THE REGULATION OF GONADOTROPHIN SECRETION FOLLOWING DIVERGENT SELECTION FOR PITUITARY RESPONSIVENESS TO GnRH.

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ABSTRACT

Divergent selection based on the LH response to a 5μg dose of GnRH, has created two lines of sheep which differ in their ability to release gonadotrophins in response to a GnRH challenge in both male and female lambs. Significantly correlated between-line differences have also been reported in female reproductive performance. The aim of this project was to investigate the regulation of gonadotrophin secretion in animals from the two lines, and to elucidate the primary site of the selected difference/s.

Physiological studies of adult ewes and prepubertal ram lambs demonstrated that despite similar peripheral steroid concentrations, endogenous and exogenously-stimulated gonadotrophin secretion differed significantly between the two lines. Mean LH and FSH concentrations in the prepubertal male lambs were significantly higher in the High line than the Low line, due to the secretion of LH pulses of significantly greater amplitude by the High line ram lambs. Similarly, higher amplitude LH pulses were observed in the High line ewes during the follicular phase of the oestrous cycle. The age-related changes in basal LH secretion in the ram lambs and the observation of significant differences in LH pulse amplitude in the adult ewes during the follicular phase of the oestrous cycle, when progesterone negative feedback is reduced, indicate that the effects of the between-line difference in the regulation of endogenous LH secretion are regulated by gonadal negative feedback. However studies in prepubertal ram lambs demonstrated that the primary site of the selected difference was at the level of the hypothalamo-pituitary gland complex.

Studies of the regulation of LH secretion by the hypothalamo-pituitary gland complex demonstrated that the High line lambs appeared to secrete significantly less GnRH than the Low line and that the pituitary glands of the High line were 5-fold more sensitive to

GnRH than the Low line. Pituitary sensitivity encompasses a large number of variables, including gonadotroph and GnRH-receptor number, the intracellular events which follow receptor activation and the amount of releasable LH stored in the pituitary gland, the individual or combined effects of which could result in differences in pituitary sensitivity. Pituitary gonadotroph number/size was studied indirectly as a function of pituitary gland weight. The pituitary glands obtained from the High line tended to be heavier than those obtained from the Low line; however, this difference was not statistically significant. The pituitary glands of the High line were also found to contain significantly more GnRH-receptors/mg of protein than the Low line. The importance of this difference with regard to pituitary sensitivity was questioned however, following the demonstration that the between-line difference in the magnitude of the LH response was maintained *in vitro* following either GnRH-stimulated LH release or the direct stimulation of both the Ca^{2+}-calmodulin and Protein Kinase C second messenger systems. Examination of the pituitary stores of LH in the two lines demonstrated that the 5μg dose of GnRH used in the selection programme stimulated a maximal release of LH in both lines, and that the High line stored significantly greater quantities of releasable LH compared with the Low line. The results also indicated that the two lines may differ in their ability to synthesis LH in response to GnRH stimulation.

In conclusion indirect selection in 10-week old male lambs has created two lines of sheep which differ in the selected character, the reproductive performance of the adult ewes and a number of correlated physiological characters. The primary site of action of selection appears to be the pituitary gland, where selection has not only altered the amount of stored LH (the selected character) but has also affected a number of other diverse aspects of pituitary function.

DECLARATION

I hereby declare that this thesis has been composed by myself, and has not been submitted for any other degree, in Edinburgh or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the preparation of this thesis.

Neil P. Evans

PUBLICATIONS ARISING FROM THIS THESIS

1. Abstracts of spoken papers

N.P. Evans, M. Fordyce, J.R. McNeilly, R.B. Land and R. Webb. Differences in the LH response to LH-RH in castrated and entire lambs from the LH-RH selection line.J. Reprod. Fert. Abstract Series No.1,1988, p30

N. P. Evans, J.R. McNeilly, M. Ritchie, W. A. R. Ritchie, R. Webb and S. B. Wilson. An Investigation into possible differences in the pituitary sensitivity of two lines of lambs known to differ in their LH response to a given dose of GnRH. J. Reprod. Fert Abstract Series No.3,1989, p 40

N. P. Evans, T. A. Bramley, J. R. McNeilly and R. Webb. Extra- and Intra-cellular effects of divergent selection for pituitary responsiveness to GnRH in sheep. Society for the Study of Reproduction, Summer meeting 1991 (submitted).

2. Conference Proceedings

N. P. Evans, C. S. Haley, R. B. Land. G. J. Lee, J. R. McNeilly. and R. Webb. Two possible male predictor traits for female reproduction. 11th International Congress on Animal Reproduction and Artificial Insemination. Vol 3 , 369, 1988.

N. P. Evans, C. S. Haley, J. R. McNeilly, R. B. Land, and R. Webb. Predictor traits in males reflecting fertility in females. Proc. Society of Animal Breeding 1988/89 p 18-22

3. Posters

N. P. Evans, and J. R. McNeilly. Plasma gonadotrophin levels in adult ewes from lines selected on their response to exogenous LH-RH. Society for the Study of Fertility, Winter meeting 1987, University of Reading.

N. P. Evans, C. S. Haley, G. J. Lee, J. R. McNeilly and R. Webb. Selection to alter reproductive performance. Proc. 4th World Congress on Genetics Applied to Livestock Production. 1990. Edinburgh.

4 Refereed Papers

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ABBREVIATIONS

AA - Arachidonic acid
Arg -Arginine
BSA-GAD - Bovine serum albumin general assay dilutent
Ca^{2+} - Calcium ion
cAMP - cyclic Adenosine 3',5'- monophosphate
CNS - Central nervous system
DAG - Diacylglycerol
DARS - Donkey anti-rabbit serum
DBB - Diagonal Band of Broca
DMEM - Dulbecco's Modified Eagles Medium
DNA - Deoxyribonucleic acid
DPBS- Dulbecco's phosphate buffered saline
DPBS^{--} - DPBS without Ca^{2+} and Mg^{2+}
FSH - Follicle-stimulating hormone
GABA - gamma-aminobutyric acid
GAP - GnRH-associated protein
Glu - Glutamic acid
Gly - Glycine
GnRH - Gonadotrophin-releasing hormone
$GnRH_A$ - GnRH agonist
hCG - Human chorionic gonadotrophin
His - Histidine
IP_3 - Inositol 1, 4, 5-triphosphate
JTT - Javanese Thin tailed sheep
LH - Luteinizing hormone
Lys - Lysine
mRNA - messenger Ribonucleic acid
NRS - Normal rabbit serum
OVLT - Organum vasculosum of the lamina terminalis
$PGF2\alpha$ – Prostaglandin $F2\alpha$
Phosgel - phosphate gelatin assay buffer
PDB - Phorbol 12, 13, dibutyrate
PI - Phosphoinositide
PKC - Protein Kinase C
PMSG - Pregnant mares' serum gonadotrophin
Pro - Proline
Pyro -Pyrosine
RNA - Ribonucleic acid
Ser - Serine
Trp - Tryptophan
TSH - Thyroid-stimulating hormone
Tyr - Tyrosine

CHAPTER 1. - Literature review

1.1. General reproductive strategy of the sheep

Following birth, ewe and ram lambs are reproductively incompetent. Full sexual reproductive function (or puberty) only occurs after a period of physical development and maturation. The age at which puberty is attained is influenced by a number of genetic effects and environmental variables such as nutrition and photoperiod (Southam, Hullet and Bolki, 1971). Nutrition affects the age of puberty in both sexes (Allen and Lamming, 1961), a critical weight or size having to be reached in both male and female lambs, before puberty can occur. The effects of photoperiod however are partially sex-dependent, photoperiod having a greater effect on the age at which puberty is attained in the female lamb, than in the male. Puberty ~~only~~ only occurs in the female, following exposure of physiologically competent female lambs to a decreasing photoperiod. Therefore spring-born female lambs exhibit their first oestrus during the following autumn/winter, while lambs born late in the season or slow-growing females which do not attain the critical body weight before the autumnal decrease in photoperiod, delay puberty until the following autumn/winter (Hafez, 1952; Mallampati, Pope and Casida, 1971; Dyrmundsson and Lees, 1972; Keane, 1974; Foster and Ryan, 1979a, 1979b; Yellon and Foster, 1985). Puberty in the female lamb normally occurs after the start of the breeding season in the adult ewe (Hafez, 1952; Dyrmundsson, 1973). This occurs either due to the extra time needed for the completion of reproductive development in the ewe lambs, or to a requirement for a shorter photoperiod to activate reproductive activity in the ewe lambs, due to their limited photoperiodic history.

Reproductive activity in the adult ewe, unlike that of other domestic species such as cattle and pigs, is characterised by periods of reproductive

activity separated by periods of reproductive quiescence or anoestrus (Lees, 1965; Rowlands and Weir, 1984). In the female, the periods of reproductive activity consist of cycles of ovarian activity and the occurrence of behavioural oestrus. In the male, coincident periods of reproductive activity and anoestrus also occur (Lees, 1965) and are characterised by increased testicular size and the occurrence of physical and behavioural changes, including rutting behaviour, increased aggression, flehmen behaviour, an increase in the degree of cutaneous hyperaemia in the inguinal region ("the sexual flush") and the development of the muscles of the neck (Lincoln and Davidson, 1977; Lincoln and Short, 1980).

The length of the reproductively active period, or breeding season, is dependent on breed and is primarily determined by the length of the period of ovarian activity in the female. Primitive breeds of sheep are naturally mono-oestrus and experience a very short breeding season. Domesticated breeds are normally poly-oestrus, the number of oestrous cycles varying between 2-3 cycles in Scottish Blackface sheep, 5-6 cycles in some lowland breeds and almost continuous ovarian activity in breeds such as the Dorset Horn (Webster and Haresign, 1983), the Booroola Merino (Bindon and Piper, 1976) and the D'man (Bouix and Kadiri, 1975). Within breed variation in the duration of the breeding season is dependent on factors such as nutritional status and latitude. Scottish Blackface sheep when maintained in the Scottish highlands have 2-3 oestrous cycles per breeding season, but if maintained at a lower latitude in southern Britain can have between 3-5 oestrous cycles.

