days. Therefore, in that species, the maturational changes in testicular temperature are not completed at the onset of spermatogenesis. Indeed, a mature rectal-testis temperature gradient it is not yet achieved when the Sertoli blood-testis barrier is formed and the first primary spermatocytes appear (at the end of the third week of life) but is rather established during the late juvenile phase (D21-D40) and before achievement of puberty (D40-D50) (see Adams and Steiner, 1988).

Kormano (1967a) also reported that this increase in rectal-testis temperature gradient was accompanied by a striking increase in the capillary network on the testis surface and attainment of full scrotal development. In ram lambs, although the testicular capillary blood flow (TCBF) (per testis) increases as testis size increases, TCBF per unit gram of testis has been shown not to vary between 50-day old and 120-day old ram lambs (Courot and Joffre, 1977). Therefore, a decrease in testicular temperature does not appear to relate to a change in this parameter in ram lambs although possible changes in the architecture of testicular microvessels and other differences in testicular microcirculation occurring during pubertal development could favour a better heat exchange within the testis which probably generate more heat as its spermatogenic and steroidogenic activities become well established.

Since the scrotum of the ram lambs is well developed at birth and since its surface area increases during testicular development (covering the increasing surface area of the developing testis), increasing heat lost through the scrotum must participate to some extent in the reduction in subcutaneous scrotal temperature taking place during pubertal development. However, our data obtained in the older lambs (group 1), shows that an increase in testicular size is not necessarily accompanied by a decrease in subcutaneous scrotal temperature, even though testicular volumes range from 52.2 cm^3 to 131.5 cm^3 in that group. Nevertheless, the highest subcutaneous scrotal temperatures (36.8 °C and 37.3 °C) were measured in the younger lambs with the smallest testes (4.58 cm^3 and 4.92 cm^3).

In the previous chapter, we have reported that total testicular blood plasma flow (TTBPF) expressed per unit weight of testis is much higher at an early stage of testicular development in ram lambs (see chapter 3). Indeed, we suspect that a higher percentage of arterial blood ("warmer" blood) transfers to the venous blood ("cooler" blood) of the small prepubertal testis (see fig. 3.8.), and consequently that the temperature gradient between the spermatic artery and vein must be low at that stage of development. The thermoregulatory mechanism would become more efficient in a more developed testis since the percentage of arterial blood transferring to the pampiniform plexus would be reduced and the result would be a more pronounced temperature gradient in the spermatic cord.

> One must remember that the testicular temperatures were not recorded during the testicular blood flow measurements done in the previous experiment (see chapter 3). Since the testes were kept outside the scrotum during these measurements (the temperature surrounding the testis was lower than the subcutaneous scrotal temperature measured in the present experiment), it is likely that the testicular temperatures were slightly below the temperatures found in lambs with an intact scrotum. However, since TTBPF did not vary greatly over the 140 minutes of measurement, we assume that a new "thermic equilibrium" was created between the testis and the surrounding environment and that the difference in the amount of blood

circulating through the arterio-venous anastomoses still reflected the difference in the spermatic vasculature existing between the prepubertal and the postpubertal testes.

A recent observation by Noordhuizen-Stassen et al. (1988) would support our idea of an meaningful relationship between the temperature of the testis and the overflow of blood through arterio-venous anastomoses since they observed that this flow was more important in the abdominal testes (which are relatively undeveloped and warm) of the cryptorchid pigs than in the scrotal testes (more developed and cooler).

In conclusion the data presented in this chapter suggest that a more pronounced rectal-testis temperature gradient is established early during testicular development in ram lambs. However, to confirm this finding, this experiment need to be repeated using more lambs of the same breed and taking repeated measurements in each animal at regular interval during their pubertal development. Furthermore, to minimize the variation due to the environment, the measurements should be recorded in a room with constant ambient temperature.

Although we have suggested that the counter-current heat exchange mechanism taking place within the spermatic cord becomes more efficient early during testicular maturation, the exact nature of the maturational modification in the thermoregulatory mechanism of the testis remains to be demonstrated. A better understanding of the relationships between the relative amount of arterial blood transferring to the venous blood in the spermatic cord and the changes in testicular temperature occurring during testicular maturation in ram lambs would certainly help us identify the exact process by which the optimal thermal conditions for spermatogenesis are established.

CHAPTER 5.

ACTIVE IMMUNIZATION AGAINST OESTRADIOL-17ß AND PUBERTAL DEVELOPMENT IN RAM LAMBS.

5.1. INTRODUCTION

Considerable experimental evidence now supports the hypothesis that oestradiol regulates many processes of the male reproductive system under physiological conditions. The presence of oestradiol receptors within the hypothalamic-pituitary-testicular axis suggest that this steroid has direct effects on this axis in many species including the ram (Mulder et al., 1974; Wise et al., 1975; de Boer et al., 1976; Thieulant and Pelletier 1979; Pelletier and Caraty, 1981; Glass et al., 1984; Tsai-Morris et al., 1985a). Furthermore, local formation of oestrogens by neuroendocrine tissues has been demonstrated in the male of many species (Naftolin et al., 1975). Indeed, it seems necessary for testosterone to be aromatized before it can exert its biological effect in many target organs including the hypothalamus (Naftolin et al 1975; D'Occhio et al 1983, 1985). In the ram, oestradiol production by the testis parallels the onset of spermatogenesis and testosterone secretion (Barenton and Pelletier, 1983; Barenton et al., 1983).

Before puberty, the hypothalamic-pituitary axis appears to be extremely sensitive to gonadal steroid inhibition, resulting in low tonic LH and FSH secretion. As puberty proceeds, there is a decrease in responsiveness to the inhibitory actions of gonadal steroids, resulting in increased gonadotropin secretion sufficient to initiate reproductive activity (Ramirez and McCann 1963; 1965; Ramirez, 1973). This developmental concept, known as the "gonadostat" hypothesis, has been used as an explanation for the initiation of puberty in the male sheep (Olster and Foster, 1986; 1988). Oestrogens might play an important role in that mechanism since the picomolar concentration of oestradiol circulating in the ram has been shown to provide negative-feedback signals to the hypothalamus-pituitary axis and thus to reduce gonadotropin secretion (Schanbacher, 1979; 1984a; Sanford, 1985; 1987a; 1987b; Schanbacher et al., 1987; Monet-Kuntz et al., 1988). In the developing male lamb, manipulation of circulating oestradiol concentration by steroid implantation (Jenkins and Waites, 1983) or by immunization (Land et

al., 1981; Jenkins et al., 1986) has also provided evidence for a physiological role for this steroid. That oestradiol can directly inhibit testicular androgen production in laboratory rodents by affecting the metabolism of interstitial cells has been suggested by many investigators (Dorrington et al., 1978; Hsueh et al., 1978; Kalla et al., 1980; Moger 1980; van der Molen et al., 1981). It is still unclear whether oestradiol exerts a direct effect on the seminiferous tubules since there is controversy concerning the presence of oestrogen receptors in Sertoli cells and no oestrogen receptors have been identified in the germinal cells so far.

Land et al (1981) have shown that the rate of growth of the testis were greater in lambs passively immunized against oestrogens (14-26 week old Merino ram lambs), however this effect was not observed by Jenkins et al. (1986) working with younger crossbred lambs (2-16 week old), using the same approach. To our knowledge, the use of active immunization against oestradiol-17ß in prepubertal ram lambs has not been reported.

Therefore, the aim of the present experiment was to explore the possibility of accelerating testicular maturation in prepubertal Merino ram lambs by active immunization against oestradiol-17ß. We hypothesize that if circulating oestradiol-17ß restrains gonadotropin secretion during the prepubertal period and if oestradiol-17ß is also capable of acting directly on the testis to inhibit testosterone synthesis therefore, circulating oestradiol-17ß must retard the establishment of spermatogenesis in ram lambs. By early active immunization against oestradiol-17ß, we hope to neutralize those inhibitory effects, resulting in increased gonadotropin stimulation with concomitant activation of the steroidogenic potential of the testes, early establishment and improvement of the spermatogenic function and consequently, precocious testicular development. An additional outcome of this experimental design was to evaluate the effect of biopsy sampling on testicular growth in ram lambs.

5.2. EXPERIMENTAL PROCEDURE

5.2.1. Experimental animals

Eighteen South Australian Merino ram lambs born at the beginning of June 1987 were used in this experiment. The lambs were kept outdoors with their mothers until weaning at 12 weeks of

age. The lambs were then transferred to a room with controlled light (12 hours light: 12 hours dark).

5.2.2. Pen restriction

Lambs were grouped in each pen according to their body weight at their arrival in the controlled environment (section 2.2.1). Each pen received at least two control lambs and two E_2-immunized lambs.

5.2.3. Treatment

Nine lambs received a primary injection of 17ß-oestradiol-6(o-carboxymethyl)oxime-bovine serum albumin conjugate (E_2-6-BSA) (1 mg/lamb) in Freund's complete adjuvant (FCA) at 14 weeks of age (see section 2.3.1) (E_2-immunized, n=9). The remaining lambs were treated identically, but using bovine serum albumin (BSA) (1 mg/lamb) in FCA (Controls, n=9). All lambs received a booster injection four weeks later. Freund's incomplete adjuvant (FIA) was used instead of FCA for this second injection. The immunogens were prepared and injected as described in section 2.3.1.

Testicular biopsies were taken from four lambs in each immunization group (four controls "CB" and four E_2-immunized "EB" ram lambs) at 22 and 26 weeks of age. For practical reasons, the heaviest lambs were deliberately selected for this surgical procedure. The other lambs (five controls "CN" and five E_2-immunized "EN" ram lambs) were used as 'biopsy-controls' to evaluate the effect of biopsy sampling on subsequent testicular development. (see section 5.2.9.)

5.2.4. Body weight and testicular volume measurement

Body weight and testicular diameter and length were recorded weekly as described in sections 2.4 and 2.5. Scrotal circumference was also measured using a flexible tape around the widest part of the testis and no correction for skin thickness was made.

5.2.5. Intensive bleeding

Before the beginning of the immunization, at 14 weeks of age, the lambs were intensively bled (every 20 min.for 6 hours) as described in section 2.6.1. Intensive bleedings were repeated at 22, 26 and 30 weeks of age.

5.2.6. Measurement of testicular blood plasma flow and response to hCG.

TTBPF was measured on anaesthetized lambs before castration as described in section 2.7.2 in three controls (CN) and three E_2-immunized (EN) ram lambs (no testicular biopsies have been taken from these lambs). Testicular responsiveness to a single hCG injection (20 I.U./kg body weight) was simultaneously evaluated as described in section 2.6.3.1. PAH concentration was assayed in each blood sample collected as described in section 2.8.3. The production of testosterone by each testis (defined as the product of plasma flow per testis and the veno-arterial concentration difference for testosterone, i.e.: (ISV [T] - JUG [T]) x TTBPF) was also calculated.

5.2.7. Titre check and specificity

One blood sample for each ram lamb was collected before and fourteen days after the primary and booster injections and immediately after each intensive bleeding. Plasma from these samples were assayed for titre as described in section 2.8.2.1.

Samples from each E_2-immunized ram lambs collected at 30 weeks of age were tested for cross-reactivity. Specificity was examined by incubating the diluted antiserum (dilution that bind 50 % of labelled oestradiol) with tritiated oestradiol (^3H-E_2) in the absence or presence of graded doses of nonlabelled oestradiol, oestrone, oestrone-sulphate, testosterone, DHT, or androstenedione. Relative cross-reactivity between oestradiol and other steroids was calculated at 50% inhibition of the initial binding as described in section 2.8.2.2.

5.2.8. Hormone assays

Plasma samples were assayed for LH, FSH, PRL and testosterone as described in section 2.8.1.2.2., 2.8.1.2.3., 2.8.1.2.4. and 2.8.1.3.2., respectively. Mean hormone levels were determined by assaying a plasma pool representing the 6-h sampling period (section 2.6.1.2.). Pool samples collected before and after a hCG challenge (section 2.6.3 2.) were assayed for testosterone.

5.2.9. Testicular biopsy

Testicular biopsies were taken from four controls (CB) and four E_2-immunized (EB) ram lambs at 22 weeks of age (left testis) and at 26 weeks of age (right testis) as described in section 2.7.3.

The other lambs ('biopsy-controls': 5 CN and 5 EN) were not sham-operated and were not sedated with Xylazine 2% (Rompum) at any time during the whole experiment.

5.2.10. Castration

All ram lambs were castrated at 30 weeks of age (their body weight being well above 40 kilos at that time) with the exception of the two lightest lambs (1 CN and 1 EN) castrated at 34 weeks of age since they did not grow as quickly as the other ones and needed an extra month to reach 40 kilos of body weight. Castration was performed as described in section 2.7.4. The testis and the epididymis were not initially separated from one another (see 5.2.12) but weighed together ('testis-epididymis' weight) and then perfused with a fixative as described in section 2.8.4.1. The 'fixed testis-epididymis' were kept at -20 °C until DSP were evaluated.

5.2.11. Testicular histology

Testicular biopsies and other blocks of testicular tissue taken at castration were prepared as described in section 2.9.

5.2.12. Daily sperm production

The 'fixed testis-epididymis' were transferred to 4 °C, 24 to 48 hours before DSP evaluation. Immediately before tissue preparation for DSP evaluation, the epididymis was separated from the testis and both organs weighed separately ('fixed testis' weight and 'fixed epididymis' weight). DSP evaluation was done as described in section 2.8.4.

5.2.13. Analysis of LH pulses

The LH profiles were analyzed for pulses using a computer algorithm program developed by Philip L. Taylor (MRC Reproductive Biology Unit, Edinburgh, Scotland) for the Apple Mackintosh microcomputer (Munro; Elsevier-BIOSOFT, Cambridge, UK). see section 2.10. Munro is a modified version of 'Pulsar' method by Merriam & Watcher (1982). The same principle of testing for significant excursions from a baseline was used, but the baseline at each point sample time was calculated as a moving average over a 200 min window and then individual peaks detected by this system were tested further with a threshold which dictated that a pulse was not accepted unless the concentration at the peak exceeded the concentration at the

previous nadir by more than two standard deviations and the interval to the previous pulse was 20 min or more. The G parameters (the number of standard deviations by which a peak must exceed the baseline in order to be accepted) were 3.98, 2.40, 1.68, 1.24 and 0.93 for G1-G5, these being the requirement for pulses composed of one to five samples which exceed the baseline respectively. The Baxter parameters describing the parabolic relationship between the concentration of a hormone in a sample and the standard deviation (assay variation) about that concentration were 0.09129 (b_1, the y intercept), 0.01235 (b_2, the x coefficient) and 0.01010 (b_3, the x^2 coefficient). These were determined by repeated assay of pooled samples (6 replicates/ sample) containing three different concentration of LH spanning the range expected in the experimental samples (Martin et al., 1987).

Nadirs (minimum concentration observed up to 60 min before the peak of a pulse), pulse intervals and pulse amplitudes (the difference between pulse peak and preceding nadir) were calculated for each pulse and mean values for each profile were used in the analysis of treatment effects (see section 5.2.14.).

5.2.14. Statistical analysis.

Data for characteristics involving repeated measurements over time (body weight, testicular volume, testicular circumference, LH, FSH, PRL, testosterone) were analyzed by analysis of variance for repeated measures to detect differences due to immunization, biopsy, age or their interactions. LH pulse characteristics, TTBPF and T production were analyzed by analysis of variance for repeated measures to detect differences due to immunization, time or their interactions. Daily sperm production, 'testis-epididymis' weight and volume at castration, 'fixed testis' and 'fixed epididymis weight' were analyzed using a two-factor analysis of variance to localize differences due to immunization, biopsy and their interaction. Titres for each period of measurement were analyzed using a one-factor analysis of variance. All these analyses were done using the CLR ANOVA computer program (Clear Lake Research, Morningside, Houston, TX, 77005).

If statistical significance was found in the analysis of variance for repeated measures, a two-factor analysis of variance for each time period was also carried out. Treatment differences for

the four groups (CN, EN, CB, EB) were then tested by Duncan's New Multiple Range test. Single pairwise comparisons were made using student-*t* -test. A logarithmic transformation [log (x+1)] was applied to some hormone data (in this experiment: mean testosterone and mean FSH) to equalize variances. The data presented in tables and graphs are the actual ones.

5.3. RESULTS

5.3.1. Health of animals

During the whole experiment, all lambs remained healthy except one E_2-immunized lamb (1 EN) who never adjusted to the controlled environment, had no appetite and finally was eliminated from the study (at 18 weeks of age). The data from this lamb was excluded from all analyses.

5.3.2. Titre check and specificity

The titres are presented in table 5.1. The percentage binding of oestradiol-17ß remained negligible (less than 4 %) during the whole experiment in the control lambs. All E_2-immunized lambs responded well to the immunization protocol used. The titres started to rise already 14 days after the primary injection and were considerably more elevated 14 days after the booster injection. Thereafter, oestradiol binding in diluted plasma (1:5000) varied between 40 and 60 % in the E_2-immunized lambs.

The antisera from each E_2-immunized ram lamb at 30 weeks of age demonstrated very low cross reactivity towards testosterone, DHT and androstenedione (< 2.5 % at the highest concentration tested: 1000 pg/ml). The cross reactivities of the antisera towards oestrone and oestrone-sulphate averaged 15.0 ± 3.1 % (min: 5 % and max: 25 %) and 3.2 ± 0.5 % (min: < 2.5 % and max: 10 %), respectively.

5.3.3. Body weight

Liveweight increased significantly ($p < 0.01$) with age in all groups of lambs as shown in fig. 5.1. As expected, body weights were more elevated ($p < 0.02$) in ram lambs in which testicular biopsies were taken (CB and EB) since the heaviest lambs of both immunization groups have been deliberately selected for this surgical procedure. The immunization treatment did not affect

Note : Titres are reported as percentage binding of 10000 cpm of ^{3}H-oestradiol for a given dilution of plasma (1:100 or 1:5000).

Table 5.1.

Titre check

Percentage binding (% ± sem) of ^{3}H-oestradiol-17ß

in plasma from control and E_2-immunized lambs,

at 14, 16, 20, 22, 26 and 30 weeks of age

	before immunization [a] (14 weeks)	14 days after primary injection [a] (16 weeks)	14 days after booster injection [a] (20 weeks)	22 weeks [b]	26 weeks [b]	30 weeks [b]
control (n=9)	2.50 ± 0.07	1.81 ± 0.09	2.18 ± 0.35	2.70 ± 0.08	3.12 ± 0.14	3.63 ± 0.21
E_2-immunized (n=8)	2.70 ± 0.06	8.65 ± 3.07*	60.64 ± 6.77**	43.51 ± 6.11**	57.10 ± 5.56**	55.74 ± 6.09**

[a]: plasma samples diluted at 1:100 for control and E_2-immunized lambs

[b]: plasma samples diluted at 1:100 for controls and at 1:5000 for E_2-immunized lambs

*: $p < 0.05$, significantly different from controls

**: $p < 0.01$, significantly different from controls

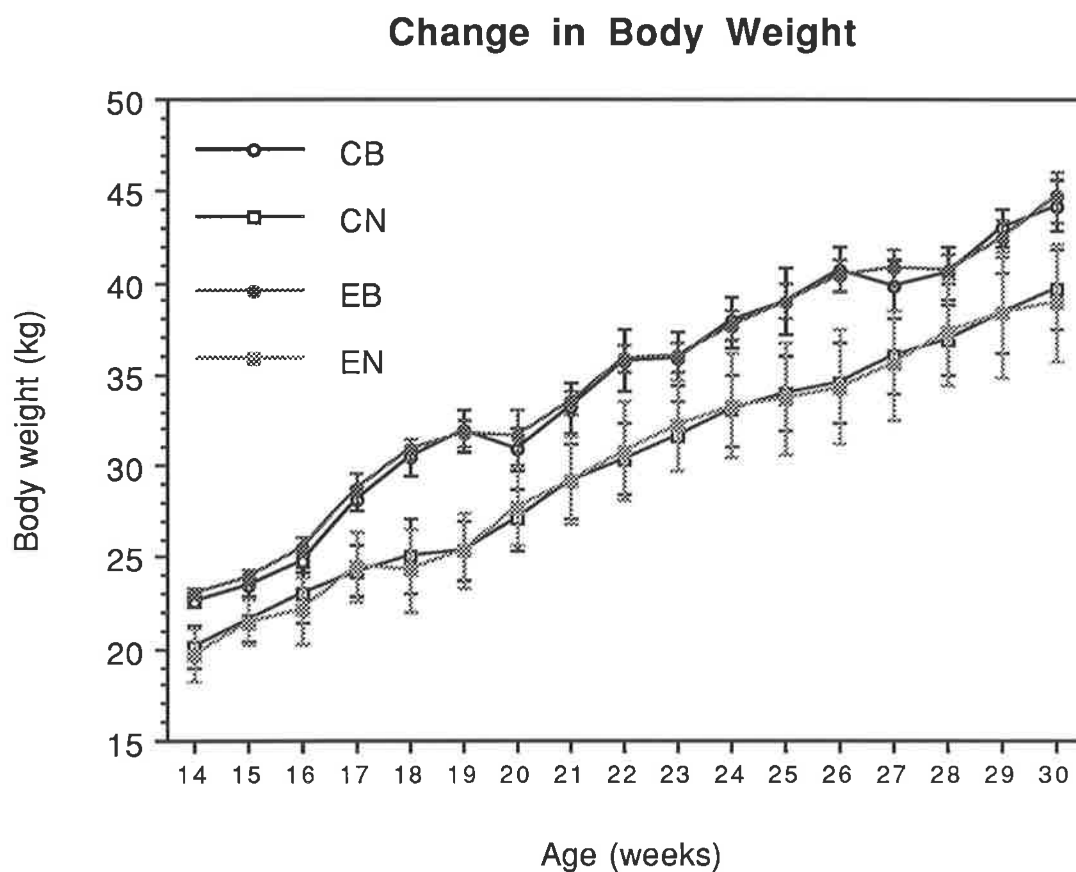


Fig. 5.1.: Changes in mean body weight (kg ± sem) from 14 to 30 weeks of age in ram lambs actively immunized against BSA (C) or against E_2-6-BSA (E) and submitted or not to testicular biopsy (B: biopsies taken; N: no biopsy taken).
(CB: n=4, CN: n=5, EB: n=4 and EN: n=4).

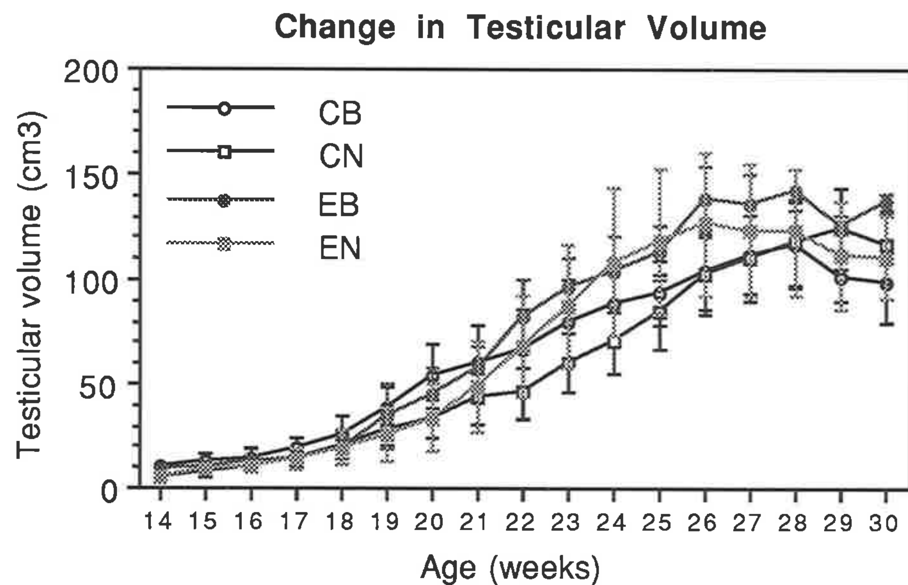
total body weight gain over the 16 weeks-study (controls [CN and CB]: 20.48 ± 0.89 kg and E_2-immunized [EN and EB]: 20.45 ± 1.15 kg).

5.3.4. Testicular volume

Testicular volume increased progressively in the four groups of lambs until 26 weeks of age. From 27 weeks of age until the end of the experiment, testicular volumes did not increase anymore but rather tended to decrease in all groups (fig. 5.2.). Overall testicular growth patterns were comparable in all groups whether or not biopsies were taken. Testicular volumes tended to be enhanced between 22 and 26 weeks of age in the E_2-immunized lambs however the differences between means were not statistically significant at any age. The analysis of variance showed a significant interaction ($p < 0.07$) between immunization treatment and age. We have noticed that testicular volume was decreasing towards the end of the experiment in two lambs in each group (2/5 CN, 2/4 CB, 2/4 EN, 2/4 EB). In fig. 5.3., we present the testicular growth pattern of one lamb selected from each group because it exhibited the most pronounced decline in testicular volume towards the end of the experiment. Lamb # 15 (EN) exhibited the most important increase in testicular volume between 23 and 25 weeks of age and the steepest decline thereafter (fig. 5.3.).

5.3.5. Testicular circumference

Changes in testicular circumference were comparable in all groups during the whole study (fig. 5.2.). Testicular circumferences increased progressively until 27 weeks then remained stable. The analysis of variance indicates a significant interaction between age and immunization treatment ($p < 0.01$). In fact, as for testicular volume, between 23 and 26 weeks of age, testicular circumferences tended to be slightly enlarged in the E_2-immunized groups but the differences were not statistically significant. Significant interactions between age and biopsy treatment ($p < 0.05$), and between age, immunization and biopsy treatments ($p < 0.06$) were also found although, at any specific age, we had observed a significant difference due to biopsy sampling. A final decline in testicular circumference was observed in 4 lambs only (2 CB, 1 EB and 1 EN).



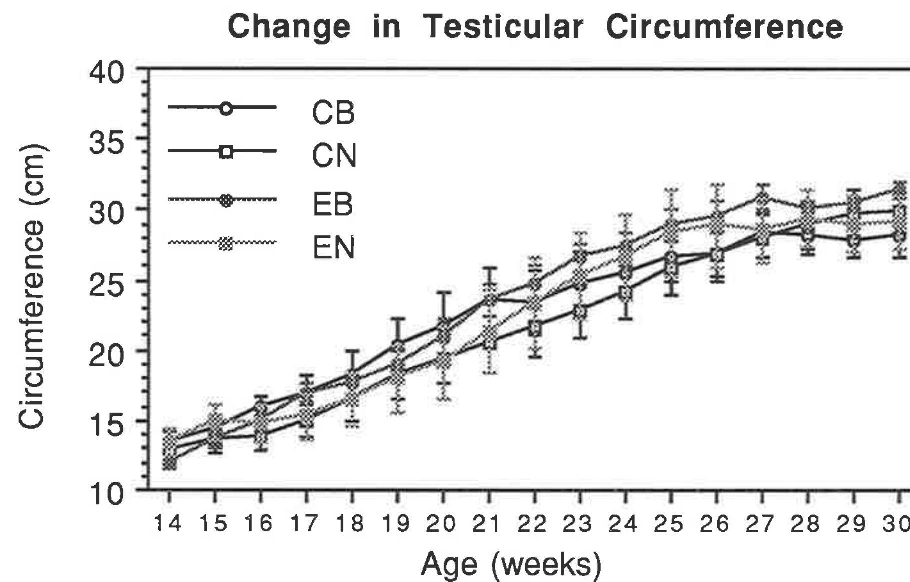
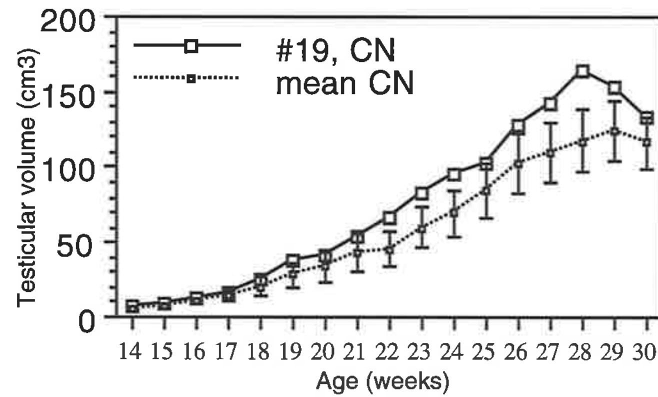
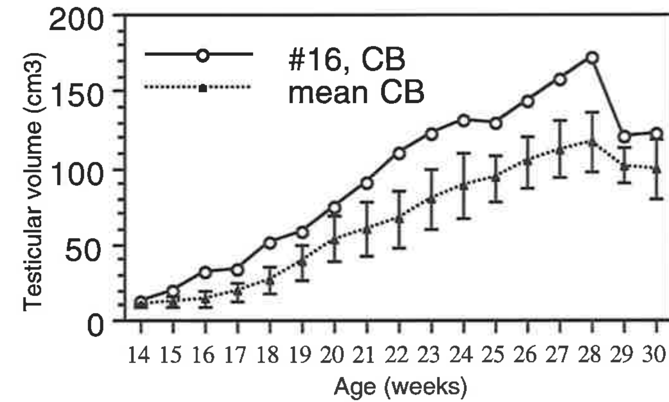
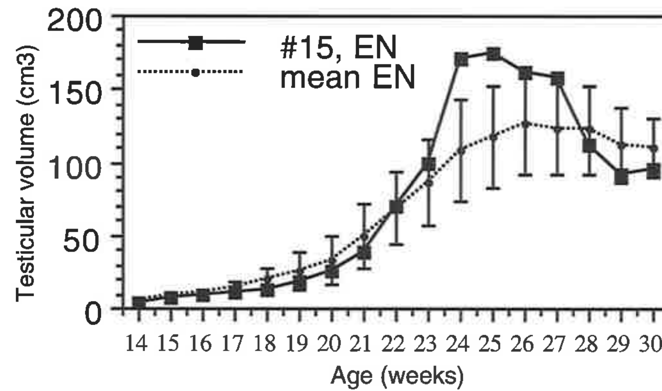


Fig. 5.2.: Changes in mean testicular volume ($cm^3 \pm$ sem, left) and mean circumference (cm ± sem, right) from 14 to 30 weeks of age in ram lambs actively immunized against BSA (C) or against E_2-6-BSA (E) and submitted or not to testicular biopsy (B: biopsies taken; N: no biopsy taken). (CB: n=4, CN: n=5, EB: n=4 and EN: n=4).







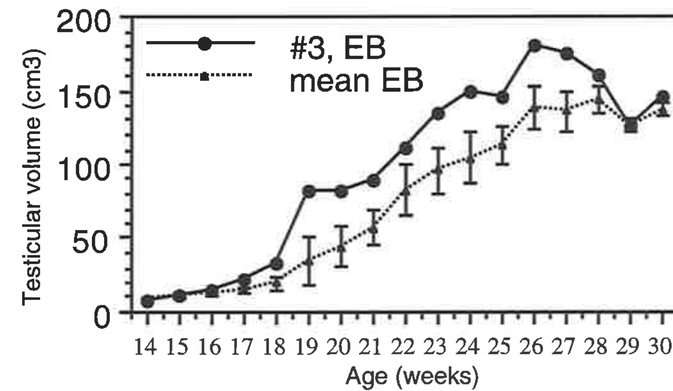


Fig. 5.3.: Changes in testicular volume (cm^3) from 14 to 30 weeks of age in one ram lamb selected in each group and exhibiting the most pronounced decline in testicular volume towards the end of the experiment. The lambs were actively immunized against BSA (C) or against E_2-6-BSA (E) and submitted or not to testicular biopsy (B: biopsies taken; N: no biopsy taken). The mean testicular volume ($cm^3 \pm$ sem) for the corresponding group is represented by dotted line. (CB: n=4, CN: n=5, EB: n=4 and EN: n=4).

5.3.6. Testosterone concentration

Mean testosterone concentrations in plasma at different ages for each group are shown in fig. 5.4. Testosterone concentration increased significantly ($p < 0.01$) with age in all lambs. The level of testosterone was significantly higher in E_2-immunized lambs (EN and EB) than in controls at 22, 26 and 30 weeks of age ($p < 0.01$). There is a significant interaction between age and biopsy treatment ($p < 0.02$). Testosterone concentrations in EN and CN lambs increased between 22 and 30 weeks while testosterone levels did not vary much in the CB and the EB lambs during that same period. At 30 weeks of age, a significantly higher level of testosterone was found the EN lambs than in the EB lambs ($p < 0.02$) while the level of testosterone was still comparable between CN and CB at that age.

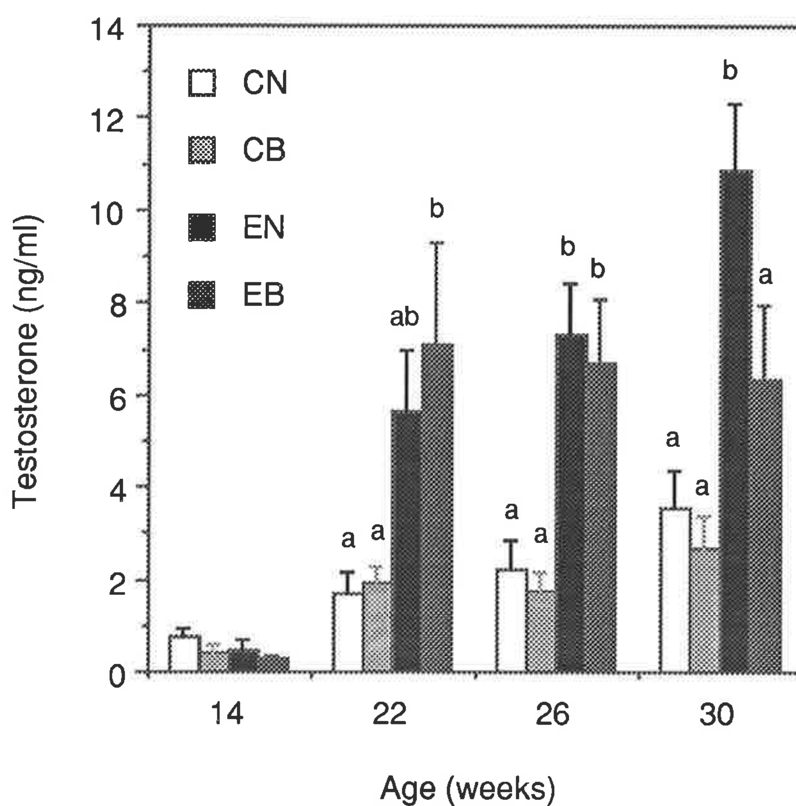
5.3.7. LH concentration

LH concentrations in plasma tended to decrease with age in control lambs with the exception of the relatively high level of LH measured in CN lambs at 30 weeks of age (fig. 5.5.). The immunization treatment significantly affected LH levels in plasma ($p < 0.01$). In E_2-immunized lambs (EN and EB), mean LH concentrations were significantly higher at 22 and 26 weeks of age ($p < 0.01$ at both ages). At 30 weeks of age, mean LH concentrations still tended to be higher in E_2-immunized lambs but the differences were not statistically significant since mean LH concentrations were elevated in the CN lambs at that age.

Since no significant effects on LH concentration were found due to biopsy treatment or "biopsy x age" interaction, the data from CN and CB lambs (controls) have been pooled at each age as well as the data from EN and EB (E_2-immunized) to compare the LH pulse characteristics during the four intensive bleedings (Table 5.2).

Pulse frequency did not vary with age in the control lambs. The number of peaks found within a 6 h-sampling period at four different ages was not significantly different between the 2 immunization groups, however, a tendency towards more peaks in E_2-immunized lambs was present. In conjunction with this observation, overall mean pulse interval was found to be of shorter duration in the E_2-immunized lambs ($p < 0.05$). A significant low interpulse interval was observed at 26 weeks of age in E_2-immunized lambs ($p < 0.07$).

(Overall mean pulse interval: average of the duration of each pulse interval measured during each 6-h sampling period done between 14 and 30 weeks of age.)



values with different superscripts are significantly different at 95%

Fig. 5.4.: Mean testosterone concentration (ng/ml ± sem) in plasma collected at 14, 22, 26 and 30 weeks of age from ram lambs actively immunized against BSA (C) or against E_2-6-BSA (E) and submitted or not to testicular biopsy (B: biopsies taken; N: no biopsy taken). (CB: n=4, CN: n=5, EB: n=4 and EN: n=4).

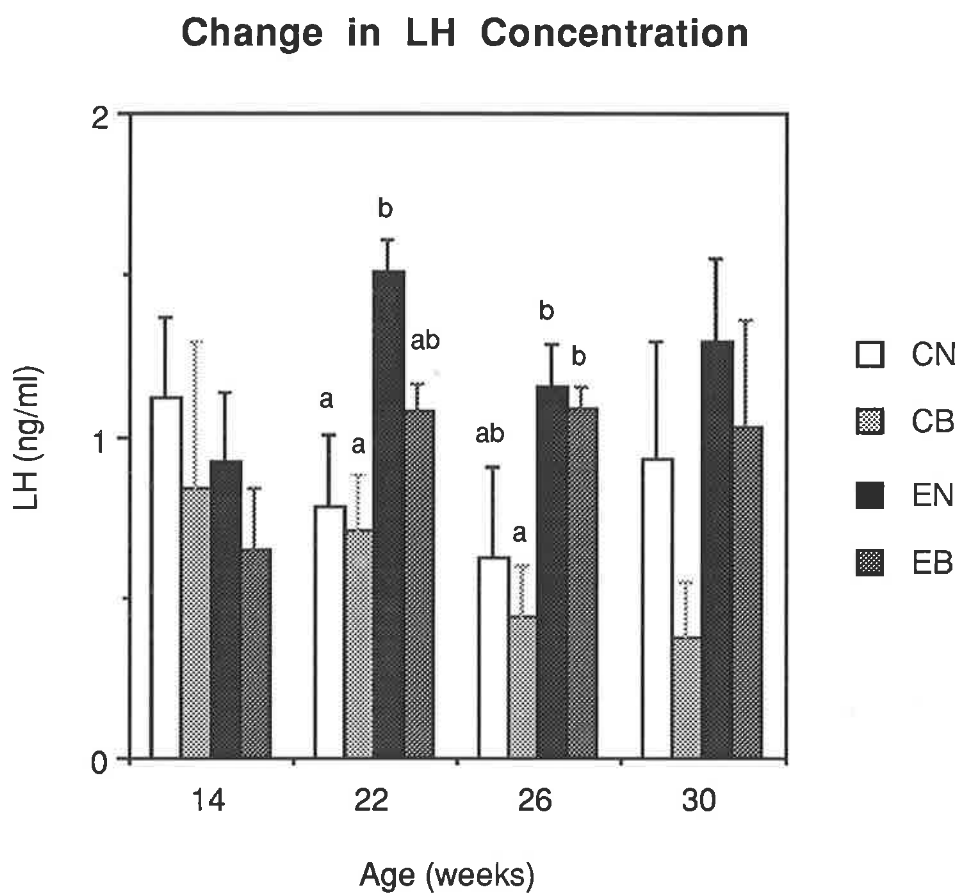


Fig. 5.5.: Mean LH concentration (ng/ml ± sem) in plasma collected at 14, 22, 26 and 30 weeks of age from ram lambs actively immunized against BSA (C) or against E_2-6-BSA (E) and submitted or not to testicular biopsy (B: biopsies taken; N: no biopsy taken). (CB: n=4, CN: n=5, EB: n=4 and EN: n=4).

Table 5.2

LH pulse characteristics in control (CN and CB) and E_2-immunized ram lambs (EN and EB) during intensive bleedings (every 20 minutes for 6 hours) at 14, 22, 26 and 30 weeks of age.

	age wks	mean[a] LH level (ng/ml)	number[a] of peaks per 6 hrs	mean[a] pulse interval (min.)	mean[a] pulse amplitude (ng/ml)	mean[a] pulse nadir (ng/ml)
Control (n=9)	14	1.00 ± 0.23	1.89 ± 0.31	188.14 ± 47.72	4.26 ± 1.45	0.39 ± 0.10
	22	0.75 ± 0.14	2.67 ± 0.58	172.88 ± 40.71	1.86 ± 0.27	0.44 ± 0.14
	26	0.54 ± 0.17	2.44 ± 0.71	194.66 ± 47.08	0.90 ± 0.15	0.80 ± 0.09
	30	0.70 ± 0.22	1.77 ± 0.49	242.77 ± 46.71	1.03 ± 0.25	0.96 ± 0.19
E_2-immunized (n=8)	14	0.75 ± 0.15	2.25 ± 0.25	155.00 ± 31.74	3.10 ± 0.69	0.33 ± 0.08
	22	1.30 ± 0.10 **	3.50 ± 0.54	109.70 ± 15.80	2.42 ± 0.32	0.67 ± 0.12
	26	1.12 ± 0.07 **	3.00 ± 0.33	95.41 ± 11.36	1.60 ± 0.14 **	1.07 ± 0.03 *
	30	1.16 ± 0.20	2.88 ± 0.44	145.00 ± 37.61	1.40 ± 0.18	1.02 ± 0.07

[a]: mean ± s.e.m.

*: $p < 0.05$, significantly different from controls

**: $p < 0.01$, significantly different from controls

Mean pulse amplitude decreased with age in controls ($p < 0.01$) and was significantly affected by immunization treatment ($p < 0.01$). Pulse amplitudes were significantly elevated in E_2-immunized lambs at 26 weeks of age ($p < 0.01$) and also tended to be higher at 22 and 30 weeks of age.

Mean pulse nadir increased with age in all lambs ($p < 0.001$). Pulse nadirs were significantly higher in E_2-immunized lambs at 26 weeks of age ($p < 0.05$) but not significantly different from controls at 22 and 30 weeks of age.

5.3.8. FSH concentration

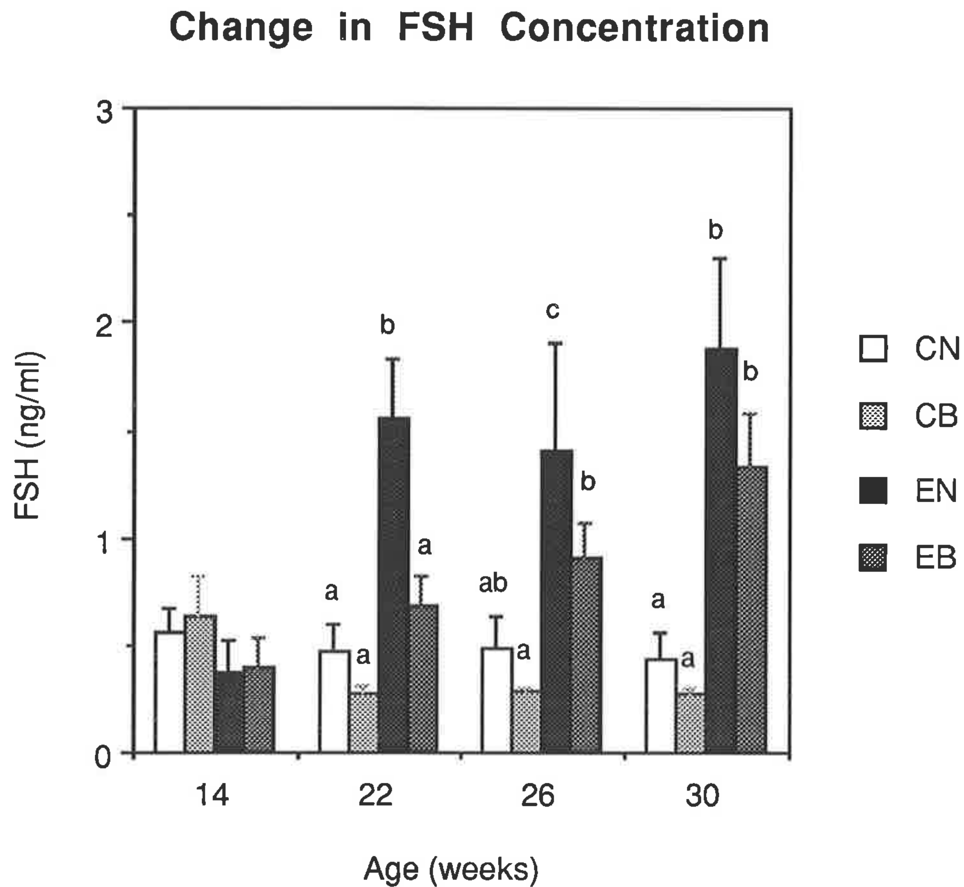
Mean FSH concentrations in plasma are shown in fig. 5.6. In control lambs (CN and CB), mean FSH levels did not vary significantly with age although mean FSH level tended to be more elevated at 14 weeks of age than at older ages in CB lambs. Mean FSH concentrations were significantly more elevated in E_2-immunized than in controls at 26 weeks and 30 weeks of age ($p < 0.01$) and in the EN lambs at 22 weeks of age ($p < 0.01$). FSH level was more elevated in EN lambs than in EB lambs at 22 weeks of age ($p < 0.01$) but at that age this difference was not due to the biopsy procedure since this measurement was done before surgery was initiated. This tendency for higher FSH levels in the EN lambs remained until the end of the experiment however at 30 weeks of age the difference in means between EN and EB lambs was not statistically significant. Mean FSH concentrations were comparable between CB and CN lambs at all ages.

5.3.9. PRL concentration

Prolactin concentrations in plasma were lower at 14 weeks of age than at any other age in all groups (fig. 5.7.). Prolactin tended to increase between 22 and 30 weeks of age in lambs in which biopsies had been taken ("biopsy x age" interaction, $p < 0.08$). Mean PRL concentrations in plasma were not affected by immunization treatment .

5.3.10. Testosterone concentration after a hCG challenge

The testosterone response to a single dose of hCG (measured in three CN and three EN lambs) is summarized in fig. 5.8. Before the hCG injection, no significant differences between CN and EN lambs were found in the testosterone concentration measured in the jugular (JUG), the right



values with different superscripts are significantly different at 95%

Fig. 5.6.: Mean FSH concentration (ng/ml ± sem) in plasma collected at 14, 22, 26 and 30 weeks of age from ram lambs actively immunized against BSA (C) or against E_2-6-BSA (E) and submitted or not to testicular biopsy (B: biopsies taken; N: no biopsy taken). (CB: n=4, CN: n=5, EB: n=4 and EN: n=4).

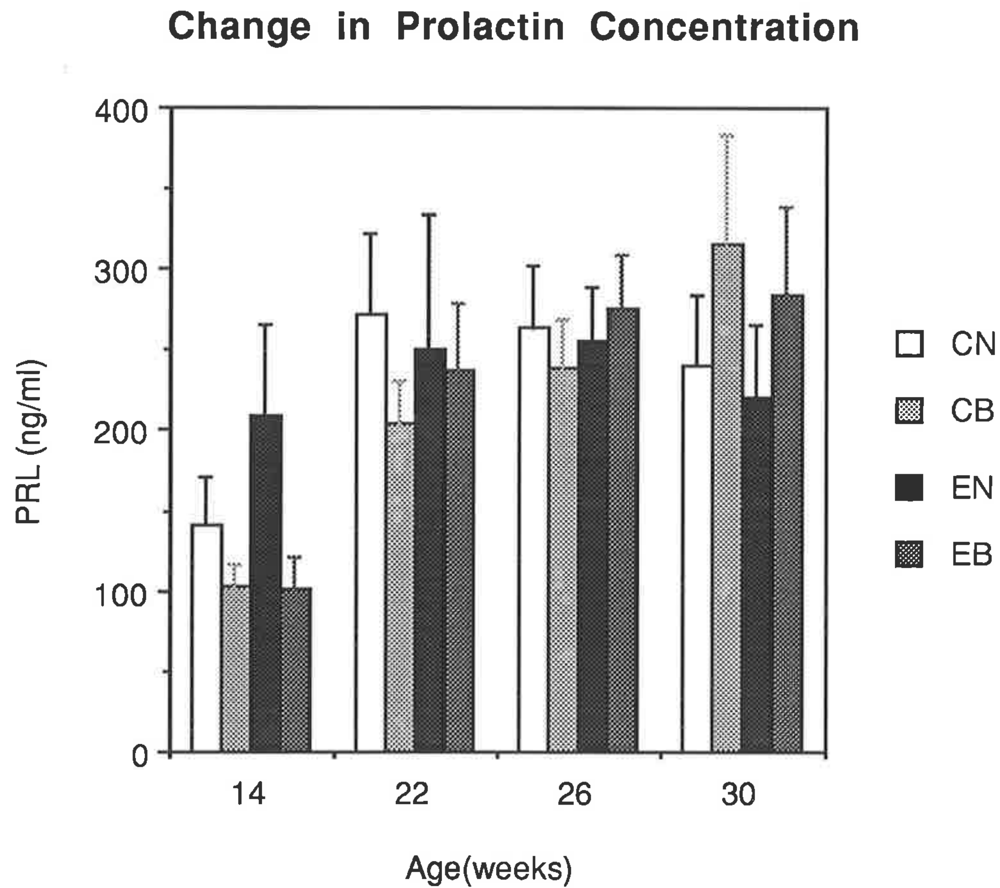
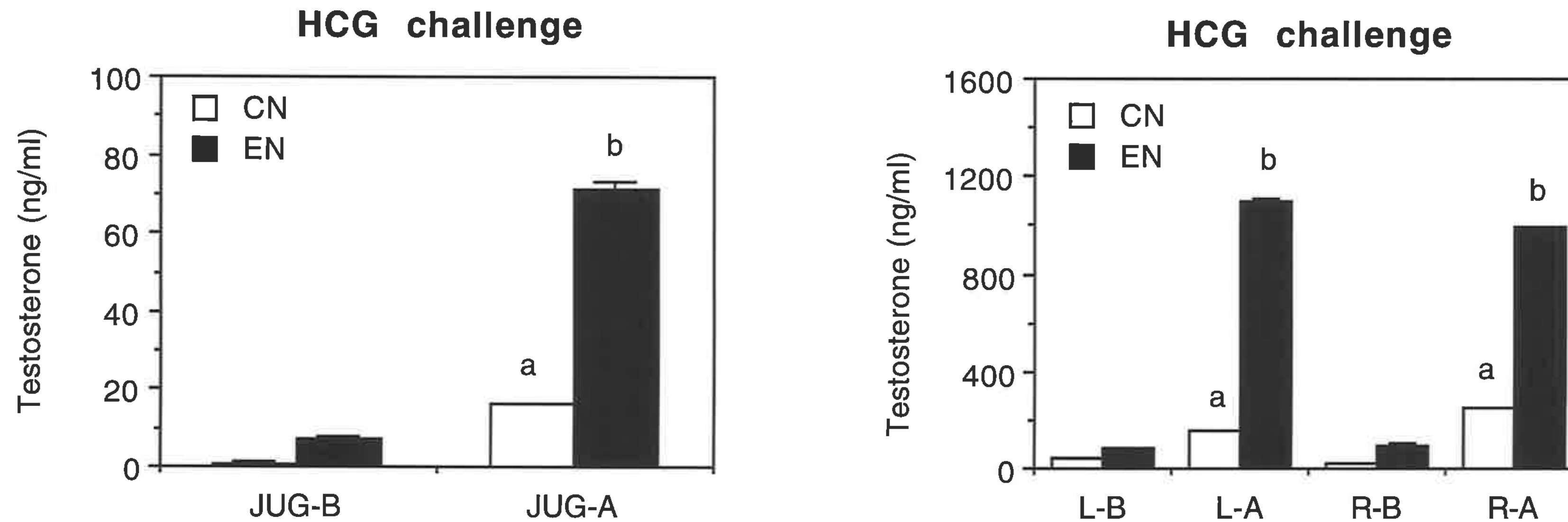


Fig. 5.7.: Mean prolactin concentration (ng/ml ± sem) in plasma collected at 14, 22, 26 and 30 weeks of age from ram lambs actively immunized against BSA (C) or against E_2-6-BSA (E) and submitted or not to testicular biopsy (B: biopsies taken; N: no biopsy taken). (CB: n=4, CN: n=5, EB: n=4 and EN: n=4).



values with different superscripts are significantly different at 95%

Fig. 5.8.: Mean testosterone concentration (ng/ml ± sem) measured in the jugular (JUG) and in the left (L) and right (R) internal spermatic vein before (B) and after (A) a single i.v. injection of hCG (20 I.U./kg BW) to ram lambs actively immunized against BSA (CN) or against E_2-6-BSA (EN) (n=3 lambs per group). Blood samples were drawn at 10-min intervals for 40 minutes before the hCG injection and for 100 minutes after the hCG injection and "pre hCG" and "post hCG" pools were made before assessment.

and left internal spermatic vein (R-ISV and L-ISV) samples. Testosterone level was significantly increased after the hCG challenge in the JUG and in the R-ISV and L-ISV pool samples ($p < 0.05$ for the three samples). Testosterone concentrations in the JUG and in the R-ISV and L-ISV pool samples were significantly higher in the EN lambs following the hCG injection ($p < 0.05$, $p < 0.05$ and $p <.0.01$, respectively).

5.3.11. Testicular weight and volume at castration

Testicular volume and 'testis-epididymis' weight at castration are summarized in table 5.3. Immunization treatment did not affect significantly 'testis-epididymis' weight at castration but tended to increase testicular volume ($p < 0.08$). Biopsy treatment also had a significant effect on testicular volume ($p < 0.03$). Testicular volume was significantly smaller ($p < 0.05$) in CB lambs compared to CN lambs and EN lambs. The correlation coefficient between calculated testicular volume and 'testicular-epididymis' weight at castration was r: 0.898 ($p < 0.01$). Biopsy sampling and immunization treatment did not influence the 'fixed testis' weights and the 'fixed epididymis' weights (fig. 5.9.).

5.3.12. Daily sperm production

Immunization or biopsy treatment did not significantly affect daily sperm production (DSP/gram testis or DSP/testis) as shown in table 5.4. The minimum DSP value (0.7×10^6 sperm per gram testis) was found in the lamb # 15 (EN) which had exhibited the most pronounced decline in testicular volume towards the end of the experiment (see fig. 5.3.). The maximum DSP value (49.05×10^6 sperm per gram testis) was found in a control lamb (lamb #2, CN).

5.3.13. Total testicular blood plasma flow (TTBPF)

TTBPF has been successfully measured in 10 testes (5 testes from 3 CN lambs and 5 testes from 3 EN lambs). TTBPF per testis (ml/min} and TTBPF per unit weight of testis (µl/g/min) did not change significantly following a hCG injection. TTBPF per testis tended to be more elevated in controls but the difference between the two groups was not statistically significant at any time (fig. 5.10.). However, TTBPF per unit weight of testis was significantly higher ($p < 0.05$) in controls than in E_2-immunized lambs (fig. 5.10.). Both TTBPF measurements

Table 5.3

'Testis-epididymis' weight and volume at castration in lambs immunized against BSA (C) or oestradiol-17ß-6-CMO-BSA (E) and submitted or not to biopsy sampling (B or N).

Treatment	'Testis-epididymis' Weight[a] (g ± s.e.m.)	Testicular Volume[b] (cm^3 ± s.e.m.)
CN (n=5)	203.62 ± 25.75	163.50 ± 18.39[a]
CB (n=4)	155.70 ± 20.59	110.13 ± 14.49[b]
EN (n=4)	206.34 ± 31.64	188.08 ± 23.37[a]
EB (n=4)	202.26 ± 13.80	153.07 ± 11.58[a b]

values with different superscripts are significantly different at 95%

[a] : The testis and the epididymis were not separated from one another but weighed together immediately after castration (before perfusion with a fixative).

[b] : Testicular diameter and length were measured with calipers immediately after castration and the volume was estimated by assuming that the testis is a prolate spheroid (Setchell and Waites, 1964). It was calculated according to the formula $1/6\ \pi\ a^2b$ (a=largest width; b=length, of the testis).

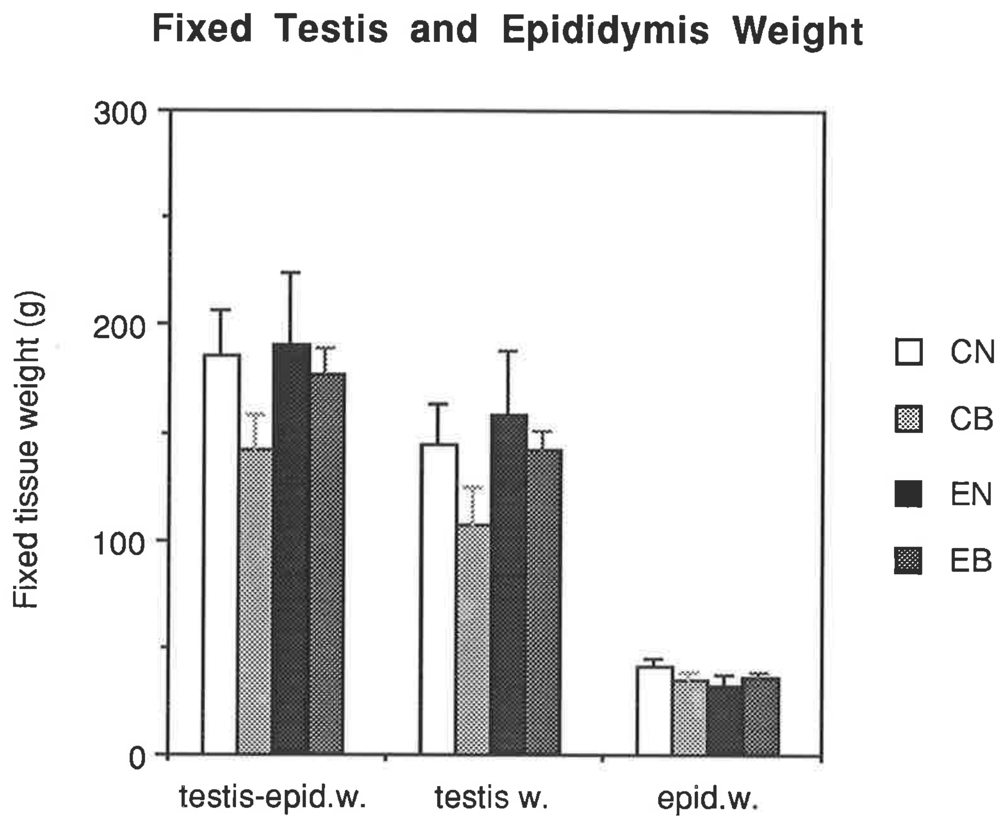


Fig. 5.9.: Mean weight (g ± sem) of the testis attached to the epididymis and fixed with a special solution (2% glutaraldehyde and 0.1 M. cacodylate buffer), and mean weight (g ± sem) of the 'fixed testis' and 'fixed epididymis' after their separation, in ram lambs actively immunized against BSA (C) or against E_2-6-BSA (E) and submitted or not to testicular biopsy (B: biopsies taken; N: no biopsy taken).

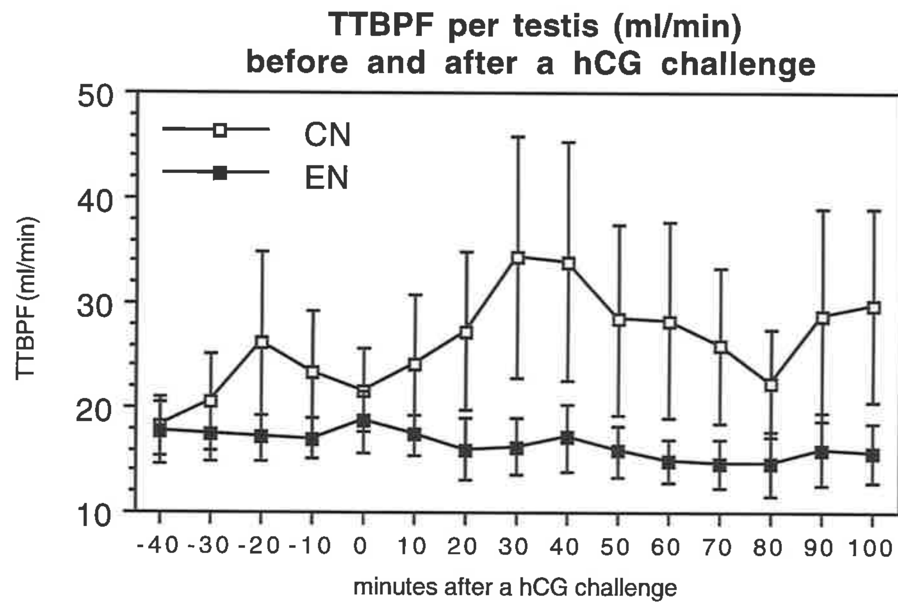
(CB: n=4, CN: n=5, EB: n=4 and EN: n=4).

Table 5.4

Daily sperm production (DSP) at castration in lambs immunized against BSA (C) or oestradiol-17ß-6-CMO-BSA (E) and submitted or not to biopsy sampling (B or N).

Treatment	Daily sperm production* No sperm per testis (x 10^9)	Relative DSP* No sperm /g testis (x 10^6)
CN (n=5)	4.85 ± 1.32	30.42 ± 7.01
CB (n=4)	3.16 ± 1.13	24.84 ± 8.36
EN (n=4)	2.68 ± 2.22	12.24 ± 8.86
EB (n=4)	4.00 ± 0.47	27.70 ± 1.82

*: mean ± s.e.m.



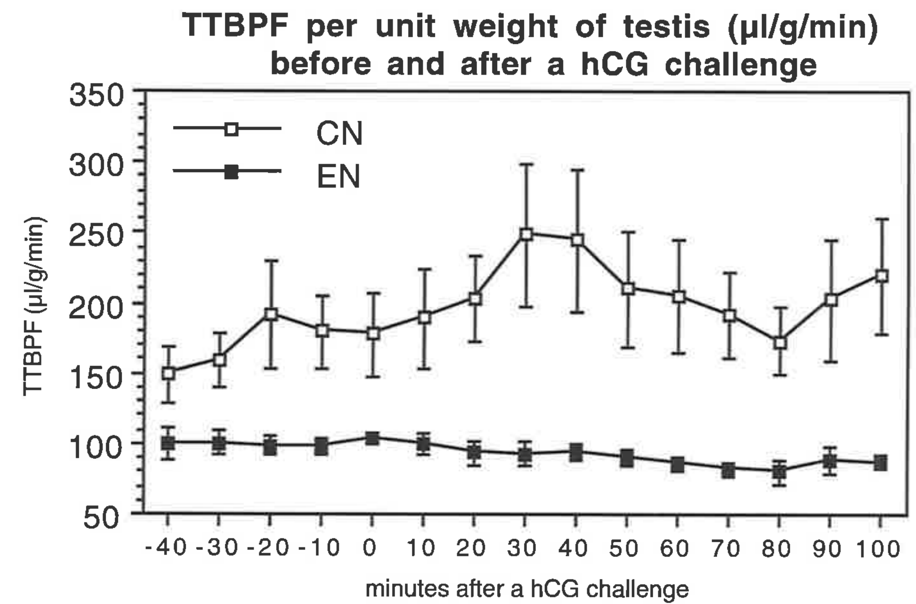


Fig. 5.10.: Total testicular blood plasma flow (TTBPF) per testis (ml/min; left) and per unit weight of testis (µl/g/min; right) before and after a hCG injection (20 I.U./kg BW) in ram lambs actively immunized against BSA (CN) or against E_2-6-BSA (EN). Values are the means ± sem (n=5 testes belonging to 3 lambs in each group).

fluctuated significantly ($p < 0.01$) during the 150 min-sampling period in controls but did not change significantly with time in E_2-immunized lambs.

5.3.14. Testosterone production.

The production of testosterone has been calculated for 10 testes (5 testes from 3 CN lambs and 5 testes from 3 EN lambs). For the EN lambs, mean testosterone production by one testis (ng/min) increased significantly ($p < 0.01$) after the hCG injection. For the CN lambs, although mean testosterone production by one testis (ng/min) tended to increase after the hCG injection, the difference was not statistically significant. Before stimulation with hCG, mean testosterone production by one testis tended to be more elevated in the EN lambs but the difference between EN and CN lambs was not statistically significant (table 5.5.). After the hCG injection, mean testosterone production by one testis was significantly higher ($p < 0.05$) in the EN lambs compared with the CN lambs (table 5.5.).

5.3.15. Spermatogenesis and testicular histology

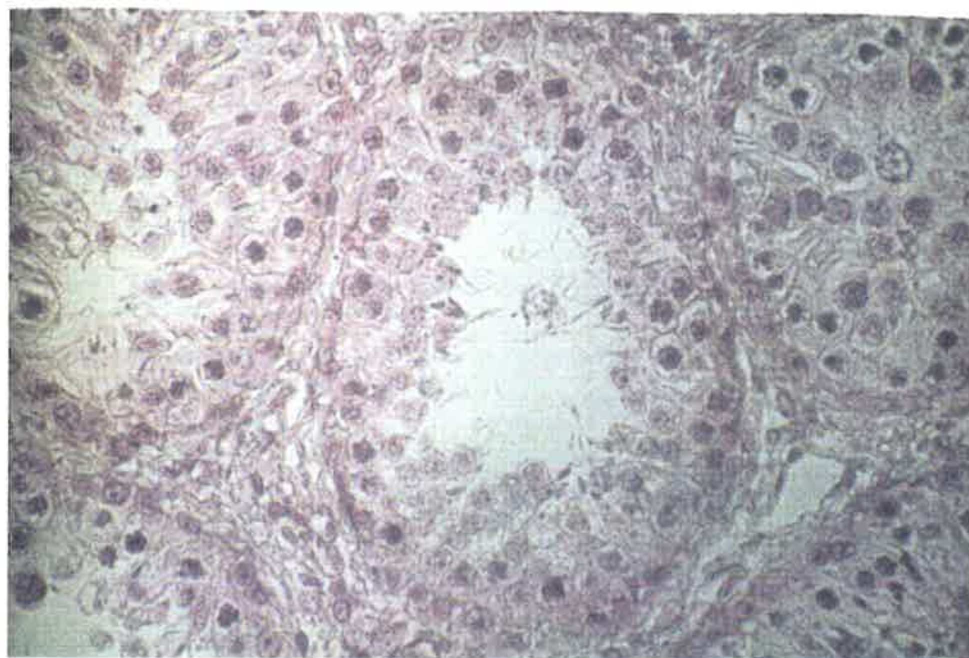
Testicular biopsy samples which were collected at 22 and 26 weeks of age as well as all the testicular tissues collected at castration were examined under light microscopy. At 22 weeks of age, three out of four control (CB) lambs and two out of four E_2-immunized (EB) lambs had spermatozoa present in the lumen of the seminiferous tubules. We noted that the testes that were not producing spermatozoa at 22 weeks of age were of small size (< 43 cm^3) with the exception of the testes of one EB lamb (lamb #1) which were relatively large (~91 cm^3). At 26 weeks of age, all EB lambs and all CB lambs have achieved puberty. Finally, at the end of the experiment, mature spermatozoa were present in all controls (CN and CB) and E_2-immunized lambs (EN and EB). At that time, the smallest testis weighed 42.22 g and was found in a CB lamb (lamb #10). At 22 and 26 weeks of age, large vacuoles were observed in the epithelium of the seminiferous tubules of one E_2-immunized lamb (lamb # 3, EB). At 30 weeks of age, nearly complete absence of germ cells was observed in lamb #15 with other apparent signs of degeneration in the seminiferous tubules such as those observed in lamb #3 at 22, 26 and 30 weeks of age (fig. 5.11.). In the other lambs at 30 weeks of age, the general aspect of the seminiferous tubules appeared normal and contained numerous meiotic figures and apparently

Table 5.5.

Mean testosterone production (μg/min) by one testis before and after a hCG injection (20 I.U./kg BW) in ram lambs actively immunized against BSA (CN) or against E_2-6-BSA (EN).

Treatment	T production per testis (μg/min ± s.e.m.) before hCG (number of testis measured)	T production per testis (μg/min ± s.e.m.) after hCG (number of testis measured)
CN	**0.525 ± 0.260^a** (n=5 testes)	**5.064 ± 2.088^a** (n=4 testes)
EN	**1.503 ± 0.686^a** (n=5 testes)	**15.145 ± 3.263^b** (n=5 testes)

values with different superscripts are significantly different at 95%



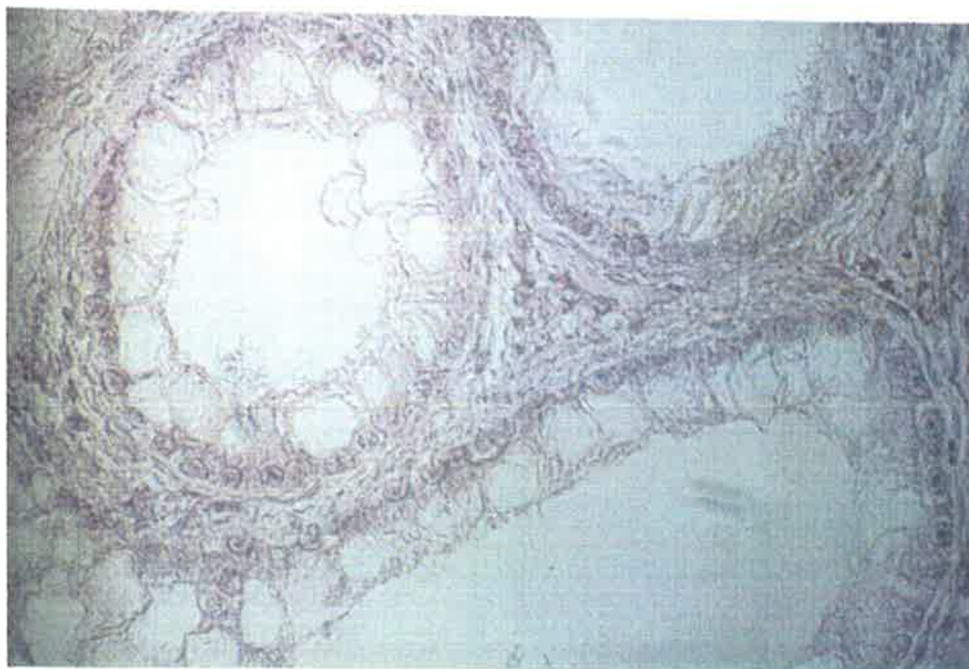


Fig. 5.11.: Normal appearance of the seminiferous tubules of a 30 week old control lamb (above) compared with the seminiferous tubules observed in one E_2-immunised lamb (lamb #15, EN) (below). Note the large vacuoles within the epithelium and the absence of spermatogenesis. Haematoxylin and eosin staining (x 50).

L. 22-24: The finding that a decline in testis weight can occur when liveweight continues to increase was also noticed by other researchers working with developing Merino ram lambs kept on pasture (C. M. Oldham, personal communication).

L. 25: Although there was a clear change in the testis growth curve.

normal spermiogenesis with round and/or elongated spermatids (fig. 5.11.). No abnormalities were observed in the interstitial tissues of the control and E_2-immunized lambs.

5.4. DISCUSSION

To our knowledge, this is the first time that active immunization against oestradiol-17ß used to study pubertal development in ram lambs. We have shown that the prepubertal Merino ram lambs responded well to the immunization procedure used and produced a amount of oestradiol antibodies. However, the presence of these antibodies in circulation did not result in an improvement in testicular maturation. In fact, although we have observed a tendency for larger testicular volumes in the E_2-immunized lambs.between 22 and 26 weeks of age, the difference between the control and the E_2-immunized lambs did not persist until 30 weeks of age. At that age, the more precise measurement of diameter and length done on the removed testis had also pointed out a tendency for larger testes in E_2-immunized lambs but, no concomitant increase in 'testis-epididymis' weight or in 'fixed testis' weight and 'fixed epididymis' weight have been found to support this observation.

Our results, therefore, resemble more obtained by Jenkins et al. (1986) than obtained by Land et al. (1981). In fact, using passive immunization against oestrogens, Jenkins et al. did not find any increase in testicular growth, between 2 and 16 weeks of age, in crossbred lambs; while Land et al. have shown that the rate of growth of the testis was greater in immunized Merino lambs, between 14 and 26 weeks of age. Unfortunately, the lambs used in these two studies were not studied beyond these ages (16 and 26 weeks of age, respectively) and the histological appearance of the testes at the end of these experiments has not been reported.

One of the most striking observation in the present experiment, is the final decline in testicular volume observed in about half of our lambs (whether or not they were submitted to biopsy sampling or immunized against E_2). Using the testicular circumference measurements, however, this decrease in testicular volume was not very apparent.

One possible explanation for this decline in testicular volume is that the environmental conditions, perhaps the lighting regimen, were not quite appropriate, for these winter born

lambs never exposed to decreasing day length, to favour continuous growth of their testes. Indeed, based on the PRL data (rather high PRL levels), it seems that the lambs have interpreted the 12L:12D light cycle as long days (which are not very stimulating for the reproductive system of ram lambs - see Colas et al. 1987-) by contrast with the autumn born lambs presented in chapter 3 (rather low PRL levels) or the autumn born crossbred ram lambs studied by Klindt et al.(1985) which interpreted the same artificial cycle more like short days.

Another important observation was that the decrease in testicular size was very pronounced in some E_2-immunized lambs in which major histological alterations of the testis have been noticed. Although a singular testicular growth pattern was already apparent at 22 weeks of age in one E_2-immunized lamb (e.g. slight reduction in the rate of testicular growth in lamb #3 EB), most detrimental effects on the testes have been essentially detected by histological examination of the testicular tissue taken during biopsy procedures carried out at 22, 26 and 30 weeks of age. Indeed, large vacuoles were observed within the seminiferous tubules of two E_2-immunized lambs (#15 EN and #3 EB), and spermatogenesis was markedly impaired in lamb #15 (EN). Surprisingly, we have found that one 22 week old EB lamb (lamb #1) had not started releasing spermatozoa although the volume of each of its testes was already quite large (~ 91 cm^3). Indeed, in this experiment and in the previous experiment presented in chapter 3, testes of smaller size (< 61 g; ~ 80 cm^3) were found to be releasing spermatozoa. Similarly, Papachristoforou (1987), studying South Australian Merino lambs grown outdoors, has also found that most testes weighing 50-60 g had started releasing spermatozoa. Other deleterious effects have been also identified in some E_2-immunized lambs, at the end of the experiment, by DSP evaluation (very low DSP in lamb #15 EN) and by measurement of TTBPF (significantly lower TTBPF per unit weight of testis in E_2-immunized lambs).

Important age and species differences seem to exist with regard to the consequences of immunoneutralization of oestrogens. In a 6 months-study with Ile de France adult rams, testis weight was shown to increase following active immunization against oestrogen (Schanbacher et al., 1987), however, no improvement in sperm production and no abnormalities in the histological appearance of seminiferous tubules and Leydig cells have been found in those rams

L. 5: Although nuclear and cellular volumes of a single Leydig cell were decreased and an increase in lipid content per Leydig cell was observed (Wrobel et al., 1990).

L. 10-17: It is perhaps relevant that testis blood flow was lower in surgically hypophysectomized rams treated with high doses of pituitary extract, sufficient to produce LH and FSH concentrations about 5 times higher than normal, than in intact controls (Setchell *et al.*, 1991). Interestingly, our data has clearly shown that testicular blood flow was not affected, in short term, by a single hCG injection. This lack of immediate response of testis blood flow to hCG is similar to the situation in rats, although, in this species, there is first a fall and then a rise in testis blood flow at longer times after hCG injection (see Setchell, 1990 for review).

L. 28: High intratesticular testosterone (T) concentration could perhaps have some inhibitory effects on the testicular function (e.g. a higher level of 'adverse' metabolites could be produced due to an increase in substrate (T) availability within various metabolic pathways. Indeed, T and DHT can be enzymatically converted into several other derivatives with diminished or altered types of biological activities in many tissues [Coffey, 1988]). Another postulate is that high intratesticular testosterone (T) concentration might lead to ~~exhaustion~~ inhibition of testicular functions.

(Monet-Kuntz et al., 1988). Thompson and Honey (1984) reported that active immunization against oestrone in prepubertal stallions has led to an increase in testicular weight and DSP at 27 months of age. On the other hand, DSP was not affected following active immunization against oestradiol in prepubertal bulls (D'Occhio et al., 1987) and the testes of these bulls did not present any major histological abnormalities (Wrobel et al., 1990). see INSERT 5.

The lower TTBPF (per unit weight of testis) observed in our E_2-immunized lambs does not support the observations reported by other investigators. In fact, Monet-Kuntz et al (1988) reported that the blood and lymphatic vessels within the interstitium had a larger volume in E_2-immunized rams than in controls. Similarly, Nieschlag et al. (1975c) observed a higher percentage volume of blood vessels in the E_2-immunized rabbit testis. At the moment, it is unclear whether the important hormonal changes occurring following immunization against oestradiol could be responsible for a change in testicular blood flow but severe alteration of the spermatogenic function such as that observed in some E_2-immunized lambs (e.g. #15 EN) would certainly influence this parameter. Indeed, it is generally accepted that the amount of blood flowing through the testis is determined largely by the mass of the tubules (Setchell and Brooks, 1988). In agreement with our previous experiment (chapter.3), we have again shown that testicular blood flow was not affected by a hCG injection in short term. see INSERT 10 - 17

The steroidogenic function of the testis of the E_2-immunized lambs was markedly enhanced. For instance, we have demonstrated that the amount of circulating testosterone is more than doubled in E_2-immunized lambs and that the testicular responsiveness to a hCG challenge was significantly more pronounced in the EN lambs. Although it is not clear if the intratesticular LH receptors increase after immunoneutralization of oestrogens (Monet-Kuntz et al , 1988; Sanford, 1989), the increase in the testosterone secretion observed in the E_2-immunized lambs is likely to reflect a change in the activity of key regulatory enzymes involved in the conversion of progesterone to androgens as suggested by many authors (Moger 1980; Navickis et al., 1981; Nozu et al., 1981b; Keel and Abney 1982). Whether the high level of testosterone produced in our E_2-immunized lambs could have contributed to the decline in testicular volume observed towards the end of the experiment is not known. see INSERT 28. However, that a tremendous testosterone

L. 11: The fact that LH pulse frequency was elevated in our E_2-immunized ram lambs compared with controls, strongly suggests that E_2 acts at the level of the hypothalamus. Indeed, since LH pulse frequency largely reflects the frequency of GnRH pulses discharged into the pituitary portal vessels, we assume that the activity of the GnRH pulse generator is increased due to immunoneutralization of E_2 (see P. 8a). Furthermore, the fact that LH pulse amplitude was also higher in our E_2-immunized ram lambs compared with controls, also suggests that the pituitary gland is an important site of action for E_2. Supporting this view, Olster and Foster (1986).reported that the amplitude of LH pulses in oestradiol-replaced castrated ram lambs were lower than those observed in castrated and testosterone-treated castrated lambs, even at similar frequencies of LH release. Thus, the possiblity of a direct inhibitory effect of E_2 on the pituitary gland to decrease responsiveness of the gonadotropes to GnRH is supported in both cases.

response to immunization could have been detrimental to the testicular function in long term.has already been suggested by Sanford (1987a). For instance, this author has reported that, for the adult crossbred rams living in Canada under natural lighting, the testicular regression occurring in early winter was more apparent for the rams passively immunized against E_2. Surprisingly, the high level of circulating testosterone observed in our E_2-immunized lambs did not have any positive effect on the overall body weight gain. Perhaps, an essential amount of oestrogens is required with testosterone to obtain a notable anabolic effect and continuous maturation of the testis in the ram lambs ("synergistic effect").

There is no doubt that the increase in testosterone level is related to the corresponding increase in mean LH level (~ 2-fold; reflecting more peaks and higher mean pulse amplitude) in our E_2-immunized lambs. Simultaneous increases in LH and testosterone secretion have been, in fact, frequently observed in oestradiol-immunized adult rams (Sanford, 1985; 1987a; Schanbacher; 1979; 1984a; Schanbacher et al., 1987; Monet-Kuntz et al., 1988). However, in other studies, oestradiol and/or oestrone immunization did not affect LH concentrations in ram lambs (Land et al., 1981; Jenkins et al., 1986) and adult rams (Sanford, 1989; Sanford et al., 1991). see INSERT 11

In the present experiment, FSH concentrations were more elevated in the E_2-immunized lambs (> 2-fold) which support the idea that this pituitary hormone is also regulated by oestrogens (possibly acting in synergy with inhibin; see Price, 1991) during pubertal development. Similarly, in adult rams, immunoneutralization of circulating oestradiol also resulted in elevated plasma FSH (Schanbacher, 1979; Sanford, 1987a; Schanbacher et al., 1987; Monet-Kuntz et al., 1988). However, no significant changes in plasma FSH values were observed, during passive immunization against oestrogens in ram lambs (Land et al., 1981; Jenkins et al., 1986).

In agreement with other reports, we did not observe a change in PRL levels in the E_2-immunized lambs (Jenkins et al., 1986; Sanford 1987a; 1989; 1991).

The level of oestradiol binding in plasma obtained from our E_2-immunized lambs compared well with that obtained in adult rams actively immunized against E_2-6-BSA (Schanbacher, 1984a; Sanford, 1987b). Two weeks after the booster injection, the titres reached were already well above the titres obtained in other studies using passive transfer of oestrogen antiserum in ram lambs (Land et al., 1981;

Jenkins et al., 1986). As the antisera cross-reacted slightly with oestrone and oestrone sulphate (approximately 15% and 3 % respectively) at 30 weeks of age we consider that these oestrogens, also, might have been neutralized by the circulating antibodies directed against oestradiol-17ß. It is unlikely that androgens were bound to the antibodies since their binding were negligible *in vitro* even when a high concentration of non-labelled testosterone, DHT or androstenedione was present in the assay tube (1000 pg/ml). In support of this view, testosterone metabolic clearance rate (related to the unbound form of T) has been shown not to be affected by immunization against oestradiol in rats (Nishihara and Takahashi, 1983) and rams (Schanbacher et al., 1987) which suggests that the E_2-antibodies do not interfere with the metabolism and activities of testosterone.

In the control lambs, the pattern of hormonal secretions observed during the 16-week study period resemble that observed in our previous experiment (chapter.3). Mean LH concentrations in plasma tended to decrease with age while mean testosterone concentrations increased. We have also determined that LH pulse frequency did not vary significantly during that period, but that mean pulse amplitude decreased and mean pulse nadir increased slightly. These results are comparable to the data reported by Yarney and Sanford (1989) except that they did not find a change in baseline LH concentration during pubertal development in crossbred ram lambs. On the other hand, Klindt et al (1985) reported a decline in all parameters of LH secretion (mean, baseline, frequency and amplitude) during pubertal development in crossbred lambs.

In agreement with our previous experiment (chapter 3), we did not observe significant variations in mean FSH levels between 14 and 30 weeks of age. In contrast, Yarney and Sanford (1989) have found that FSH and LH levels follow a similar pattern between 30 and 200 days of age.

An important rise in PRL occurred between 14 and 22 weeks of age, however, since PRL levels remained elevated thereafter, we could not interpret it as a short sharp elevation similar to that observed in our previous experiment (see chapter 3) or similar do that reported by other investigators (Ravault and Courot; 1975; Klindt et al. 1985) working with autumn born lambs. Interestingly, PRL levels tended to be more elevated between 22 and 30 weeks of age in lambs in which biopsies had been taken. Since "stress" has been shown to affect PRL concentration

(Raud et al., 1971), we suggest that this slight elevation may reflect the additional stress brought about by the surgical procedure and subsequent healing.

Finally, biopsy sampling did not impair testicular development whether it was monitored by measuring testis circumference or by estimation of testicular volume or by comparing the 'testis-epididymis' weights evaluated immediately after castration, as well as the 'fixed testis' weights and the 'fixed epididymis' weights. Only the estimation of testicular volume done on the removed testis has shown that the testes of the CB lambs were significantly smaller than the testes of the other lambs in which no biopsy were taken (CN and EN). This last observation may reflect a slight change in the shape of the testes at the site of the biopsy subsequent to healing although neither important adhesions between the tunica vaginalis and the albuginea nor obvious scar tissues were observed at castration in any lambs. DSP was not affected by biopsy sampling.

Successful repetitive testicular biopsies have already been reported in rams during pubertal development (Lunstra and Echternkamp, 1988) and in adult bulls (Pimentel et al., 1984). It seems that the use of a careful procedure that minimizes damage to the vascular layer of the tunica albuginea and postoperative inflammation explains the success of the procedure. However, we cannot conclude that this procedure is totally without consequences as we have observed some significant hormonal modifications following biopsy sampling. Indeed, in addition to PRL differences already mentioned, we have observed that the level of circulating testosterone reached at 30 weeks of age was not as high in the lambs submitted to biopsy sampling as in the other lambs in which no biopsy were taken (although this effect was statistically significant in the EB lambs only). A tendency for a lower FSH concentration in plasma from lambs submitted to testicular biopsy was also present, but since this difference has been observed before the surgical procedure was initiated it is more likely that this difference was not due to biopsy sampling. Biopsy sampling did not influence circulating LH concentrations. Thus, even if repetitive testicular biopsies did not seem to impair subsequent testicular development, the fact that hormonal changes are still evident one month after the

surgery in some lambs, leads us not to recommend the use of this surgical procedure in an endocrinological study on pubertal development.

In conclusion, this study has provided additional evidence that oestradiol-17ß in the blood circulation plays an important role in the regulation of LH and FSH secretion in the ram lambs during pubertal development but also that the impairment of the biological functions of this steroid through antibody binding can have marked effects on the structures and functions of the testis. In fact, the most striking observation in the present experiment was that a decline in testicular volume occurred towards the end of the experiment and was more pronounced in E_2-immunized lambs. We also reported that relative TTBPF was significantly reduced following immunization against oestradiol-17ß and finally, that testicular biopsies taken at 22 and 26 weeks of age did not affect testicular development, but affected circulating testosterone and prolactin levels at 30 weeks of age.

CHAPTER 6.

PASSIVE IMMUNIZATION AGAINST OESTROGENS AND PUBERTAL DEVELOPMENT IN RAM LAMBS.

6.1. INTRODUCTION

Our previous experiment using an active immunization approach has provided additional evidence that oestradiol-17ß in the blood circulation plays an important role in the regulation of LH and FSH secretion in the ram lambs during pubertal development. However, this experiment also that the impairment of the biological functions of this steroid through an important antibody binding (high titre) could be detrimental to the structure and function of the testis in the long term (see chapter 5).

Immunization against steroids can also be achieved when antibodies, that have been produced in an actively-immunized animal, are administered exogenously to recipients (the experimental animals in the passive immunization study). In comparison with active immunization, the passive immunization approach allows more control of antisera specificity and titre. This later advantage is important since the magnitude of the endocrine changes subsequent to neutralization of a given hormone seems to relate to the titre of the antiserum in the circulation (Sharpe and Fraser, 1983; Roberts and Reeves, 1988). Moreover, since the passive transfer of anti-steroid antibodies provides a fast method of neutralizing this class of hormone and since our interest is to study a maturational process that occurs in a relatively short period of time in the male sheep, the use of this approach would appear more judicious. In fact, this method has already proven itself useful in determining the biological actions of endogenous circulating oestrogens in the prepubertal ram lambs (Land et al , 1981; Jenkins et al., 1986) as well as in adult rams (Sanford, 1985; 1987a; 1989; Sanford et al., 1991). Surprisingly, very few researchers have used purified immunoglobulins along with the passive immunization approach (Zeleznik et al., 1985; Dubey et al., 1987; Roberts and Reeves, 1988).

Since, the anti-oestradiol antisera generally cross-react slightly with oestrone and oestrone-sulphate (e.g. see section 5.3.2.), it is generally considered that these "oestrogens" are also

L. 9: D'Occhio and Brooks (1980) have shown that oestrone, oestradiol-17ß and the synthetic oestrogen, diethylstibestrol, were the most effective oestrogens in eliciting mounting behaviour in adult castrated rams.

L. 23: The exact concentration of oestrone or oestrone sulphate in the circulation of ram lambs or adult rams has not yet been determined.

neutralized by the circulating antibodies. While the role of oestradiol-17ß in the regulation of the hypothalamic-pituitary-testicular axis in the ram has been principally scrutinized (Bolt, 1971; Rigg and Malven, 1974; Edgerton and Baile, 1977; Schanbacher and Ford 1977; Parrott and Davies, 1979; Schanbacher 1980a; Jenkins and Waites, 1983; D'Occhio et al., 1983b; 1985; Olster and Foster, 1986; Olster and Foster, 1988; Sanford and Robaire, 1990; see also chapter 5), attempts to define the physiological role of oestrone in the male have been scarce.

Land et al. (1981) have shown that the concentration of FSH was correlated with the oestrone antibody titre in passively immunized Merino ram lambs suggesting that oestrone may have a particular part to play in the feedback control of FSH release in the ram lamb. Thompson and Honey (1984) reported that active immunization against oestrone in prepubertal stallions led to an increase in testicular weight and daily sperm production (DSP) at 27 months of age. In boars, although the titres obtained following active immunization against oestrone were very low, direct testicular local effects were observed including lower Leydig cell numbers, increased Leydig cell size and lower seminiferous tubules diameter (Wise et al., 1991). In the laboratory species, it was found that oestrone might exert a positive feedback action on the pituitary since E_1-immunized male rats had lower FSH and normal LH levels (Wuttke et al., 1975). On the other hand, D'Occhio et al. (1982) have shown that infusion with oestrone had no effect either on the pattern of LH secretion or on the pituitary response to GnRH in castrated young bulls.