The breeding season occurs during the autumn and appears to be initiated in both sexes by a decrease in the prevailing photoperiod (Marshall, 1937; Ortavant, Mauleon, Thibault, 1964; Lincoln and Davidson, 1977). Animals moved between the northern and southern hemispheres

experience a 6-month shift in the onset of their breeding season (Marshall, 1936). The importance of photoperiod in the regulation of the breeding season is further demonstrated by the artificial induction of seasonal changes in both ewes (Yeates, 1949; Hafez, 1952; Thwaites, 1965; Wodzicka-Tomaszewska, Hutchinson and Bennet, 1967; Legan and Karsch, 1980) and rams (Lincoln, 1976a; Lincoln and Davidson, 1977; Lincoln, Peet and Cunningham, 1977) by the use of artificial photoperiods; reproductive activity being induced by decreasing day length and terminated by increasing day length (Pelletier and Ortavant, 1975a; Legan and Karsch, 1980). Sheep maintained in equatorial conditions, where the light:dark ratio is similar, are not subjected to pronounced change in photoperiod; however, although seasonality is not as distinct, cycles of breeding activity do occur due to the presence of a natural rhythm of breeding activity (Wodzicka-Tomaszewska *et al.*, 1967), and the utilization of environmental cues, such as temperature (Thwaites, 1965). Evidence for the importance of a natural rhythm of breeding activity is now increasing, the effects of photoperiod being important for the synchronization of this endogenous rhythm (Thwaites, 1965; Bittman, Karsch and Hopkins, 1983; Almeida and Lincoln, 1984; Farner, 1985; Lincoln, Libre and Merriman, 1989; Karsch, Robinson, Woodfill and Brown, 1989). If mating is unsuccessful, at the end of the breeding season the ewes become less responsive to the stimulatory effects of the natural ambient daylength and become photorefractory (Lincoln, 1980; Worthy and Haresign, 1983; Robinson and Karsch, 1984 Malpaux, Robinson, Brown and Karsch, 1987). The cycles of ovarian activity cease and the ewes enter anoestrus. If mating is successful and pregnancy occurs, breeding activity is abolished for the duration of the gestational period. In most breeds of sheep, gestational anoestrus is followed by a further period of involuntary (lactational) anoestrus, which extends into seasonal anoestrus and limits breeding capacity to one litter per year.

However in some breeds of sheep such as the Dorset Horn and the Merino, the occurrence of a fertile *post-partum* oestrus provides the opportunity for a second pregnancy and allows the production of two crops of lambs per year.

The mean length of the gestational period in sheep is 147 days (Haresign, Foxcroft and Lamming, 1983), but varies with breed between 144 and 154 days (Rowlands and Weir, 1984). Litter size also varies according to breed and the environment; the average litter size being between 1-3 lambs. However, some breeds such as the Booroola Merino and the Cambridge are renowned for their high prolificacy and litters of up to 7 lambs have been recorded (see sections 1.4.2.1. and 1.4.2.4.).

1.2 Endocrine control of reproduction

1.2.1 Physiology of the female

1.2.1.1. Birth to puberty

1.2.1.1.1. LH

In the female lamb, the mean LH concentration in the peripheral circulation increases between birth and puberty (Foster, Jaffe and Niswender, 1975a; Foster, Lemons, Jaffe and Niswender, 1975b; Fitzgerald and Butler, 1978; Foster and Ryan, 1979a, 1979b; Keisler, Inskeep and Dailey, 1985). During the immediate postnatal period, LH is secreted in a continuous fashion and the concentration of LH in the circulation is low. As the lambs mature, LH secretion becomes pulsatile (Foster, Cook and Nalbandov, 1972; Foster *et al.*, 1975a, 1975b; Echternkamp and Laster, 1976). The initiation of pulsatile LH secretion occurs between 2 (Foster *et al.*, 1972) and 4-9 weeks of age (Foster *et al.*, 1975a). The low secretion rate of LH and the absence of pulses in the early postnatal period are postulated to be due to the suppression of LH secretion by materno-placental steroids in the peripheral circulation of the neonatal lamb (Findlay and Cox, 1970) and/or

to postnatal maturational changes in the hypothalamo-hypophyseal complex which must occur before pulsatile LH secretion is initiated (Foster *et al.*, 1975a). Ovariectomy of adult ewes results in an immediate increase in LH secretion, However in neonatal lambs an increase in LH secretion is not seen until the animals are 4-6 weeks of age, coincident with the onset of endogenous LH pulsatility in intact controls. Therefore it would appear that a steroid-independent change occurs in the regulation of LH secretion, at the level of the hypothalamo-hypophysial complex, which is associated with the onset of pulsatile LH secretion (Foster *et al.*, 1975a).

The increase in the mean LH concentration in the prepubertal period is not continuous. An initial increase occurs between birth and 5 weeks of age and is followed by a transient decrease (Bindon and Turner, 1974; Foster *et al.*, 1975a), with further peaks of LH secretion occurring at approximately 11 and 17-20 weeks of age (Foster *et al.*, 1975b; Claypool and Foster, 1990). Thereafter, the mean LH concentration decreases until puberty, which occurs at 25-35 weeks of age. After puberty LH secretion increases and cycles of ovarian and hormonal activity begin. The concentration of LH in the prepubertal female is significantly higher than that seen in cycling adult ewes and remains elevated throughout the first breeding season (Foster *et al.*, 1975b). Ovarian activity in the prepubertal lamb is associated with a pattern of LH secretion similar to that seen in the adult. Changes in peripheral LH concentrations occur due to changes in LH pulse amplitude and LH pulse frequency. The changes in the mean LH concentration during the prepubertal period have been reported to parallel the changes in LH pulse frequency (Foster *et al.*, 1975a) and to be inversely related to the changes in LH pulse amplitude (Bindon and Turner, 1974; Foster *et al.*, 1975b). However other studies maintain that the changes in the mean LH concentrations are principally due to changes in LH pulse amplitude (Keisler *et al.*, 1985; Echternkamp and Laster, 1976).

During the prepubertal period, tonic LH secretion is sensitive to regulation by both progesterone and oestradiol. Progesterone is capable of suppressing LH secretion, even though high concentrations of endogenous progesterone are not seen until after puberty and the formation of a functional corpus luteum. Progesterone treatment prevents the increase in LH secretion which occurs following ovariectomy in prepubertal female lambs (Foster and Karsch, 1976). In spring-born lambs, the effectiveness of oestradiol at inhibiting LH secretion decreases dramatically between 25 and 35 weeks of age in both ovariectomised (Foster and Ryan, 1979a, 1979b, 1981; Claypool and Foster, 1990) and entire lambs (Foster and Karsch, 1975). Following the decrease in the negative feedback capabilities of oestradiol, the mean LH concentration increases, due to an increase in LH pulse frequency (Huffman, Inskeep and Goodman, 1987; Claypool and Foster, 1990). Oestradiol can therefore be seen to play an important role in the regulation of LH pulse frequency in young female lambs (Foster *et al.*, 1975a; Foster and Ryan, 1981). The increases in the concentration of LH observed in entire, ovariectomised and oestradiol-treated ovariectomised lambs prior to puberty, indicate that two mechanisms are involved in the regulation of LH secretion at this time:- firstly, a decrease in the negative feedback effects of oestradiol and secondly, a direct stimulation of increased gonadotrophin secretion at the level of the hypothalamus/pituitary gland (Foster and Ryan, 1979a, 1979b).

1.2.1.1.2. FSH

Unlike LH, FSH is not released in a pulsatile manner. However FSH secretion during the early post-natal period appears to be closely linked with LH secretion, high concentrations of FSH being observed during periods of elevated LH secretion (Foster *et al.*, 1975a). The concentration of FSH increases during the first 5 weeks after birth, then decreases

transiently, before increasing gradually until puberty (Foster *et al.*, 1975a, 1975b). At puberty the concentration of FSH is similar to that seen in the adult. As no significant changes are observed in peripheral FSH concentrations immediately prior to the attainment of puberty (Foster *et al.*, 1975a; Foster *et al.*, 1975b; Fitzgerald, 1978 cited by Foster and Ryan, 1981) and the administration of exogenous LH alone is capable of inducing ovarian activity (Ryan and Foster, 1980), it was concluded that prepubertal FSH concentrations were sufficient for the initiation of oestrous cycles and that it was the changes in the pattern of LH secretion which controlled the attainment of puberty (Foster and Ryan, 1981).

1.2.1.2. Puberty

The sequence of events leading to the onset of cyclical patterns of follicular growth and ovulation, and the attainment of puberty in the female lamb, are very similar to those seen at the transition between the anoestrous season and the breeding season in the adult ewe. Silent ovulations occur prior to the onset of clearly defined oestrous cycles. The physiology of the pubertal period is characterised by the appearance of preovulatory-type surges of gonadotrophin secretion. As preovulatory-type patterns of gonadotrophin and oestradiol secretion can be induced in prepubertal female lambs prior to the occurrence of endogenous surges by the administration of oestradiol or PMSG (Squires, Scaramuzzi, Caldwell and Inskeep, 1972; Foster and Karsch, 1975), it was proposed that their absence during the prepubertal period was not due to the inability of the hypothalamus/pituitary gland to secrete sufficient quantities of gonadotrophins, but due to inhibition of the gonadotrophin surge mechanism, which remains functional, but inactive until after puberty (Foster and Ryan, 1981). The effectiveness of oestradiol at inducing a preovulatory-type surge of gonadotrophin secretion increases as puberty

approaches, coincident with a decrease in the negative feedback capabilities of oestradiol (Foster and Karsch, 1975). Therefore it was proposed that as tonic LH secretion is acutely sensitive to oestradiol negative feedback and that the peripheral LH concentration is involved with the positive regulation of oestradiol secretion, that prior to puberty the concentration of oestradiol never reaches the concentration required to stimulate a preovulatory gonadotrophin surge. This proposal was substantiated by the demonstration that an appropriate pattern of exogenous LH induced a preovulatory gonadotrophin surge in prepubertal female lambs (Ryan and Foster, 1980; Keisler *et al.*, 1985).

The initiation of cyclical ovarian activity in the pubertal female lamb, is characterised by an increase in the peripheral concentrations of both LH and progesterone, 2-6 days prior to the observation of the first LH surge (Walton, McNeilly, McNeilly, and Cunningham, 1977; Foster and Ryan, 1981; I'Anson and Legan, 1988a). This is followed by a short period during which the peripheral concentration of progesterone is elevated (Thorburn, Bassett and Smith, 1969; Walton *et al.*, 1977; Yuthasastrakosol, Palmer and Howland, 1975; I'Anson and Legan, 1988a, 1988b) and a second gonadotrophin surge occurs 6-7 days later (Walton *et al.*, 1977). This second surge of LH secretion induces ovulation but is not always accompanied by normal oestrous behaviour, "a silent ovulation" (Foster, 1988). The number of silent ovulations varies (Downing, 1980 cited Dyrmundsson, 1987; Hare and Bryant, 1982; Quirke, Stanbenfeldt and Bradford, 1985), but is normally greater in the pubertal female than in the adult. The silent ovulation is followed by a luteal phase of normal duration and a cycle of follicular development which culminates in ovulation accompanied by oestrous behaviour.