The significance of oestrone-sulphate is not known at present but may be important in testicular development in some species. For instance, in the boar, an approximate 5-fold increase in the secretion of this steroid occurs during pubertal development (Setchell et al., 1983). Whether this sulphated steroid participates in pubertal development in the male sheep has not yet been determined.

From the beginning, the essential requirement for the two major experiments presented in this chapter was to obtain an adequate amount of specific anti-oestradiol and anti-oestrone antibodies from actively-immunized wethers. Therefore, the preliminary steps including development, collection, purification and characterization of the antibodies are described in details in the first section (section 6.2.) of this chapter.

L. 1-10: One of the main objective of the experiment presented in section 6.3 of chapter 6 was to verify if similar deleterious effects (as observed in our actively immunized ram lambs described in chapter 5) would be observed is ram lambs passively immunized against oestradiol even though a much lower level of anti-oestradiol antibodies would be present in their circulation.

The two passive immunization studies (sections 6.3 and 6.4) presented in chapter 6 differ in many ways from the two studies (using passive immunization against oestradiol in ram lambs) that were previously reported (Land et al, 1981; Jenkins and Waites, 1983).

•The main differences were:

-two different immunogens were used (oestradiol-3-HSA and oestrone-3-BSA) to produce the antisera.

-the type of treatment used:

purified IgG and complete antiserum.

-the experimental design:

complete endocrinological investigation including: regular intensive bleedings; GnRH challenge; hCG challenge; histological analysis of testicular tissue; daily sperm production.

-the controlled environment where the lambs were studied:

artificial lighting(12L:12D); pen restriction, handfeeding.

-the duration of the study:

16 weeks.

-the age of the ram lambs studied:

14-30 weeks of age or 10-26 weeks of age

•It was also very important to establish a link with other studies:

comparisons with other experiments presented in chapter 3 and in chapter 5 (ram lambs of same age, same breed; kept in the same environment, same feeding).

The aim of the first experiment (section 6.3.) was to determine if the effects obtained with active immunization against oestradiol-17ß could be reproduced with the use of a passive immunization approach. Our hypothesis is that regular administration of exogenous anti-oestradiol-17ß antibodies should neutralize the inhibitory effects of this hormone in prepubertal Merino ram lambs and should lead to an increase in gonadotropin secretion along with a concomitant activation of the steroidogenic and spermatogenic functions of the testes. However, contrary to what has been observed with the use of active immunization against oestradiol-17ß, a lower level of antibodies in circulation should not lead to an overstimulation of the hypothalamic-pituitary-testicular axis but should rather moderately improve testicular maturation without detrimental consequences on the spermatogenic function. Another objective of this experiment was to verify if purified IgG directed against oestradiol-17ß are as effective as the corresponding complete (non-purified) antiserum in neutralizing this hormone during pubertal development in the ram lambs. (see INSERT 1-10)

Ultimately, since oestrone and oestrone-sulphate are also possibly involve in the regulation of the hypothalamic-pituitary-testicular axis, in the second experiment of this chapter (section 6.4.), we hypothesized that passive immunization against oestrone (and oestrone-sulphate due to cross-reaction) should also stimulate testicular maturation in similar ways as passive immunization against oestradiol-17ß in Merino ram lambs.

6.2. PRELIMINARY : DEVELOPMENT, PURIFICATION AND CHARACTERIZATION OF ANTIBODIES DIRECTED AGAINST OESTRADIOL-17ß, OESTRONE OR BOVINE SERUM ALBUMIN.

6.2.1. EXPERIMENTAL PROCEDURE

6.2.1.1. Experimental animals

Nine adult castrated males (wethers) were used for antibody production. They were kept indoors in individual pens and were fed with commercial sheep pellets and oat hay according to their maintenance requirements.

6.2.1.2. Treatment

The wethers were immunized against BSA (n=2), oestradiol-17ß-3-CMO:HSA (E_2-3-HSA, n=3), or oestrone-3-BSA (E_1-3-BSA, n=4). The primary injection was prepared in Freund's complete adjuvant (FCA). Freund's incomplete adjuvant (FIA) was used instead of FCA in booster injections. The first booster injection was given 4 months after the primary injection. Others boosters were repeated every month during one year. The immunogen preparation was injected as described in section 2.3.1.

6.2.1.3. Blood collection

Fourteen days after each booster injection, approximately 750 ml of blood (jugular venepuncture) were collected from each wether into sterile bottles, using 14G sterile needles and cannulae. The blood was kept at room temperature for 4 hours then transferred into a cold room for an extra 20 hours to allow clot formation. The following morning, the blood was centrifuged at 4°C at 2000 g for 20 minutes and the supernatant (the antiserum) was decanted. This operation was repeated a second time to remove all blood clots and the antiserum was finally stored at -20 °C until utilization. Small aliquots (~5 ml) of the whole antiserum were stored at -20°C for titre check.

6.2.1.4. Titre check and pool of antisera

Each antiserum collected was assayed for titre check as described in section 2.8.2.1.

The antisera collected from one E_2-immunized wether (W82, the one producing the antisera with the highest titre) following the first, second and third booster injections were pooled to give about two litres of E_2-antiserum. Similarly, the antisera collected from one E_1-immunized wether (X4, the one producing the antisera with the highest titre) following the first, second, third and fourth booster injections were pooled to give about two litres of E_1-antiserum.

The antisera collected from two BSA-immunized wethers (W76, W79) following the first, second, third and fourth booster injections were also pooled to give about four litres of BSA-antiserum to be used for the controls.

Eventually, each pool of anti-oestrogen antisera (anti-oestradiol, anti-oestrone) was characterized for titre and specificity.

6.2.1.5. Specificity of antiserum

Specificity of the anti-oestrogen antisera and/or specificity of the IgG obtained after purification (see section 6.2.1.6.) were examined as described in section 2.8.2.2. with the exception that a larger variety of unlabelled steroids and other compounds were tested (see table 6.2.1.).

6.2.1.6. Purification of antiserum

One to two litres of the pooled antisera (pooled antiserum directed against oestrone, oestradiol or BSA) were purified as described in section 2.3.2.1.

The degree of purity of the IgG obtained were verified by electrophoresis (using 8% non reducing sodium duodecylsulfate polyacrylamide gel).

6.2.1.7. Apparent affinity of purified IgG directed against Oestradiol-17ß

The "apparent" affinity constant (K) characterizing the hormone-antibody system can be derived from saturation (or binding-inhibition) curve. For the purified IgG directed against E_2-3-HSA (W82), the K was determined by incubating 100 µl of labelled E_2 (~10000 cpm/100µl) and 100 µl of various concentrations (0 pg/ml to 200 pg/ml) of unlabelled E_2 in gel-PBS with 100 µl of purified IgG diluted in saline (dilution that binds 50% of the labelled steroid). The tubes were left overnight at 4°C, then processed as for titre check (section 2.8.2.1.). Labelled oestradiol-

17ß used was described in section 2.8.2.1. Scatchard (1949) analysis of the hormone binding data, which relates the bound hormone concentration to the bound over unbound ratios at equilibrium, was performed to obtain an estimate of the affinity constant. The slope of the line yields "-K".

6.2.2. RESULTS

6.2.2.1. Health of animals

Abscess formation (1-2 cm diameter) was almost always noted at the immunization sites following the primary immunization. However, following booster immunizations, only a few abscesses were present and were of small size (< 1 cm). The animals remained in good condition otherwise throughout the antibody production, and showed no adverse reactions to either immunizations or bleedings. The body weight of the wethers were not affected by the immunization and bleeding procedures.

6.2.2.2. Titre check

The titre for each anti-oestrogen antiserum was: 1:50000 for the E_2-antiserum (W82) and 1:40000 for the E_1-antiserum (X4). When 0.332 g of purified IgG directed against oestradiol or oestrone was diluted in 5 ml of saline, the titre obtained was: 1:80000 and 1:70000, respectively.

6.2.2.3. Specificity

For the E_2-antiserum (W82) and the corresponding purified IgG preparation (see table 6.2.1.), we have seen that the only steroids cross-reacting to any extent with the antibodies were oestrone and oestrone-sulphate. Similarly, the only steroids that cross-react to any extent with the purified IgG directed against E_1-3-BSA (X4) were oestradiol-17ß and oestrone-sulphate (see table 6.2.1.).

6.2.2.4. Apparent affinity

Data on the assessment of the affinity constant of the purified IgG directed against oestradiol (W82) are presented in fig. 6.2.1. The binding of radiolabelled oestradiol-17ß was effectively displaced by graded doses of unlabelled oestradiol-17ß (binding-inhibition curve). Analysis of

Table 6.2.1.
Specificity

Cross-reacting steroids and others	Cross-reactivity[1] (%) Pur. IgG against E_2-3-HSA (W82)	Cross-reactivity[1] (%) Pur. IgG against E_1-3-BSA (X4)
oestradiol-17ß	100	< 4
oestrone	11	100
oestrone-sulphate	< 7.5	14
oestradiol-17α	n	n
oestriol	n	n
testosterone	n	n
dihydrotestosterone	n	n
dehydroepiandrosterone	n	n
androstenedione	n	n
pregnenolone	n	n
progesterone	n	n
corticosterone	n	n
cortisone	n	n
cholesterol	n	n
hydrocortisone	n	n
diethylstilboestrol	n	n
1-4-6-androstatrien 3-17 dione	n	n
Δ^5-androsten 3ß-17ß diol	n	n
5 α-androstan 3ß-17ß diol	n	n

[1] :The relative inhibitory activity of each steroid for the antibody preparation was calculated from the ratio of the mass of immunogenic steroid [E_2 or E_1] required to displace 50% of the radiolabeled immunogenic steroid [^{3}H-E_2 or ^{3}H-E_1] to the mass of the cross-reacting steroid (or other compound) required to displace the same fraction of the labeled steroid.

n: negligible displacement at the highest concentration tested (1000 pg/100 μl).

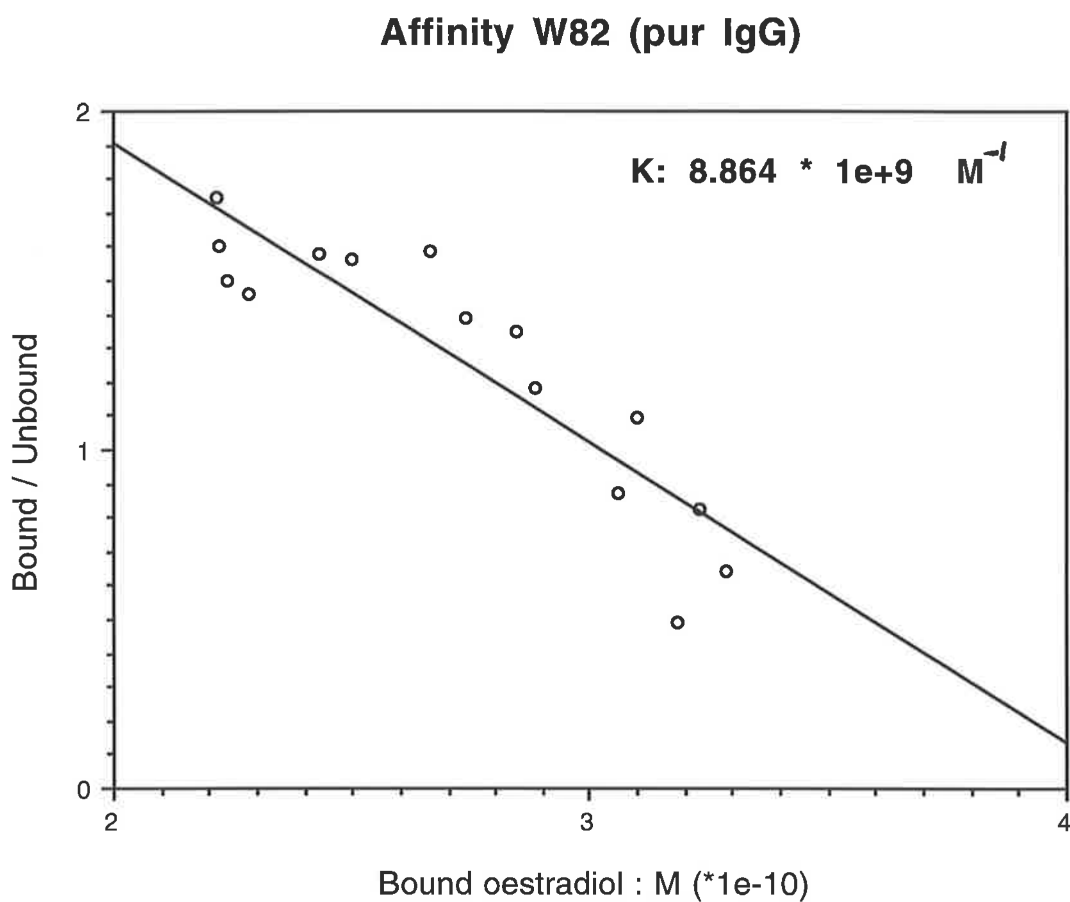


Fig. 6.2.1.: Saturation curve of a preparation of purified IgG directed against oestradiol-17ß (W82) obtained, at 4°C, by incubating a constant amount of the immunoglobulin preparation with increasing concentration of oestradiol-17ß (constant amount of ^{3}H-oestradiol with increasing amount of unlabeled oestradiol). The final incubation volume was 0.3 ml. The solid line represents the simple regression that best fit the data. The slope of that line yields -K (K being the "apparent" affinity constant).

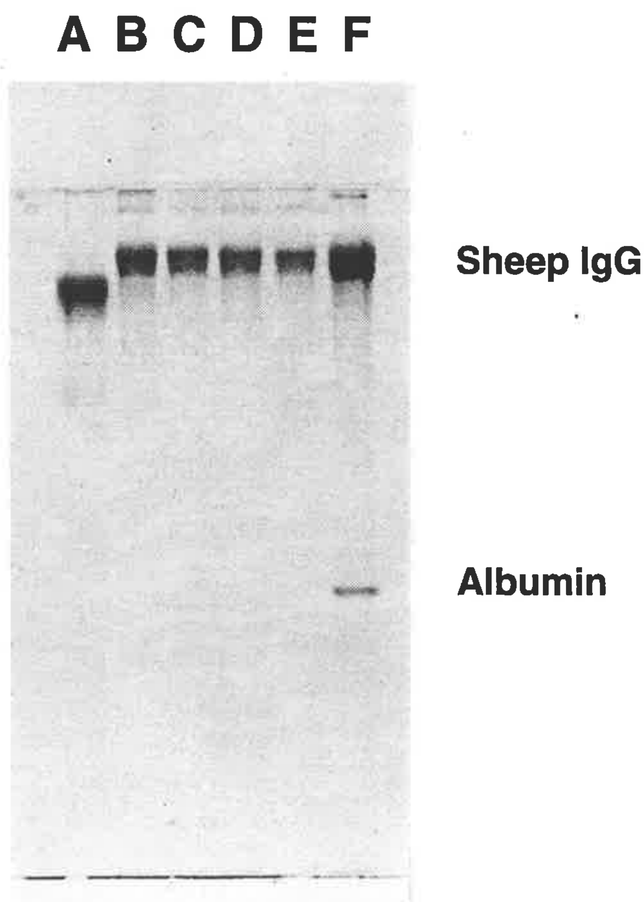


Fig. 6.2.2.: Electrophoresis (using 8% non reducing sodium duodecylsulfate polyacrylamide gel) showing the degree of purity of the IgG directed against oestrone "X4" (**C**), oestradiol-17ß "W82" (**D**) and BSA (**E**), purified by caprylic acid precipitation. Note the differences in purity by comparison with pure rabbit IgG (**A**), partially purified IgG originating from sheep plasma (**B**) and a mixture of sheep IgG+IgA+Albumin (**F**).

data by Scatchard plot allowed the estimation of the apparent affinity constant (K) which was found to be 8.864 x 10^9 M^{-1} (fig. 6.2.1.).

6.2.2.5. Purity of the IgG preparation

The isolation of IgG from the antisera directed against oestradiol, oestrone and BSA using the caprylic acid method has led to a relatively pure preparation of IgG as confirmed by electrophoresis (fig. 6.2.2.), comparable to what has been reported by Steinbuch and Audran (1969). The procedure of purification gave repeatable results since our three preparations of IgG (oestradiol "W82", oestrone "X4", and BSA) exhibited a comparable well defined band on the electrophoretic gel, located at the level of sheep IgG.

6.2.3. DISCUSSION

The specificity of the anti-oestradiol antisera produced in those wethers was comparable to the specificity of similar antisera generated in sheep by other investigators (Scaramuzzi et al., 1975; Land et al., 1981; Jenkins et al., 1986; Sanford, 1985; 1987a; 1989; Sanford et al., 1991; Schanbacher et al., 1987; our previous experiment presented in chapter 5.). The purified IgG directed against oestrone shows slight cross-reactivity with oestrone-sulphate (14%) and oestradiol-17ß (< 4%) which also compares well to the specificity of other anti-oestrone antisera generated in sheep (Martensz et al., 1979; Land et al., 1982). The apparent affinity constant (K) of the purified IgG directed against oestradiol was in the same range as that reported by other authors (Scaramuzzi et al., 1975; Nieschlag and Wickings, 1978; Zeleznik et al., 1985).

Thus, since we have obtained an adequate amount of antisera containing a relatively large amount (high titres) of specific antibodies (e.g. negligible cross-reactivity with androgens) and because the isolation of IgG has also been successful, leaving very few contaminants in the preparation, it has been possible to carry out the passive immunization studies planned (see sections 6.3. and 6.4.).

6.3. PASSIVE IMMUNIZATION AGAINST OESTRADIOL-17ß AND PUBERTAL DEVELOPMENT IN RAM LAMBS.

6.3.1. EXPERIMENTAL PROCEDURE

6.3.1.1. Experimental animals

Nineteen South Australian Merino ram lambs born between 17th and 29th April 1988 were used in this experiment. The lambs were kept outdoors with their mothers until weaning at 8 weeks of age. The lambs were then transferred to a room with controlled light (12 hours light: 12 hours dark).

6.3.1.2. Pen restriction

Lambs were grouped in each pen according to their body weight at their arrival in the controlled environment (section 2.2.1). Each pen contained at least two control lambs and two E_2-immunized lambs.

6.3.1.3. Treatment

Ten control lambs (Control, n=10) received intravenous saline injections (subgroup "S", n=6) or preparations of purified IgG directed against BSA diluted in saline (subgroup "Anti-BSA", n=4). Nine lambs were passively immunized against oestradiol-17ß-3-CMO:HSA (E_2-3-HSA) (E_2-immunized, n=9). Five of these lambs received injections of purified IgG directed against oestradiol-17ß (W82) (subgroup "Anti-E-G", n=5). The remaining four lambs received injections of the corresponding complete antiserum directed against oestradiol-17ß (subgroup "Anti-E-S", n=4). The immunization protocol consisted of fortnightly i.v. injections of the appropriate treatment, starting at 10 weeks of age, ending at 20 weeks of age.

With only approximate information regarding the ultimate titre desired, the dose of anti-oestradiol antiserum or purified IgG diluted in saline was a compromise between that considered effective on the basis of earlier experiments (Land et al., 1981; Jenkins et al., 1986; Sanford, 1985; 1987a) and the number of animals available for treatment. Therefore, the following doses were injected each time:

L. 4: Using the caprylic acid precipitation method of purification (described in section 2.3.2.1.), we can obtain 13 g of purified IgG (~0.26g/ml) from 500 ml of sheep serum (with minimum lost during the procedure). Therefore, we used 12.9 ml of complete E_2-antiserum which is, in weight, about equivalent to 0.332 g of purified IgG (and which give a titre that is equivalent to the titre obtained following injection of 0.332 g of purified IgG against E_2).

subgroup "S": 5 ml saline per lamb

subgroup "Anti-BSA": 0.332 g of purified IgG against BSA diluted in 5 ml saline per lamb

subgroup "Anti-E-G": 0.332 g of purified IgG against E_2 diluted in 5 ml saline per lamb

subgroup "Anti-E-S": 12.8 ml complete E_2-antiserum per lamb (see INSERT 4)

6.3.1.4. Body weight and testicular volume measurement

Body weight, testicular diameter and length were recorded weekly as described in sections 2.4. and 2.5.

6.3.1.5. Intensive bleeding

Before the beginning of the immunization, at 10 weeks of age, the lambs were intensively bled (every 20 min. for 6 hours) as described in section 2.6.1. Intensive bleedings were repeated at 14, 18, 22, 26 and 30 weeks of age.

6.3.1.6. Pituitary responsiveness to GnRH.

At the end of the intensive bleeding at 30 weeks of age, all lambs received a single GnRH injection (5 ng/kg body weight) and additional blood samples were collected as described in section 2.6.2.

6.3.1.7. Testicular responsiveness to hCG.

Testicular responsiveness to a single hCG injection (20 I.U./kg body weight) was evaluated at 30 weeks of age in nine anaesthetized lambs (two "S", two "Anti-BSA", two "Anti-E-G", three "Anti-E-S") as described in section 2.6.3.1. This parameter has been determined exclusively in the lambs in which the surgical cannulations of the internal spermatic veins (and therefore blood samplings) have been totally successful (n=9).

6.3.1.8. Titre check

One blood sample for each lamb was collected immediately before and after an immunization and these samples were assayed for titre check.as described in section 2.8.2.1.

6.3.1.9. Hormone assays

Plasma samples were assayed for LH, FSH, PRL and testosterone as described in section 2.8.1.2.2., 2.8.1.2.3., 2.8.1.2.4. and 2.8.1.3.2., respectively. Mean hormone levels were determined by assaying each plasma pool representing the 6-h sampling period (section

2.6.2.2.). Each blood sample collected following a GnRH challenge was assayed for LH. Pool samples collected before and after a hCG challenge (section 2.6.3.2.) were assayed for testosterone.

6.3.1.10. Castration

All ram lambs were castrated at 30 weeks of age with the exception of the two 'heavy' control lambs ("S") castrated at 26 weeks of age (only these 2 lambs weighed more than 20 kg at the beginning of the experiment and more than 35 kg at 26 weeks of age). Castration was performed as described in section 2.7.4.

6.3.1.11. Testicular histology

Blocks of testicular tissue taken at castration were prepared as described in section 2.9.

6.3.1.12. Statistical analysis.

Data for characteristics involving repeated measurements over time (body weight, testicular volume, FSH, LH, PRL and testosterone) were analyzed by a analysis of variance for repeated measures to detect differences due to immunization treatment, time or their interactions. Titres before and after each immunization, and testicular weight and volume at castration were analyzed using a one factor analysis of variance to localize differences among and within the treatment groups. All these analyses were done using the CLR ANOVA computer program (Clear Lake Research, Morningside, Houston, TX, 77005).

If statistical significance was found in the analysis of variance for repeated measures, a one factor analysis of variance for each time period was carried out. Mean differences for the 4 treatment groups were then tested by Duncan's New Multiple Range test. Single pairwise comparisons were made using student-*t* -test. A logarithmic transformation [log (x+1)] was applied to some data (in this experiment: testicular weight, mean testosterone and mean PRL) to equalize variances. The data presented in tables and graphs are the actual ones.

6.3. RESULTS

6.3.2.1. Health of animals

During the whole experiment, all lambs remained healthy; however the smallest lambs did not show a very good appetite at the beginning of the experiment and took a long time to get use to the commercial pellets.

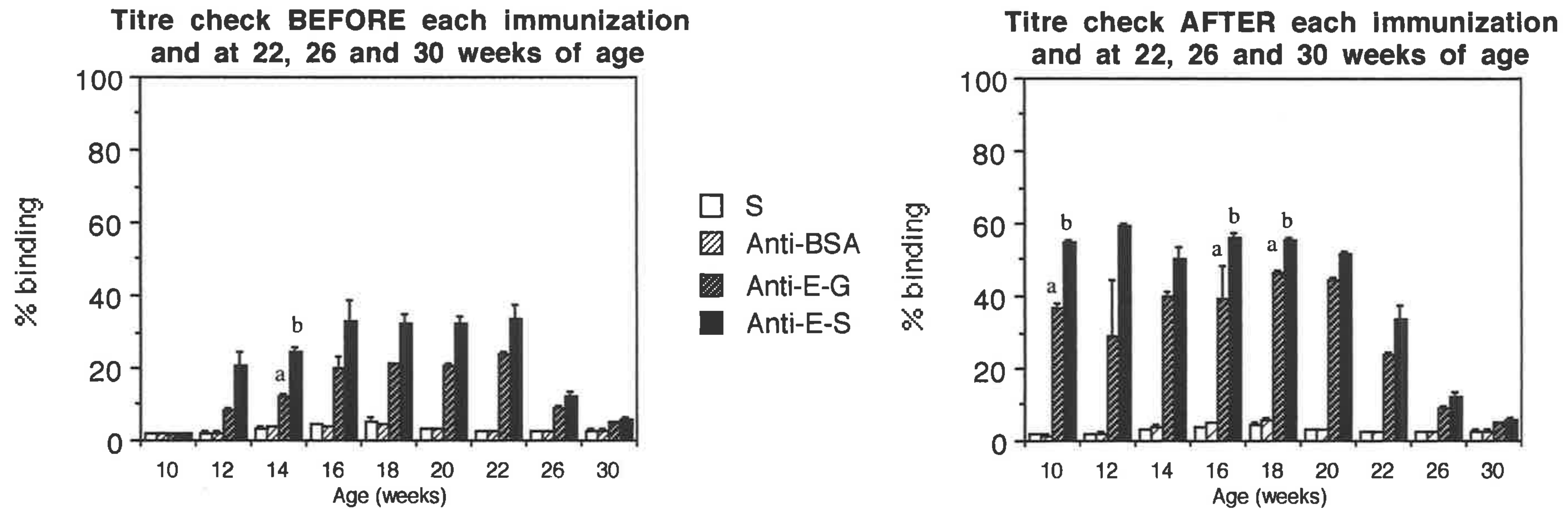
6.3.2.2. Titre check

The percentage binding of oestradiol-17ß remained negligible (less than 6 %) during the whole study in the control lambs (S and Anti-BSA lambs). In E_2-immunized lambs (Anti-E-G and Anti-E-S lambs), plasma collected immediately after an antibody injection (highest concentration of antibodies in circulation, between 10 and 20 weeks of age) could bind between 54 and 65 % of radioactive oestradiol-17ß (at a dilution of 1:100) (fig. 6.3.1.). This percentage binding was comparable in animals receiving complete antiserum (Anti-E-S lambs) and in those receiving the preparation of purified IgG directed against oestradiol (Anti-E-G lambs). When the titre was determined in samples collected immediately before an immunization (fig.6.3.1.), the Anti-E-S lambs had a higher percentage binding ($p < 0.05$) than the Anti-E-G lambs at 12, 14 and 18 weeks of age. The percentage binding varied between 46 and 59 % in Anti-E-S lambs and between 28 and 52 % in Anti-E-G lambs. The percentage binding slowly decreased after the last immunization (at 20 weeks) and reached values ranging between 27 and 39 % at 26 weeks of age and between 10 and 17 % at 30 weeks of age. Therefore, six and ten weeks after the last immunization, the percentage binding of oestradiol-17ß was still significantly increased ($p < 0.001$) in all E_2-immunized lambs and there was no significant difference between Anti-E-S and Anti-E-G lambs.

6.3.2.3. Body weight

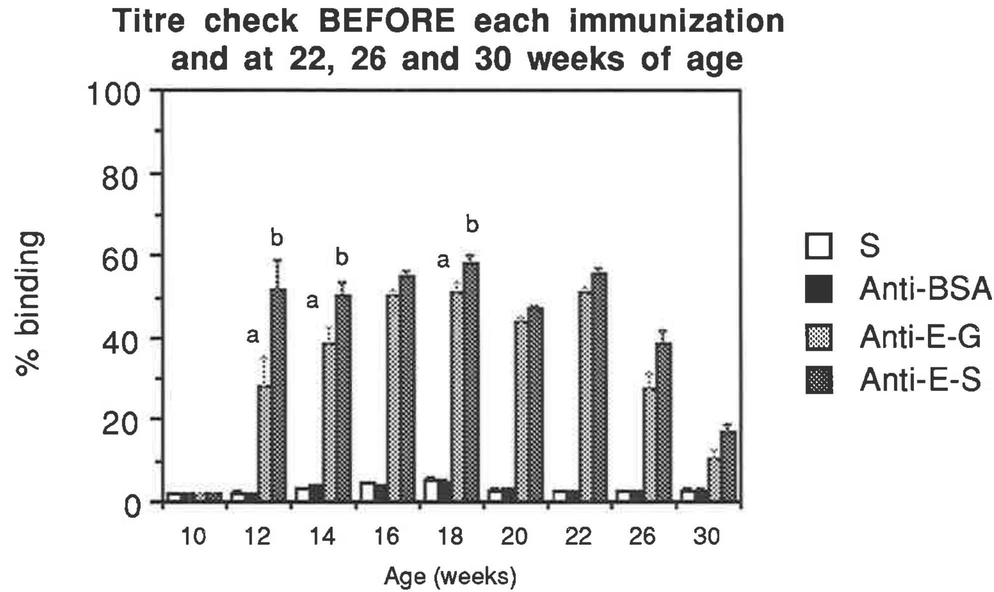
Liveweight patterns for control (S and Anti-BSA) and E_2-immunized lambs (Anti-E-G and Anti-E-S) are shown in fig. 6.3.2. At the beginning of the experiment, the range in body weight among ram lambs was large (BW ranged between 12.6 and 19.9 kg, except for two additional S lambs weighing more than 20 kg), however, mean body weight for each treatment group remained comparable during the whole study (no treatment effect). Gain in body weight was

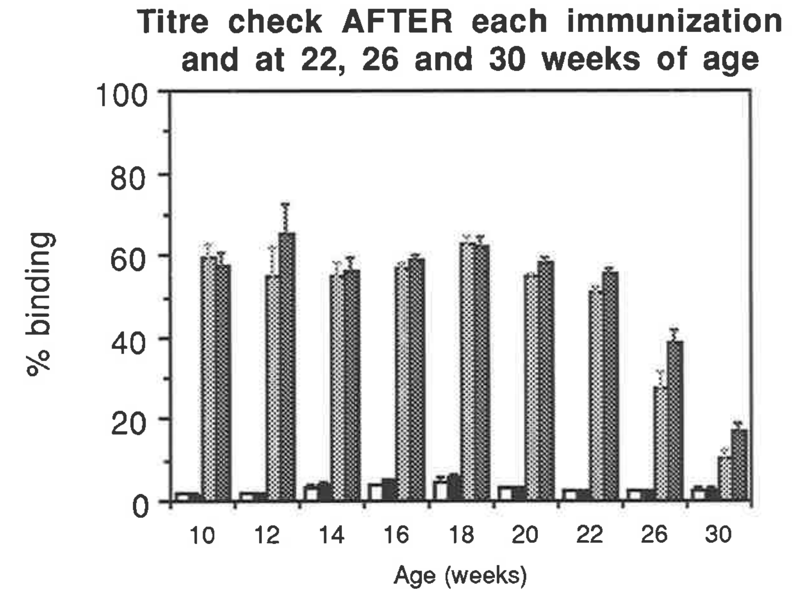
One should note that the difference in oestradiol binding between plasma samples collected before an immunization and those collected after an immunization is more pronounced when samples are diluted at 1:500 (fig. 6.3.1.a.; below) than when they are diluted in 1:100 (fig. 6.3.1.; P. 181).



for the E_2-immunized lambs (Anti-E -G and Anti-E-S): values with different superscripts are significantly different at 95%

Fig. 6.3.1.a: Mean percentage binding (% ± sem) of ^{3}H-oestradiol-17ß in diluted plasma (1:500) collected before (left fig.) and after (right fig.) each immunization (at 10, 12, 14, 16, 18, 20 weeks of age), and at 22, 26 and 30 weeks of age thereafter, from ram lambs receiving saline (S), purified IgG against BSA (Anti-BSA), purified IgG against E_2-3-HSA (Anti-E-G) or complete antiserum directed against E_2-3-HSA (Anti-E-S). (S: n=6, Anti-BSA: n=4, Anti-E-G: n=5 and Anti-E-S: n=4).





for the E_2-immunized lambs (Anti-E -G and Anti-E-S): values with different superscripts are significantly different at 95%

Fig. 6.3.1.: Mean percentage binding (% ± sem) of ^{3}H-oestradiol-17ß in diluted plasma (1:100) collected before (left fig.) and after (right fig.) each immunization (at 10, 12, 14, 16, 18, 20 weeks of age), and at 22, 26 and 30 weeks of age thereafter, from ram lambs receiving saline (S), purified IgG against BSA (Anti-BSA), purified IgG against E_2-3-HSA (Anti-E-G) or complete antiserum directed against E_2-3-HSA (Anti-E-S). (S: n=6, Anti-BSA: n=4, Anti-E-G: n=5 and Anti-E-S: n=4).

very low during the first ten weeks of the experiment then rose significantly until the end of the experiment in all lambs. All groups of lambs had gained approximately 16 kg during the study (S: 14.2 ± 1.3 kg, Anti-BSA: 16.6 ± 2.0 kg, Anti-E-G: 16.8 ± 1.9 kg, Anti-E-S: 16.0 ± 2.6 kg).

6.3.2.4. Testicular volume

Changes in testicular volume are shown in fig. 6.3.3. As for body weight, there was very minor change in testicular volume up to 21 weeks of age, then a very pronounced increase in testicular volume occurred and continued until the end of the experiment. The immunization treatment did not influence the pattern of testicular development.

6.3.2.5. Testosterone concentration

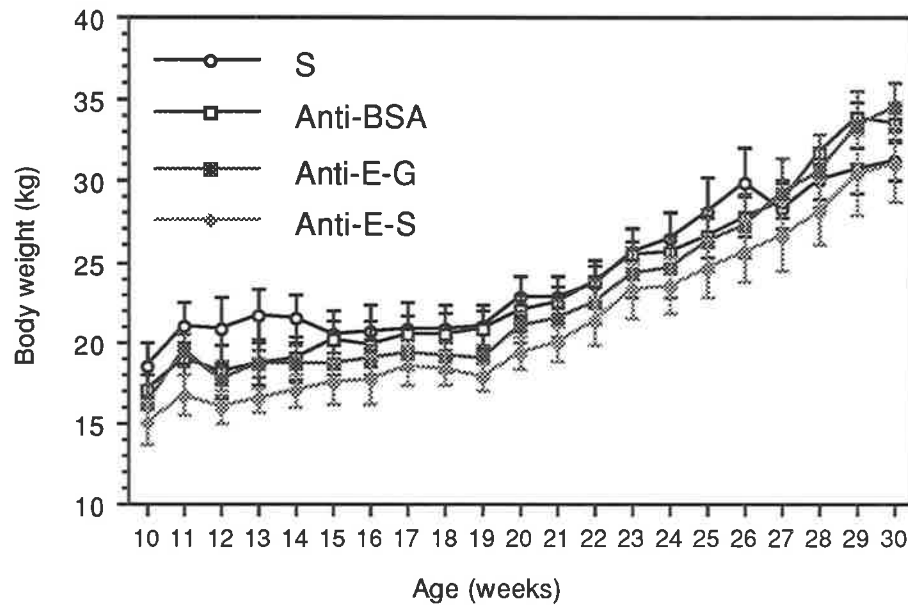
Mean circulating testosterone concentrations at different ages for the four groups of lambs are shown in fig. 6.3.4. Until 18 weeks of age, testosterone concentrations remained low. In all groups, testosterone levels started rising at 22 weeks of age and increased significantly thereafter ($p < 0.01$). Testosterone level was significantly more elevated ($p < 0.05$) in the S lambs at the beginning of the experiment and was significantly lower ($p < 0.05$) in the Anti-E-G lambs at 18 weeks of age. Overall mean testosterone levels were not affected by the immunization treatment.

6.3.2.6. LH concentration

Changes in mean LH concentrations are shown in fig. 6.3.5. At 10 weeks of age, LH level was more elevated in S lambs ($p < 0.05$) however no significant differences between groups were observed after the beginning of the immunization until the end of the experiment. LH concentrations increased slightly but significantly between 22 and 30 weeks of age in all four groups of lambs ($p < 0.05$).

6.3.2.7. FSH concentration

Mean FSH concentrations are shown in fig. 6.3.6. Mean FSH levels were significantly more elevated at 10 weeks of age than at any other age in all groups of lambs ($p < 0.05$). No significant differences in FSH concentration were found between the four groups of lambs at all ages.



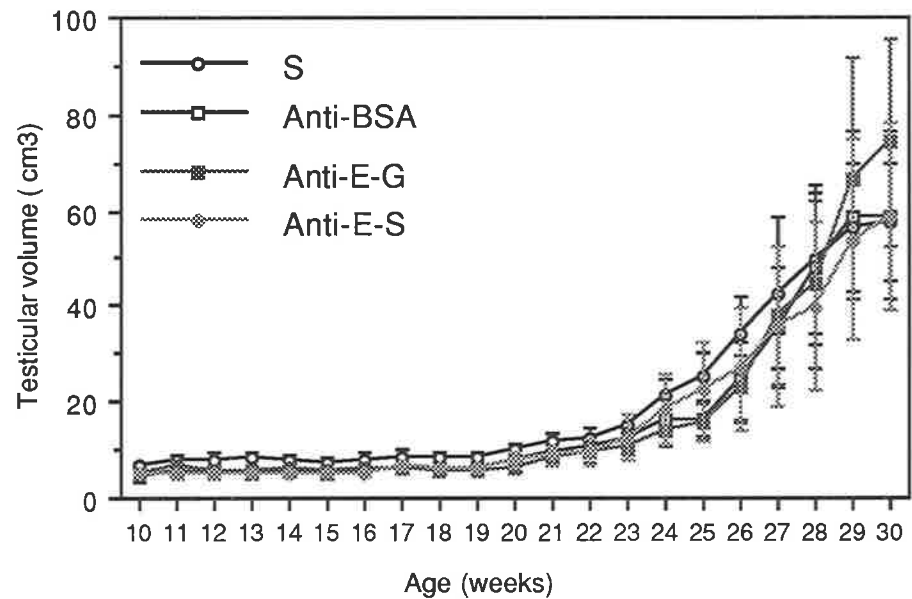
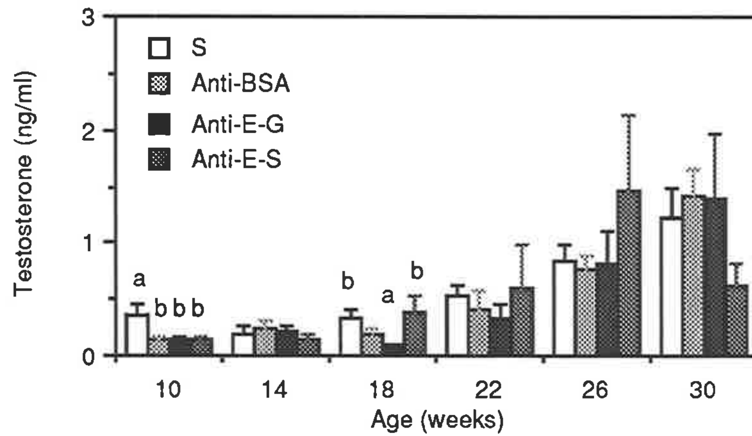
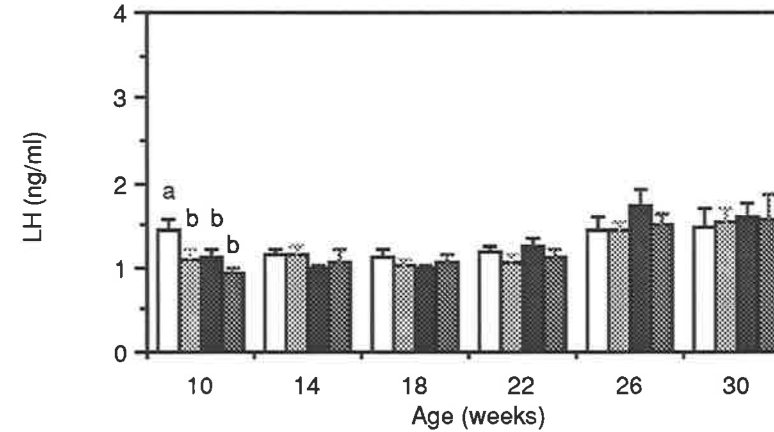
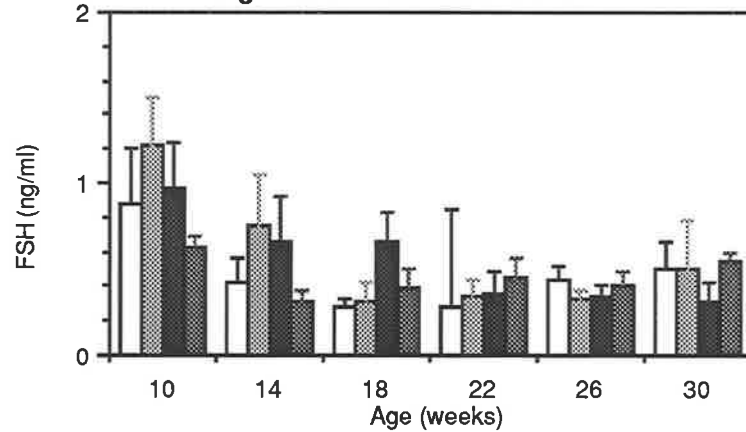


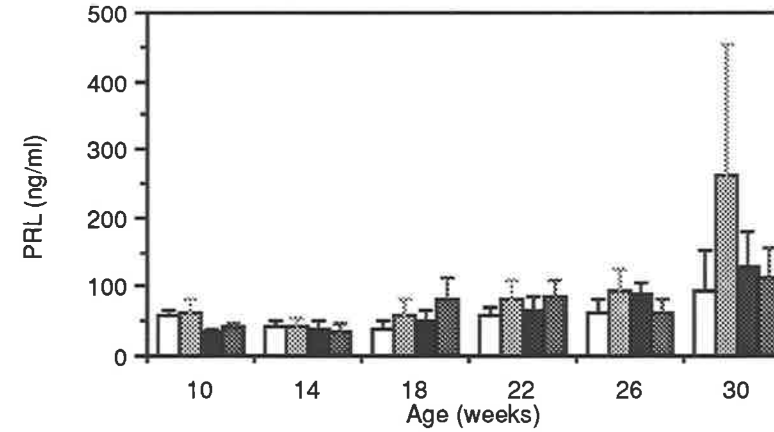
Fig. 6.3.2.and 6.3.3.: Changes in mean body weight (kg ± sem) and mean testicular volume (cm^3 ± sem) from 10 to 30 weeks of age in ram lambs treated with saline (S), purified IgG against BSA (Anti-BSA), purified IgG against E_2-3-HSA (Anti-E-G) or complete antiserum directed against E_2-3-HSA (Anti-E-S).

(S: n=6, Anti-BSA: n=4, Anti-E-G: n=5 and Anti-E-S: n=4).









values with different superscripts are significantly different at 95%

Fig. 6.3.4, 6.3.5, 6.3.6 and 6.3.7 : Mean testosterone, LH, FSH and PRL concentrations (ng/ml ± sem) in plasma collected at 10, 14, 18, 22, 26 and 30 weeks of age from ram lambs treated with saline (S), purified IgG against BSA (Anti-BSA), purified IgG against E_2-3-HSA (Anti-E-G) or complete antiserum directed against E_2-3-HSA (Anti-E-S). (S: n=6, Anti-BSA: n=4, Anti-E-G: n=5 and Anti-E-S: n=4).

6.3.2.8. PRL concentration

Mean PRL concentrations are shown in fig. 6.3.7. PRL concentrations tended to increase with age in all groups of lambs but this was significant only for the Anti-E-G lambs ($p < 0.05$). A large variation in PRL concentrations, including very high values, was observed in the Anti-BSA lambs at 30 weeks of age. The immunization treatment did not affect the level of prolactin at any time during the whole experiment.

6.3.2.9. LH concentration after a GnRH challenge

The pituitary responsiveness to a GnRH challenge at 30 weeks of age is shown in fig. 6.3.8. The maximal increase in LH was observed ten minutes after the GnRH injection in all lambs. LH concentrations associated with the response curve was not affected by the immunization treatment.

6.3.2.10. Testosterone concentration after a hCG challenge

The testosterone response to a single dose of hCG is summarized in fig. 6.3.9. For the analysis of these data, two S lambs and two Anti-BSA were simply considered as "Controls" (n= 4) while two Anti-E-G and three Anti-E-S lambs were considered as a single group called "E_2-immunized" lambs (n= 5).

Before the hCG injection, no significant difference between control and E_2-immunized lambs were found in the testosterone concentration measured in the jugular (JUG), the right and left internal spermatic vein (L and R). Testosterone levels were significantly increased in all pool samples after the hCG challenge ($p < 0.10$ for the JUG samples of the control lambs and $p < 0.05$ for all the other samples). Testosterone concentrations in JUG, L and R pool samples were comparable following the hCG injection in the control and E_2-immunized lambs.

6.3.2.11. Testicular weight and volume at castration

Testicular volume and weight at castration are shown in table 6.3.1. Immunization treatment did not affect significantly testicular weight or volume at castration. The correlation coefficient between calculated testicular volume and testicular weight at castration was r: 0.987 ($p < 0.01$).

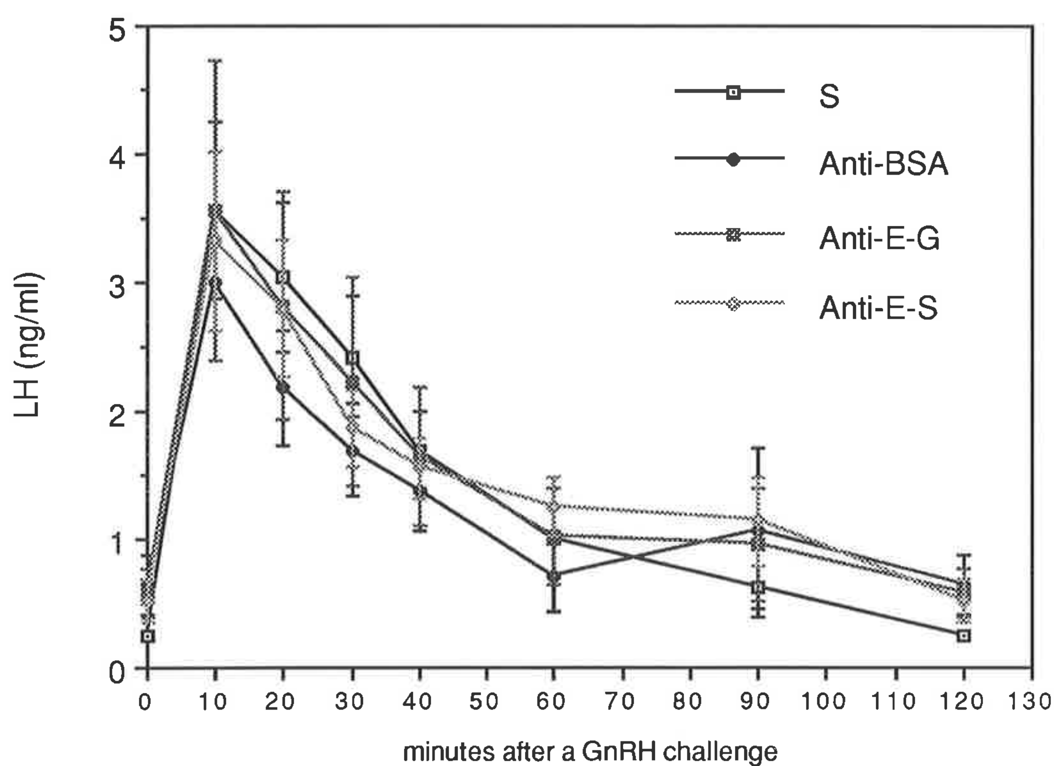


Fig. 6.3.8.: Time course of circulating LH (mean: ng/ml ± sem) after a single i.v. injection of GnRH (5 ng/kg BW) at 30 weeks of age in ram lambs treated with saline (S), purified IgG against BSA (Anti-BSA), purified IgG against E_2-3-HSA (Anti-E-G) or complete antiserum directed against E_2-3-HSA (Anti-E-S).
(S: n=6, Anti-BSA: n=4, Anti-E-G: n=5 and Anti-E-S: n=4).

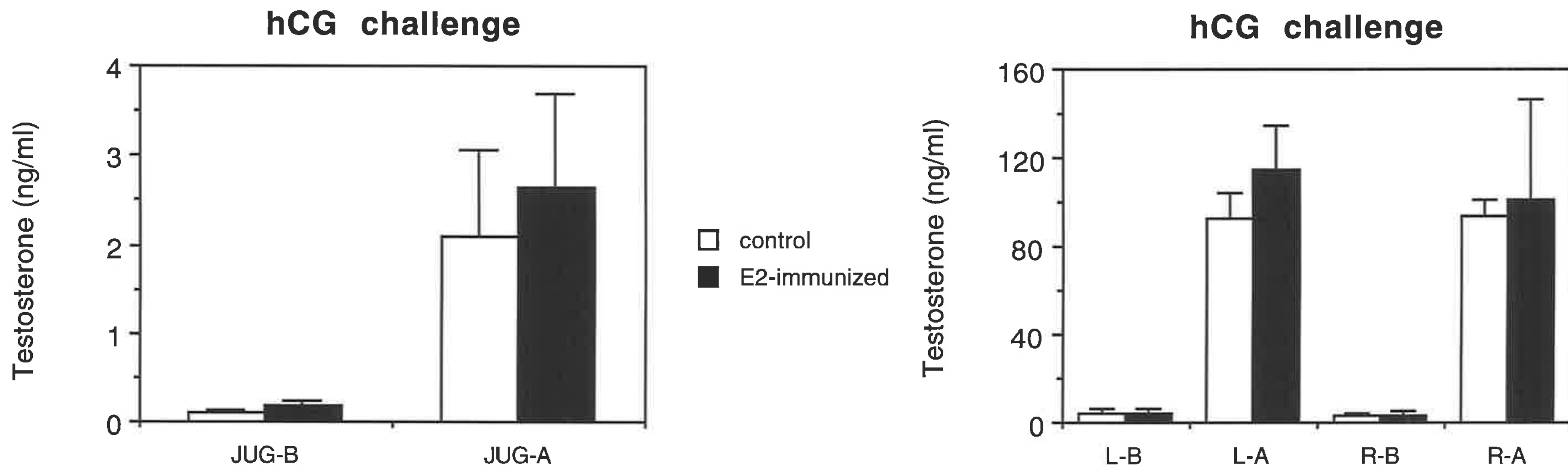


Fig. 6.3.9.: Mean testosterone concentration (ng/ml ± sem) measured in the jugular (JUG) and in the left (L) and right (R) internal spermatic vein before (B) and after (A) a single i.v. injection of hCG (20 I.U./kg BW) to 4 controls (2 "S" and 2 "Anti-BSA") and 5 E_2-immunized lambs (2 "Anti-E-G" and 3 "Anti-E-S"). Blood samples were drawn at 10-min. intervals for 40 minutes before the hCG injection and for 100 minutes after the hCG injection and "pre hCG" and "post hCG" pools were made before assessment.

Table 6.3.1.

Testicular weight and volume at castration in lambs treated with saline (S), purified IgG against BSA (Anti-BSA), purified IgG against oestradiol-17ß (Anti-E-G) or complete oestradiol antiserum (Anti-E-S).

Treatment	Testicular Weight[a] (g ± s.e.m.)	Testicular Volume[b] (cm^3 ± s.e.m.)
S (n=6)	47.75 ± 8.13	51.37 ± 8.48
Anti-BSA (n=4)	58.50 ± 17.90	63.87 ± 19.11
Anti-E-G (n=5)	49.76 ± 16.19	53.80 ± 17.26
Anti-E-S (n=4)	58.55 ± 22.03	70.56 ± 22.97

[a] : The testis and the epididymis were separated from one another immediately after castration and weighed separately.

[b] : Testicular diameter and length were measured with calipers immediately after castration and the volume was estimated by assuming that the testis is a prolate spheroid (Setchell and Waites, 1964). It was calculated according to the formula $1/6\ \pi\ a^2b$ (a=largest width; b=length, of the testis).

6.3.2.12. Spermatogenesis

Testicular tissue from all lambs was examined under light microscopy. Three control lambs (two S and one Anti-BSA) out of ten and three E_2-immunized lambs (two Anti-E-G and one Anti-E-S) out of nine had achieved puberty at the end of the experiment as determined by the presence of spermatozoa in the lumen of their seminiferous tubules. In all the other lambs, spermatogenesis had already started as observed by the presence of few meiotic figures within the seminiferous tubules, however, no elongated spermatids or spermatozoa were yet apparent (fig. 6.3.10.). No abnormalities in the appearance of the seminiferous tubules or the interstitial tissues were noted in lambs of all groups.

The largest "infertile" testis weighed 64.48 g and was found within the S group. The smallest testis in which we have found spermatozoa weighed 61.02 g and was also found within the S group. 'Infertile testes" were those where no spermatozoa were observed in the lumina of the seminiferous tubules.

6.3.3. DISCUSSION

Although we have maintained a significant titre (~1:100) during the whole study whether purified IgG or a complete antiserum against oestradiol were used, the presence of passively transferred antibodies in circulation did not influence any of the parameters measured in these ram lambs. It is possible that the amount of antibodies injected was not sufficient to completely neutralize the circulating oestradiol.

Therefore, our results do not agree with those reported by Land et al. (1981) who have shown that a comparable titre was effective and could lead to a significant increase in the rate of growth of the testis in E_2-immunized Merino lambs. Similarly, our results do not support those of Jenkins et al. (1986) who have found a significant increase in testosterone levels at 12 and 16 weeks of age in younger crossbred ram lambs passively immunized against E_2.

It seems unlikely that a low affinity of the antibodies used were responsible for the lack of effect observed in the present experiment since, in an *in vitro* situation (radioimmunoassay procedure), we have demonstrated that the purified IgG directed against oestradiol could bind to the homologous hormone in the same order ($8.9 \times 10^{+9}$ M^{-1}) of similar effective antibodies

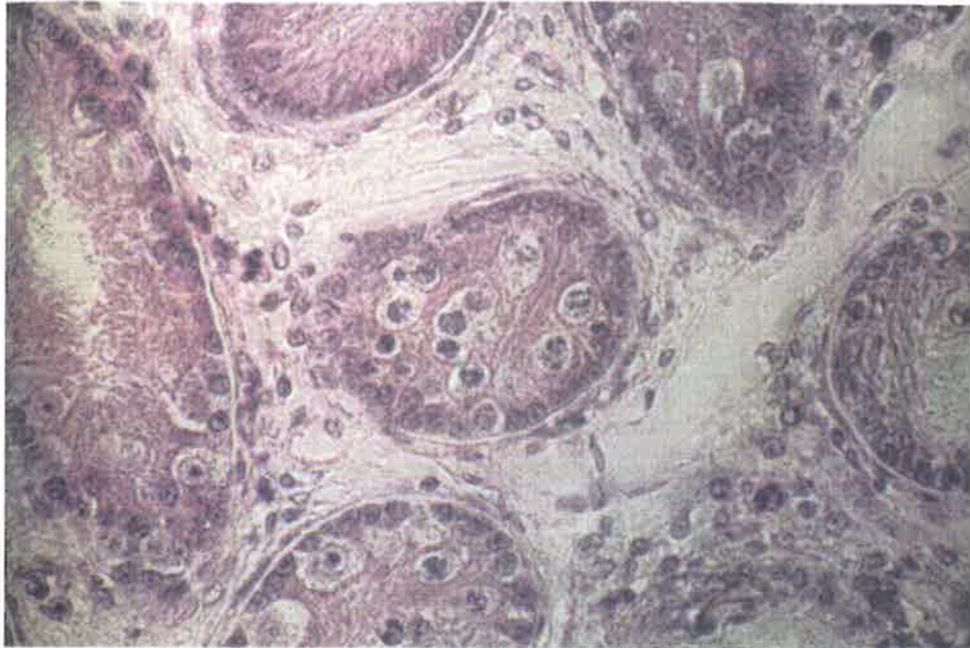


Fig. 6.3.10.: Normal appearance of the seminiferous tubules of a lamb who had not achieved puberty yet. Note the presence of few meiotic figures within the seminiferous tubules but the absence of elongated spermatids or spermatozoa. Haematoxylin and eosin staining (x 50).

previously used by other authors (Nieschlag and Wickings, 1978; Zeleznik et al., 1985). Nevertheless, this possibility will be considered further in the general discussion presented at the end of this chapter (section 6.5.).

Thus, since the *in vivo* effectiveness of the oestradiol-antisera or of the purified IgG directed against oestradiol has not been demonstrated in this experiment, we cannot conclude that the presence of oestradiol is not required for a normal pubertal development in the Merino ram lambs.

Our observations confirmed that successive injections of purified IgG preparation do not alter the health of the recipients and therefore, that the use of IgG isolated by caprylic acid precipitation is safe. Indeed, caprylic acid is a liquid material that is relatively innocuous considering that it has been safely used for many years as a stabilizer of intravenously injected albumin preparations (Steinbuch and Audran, 1969). Other authors, using the passive immunization approach, have reported using purified immunoglobulins obtained with the aid of ammonium sulphate precipitation method (Zeleznik et al., 1985; Dubey et al., 1987; Roberts and Reeves, 1988), however, the major advantage of the caprylic acid precipitation method is represented by the markedly reduced formation of aggregates, as compared with the ammonium sulphate precipitation method (Russo et al., 1983). This last point is very important to consider for a passive immunization study, since the presence of aggregates may favour the formation of anti-sheep IgG immunoglobulins (e.g. anti-idiotype antibodies) in the recipients, which, thereafter, may interfere with the binding capacity of the antibodies administered. In the present study, we had no reason to suspect the presence of such anti-sheep IgG immunoglobulins (see general discussion for chapter 6., section 6.5.).

An important observation of this experiment is that these lambs did not grow at an adequate rate at the beginning of the experiment and that their development was considerably retarded compared with other lambs kept in the same environmental conditions (see chapters 3 and 5). In fact, only a few lambs (six out of nineteen) had achieved puberty at the end of the experiment. One explanation for this difference is that these lambs were born in autumn, at the end of the dry season (in South Australia) and were kept outdoors with their mothers at a time when the quality

The poor growth rate exhibited during the first ten weeks of study by the lambs studied in section 6.3. has been mentioned in the discussion. The maturation of the reproductive system was certainly retarded in those lambs compared with the lambs presented in chapters 3 and 5 and in section 6.4. This "slower" maturation rate could explain some of the endocrinological differences that were observed (e.g. low percentage of lambs that had reached puberty at 30 weeks of age):

- plasma testosterone concentrations measured in lambs of section 6.3 remained low (< 1.5 ng/ml; from 14 to 30 weeks of age) compared with the lambs of chapter 3.(see fig. 3. 1: < 2 ng/ml up to 26 weeks of age, and ~4 ng/ml at 30 weeks of age)
- plasma LH concentrations remained stable or increased slightly in lambs of section 6.3 (~1 ng/ml), however, plasma LH concentrations decreased in lambs of chapter 3 (see fig. 3.2)
- plasma FSH concentrations were slightly elevated at 10 and 14 weeks of age in lambs of section 6.3, however, plasma FSH concentrations were slightly elevated only in the 26 week old lambs of chapter 3 (see fig. 3.3). Nevertheless, in all cases, FSH values were always below 1 ng/ml.

Finally, many endocrinological similarities, between the lambs described in section 6.3 and those presented in other chapters, were also found and were discussed. Interestingly, various variables measured in the lambs of section 6.3 (at 30 weeks of age) are comparable to those measured in the 26 week old lambs of chapter 3 (e.g. testis weight-table 3.1; GnRH challenge-fig. 3.5; hCG challenge-fig. 3.6).

of the pasture was particularly poor. Since we have weaned these lambs at an earlier age, some of them were relatively immature (skinny) on their arrival in the room with controlled light and took a very long time to adjust to the new environmental and nutritional conditions. Therefore, the pattern of hormonal secretion observed in these lambs did differ slightly from that observed in our previous experiments (chapter 3 and 5). For instance, mean LH concentrations did not tend to decrease with age but rather tended to increase. Mean FSH levels did not vary much between between 14 and 30 weeks of age but was more elevated at 10 weeks of age in all lambs. However, as expected, mean testosterone concentrations slowly increased with age and followed testicular development. It seems that these autumn born lambs have interpreted the 12L:12D light cycle as short days similarly to the autumn born lambs studied in chapter 3. In fact, PRL levels were relatively low between 10 and 26 weeks of age and tended to rise slightly at 30 weeks of age. Whether this late rise corresponds to the elevation in PRL observed at an earlier age in other ram lambs which have grown more rapidly (Ravault and Courot; 1975; Klindt et al., 1985; see also chapter 3) can only be speculated. (see facing page)

6.4. PASSIVE IMMUNIZATION AGAINST OESTRONE AND PUBERTAL DEVELOPMENT IN RAM LAMBS.

6.4.1. EXPERIMENTAL PROCEDURE

6.4.1.1. Experimental animals

Nineteen South Australian Merino ram lambs born between 2nd and 11th October 1988 were used in this experiment. The lambs were kept outdoors with their mothers until weaning at 12 weeks of age. The lambs were then transferred to a room with controlled light (12 hours light: 12 hours dark).

6.4.1.2. Pen restriction

Lambs were grouped in each pen according to their body weight at their arrival in the controlled environment (section 2.2.1). Each pen received at least two control lambs and two E_1-immunized lambs.

6.4.1.3. Treatment

Nine control lambs received preparations of purified IgG directed against BSA diluted in saline (control, n=9). Ten lambs received preparations of purified IgG directed against oestrone (X4) diluted in saline (E_1-immunized, n=10). The immunization protocol consisted of fortnightly i.v. injections of the appropriate treatment, starting at 14 weeks of age, ending at 24 weeks of age. To obtain a titre approximating 1:100 during the whole study, the following doses were injected to each lamb:

<u>weeks: 14, 16 and 18</u>

Control: 0.332 g of purified IgG against BSA diluted in 5 ml of saline

E_1-immunized: 0.332 g of purified IgG against E_1 diluted in 5 ml of saline

Thereafter, the amount of purified IgG injected was increased as body weight was increasing considerably:

<u>weeks: 20, 22 and 24</u>

Control: 0.398 g of purified IgG against BSA diluted in 6 ml of saline

E_1-immunized: 0.398 g of purified IgG against E_1 diluted in 6 ml of saline

6.4.1.4. Body weight and testicular volume measurement

Body weight, testicular diameter and length were recorded weekly as described in sections 2.4 and 2.5.

6.4.1.5. Intensive bleeding

Before the beginning of the immunization, at 14 weeks of age, the lambs were intensively bled (every 20 min. for 6 hours) as described in section 2.6.1. Intensive bleedings were repeated at 18, 22 and 26 weeks of age.

6.4.1.6. Pituitary responsiveness to GnRH.

Pituitary responsiveness to a GnRH challenge was tested at the end of the intensive bleeding at 26 weeks of age as described in section 2.6.2.

6.4.1.7. Testicular responsiveness to hCG.

Testicular responsiveness to a single hCG injection was evaluated at 26 weeks of age in anaesthetized lambs as described in section 2.6.3.1.

6.4.1.8. Titre check

see section 6.3.1.8.

6.4.1.9. Hormone assays

see section 6.3.1.9.

6.4.1.10. Castration

All ram lambs were castrated at 26 weeks of age as described in section 2.7.4.

6.4.1.11. Testicular histology

Blocks of testicular tissue taken at castration were prepared as described in section 2.9.

6.4.1.12. Statistical analysis.

Statistical analyses were done as described in section 6.3.1.12. except that all pairwise comparisons were made using student-*t* -test. A logarithmic transformation [log (x+1)] was applied to some data (in this experiment: mean FSH, and testosterone values after a hCG challenge) to equalize variances. The data presented in tables and graphs are the actual ones.

6.4.2. RESULTS

6.4.2.1. Health of animals

During the whole experiment, all lambs remained healthy.

6.4.2.2. Titre check

The percentage binding of oestrone remained negligible (less than 4 %) during the whole experiment in the controls. In E_1-immunized lambs, plasma collected immediately after an antibody injection (highest concentration of antibodies in circulation) could bind between 65 and 80 % of radiolabelled oestrone (at a dilution of 1:100) (fig. 6.4.1.). The percentage binding had decreased within 2 weeks after an immunization and had reached values ranging between 24 and 36 % at the time preceding a new injection and at the end of the experiment (at 26 weeks of age).

6.4.2.3. Body weight

Liveweight patterns for control and E_1-immunized lambs are shown in fig. 6.4.2. Body weight increased continuously during the 12 weeks of study. Immunization treatment did not affect body weight at any time. Both groups had gained approximately 12 kg in 12 weeks (Control: 12.41 ± 1.40 kg and E_1-immunized: 12.09 ± 1.34 kg).

6.4.2.4. Testicular volume

Changes in testicular volume in controls and E_1-immunized lambs are shown in fig. 6.4.3. Testicular volume increased slowly up to 22 weeks of age, thereafter a more pronounced increase in testicular volume occurred and continued until 25 weeks of age. The pattern of testicular development was similar in both groups.

6.4.2.5. Testosterone concentration

Mean circulating testosterone concentrations at different ages for both groups are shown in fig. 6.4.4. In both groups, testosterone level had increased significantly with age ($p < 0.01$). Immunization treatment did not affect testosterone level at any time.

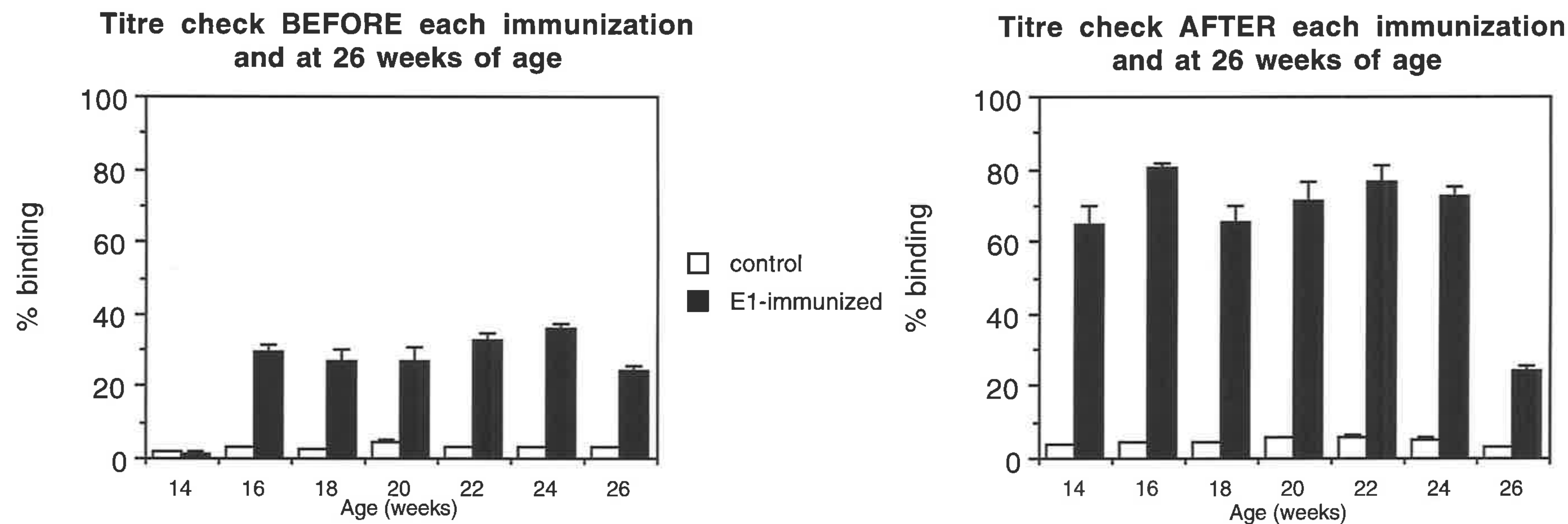
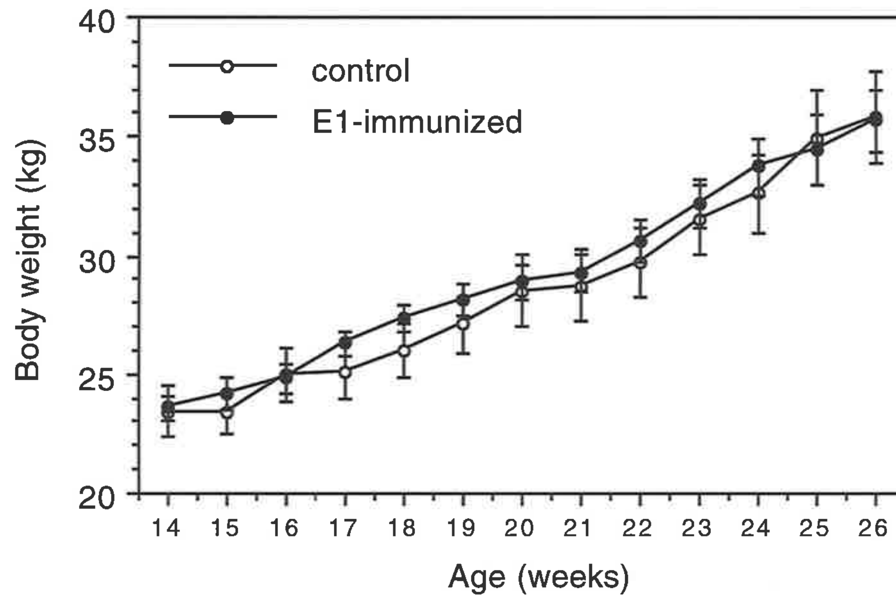


Fig. 6.4.1.: Mean percentage binding (% ± sem) of ^{3}H-oestrone in diluted plasma (1:100) collected before (left fig.) and after (right fig.) each immunization (at 14, 16, 18, 20, 22 and 24 weeks of age), and at the end of the experiment (at 26 weeks of age) from ram lambs receiving purified IgG directed against BSA (control, n=9) or purified IgG directed against E_1-3-BSA (E_1-immunized, n=10).



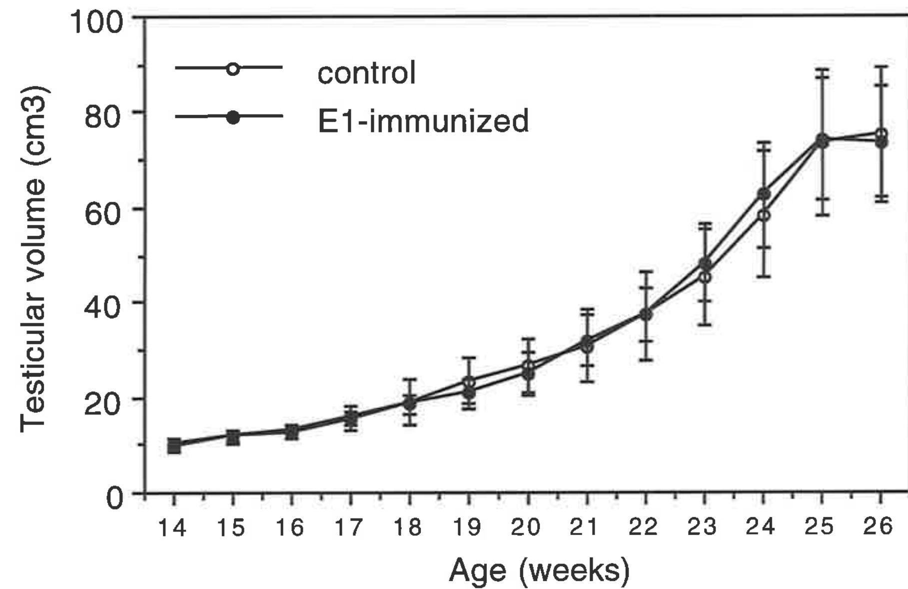


Fig. 6.4.2.and 6.4.3.: Changes in mean body weight (kg ± sem) and mean testicular volume (cm^3 ± sem) from 14 to 26 weeks of age in ram lambs treated with purified IgG directed against BSA (control, n=9) or purified IgG directed against E_1-3-BSA (E_1-immunized, n=10).

6.4.2.6. LH concentration

Changes in mean LH concentration are shown in fig. 6.4.5. No significant difference between groups was found during the whole experiment. The level of LH did not vary with age in either groups of lambs.

6.4.2.7. FSH concentration

Mean FSH concentrations are shown in fig. 6.4.6. FSH had decreased slightly but significantly with age in control ($p < 0.01$) and in E_1-immunized lambs ($p < 0.05$). No significant difference in mean FSH concentration was found between the 2 groups at any time.

6.4.2.8. PRL concentration

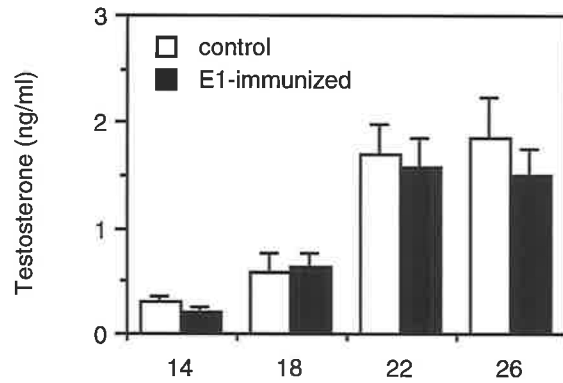
Mean PRL concentrations are shown in fig. 6.4.7. PRL level did not vary with age in controls but was significantly lower at 26 weeks of age than at 14 and 18 weeks of age in E_1-immunized lambs ($p < 0.05$). At 18 weeks of age, a higher concentration of PRL was found in E_1-immunized lambs ($p < 0.01$). PRL levels were comparable between the 2 groups at 14, 22 and 26 weeks of age.

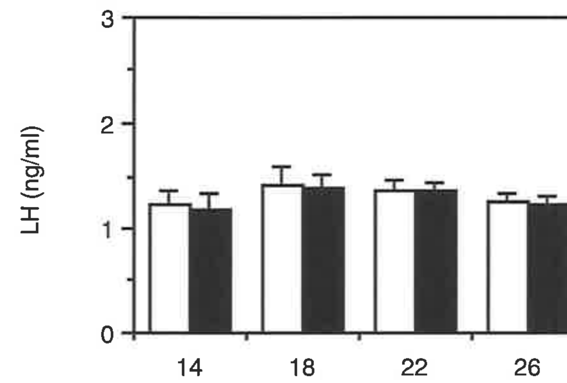
6.4.2.9. LH concentration after a GnRH challenge

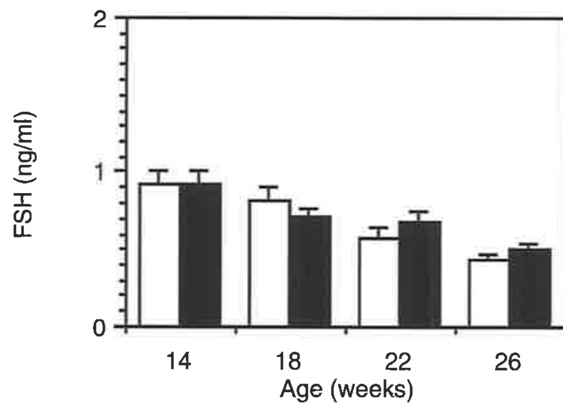
The pituitary responsiveness to a GnRH challenge at 26 weeks of age in controls and E_1-immunized lambs is shown in fig. 6.4.8. The maximal increase in LH was observed ten minutes after the GnRH injection in all lambs. LH concentrations associated with the response curve were not affected by the immunization treatment.

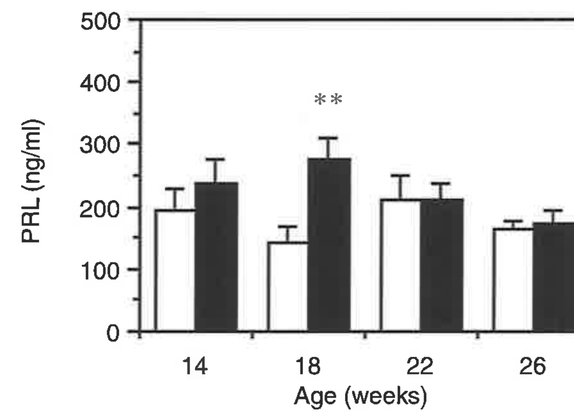
6.4.2.10. Testosterone concentration after a hCG challenge

The testosterone response to a single dose of hCG is summarized in fig 6.4.9. Before the hCG injection, no significant difference between controls and E_1-immunized lambs were found in the testosterone concentration measured in the jugular (JUG), the right and left internal spermatic vein (L and R). Testosterone levels were significantly increased in all pool samples after the hCG challenge ($p < 0.05$ for the L samples of the control lambs and $p < 0.01$ for all the other samples). Testosterone concentrations in JUG, L and R pool samples were comparable following the hCG injection in control and E_1-immunized lambs.









**: $p < 0.01$, significantly different from controls

Fig. 6.4.4, 6.4.5, 6.4.6 and 6.4.7 : Mean testosterone, LH, FSH and PRL concentrations (ng/ml ± sem) in plasma collected at 14, 18, 22 and 26 weeks of age from ram lambs treated with purified IgG directed against BSA (control, n=9) or purified IgG directed against E_1-3-BSA (E_1-immunized, n=10).

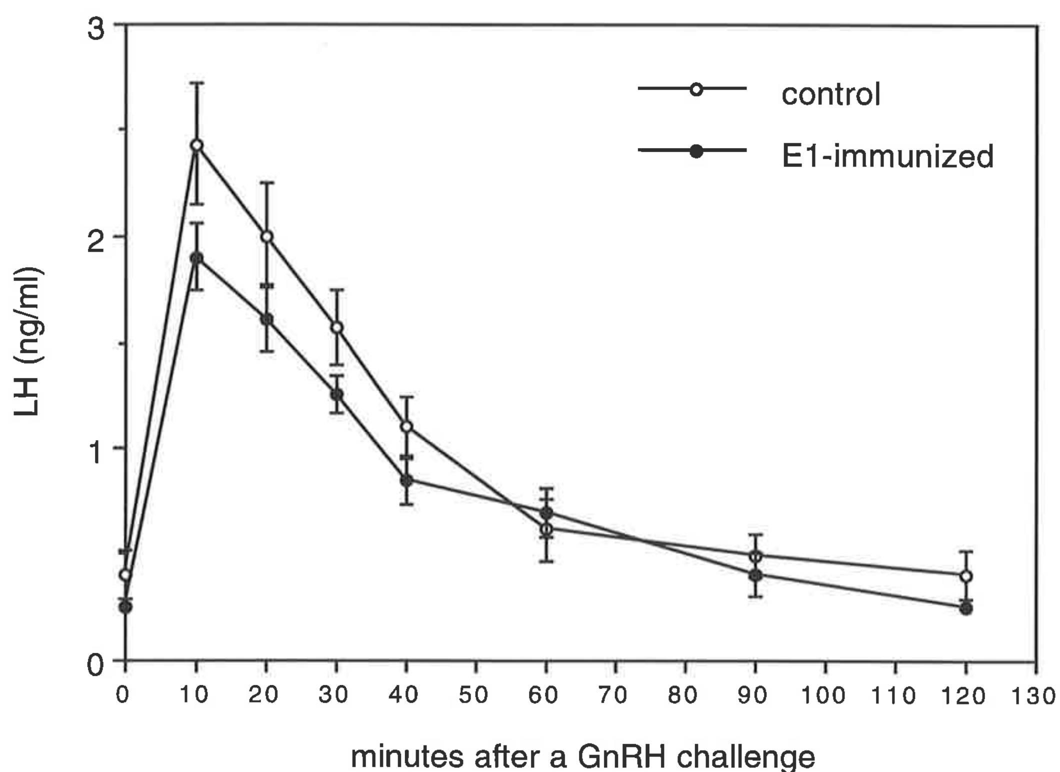


Fig. 6.4.8.: Time course of circulating LH (mean: ng/ml ± sem) after a single i.v. injection of GnRH (5 ng/kg BW) at 26 weeks of age in ram lambs treated with purified IgG directed against BSA (control, n=9) or purified IgG directed against E_1-3-BSA (E_1-immunized, n=10).

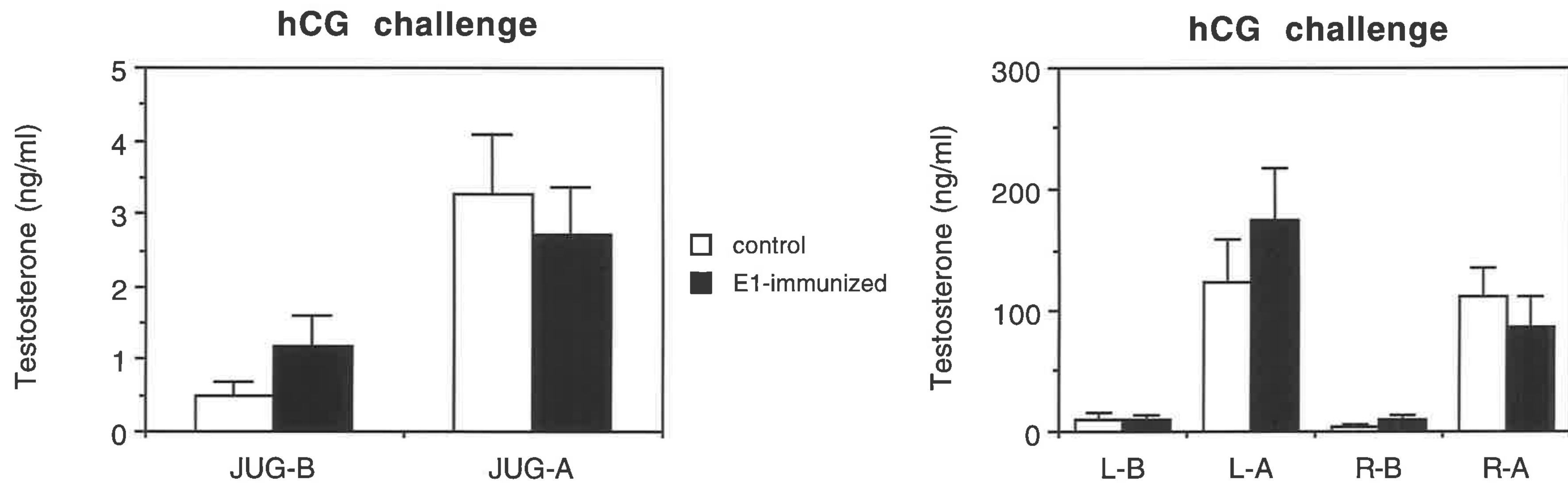


Fig. 6.4.9.: Mean testosterone concentration (ng/ml ± sem) measured in the jugular (JUG) and in the left (L) and right (R) internal spermatic vein before (B) and after (A) a single i.v. injection of hCG (20 I.U./kg BW) to ram lambs treated with purified IgG directed against BSA (control, n=9) or purified IgG directed against E_1-3-BSA (E_1-immunized, n=10). Blood samples were drawn at 10-min. intervals for 40 minutes before the hCG injection and for 100 minutes after the hCG injection and "pre hCG" and "post hCG" pools were made before assessment.

6.4.2.11. Testicular weight and volume at castration

Testicular volume and weight at castration are summarized in table 6.4.1. Immunization treatment did not affect significantly testicular weight or volume at castration. The correlation coefficient between calculated testicular volume and testicular weight at castration is r: 0.983 ($p < 0.01$).

6.4.2.12. Spermatogenesis

Testicular tissue from all lambs was examined under light microscopy. Eight control lambs out of nine lambs and eight E_1-immunized lambs out of ten had achieved puberty at the end of the experiment as determined by the presence of spermatozoa in the lumen of the seminiferous tubules examined. No abnormalities in the appearance of seminiferous tubules or the interstitial tissues were observed in all lambs.

The largest "infertile" testis weighed 31.29 g and was found within the E_1-immunized group. The smallest testis in which we have found spermatozoa weighed 35.55 g and was found among the control lambs.

6.4.3. DISCUSSION

The presence of the purified IgG directed against oestrone in circulation did not influence any of the parameters measured in the ram lambs and therefore, as for the preceding passive immunization study (see section 6.3.), the *in vivo* effectiveness of these antibodies could not be demonstrated. However, it is clear that the IgG used in the present study were able to bind effectively radiolabelled oestrone within an *in vitro* radioimmunoassay as determined by the regular titre check done. In fact, the titre reached after each injection of purified IgG directed against oestrone (> 1:100) was slightly higher than the titre obtained with the passive transfer of anti-oestradiol antibodies (see section 6.3.; ~1:100), however, the percentage binding had decreased considerably within two weeks and was finally lower (< 1:100) than that obtained before each new injection in our previous study. Therefore, it is possible that the amount of antibodies present in circulation was not sufficient to stop all oestrone from reaching its receptors, assuming that the binding affinity of the IgG were higher than the binding affinity of

Table 6.4.1.

Testicular weight and volume at castration in lambs treated with purified IgG directed against BSA (control) or purified IgG directed against E_1-3-BSA (E_1-immunised).

Treatment	Testicular Weight[a] (g ± s.e.m.)	Testicular Volume[b] (cm^3 ± s.e.m.)
control (n=9)	68.52 ± 12.64	72.07 ± 15.38
E_1-immunised (n=10)	62.69 ± 7.82	68.83 ± 9.67

[a] : The testis and the epididymis were separated from one another immediately after castration and weighed separately.

[b] : Testicular diameter and length were measured with calipers immediately after castration and the volume was estimated by assuming that the testis is a prolate spheroid (Setchell and Waites, 1964). It was calculated according to the formula $1/6\ \pi\ a^2b$ (a= largest width; b=length, of the testis).

the oestrone receptors within the target tissues of the ram lambs (see general discussion for chapter 6, section 6.5.). Thus, here again, we cannot conclude that the participation of oestrone is not required for normal pubertal maturation in the Merino ram lambs. Furthermore, we cannot support the observation made by Land et al (1981) indicating that the concentration of FSH was correlated with the oestrone antibody titre in passively immunized Merino ram lambs. Our results differ from those of other investigators who have reported some significant effects following immunization against oestrone (in prepubertal stallions: Thompson and Honey (1984); in boars: Wise et al., 1991: in laboratory species: Wuttke et al., 1975).

The present lambs were born in spring and were kept outdoors with their mothers at a time of the year when the quality of the pasture was excellent. At weaning time (12 weeks of age), these lambs were more mature than the lambs studied in the preceding experiment (see section 6.3.). Body weight gain was adequate and continuous, allowing most of the lambs (84%) to achieve puberty by 26 weeks of age. The increase in testicular volume started slowly then progressed more rapidly until 25 weeks of age. Unfortunately, since we ended this experiment at 26 weeks of age, we do not know whether, the testicular volume would have continued to increase, reach a plateau, or decline, thereafter (as for the lambs studied in chapter 5).

Since, the present lambs had only experienced increasing day length (from 0 to 12 weeks of age) and then constant day length, they have probably interpreted the 12L:12D light cycle as long days which have been shown not to be very stimulatory for testicular growth (Alberio, 1976; Alberio and Colas, 1976; Colas et al. 1987; see chapter 5). Therefore, a decline in testicular volume occurring later on during pubertal development, similar to that observed in the lambs studied in chapter 5 could have been observed. In fact, although the PRL concentrations were not as elevated as for the winter born lambs presented in chapter 5, the PRL values were more elevated than those observed in the autumn born lambs (chapter 3 and section 6.3). PRL levels tended to be significantly more elevated at 18 weeks of age only in the E_1-immunized lambs.

The other hormonal changes that occurred during this 12-week study period differ only slightly or were consistent with other observations reported for developing lambs of those ages (see

chapters 3 and 5, and section 6.3.). For instance, mean LH concentration did not vary with age, mean FSH level decreased slightly with age and mean T concentration increased with age.

6.5. GENERAL DISCUSSION FOR CHAPTER 6

Since we did not observe any significant effects following either passive immunization treatments while important hormonal changes occurred following active immunization against oestradiol-17ß, we concluded that the passive transfer of oestrogen antibodies (fortnightly injections maintaining a titre at about 1:100) may not have been sufficient to neutralize this class of hormone in developing ram lambs. Indeed, the two experiments presented in this chapter have pointed out the difficulties encountered in determining precisely the effectiveness of the antibodies *in vivo*, although the antibodies used have been shown to possess excellent characteristics *in vitro* (high specificity and high affinity). Thus, some possible explanations for this lack of effect could depend on the *in vivo* characteristics of the antibodies administered. For instance, not enough binding sites in circulation (low titre), or antibodies with lower affinity than that of the oestrogen receptors within target cells, or antibodies cross-reacting strongly with circulating androgens (low specificity for oestrogens), would explain why the immunoneutralization of oestrogens was not successful.