To summarise, in the developing prepubertal female lamb, although the hypothalamus/pituitary gland is capable of producing LH pulses of

high frequency (1 per hour) (Foster *et al.*, 1975a) the high sensitivity of the hypothalamus to oestradiol negative feedback suppresses LH pulse frequency and basal LH concentrations remain low. The pulses of LH that occur are infrequent and only capable of eliciting small transient increases in ovarian oestradiol secretion. The LH surge mechanism remains inactive. Associated with the onset of puberty the sensitivity of the hypothamus to oestradiol negative feedback decreases and LH pulse frequency increases, causing a gradual increase in basal LH concentrations (Foster and Ryan, 1981). 2-6 days prior to the first preovulatory gonadotrophin surge, the peripheral LH concentration increases due to a reduction in the effectiveness of oestradiol negative feedback and a steroid- independent upregulation of LH secretion. The increase in LH secretion stimulates a sustained increase in oestradiol secretion and activates the dormant gonadotrophin surge mechanism. The resultant gonadotrophin surge is followed by a short luteal phase, a second surge of gonadotrophin secretion and a silent ovulation. The silent ovulation is thereafter followed by a cycle of ovarian activity, which culminates in an ovulation, which is accompanied by a period of behavioural oestrus (Foster and Ryan, 1981).

1.2.1.3. The oestrous cycle

The oestrous cycle is the outward manifestation of a cycle of ovarian development, of approximately 17 days duration, which is synchronized by a series of hormone systems. Oestrogen, progesterone and androgens are produced by the ovary and are responsible for the manifestation of behavioural oestrus and the physical changes that occur in the reproductive tract during the oestrous cycle. LH, FSH and prolactin are secreted by the pituitary gland and are involved in the control of follicular growth, development and ovulation. Proteins such as inhibin and activin

are also produced by the ovary, but their role in the control of reproduction is not fully understood. In rodents, the oestrous cycle can be divided into pro-oestrus, oestrus, met-oestrus, and di-oestrus; however, these stages are not well defined in large mammals. Therefore the oestrous cycle in sheep (Day 0= oestrus) is divided into a luteal phase which lasts from Day 2 to Day 13 and a follicular phase which lasts from Day 14 to Day 1 (Baird and McNeilly, 1981).

1.2.1.3.1 Luteal phase

The luteal phase lasts from the formation of the corpus luteum in the ovary, until either the corpus luteum regresses, or pregnancy is established. The physiology of the luteal phase is dominated by the high concentration of the ovarian steroid progesterone. The concentration of progesterone in the peripheral circulation increases gradually from Day 1 to Day 12 of the oestrous cycle (Herriman, Harwood, Mallinson and Heitzman, 1979), as the granulosa and thecal cells of the ruptured follicle luteinize and begin to secrete progesterone (Hauger, Karsch and Foster, 1977; Hansel and Convey, 1983; Rowlands and Weir, 1984). The concentration of progesterone in the peripheral circulation thereafter remains high, until approximately Day 12 after which, in conjunction with the decline in the corpus luteum, it declines precipitously (Pant, Hopkinson and Fitzpatrick, 1977).

Progesterone is a potent inhibitor of LH secretion in both intact and ovariectomised ewes (Baird and Scarramuzzi, 1976; Foster and Karsch, 1976; Hauger *et al.*, 1977; Karsch, Legan, Hauger and Foster, 1977; Karsch, Foster, Legan, Ryan and Peter, 1979; Karsch, Goodman and Legan, 1980a; Karsch, Legan, Ryan and Foster, 1980b). Therefore as the concentration of progesterone in the peripheral circulation increases, the mean LH concentration decreases. LH secretion during the mid-luteal phase

consists of low frequency, high amplitude LH pulses (Foster *et al.*, 1975b; Baird, Swanston, and Scaramuzzi, 1976; Hauger *et al.*, 1977; Baird, 1978a; Karsch, Bittman, Foster, Goodman, Legan and Robinson, 1984).

During the early post-ovulatory period, after the rupture of the large dominant ovarian follicle and before the formation of the corpus luteum, steroid negative feedback is absent; however, the concentration of LH remains low. Initially, it was proposed that the concentration of LH remained low due to depletion of the pituitary stores of LH (Hansel and Convey, 1983) or desensitisation of the pituitary to GnRH secretion. However subsequent studies have shown that, although pituitary LH content and GnRH receptor numbers do indeed decline at the end of the LH surge (Roche, Foster, Karsch, Cook and Dziuk, 1970; Crowder and Nett, 1984), the pituitary gland can be stimulated to release significant quantities of LH with exogenous GnRH, within 6 hours of the preovulatory gonadotrophin surge (Dobson, Pant and Ward, 1974). Therefore it would appear that the low concentration of LH which occurs during the post-ovulatory period occurs due to a reduction in the hypothalamic stimulation of LH secretion.

During the luteal phase, waves of follicular growth can be observed in the ovary (Smeaton and Robertson, 1971; Holst, Braden and Mattner, 1972; Brand and De Jong, 1973). The first wave of follicular growth occurs during the early luteal phase (Smeaton and Robertson, 1971; Holst *et al.*, 1972), with a second wave occurring during the mid-luteal phase (Smeaton and Robertson, 1971). During the early- and mid-luteal phase, follicular growth is arrested before the follicles reach ovulatory size and the growing follicles become atretic and regress. In association with the waves of follicular growth, changes are seen in peripheral concentrations of oestradiol and LH (Cox, Mattner and Thorburn, 1971; Holst *et al.*, 1972; Hauger *et al.*, 1977; Baird and Scaramuzzi, 1976). As the follicles mature and increase in size, they secrete increasing concentrations of oestradiol;

however, as the concentration of progesterone in the peripheral circulation is high, these increases in the peripheral oestradiol concentration only stimulate small transient increases in the LH secretion. Fluctuations are also seen in the peripheral FSH concentrations during the early and mid-luteal phase, however these fluctuations do not appear to coincide with either follicular growth or LH secretion (Salamonsen, Jonas, Burger, Buckmaster, Chamley, Cumming, Findlay and Goding, 1973; Findlay and Clarke, 1987). Therefore, it was proposed that the first two waves of follicular growth are not completed due to a lack of gonadotrophic support. This proposal is supported by the demonstration that follicular atresia does not occur due to the inability of the follicles to complete development, since the removal of progesterone during the early- or mid-luteal phase by the induction of luteolysis (Baird and Scarrammuzi, 1976; Driancourt, Philipon, Locatelli, Jacques and Webb, 1988), or the removal of the corpus luteum (Smeaton and Robertson, 1971), allows complete follicular development and ovulation.

Luteolysis in the ewe is triggered on approximately Day 12 of the oestrous cycle by prostaglandin $F2_{\alpha}$ ($PGF2_{\alpha}$) (Baird, 1978b; Baird and McNeilly, 1981), secreted from the endometrium (Flint and Hillier, 1975). In some species, oxytocin also exhibits luteolytic activity. In the sheep, oxytocin secreted by the corpus luteum, although not directly involved with the stimulation of luteolysis, has been implicated in the control of $PGF2_{\alpha}$ secretion, as the concentration of endometrial oxytocin receptors increases at the time of luteolysis and oxytocin has been shown to stimulate $PGF2_{\alpha}$ secretion (Roberts, McCracken, Gavagen and Soloff, 1976; Sheldrick and Flint, 1985; Hooper, Watkins and Thorburn, 1987). Oxytocin binding is regulated by progesterone and oestradiol (Vallet, Lamming and Batten, 1990). Therefore oxytocin is involved in the up-regulation of $PGF_{2\alpha}$

secretion from the endometrium at the end of the luteal phase (McCracken, 1980).

1.2.1.3.2. Follicular phase

As discussed previously, during the luteal phase the high concentration of progesterone inhibits the secretion of preovulatory-type surges of gonadotrophin secretion, by preventing the positive feedback effects of oestradiol on LH secretion (Scarramuzzi, Tillson, Thorneycroft and Caldwell, 1971). At the end of the luteal phase, the corpus luteum regresses and the concentration of progesterone in the peripheral circulation falls. This decline in the peripheral progesterone concentration occurs at about Day 12 of the oestrous cycle and coincides with a third wave of follicular growth in the ovary (Smeaton and Robertson, 1971). This wave of follicular growth contains the follicle/s which subsequently complete development and ovulate (Herriman *et al.*, 1979) and is associated with an increase in the peripheral oestradiol concentration (Webb and England, 1982). The increase in the concentration of oestradiol and the decrease in the inhibitory effects of progesterone (Karsch *et al.*, 1984) at this time, stimulate increased LH secretion, as shown by the increase in the LH concentration in the peripheral circulation during the early follicular phase (Baird *et al.*, 1976; Pant *et al.*, 1977; Baird, 1978a; Wallace, Martin and McNeilly, 1988). During the early follicular phase the concentration of FSH decreases (L'Hermite, Niswender, Reichert and Midgley, 1972; Salamonsen *et al.*, 1973; Wallace *et al.*, 1988; Campbell, Mann, McNeilly and Baird, 1990a) due to the negative feedback effects of the increased concentrations of oestradiol (Baird and McNeilly, 1981; Fraser, Clarke and McNeilly, 1981; Platt, Foster, Tarnavsky and Reeves, 1983; Clarke, Cummins and de Krester, 1983; McNeilly, 1984; Martin, Price, Thiery and Webb, 1988; Mann, Campbell, McNeilly and Baird,

1990) and inhibin, which increase in parallel with the size of the preovulatory follicle (Tsonis, Quigg, Lee, Leversha, Trounson and Findlay, 1983; Findlay, Clarke and Robertson, 1990; Campbell *et al.*, 1990a, Mann *et al.*, 1990). The role of inhibin in reducing the concentration of FSH in the peripheral circulation during this period has been demonstrated by the decrease in the concentration of FSH following the administration of bovine follicular fluid during the preovulatory period (Millar, Crister, Rowe and Ginther, 1979; McNeilly, 1984).