With regard to the first possibility, an essential condition that needs to be fulfilled in such passive immunization study is that a sufficient quantity of anti-steroid antibodies must be injected into the animal to obtain enough sites capable of binding the total mass of the homologous steroid in circulation. We believed that, in both experiments, there was an excess in unoccupied antibody binding sites in the system throughout the whole study since a significant titre (~1:100) was measured before and after each injection of antibodies (e.g. some plasma samples diluted at 1:100 were able to bind 50 % of a total of ~20 pg of added oestrogenic tracer in a total assay volume of 300 µl, which represents about eight times the plasma concentration of oestrogens found in ram lambs). Thus, the oestrogen binding capacity was unlikely to be saturated since the antibodies were still able to bind *in vitro* approximately eight times the plasma oestrogen concentration found *in vivo* (even after an important dilution - e.g.

Various methodology problems that can occur with passive immunization have been mentioned in chapter (see section 1.10. 4. ii).

100 X). Furthermore, the titres maintained in our studies and the quality of our antibodies compared well with that considered effective in other passive immunization studies (Land et al., 1981; Jenkins et al., 1986; Sanford, 1985; 1987a, 1989; Sanford et al., 1991). Nevertheless, it is obvious that the complete neutralization of the biological activities of endogenous oestradiol or oestrone may not have been produced despite the fact that a creditable quantity of antibodies were administered in both experiments.

Another possible explanation for the lack of effect of our treatments, could be that an increased secretion rate of oestrogens had occurred in response to the passive immunization. Indeed, since testicular oestrogen secretion appears to be controlled by LH and since oestrogens can regulate LH secretion, interference by antibodies with an element of the negative feedback loop would tend to produce an increase in LH secretion and hence oestrogens. With time, there would be a tendency to re-establish the pre-immunization equilibrium between oestradiol and LH, since the system would eventually saturate the limited amount of antibody binding sites with oestrogens, leaving the excess as free hormones to "feedback" on the pituitary and reduce LH secretion at a much higher level of circulating (free plus bound) oestrogens (Martin, 1984). Although we have not measured the level of oestrogens in our ram lambs, the levels of LH, FSH and testosterone were always similar to the ones measured in the controls, at various occasions during the immunization period. Therefore, it is unlikely that hypersecretion of LH had occurred in our passively immunized lambs. However, since an intensive bleeding was always done 14 days after an injection of antibodies (before a new injection), it remains possible that a difference between the control and the immunized lambs would have been observed immediately after the arrival of new antibodies in circulation (before a new equilibrium is re-established). Nevertheless, as mentioned before, the titres measured before and after a new injection of antibodies, had always indicated that an excess in binding sites was present in each E_2- or E_1-immunized lamb.

It is unlikely that all oestrogens formed within the system would have been neutralized by the circulating antibodies since some androgen aromatization seems to occur in different areas where the antibodies have no access (e.g. beyond the blood-brain barrier and the blood-testis barrier).

L. 5-9: In an actively immunized animals, there are many cells of the immune system (macrophages, T-cells, B-cells, killer cells) that become 'activated' and proceed to eliminate the antigens. These cells can secrete a variety of substances (e.g. lymphokines, cytokines) which can be recognized by specific immune cells but also by other non specific cells (cells that possess homologous receptors) and therefore, cause the appearance of "non-specific" effects that are not necessary for the elimination of the antigens. Some researchers have started to identify what are the various cytokines that can affect the reproductive system and what are their potential actions (see recent review by Ben-Rafael and Orvieto, 1992)

We postulate that in passively immunized animals, the elimination of the antigen after its binding with an antibody does not not require a very drastic response by the immune system, and therefore, that the probability to observe "non-specific" effects (that could affect the reproductive tissues) are considerably reduced in those animals. Whether these "non-specific" effects account for the different responses obtained in passively and actively immunized animals still remain to be demonstrated.

However, since oestrogen formation must also take place in the actively immunized ram lambs which nevertheless exhibited obvious hormonal changes (see chapter 5), we believe that this fact does not explain the different results obtained in passively and actively immunized lambs. Here again, the smaller amount of antibodies circulating (lower titre) in passively immunized animals is more likely to explain the absence of hormonal changes in these lambs. Furthermore, one must keep in mind that in actively immunized ram lambs, the immune response is strongly stimulated by a lot of substances secreted by activated lymphocytes T (lymphokines) and macrophages (cytokines) which might also participate in a more efficient neutralization of the hormone (Roitt et al., 1989).

As mentioned above, in addition to the titre that needs to be consider, the degree to which the antibodies can bind to the homologous hormone and stop it from reaching its receptors will also determine the degree to which the steroid is biologically neutralize. High affinity binding in blood and extracellular fluids would tend to draw steroid out of target tissues, however, if the affinity of the antibody is lower than the affinity of the receptors, the free steroid will still be able to enter the target cells and bind to its receptors. In the first experiment (section 6.3.), although we have found a high apparent affinity constant (K) for our anti-oestradiol antibodies *in vitro* using radioimmunoassay method, this has not been demonstrated *in vitro* in a situation where the oestrogens receptors present in the target cells will be in competition for the hormones, or directly *in vivo*. Therefore, we cannot definitely demonstrate the lack of effect of our treatments was due to the antibodies because of their relatively low *in vivo* affinity. Furthermore, our data did not suggest either that the antibodies were in fact acting as protein carrier, enhancing the oestrogenic potential within the animal, a possibility that has been mentioned by some authors (e.g. Martin, 1984).

The possibility also exists that the binding sites were occupied by the "predominant" circulating androgens *in vivo*, however, extremely low degree of cross-reactivity with these steroids demonstrated *in vitro* does not support this idea. In agreement with this view, it has been shown that, in actively immunized rams, the presence of E_2-antibodies does not affect

L. 4-10: Since oestradiol (as any other steroid) needs to enter a cell to reach its receptor and to affect this cell, it is unlikely that an anti-idiotype that could bind specifically to an anti-oestradiol antibody would affect this cell and mimic the action of oestradiol (assuming that an oestradiol-antibody cannot enter a cell).

L. 11-12: Between May and August 1993, (at the University of Western Australia; in collaboration with G. B. Martin, Animal Science Group), we used two immunological techniques (passive haemagglutination and double immunodiffusion) to detect the presence of anti-IgG antibodies in plasma samples from the lambs studied in section 6.3. Using both techniques, no anti-IgG antibodies were found in the plasma of the lambs that have received either complete antiserum or purified IgG (see Appendix: Chapter 6, section 6.3).

testosterone metabolic clearance rate, indicating that the same amount of unbound T was still available for metabolism and clearance by the intestines and the liver (Schanbacher et al., 1987). Finally, another possible explanation that depends on the immune response of the recipient to the foreign proteins injected into its jugular vein need to be considered. Indeed, the production of anti-sheep antisera (or anti-sheep IgG) immunoglobulins by the passively immunized animal in reaction to the successive arrivals of foreign antibodies, could then lead to an important reduction in the binding capacity of the antibodies (e.g. anti-idiotype antibodies blocking the binding sites on the foreign antibodies) or could accelerate the clearance of the foreign antibodies (e.g. anti-allotype antibodies binding to the foreign antibodies and activating their elimination by the immune system) (Roitt et al., 1989; Madon et al., 1991). See INSERT 4-10

We believe that our lambs have not produced antibodies (see Madon et al., 1991) against the antiserum or the IgG that they have received (see INSERT 11-12). In fact, a lot of attention has been taken in each experiment to minimize the risk of activating the immune system of the recipient by the antibody injections (e.g. large amount of foreign proteins - that have been sterilized after ultracentrifugation at high speed to eliminate aggregates - were injected intravenously fortnightly). Furthermore, since a significant titre was measurable fourteen days after each antibody injection, it shows that the antibody binding sites present in the plasma collected from the immunized lambs have kept their ability to bind the labelled hormone indicating that no "anti-idiotype" antibodies were interfering at least in the *in vitro* assay. Furthermore, no evidence of serum sickness or anaphylactic reactions following the intravenous injections was observed in these experimental ram lambs, which also supports the idea that the type of immunoglobulins usually involved in this sort of sickness (e.g. IgE able to recognize the foreign antigens) were not produced in response to many antibody injections.

Thus, because of the lack of effect of our treatments, the initial hypothesis has not been not verified and will have to be reformulated in another project. However, both studies confirmed that repeated injections of purified IgG obtained with the aid of caprylic acid are not detrimental to the health of the recipients and could eventually become an interesting alternative to the use of complete antiserum in passive immunization studies.

Further work should be done using the same antibodies to see whether increasing doses of antibodies (higher titre) could be effective in neutralizing oestrogens in developing ram lambs. In two short-term experiments presented in the following chapter (chapter 7), we will try to demonstrate the *in vivo* effectiveness of the antibodies used in the experiments presented above.

CHAPTER 7.

OESTRADIOL-17ß IMPLANT STUDIES AND IMMUNIZATION AGAINST OESTRADIOL-17ß IN CASTRATED LAMBS.

7.1. INTRODUCTION

Several observations support the idea that oestrogens may still be biologically active in the castrated rams (Schanbacher et al., 1984). For instance, detectable levels of oestradiol-17ß have been measured in plasma of castrated ram lambs (< 1 pg/ml) (Pope et al.,1990) and of adult wethers (Sanford and Robaire, 1990) and the oestrogen receptors within the hypothalamic-pituitary axis have been shown not to decrease in number following castration (Thieulant and Pelletier, 1979). Furthermore, it has been shown that oestradiol replacement therapy was more efficient than testosterone treatment in reducing gonadotropin secretion in adult castrated rams (Edgerton and Baile, 1977; Schanbacher and Ford 1977; Parrott and Davies, 1979; Schanbacher 1979; D'Occhio et al., 1985). Silastic capsules containing oestradiol-17ß have been often used to maintain serum levels in castrated rams at concentrations similar to that found in intact rams and to suppress gonadotropin secretion (Schanbacher, 1979; 1980a; D'Occhio et al., 1983b; 1985; Sanford and Robaire, 1990). In hemicastrated and castrated ram lambs, oestradiol implantation has also been shown to be very effective in reducing gonadotropins (Jenkins and Waites, 1983; Olster and Foster, 1986; 1988). Finally, Sanford (1987b) has found an increase in LH pulse frequency in castrated rams after prolonged immunization against oestradiol.

In the previous experiments described in chapter 6, we have observed no significant effect following administration of anti-oestrogen antibodies to ram lambs studied during the period of transition into puberty (see chapter. 6). Therefore, in one of the next experiments using the castrated ram lambs implanted with oestradiol-17ß as model, we intended to verify if passively transferred anti-oestradiol antibodies would be more efficient in a short-term study that will not require repeated injection of antibodies. We have also actively immunized some wethers against oestradiol-17ß using a similar experimental protocol for comparison of the efficiency of both immunization approaches.

The hypothesis of these two experiments is that oestradiol-17ß released from a Silastic capsule should be neutralized in castrated ram lambs actively immunized (section 7.2.) or passively immunized against oestradiol-17ß (section 7.3.)and consequently, that the reduction in LH and FSH secretions inflicted by the oestradiol implant should no longer take place.

Another objective of the second experiment (section 7.3.), was to demonstrate the *in vivo* effectiveness of the antibodies used in the experiment reported in the section 6.3.of chapter.6, using an equivalent dose of purified antibodies but a slightly different animal model (wether treated with oestradiol) in which the large majority of the competing steroids have been eliminated by castration.

7.2. OESTRADIOL-17ß IMPLANTATION AND ACTIVE IMMUNIZATION AGAINST OESTRADIOL-17ß IN CASTRATED LAMBS.

7.2.1 EXPERIMENTAL PROCEDURE

7.2.1.1. Experimental animals

Eight South Australian Merino ram lambs born in September 1989 were used in this experiment. The lambs had been castrated using elastrator bands at approximately 2 weeks of age. The castrated lambs (wethers) were kept outdoors until 30 weeks of age. The wethers were then transferred to a room with controlled light (12 hours light: 12 hours dark). At the beginning of the experiment, the average liveweight was 40.39 ± 0.98 kg.

7.2.1.2. Treatment

Four wethers were actively immunized against BSA (n=4) while the other ones (n=4) were actively immunized against 17ß-oestradiol-3(O-carboxymethyl)oxime-human serum albumin (E_2-3-HSA). The primary immunization was done as described in section 2.3.1. at approximately 30 weeks of age (day 1). The booster injection was given 4 weeks later (day 28). Seven days after the booster injection (day 35), two of the four BSA-immunized wethers were implanted with an empty Silastic capsule as described in section.2.7.1 (BSA / 0, n=2) The other two BSA immunized wethers were implanted with a Silastic capsule filled with oestradiol-17ß (BSA / E_2, n=2). Two E_2-immunized wethers received an empty Silastic capsule (E / 0 n=2) and the other E_2-immunized wethers received a Silastic capsule filled with oestradiol-17ß (E / E_2, n=2).

7.2.1.3. Intensive bleeding

One day before the primary immunization (day 0), the wethers were intensively bled (every 10 minutes for 4 hours) as described in section 2.6.1. Intensive bleeding was repeated fourteen days after the booster immunization (seven days after the implantation) (day 42).

7.2.1.4. Titre check

One blood sample from each wether was collected at the end of the two intensive bleedings (day 0 and day 42) and these samples were assayed for titre check,.as described in section 2.8.2.1.

7.2.1.5. Hormone assays

Plasma samples were assayed for LH, FSH as described in sections 2.8.1.2.2. and 2.8.1.2.3., respectively. Mean hormone levels were determined by assaying a plasma pool (25 aliquots/ wether/ pool) representing the 4-hour sampling period (section 2.6.1.2.).

7.2.1.6. Statistical analysis.

Hormone data (mean LH and mean FSH) collected before the beginning of the treatment (control bleeding) were compared with mean LH and mean FSH values obtained from intact Merino ram lambs intensively bled at 30 weeks of age (control lambs used in the experiments presented in chapter 3 [n=4], chapter 5 [n=9] and section 6.3. of chapter 6 [n=8]) using one-factor analysis of variance.

Overall hormonal changes for each intensive bleeding were analyzed by two-factor analysis of variance to detect differences due to immunization, implantation and their interactions. Titres for each period of measurement were analyzed using a one-factor analysis of variance considering the immunization treatment only. All these analyses were done using the CLR ANOVA computer program (Clear Lake Research, Morningside, Houston, TX, 77005). However, because of the very small number of animals per 'immunization / implant' group (n=2 / group), the results obtained for each wether are presented as such in the tables and are discussed individually in the result section (statistical mean comparisons were not considered). Furthermore, to facilitate the interpretation of the data, for each wether, LH and FSH concentrations measured after the initiation of the immunization treatment (post-treatment values) were also considered as percentages of the values obtained during the first bleeding (pre-treatment values) :

$$\frac{\text{post-treatment values}}{\text{pre-treatment values}} \times 100$$

7.2.2. RESULTS

7.2.2.1. Titre check

Titres for each wether are presented in table 7.2.1. As expected, the percentage binding of oestradiol-17ß in diluted plasma (1:100) remained negligible (less than 8 %) in the BSA-immunized wethers. Fourteen days after the booster injection (day 42), oestradiol binding was significantly higher ($p < 0.05$) in the E_2-immunized (dilution 1:1000) than in the BSA-immunized group (dilution 1:100). Indeed, at that time (day 42), we noticed that three out of four E_2-immunized wethers responded well to the immunization protocol used (26, 42 and 54 % binding at dilution 1:1000). The fourth E_2-immunized wethers exhibited a lower percentage binding (9.8 % binding at dilution 1:1000).

7.2.2.2. LH concentration

LH concentrations in plasma from each wether are shown in table 7.2.2. Before the beginning of the treatment, LH levels were significantly more elevated ($p < 0.01$) in all wethers (mean: 2.489 ± 0.319 ng/ml, n=8) than in 30 week old intact Merino ram lambs (mean: 0.917 ± 0.151 ng/ml, n=21). The analysis of variance shows that implant treatment significantly affected mean LH levels ($p < 0.05$). It also points out a significant interaction between the implant and the immunization treatment ($p < 0.06$).

In fact, in all wethers receiving an empty implant (BSA / 0 and E / 0), LH levels remained elevated (> 2.1 ng/ml; 86 %-140 % of pre-treatment values) while very low LH concentrations (< 0.195 ng/ml; < 13% of pre-treatment values) were found in the two wethers immunized against BSA and receiving an oestradiol implant (BSA / E_2). In the E_2-immunized wethers receiving an oestradiol implant (E / E_2), one had an elevated LH level (3.304 ng/ml; 174 % of pre-treatment value) while the other one (exhibiting a relatively low titre) had an intermediate LH level (0.884 ng/ml) that compared well with the values normally found in intact ram lambs and that was reduced compared with the pre-treatment value (49 % of pre-treatment value).

7.2.2.3. FSH concentration

FSH concentrations in plasma from each wether are shown in table 7.2.3. Before the beginning of the treatment, FSH levels were significantly more elevated ($p < 0.01$) in all wethers (mean:

Table 7.2.1.

Titre Check

Percentage binding (%) of ^{3}H-oestradiol-17ß before the beginning of treatment (day 0) and 14 days after the booster injection (day 42) in plasma from each wether (n=2/group) actively immunized against BSA or oestradiol-17ß (E_2).

	day 0		day 42	
	BSA[a] immunized	E_2[a] immunized	BSA[a] immunized	E_2[b] immunized
empty implant	2.77 4.89	3.64 5.31	7.53 3.65	54.32 * 26.28 *
E_2 implant	2.86 3.50	2.69 3.05	3.60 5.23	9.83 * 41.72 *

[a]: % binding at dilution 1:100

[b]: % binding at dilution 1:1000

* : $p < 0.05$, significantly different from BSA-immunized wethers

Table 7.2.2.

LH concentrations (ng/ml) in plasma from each wether (n=2/group) before the beginning of treatment (day 0) and 14 days after the booster injection (day 42) (7 days after oestradiol implantation)

	day 0		day 42	
	BSA immunized	E_2 immunized	BSA immunized	E_2 immunized
empty implant	3.557 (100)	1.548 (100)	4.075 (114.56)	2.141 (138.31)
	2.758 (100)	3.424 (100)	3.865 (140.14)	2.934 (85.69)
E_2 implant	1.480 (100)	1.809 (100)	< 0.195 (< 13.18)	0.884 (48.87)
	3.437 (100)	1.901 (100)	< 0.195 (< 5.67)	3.304 (173.80)

values between brackets (...): percentage of pre-treatment values

Table 7.2.3.

FSH concentrations (ng/ml) in plasma from each wether (n=2/group) before the beginning of treatment (day 0) and 14 days after the booster injection (day 42) (7 days after oestradiol implantation)

	day 0		day 42	
	BSA immunized	E_2 immunized	BSA immunized	E_2 immunized
empty implant	110.183 (100)	57.503 (100)	69.584 (63.15)	44.001 (76.52)
	42.854 (100)	45.433 (100)	62.823 (146.60)	67.989 (149.65)
E_2 implant	28.030 (100)	39.777 (100)	1.727 (6.16)	11.351 (28.54)
	48.501 (100)	49.789 (100)	2.395 (4.94)	54.946 (110.36)

values between brackets (...): percentage of pre-treatment values

52.759 ± 8.741 ng/ml, n=8) than in 30 week old intact Merino ram lambs (mean: 0.412 ± 0.064 ng/ml, n=21). The analysis of variance shows that only the implant treatment significantly affected mean FSH levels ($p < 0.05$).

Indeed, in each wether receiving an empty implant (BSA / 0 and E / 0), FSH concentrations remained elevated ($>$ 44 ng/ml; 63 %-150 % of pre-treatment values) while low FSH levels ($<$ 2.4 ng/ml; 5-6 % of pre-treatment values) were found in the two wethers immunized against BSA and receiving an oestradiol implant (BSA / E_2). These low FSH concentrations were still more than two-fold above the values normally found in intact ram lambs. In the E_2-immunized wethers receiving an oestradiol implant (E / E_2), one had an elevated FSH level (54.946 ng /ml; 110 % of pre-treatment value) while the other one (exhibiting a relatively low titre check) had an intermediate FSH level (11.351 ng/ml) which was more than ten-fold above the values normally found in intact ram lambs but reduced compared with the pre-treatment value (29 % of pre-treatment value).

7.2.3. DISCUSSION

In the oestradiol-treated wethers immunized against BSA (BSA / E_2), we have observed a maintenance of plasma LH at a lower level than that of normal intact ram lambs, while FSH level was slightly above that of normal intact ram lambs used in other experiments. This indicates that oestradiol released from the implant can effectively reduce pituitary gonadotropin secretion, this effect being more pronounced for LH while not complete for FSH. This greater reduction in LH secretion can be explained by the fact that we suspect that the concentration of circulating oestradiol obtained with the implant was slightly above physiological level (e.g. based on the data reported in other studies in which similar silastic capsules were implanted in wethers - see D'Occhio et al., 1983b; 1985). Furthermore, the incomplete reduction in FSH secretion supports the view that testicular inhibin (absent in wether) is also an important regulator of FSH in the male sheep and that it might be required with oestrogens for a complete control of FSH in that species (Price, 1991).

On the other hand, in one of the E_2-immunized (oestradiol-treated) wethers (E / E_2), both plasma LH and FSH remained increased (values comparable to the pre-treatment values) which shows

that the immunization treatment can effectively stop the oestradiol released by the implant from acting on the hypothalamic-pituitary axis. In the other E / E_2 wether, LH and FSH levels were slightly reduced by the oestradiol implant (48 % and 28 % of pre-treatment values, respectively). In this wether, the LH value obtained 14 days after the booster injection remained comparable to the values normally found in intact ram lambs while the FSH value measured at that time was well above the values normally found in intact ram lambs. Interestingly, this difference in response between the two E_2-immunized wethers could reflect the difference in titres observed in these two animals. Therefore, this study also seems to support the idea that the amount of antibodies present in circulation influence the magnitude of the endocrine changes subsequent to the neutralization of a given hormone (Sharpe and Fraser, 1983; Roberts and Reeves, 1988). Unfortunately, the relative specificity and affinity of the antibodies present in the circulation of these two E_2-immunized wethers have not been estimated. Nevertheless, since the relevance of the *in vitro* techniques for the characterization of antibodies has been extensively questioned (Martin , 1984) and discussed in the preceding chapter (see section 6.5), we have no reason to believe that, in the present experiment, the *in vivo* characteristics (affinity and specificity) of the anti-oestradiol antibodies varied significantly between these two E_2-immunized wethers. The fact that the same immunization protocol and the same immunogen (that had served to develop the antibodies used in the experiment presented in section 6.4. of chapter 6 and in the experiment that will be presented in section 7.3) have been used at the same time for each immunized wethers also increases the likelihood that the antibodies produced were all similar from one wether to another.

Finally, in the wethers receiving an empty capsule, we did not observe any tendency for an increase in LH levels in E_2-immunized compared with the BSA-immunized group. Therefore, this result does not support the finding that an increase in LH pulse frequency occurs in wethers actively immunized against oestradiol (Sanford, 1987b). However, since very few animals per treatment group were used in our study, we think that it would be premature to conclude that the low level of oestradiol found in the young wethers does not influence the hypothamic-pituitary axis at all.

7.3. OESTRADIOL-17ß IMPLANTATION AND PASSIVE IMMUNIZATION AGAINST OESTRADIOL-17ß IN CASTRATED LAMBS.

7.3.1 EXPERIMENTAL PROCEDURE

7.3.1.1. Experimental animals

Eight South Australian Merino ram lambs born in April 1989 had been used in the experiment described in chapter 3 and were used thereafter in this experiment . The selected lambs had been castrated between 22 and 30 weeks of age. The castrated rams (wethers) were kept in the controlled light room (12 hours light: 12 hours dark) during the whole experiment. At the beginning of the experiment (at 34 weeks of age), the average liveweight was 33.6 ± 0.68 kg.

7.3.1.2. Treatment

At 34 weeks of age, four wethers were injected with 0.664 g of purified IgG directed against BSA diluted in 10 ml of saline. Four wethers received the same amount of purified IgG directed against oestradiol-17ß-3-HSA (E_2-3-HSA) (W82; see section 6.2) diluted in 10 ml of saline.

Immediately before the IgG injection, two of the four BSA immunized wethers were implanted with an empty Silastic capsule as described in section 2.7.1.(BSA / 0, n=2). The other BSA immunized wethers were implanted with a Silastic capsule filled with oestradiol-17ß (BSA / E_2, n=2). Two E_2-immunized wethers received an empty Silastic capsule (E / 0, n=2) and the other E_2-immunized wethers received a Silastic capsule filled with oestradiol-17ß (E / E_2, n=2).

7.3.1.3. Intensive bleeding

One day before the implantation and the passive transfer of antibodies, the lambs were intensively bled (every 10 minutes for 4 hours) as described in section 2.6.1. Intensive bleedings were repeated seven days and fourteen days after the beginning of treatments.

7.3.1.4. Titre check

One blood sample from each lamb was collected at the end of the three intensive bleedings and these samples were assayed for titre check,.as described in section 2.8.2.1.

7.3.1.5. Hormone assays

As described in section 7.2.1.5.

7.3.1.6. Statistical analysis.

As described in section 7.2.1.6, except that, overall hormonal changes for the two intensive bleedings done after the start of the treatment were analyzed by analysis of variance for repeated measures to detect differences due to immunization, implantation, time and their interactions.

7.3.2. RESULTS

7.3.2.1. Titre check

Titres for each wether are presented in table 7.3.1. As expected, the percentage binding of oestradiol-17ß in diluted plasma (1:100) remained negligible (less than 7 %) during the whole study in the wethers receiving purified IgG against BSA. Seven days after the injection of IgG, the percentage binding of oestradiol in diluted plasma (1:100) was significantly higher ($p < 0.01$) in the E_2-immunized wethers (~50 % at 1:100). Seven days later, the titre had decreased only slightly (~47 % at 1:100).

7.3.2.2. LH concentration

LH concentrations in plasma from each wether at three different times are shown in table 7.3.2. Before the beginning of the treatment, LH levels was significantly more elevated ($p < 0.01$) in all wethers (mean: 4.537 ± 0.277 ng/ml, n=8) than in 30 week old intact Merino ram lambs (mean: 0.917 ± 0.151 ng/ml, n=21). The analysis of variance shows significant effects due to the implant treatment ($p < 0.01$) and time ($p < 0.01$) as well as significant "implant x time" interaction ($p < 0.02$) and significant "implant x immunization x time" interaction ($p < 0.02$).

In fact, in all wethers receiving an empty implant (BSA / 0 and E_2 / 0), LH concentrations remained elevated (> 2.976 ng/ml; 68 %-125 % of pre-treatment values). Low LH concentrations were found in wethers immunized against BSA and receiving an oestradiol implant (BSA / E_2; < 1.122 ng/ml) and in wethers immunized against oestradiol and receiving an oestradiol implant (E / E_2; < 1.925 ng/ml) however, in terms of percentage of the pre-treatment values, the reduction in LH concentration was more pronounced for the BSA / E_2 wethers (13 %-22 % of pre-treatment values) than for the E / E_2 wethers (27 %-45 % of pre-treatment values).

Table 7.3.1.

Titre Check

Percentage binding (% at dilution 1:100) of ^{3}H-oestradiol-17ß in plasma from each wether (n=2/group) before the beginning of treatment and, 7 and 14 days after injection of purified IgG against BSA or purified IgG against oestradiol-17ß (E_2).

	Before	treatment	7 days	after	14 days	after
	Pur. IgG against BSA	Pur. IgG against E_2	Pur. IgG against BSA	Pur. IgG against E_2	Pur. IgG against BSA	Pur. IgG against E_2
empty implant	4.27 4.45	4.87 4.31	6.35 3.60	52.72 ** 52.82 **	5.57 6.29	47.19 ** 47.54 **
E_2 implant	4.24 4.36	4.34 4.37	5.93 6.94	50.83 ** 50.16 **	5.83 6.51	47.93 ** 42.37 **

** : $p < 0.01$, significantly different from wethers receiving Pur. IgG against BSA

In these wethers (BSA / E_2 and E / E_2), the LH concentrations measured after implantation compared well with the values normally found in intact ram lambs in other experiments and, for each of these wethers, tended to be lower fourteen days than seven days after the start of the treatment.

7.3.2.3. FSH concentration

FSH concentrations in plasma from each wether at three different times are shown in table 7.3.3. Before the beginning of the treatment, FSH levels were significantly more elevated ($p < 0.01$) in all wethers (mean: 19.242 ± 3.161 ng/ml, n=8) than in 30 week old intact Merino ram lambs (mean: 0.412 ± 0.064 ng/ml, n=21). The analysis of variance shows significant effects due to the implant treatment ($p < 0.01$) and time ($p < 0.01$) as well as significant "implant x time" interaction ($p < 0.01$), significant "immunization x time" interaction ($p < 0.01$) and significant "implant x immunization x time" interaction ($p < 0.01$).

Indeed, in wethers receiving an empty implant (BSA / 0 and E / 0), FSH concentrations remained elevated (> 12.303 ng/ml, 78 %-169 % of pre-treatment values). Relatively low FSH concentrations were found in wethers immunized against BSA and receiving an oestradiol implant (BSA / E_2; < 2.228 ng/ml) and in wethers immunized against oestradiol and receiving an oestradiol implant (E / E_2; < 3.787 ng/ml) however, in terms of percentage of the pre-treatment values, the reduction in FSH concentration was more pronounced for the BSA / E_2 wethers (1.5 %-9 % of pre-treatment values) than for the E / E_2 wethers (12 %-37 % of pre-treatment values). The FSH concentrations measured after implantation in three out of four E_2-implanted wethers (one BSA / E_2 and two E / E_2) were more than two-fold above the values normally found in intact ram lambs while the FSH concentrations in the fourth E_2-implanted wether (BSA / E_2) compared well with the values normally found in intact ram lambs. For each E_2-implanted wethers, the FSH values tended to be lower fourteen days than seven days after the start of the treatment.

Table 7.3.2.

LH concentrations (ng/ml) in plasma from each wether (n=2/group). before the beginning of treatment and 7 and 14 days after immunization and implantation

	Before treatment		7 days after		14 days after	
	Pur. IgG against BSA	Pur. IgG against E_2	Pur. IgG against BSA	Pur. IgG against E_2	Pur. IgG against BSA	Pur. IgG against E_2
empty implant	4.653 (100)	4.405 (100)	5.174 (111.20)	3.572 (81.09)	3.941 (84.70)	2.976 (67.56)
	4.723 (100)	5.130 (100)	5.892 (124.75)	6.002 (117.00)	4.520 (95.70)	5.504 (107.29)
E_2 implant	4.917 (100)	4.255 (100)	1.122 (22.82)	1.925 (45.24)	0.927 (18.85)	1.150 (27.03)
	5.385 (100)	2.825 (100)	0.899 (16.69)	1.184 (41.91)	0.722 (13.41)	0.966 (34.19)

values between brackets (...): percentage of pre-treatment values

Table 7.3.3.

FSH concentrations (ng/ml) in plasma from each wether (n=2/group). before the beginning of treatment and 7 and 14 days after immunization and implantation

	Before treatment		7 days after		14 days after	
	Pur. IgG against BSA	Pur. IgG against E_2	Pur. IgG against BSA	Pur. IgG against E_2	Pur. IgG against BSA	Pur. IgG against E_2
empty implant	11.903 (100)	16.642 (100)	12.303 (103.36)	14.875 (89.38)	20.173 (169.48)	15.996 (96.12)
	25.704 (100)	36.565 (100)	22.962 (89.33)	28.500 (77.94)	31.567 (122.81)	29.177 (79.79)
E_2 implant	17.089 (100)	10.206 (100)	0.481 (2.81)	3.787 (37.10)	0.250 (1.46)	1.573 (15.41)
	23.718 (100)	12.108 (100)	2.228 (9.39)	3.605 (29.77)	0.996 (4.20)	1.474 (12.17)

values between brackets (...): percentage of pre-treatment values

7.3.3. DISCUSSION

In this second experiment. in the oestradiol-treated wethers immunized against BSA, we have observed maintenance of plasma LH in all animals at a level that compared well with that of normal intact ram lambs, while FSH levels were generally above that of normal intact ram lambs (in 3 out of 4 wethers). As in the preceding experiment (section 7.2), the FSH levels obtained also support the view that oestrogens require the presence of testicular inhibin (absent in wethers) for a complete control on FSH secretion in that species (Price, 1991).

Our results also indicate that oestradiol released from an implant can reduce pituitary gonadotropin secretion to some extent whether purified IgG directed against BSA or purified IgG directed against oestradiol-17ß are present in circulation. However, a difference between these two treatments becomes apparent when the LH and FSH concentrations are expressed as a percentage of pre-treatment values. Indeed, the reduction in gonadotropin secretion was found to be more pronounced in the BSA-immunized than in the E_2-immunized wethers which shows that although some oestradiol released by the implant was still able to act on the hypothalamic-pituitary axis in the E_2-immunized wethers, a reduction in the intensity of oestradiol action could have been due to the presence of the antibodies in circulation. Moreover, since the LH and FSH levels measured seven days after the start of the treatment were slightly above that observed one week later, it may be that oestradiol was more efficient in reducing gonadotropin secretion after a slight reduction in titre had occurred in the E_2-immunized wethers (due to normal clearance of antibodies and/or progressive occupation of the binding sites by the endogenous hormones). In fact, other studies have also indicated that a slight change in titre can influence the magnitude of the endocrine changes subsequent to the neutralization of steroid hormone (Sharpe and Fraser, 1983; Roberts and Reeves, 1988; see also section 7.2.). However, in the present experiment, other possibilities need to be considered to explain this reduction in gonadotropins taking place within a week since such a reduction has also occurred in wethers immunized against BSA. For instance, it is possible that temporal modifications occurring at the level of the hypothalamo-pituitary axis would have resulted in a higher sensitivity to oestradiol after 14 days of treatment than after 7 days. However, since the exact mechanism of action of oestradiol on this axis is

One must keep in mind that the value used for comparison was not obtained from contemporary intact ram lambs.

still very much unknown, the nature of these temporal modifications can only be speculated at the moment. Furthermore, other investigators have observed that the release of steroid hormone from Silastic capsules decreases with time, due to the formation of fibrous connective tissues surrounding the capsule (Smith et al., 1977; D'Occhio and Brooks, 1980). Nevertheless, because plasma oestradiol concentrations have not been measured in the present study, we cannot completely eliminate the possibility that more oestradiol was released from the Silastic capsule during the second bleeding.

It is important to remember that the reduction in gonadotropin secretion observed in E / E_2 wethers must be due to the presence of free oestradiol within the hypothalamic-pituitary axis, since there is no possible concomitant increase in testicular hormones in the castrated ram lambs that could also affect this axis and the adrenal contribution should not change. Furthermore, we assume that the extremely low level of endogenous steroids present in this animal model could not interfere significantly with the E_2-antibodies in circulation, contrary to the situation found in intact ram lambs.

We conclude that the dose of antibodies (maintaining a titre at ~1:100) used in this experiment (equivalent to the dose used in the study described in section 6.3. of chapter 6) was not sufficient to completely neutralize oestradiol-17ß released by the Silastic capsule and therefore that this hormone was still able to act on the hypothalamic-pituitary axis and reduce LH and FSH secretions. Nevertheless, the *in vivo* effectiveness of the antibodies has been partly demonstrated since the action of oestradiol on gonadotropin secretion was less pronounced in the wethers that have received an injection of purified IgG directed against oestradiol than in wethers that have received an injection of purified IgG directed against BSA.

7.4 GENERAL DISCUSSION FOR CHAPTER 7

In both experiments we have shown that the oestradiol replacement therapy was very effective in reducing circulating LH and FSH levels in all wethers immunized against BSA.

Since the steroid-filled Silastic capsule seems to maintain essentially constant serum steroid concentration (Schanbacher, 1980b) and since constant blood hormone levels seem to provide a stronger feedback signal on LH than do similar mean concentration derived from episodic pulses

in the intact animals (D'Occhio et al., 1983a), we assume that the "suppressive" effect on the hypothalamic-pituitary axis obtained with the oestradiol implant was very efficient in wethers immunized against BSA. Indeed, the LH levels measured in BSA / E_2 wethers, in the first experiment (section 7.2.), were found to be lower than that of normal intact ram lambs. although no strictly comparable data were available. Nevertheless, one must keep in mind that oestradiol-treated castrated ram lambs still differ considerably from intact ram lambs. For instance, we suspect that the plasma oestradiol level obtained in this study were somewhat higher than the levels found in intact rams lambs, and it is obvious that the level of other steroids normally present in intact animals are extremely reduced in castrated animals.

The fact that the oestradiol therapy never completely reduced FSH concentration to values comparable to that found in intact ram lambs (except in one wether passively immunized against BSA) supports the idea that other testicular hormones are involved in the regulation of this gonadotropin. For instance, inhibin has been implicated in the control of FSH secretion in adult rams (Blanc and Terqui, 1976; Walton et al., 1978; Walton et al., 1980; Voglmayr et al., 1990; Clarke et al., 1991) and in prepubertal ram lambs (Al-Obaidi, 1987). In fact, inhibin could act in synergy with oestrogens to regulate FSH secretion in the male sheep (for review see Price, 1991). The possibility also remains that a more complete reduction in FSH levels would have occurred in our oestrogen-treated wethers if supplemented in the same time with a physiological dose of testosterone since a synergistic effect may also occur with both steroids (Parrott 1978; Mawhinney and Neubauer,.1979; Schanbacher, 1980a; D'Occhio and Brooks, 1980, Mainwaring et al., 1988).

These two experiments have also shown that the active immunization approach is more effective than the passive immunization approach which was not very efficient in neutralizing oestradiol released by the implant. Thus, this result agrees with the ones obtained in chapters 5 and 6. Among our observations, three important results support the idea that a relatively high titre (between 1:100 and 1:1000) is required in the castrated ram lambs (weighing about 35 kg) supplemented with one 24-cm Silastic capsule packed with crystalline oestradiol-17ß, even though very few cross-reacting steroids are likely to interfere with the antibodies in this animal model.

L. 13: Since the suppression by oestradiol was also more pronounced fourteen days than seven days after the injection of purified IgG directed against BSA, we cannot eliminate the possibility that a larger amount of oestradiol was released by the implant with time and therefore that the amount of unoccupied binding sites (on passively transferred antibodies) available for the exogenous hormone became considerably reduced with time.

First, in E_2-implanted wethers actively immunized against oestradiol, the inhibiting effect of oestradiol on FSH and LH secretion was more efficient in the subject which had a relatively low titre (~10% binding in plasma diluted at 1:1000) than in the one which had a higher titre (~50% binding in plasma diluted at 1:1000).

Second, in the two E_2-implanted wethers that had received an injection of purified IgG directed against oestradiol (low titres, ~50% binding in plasma diluted at 1:100), the inhibiting effect of oestradiol on FSH and LH secretion was less pronounced than for the E_2-implanted wethers that had no E_2-antibodies in their circulation (e.g. wethers that had received an injection of purified IgG directed against BSA).

Third, the LH and FSH suppression by oestradiol was more pronounced fourteen days than seven days after the injection of purified IgG directed against oestradiol (e.g. after a slight reduction in titre had occurred due to normal clearance of the antibodies and/or progressive occupation of the binding sites by the endogenous hormones). (see INSERT 13)

We conclude that active immunization against oestradiol (section 7.2.) can effectively neutralize the oestradiol released from a Silastic capsule. Furthermore, even though, the *in vivo* effectiveness of the purified IgG directed against oestradiol has been partly demonstrated in the second experiment using oestradiol-treated castrated ram lambs (section 7.3.), we cannot exclude the possibility that the *in vivo* affinity of the passively transferred antibodies were often too low to compete efficiently with the oestrogen receptors within the target tissues in the hypothalamic-pituitary axis of these wethers (as for the intact ram lambs studied in the experiment reported in section 6.4. of chapter 6).

The data obtained in these two experiments strongly suggest that the magnitude of the neutralization varies according to the titre obtained. Indeed, it seems that a high titre (that reflects the presence of a large quantity of binding sites directed against oestradiol) can lead to a successful neutralization of oestradiol-17ß and consequently, to complete suppression of its effects on gonadotropin secretion.

CHAPTER 8.

ACTIVE IMMUNIZATION AGAINST TESTOSTERONE IN ADULT MERINO RAMS

8.1. INTRODUCTION

The effects observed in ram lambs (chapter 5) and young wethers (section 7.2. of chapter 7) actively immunized against oestradiol-17ß indicate that the biological activity of this steroid can be neutralized, at least partly, by antibodies. Other investigators have also demonstrated that similar treatment applied to mature rams resulted in significant modifications within the hypothalamic-pituitary-testicular axis reflected by increased gonadotropin and testosterone secretion, and increased testicular weight and daily production of round spermatids; these effects being noticeable after 6 months (Schanbacher et al., 1987; Monet-Kuntz et al., 1988) and 12 months of immunization (Monet-Kuntz et al., 1988).

At the moment, the course of events following active immunization against steroids is still not totally understood since very few studies have been carried out including repeated measurements during a relatively long period of time. Therefore, since most researchers have described the effects obtained at only one point during the course of immunization, it is likely that we know more about temporary and brief responses rather than about the dynamic process that continuously tries to establish a "new equilibrium" within the immunized animals (for review see: Nieschlag and Wickings, 1977, 1978; Haynes and Southee, 1984).

Recently, it has been suggested that immunity against testosterone early in life may confer lifetime reproductive advantages in the bull (D'Occhio et al., 1987). In fact, daily sperm production has been shown to be increased in young bulls actively immunized against testosterone without detrimental effects of this treatment on the weight of the epididymis, and other androgen-dependent organs important for sperm maturation and storage. Similarly, an increase in sperm production as well as normal seminal characteristics have been reported for young testosterone-immunized bulls (Walker et al., 1984). Interestingly, immunity against testosterone appears not to diminish sexual behaviour in young rams (Haynes and Southee,

1984) or in young boars (Thompson et al., 1985). However, untoward side-effects, following this treatment, have also been reported in laboratory animals such as loss of sexual activity (Nieschlag and Kley, 1974; Nieschalg and Wickings, 1977, 1978) and atrophy of the accessory reproductive glands (Hillier et al., 1975a, b , Nieschlag et al., 1975c; see appendix table 6). In ram lambs, decreased anabolic effect with no improvement in testicular weight (Schanbacher, 1982) have also been described.

Since no long-term study using active immunization against testosterone in adult rams have been reported yet (see appendix: table 7) and since it is unknown whether deleterious side-effects would occur in these animals following such treatment, we intended to carry out an experiment of one year duration with postpubertal Merino rams.

The aim of this experiment was to explore the possibility of improving sperm production in rams by immunoneutralization of testosterone. Indeed, considering that, in the adult ram, testosterone and its metabolites (oestradiol and DHT) restrain gonadotropin secretion by negative feedback action on the hypothalamic-pituitary axis (Schanbacher, 1984b), our hypothesis is that the antibodies directed against testosterone should neutralize this hormone in the peripheral circulation and should lead to enhancement of gonadotropin secretion. The testis receiving more stimulation from gonadotropins should, therefore, improve its steroidogenic and spermatogenic functions.

Apart from repeated endocrinological investigations of the hypothalamic-pituitary-testicular axis, various components of the male reproductive system (sexual behaviour, sperm concentration in ejaculate, testicular blood flow and epididymal weight) have been evaluated in this experiment to verify whether the treatment caused any detrimental effect on the whole system.

An additional objective of this experiment was to determine if the hypothalamic-pituitary axis was still affected by the immunization procedure after removal of the gonads.

8.2 EXPERIMENTAL PROCEDURE

8.2.1. Experimental animals

Eighteen month old South Australian Merino rams were used in this experiment. The rams were kept outdoors in paddocks adjacent to the Waite Agricultural Research Institute (see section 2.2.2.). The study started in August 1988 and ended in July 1989. However, additional information on these rams has also been collected after castration (in October 1989).

8.2.2. Treatment

Five rams (Control, n=5) received a primary injection of bovine serum albumin (BSA) in Freund's complete adjuvant (FCA) in August 1988. The other rams (T-immunized, n=5) were treated identically, but using testosterone-3(o-carboxymethyl)oxime-BSA (T-3-BSA) in FCA. All rams received a first booster injection in November. Other boosters were given in January, March, May and July. Freund's incomplete adjuvant (FIA) was used instead of FCA for each booster injection. The immunogens were prepared and injected as described in section 2.3.1. An additional booster injection has been given in October 1989, after castration.

8.2.3. Body weight and testicular size measurement

Body weight, testicular diameter and length were recorded monthly until November then every second month, as described in sections 2.4. and 2.5. Scrotal circumference was also measured using a flexible tape around the widest part of the testis; no correction for skin thickness was made.

8.2.4. Intensive bleeding

Before the beginning of the immunization, in August, the rams were intensively bled (every 20 minutes for 6 hours) as described in section 2.6.1. Intensive bleedings were repeated fourteen days after each booster injection (in November, January, March, May and July). On the day prior to sampling, rams were kept indoors in individual pens and one of their jugulars was cannulated with indwelling polyethylene cannulae (1.5 mm O.D., 1.0 mm I.D.). At these occasions, they were fed with sheep commercial pellets and lucerne hay.

8.2.5. Pituitary responsiveness to GnRH

At the end of the intensive bleedings done in November, March and July, all rams received a single GnRH injection (5 ng/kg body weight) and additional blood samples were collected as described in section 2.6.2.

Furthermore, in October 1989, the castrated rams were bled at 10 minute intervals for 4 hours and received thereafter a single GnRH injection (5 ng/kg body weight). Additional blood samples were collected at 10 minute intervals during the following two hours.

8.2.6. hCG challenge

Immediately after the intensive bleeding done in January (in conscious rams), testicular responsiveness to a single hCG injection (20 I.U./kg body weight) was evaluated. Additional blood samples were collected at 10, 20, 30 40, 50, 60 , 80, 100 and 120 minutes after the hCG injection, to assess the responsiveness of the testes to a standard challenge.

8.2.7. Measurement of testicular blood plasma flow and response to hCG

TTBPF was measured in anaesthetized rams before castration as described in section 2.7.2. Testicular responsiveness to a single hCG injection was simultaneously evaluated as described in section 2.6.3.1. (in anaesthetized rams). PAH concentration was assayed in each blood sample collected as described in section 2.8.3. The production of testosterone by each testis (defined as the product of plasma flow per testis and the veno-arterial concentration difference for testosterone, i.e.: (ISV [T] - JUG [T]) x TTBPF) was also calculated.

8.2.8. Titre check

One blood sample for each ram was collected immediately after each intensive bleeding. Plasma from these samples were assayed for titre as described in section 2.8.2.1.

8.2.9. Hormone assays

Plasma samples were assayed for LH, FSH, PRL and testosterone as described in section 2.8.1.2.2., 2.8.1.2.3., 2.8.1.2.4. and 2.8.1.3.2., respectively. Vigorous extraction of samples into toluene:hexane (2:1 V/V) cocktail ensured that anti-testosterone antibodies did not influence recoveries and that total testosterone (bound and unbound) was measured. Mean hormone levels were determined by assaying plasma pools representing the 6-hour sampling

period (section 2.6.1.2.). Each blood sample collected following a GnRH challenge was assayed for LH (November, March, July), however, after castration (in October 1989), the pool samples collected before and after the GnRH injection were assayed for LH ("pre" and "post" GnRH pool samples). Each blood sample collected following the hCG challenge, in January, was assayed for testosterone. Pool samples collected before and after the hCG challenge, in July, were assayed for testosterone (see section 2.6.3.2.: "pre" and "post" hCG pool samples).

8.2.10. Libido trials

The rams had not been used previously as flock sires and were considered inexperienced at the start of the experiment. Sexual activity was determined by recording the number of times that each of the following aspects of behaviour: sniffs, nudges, mounts and intromissions followed by ejaculations, was displayed by each ram during a ten minute exposure to a teaser ewe. These components of mating behaviour have been fully described by Banks (1964) and have been recorded in other libido trials (D'Occhio and Brooks 1980; D'Occhio et al., 1985). Each trial was done in a large yard (5.0 x 8.5 m) adjacent to a pen where the other rams were temporarily held. The teaser ewe was secured in a collection bail (as used for semen collection with artificial vagina) which was placed in one corner of the service yard. Oestrous has been induced by daily intramuscular injection of 50 mg of oestradiol benzoate in one ml of peanut oil, in the teaser ewes used for the first (October) and second (January) libido trials however this treatment has not been necessary for the third (June) and fourth (July) trials (since most rams were mounting the teaser ewes not prepared with hormones).

8.2.11. Semen collection and evaluation

Semen was collected on four occasions (2 November, 29 May, 4 July, 11 July) by use of an artificial vagina (Evans and Maxwell, 1987). On these occasions, the rams that did not mount the teaser ewe were not included in the data (two T-immunized rams in the first collection and one T-immunized ram in the second, third and fourth collections). The same method was used for the final collection (26 July) except that two rams (one control and one T-immunized) that did not mount the teaser ewe at that time had to be collected by electroejaculation. The rams had

been trained for the artificial vagina method of collection a few weeks before the first collection (in October) and at various occasions between January and May.

The ejaculates were examined under light microscope and the number of spermatozoa per unit volume of ejaculate were estimated using a haemacytometer.

8.2.12. Castration

All rams were castrated at the end of July 1989 as described in section 2.7.4. The weight of the epididymis and the testes were precisely recorded.

8.2.13. Daily sperm production

Pieces of the left and right testis (approximately 5 g / testis) were taken immediately following castration (no fixation) and placed at -20 °C until DSP were evaluated. The testicular tissues were transferred to 4 °C, one day before DSP evaluation. DSP evaluation was done as described in sections 2.8.4.2. and 2.8.4.3.

8.2.14. Testicular histology

Blocks of testicular tissue taken at castration were prepared as described in section 2.9. and were examined under light microscopy. Any abnormalities in the general appearance of the seminiferous tubules and the interstitial tissues were recorded.

8.2.15. Analysis of LH pulses

As described in section 5.2.13. (see chapter 5).

8.2.16. Statistical analysis.

Data for characteristics involving repeated measurements over time (body weight, testicular volume, testicular circumference, LH, FSH, PRL, testosterone, LH pulse characteristics, testosterone concentrations preceding and following a hCG injection, LH concentrations preceding and following a GnRH injection, sexual activity, TTBPF, testosterone production and titre check) were analyzed by analysis of variance for repeated measures to detect differences due to immunization, time and their interactions. Daily sperm production, sperm concentration for each collection, testicular weight, testicular volume, and epididymis weight at castration were analyzed using a one factor analysis of variance to localize differences among and within

groups. All these analyses were done using the CLR ANOVA computer program (Clear Lake Research, Morningside, Houston, TX, 77005).

Pairwise comparisons of the means were made using student-*t* -test. A logarithmic transformation [log (x+1)] was applied to some data (in this experiment: testosterone, LH, FSH, LH concentrations preceding and following a GnRH injection, testosterone concentrations preceding and following a hCG injection, TTBPF) to equalize variances. The data presented in tables and graphs are the actual ones.

8.3. RESULTS

8.3.1. Health of the animals

The rams remained healthy during the whole experiment. Abscess formation was almost always noted at the immunization sites following the primary immunization; then, following booster immunizations, only a few abscesses were present and were of small size (< 1 cm).

8.3.2. Titre check

Titres are presented in table 8.1. The percentage binding of radioactive testosterone remained negligible (less than 5 %) during the whole experiment in the control rams. All T-immunized rams responded well to the immunization protocol used. The percentage binding of testosterone in diluted plasma (1:5000) collected fourteen days after each booster injection varied between 17 and 34 % in T-immunized rams.

8.3.3. Body weight

Body weight increased significantly ($p < 0.01$) between August and March then decreased slightly but significantly ($p < 0.01$) until the end of the experiment in both groups as shown in fig. 8.1. There was no significant difference in body weight between control and T-immunized rams during the whole study.

8.3.4. Testicular volume and circumference

Changes in testicular volume and circumference in control and T-immunized rams are shown in fig. 8.2. Between August and October, a continuous increase in both parameters occurred in both groups ($p < 0.01$). Both parameters varied slightly between November and May with a

Table 8.1.

Titre check

Percentage binding (% ± s.e.m.) of ^{3}H-testosterone in plasma from control (C) and T-immunized rams (T). in August, November, January, March, May and July

	Aug.[a]	Nov.[b]	Jan.[b]	Mar.[b]	May.[b]	Jul.[b]
C (n=5)	4.7 ± 0.2	3.3 ± 0.5	2.7 ± 0.2	4.2 ± 0.4	2.8 ± 0.5	2.6 ± 0.5
T (n=5)	4.7 ± 0.1	17.0 ± 3.6**	20.1 ± 4.6**	33.4 ± 5.6**	20.4 ± 2.8**	26.4 ± 6.5**

[a].: plasma samples diluted at 1:100 for control and T-immunized rams

[b] : plasma samples diluted at 1:100 for controls and at 1:5000 for T-immunized rams

**: $p < 0.01$, significantly different from controls

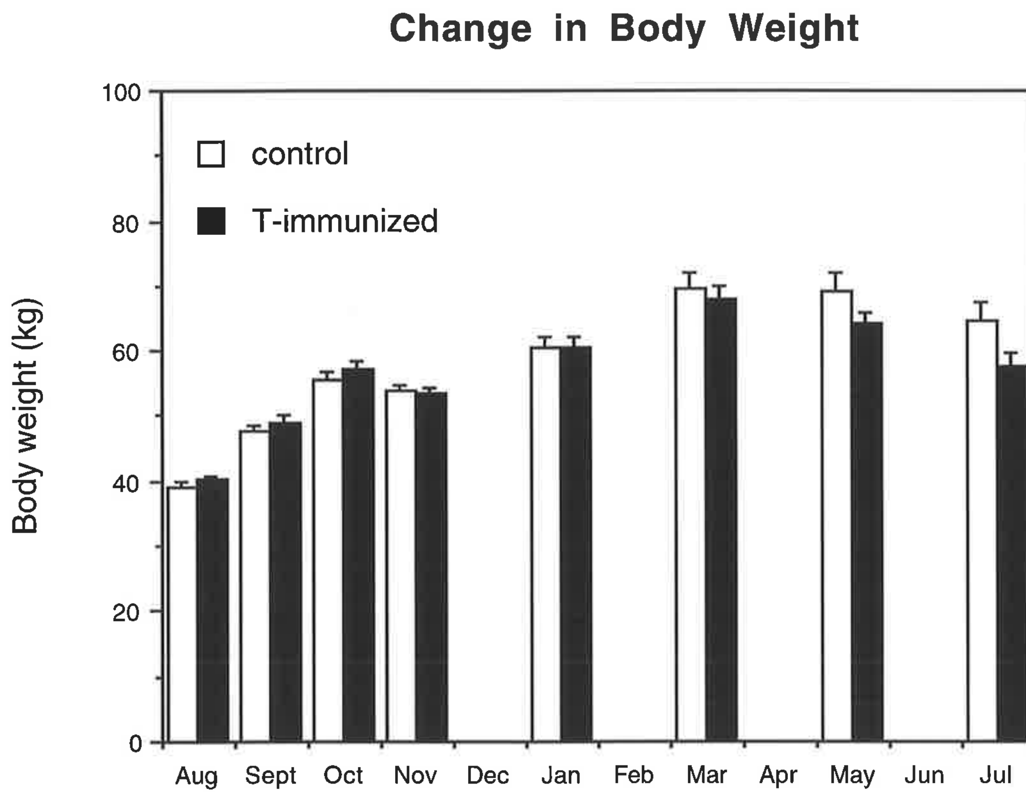
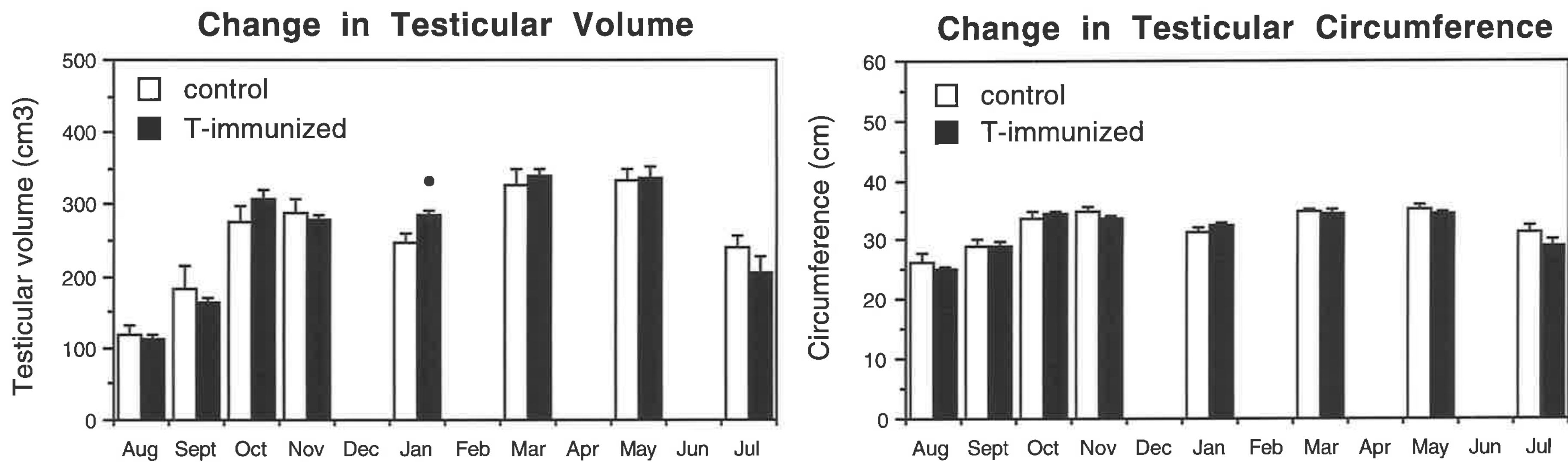


Fig. 8.1.: Changes in mean body weight (kg ± sem) from August to July in adult Merino rams actively immunized against BSA (Control, n=5) or against testosterone-3-BSA (T-immunized, n=5).



•: $p < 0.08$, significantly different from controls

Fig. 8.2.: Changes in mean testicular volume ($cm^3 \pm$ sem, left) and mean circumference (cm ± sem, right) from August to July in adult Merino rams actively immunized against BSA (Control, n=5) or against testosterone-3-BSA (T-immunized, n=5).

significant rise ($p < 0.05$) occurring between January and March. Finally, testicular volume and circumference decreased significantly between May and July ($p < 0.01$). There was no significant difference in testicular circumference between control and T-immunized rams during the whole study, however, a higher testicular volume ($p < 0.08$) in T-immunized rams was observed in January.

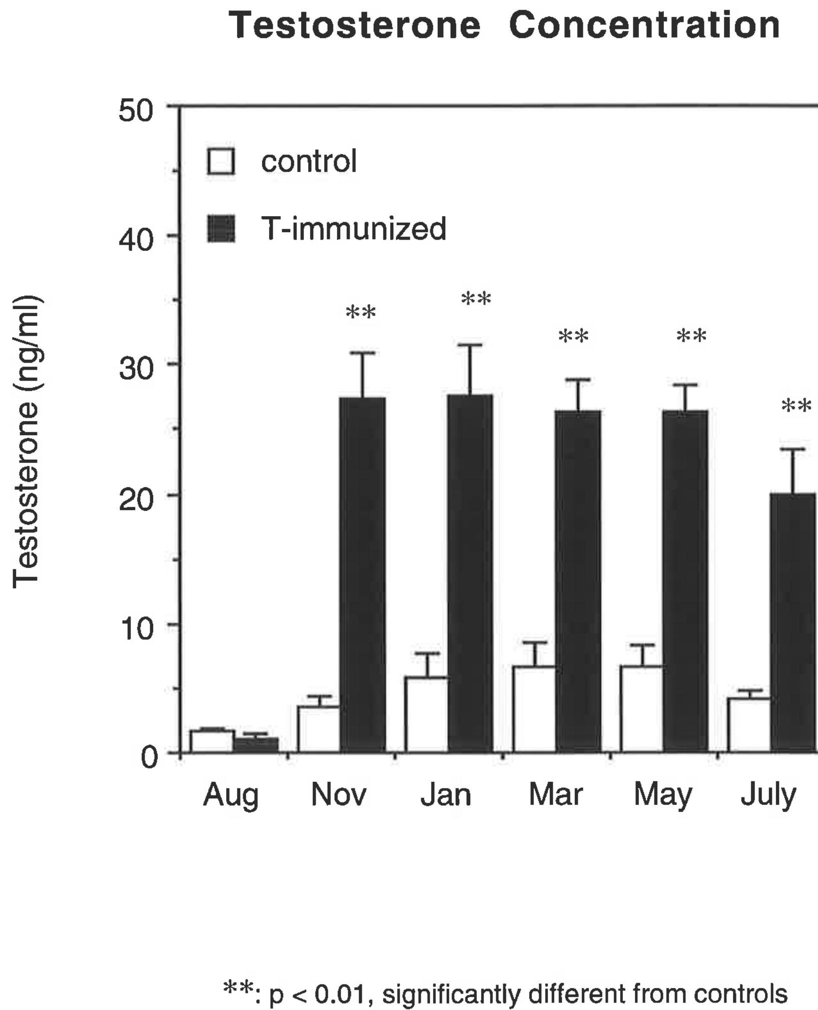
8.3.5. Testosterone concentration

Mean testosterone concentrations in plasma during six different intensive bleedings are shown in fig. 8.3. Before the beginning of the immunization in August, no difference in testosterone concentration was found between the two groups. The T-immunized rams had significantly ($p < 0.01$) higher testosterone concentrations than the controls during the other intensive bleedings done between November and July. In controls, mean testosterone level increased between August and January, then remained stable until May and finally decreased in July. In T-immunized rams, mean testosterone level did not vary significantly between November and May and there was a tendency for a decrease in testosterone concentration in July.

8.3.6. LH concentration

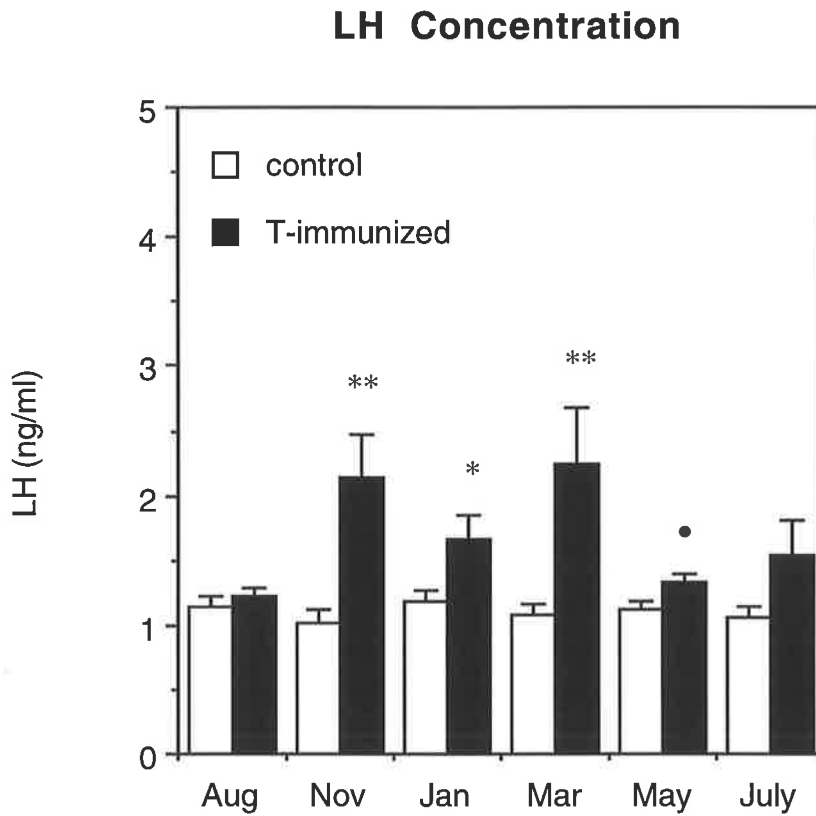
Mean LH concentrations in plasma are shown in fig. 8.4. In controls, mean LH concentrations did not vary significantly with time. In T-immunized rams, mean LH concentrations varied significantly with time ($p < 0.01$), the highest values being found between November and March. Mean LH concentrations in T-immunized rams were significantly higher in November, January, March and May ($p < 0.01$, $p < 0.05$, $p < 0.01$ and $p < 0.07$, respectively) than in controls (Table 8.2). In July, although LH level was still higher in T-immunized than in control rams, at that time, the difference was not statistically significant (Table 8.2).

Table 8.2. shows LH pulse characteristics in control and T-immunized rams during the six intensive bleedings. The number of peaks found within a 6-hour sampling period was significantly higher in T-immunized rams than in controls in November and March ($p < 0.01$). Mean pulse interval was also found to be of shorter duration in T-immunized rams in November and March ($p < 0.01$). Mean pulse amplitude was significantly more elevated in T-immunized rams in November ($p < 0.01$), January ($p < 0.07$) and March ($p < 0.07$). Mean pulse nadir was



**: $p < 0.01$, significantly different from controls

Fig. 8.3.: Mean testosterone concentration (ng/ml ± sem) in plasma collected in August, November, January, March, May and July from adult Merino rams actively immunized against BSA (Control, n=5) or against testosterone-3-BSA (T-immunized, n=5).



**: p < 0.01, * : p < 0.05, • : p < 0.07; significantly different from controls

Fig. 8.4.: Mean LH concentration (ng/ml ± sem) in plasma collected in August, November, January, March, May and July from adult Merino rams actively immunized against BSA (Control, n=5) or against testosterone-3-BSA (T-immunized, n=5).

Table 8.2.

LH pulse characteristics in control (C) and T-immunized rams (T) during intensive bleedings (every 20 minutes for 6 hours) done in August, November, January, March, May and July.

		mean[a] LH level (ng/ml)	number[a] of peaks per 6 h.	mean[a] pulse interval (min.)	mean[a] pulse amplitude (ng/ml)	mean[a] pulse nadir (ng/ml)
C (n=5)	Aug	1.14 ± 0.09	0.40 ± 0.25	360.0 ± 0.0	0.78 ± 0.48	1.38 ± 0.08
	Nov	1.02 ± 0.09	0.60 ± 0.25	360.0 ± 0.0	0.48 ± 0.27	1.01 ± 0.06
	Jan	1.18 ± 0.09	1.40 ± 0.51	294.0 ± 42.9	0.62 ± 0.18	0.74 ± 0.08
	Mar	1.07 ± 0.09	0.60 ± 0.25	360.0 ± 0.0	0.44 ± 0.19	0.32 ± 0.10
	May	1.11 ± 0.08	1.60 ± 0.25	228.0 ± 56.4	1.09 ± 0.09	0.42 ± 0.13
	Jul	1.06 ± 0.09	2.00 ± 0.55	198.7 ± 67.2	1.44 ± 0.57	0.85 ± 0.19
T (n=5)	Aug	1.22 ± 0.057	0.40 ± 0.25	360.0 ± 0.0	0.26 ± 0.19	1.37 ± 0.10
	Nov	2.13 ± 0.34 **	3.20 ± 0.58 **	113.3 ± 17.3 **	2.06 ± 0.34 **	1.65 ± 0.29 *
	Jan	1.65 ± 0.20 *	2.20 ± 0.58	215.3 ± 59.7	1.09 ± 0.13	1.12 ± 0.03 **
	Mar	2.25 ± 0.43 **	3.20 ± 0.58 **	83.3 ± 15.0 **	0.90 ± 0.12	1.38 ± 0.19 **
	May	1.32 ± 0.06 •	1.60 ± 0.51	194.0 ± 68.1	0.86 ± 0.30	1.02 ± 0.23 *
	Jul	1.53 ± 0.27	1.60 ± 0.25	224.0 ± 64.0	1.04 ± 0.21	1.58 ± 0.19 *

[a]: mean ± s.e.m.

• : $p < 0.07$, significantly different from controls

* : $p < 0.05$, significantly different from controls

**: $p < 0.01$, significantly different from controls

significantly increased in T-immunized rams during the five bleedings done between November and July ($p < 0.07$).

8.3.7. FSH concentration

Mean FSH concentrations in plasma are shown in fig. 8.5. In controls, mean FSH levels did not vary significantly with time. In T-immunized rams, mean FSH concentrations varied significantly with time ($p < 0.01$), the highest values being found between November and March. FSH concentrations were significantly elevated ($p < 0.05$) in T-immunized rams controls during the five bleedings done between November and July.

8.3.8. PRL concentration

Mean PRL concentrations in plasma varied significantly ($p < 0.001$) over the year in both groups of rams (fig. 8.6). As expected, PRL secretion increased with day length. The lowest values were found during the short-days period (in May, July and August) and the highest values during the long-days period (in January and March). The immunization treatment did not affect PRL secretion at any time.

8.3.9 a. LH concentration after a GnRH challenge

The LH responses to exogenous GnRH in November, March and July are shown in fig. 8.7. The maximal increase in circulating LH was generally observed ten minutes after the GnRH injection in all rams and LH level had returned to pre-treatment value within 120 minutes. Overall LH response following a GnRH challenge were significantly more pronounced in T-immunized rams than in controls, in November ($p < 0.01$) and in March ($p < 0.01$). However, in July, LH concentrations associated with the response curve were not significantly different between the two groups of rams. Therefore, in T-immunized rams, the pituitary responsiveness to GnRH changed significantly with time while, in controls, the LH response remained comparable between the three GnRH challenges.

8.3.9 b. LH and FSH concentrations after a GnRH challenge, after castration.

The LH and FSH responses to exogenous GnRH in October 1989 are shown in fig. 8.8. Plasma LH and FSH concentrations increased significantly after a GnRH injection in control and in T-immunized castrated rams. The hormonal increase was significantly more pronounced for

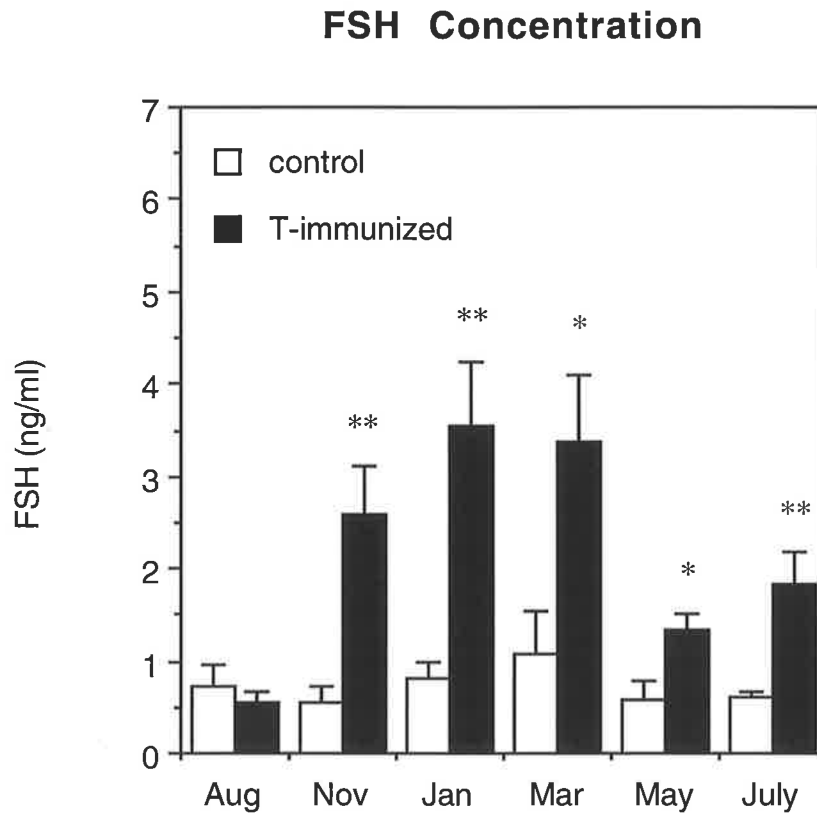


Fig. 8.5.: Mean FSH concentration (ng/ml ± sem) in plasma collected in August, November, January, March, May and July from adult Merino rams actively immunized against BSA (Control, n=5) or against testosterone-3-BSA (T-immunized, n=5).

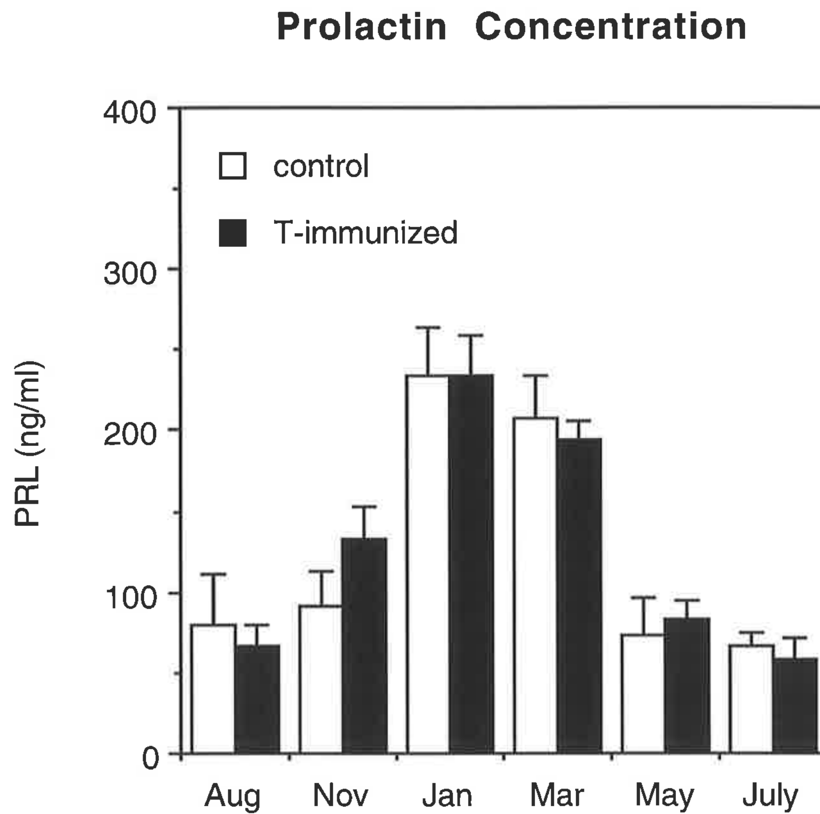


Fig. 8.6.: Mean prolactin concentration (ng/ml ± sem) in plasma collected in August, November, January, March, May and July from adult Merino rams actively immunized against BSA (Control, n=5) or against testosterone-3-BSA (T-immunized, n=5).

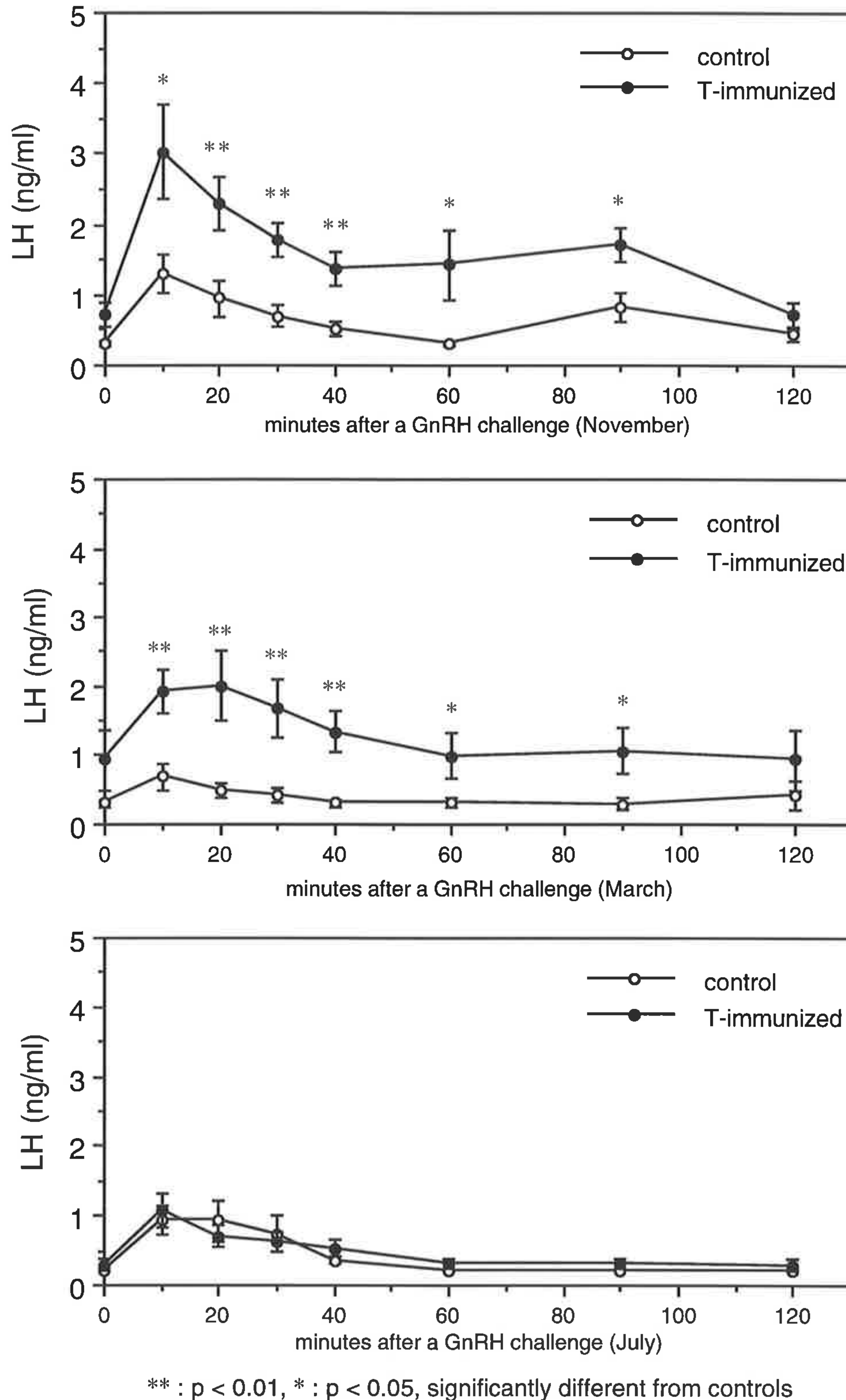
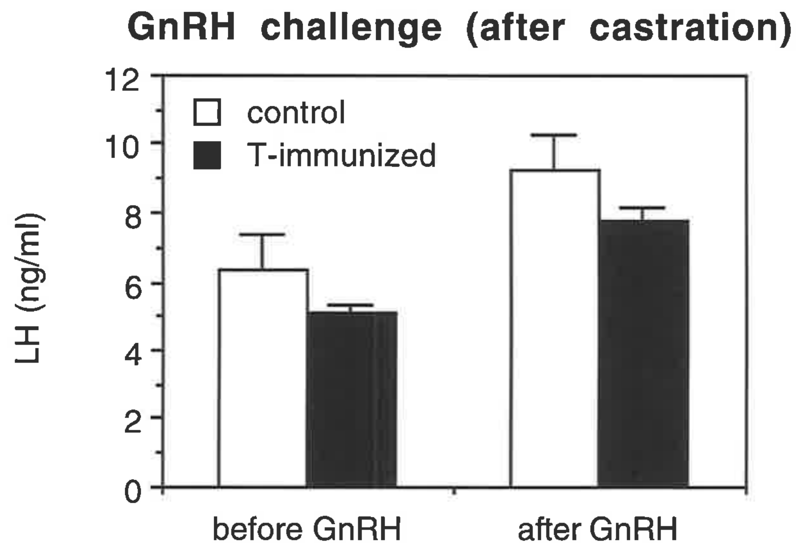


Fig. 8.7.: Time course of circulating LH (mean: ng/ml ± sem) after a single i.v. injection of GnRH (5 ng/kg BW) in November (top), March (middle) and July (bottom) in adult Merino rams actively immunized against BSA (Control, n=5) or against testosterone-3-BSA (T-immunized, n=5).



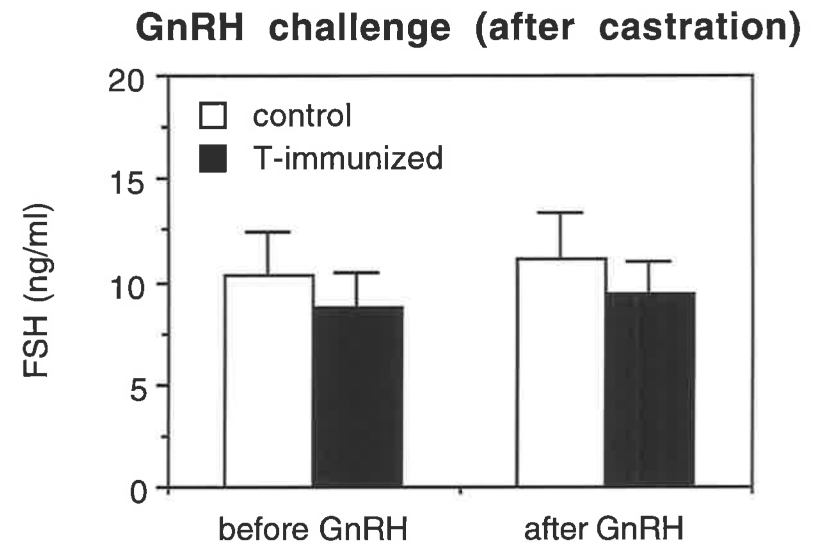


Fig. 8.8.: Mean LH concentration (ng/ml ± sem; left fig.) and mean FSH concentration (ng/ml ± sem; right fig.) measured in the jugular venous plasma before and after a single i.v. injection of GnRH (5 ng/kg body weight) to castrated Merino rams actively immunized against BSA (Control, n=5) or against testosterone-3-BSA (T-immunized, n=5). Blood samples were drawn at 10-min intervals for 4 hours before the GnRH injection and for 2 hours after the GnRH injection and "pre GnRH" and "post GnRH" pools were made before assessment. (GnRH challenge done in October).

LH (~1.6 fold increase) than for FSH (~1.1 fold increase). No significant differences were found between the mean values measured in control and in T-immunized castrated rams, before or after the GnRH challenge.

8.3.10. Testosterone concentration after a hCG challenge

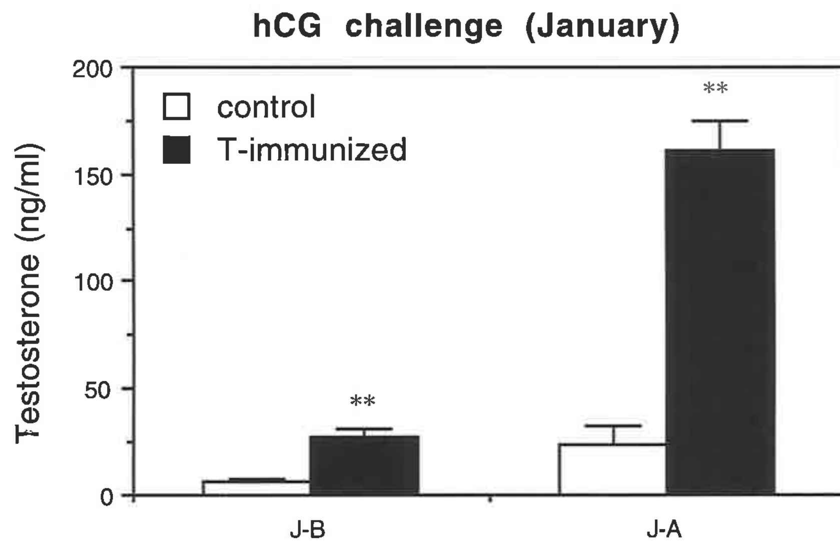
The testicular responsiveness to a hCG challenge evaluated in January (in conscious rams) is shown in fig. 8.9. Mean circulating testosterone concentrations before and after the hCG injection were significantly more elevated ($p < 0.01$) in T-immunized rams than in controls (N.B: in this assay, the highest testosterone concentration which has been measured was 200 ng/ml and if the value found in samples collected after a hCG challenge from T-immunized rams was above that value, these plasma samples were given the value of 200 ng/ml). The overall testosterone response following the hCG injection was significantly more pronounced ($p < 0.01$) in T-immunized rams (average: 6.24 ± 0.93 -fold increase in testosterone) than in controls (average: 4.47 ± 1.29 -fold increase in testosterone) In both groups, testosterone concentrations remained above pre-treatment values for at least 120 minutes after the hCG injection.

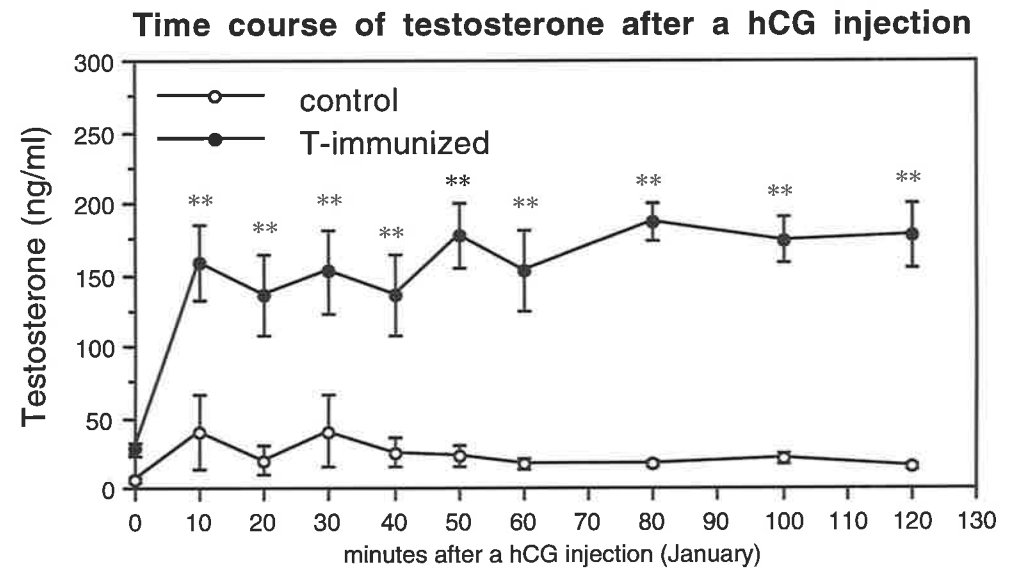
The testosterone response to a single dose of hCG measured at the end of the experiment in July (in anaesthetized rams) is summarized in fig. 8.10. Before and after the hCG injection, T-immunized rams had significantly ($p < 0.01$) more testosterone in the jugular as well as in the right and left internal spermatic vein samples than the controls. Testosterone level in the right and left internal spermatic vein increased significantly after the hCG challenge in both groups ($p < 0.05$). However, in the jugular plasma of three T-immunized and in the five controls, the rise in testosterone level was low (one to two-fold increase in testosterone) so that mean values measured after the hCG injection were not significantly different than that measured before the hCG injection. In the remaining two T-immunized rams (#40 and #44), the rise in testosterone level following the hCG injection was still very pronounced (more than 15-fold increase in testosterone).

8.3.11. Total testicular blood plasma flow (TTBPF)

TTBPF has been successfully measured in 18 testes (9 testes from 5 controls and 9 testes from 5 T-immunized rams). TTBPF per testis (ml/min) or per unit weight of testis (µl/g/min) did not

As the main point of this study was to demonstrate a difference between the immunized and control rams, not to determine precisely the response of the immunized rams, these measurements were not repeated at a higher dilution





**: p < 0.01, significantly different from controls

Fig. 8.9.: Left fig.: Mean testosterone concentration (ng/ml ± sem) measured in the jugular venous plasma (J), in January, before (B) and after (A) a single i.v. injection of hCG (20 I.U./kg BW) to adult Merino rams actively immunized against BSA (Control) or against testosterone-3-BSA (T-immunized). The "pre hCG" value for each group is the mean of 19 samples/ram x 5 rams, while the "post hCG" value is the mean of samples collected at 10-min intervals for one hour, then at 20-min intervals for the next hour.