During the late follicular phase, the frequency of LH pulses increases to 1 per 30 mins (Foster *et al.*, 1975b; Baird and Scaramuzzi, 1976; Baird *et al.*, 1976; Baird, 1978a; Karsch, Foster, Bittman and Goodman, 1983; Karsch *et al.*, 1984) and stimulates further oestradiol secretion from the preovulatory follicles (Baird and Scaramuzzi, 1976; Hauger *et al.*, 1977; Goodman, Legan, Ryan, Foster and Karsch, 1981a; McNatty, Gibb, Dobson, Thurley and Findlay, 1981a; Baird and McNeilly, 1981; McNeilly, O'Connell and Baird, 1982; McLeod, Haresign and Lamming, 1982; Campbell, McNeilly, Picton and Baird, 1990b). The thecal cells of the preovulatory follicles secrete increasing quantities of androgens, which are converted to oestradiol by aromatisation within the granulosa cells (McNatty, 1981; Baird, 1977). The increase in the oestradiol concentration which occurs during the late follicular phase is important for the stimulation of the preovulatory gonadotrophin surge, as abolition of the increase by either passive immunization against oestradiol (Fairclough, Smith and Peterson, 1976) or by ovariectomy (Webb, England and Fitzpatrick, 1981) delays or inhibits the preovulatory gonadotrophin surge, while administration of exogenous oestradiol stimulates the secretion of a preovulatory surge of gonadotrophin secretion.

The effects of oestradiol are mediated partly by increasing the responsiveness of the pituitary gland to GnRH (Convey, 1973; Reeves,

Arimura, and Schally, 1971a, 1971b) and partly by increasing GnRH secretion from the hypothalamus (See Section 1.3.4.1). The mutual positive feedback effects exerted by oestradiol and LH during the late follicular phase drive a cascade reaction which results in the preovulatory LH surge. A correlated surge of FSH secretion also occurs at this time (Jonas, Salamonsen, Burger, Chamley, Cumming, Findlay and Goding, 1973; L'Hermite *et al.*, 1972; Salamonsen *et al.*, 1973).

The high concentration of oestradiol during the late follicular phase triggers behavioural oestrus, which occurs at a similar time to the preovulatory LH surge (Hauger *et al.*, 1977; Pant *et al.*, 1977), ovulation occurring approximately 24 hours later (Cumming, Brown, Blockley, Wilfield, Baxter, and Godling, 1971) during the second half of behavioural oestrus. During the preovulatory gonadotrophin surge, prior to ovulation, the peripheral oestradiol concentration decreases (Baird and Scaramuzzi, 1976; Webb *et al.*, 1981) as the main source of oestradiol secretion is removed due to luteinization of the ovulatory follicle and the inhibitory effects of high LH concentrations on oestradiol secretion (Moor, 1974).

A second surge of FSH secretion occurs 20-30h after the preovulatory gonadotrophin surge, independent of any change in the peripheral LH concentration (L'Hermite *et al.*, 1972; Salamonsen *et al.*, 1973; Pant *et al.*, 1977; Dobson and Ward, 1977; Baird and McNeilly, 1981; Cahill, Saumande, Ravault, Blanc, Thimonier, Mariana, and Mauleon, 1981; Campbell *et al.*, 1990a). The second FSH surge is proposed to occur due to the removal of oestradiol negative feedback (Campbell *et al.*, 1990a) and is thought to be important in the recruitment of oestrogenic follicles to form the first wave of follicular growth for the next oestrous cycle (Smeaton and Robertson, 1971; Pant *et al.*, 1977; McNatty, Gibb, Dobson and Thurley, 1981b, Lahlou-Kassi, Schams, and Glatzel, 1984) and preantral follicles for the next ovulatory cycle (Cahill *et al.*, 1981). FSH secretion during the oestrous cycle

is controlled by a combination of oestradiol and inhibin. While the role played by oestradiol in the generation of the second surge of FSH secretion has been discussed, the role of inhibin remains unclear, with both negative (Hasegawa, Miyamoto, Igarashi, Yanaka, Sasaki and Iwamura, 1987; Findlay *et al.*, 1990) and positive correlations (Campbell *et al.*, 1990a) between the concentrations of FSH and inhibin being reported. Recent evidence however, suggests that the effects of inhibin are secondary to the effects of oestradiol, with oestradiol determining the pattern of FSH secretion and inhibin regulating the amount of FSH secreted (Campbell *et al.*, 1990a, 1990b; Campell, Mann, McNeilly and Baird, 1990c).

1.2.1.4 Seasonal anoestrus

1.2.1.4.1. Progesterone

During the breeding season the control of gonadotrophin secretion is dominated by the presence or absence of a corpus luteum on the ovary and the concentration of progesterone in the peripheral circulation. At the end of the final luteal phase of the breeding season the concentration of progesterone decreases and remains low throughout the anoestrous period, until the first ovulation of the following breeding season (Thorburn *et al.*, 1969; Yuthasastrakosol *et al.*, 1975). Progesterone alone does not control the cycle of reproductive activity/anoestrus, as administration of exogenous progesterone to ewes at the end of the breeding season in a pattern similar to that seen during the ovarian cycle (11 days out of 17) is insufficient to prevent the advent of anoestrus (Fletcher and Lindsay, 1971) and administion late in seasonal anoestrus can only advance the onset of ovarian cyclicity (Wishart, 1966), with complete follicular development and ovulation, when accompanied by the administration of exogenous gonadotrophins (Dutt, 1955; Raeside and Lamond, 1956).

1.2.1.4.2. Gonadotrophins

The most striking feature of the patterns of gonadotrophin secretion during anoestrus is the absence of surges of gonadotrophin secretion similar to those observed prior to ovulation. However, treatment of anoestrous animals with exogenous LH in a pattern similar to that seen during the follicular phase, or administration of either oestradiol or GnRH, both of which stimulate LH secretion, will stimulate the secretion of a preovulatory gonadotrophin surge and ovulation (Raeside and McDonald, 1959; Goding, Catt, Brown, Kaltenbach, Cumming and Mole, 1969; Fletcher and Lindsay, 1971; Symons, Cunningham and Saba, 1973; Nett, Akbar, and Niswender, 1974; Reeves, Tarnavsky and Chakraborty, 1974; Martensz, Baird, Scaramuzzi and Van Look, 1976; Land, Wheeler and Carr, 1976; Legan and Karsch, 1979; Goodman *et al.*, 1981a; McNeilly *et al.*, 1982; Goodman and Karsch, 1981). Therefore it was concluded that LH is important in the control of seasonal reproductive activity and that the changes in the pattern of tonic LH secretion are necessary for the seasonal initiation and termination of reproductive activity (Legan, Karsch and Foster, 1977).

Changes in LH secretion are proposed to occur following alterations in the sensitivity of the hypothalamus to gonadal steroids which are induced by changes in photoperiod (Hoffman, 1973). As progesterone does not appear to be actively involved in the control of seasonal reproductive activity, studies have concentrated on oestradiol as the principle factor regulating seasonality in the sheep. Following the final gonadotrophin surge of the breeding season the mean LH concentration decreases and does not increase at the time of the next expected follicular phase. The concentration of LH thereafter remains low, except for the occurrence of large infrequent pulses of LH (Roche *et al.*, 1970; Martensz *et al.*, 1976; Scaramuzzi and Baird, 1977; Yuthasastrakosol *et al.*, 1975). Estimates of LH pulse frequency during anoestrus range between 1 per 5hrs and 1 per

24hrs (Martensz *et al.*, 1976; Scaramuzzi and Baird, 1977; Walton, Evins, Fitzgerald and Cunningham, 1980). These variations in pulse frequency are possibly due to the different breeds used in these studies. This pattern of LH secretion is maintained until approximately 24 days before the detection of oestrous behaviour at the start of the following breeding season. The concentration of FSH varies throughout the anoestrous period, but no discernible pattern exists in the fluctuations, and mean FSH concentrations are not significantly different from those seen during the breeding season (Walton *et al.*, 1977, 1980).

1.2.1.4.3. Follicular growth and oestradiol secretion

During seasonal anoestrus, waves of follicular growth are observed on the ovary, with follicles achieving diameters of approximately 5-6mm before undergoing atresia. Follicles of this size are capable of secreting steroids, but the concentration of oestrogens in the peripheral circulation remain low, comparable to those observed during the mid-luteal phase of the oestrous cycle (Yuthasastrakosol *et al.*, 1975). Termination of follicular growth during anoestrus appears to occur due to a lack of LH support, as administration of exogenous LH, GnRH, or oestradiol in a suitable pattern, stimulates follicle development, ovulation and behavioural oestrus, as mentioned previously.

If ovulation is induced during anoestrus, LH and FSH concentrations fall rapidly to basal concentrations following the gonadotrophin surge and a functional corpus luteum seldom forms (Haresign *et al.*, 1983). This indicates that although the positive feedback effects of oestradiol are maintained out-with the breeding season, other factors are important for the development of "normal cyclic" ovarian function. Comparative studies on the effectiveness of oestradiol in stimulating LH release indicate that there is no difference between anoestrus and the breeding season (Beck

and Reeves, 1973; Legan and Karsch, 1979; Goodman *et al.*, 1981a). The results from these studies disagree with the results of Land *et al.*, (1976) who found that sensitivity to oestradiol positive feedback decreased during seasonal anoestrus. However the results of Land *et al.*, (1976) may not reflect the true endogenous situation, as the ewes used in this study were long-term ovariectomised ewes and pituitary sensitivity may therefore have been affected. It is well documented that oestradiol also exerts a negative feedback effect on gonadotrophin secretion, its administration resulting in an initial depression in the concentration of gonadotrophins in the peripheral circulation (Nett *et al.*, 1974; Martensz *et al.*, 1976; Legan and Karsch, 1979). The effectiveness of oestradiol in suppressing LH secretion appears to change with the reproductive state of the animal, being ineffective during the follicular phase, but suppressing tonic LH secretion in the seasonally anoestrous ewe.

Changes have also been observed in the negative feedback capabilities of oestradiol in ovariectomised ewes fitted with oestradiol implants, LH secretion decreasing in the ovariectomised ewes, coincident with the anoestrous period in the intact controls and increasing again at the beginning of the breeding season (Legan *et al.*, 1977). As the release of oestradiol from the implants was constant, the effectiveness of oestradiol in suppressing LH release must decrease at the start of the breeding season (Legan *et al.*, 1977). Oestradiol affects the regulation of LH secretion at both the hypothalamus and the pituitary gland (See Section 1.3.4.1.); however, since the administration of GnRH to anoestrous ewes stimulates LH and FSH secretion, the effects of oestradiol during anoestrus must occur at the level of the hypothalamus, preventing the release of GnRH (Symons *et al.*, 1974).