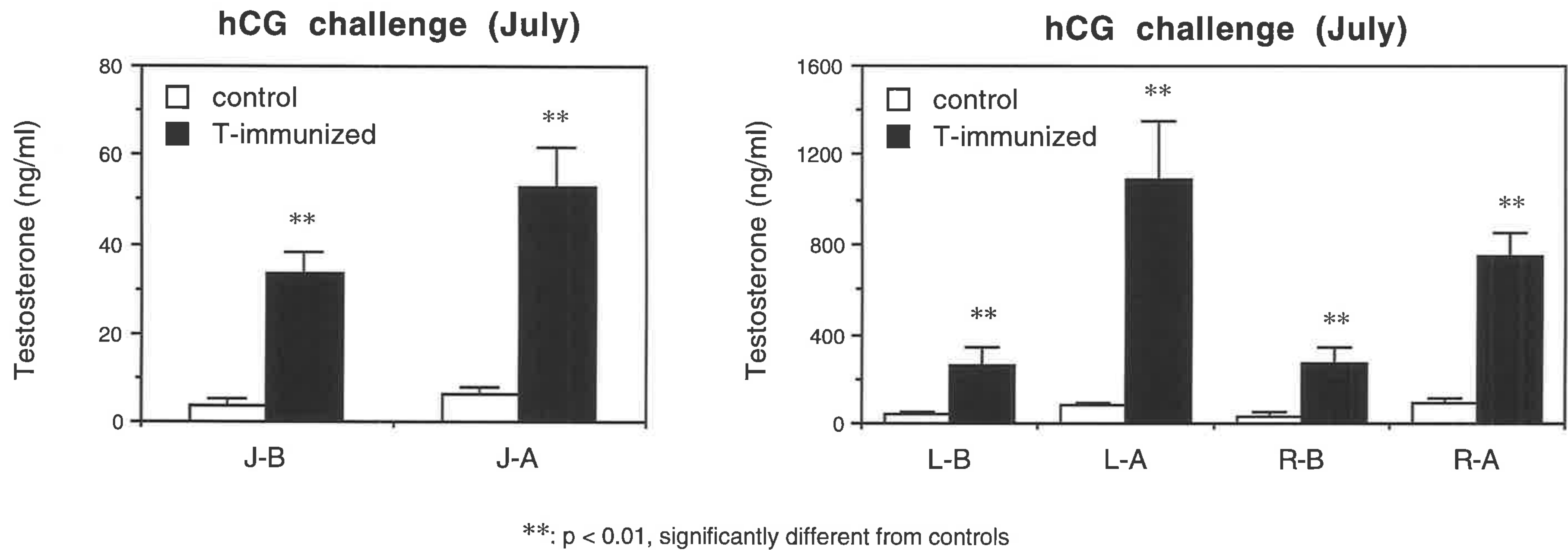


Fig. 8.10.: Mean testosterone concentration (ng/ml ± sem) measured in the jugular (J) and in the left (L) and right (R) internal spermatic vein before (B) and after (A) a single i.v. injection of hCG (20 I.U./kg BW) to adult Merino rams actively immunized against BSA (Control, n=5 rams) or against testosterone-3-BSA (T-immunized, n=5 rams). Blood samples were drawn at 10-min intervals for 40 minutes before the hCG injection and for 100 minutes after the hCG injection and "pre hCG" and "post hCG" pools were made before assessment. (hCG challenge done in July)

change significantly following a hCG injection (fig. 8.11). TTBPF per testis did not vary significantly during the 140 minutes sampling period in either group but TTBPF per unit weight of testis, in controls, decreased significantly ($p < 0.05$) towards the end of the sampling period. Overall, TTBPF per testis was significantly higher in controls than in T-immunized rams ($p < 0.05$), however, for TTBPF per unit weight of testis, the difference between the two groups was significant ($p < 0.07$) only on two occasions during the sampling period although the other values also tended to be higher in controls.

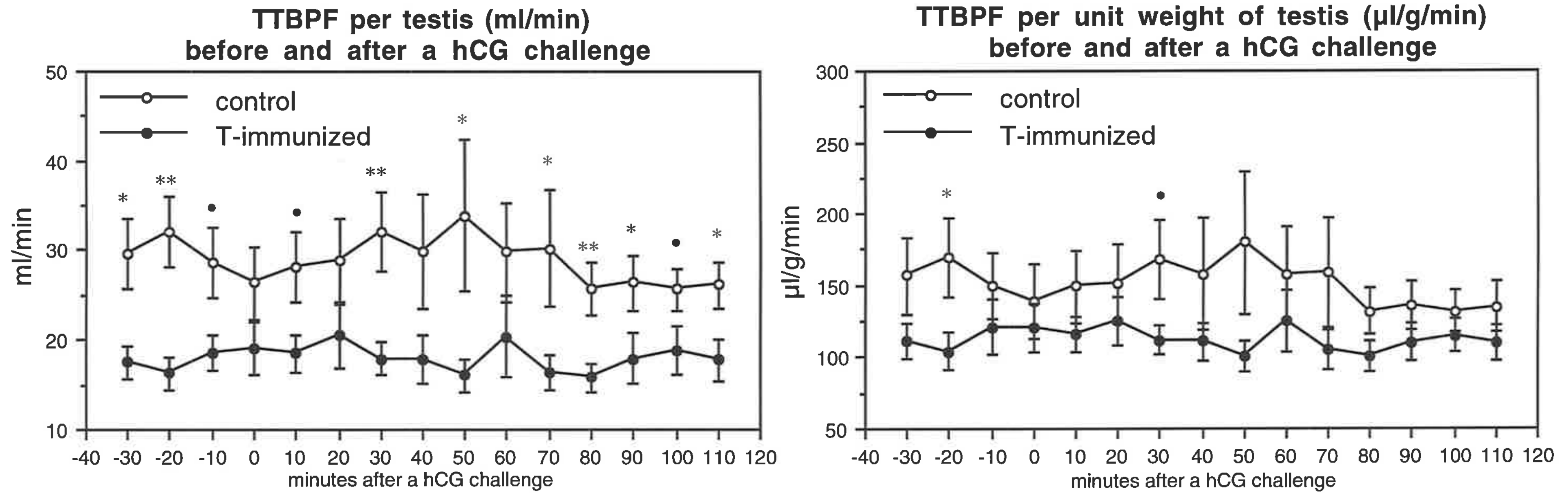
8.3.12. Testosterone production.

The production of testosterone has been calculated for 18 testes (9 testes from 5 controls and 9 testes from 5 T-immunized rams). For the T-immunized rams, mean testosterone production by one testis (ng/min) increased significantly ($p < 0.01$) after the hCG injection. For the control rams, although mean testosterone production by one testis (ng/min) tended to increase after the hCG injection, the difference was not statistically significant. Before and after stimulation with hCG, mean testosterone production by one testis was significantly higher ($p < 0.05$) in T-immunized rams compared with the controls (table 8.3.).

8.3.13. Sperm concentration in ejaculates

Mean sperm concentrations in ejaculates collected in November (before the first booster injection), and later on in May and July were not significantly different between the two groups of rams (fig. 8.12). However, one must note that for the first collection (November), two T-immunized rams (#40 and #48) were not included in the data since they did not mount the teaser ewe and no ejaculate had been obtained from them. Furthermore, for the second (May 29) , third (July 4) and fourth (July 11) collection, again one T-immunized ram (#40) was not included in the data for the same reason.

For the last collection (July 26), all rams have been included in the data however the semen from one control (#43) and one T-immunized ram (#40) had to be collected by electroejaculation. Although the mean value obtained for the T-immunized rams, at that time, was slightly below the value obtained for the controls, the difference between means was not significantly different



**: $p < 0.01$, * : $p < 0.05$, • : $p < 0.07$; significantly different from controls

Fig. 8.11.: Total testicular blood plasma flow (TTBPF) per testis (ml/min; left) and per unit weight of testis (µl/g/min; right) before and after a hCG injection (20 I.U./kg BW) in adult Merino rams actively immunized against BSA (Control) or against testosterone-3-BSA (T-immunized). Values are the means ± sem (n=9 testes belonging to 5 rams in each group).

Table 8.3.

Mean testosterone production (μg/min) by one testis before and after a hCG injection (20 I.U./kg BW) in rams actively immunized against BSA (C) or against testosterone-3-CMO:BSA (T).

Treatment (number of testes)	T production per testis (μg/min ± s.e.m.) before hCG	T production per testis (μg/min ± s.e.m.) after hCG
C (n=9 testes)	1.163 ± 0.266^{a}	2.469 ± 0.236^{a}
T (n=9 testes)	4.664 ± 1.060^{b}	15.076 ± 2.172^{c}

values with different superscripts are significantly different at 95%

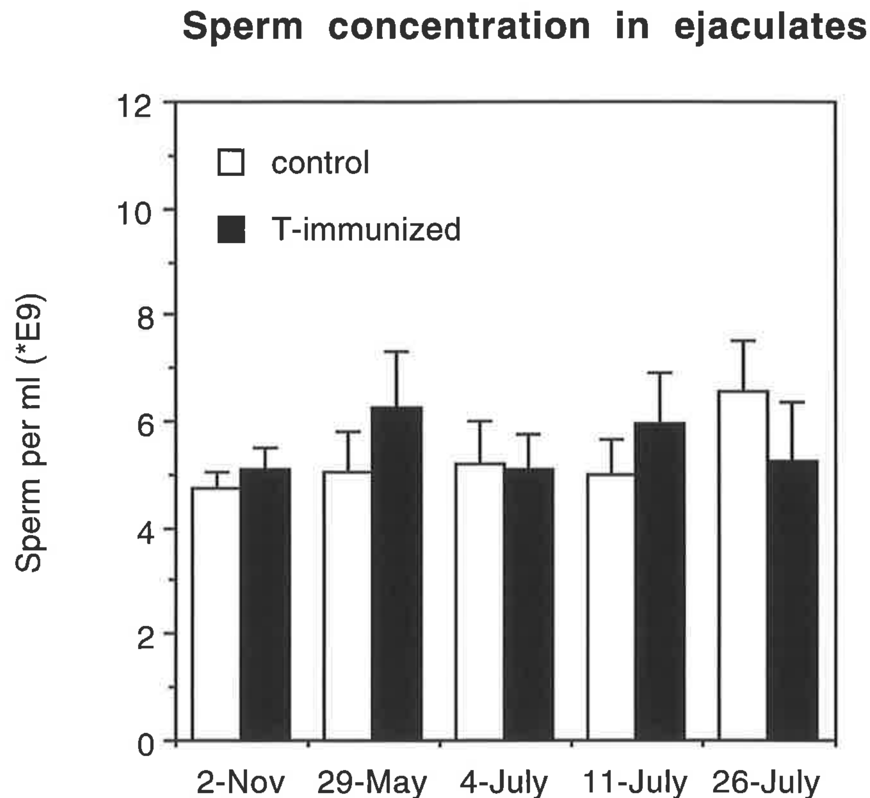


Fig. 8.12.: Mean sperm concentration (sperm/ml x $10^9 \pm$ sem) in ejaculates of adult Merino rams actively immunized against BSA (Control: n=5) or against testosterone-3-BSA (T-immunized: n=3 in November, n=4 in May and July 4 and 11 and finally, n=5 in July 26). The ejaculates were collected with an artificial vagina on each occasion for all rams except for one control and one T-immunized rams for which we had to use electroejaculation for the last collection (July 26).

(fig. 8.12). The lowest value was found in ram #40 (1.875 x 10^9 sperm/ml) during the last collection.

8.3.14. Sexual activity

The number of times that each of the following aspects of behaviour: sniffs, nudges, mounts and intromissions followed by ejaculations, was displayed by each ram within ten minutes exposure to a teaser ewe, in October, January, June and July, are shown in fig. 8.13.
Unfortunately, no tests were done on the rams before immunization.
The number of sniffs was not significantly different between control and T-immunized rams and did not vary significantly with time in both groups although it tended to decrease slightly in controls in July. The number of nudges also tended to decrease with time in controls although this was not statistically significant. On the other hand, the number of nudges tended to increase in T-immunized rams but, here again, this rise was not statistically significant. The number of nudges was nevertheless, significantly lower in October and significantly higher in July in T-immunized rams. The analysis of variance of the number of mounts did not show a significant treatment effect although, a significant time effect ($p < 0.01$) and a significant "treatment x time" interaction ($p < 0.01$) were found. Indeed, the number of mounts increased significantly with time in T-immunized rams while it remained constant in controls. Considering the number of mounts culminating in ejaculation (intromission and ejaculation), the control rams were more successful than the T-immunized rams, the mean difference being significant in October ($p< 0.01$) and in June ($p < 0.07$). In both groups, the number of mounts accompanied by ejaculation did not vary significantly with time.

Few T-immunized rams did not perform any mount culminating in ejaculation during the various trials (2 in October, 1 in January, 3 in June and 1 in July). Ram #40 was especially very inactive during the first three trials (no nudges or mounts) however, in July, this ram performed the highest number of sniffs, and a high number of nudges and mounts (all incomplete).

8.3.15. Testicular weight and volume, and epididymis weight at castration

Testicular weight and volume and, epididymis weight at castration are summarized in table 8.4. Immunization treatment did not significantly affect testicular weight or volume at castration. However, epididymis weights were significantly reduced in T-immunized rams ($p < 0.05$). The

The ratio obtained between epididymis weight and testicular weight (EW/TW) was not significantly affected by the immunization treatment.

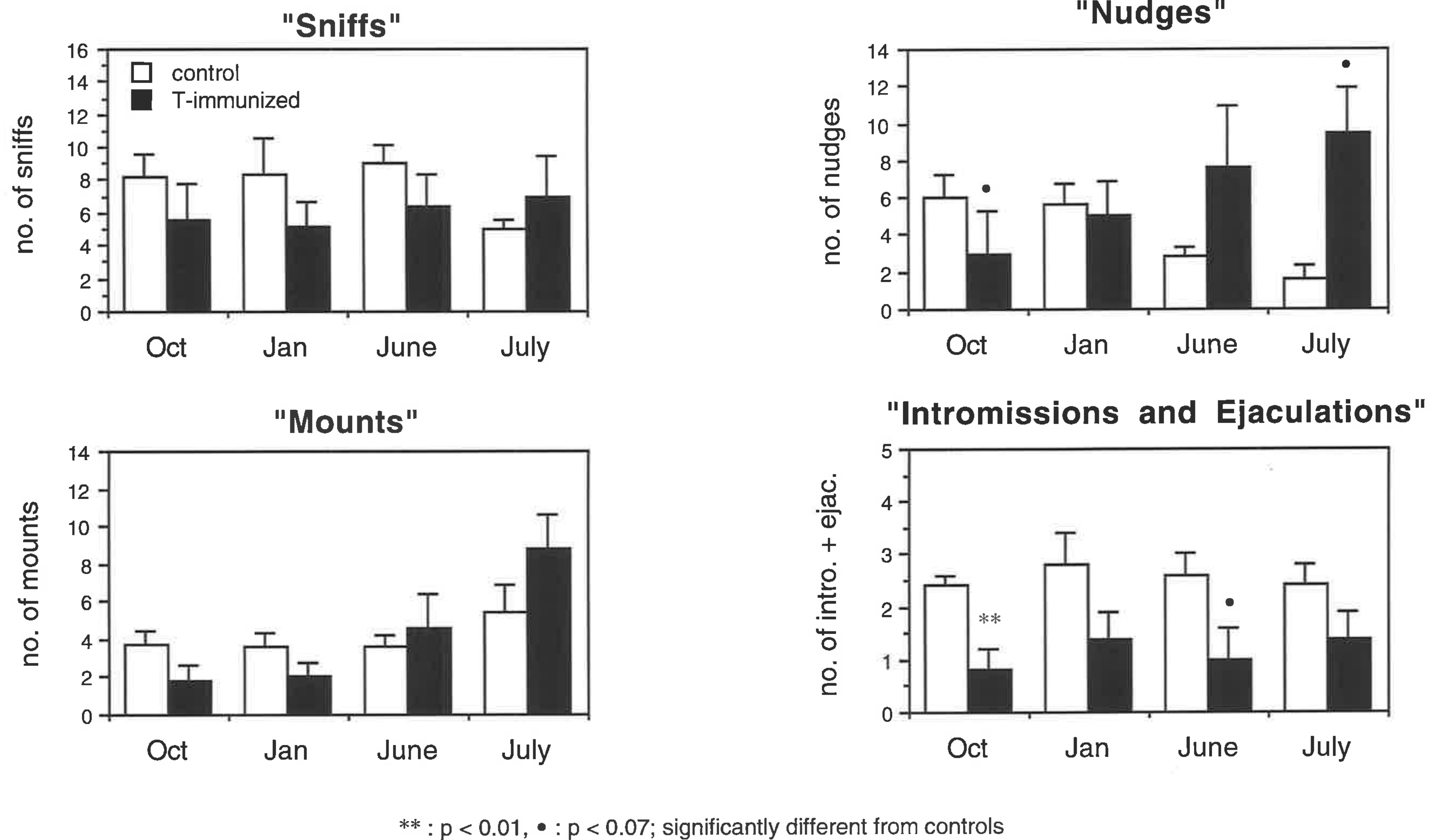


Fig. 8.13.: Sexual activity (number of sniffs, nudges, mounts and intromissions followed by ejaculations) in adult Merino rams actively immunized against BSA (Control) or against testosterone-3-BSA (T-immunized) within ten minutes exposure to a teaser ewe. Libido trials were repeated at four occasions for each ram. Values are the means ± sem (n=5 rams in each group).

Table 8.4.

Testicular volume, testicular weight (TW) and epididymis weight (EW) at castration, and EW/TW ratio, in rams actively immunized against BSA (C) or against testosterone-3-CMO:BSA (T).

Treatment	Testicular Volume[b] (cm^3 ± s.e.m.)	Testicular Weight[a] (g ± s.e.m.)	Epididymis Weight[a] (g ± s.e.m.)	EW/TW ratio (% ± s.e.m.)
C (n=5)	238.84 ± 22.65	195.62 ± 15.96	40.51 ± 2.96	21.3 ± 1.0
T (n=5)	197.91 ± 27.03	162.39 ± 21.37	30.99 ± 2.01 *	20.0 ± 1.7

*: $p < 0.05$, significantly different from controls

[a] : The testis and the epididymis were separated from one another immediately after castration and weighed separately.

[b] : Testicular diameter and length were measured with calipers immediately after castration and the volume was estimated by assuming that the testis is a prolate spheroid (Setchell and Waites, 1964). It was calculated according to the formula $1/6\ \pi\ a^2 b$ (a= largest width; b=length, of the testis).

lowest testicular weight (92.9 g), testicular volume (102.9 cm^3) and epididymis weight (23.72 g) were found in one T-immunized ram (#40). The correlation coefficient between calculated testicular volume and testicular weight at castration was r: 0.982 ($p < 0.01$).

8.3.16. Daily sperm production

The immunization treatment did not significantly affect daily sperm production in July although both values (DSP/gram testis or DSP/testis) tended to be lower in T-immunized rams (table 8.5.). The lowest values (DSP/gram testis : 2.68 x 10^6 and DSP/testis: 0.25 x 10^9) were found in one T-immunized ram (#40).

8.3.17. Spermatogenesis and testicular histology

In control rams, the general aspect of the seminiferous tubules appeared normal and contained numerous meiotic figures and apparently normal spermiogenesis with round and/or elongated spermatids. Spermatozoa were released in the lumen of many seminiferous tubules in all these rams. In four out of five T-immunized rams, the general aspect of the seminiferous tubules also appeared normal and spermatozoa were released into the lumen of a large number of tubules. However, in the fifth T-immunized ram (# 40), we have observed disorganization of many seminiferous tubules in which the number of spermatids and spermatozoa seemed considerably reduced. No obvious abnormalities were observed in the interstitial tissues of the control and T-immunized rams.

8.4. DISCUSSION

We have clearly demonstrated, in that experiment, that the hypothalamic-pituitary system of the postpubertal Merino ram was able to react adequately with increased gonadotropin hormone release, following active immunization against testosterone-3-BSA, and that the level of circulating testosterone was consequently markedly elevated. Furthermore, we have shown that these hormonal changes could be observed over a relatively long period of time (from November until July) probably because a high level of binding sites (titre: ~ 23% at dilution 1:5000) have been maintained in circulation with the help of regular booster injections. However, the improvement in sperm production that we have predicted in our hypothesis has not been

Table 8.5.

Daily sperm production (DSP ± s.e.m.) at castration in rams actively immunized against BSA (C) or against testosterone-3-CMO:BSA (T)

Treatment	Daily sperm production No sperm per testis (x 10^9)	Relative DSP No sperm /g testis (x 10^6)
C (n=5)	5.90 ± 0.62	30.53 ± 2.89
T (n=5)	4.22 ± 1.29	23.29 ± 6.03

demonstrated at the times of semen collection (in November, in May and on three occasions in July) and at the end of the experiment as shown by statistically comparable sperm concentration in ejaculates and comparable DSP values between control and T-immunized rams.

Our data on mean gonadotropin concentrations (especially LH) measured at regular intervals indicated that the differences observed between T-immunized and control rams were most apparent between November and March (3 to 6 months after the start of the immunization) and less pronounced thereafter. Moreover, we have observed that the pituitary responsiveness to GnRH was significantly more pronounced in T-immunized rams than in controls in November and March but was comparable in July. In controls, mean gonadotropin levels and pituitary responsiveness to a GnRH challenge did not vary significantly from one bleeding to another. Therefore, it seems that the temporal changes in gonadotropin secretion observed in T-immunized rams do not reflect seasonal changes within the hypothalamic-pituitary axis, but more likely reflect variation in the amount of free testosterone able to act on this axis during the course of immunization. Similar transient changes in LH concentrations have also been observed in other animals actively immunized against testosterone over a long period of time (in rabbits: Thorneycroft et al., 1975; in Rhesus monkeys: Wickings and Nieschlag, 1978; in bulls: D'Occhio et al., 1987).

Interestingly, after castration, we did not find any significant differences in LH and FSH secretion before and after a GnRH injection, between the two groups of rams. Thus, it seems that no persistent changes in the functioning of the hypothalamic-pituitary axis had occurred in the T-immunized wethers (e.g. pituitary gonadotropes more responsive to GnRH) that would maintain a more pronounced response of these rams after the removal of their testes. This observation supports the view that the hypothalamic-pituitary axis may become adjusted to the presence of anti-testosterone antibodies after a long period of immunization since the pituitary responsiveness to a GnRH challenge was already comparable between both groups of rams in July. Furthermore, this finding supports the view that the increase in gonadotropin secretion observed before castration in T-immunized rams is mainly due to antibody interference with the

L. 9: D'Occhio and Brooks (1983) reported definite seasonal modifications of the hypothalamic-pituitary-testicular axis of Merino rams kept on pasture at the Waite Agricultural Research Institute (Adelaide, South Australia).

testicular products (e.g. binding of T --> reduction in free T) participating in gonadal negative feedback (Nieschlag and Wickings, 1977, 1978; Haynes and Southee, 1984).

We have noticed that the testicular responsiveness to hCG, in controls and in three T-immunized rams, was more pronounced in January than in July (although no significant difference was found) while, in the remaining T-immunized rams (#40 and #44), the response to hCG was still very pronounced in July. In controls, the lower response observed in July may possibly be due to the presence of Nembutal™ (sodium pentobarbitone) within the system (side-effect in anaesthetized rams), but could also reflect a seasonal change within the testis as indicated by the decrease in testicular volume observed towards the end of the experiment. Apart from a seasonal change in Leydig cells sensitivity to hCG (e.g. seasonal decrease in testicular gonadotropin receptors -see Barenton and Pelletier, 1983) that could have occurred in our rams, one could think that some factors, other than LH, could have been involved in maintaining testosterone production at a higher level in January For instance, it is possible that high level of prolactin measured in this month had exerted a stimulatory influence on the testis (e.g. synergism with LH and T - see Ravault et al., 1977; Klindt et al., 1985; Yarney and Sanford, 1989). (see INSERT 9)

For the T-immunized rams exhibiting a lower testosterone response to hCG in July, it is likely that their system had started to counteract the effects of the anti-testosterone antibodies at that time in contrast to the other T-immunized rams (#40 and #44) which were still very much affected by the presence of the anti-testosterone antibodies (as indicated by their pronounced response to the hCG injection and the low performances displayed by ram #40 - see below-). Thus, as for the decrease in pituitary responsiveness to GnRH and in gonadotropin secretion occurring towards the end of the experiment in T-immunized rams, the temporal change in the testicular responsiveness to hCG also seems to indicate that the system become adjusted to the presence of anti-testosterone antibodies after a long period of immunization (re-establishment of a new equilibrium). Indeed, if the amount of testosterone being produced becomes sufficient to saturate the antibody-binding sites in circulation, the free (biologically active) fraction may

become large enough in quantity to exert its effect on target organs and therefore, re-establish the pre-treatment status (see Martin, 1984).

Nevertheless, our observations on testosterone secretion in the present experiment seem to demonstrate an overall improvement of the steroidogenic capacity of the testis in T-immunized rams. In support of this view, it has been demonstrated that an increase in the number and/or size of Leydig cells (hyperplasia and/or hypertrophy) occurs following active immunization against testosterone in rabbits (Nieschlag and Wickings, 1977, 1978) and in bulls (Wrobel et al., 1990). Furthermore, since a very pronounced rise in plasma testosterone had occurred as early as ten minutes after the hCG injection in our T-immunized rams, in January, we believe that a simple change in metabolic clearance rate would not be sufficient to explain the high level of circulating testosterone found at that time. We, indeed, believe that the capacity to produce testosterone was really enhanced. A similar initial sharp rise in testosterone level following a hCG injection has also been found in bulls actively immunized against testosterone (D'Occhio et al., 1987).

Because the elevated concentrations of gonadotropins measured in T-immunized rams did not reach the values found in castrated rams (see values measured in October after castration, fig. 8.13.), it seems that some hormone was still able to act on the hypothalamic-pituitary system during the whole study. Indeed, one should consider that the testis can secrete hormones other than testosterone (e.g. inhibin, oestradiol-17ß) which could participate in negative feedback (see oestradiol effects demonstrated in chapters 5 and 7) and which could still affect the secretion of gonadotropins even if testosterone has been neutralized by specific antibodies. Moreover, an increase in various steroids are likely to occur in T-immunized animals as a result of increased substrate availability subsequent to an increase in gonadotropin stimulation (Nieschlag and Wickings, 1978; Haynes and Southee, 1984). For example, an increase in plasma oestrogen level in prepubertal boars actively immunized against testosterone has been observed six months after the start of the treatment (Thompson et al., 1985). Similarly a 3- to 4-fold increase in Δ^4-androstenedione has been observed in T-immunized rabbits which indicated a stimulation of the

biosynthetic pathways for testosterone (Nieschlag and Wickings, 1978). In male monkeys immunized against testosterone, serum DHT levels were 5-to 10-fold higher than control levels which reflected increased 5 α-reduction by testicular tissues although, in this case, it is likely that the increase in DHT also reflected increased binding to the antibodies that cross-react with DHT (Wickings and Nieschlag, 1978). Unfortunately, we have not measured the plasma level of oestrogens, DHT or any other androgens in the present experiment and therefore, we can only assume that similar hormonal changes had occurred in our T-immunized rams and had helped maintain gonadotropin secretion at a lower level than that found in castrated rams.

The significant increase in testicular volume observed in January, in T-immunized rams, may indicate that, during this month, an improvement in sperm production had occurred but since we did not collect any semen during that month, this has not been confirmed. Thereafter, the increase in testicular volume did not persist and, on the contrary, a slight tendency for a lower testicular volume and weight in T-immunized rams has been observed at the end of the experiment. Nevertheless, it seems that all rams produced spermatozoa during the whole study as reflected by the initial increase in testicular volume (between August and October) and by the maintenance of a respectable testicular volume thereafter. This was, in fact, confirmed (in five controls and in four T-immunized rams) by semen collections done in November, in May and in July. Complete spermatogenesis has also been confirmed in each ram, including the fifth T-immunized ram (#40), at the end of the experiment by semen collection and by histological examination of testicular tissues.

Since spermatogenesis is considered to be testosterone-dependent, it is likely that some testosterone must escape from the antibodies when passing through the interstitial fluid or lymph that bathes the Leydig cells, allowing this essential hormone to reach its homologous receptors within the seminiferous tubules. However, it is also possible that some testosterone was formed within the Sertoli cells which can convert progesterone or other substrates coming from highly stimulated Leydig cells, into testosterone (Bardin et al., 1988). In both cases, testosterone presumably can still exert its biological action within the seminiferous tubules and permit maintenance of spermatogenesis even in the presence of circulating anti-testosterone antibodies

(Nieschlag and Wickings, 1978). Whether spermatogenesis can be maintained by steroids other than testosterone (e.g. DHT) in T-immunized animals, remains to be demonstrated. Interestingly, the results form our previous experiment (see chapter 5) have indicated that even in the presence of a high concentration of testosterone, LH and FSH, spermatogenesis may not be adequate and may indeed require the presence of oestrogens for a synergistic effect with other essential hormones.

Thus, our results demonstrating maintenance of spermatogenesis following active immunization against testosterone are in agreement with that reported by other authors (Nieschlag et al., 1975c; Walker et al., 1984; Haynes and Southee, 1984; D'Occhio et al., 1987).

In one of the T-immunized ram (#40), we have observed important disorganization within the seminiferous tubules which probably explains the extremely low DSP values found in that ram, at the end of the experiment. Moreover, in that particular ram, many other observations suggest that the level of biologically active testosterone was insufficient to allow good performance of many target organs (lowest final testicular weight and volume, lowest sperm concentration in ejaculate at the end of the experiment, lowest epididymal weight, no mount culminating in ejaculation). In agreement with these findings, we have also found that the level of activity of the hypothalamic-pituitary-axis was generally very elevated in that ram (highest mean gonadotropin concentration found during each intensive bleeding except in May [for LH and FSH] and in July [for FSH], highest LH response to GnRH in November and March). Moreover, the steroidogenic function in that ram was also markedly enhanced (highest mean testosterone concentration found in January and July, highest testosterone response to a hCG in January).

Since the titres measured in ram #40 were comparable to those found in the other T-immunized rams, we assume that the affinity of some antibodies produced by that particular ram must have been higher and had allowed a more efficient neutralization of circulating testosterone rather than the antibodies circulating in the other rams. However, since we did not perform any *in vivo* or *in vitro* binding studies that would allow us to make precise comparisons between the characteristics of the antisera produced by the five T-immunized rams, this interpretation remains

L. 12: In the present study, testicular weight and the ratio obtained between epididymis weight and testicular weight (EW/TW) were not significantly affected by the immunization treatment. Nevertheless, we believe that the significant reduction in epididymis weight observed in T-immunized rams clearly indicated that this organ was more affected by a reduction in T bioavailability following immunoneutralization of T. This may indicate that the threshold regarding T requirement within the epididymis is higher than that within the testis itself. Interestingly, one must remember that, for a better efficiency, T needs to be converted to DHT in the epididymis (but not in the testis) and that this fact could perhaps explain the higher T requirement within the that organ. Another explanation could be that the neutralization of T by the antibodies was perhaps less efficient within the testis (e.g. due to the presence of the blood-testis barrier and/or due to higher affinity of the intratesticular T receptors) than within the epididymis.

Furthermore, as the ratio obtained between epididymis weight and testicular weight (EW/TW) is an "indirect" parameter, it is difficult to give a "physiological" interpretation of such parameter (e.g. the physiological significance of this ratio remains uncertain; it may never be affected even in a situation where both variables (EW and TW) are considerably reduced or increased).

entirely speculative. Furthermore, although the specificity of the antisera has not been estimated in this experiment, we assume that the antibodies produced by each T-immunized ram cross-reacted with other steroids in the same way as the antibodies produced in other animals using the same conjugate (i.e. high degree of cross-reactivity with DHT and negligible cross-reactivity with oestrogens; see Nieschlag and Wickings, 1977; 1978) and, therefore, that the results obtained in these rams could be due to the neutralization of both androgens: testosterone and DHT.

Our results indicate that some deleterious effects could appear in adult rams following active immunization against testosterone. Indeed, the number of mounts accompanied by ejaculation (the consummatory phase of sexual behaviour) was significantly reduced in T-immunized rams, in October and in July, and the weight of their epididymis was significantly lower at the end of the experiment. (see INSERT 12) These results disagree with that from other investigators indicating that sexual activity did not change in young rams (Haynes and Southee, 1984) or young boars (Thompson et al., 1985) actively immunized against testosterone and also disagree with that from D'Occhio et al. (1987) reporting comparable epididymis weight in T-immunized and control bulls. However, since the immunization treatment was initiated before puberty in these studies, the difference with our results may indicate variation due to the age of the animal models. It is also likely that the different results obtained reflect important differences in the characteristics of the antibodies produced by these animals (e.g. very low titre obtained in bulls vs high titre in our T-immunized rams; important genetic differences in the immune response).

Finally, as for the ram lambs actively immunized against oestrogens (see chapter 5), we have found an important reduction in testicular blood flow (TTBPF per testis being significantly reduced in T-immunized rams) at the end of the experiment. Although it is still unknown whether the major hormonal changes occurring following immunization against testosterone are sufficient to produce a change in testicular blood flow, an alteration in the spermatogenic function such the that observed one T-immunized ram (#40) will certainly affect the amount of blood circulating through the testis (Setchell and Brooks, 1988). As in the previous

studies (chapters 3 and 5), we have shown that testicular blood flow was not affected by the hCG injection in the short-term (within 100 minutes).

In conclusion, our data shows that, in Merino rams, the epididymis and the centre responsible for sexual behaviour are more likely to be negatively affected by long-term immunity against testosterone. Furthermore, although we can observe persistent increments in gonadotropin and testosterone secretion in rams actively immunized against testosterone, these hormonal changes are not beneficial to the spermatogenic function and can be associated with a significant reduction in testicular blood flow.

L. 23-...

Since immunoneutralization of oestradiol was very effective as shown by a marked rise in gonadotrophin secretion (i.e. suppression of the negative feedback action on the hypothalamic-pituitary axis), and since this neutralization has not led to an increase in the rate of testicular maturation, we conclude that circulating oestradiol does not restrain testicular development in ram lambs. Moreover, the fact that we have observed a very important decline in testicular size after three months of immunization in nearly all our E_2-immunized lambs (as well as other deleterious effects in some E_2-immunized lambs- see chapter 5), suggests that oestradiol might even have a positive role to play within the testis in developing ram lambs.

CHAPTER 9. GENERAL DISCUSSION

The purpose of this chapter is to discuss and place into perspective our main observations on pubertal development, and the various consequences of immunizing developing ram lambs against oestrogens. The effects of immunoneutralization of testosterone in adult rams over a period of twelve months will also be discussed. Indeed, all along this discussion, some comparisons will be made between the observations made in our E_2-immunized ram lambs, the ones obtained in our T-immunized adult rams, and others reported by various authors.

About our working hypotheses:

The period of transition into puberty of the South Australian Merino ram lambs has been the main subject of interest of this thesis. We have shown that the negative effects of oestrogens on the hypothalamic-pituitary-testicular axis of the developing rams could be efficiently neutralized by antibodies produced actively, but less efficiently by the antibodies received passively. However, since the immunoneutralization of oestrogens during prepuberty has not led to an improvement of testicular maturation, and therefore, since the time of achievement of puberty has not been advanced, our starting hypothesis has to be rejected.

In addition, another working hypothesis has to be rejected since long-term immunoneutralization of testosterone in adult Merino rams has not led to an improvement of the spermatogenic function, in spite of enhancement of the activity of the hypothalamic-pituitary-testicular axis.

About our observations in the actively immunized sheep:

To our knowledge, this is the first time that active immunization against oestradiol-17ß has been used to study pubertal development in ram lambs. Our data clearly show that the prepubertal Merino ram lambs can respond well to a standardized immunization protocol and can produce an adequate amount of oestradiol antibodies during a 16-week study period (see chapter 5). (to INSERT 23)

L. 3-7: One must be cautious when using comparisons with other species like rats (since in this species the major androgen produced during the prepubertal period is not testosterone). Indeed, the predominance of testosterone production throughout prepubertal development distinguishes male sheep from other mammals, in which androstenedione (e.g. bulls) and other 5α-reduced (e.g. rats) products are preferentially secreted during juvenile life (Setchell and Brooks, 1988).

It is still unclear whether oestrogens exert a direct effect on the seminiferous tubules since there is still controversy concerning the presence of oestrogen receptors in Sertoli cells and since no such receptors have been identified in the germinal cells so far (Bardin et al., 1988). However, the fact that some observations indicate that the conversion of testosterone into oestrogen by rat Sertoli cells (aromatization) is modulated by the stage of the spermatogenic cycle, suggests that the formation of this gonadal steroid needs to be closely monitored in order to obtain the special requirements of each stage of the spermatogenic cycle (Boitani et al., 1981; Saez et al., 1987).

(see INSERT 3-7)

Several studies have also shown that oestrogens can influence directly testicular androgen production *in vivo*, in various species (Moger 1980; Sanford 1985; 1987a; D'Occhio et al., 1987; Sanford et al., 1991). Whether oestrogens act on other testicular cells such as the myoid cells surrounding the seminiferous tubules has not been determined yet. Overall, one must keep in mind that important age, season, and species differences may exist with regard to the physiological role played by oestrogens in the regulation of spermatogenesis and other testicular functions.

In our active E_2-immunization study, the most striking observation was that a pronounced decline in testicular volume had occurred after 8-16weeks of treatment in some of the E_2-immunized ram lambs and that important abnormalities in their seminiferous tubules could also be observed (e.g. large vacuoles within the seminiferous epithelium and nearly complete absence of germ cells - see fig. 5.11-). Other deleterious effects have also been identified, at the end of the experiment, by evaluation of daily sperm production (e.g. very low DSP in one particular E_2-immunized lamb) and by measurement of testicular blood flow (e.g. TTBPF per unit weight of testis was significantly reduced in E_2-immunized lambs - see fig. 5.10-). We do not know whether the important hormonal changes (increased circulating LH, FSH and T levels) observed following active immunization against oestradiol have led, in the long term, to the major changes in TTBPF and to alteration of the spermatogenic function. However, since the amount of blood flowing through the testis is determined largely by the mass of the tubules (Setchell and Brooks, 1988), we think that the reduction in TTBPF reflected, in part, the lost of

TTPBF in

L. 1: The low variability seen in TTPBF in E_2-immunized (EN) ram lambs (Fig. 5.10, P.156), suggest an homogenous response of all animals for this parameter, whereas abnormalities of seminiferous tubules have been observed only in two ram lambs (#15 and #3; in which TTPBF has not been measured). Thus, it is possible that the change in testis blood flow occurs well before any obvious abnormalities could be observed in the histology of the testis. Indeed, we believe that an important decrease in testicular blood flow will eventually lead to a lack of oxygen (and nutrients) available for the seminiferous tubules which are avascular and must rely on oxygen diffusing in through tubules walls (Setchell, 1978). Consequently, some signs of degeneration will become apparent within the seminiferous tubules due to this decrease in oxygen and nutrient supplies.

Still, at the moment, we agree that all interpretations of these alterations in testicular functions observed in E_2-immunized (EN) ram lambs remain highly speculative and more work will certainly be required to clarify the situation and to obtain a clear explanation of the physiological mechanisms involved in the testicular response following immunization against oestradiol.

cellular components within the epithelium of the seminiferous tubules observed in some E_2-immunized lambs. (see INSERT)

A considerable body of evidence now supports the view that spermatogenesis is under the control of a highly complex regulatory network which might vary considerably from one species to another. Generally , it is considered that the two hormones that are proximately required by Sertoli cells to support spermatogenesis in all its phases are FSH and testosterone (Bardin et al., 1988). Still, the mechanism whereby the combination of FSH and testosterone maintains spermatogenesis remains to be clarified. The main function of LH in the males is to stimulate *de novo* synthesis by Leydig cells of testosterone, high concentrations of which must be present in Sertoli cells for sperm production to occur.

The data obtained from our E_2-immunized ram lambs clearly indicate that a marked increase in LH, FSH and testosterone secretions occurring during the period of transition into puberty is not sufficient to improve the spermatogenic function and to accelerate testicular maturation. We can speculate that, in these ram lambs, a highly favorable intratubular milieu has not been established since most individuals have not achieved a superior rate of reproductive maturation. Thus, oestrogens may not affect testicular functions in an entirely negative way but may indeed be an essential element required to maintain the proper milieu for the spermatogenic process in ram lambs (e.g. oestrogen participation within a complex interplay of signals from the local environment that regulates the functions of FSH, LH and T on testicular cells). To support this view, there is now increasing evidence that oestrogens could play an important role in developing tissues. For instance, oestrogens have been found to modulate the synthesis of growth factors which are thought to function as autocrine or paracrine regulators of both normal and abnormal growth and differentiation (Sutherland et al., 1988). Certain growth factors have been localized to and are known to be produced by Sertoli cells, including seminiferous growth factor (SGF), somatomedin C / insulin-like growth factor (IGF-1), Sertoli cell-secreted growth factor (SCSGF), transforming growth factor α and ß (TGFα, TGFß) and interleukin-1α (1L-1α) (Bellvé and Zheng, 1989). Thus, for a better understanding of the mechanisms regulating spermatogenesis, we believe that this kind of molecular link should be looked at very attentively.

At the moment, we do not know whether oestrogens regulate the transcription of specific growth factor genes in testicular cells of male sheep and the exact nature of the crucial growth factors as well as their mechanisms of action remain to be determined.

Interestingly, in adult Merino rams actively immunized against testosterone, we have also found a marked improvement in LH, FSH and testosterone secretions that was more pronounced after 3 to 6 months of immunization, and a significant decrease in TTPBF after one year of immunization (see chapter 8). Similarly, this enhancement in hormonal secretion did not result in a persistent improvement of the spermatogenic function. In this experiment, however, although the amount of total circulating testosterone was significantly elevated, the amount of free testosterone (presumably available for target tissues) was assumed to be very reduced (due to the presence of specific antibodies directed against T). This reduction in free "bioavailable" testosterone could, therefore, explain the lack of improvement of the spermatogenic function. However, since spermatogenesis did not seem to be affected by the treatment in most T-immunized rams, it is likely that the neutralization of intragonadal testosterone was never totally complete. In one particular ram that had exhibited a very pronounced response to T-immunization (e.g. most pronounced hormonal changes and lowest level of sexual activity), we have also observed some anomalies in the appearance of its testicular tissues taken after one year of treatment (e.g. disorganization of many seminiferous tubules in which the number of germ cells were considerably reduced). Thus, as for the E_2-immunized ram lambs, we suspect that an optimal intratubular environment has not been established following the immunization treatment and that most T-immunized rams have not expressed their maximal spermatogenic potential.

Androgens and oestrogens are well known to be very potent inhibitors of the activity of the GnRH pulse generator in intact and castrated sheep at various ages (Thiéry and Martin, 1991). Following a reduction in feedback by gonadal steroids due to the presence of neutralizing antibodies, both the frequency and amplitude of GnRH pulses is likely to increase. Therefore, the marked increase in LH and FSH secretion observed following immunoneutralization of oestrogens or testosterone must reflect partly the increase in the activity of the GnRH pulse generator. In the ram, the demonstration of specific oestrogen receptor sites in the

hypothalamus supports a role for oestrogens within the CNS (Pelletier and Caraty, 1981; Glass et al., 1984). Interactions between oestrogens and catecholaminergic, opioidergic and other neurotransmittory systems could be of crucial importance in the control of gonadotropin secretion (for review see Thiéry and Martin 1991). A better understanding of the mechanism of action of testosterone and oestrogens in the CNS, will certainly help us determine the way the GnRH pulse generator is regulated in control and immunized animals.

The increase in pituitary responsiveness to a GnRH challenge (observed in our T-immunized animals) suggests that some important changes may also occur at the pituitary level (such as increase in the number of gonadotropes; increase in GnRH receptors; enzymatic improvement of the synthetic pathways, etc.) following immunoneutralization of gonadal steroid. The nature of these changes has not been determined in the present studies and will have to be specified in future experiments. Although the exact mechanism of steroid actions at the pituitary level also remains to be clarified (e.g. the nature of the genes that are transcribed in presence of E_2 and the ones that are turned off; post-transcriptional regulation, etc.), the confirmation that androgenic and oestrogenic receptors are also present in the ram pituitary (Pelletier et al., 1981) supports the view that both steroids can have direct effects on this gland. Our data clearly show that plasma PRL levels are not affected by immunoneutralization of oestrogens in developing ram lambs or by immunoneutralization of testosterone in adult rams.

In our E_2-immunized ram lambs and in our T-immunized rams, apart from a considerable enhancement in mean plasma testosterone levels reflecting the increased LH stimulation, we have also observed an increase in testicular responsiveness to a single hCG challenge. Therefore, here again, some important testicular changes must have occurred, leading to an improvement of the steroidogenic function (such as increase in the number of the Leydig cells; increase in LH/hCG receptors; enzymatic improvement of the steroidogenic pathways, etc.). The nature of these modifications has not been clarified yet and will also have to be specified in the near future. There is no doubt that our results have pointed out the remarkable steroidogenic capacity of the testes of either the prepubertal Merino ram lambs or the adult Merino rams.

At the moment, the complete mechanism by which LH stimulates steroidogenesis is not known, and it is not yet possible to determine with certainty the site(s) at which oestrogens could antagonize the action of LH, assuming that they do. It is possible that oestradiol induces the production of a protein that can inhibit some important steps in the steroidogenic pathways (Aquilano and Dufau, 1983). The nature of this protein and the mechanism of inhibition need to be determined. On the other hand, it has been shown recently that oestradiol *in vitro* can induce a marked dose-dependent inhibition of RNA synthesis by purified Leydig cells of mature rats as well as impairment of testosterone synthesis after hCG stimulation (Ronco et al., 1988). For the male sheep, the physiologic importance of all these recent *in vitro* observations still remains to be demonstrated. Indeed, the direct effect of oestrogens on Leydig cells has been unequivocally demonstrated in the rat although Daehling et al. (1985) had also reported some direct inhibitory effects of natural and synthetic oestrogens in testosterone release from human testicular tissue *in vitro*. One must, therefore, keep in mind that the improvement in steroidogenic function observed in our E_2-immunized ram lambs could also reflect the neutralization of direct inhibitory effects of oestrogens on Leydig cells (along with increased LH stimulation).

Surprisingly, the high level of circulating testosterone observed in our E_2-immunized lambs did not have any positive effect on the overall body weight gain. This observation suggests that, perhaps, an essential amount of oestrogens is required with testosterone to obtain a notable anabolic effect ("synergistic effect"). Indeed, exogenous oestrogens are commonly used as growth promotants for farm animals (Schanbacher, 1984c; O'Callaghan et al., 1986; Roche et al., 1986; Wagner et al., 1988; Goldspink, 1991; Owens et al., 1993), and may have direct anabolic effects.

As mentioned previously, recent *in vitro* studies on oestrogen-mediated cell growth indicate that oestradiol induces the secretion of specific growth factors. These growth factors may, in turn, affect cell growth by autocrine or paracrine mechanisms and form an obligate link between activation of transcription and oestrogen-induced cellular replication (Bellvé and Zheng, 1989). Proliferation may also be mediated directly through oestrogen receptor-mediated changes in the transcription of specific genes critical to cell cycle progression and DNA synthesis, or indirectly by the release from inhibition by serum-borne growth inhibitory factors. The direct effects of oestrogens on cell

replication still remain speculative and controversial (Sutherland et al., 1988). That oestrogens and androgens (T or DHT) act synergistically in various target tissues (including bones, muscles) is likely for the male sheep. For instance, such synergistic effect has been recognized within the brain of the ram (e.g. to elicit mating behaviour -see Parrott, 1978-) and in tissues of other species (Naftolin et al., 1975; Mawhinney and Neubauer, 1979).

Our results obtained from adult rams actively immunized against T, indicated that the epididymis and the centre responsible for sexual behaviour are more likely to be affected by this treatment in the long term (e.g. significantly reduced number of mounts accompanied by ejaculation, and significantly lower epididymis weight have been observed in T-immunized rams). It is well known that epididymal development and function are strictly regulated by androgens and that sperm maturation itself is dependent upon androgen-controlled secretory products of the epididymal epithelium.(Orgebin-Crist, 1986; Amann, 1987; Brooks; 1987; Fournier-Delpech and Courot, 1987). Furthermore, androgens are essential for the establishment of male courtship, coital behaviour patterns, and maintenance of libido. In this regard, our results confirm that the epididymis do require an adequate amount of androgens for optimal functioning and that immunoneutralization of testosterone can reduce this amount significantly in the long term. Our results disagree with that from D'Occhio et al. (1987) reporting comparable epididymis weight in T-immunized and control bulls. Our results also disagree with that from other investigators indicating that sexual activity did not change in young rams (Haynes and Southee, 1984) or young boars (Thompson et al., 1985) actively immunized against testosterone. It is likely that these different findings reflect species differences or differences in the experimental protocol.

In most of our T-immunized rams, a return to moderate pituitary responsiveness to GnRH, to average gonadotropin levels, and to moderate testicular responsiveness to hCG, after 8-12 months of immunization, indicated that their system was able, with time, to re-establish a new equilibrium within the hypothalamic-pituitary-testicular axis. Indeed, if the amount of testosterone being produced becomes sufficient to saturate the antibody-binding sites in

circulation, the free ("biologically active") fraction may become large enough in quantity to exert its effect on target organs and therefore, re-establish the pre-treatment status (see Martin, 1984). The results obtained after castration of control and T-immunized adult rams support the view that the increase in gonadotropin secretion that persisted in T-immunized rams after many months of immunization was mainly due to antibody interference with testosterone (e.g. reduction in free T), and consequently, to a reduction in the negative feedback action of testosterone (Nieschlag and Wickings, 1977, 1978; Haynes and Southee, 1984). For instance, we have found comparable levels of gonadotropins between groups of castrated rams, before or after a GnRH injection, even though a high level of antibodies were still present in circulation. Thus, it seems that no persistent changes in the functioning of the hypothalamic-pituitary axis had occurred in the T-immunized animals that would have maintained a more pronounced secretion of hormones by the pituitary after the removal of the testes. Moreover, this finding indicates that the hypothalamic-pituitary axis is not damaged by the long-term presence of antibodies directed against testosterone and that it is still able to adjust normally to a new endocrine situation (such as absence of gonads).

Comparisons with other immunization studies

In retrospect, our results on active immunization against oestradiol in developing ram lambs resemble more those obtained by Jenkins et al. (1986) than those obtained by Land et al. (1981). In fact, using passive immunization against oestrogens, Jenkins et al. had not found any increase in testicular growth, between 2 and 16 weeks of age, in crossbred lambs; while Land et al. (1981) had shown that the rate of growth of the testis was greater in E_2-immunized Merino lambs, between 14 and 26 weeks of age. Unfortunately, the lambs used in both experiments, were not studied beyond those ages and the histological appearance of the testes of these lambs at the end of these experiments has not been reported. Neither team had found major changes in hormone secretion such as those observed in our experiment. Of course, since these authors have used a different approach (passive instead of active immunization) for studying the role of oestrogens in the development of the reproductive system, and since they have used ram lambs of different

breeds or strains, kept under very different experimental conditions, any comparison between their studies and the ones presented in this thesis need to be done with caution and must remain general.

In this thesis work, we did not study the effects of immunization against oestrogens in adult rams since this has already been examined by other authors (passive immunization: Sanford 1985, 1987a, 1989, 1991; active immunization: Schanbacher 1979, 1984; Schanbacher et al., 1987; Monet-Kuntz et al., 1988; see appendices). We will simply stress that the consequences of the immunoneutralization against oestrogens in developing rams have been more drastic than in mature rams in which the spermatogenic function was not or only slightly affected and in which the steroidogenic function was also significantly enhanced. There is no doubt that the different results obtained in E_2-immunized rams point out important age differences that should be considered attentively.

Passive vs active immunization

We must recall that the immune response in the actively immunized sheep is far different from that of the passively immunized sheep. In the actively immunized animals, although the production of antibodies of high specificity and high affinity is essential and central for effective neutralization of the antigens, a lot of other mechanisms are participating (e.g. production of lymphokines by activated T-cells, multiples actions by activated macrophages and by other cells of the immune system) and could have major impacts on the overall clearance of the antigens. On the other hand, in the passively immunized animals, the neutralizing effects produced depend almost exclusively on the characteristics of the antibodies injected into their circulation.

For this reason, we have repeated our previous experiment (active immunization against oestradiol-17ß), but this time, studying other Merino ram lambs passively immunized against oestradiol-17ß (see section 6.3) or passively immunized against oestrone (see section 6.4.).

About our observations in the passively immunized sheep:

Unfortunately, we did not observe any significant effects following passive immunization against oestradiol-17ß or oestrone in our ram lambs. Therefore, with these experiments, we have not been able to support the postulate that oestrogens influence pubertal development of the Merino ram lambs and to confirm the observations made in our preceding experiment using actively E_2-immunized ram lambs. Since Land et al. (1981) and Jenkins et al. (1986) had reported significant effects following passive immunization against oestrogens in developing ram lambs (with titres comparable to those maintained in our experiments), we could not simply argue that this approach was not effective in neutralizing oestrogens in ram lambs.

Considering all possible explanations, we suspect that the lack of effect of our passive immunization treatments must have been due to an insufficient amount of antibodies injected into the animals (e.g. low titre) and/or incomplete neutralization of circulating oestrogens by the antibodies (e.g. low affinity of the antibodies compare to the oestrogenic receptors within the target cells). We do not think that the recipient had produced an immune response against the foreign antibodies (e.g. formation of anti-sheep IgG immunoglobulins such as anti-idiotype antibodies) which, could have interfered with the binding capacity of the transferred antibodies. (see general discussion for chapter 6). Nevertheless, both passive immunization experiments have shown that repeated injections of purified IgG obtained with the aid of caprylic acid have no detrimental effects on the general health of the animal and could possibly become an interesting alternative to the use of complete antiserum in other passive immunization studies.

About our observations in E_2-treated wethers immunized against oestrogens:

Although we have kept in mind that E_2-treated castrated ram lambs differ considerably from intact ram lambs, using this animal model, we have been able to demonstrate the efficiency of both passive and active immunization approaches in neutralizing exogenously administered oestradiol (released from a Silastic capsule implanted under the skin). Here again, we have shown that the active immunization approach (section 7.2. of chapter 7) is more effective than the passive immunization approach (section 7.3. of chapter 7). Although very few animals were

used in this study, our observations indicated that some oestradiol released by the implant was always able to act on the hypothalamic-pituitary axis in most wethers, but that a reduction in the intensity of oestradiol action could occur because of the presence of the E_2-antibodies in circulation. Moreover, the data obtained in these studies strongly suggest that the magnitude of the neutralization varies according to the titre obtained in each animal. For instance, we have found that the reduction in gonadotropin secretion was slightly less pronounced in the E_2-treated castrated ram lambs receiving purified IgG directed against oestradiol (low titre) than in BSA-immunized castrated ram lambs implanted with oestradiol (titre: negligible), while in actively E_2-immunized wethers (higher titre), the suppression of oestradiol action on gonadotropin secretion was very marked.

We must recall that for each passive immunization study that we have conducted, we could not exclude the possibility that the passively transferred antibodies were not always able to compete efficiently with the oestrogen receptors within the target tissues in the hypothalamic-pituitary-testicular axis (because of lower *in vivo* affinity for oestrogen). Indeed, it is now unclear whether antibody-bound steroids are biologically inactive since the exact mechanism of action of antibodies is only partly understood. Moreover, the fact that various authors have reported that plasma protein-bound steroids are not necessarily biologically inactive (Siiteri et al., 1982; Siiteri and Simberg, 1986 ; Pardridge, 1985, 1986, 1987) suggests that the situation with antibodies might also be very complex. Hopefully, these questions will soon be answered and precise ways to evaluate what fraction of the hormone is neutralized by antibodies *in vivo* will become available.

About our observations on pubertal development:

All ram lambs that we have extensively studied during their period of transition into puberty were of the South Australian Merino breed and were kept under the same well defined experimental conditions. Under these conditions, all non-immunized and immunized ram lambs had generally achieved puberty at a relatively early age (between 22 and 26 weeks of age). Nevertheless, we believe that, under these relatively favorable environmental conditions, most

of our lambs did not achieve their fastest possible rate of reproductive maturation since a variety of uncontrolled factors could, still, have slowed down the developmental process before and during the experiment. For example, we have observed that advancement of the weaning time by four weeks (e.g. at 8 weeks of age instead of 12 weeks of age) could considerably retard body growth, testicular development and initiation of spermatogenesis (see section 6.3. of chapter 6). The fact that our ram lambs were studied in the same environment certainly gave more value to the comparisons made between our experiments.

The increase in LH pulse frequency and mean LH level, which generally occurs early during pubertal development and which are likely to reflect the reactivation of the hypothalamic pulse generator and the major signal for the initiation of spermatogenesis (Adams and Steiner, 1988), may not apply to ruminants, or else it must have occurred before the start of our experiments since it has never been observed during the period of investigation. To the contrary, we have generally observed a slight decrease in mean LH level which has been reported to occur later on during pubertal development, when the steroidogenic function of the testis become considerable. It would be very interesting to verify whether the response to active immunization would change if the immunization treatment was initiated in younger ram lambs (well before the reawakening of the GnRH pulse generator).

In our studies, the maturational changes in the secretion of FSH were not pronounced and did not follow the pattern of LH secretion. These observations support the idea that FSH release is regulated by the feedback action of some peptidic hormones of testicular origin (e.g. inhibin, activin; for review see de Kretser et al., 1987) as well as by gonadal steroids (for review see Price, 1991) while LH release is mainly under the control of gonadal steroids (Schanbacher, 1980; 1984a, b). Indeed, using castrated ram lambs we have also shown that exogenously administered oestradiol-17ß was able to suppress LH to a level that compared well with that found in intact ram lambs while the suppression of FSH was always incomplete (see chapter 7).

We have found that total testicular blood plasma flow (TTBPF) when expressed per unit weight of testis (µl/g/min) decreased as the testis grows, although total blood flow per testis (ml/min) is increasing (see chapter 3). The latter observation was expected as more blood is required to feed the increasing mass of tissues present in the developing testis. On the other hand, the decline in

TTBPF per unit weight of testis that we have observed was surprising, and seemed to occur before the rapid increase in testicular weight and thus before the establishment of spermatogenesis. In an attempt to explain these slightly "opposite" results, we have combined our data on TTBPF together with the data on capillary testicular blood flow (CTBF) obtained from developing lambs, by Courot and Joffre (1977) (see fig. 3.8. in chapter 3). We have postulated that while an increasing amount of blood reaches the testis during maturation, the relative amount of arterial blood transferring to the venous blood in the spermatic cord decreases and consequently the exchange of heat between the vein and the artery at the level of the spermatic cord becomes more pronounced. This postulate also supports our finding that a notable decrease in subcutaneous scrotal temperatures seems to occur early during testicular maturation in ram lambs (presumably due to an increased counter-current heat exchange) (see chapter 4). Indeed, our data tend to indicate a more pronounced rectal-testis temperature gradient in the pubertal than in the impubertal ram lambs, similar to what has been found in rats (Kormano, 1967b).

We made some interesting observations suggesting a major influence of photoperiod during pubertal development in South Australian Merino ram lambs. Indeed, our data demonstrate that ram lambs born in autumn grew well under a 12 L: 12 D lighting regimen (see chapter 3 and section 6.4 of chapter 6). However, we have observed that some winter born ram lambs may exhibit a rate of testicular maturation that is quite slow or slightly disturbed under the same lighting condition (see chapter 5 and section 6.3 of chapter 6). For instance, in some of the winter born lambs we have observed a slight decline in testicular volume towards the end of the experiment (see chapter 5). One possible explanation for this final decline is that the lighting was not quite appropriate for these lambs since they have never been exposed to decreasing day length during their development. According to Colas and co-workers (1987), the optimal lighting for testicular maturation in ram lambs should be exposure to long days and then to short days. Plasma PRL concentrations measured at regular intervals were generally relatively low in autumn born lambs while they were more elevated in lambs born in winter even though all lambs were kept in exactly the same controlled environment after weaning. It seems therefore that our

winter born lambs had interpreted the 12L:12D light cycle as long days by contrast with our autumn born lambs (and the autumn born crossbred ram lambs studied by Klindt and co-workers -1985-) which interpreted the same artificial cycle more like short days. In the lambs presented in chapter 3, in which PRL levels were maintained at lower levels, we have observed a sharp rise in PRL secretion occurring approximately at the time of onset of spermatogenesis (a similar observation has also been reported by Ravault and Courot -1975). Since we do not know the exact function of PRL in male sheep, the significance of this prepubertal rise remains unclear. It has been suggested that PRL, in ram lambs, can possibly be involved in the establishment and maintenance of testicular receptors (Yarney and Sanford, 1989) or that it may play a role in the secretory activity of the seminal glands (Ravault et al., 1977).

Finally, we have shown that testicular biopsies taken at 22 and 26 weeks of age did not impair testicular development and sperm production, using a careful procedure that minimize damage to the vascular layer of the tunica albuginea and postoperative inflammation (see chapter 5). This observation is in agreement with that reported by other investigators (Pimentel et al., 1984; Lunstra and Echternkamp, 1988). However, we cannot conclude that this procedure is totally without negative consequences as we have observed some significant hormonal modifications following biopsy sampling. For instance, the lower plasma testosterone level measured at 30 weeks of age indicates that the steroidogenic function of the testis might have been slightly affected by biopsy sampling and subsequent healing. The additional stress brought about by the surgical procedure and healing was also reflected by a slight elevation in circulating PRL level between 22 and 30 weeks of age. Thus, even if repetitive testicular biopsies did not seem to impair subsequent testicular development, the fact that hormonal changes were still evident one month after the surgery in some lambs, lead us not to recommend the use of this surgical procedure in an endocrinological study on pubertal development.

Conclusions:

In summary, our observations provided additional evidence that oestradiol-17ß in the blood circulation plays an important role in the regulation of gonadotropin secretion during pubertal development in ram lambs. Perhaps, low levels of oestrogens synergize with testicular inhibin to regulate FSH and with androgens to regulate LH. Oestrogens may also possibly synergize with testosterone within the seminiferous tubules to allow normal establishment of spermatogenesis, and may also participate in any anabolic effects.

The endocrinological changes that have occurred following active immunization against oestradiol in ram lambs or following active immunization against testosterone in adult Merino rams must certainly have implied very dynamic adjustments within the reproductive system. Our observations in T-immunized rams certainly support the view that a new equilibrium within the hypothalamic-pituitary-testicular axis is likely to take place after a long period of immunization (with a lot of readjustments within each cell of the system).

Most importantly, we have shown that, in the long term, active immunization against a steroid, can lead to deleterious effects within the testis (immunization against T or E_2), on accessory male organs (immunization against T) and on sexual behaviour (immunization against T), especially in the animals which seem to produce a very pronounced immune response (e.g. high titre; more pronounced changes compared to controls).

Even though not all endocrinological questions will be answered with the help of withdrawal techniques, we believe that, as the complexity of the immune response become revealed (e.g. better understanding of the fate of the antigen bound to the antibody), the immunological approaches will eventually offer very precise means of investigating the hormonal requirements of the male reproductive system. There is no doubt that investigations on immunized animals should continue and should even be encouraged.

Hopefully, a better understanding of the various processes involved in the maturation of the hypothalamic-pituitary-testicular axis of the sheep, including the participation of oestrogens within these processes, will eventually unravel the complex passage from an infertile state to a fertile one, in this species and in many others.

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APPENDICES

Appendix: table 1

Passive immunization against oestrogens in ram lambs

Authors (breed) (age)	Immunogen (no. of lambs) treatment, duration, immunogen, titre, lighting	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
Land et al. 1981 (Merino) (14-26 wks)	control-HSA...(n=5) E_2-6-CMO-BSA (n=6) E_1-6-CMO-BSA (n=6) 5 ml antiserum i.v. at 14, 16, 18 weeks; titre ~ 1:150 natural lighting (summer-autumn; from June to Nov.)	No change in LH production 50% increase in FSH in E_2-imm. (but not statistically significant) Positive correlation between FSH and anti-oestrone titre (r: 0.5, p ~0.05)	The rate of growth of the testis in E_1- and E_2-imm. was greater than controls ($p < 0.01$) No change in testosterone production
Jenkins et al. 1986 (Crossbred) (2-16 wks)	control-untreated-(n=6) E_2-3-CMO-BSA(n=6) 1 ml antiserum i.v. at two weeks interval titre ~ 1:230 constant lighting (12L:12D)	Greater dopamine content of median eminence in controls than in E_2-imm. 60% more GnRH in median eminence in controls (but not statistically significant) No change in LH, FSH, PRL	No change in testis size 74% increase in androgen level at 12 weeks and 37% increase at 16 weeks in E_2-imm. ($p < 0.05$) No change in testicular responsiveness to GnRH (due to endogenous LH pulse)

Appendix: table 2 (part 1)

Passive immunization against oestrogens in adult rams

Authors (breed) (age)	Immunogen (no. of rams) treatment, duration, immunogen, titre, lighting	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
Sanford, L.M. 1985 (Crossbred) (18 months)	control: non immun. serum (n=4) E_2-6-CMO-BSA (n=4) [Tamoxifen, n=4] 30 ml antiserum i.v. every 3 or 4 days for 2 weeks titre: < 1:200 natural lighting (autumn)	2-fold increase in LH in E_2-imm. (increased amplitude, baseline, frequency) No significant diff. in FSH Decreased PRL in E_2-imm. Pituitary responsiveness to GnRH is increased in E_2-imm. (more LH released)	No change in testis size 8-fold increase in testosterone in E_2-imm. (increased baseline and peak magnitude) (tendency for increased frequency but not significantly different) Testicular responsiveness to LH is increased in E_2-imm.(more testosterone released)
Sanford, L.M. 1987a (Crossbred) (16 months)	control: non immun. serum (n=4) E_2-6-CMO-BSA (n=3) [Tamoxifen; n=3] 15 ml antiserum i.v. every 3 or 4 days for 8 weeks titre: < 1:100 natural lighting (summer)	Increased LH in E_2-imm.(week 2 and 8) (increased frequency and baseline -week 8) Increased FSH in E_2-imm. No significant diff. in PRL Pit. responsiveness to GnRH: reduced LH and FSH in E_2-imm. (week 2 only)	No change in testis size (the rate of testicular regression in early winter was more pronounced in E_2-imm.) Increased testosterone (~130%) in E_2-imm. (increased baseline - week 2 and 8) (increased frequency - week 8)
Sanford, L.M. 1989 (Crossbred) (3.5 years)	control: non immun. serum (n=5) E_2-6-CMO-BSA(n=5) 50 ml antiserum i.v. every 3 or 4 days for 4 weeks titre: < 1:200 natural lighting (summer)	No significant diff. in LH, FSH, PRL Pit. responsiveness to GnRH: first peak: no change in LH (second peak: smaller in E_2-imm.)	No change in testis size Increased testosterone (125% day 10 and 150% day 24) in E_2-imm. (increased peak amplitude and baseline) No change in LH-receptor Decreased FSH-receptor in E_2-imm. (day 14)

Appendix: table 2 (part 2)

Passive immunization against oestrogens in adult rams

Authors (breed) (age)	Immunogen (no. of rams) treatment, duration, immunogen, titre, lighting	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
Sanford, L.M. 1991 (Crossbred) (one year)	control: non immun. serum (n=5) E_2-6-CMO-BSA(n=5) 50 ml antiserum i.v. every 3 or 4 days for 13 weeks titre: ~ 1:200 artificial lighting (4 month periods of 16 L: 8D and 8L: 16D, each)	No significant difference in FSH and PRL No significant difference in LH (except for a subtle increase in LH-pulse amplitude - i.e. LH pulses were, overall, 50% larger in the E_2-imm.)	Testicular regression partially prevented (i.e. larger scrotal circumference during the regression stage) No change in daily sperm output early in the redevelopment stage Increased testosterone in E_2-imm. (increased peak amplitude, pulse frequency and baseline) (T values remain at "breeding season" values)

Appendix: table 3 (part 1)

Active immunization against oestrogens in adult rams

Authors (breed) (age)	Immunogen (no. of rams) treatment, duration, immunogen, titre, lighting	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
Schanbacher B.D. 1979 (?) (?-adult)	control (n=6) E_2-6-CMO-BSA (n=6) T-3-BSA (n=6) imm. procedure:? titre: ? lighting: ?	Increased LH and FSH in E_2-and T-immunized rams	
Schanbacher B.D. 1984 (Suffolk) (?-adult)	control (n=5) E_2-6-CMO-BSA(n=5) immunized at week 0, 4 and 8 titre: between 1:2200 and 1:16500 (week 12) artificial lighting (16L:8D) and (8L:16D) for 12 weeks each	Increased LH in E_2-imm. (increased frequency)	Increased testosterone in E_2-imm. combined effects of photoperiod and E_2-immunization
Schanbacher B.D. et al. 1987 (Ile-de-France) (4 years)	control (n=5) E_2-6-CMO-BSA(n=5) castration in spring (April) immun. in Oct., Dec. and Feb. titre: <1:1000 natural lighting (autumn-spring; from Oct. until April)	<u>in spring (April)</u> Increased LH in E_2-imm. (increased LH frequency and amplitude) Increased FSH in E_2-imm.	<u>In spring (April)</u> Increased testicular weight in E_2-imm. Increased nuclear and cytoplasmic volume of Leydig cells and, increased total volume of Leydig cells in E_2-imm. No change in number of Leydig cells 10-fold increase in testosterone (no change in metabolic clearance rate)

Appendix: table 3 (part 2)

Active immunization against oestrogens in adult rams

Authors (breed) (age)	Immunogen (no. of rams) treatment, duration, immunogen, titre, lighting	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
Monet-Kuntz, et al. 1988 (Ile-de-France) (4 years)	control (n=10) E_2-6-CMO-BSA (n=10) for each treatment: (5 castrated in spring-April) (5 castrated in autumn-Sept.) immunized in Oct., Dec., Feb., and immunized in June and Aug. titre: < 1:1000 natural lighting (autumn-spring; from Oct. until April) or (autumn-autumn; from Oct. until Sept.)	In spring (April) LH and FSH: (see Schanbacher et al., 1987) In autumn (September) 2-fold increase in LH in E_2-imm. Increased FSH in E_2-imm.	In spring(April) Testicular weight and intertitial tissue: (see Schanbacher et al., 1987) No change in tubule volume 2-fold increase in number of LH-receptor per Leydig cell in E_2-imm. In spring (April) and autumn (September) Increased testosterone in E_2-imm. Increased testis content of LH receptor in E_2-imm. No change in number of FSH-receptor No change in Sertoli cell number and volume Increased volume of blood and lymph vessels in E_2-imm. In autumn (September) No change in no. of LH-receptor per Leydig cell Increased interstitial volume in E_2-imm. Increased in number of Leydig cells in E_2-imm. No change in volume of Leydig cells Increased sem. tubule volume in E_2-imm. Increased length seminiferous tubules in E_2-imm. No change in diameter of seminiferous tubules No change in daily production of A1 spermatogonia Increased daily production in leptotene spermatocytes and round spermatids in E_2-imm.

Appendix: table 4

Active immunization against oestrogens in castrated rams

Authors (breed) (age)	Immunogen (no. of sheep) treatment, duration, immunogen, titre, lighting	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
Sanford, L.M. 1987b (Crossbred) (2 years)	control: Rabbit γ globuline (n=4) E_2-6-CMO-BSA (n=4) imm.: monthly booster for 7 months (until spring-April) titre: ~ 1:5000 natural lighting (indoor)	Assessment in spring (April) Increased LH in E_2-imm. (increased frequency) Pit. responsiveness to GnRH: first peak: more LH released in E_2-imm. (second peak: no difference in LH released)	

Appendix: table 5

Active immunization against oestrogens in prepubertal bulls

Authors (breed) (age)	Immunogen (no. of bulls) treatment, duration, immunogen, titre, lighting	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
D'Occhio et al. 1987 (Shorthorn) (3-14 months)	control (n=7) E_2-6-CMO-HSA (n=7) T-3-HSA (n=9) immunized.at 3, 5, 7, 9, 11 months of age titre: ~1:12200 (E_2); ~1:730 (T) at 5 months of age then decline natural lighting (outdoor)	Increased LH and FSH in T-imm (no change in E_2-imm.) Pit. responsiveness to GnRH: tendency for higher LH (delta value) in E_2-imm. (but not significantly different)	Increased testis weight and diameter in T-imm. No change in epididymal weight Increased testosterone in T-imm. (maximum between 5 and 6 months) No change in daily sperm production (DSP/g testis) Testicular responsiveness to hCG: Increased testosterone in T- and E_2-imm. (0 to 3 hour after hCG) Testicular responsiveness to GnRH: Increased testosterone in T-and E_2-imm. (due to endogenous LH pulse)
Wrobel et al. 1990 (Shorthorn) (3-14 months)	see D'Occhio et al. 1987 control (n=3) E_2-6-CMO-HSA (n=3) T-3-HSA (n=3) Histology: 18 semithin sections per treatment group (3 locations/testis x 3 bulls/group x 2 replicates)		in T-imm Increased volume of Leydig cells Increased absolute number of Leydig cells per testis Leydig cell mitochondrial, nuclear and smooth reticulum endoplasmic masses per testis are increased in E_2-imm. Nuclear and cellular volumes of a single Leydig cell are decreased in E_2-imm. Increased lipid content in Leydig cell in E_2-imm.

Appendix: table 6

Active immunization against oestrogens in other farm animals

Authors (species) (breed) (age)	Immunogen (no. of animals) treatment, duration, immunogen, titre, lighting	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
Thompson and Honey 1984 (Stallion) (Quaterhorse) (6-27 months)	control (n=5) E_1-17-BSA (n=4) active. imm.: at 4, 8, 10, 12, 16 and 20 months of age titre: > 50% binding at serum dilution 1:10 natural lighting (outdoor)	No change in LH Increased FSH (initially only and mainly for one E_1-imm. subject)	Increased testicular weight in E_1-imm. (increased parenchymal weight) Increased daily sperm production (DSP/g testis and DSP/stallion) in E_1-imm. No change in seminal characteristics (at 27 months) Increased testosterone in E_1-imm. (bound form)
Wise et al. 1991 (Boar) (Crossbred) (5-24 weeks)	control: KLH (n=6) E_1-3-KLH (n=6) active. imm.: at 5, 9, 13 and 17 weeks of age titre: 5 % binding at serum dilution 1:100 (> 17 weeks) ? lighting	No change in LH and PRL	Tendency lower testicular, epididymal and accessory gland weights in E_1-imm. (but not significantly different) Decreased seminal vesicle weight in E_1 imm. ($p < 0.05$) Tendency decreased total Leydig cell numbers ($p < 0.09$) in E_1-imm. Increased Leydig cell volume in E_1-imm. Reduced diameter of seminiferous tubules in E_1-imm. ($p < 0.09$) Increased size and number of intra-cellular organelles in E_1-imm. No change in serum T, DHEA and ES No change in testicular P4, T and E_1

Appendix: table 7

Immunization against oestrogens in laboratory animals

Authors (species) (breed) (age)	Immunogen (no. of animals) treatment, duration, immunogen, titre, lighting	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
Nishihara and Takahashi 1983 (rat) (Wistar) (2-6 months)	control (n=4) E_2-6-CMO-BSA (n=6) active imm.: every 2 weeks (4 times) then once a month titre: < 1:250 constant lighting	Increased LH in E_2-imm.	12.5 fold increase in E_2 level after 4 immunization (bound form) Increased testosterone in E_2-imm. (no change in metabolic clearance rate; increased production rate) No effect of exogenous E_2 (intratesticular implant) in E_2-imm.
Nieschlag et al. 1974; 1975a,b,c & Nieschlag and Kley (1974) (rabbit) (New Zealand) (adult)	rabbit (E_2-imm.) E_2-17-BSA (n=2 or 3) control (n=5) titre: 1:5000-1:64000 rabbit (E_1-imm.) E_1-17-BSA (n=7) control (n=5)	rabbit (E_2-imm.) increased LH no change in FSH rabbit (E_1-imm.) normal chasing, mounting and ejaculation behaviour	rabbit (E_2-imm.) Leydig cell hyperplasia and hypertrophy No change in testicular weight Increased in testosterone rabbit (E_1-imm. and E_2-imm.) no change in accessory reproductive glands
Hillier et al. 1975 (rat) (Spague-Dawley) (adult)	control, BSA (n=) E_2-6-BSA (n=)	increase in FSH in E_2-imm.	no change in testicular morphology
Wuttke et al. 1975 (rat) (-) (adult)	control (n=?) E_1-17-BSA (n=50)	decreased FSH in E_1-imm. (decreasing FSH with increasing antibody titre) no change in LH	

Appendix: table 8 (part 1)

Immunization against testosterone in laboratory animals

Authors	Species, (no. of animals) treatment, duration, immunogen, titre	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
Lieberman et al. 1959 & Neri et al., 1964	castrated rats treated with testosterone act. imm., T-3-BSA		reduced anabolic effect on levator ani, seminal vesicles and ventral prostate
Goldman et al., 1972	pregnant rats --> male foetus pass. imm. from 12 to 15 weeks of gestation		the antibodies can pass placenta increase in T produced in vitro male foetus: sex differentiation prevented reduced anogenital distance reduced testicular weight
Nieschlag et al., 1973, 1974, 1975 a, b, c & Wickings et al., 1976 & Wuttke et al., 1975	rabbits: act. imm., T-3-BSA (control, n=6 and T-imm.,n=10) (control, n=5 and T-imm., n=4) titre: 1:5000 - 1:64000 rats: act. imm., T-3-BSA (control, n= ?, T-imm., n=50) titre: 1:8000 - 1:64000	in rabbits and rats: increase in LH and FSH positive correlation between titre and hormone level no mating in rats no increase in PRL increase in LH and FSH	in rabbits: increase in T secretion (*in vivo* and *in vitro*) Leydig cell hyperplasia and hypertrophy and increase in nuclear volume increase in testicular weight morphological degeneration of seminal vesicles and ventral prostate (33% decrease in weight) in rats: normal spematogenesis reduced testicular weight
Nieschlag and Kley, 1974	rabbits: act. imm., T-3-BSA (control, n=5, T-imm., n=6)	loss of sexual activity	
Nieschlag and Wickings 1977, 1978 (reviews)	rabbits act. imm., T-3-BSA high cross-reactivity with DHT	decrease in sexual activity increase in LH and FSH glomerulonephritis in 50% rabbits (no mortality, same health)	atrophy of accessory reproductive glands hyperplasia and hypertrophy of Leydig cells increase in testicular weight increase in testicular responsiveness to hCG increase in T (x 100), (probably decrease in free T) (increase in T production and decrease in MCR)
Wicking and Nieschlag, 1978	Rhesus monkeys act. imm., duration: one year, titre: 1:1000 - 1:50000	LH no seasonal change in T-imm.	increase in T (increase in bound T) increase in testicular weight

Appendix: table 8 (part 2)

Immunization against testosterone in laboratory animals

Authors	Species, (no. of animals) treatment, duration, immunogen, titre	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive organs, [Gonadal steroid]
Hillier et al., 1973, 1975 a,b	rats act. imm. (control and T-imm.: n=5 per group), titre: 1:20000	increase in LH and FSH no change in PRL positive correlation between titre and hormone level	increase in T (x 25) (increase in bound T) increase in testicular weight 25% decrease in weight of ventral prostate
Thorneycroft et al., 1975	rabbits act. imm., duration: 30 weeks titre: up to 1:25000	increase in LH positive correlation between titre and hormone level	increase in T (x 100)
Bidlingmaier et al. 1977	pregnants rabbits --> male foetus act. imm, titre up to 1:40000 duration: a few months		increase in T in male and female foetus <u>in male foetus</u>: undevelopped sex glands degeneration Wolfian ducts feminization of the penis no change in testis, same action of MIS
Gay and Kerlan 1978	rats pass. imm.		
Main, Davies and Setchell, 1977, 1980	rats pass. imm. (control and T-imm.: n=6 to 8 per group), T-3-BSA, from 23 days to 78 days	increase in LH at all ages (all doses) increase in FSH at 36, 50 and 78 days of age (high dose)	increase in T (probably all bound)
Sharpe and Fraser 1983	rats act. imm., (control and T-imm.: n=14 per group), duration: one year, T-15-BSA, titre: 1:50 - 1:4000	increase in LH (x 8) increase in FSH (x 2.5) positive correlation between titre and hormone level	increase in T (x 50) increase in testicular weight incubation Leydig cells: increase testicular responsiveness to hCG, increase in LH and GnRH receptors / Leydig cell no change in binding affinity

Appendix: table 9 (part 1)

Immunization against testosterone in farm animals

Authors	Species, (no. of animals) treatment, duration, immunogen, titre	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive glands, [Gonadal steroid]
Schanbacher, 1979	controls (n=6) T-3-BSA (n=6) immun. procedure: act. imm. titre: ? adult rams	increase in LH and FSH in T-immunized rams	
Schanbacher, 1982	ram lambs act. imm., (control and T-imm., n=12 per group), from 10 to 22 weeks, T-3-BSA, titre: < 1:10000	decrease in anabolic effect growth rates and carcass charact. similar to castrated animals increase in LH and FSH at 18 and 22 weeks increased pituitary responsiveness to GnRH	no change in testicular weight increase in T (probably decrease in MCR) (no increase in T after a GnRH challenge)
Haynes and Southee, 1984 (review)	ram lambs act. imm. (control and T-imm., n=14 per group) from 3 months to 28 weeks, T-3-BSA, titre: 1:5000 - 1:10000 Adult rams, act. imm.	no change in sexual behaviour increase in LH at the beginning but did not persist	increase in testicular volume increase in T (x 100) (increase in bound T) (but decrease in T and testicular volume in December, probably seasonal effect)
Thompson et al., 1985	prepubertal boars act.imm., (controls: n=6 and T-imm: n=7) from 1 to 14 months, T-3-BSA, titre decreased with time	no change in sexual activity at 13 months of age decrease in LH and FSH no effect on body weight	increase in oestrogen at 7 months of age (only) no change in seminal characteristics at 14 months no change in testicular growth and no change in DSP and testis weight at 14 months increase in T (increase in bound T) in plasma (but same intratesticular T concentration)
Walker et al., 1984	prepubertal bulls, act.imm., from 1 to 12 and 18 months	no change in LH and FSH	increase testicular growth normal seminal characteristics increase in sperm production at 12 and 18 months

Appendix: table 9 (part 2)

Immunization against testosterone in farm animals

Authors	Species, (no. of animals) treatment, duration, immunogen, titre	CNS, hypothalamic-pituitary axis [LH, FSH, PRL]	Testis, accessory reproductive glands, [Gonadal steroid]
D'Occhio et al., 1987 & Worbel et al., 1990	prepubertal bulls act. imm.(control and T-imm. n=7 per group) from 3 to 14 months, T-3-BSA, titre decreased with time (~ 1: 730)	increase in LH and FSH (between 3 and 10 months of age) pit. responsiveness to GnRH: same LH response (at 10 months of age)	increased testicular volume after 6 months of age increased testicular weight at 14 months of age increase in T between 3 and 10 months of age (then gradual decline) increase DSP at 14 months of age no change in epid. weight and other access. organs increase in Leydig cell mass per testis

Appendix: Chapter 3 (page 1).

Mean Plasma Oestradiol Concentration in Ram Lambs.

EXPERIMENTAL PROCEDURE: (see chapter 3)

Measurement of plasma oestradiol concentration and response to hCG.

Human chorionic gonadotropin (hCG) was administered to anaesthetized sheep as a single intravenous injection (20 I.U./kg body weight) at the time of testicular blood flow measurement (see section 2.7.2). Blood samples from the jugular (JUG) and the internal spermatic veins (ISV) were taken at 10 minute interval, starting 40 minutes before the hCG challenge and for another 100 minutes thereafter. The "pre-hCG" pool for each site of blood collection (JUG, left-ISV, right-ISV) consists of equal aliquots of plasma taken from the 4 serially collected blood samples. Similarly, the "post-hCG" pool for each site of blood collection consists of equal aliquots of plasma taken from the 10 serially collected blood samples. Pool samples collected before and after a hCG challenge were assayed for oestradiol in the laboratory of Dr. Graeme B. Martin at the University of Western Australia.

RESULTS AND DISCUSSION:

Plasma oestradiol concentrations measured in jugular veins and in the internal spermatic veins did not differ significantly at any age. Furthermore, plasma oestradiol concentrations measured in jugular veins or in the internal spermatic veins were never affected by the hCG injection (see fig. A, Appendix: Chapter 3, page 2).

These results seem to indicate that the testes of our ram lambs were not producing a significant amount of oestradiol. However, other researchers reported elevated oestradiol concentration in testis venous plasma of ram lambs (Watts et al., 1989; Pope et al, 1990). At the moment, we cannot eliminate the possibility that these conflicting results reflected some technical problems. Indeed, precise measurement of oestradiol concentration with normal radioimmunoassay procedure is difficult because the amount of oestradiol found in jugular plasma samples from ram lambs (and from adult rams) is very low and because other steroids may interfere with the assay (an extraction step is often necessary).

Appendix : Chapter 3 (page 2).
Mean Plasma Oestradiol Concentration in Ram Lambs.

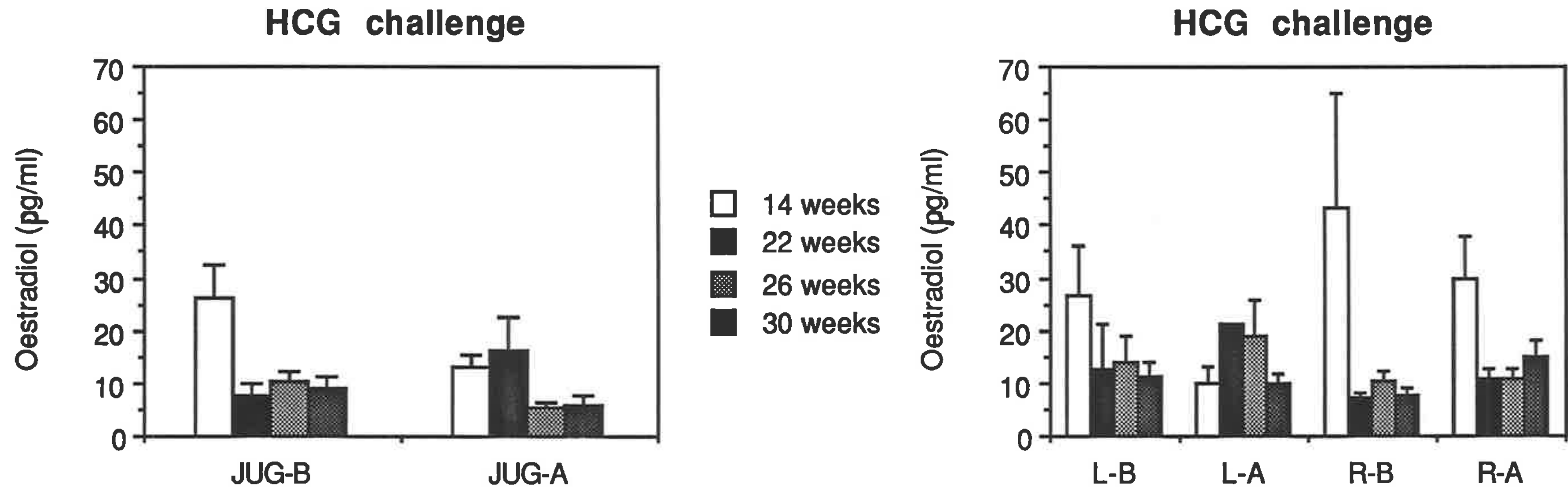

Fig. 3A Mean oestradiol concentration (pg/ml ± sem) measured in the jugular (JUG) and in the left (L) and right (R) internal spermatic vein before (B) and after (A) a single i.v. injection of hCG (20 I.U./kg BW) to South Australian Merino ram lambs at 14, 22, 26 and 30 weeks of age (mean of 4 lambs per age group).

Appendix: Chapter 6, section 6.3. (page 1)

Detection of antibodies against sheep IgG.

MATERIALS AND METHODS:

Plasma samples, collected 2 weeks after the last injection (at 22 weeks of age), were tested for antibodies against sheep IgG using two techniques and, as antigens, the same preparations of sheep purified IgG used as treatments (see section 6.3.1.3.).

i) Double immunodiffusion: Ten µl of plasma was deposited in a central well perforated in agarose gel (Life technologies, Inc., USA) covering a microscopic slide pretreated with 4 ml of agarose 1% in PBS (pH 6.8). Each plasma sample was allowed to diffuse towards five peripheral wells containing 10 µl of graded concentrations of antigen (0.125, 0.25, 0.5, 1, 2 mg/ml). The antigens used were purified IgG directed against BSA (for controls) and purified IgG directed against E_2 (for E_2-immunized lambs). Samples from E_2-immunized lambs were also allowed to diffuse towards five dilutions (1:1, 1:2, 1:4, 1:8, 1:16) of complete E_2-antiserum (W82). An arc of precipitation would be visible after 24 h (at room temperature, high humidity) if a precipitating antibody-antigen reaction took place (Crowle, 1973).

ii) Passive haemagglutination: Goat red blood cells pretreated with tannic acid (1 mg/ml in PBS; BDH chemicals, Ltd, England) were coated with either purified IgG directed against E_2 (for E_2-immunized lambs) or purified IgG directed against BSA (for controls) (Nichols and Nakamura, 1986). Normal donkey serum previously heated at 56 °C for 30 min and absorbed with goat red blood cells was used as diluent (dilution 1:100 in PBS). Serial two-fold dilutions (up to 1:2048) of each plasma were made in microtitre plates (Microdish MC96FR; CEB, France) (20 µl/well) and 20 µl of coated goat red blood cells were added to each well. The microtitre plates were left overnight at room temperature, after which haemagglutination would be observed only if anti-sheep IgG antibodies were present in plasma. Serum from a donkey actively immunized against sheep IgG was used as 'positive control' and normal donkey serum diluent was used as 'negative control'.

Appendix: Chapter 6, section 6.3. (page 2)

Detection of antibodies against sheep IgG.

RESULTS

Based on double immunodiffusion test and passive haemagglutination test, no anti-sheep IgG antibodies were present in the plasma of controls and E_2-immunized lambs at 22 weeks of age.

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