1.2.1.4.4. Androgens

In addition to oestradiol, the ovary secretes a number of other steroids including androstenedione. The secretion rate of androstenedione, relative to oestradiol, increases between the breeding season and anoestrus. Therefore, it was suggested that the increased concentration of androstenedione in the peripheral circulation affects the sensitivity of the hypothalamus to oestradiol negative feedback (Martensz *et al.*, 1976; Scaramuzzi and Baird, 1977). This theory is supported by the observation that animals immunized against androstenedione displayed an increase in both the mean LH concentration and LH pulse frequency, and that the positive feedback effects of oestradiol were abolished in the immunised animals (Martensz *et al.*, 1976). However caution must be exercised with these results as similar effects could also occur if the androstenedione antiserum cross-reacted with oestradiol.

1.2.1.4.5. Prolactin and Melatonin

During anoestrus the concentration of prolactin in the peripheral circulation is high and exhibits a marked diurnal pattern (Walton *et al.*, 1977, 1980). The mean concentration falls with the decrease in day length, approximately 25-30 days before the first ovulation (Walton *et al.*, 1977), and then remains low throughout the breeding season. The changes in the concentration of prolactin reflect the changes in photoperiod (Walton *et al.*, 1977; Thimonier, Ravault and Ortavant, 1978; Walton *et al.*, 1980) and may therefore be involved in the control of seasonal breeding (Walton *et al.*, 1980). Prolactin is known to reduce the secretion of LH in response to oestradiol, but not GnRH (Kann, Martinet and Schirar, 1976). Therefore the enhanced prolactin concentrations during seasonal anoestrus may play a role in enhancing the negative feedback effect of oestradiol, However

prolactin is not the primary determinant of the changes seen in the reproductive state of the animal.

Seasonal changes in the reproductive state of the sheep are dependent on the integrity of the pineal gland (Bittman *et al.*, 1983). In ganglionectomised ewes, where the pineal gland is isolated from optical stimuli, oestrous cycles persist even when ewes are exposed to a 'long day' inhibitory photoperiod, whereas in ewes ganglionectomised during anoestrus, a reduction in photoperiod is insufficient to induce reproductive activity (Bittman *et al.*, 1983). Melatonin is secreted by the pineal gland and the patterns of melatonin secretion are influenced by photoperiod. Therefore it was proposed that melatonin may mediate the effects of photoperiod with regard to the seasonal control of reproductive function (Rollag and Niswender, 1976). Although the photoperiodic control of ovarian cyclicity is governed by alterations in the feedback potency of oestradiol, this is in turn controlled by the concentrations of melatonin and possibly prolactin in the peripheral circulation.

To summarise, an increase in the effectiveness of oestradiol in inhibiting gonadotrophin secretion at the end of the breeding season prevents the increase in tonic LH secretion which is required for the final maturation of ovarian follicles. The concentration of oestradiol needed to trigger the preovulatory gonadotrophin surge is therefore not achieved and seasonal anoestrus ensues (Scaramuzzi and Baird, 1977; Legan *et al.*, 1977; Legan and Karsch, 1979). The negative feedback effects of oestradiol on LH secretion are heightened during anoestrus compared to the breeding season, the changes occurring at the level of the hypothalamus. These changes in the effectiveness of oestradiol in suppressing gonadotrophin secretion are primarily controlled by changes in the concentration of melatonin in the peripheral circulation, but may also be influenced by the peripheral concentrations of androstenedione and prolactin. In conclusion,

seasonal anoestrus is not a state of passive non-function, but active inhibition of the mechanism which controls tonic LH secretion.

1.2.1.5. Transition from seasonal anoestrus to the breeding season.

It is generally accepted that at the end of anoestrus, before normal ovarian cyclicity occurs, most ewes experience a silent ovulation i.e. an ovulation in the absence of behavioural oestrus (Walton *et al.*, 1976, 1980). These changes in ovarian function are accompanied by changes in gonadotrophin and steroid concentrations and are similar to those seen in prepubertal ewe lambs (see section 1.2.1.2.).

During the late anoestrous period fluctuations are seen in the concentration of LH in the peripheral circulation, prior to the initial increase in progesterone and LH, which signal the initiation of cyclical ovarian activity. These fluctuations may be important in stimulating the luteinization of ovarian follicles which subsequently secrete progesterone and cause a transient increase in the peripheral progesterone concentration. The short increase in the peripheral progesterone concentration is followed by a surge of gonadotrophin secretion and ovulation, which is not accompanied by a period of behavioural oestrus due to the absence of a regressing corpus luteum (Cole and Miller, 1933; Dutt, 1955). Following the silent ovulation, the remaining structure luteini es, forming a functional corpus luteum (Walton *et al.*, 1977, 1980) and a luteal phase of 'normal' duration occurs. This luteal phase is followed by a 'normal' follicular phase which culminates in ovulation accompanied by a period of behavioural oestrus. The concentration of oestradiol, which remains relatively low throughout anoestrus, only rises significantly coincident with the observation of the first period of behavioural oestrus (Yuthasastrakosol *et al.*, 1975; Scaramuzzi and Baird, 1977).

1.2.2. Physiology of the male

1.2.2.1 Birth to puberty

Testicular development and spermatogenesis in the prepubertal ram lamb are dependent on the secretion of gonadotrophins. Gonadotrophin treatment restores normal testes growth and function in hypophysectomised animals, where sexual function has been arrested (Courot, 1967). The mean concentrations of LH, FSH, and testosterone increase following birth until approximately 7-12 weeks of age (Lee, Cumming, de Krester, Findlay, Hudson and Keogh, 1976a; Yarney and Sanford, 1985b; Olster and Foster, 1986). The increase in LH and FSH secretion occurs due to a combination of increased maturation of the hypothalamus and an increase in the sensitivity of the pituitary gland to GnRH stimulation (Lee *et al.*, 1976a). The concentration of LH decreases thereafter and remains low throughout the remainder of the prepubertal period (Lee *et al.*, 1976a; Olster and Foster, 1986).

The changes in mean LH concentration can be studied as a function of pulse amplitude and pulse frequency. The changes which occur in LH pulse frequency appear to be more important in causing the changes in the mean LH concentrations (Yarney and Sanford, 1985a, 1985b; Olster and Foster, 1986), LH pulse amplitude exhibiting an inverse relationship to LH pulse frequency (Yarney and Sanford, 1985a, 1985b; Olster and Foster, 1986). During the early prepubertal period, LH is secreted in the form of low frequency, high amplitude pulses. The increase in the mean LH concentration between 8 and 11 weeks of age occurs due to an increase in LH pulse frequency. This appears to occur independently of any changes in the steroidal regulation of LH secretion (Claypool and Foster, 1990), the mean LH concentration increasing at the same time in both intact and testosterone-treated, castrated ram lambs (Olster and Foster, 1986).

The concentration of testosterone measured in the peripheral circulation increases with age between birth and puberty (Lee *et al.*, 1976a; Yarney and Sanford, 1985b; Olster and Foster, 1986). The increase in gonadotrophin secretion, between 8 and 11 weeks of age, occurs coincident with an increase in testicular size (Claypool and Foster, 1990), which is in turn followed by a significant increase in testosterone secretion (Lee *et al.*, 1976a; Olster and Foster, 1986). This increase in the concentration of testosterone continues throughout the remainder of the prepubertal period irrespective of any decrease in LH secretion (Lee *et al.*, 1976a; Yarney and Sanford, 1985a, 1985b; Olster and Foster, 1986). The negative feedback effects of testosterone on gonadotrophin secretion have been demonstrated in the ram in a number of physiological situations, eg. by the delay in the post-castration rise in gonadotrophin secretion by testosterone treatment and the suppression of endogenous LH secretion in testosterone-treated entire lambs (Olster and Foster, 1986). These changes in the testosterone concentration in the peripheral circulation may therefore explain the changes in gonadotrophin secretion seen during the late prepubertal period. The significant increase in the concentration of testosterone in the peripheral circulation at approximately 12 weeks of age causes a suppression of LH secretion and a consequent decrease in the peripheral LH concentration.

1.2.2.2. Puberty

Physical puberty in the ram is reached when lambs are approximately 16-20 weeks of age (Olster and Foster, 1986) and is associated with descent of the testes into the scrotum, increased testicular size and detachment of preputial adhesions which then allows free movement of the penis within the sheath (Dyrmundsson, 1972, cited by Dyrmundsson, 1987). The ability to release spermatozoa is attained before physical puberty, at

approximately 8-11 weeks of age, when the testes can be as little as half their mature size (Dyrmundsson, 1978).

Both LH (Hochereau-de-Reviers, Blanc, Courot, Garnier, Pelletier and Poirier, 1980) and FSH (Waites, Wenstrom, Crabo and Hamilton, 1983) have been demonstrated to be essential for full testicular function (reviewed by Waites and Setchell, 1990). However, when physical puberty is reached the mean concentrations of both LH and FSH are low, although as described previously, the mean FSH concentration is increasing (Lee *et al.*, 1976a; Olster and Foster, 1986). Although the mean LH concentration does not change significantly, alterations are seen in the pattern of LH secretion at and around the time of physical puberty, LH pulse frequency increasing and LH pulse amplitude decreasing (Olster and Foster, 1986). The spermatogenic cycle in the ram lasts approximately 50 days (Ortavant, 1958 cited by Olster and Foster, 1986); therefore, spermatogenesis is initiated when lambs are approximately 9-11 weeks of age i.e. soon after the initial peak of LH and FSH secretion. This peak of gonadotrophin secretion has therefore been proposed to be involved in the initiation of spermatogenesis.

The concentration of testosterone increases rapidly following puberty, coincident with a period of high-frequency, low-amplitude LH pulses (Olster and Foster, 1986) and supports the ability of LH to stimulate testosterone secretion in rams (Bremner, Findlay, Cumming, Hudson, and de Krester, 1976). The increase in the mean concentrations of testosterone and LH are accompanied by increases in the number of testicular LH-receptors and the concentration of prolactin (Yarney and Sanford, 1985b). Prolactin may therefore play a role in the induction and maintenance of testicular LH-receptors and hence the induction of puberty.

Oestradiol can affect LH secretion in the adult ram and administration of oestradiol to prepubertal ram lambs, as with the

administration of testosterone, delays the increase in LH pulse frequency and reduces LH pulse amplitude. The similar effects of oestradiol and testosterone on LH pulse frequency have led to the proposal that the effects of testosterone on LH pulse amplitude are mediated by aromatisation of testosterone to oestradiol and the subsequent inhibition of the LH pulse generator (Olster and Foster, 1986). Oestradiol does not have as great an effect on FSH secretion in the young ram. Therefore it was proposed that FSH secretion in the ram lamb is primarily controlled by testosterone (Schanbacher, 1979; Olster and Foster, 1986), or some other gonadal factor such as inhibin. Inhibin has recently been shown to be secreted from the testes and to be involved in the control of both LH and FSH secretion in the adult ram (Lincoln and McNeilly, 1989).

To summarise, sexual competence in the male is achieved a long time after the initiation of spermatogenesis, when physical changes occur in the reproductive organs, which require the presence of both gonadotrophins and testosterone. The concentrations of LH and FSH increase between birth and 8-11 weeks of age, due to maturational changes in the hypothalamus and pituitary gland, and initiate spermatogenesis. The increased LH concentration also stimulates testosterone secretion, but due to the high sensitivity of the hypophyseal axis of the young lamb to steroid negative feedback, the increase in testosterone secretion suppresses further gonadotrophin secretion and a decrease is observed in the peripheral concentrations of both LH and FSH. With the onset of puberty, the sensitivity of the hypothalamo-hypophyseal axis to steroid negative feedback decreases and an increase is seen in both LH and FSH secretion. The concentration of FSH increases in advance of the increase in LH secretion and may indicate an aspect of the differential control of LH and FSH secretion, LH secretion being reduced due to direct suppression by testosterone and indirectly by the metabolism of testosterone to oestradiol,

while FSH secretion is regulated by a combination of testosterone and other gonadal factors such as inhibin.

1.2.2.3. The sexual season in the adult ram

Changes are seen in gonadotrophin secretion throughout the seasonal cycle. Principally, there are changes in the frequency and amplitude of LH pulses, which presumably reflect changes in GnRH secretion by the hypothalamus (Katongole, Natolin and Short, 1974). The importance of GnRH/gonadotrophin secretion in regulating the seasonal cycle is demonstrated by the absence of reproductive function in adult rams which have been treated with GnRH antiserum, which abolishes LH and testosterone secretion (Lincoln and Fraser, 1979). Similarly, the administration of GnRH to seasonally quiescent rams, at a dose and frequency which stimulates a pattern of LH secretion similar to that observed during the reproductively active period, induces 'normal' seasonal changes in testicular activity and gonadotrophin secretion (Lincoln, 1979b, 1979d).

As in the female, the ram appears to have an endogenous cycle of reproductive activity which is synchronised by changes in daylength. When rams are maintained in conditions of long days for more than 2-3 months, changes occur in testicular activity and gonadotrophin secretion similar to those seen at the end of the normal reproductively active period (Lincoln and Peet, 1977). The presence of an endogenous rhythm of reproductive activity also helps explain the observation that the initiation of testicular growth and reproductive activity begins in advance of the summer solstice, when daylength is still increasing, and that testicular regression occurs in advance of the winter solstice (Lincoln and Short, 1980).

As in the ewe, photoperiod in the ram mediates its effects through the pineal gland via changes in the secretion of melatonin. In intact animals melatonin is secreted in a diurnal pattern, with increased secretion during the periods of darkness. The peak concentrations of melatonin are therefore dependent on daylength, with larger pulses being secreted following exposure of the animals to short day photoperiods (Lincoln and Short, 1980). Further support is added to the theory that melatonin plays a central role in the seasonal regulation of reproductive activity in the ram by the observation that following ganglionectomy the melatonin concentration in the peripheral circulation is undetectable, and a ram is produced which does not exhibit seasonal changes in reproductive function, 'a ram for all seasons' (Lincoln, 1979c; Barrell and Lapwood, 1979; Lincoln, Ameida and Arendt, 1981).

These seasonal changes in reproductive activity are associated with changes in testicular size and can be split into three distinct stages:- A, reproductive quiescence, when the testes are fully regressed. B, testicular growth and C, maximum testicular activity. These differences in testicular size are associated with changes in the concentrations of LH, FSH, testosterone and prolactin.

1.2.2.3.1. Reproductive quiescence

Exposure of adult rams to a sequence of short days followed by a period of long days induces testicular regression, the testes decreasing to 20% of their mature size (Lincoln, 1976a, 1976b, 1979c; Lincoln *et al.*, 1977, Lincoln, McNeilly and Cameron, 1978). This reduction in testicular size is associated with a decrease in the size of the Leydig cells and as testosterone is secreted by these cells, a decrease is also observed in the concentration of testosterone in the peripheral circulation (Lincoln *et al.*, 1977).

When the testes are fully regressed, spermatogenesis and testosterone secretion are minimal (Lincoln, 1977) and the sensitivity of the hypothalamus to testosterone negative feedback is greatly increased. Minimal doses of testosterone (1-2ng/ml) cause a significant suppression of both the mean LH concentration (Pelletier, 1974; Gallway and Pelletier, 1975; Lincoln, 1977) and LH pulse frequency (Pelletier, 1970). Therefore, the LH secretion profile during this period is characterised by low basal LH concentrations, interrupted occasionally by large pulses of LH secretion of short duration (approximately 1 pulse per day) (Lincoln, 1976a, 1976b, 1977; Lincoln and Short, 1980). The concentration of FSH in the peripheral circulation is low during reproductive quiescence, whereas prolactin is secreted at a high concentration throughout this period (Lincoln and Short, 1980).

1.2.2.3.2. Testicular Growth.

A decrease in photoperiod, whether natural or artificial, is rapidly followed by an increase in the peripheral concentrations of both LH and FSH (Lincoln, 1977; Lincoln and Peet, 1977). An increase in GnRH pulse frequency stimulates an increase in the frequency of LH pulses and an increase in basal and mean LH concentrations (Lincoln and Short, 1980). LH pulses during this period are of high amplitude and short duration (Lincoln and Short, 1980). Each LH pulse is followed by a transient increase in the concentration of testosterone (Katongole *et al.*, 1974; Lincoln, 1976b; Schanbacher and Ford, 1976). Therefore the increase in the mean LH concentration is followed by an increase in the mean testosterone concentration and an associated increase in testicular diameter (Lincoln *et al.*, 1977).

During the growth phase, FSH is secreted at a higher rate than during the quiescent period (Lincoln and Peet, 1977; Lincoln and Short,

1980). The high concentration of FSH stimulates spermatogenesis and testicular growth, mediating its effects via the Sertoli cells (Courot, 1971 cited by Lincoln and Peet, 1977; Courot and Ortavant, 1981).

Once maximum testicular size and activity are achieved, the concentrations of FSH and LH in the peripheral circulation decrease rapidly. The decrease in LH secretion may be due to high concentrations of steroids in the peripheral circulation, exerting negative feedback at both the hypothalamus and pituitary gland (Pelletier, 1970; Hopkinson, Pant, and Fitzpatrick, 1974; Pelletier, 1974; Lincoln, 1976b, 1977; Lincoln *et al.*, 1977) (See section 1.3.4.1). The decrease in FSH secretion could be due to a combination of steroid negative feedback and increased inhibin secretion by the mature testes (Franchimont, Chari and Demoulin, 1975, Lincoln and McNeilly, 1989; Lincoln, Lincoln and McNeilly, 1990). Prolactin secretion declines rapidly at the start of the testicular growth phase, significant reductions being observed after only 6 days of a reduced photoperiod (Lincoln and Short, 1980).

1.2.2.3.3. Maximum testicular activity.

During the period of maximum testicular activity, LH and testosterone secretion are high, with pronounced pulses of testosterone being observed in response to pulses of LH. Oestradiol is also present in the peripheral circulation at a relatively high concentration, due to the aromatization of testicular androgens (Schanbacher, 1984) in both gonadal and extra-gonadal tissue (Dorrington, Fritz and Armstrong, 1978). The ability of oestradiol to suppress LH secretion in castrated rams (Schanbacher, 1979), combined with the presence of specific oestradiol receptors in the hypothalamus (Pelletier and Caraty, 1981) and pituitary glands (Thieulant and Pelletier, 1979) of rams, suggests that oestradiol may

be involved in the control of LH secretion in the male and could be the primary negative feedback hormone (Schanbacher, 1979; 1984).

During the period of maximum reproductive activity, the inhibitory effects of testosterone negative feedback on LH secretion are reduced (Lincoln and Short, 1980). The frequency of GnRH/LH pulses therefore increases during this period, even in the presence of increasing concentrations of testosterone (Pelletier, 1970; Lincoln, 1977; Lincoln and Fraser, 1979). The frequency of LH pulses increases to 1 per 2 hours (Lincoln, 1979d) and are of low amplitude, but of longer duration than during the other two stages of the reproductive cycle (Lincoln, 1977) and therefore result in an overall increase in the mean LH concentration. The secretion of longer duration LH pulses during the 'active' period is proposed to be due to changes in the synthetic activity of the gonadotrophs, which is associated with priming of the pituitary gland (Lincoln and Short, 1980). FSH secretion remains low during this period (Lincoln and Short, 1980).

After a couple of months, the secretion rate of LH and testosterone declines, and this reduction in testosterone and gonadotrophin support heralds a gradual reduction in testicular size and cessation of spermatogenic activity. The concentration of prolactin remains low until the onset of testicular regression, when there is a rapid increase in the concentration of this hormone in the peripheral circulation to levels seen during reproductive quiescence (Lincoln and Short, 1980).

To conclude, during spring and early summer, or following the exposure of rams to long-day inhibitory photoperiods, the hypothalamus and pituitary gland are acutely sensitive to the negative feedback effects of testosterone. Hence the secretory activity of the hypothalamus is low (GnRH pulse frequency less than 1 per 24hrs), mean LH and FSH concentrations are low and the synthetic activity of the gonadotrophs

decline. The synthetic activity of the Leydig cells also declines due to the lack of gonadotrophic stimulation. The concentration of testosterone in the peripheral circulation is therefore low and testicular regression is evident. During late summer, the sensitivity of the hypothalamus to testosterone negative feedback decreases and the secretory activity of the hypothalamus increases. The frequency of GnRH and LH pulses increase, together with testicular diameter and the concentration of testosterone. The concentration of FSH also increases during this period but remains relatively low due to the inhibitory effects of testosterone and inhibin secreted by the testes.

1.3. Gonadotrophin - releasing hormone

1.3.1. Introduction

The regulation of seasonal breeding and reproductive activity in both males and females are controlled by the concentrations of gonadotrophins, steroids, and gonadal proteins in the peripheral circulation, the concentrations of which are controlled in turn either directly or indirectly by Gonadotrophin- releasing hormone (GnRH). GnRH is involved in the positive regulation of gonadotrophin and steroid secretion and its secretion and actions are affected by steroids. Hence, GnRH plays a pivotal role in the maintenance of cycles of ovarian/testicular seasonal reproductive activity and as such, the secretion and effects of GnRH have been studied extensively.

The control of gonadotrophin secretion by the central nervous system was initially demonstrated by Harris (1937), who showed that electrical stimulation of the hypothalamus in rabbits resulted in ovulation. Due to the absence of a direct nervous link between the hypothalamus and the pituitary gland, it was proposed that gonadotrophin secretion was controlled by the central nervous system via a neurohumoral mechanism

(Hinsey, 1937; Harris, 1955). This proposal was supported by the demonstration that gonadal function was restored in hypophysectomised rats by pituitary grafts vascularised by the hypophyseal portal vessels (Harris and Jacobson, 1952), and the demonstration that extracts of the median eminence could evoke ovulation when injected directly into the anterior pituitary (Harris, 1961). Finally, it was shown that gonadotrophin secretion was dependent on the production by the brain of a decapeptide, gonadotrophin-releasing hormone (Matsuo, Baba, Nair, Arimura, and Schally, 1971; Schally, Arimura, Kastin, Matsuo, Baba, Redding, Nair, Debeljuk, 1971). GnRH is synthesised by the hypothalamus (Silverman, 1987; Sherwood, Chiappa and Fink, 1976) and secreted into the hypophyseal portal blood stream where it passes to its principal site of action, the pituitary gland. The control of gonadotrophin secretion by GnRH has been demonstrated in a number of mammalian and submammalian species including the rhesus monkey (Carmel, Araki and Ferin, 1976; Nieill, Patton, Dailey, Tsou and Tindall, 1977) and the rat (Eskay, Mical and Porter, 1977; Sarkar and Fink, 1980). At the pituitary gland, GnRH stimulates the secretion of gonadotrophins (Dierschke, Bhattacharya, Atkinson and Knobil, 1970; Schally *et al.*, 1971; Aiyer *et al.*, 1974a; Sarkar, Chiappa, Fink and Sherwood, 1976; Clarke, Fraser and McNeilly, 1978; Lincoln and Fraser, 1979; Lincoln, 1979d: Sherwood, Chiappa, Sarkar and Fink, 1980; Kranchev, Stankov and Dobson, 1987). The ability of GnRH to stimulate the release of both LH and FSH initially led to the hypothesis that the secretion of both hormones was primarily controlled by GnRH. Subsequent studies however, have indicated that the effectiveness of GnRH at stimulating LH and FSH secretion differs in a number of physiological situations (Kalra, Ajika, Krulich, Fawcett, Quijada and McCann, 1971; Chappel and Barraclough, 1976; Ojeda, Jameson and McCann, 1977; Lumpkin and McCann, 1984; Bishop, Kalra, Fawcett, Krulich and McCann, 1972; Kranchev *et al.*, 1987) and that

the pattern of secretion of the two gonadotrophins also differs. LH release occurs in pulses (Levine, Pau, Ramirez and Jackson, 1982; Clarke and Cummins, 1982), while FSH secretion is relatively constant. These differences indicate that although GnRH is involved in the regulation of both LH and FSH secretion, some difference must exist in its ability to regulate each independently. Various mechanisms have been proposed to explain the differential secretion of the two gonadotrophins. These include;- (i) the modification of the GnRH signal by the actions of gonadal hormones and proteins at the level of the gonadotroph, e.g. oestradiol stimulating LH secretion (Clarke and Cummins, 1984), and inhibiting FSH secretion (Marshall, Case, Valk, Corley, Sauder and Kelch, 1983), and the preferential inhibition of FSH release from gonadotrophs treated with inhibin *in vitro* (Martin, Wallace, Taylor, Fraser, Tsonis and McNeilly, 1986; deGreef, Eilers, deKoning, Karels and deJong, 1987; Farnworth, Robertson, DeKretser and Burger, 1988a), (ii) that differences exist in the responsiveness of the gonadotrophs to GnRH stimulation, one gonadotrophin requiring a different pattern, or a different amount of GnRH to stimulate secretion (Yen, Rebar, Vandenberg, Naftolin, Ehara, Englbom, Ryan, Benirschke, Rivier, Amoss and Guillemin, 1972a; Yen, Vandenberg, Rebar, and Ehara, 1972b; Brown-Grant and Greig, 1975; Dalkin, Haisenleder, Ortolano, Ellis and Marshall, 1989) (See section 1.3.3), and (iii) the existence of a separate FSH-releasing hormone (Mizunuma, Samson, Lumpkin, Moltz, Fawcett and McCann, 1984; McCann and Rettori, 1987).

The abolition of endogenous GnRH secretion can be achieved in the ewe by hypothalamo-pituitary gland disconnection or immunization against GnRH. Following the removal of endogenous GnRH secretion, pulsatile LH secretion is abolished immediately; however, the concentration of FSH does not change immediately but declines linearly with time (Clarke, Cummins, Findlay, Burman and Doughton, 1984; McNeilly, Jonassen and

Fraser, 1986). This led to the hypothesis that exposure of gonadotrophes to GnRH does not stimulate FSH secretion directly as with LH (Clarke, 1987), but that FSH secretion is the result of an essentially passive process, GnRH merely stimulating FSH synthesis (Clarke, Burman, Doughton and Cummins, 1986). Differential control of gonadotrophin secretion also occurs due to the selective suppression of FSH by an ovarian protein, inhibin (Schwartz, 1986).

1.3.2. GnRH synthesis and secretion

GnRH is synthesized and secreted from neurons in the central nervous system. It is released in a regular pulsatile fashion even when neural tissue is maintained *in vitro*, which suggests that the GnRH neurons have an intrinsic rhythm of GnRH secretion, which is then coordinated by means of a separate rhythm-generating unit (Martin, 1984). The GnRH-secreting neurons are not organised into discrete areas of the central nervous system, but into numerous areas containing high concentrations of GnRH-producing cell bodies. The area of the anterior hypothalamus which encompasses the medial preoptic area and the suprachiasmatic nuclei contains an above-average concentration of GnRH neuron cell bodies (Silverman, 1987) and is therefore cited as being the primary site of GnRH synthesis and secretion. Other areas which contain a high proportion of GnRH neurons are the septal preoptic area, the diagonal band of Broca (DBB), the organum vasculosum of the lamina terminalis (OVLT), the olfactory bulb, and the preoptic area (Seeburg, Mason, Stewart and Nikolics, 1987). GnRH-producing cells and GnRH-receptors are also found in extra-hypothalamic tissues such as the gonads (Hsueh and Jones, 1981), the placenta (Khodr and Siler-Khodr, 1980; Iwasita, Evans, and Catt, 1986) and lactating mammary glands (Seeberg *et al.*, 1987).

GnRH is synthesised as part of a large precursor molecule (Millar, Aehnelt and Rossier, 1977; Gautron, Pattou and Kordon, 1981; Curtis and Fink, 1983; Curtis, Lyons and Fink, 1983). The human GnRH precursor contains 92 amino acids and has a molecular weight of 10000 (Seeburg and Adelman, 1984; Nikolics, Mason, Szonyi, Ramachandran and Seeburg, 1985). Estimates of the molecular weight of GnRH do vary however, between 1800 and 70000 (Nickolics and Seeburg, 1987, cited by Seeburg *et al.*, 1987). The GnRH precursor encodes a 23 amino acid signal sequence, which incorporates a hydrophobic centre region, which is involved in processing the peptide in the endoplasmic reticulum/Golgi apparatus, the GnRH peptide sequence, Pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 which is followed by the cleavage and amidation sequence (Gly-Lys-Arg) and a 56 amino acid extension which codes a protein called the GnRH-associated protein (GAP) (Seeburg and Adelman, 1984; Adelman, Mason, Hayflick and Seeburg, 1986; Seeburg*et al.*, 1987; Conn, Huckle, Andrews and McArdle, 1987a).

GAP is produced in conjunction with GnRH by the same neurons (Phillips, Nikolics, Branton and Seeburg, 1985). GAP immunoreactivity has been measured in hypothalamo-pituitary portal blood and the pattern of GAP and GnRH secretion were found to be very similar (Nikolics, Seeburg and Clarke, 1986 cited by Seeburg *et al.*, 1987). The physiological role of GAP appears to be complex and its importance remains unknown (Seeburg *et al.*, 1987). Low concentrations of synthetic GAP have been shown to stimulate prolactin release from rat pituitary cells, while high doses inhibit it (Seeburg *et al.*, 1987). GAP also stimulates GnRH secretion, but with a lower potency than GnRH itself (Nikolics *et al.*, 1985), and in conjunction with GnRH, can stimulate FSH secretion in hypogonadal male mice.

Detailed investigations of GnRH synthesis and secretion have proved difficult, firstly due to the low concentration of GnRH in the peripheral

circulation and secondly due to its short half-life, between 4 to 7 minutes (Redding, Schally, Arimura and Matsuo, 1972; Nett, Akbar, Niswender, Hedlund and White, 1973; Barron, Millar and Searle, 1982). The structure of porcine GnRH was elucidated by Matsuo *et al.*, (1971) and a similar structure for ovine GnRH confirmed by Burgus, Butcher, Amos, Ling, Monahan, Rivier, Fellows, Blackwell, Vale and Guillemin (1972). This then allowed the production of synthetic GnRH and the development of assays capable of measuring the low concentrations of GnRH in the peripheral circulation. The quantification of GnRH secretion however, remains difficult as methods of sampling hypothalamo-pituitary portal blood often require either the use of general anaesthesia, which can affect GnRH secretion (See Chapter 6), or invasive surgical techniques which cause physical changes in the hypothalamus and pituitary gland (Clarke and Cummins, 1982). The method of portal blood collection used by Moenter, Caraty and Karsch (1990), a modification of the method of Caraty and Locatelli (1988), is reported to provide a more accurate measure of GnRH secretion. This method involves less pituitary damage than previous methods, as demonstrated by the normal amplitude of LH pulses and surges, measured in the peripheral circulation following surgery. As these techniques for the direct measurement of GnRH have only been developed recently, most previous studies have described GnRH secretion indirectly, relying on the close relationship between LH and GnRH secretion (Clarke and Cummins, 1982).

Studies of LH and GnRH secretion have revealed that both hormones are secreted in a pulsatile fashion in many species including sheep (Butler, Bolt and Malven, 1971; Butler, Malven, Willett and Bolt, 1972; Clarke and Cummins, 1982), rats (Sarkar and Fink, 1980; Gallo, 1981) and monkeys (Carmel *et al.*, 1976; Plant, 1981). Pulsatile secretion provides the most efficient method of transmitting changes in a hormonal signal as changes

in the frequency and/or amplitude of a cyclic signal are more effective than small variations in the size of a continuous signal (McIntosh and McIntosh, 1980 cited by Martin, 1984). Pulses of LH/GnRH are secreted throughout the lifetime of most vertebrates, irrespective of the endocrinological state of the animal, changes in the frequency and amplitude of GnRH pulses governing the changes associated with cycles of reproductive development, the occurrence of seasonality (Lincoln and Short, 1980) and puberty (MacKinnon, Puig-Duran and Laynes, 1978; Lincoln and Short, 1980).

1.3.3. Physiological effects of GnRH

GnRH is involved in the control of gonadotrophin secretion at both the cellular and nuclear levels (Papavasiliou, Zmelli, Khoury, Landefeld, Chinn and Marshall, 1986a; Papavasiliou, Zmelli, Herbon, Duncan-Weldon, Marshall and Landefeld, 1986b; Saade, Clayton and London, 1987; Lalloz, Detta and Clayton, 1988a). It is documented that GnRH has three main effects:- (i) the stimulation of gonadotrophin secretion; (ii) the priming of the pituitary gland to further GnRH stimulation, by the induction of increased concentrations of GnRH receptors (Catt Loumaye, Wynn, Iwashita, Hirota, Morgan and Chang, 1985; Papavasiliou, *et al.*, 1986a) and the stimulation of an increase in the number and size of the pituitary gonadotrophes (Bogdanove, 1963; McDonald and Clegg, 1966; McDowell, Morris, Charlton, and Fink, 1982), and (iii) the stimulation of LH and FSH synthesis at a subcellular level by the stimulation of gonadotrophin subunit mRNA transcription (Liu and Jackson, 1979; Clayton, 1982; Abbot, Docherty, Roberts, Tepper, Chin and Clayton, 1985; Gharib, Bowers, Need and Chin, 1986; Papavasiliou *et al.*, 1986b; Starzec, Counis and Jutisz, 1986; Haisenleder, Katt, Ortolano, El-Gewely, Dee and Marshall, 1988) and the

glycosylation of LH (Liu and Jackson, 1978; Azhar, Reel, Pastushok and Menon, 1978; Ramey, Highsmith, Wilfinger and Baldwin, 1987).

1.3.3.1. The induction of gonadotrophin secretion

The effects of GnRH on gonadotrophin secretion are dependent on the pattern of GnRH administration. Continuous infusion of GnRH stimulates a single peak of FSH secretion and a biphasic release of LH (Bremner *et al.*, 1976; Bremner, Findlay, Lee, DeKrester and Cummings, 1980), which terminates when the pituitary gland becomes refractory. Pulsatile administration of GnRH results in the stimulation of pulses of LH secretion which can be maintained indefinitely in both sheep (Lincoln, 1979a) and primates (Wickings, Zaidi, Brabant and Neischlag, 1981) even when the pulse frequency is increased to that seen in the castrate, without inducing refractoriness. Immediately following GnRH exposure however, the gonadotrophs are less sensitive to GnRH stimulation. In the rat, this refractory period lasts between 15 and 30 minutes, the length of the refractory period being dependent on the dose of GnRH administered (Lambalk, van Rees, Schoemaker, de Koning and van Deiten, 1988). The length of the acute refractory period in the entire rat is similar to the inter-pulse interval seen in gonadectomised animals and indicates that the pulse interval in the steroid free, free-running system is dictated by the length of the acute refractory period.

The biphasic release of LH in response to the infusion of GnRH has been documented in hamsters (Arimura, Debeljuk, Matsuo and Schally, 1972), humans (Bremner and Paulsen, 1974; Hoff, Lasley, Wang and Yen 1977) sheep (Kanchev, Dobson and Stankov, 1984; Bremner *et al.*, 1976, Bremner *et al.*, 1980) and rats (Edwardson and Gilbert, 1975; DeKoning, van Dieten, and van Rees, 1976). The timing of the two phases of LH release differs between species. In hamsters, the initial release of LH occurs within

10 minutes of the start of the GnRH infusion, with the second phase of LH release occurring after approximately 90 minutes (Arimura *et al.*, 1972). In the ram, the first increase in LH secretion occurs, on average, within 5 minutes of the start of the GnRH infusion and peaks, on average, 15-30 minutes after the start of the infusion. This peak is followed by a plateau or slight decline in the peripheral LH concentration which lasts for approximately 45-60 minutes, before a second peak of LH secretion which is maximal approximately 130 minutes after the start of the GnRH infusion (Bremner *et al.*, 1976, 1980). In sheep, following the second LH peak, the gonadotroph become refractory to further GnRH stimulation and the secretion rate of LH decreases rapidly (Piper, Perkins, Tugwell and Vaught, 1973; Chakraborty, Adams, Tarnavsky and Reeves, 1974; Hooley, Baxter, Chamley, Cumming, Jonas and Findlay, 1974; Belchetz, Plant, Nakai, Keogh and Knobil, 1978; Amundson and Wheaton, 1979; Nett, Crowder, Moss and Duello, 1981). The refractory state is not caused by depletion of pituitary stores of gonadotrophins (de Koning, van Dieten and van Rees, 1978), but is due to a decrease in the responsiveness of the pituitary gland to GnRH stimulation (Chakraborty *et al.*, 1974). This occurs as a result of a combination of GnRH-receptor occupancy (Schuiling and Gnodde, 1976; Sandow, von Rechenberg, Jerzabek, Engelbort, Kuhl and Fraser, 1980; Schuiling, Pols-Valkhof, van der Schaaf-Verdonk and Koiter, 1984; Naor, Amsterdam and Catt, 1984) and receptor down-regulation (Catt, Loumaye, Katikineni, Hyde, Childs, Amsterdam and Naor, 1983) (See section 1.3.6.).

More detailed studies of the two phases of LH release (Bremner and Paulsen, 1974; Liu and Jackson, 1978) indicate that the first phase represents the release of LH which is stored in the pituitary in a releasable form, whereas the second increase in LH secretion occurs due to a combination of the release of LH, which has been stored in the pituitary gland, but requires post-translational processing and newly-synthesised

LH. This led to the development of the proposal of Yen (1977) that LH is stored in two pools, a 'readily-releasable pool' which is released immediately on exposure of the pituitary gland to GnRH, and a 'releasable pool' of LH which contains LH which is not available for immediate release, but may be mobilised following exposure to GnRH and may be supplemented with newly-synthesised LH.

The magnitude of the LH response to GnRH is controlled by a number of factors. LH pulse amplitude is directly related to the size of the GnRH pulse administered (Clarke, Thomas, Yao and Cummins, 1987), assuming that the pituitary gland contains sufficient stores of LH for release. The relationship between endogenous LH pulse amplitude and LH pulse frequency has been investigated experimentally by the measurement of LH pulse amplitude produced in response to a constant dose of GnRH administered at different frequencies (Clarke and Cummins, 1985a). These studies revealed an inverse relationship between LH pulse frequency and amplitude (Baird, 1978a; Karsch *et al.*, 1983; Clarke *et al.*, 1984; Clarke and Cummins, 1985a). Assuming the 'two pools' theory to be correct, the effect of LH pulse frequency on pulse amplitude is a reflection of the size of the readily releasable pool of LH (Clarke and Cummins, 1985a).

In entire animals, the infusion of GnRH stimulates a single pulse of FSH secretion. In gonadectomised animals however, the pattern of FSH release is sex dependent, biphasic FSH release occurring in ovariectomised ewes (Clarke and Cummins, 1985b) while in castrated rams only a single phase of FSH release occurs (Bremner *et al.*, 1976). This difference in the pattern of FSH secretion may be due to the greater concentration of FSH found in the pituitary glands of ewes when compared to rams (Robertson, Ellis, Foulds, Findlay and Bindon, 1984). The store of FSH in castrated rams may only be sufficient to provide a single phase of FSH release, whilst in ovariectomised females sufficient FSH remains after the

initial release to allow a second phase of release in response to prolonged stimulation (Findlay, Gill and Doughton, 1985).

1.3.3.2. The priming effect of GnRH

The priming effect of GnRH stimulation has been noted *in vivo* in the rat (Aiyer, Chiappa and Fink, 1974b; Fink, Chiappa, and Aiyer, 1976; Pickering and Fink, 1976a, 1979a) and the sheep (Crighton and Foster, 1976, 1977; Stelmasiak and Galloway, 1977) and *in vitro*, in cultured rat gonadotrophs (Edwardson and Gilbert, 1976; Pickering and Fink, 1976a, 1976b, 1979b). GnRH is the only neuropeptide that exhibits this self-priming ability and may reflect that apart from the release of oxytocin during parturition, the LH surge system is the only endocrine positive feedback system known to occur under physiological conditions (Fink, 1988).

Following exposure of the pituitary gland to GnRH, the responsiveness to further GnRH stimulation is enhanced. This effect lasts for up to 3 hours in sheep *in vivo* (Crighton and Foster, 1977). The priming effect of GnRH occurs when GnRH is administered, as an infusion, or as a series of pulses (Crighton, Foster, Haresign and Scott, 1975). The augmented response of pituitary gonadotrophs to a second exposure to GnRH is due to a combination of the induction of gonadotrophin synthesis (in the *hpg* mouse - Charlton, Halpin, Iddon, Rosie, Levy, McDowell, Megson, Morris, Bramwell, Speight, Ward, Broadhead, Davey-Smith and Fink, 1980; Young, Speight, Charlton and Clayton, 1983; Young, Naik and Clayton, 1984) and the mobilisation and induction of increased numbers of GnRH-receptors (Young, Detta, Clayton, Jones and Charlton, 1985a).

Major differences have been noted between the mechanisms by which GnRH stimulates pituitary priming and LH release (reviewed by

Fink, 1988). LH release occurs independently of pituitary priming when gonadotrophs are maintained *in vitro* in media deficient in Ca^{2+} (deKoning *et al.*, 1976; Pickering and Fink, 1979b), when the synthesis of protein, RNA and DNA have been blocked (Edwardson and Gilbert, 1976; deKoning *et al.*, 1976; Pickering and Fink, 1976a, 1979b) and when gonadotrophs are incubated with agents which disrupt the structure of intra-cellular microfilaments (Pickering and Fink, 1979b). Further investigations have shown the importance of some of these elements in the priming of the pituitary gland. The role of protein synthesis has been studied and shown to involve the synthesis of a novel protein of molecular weight 69000, whose purpose remains unknown (Curtis, Lyon and Fink, 1985) and the phosphorylation of two other proteins (Curtis *et al.*, 1985). Pituitary priming is also associated with a change in the orientation and length of intracellular contractile elements. Following GnRH stimulation, reorientation of these contractile elements results in a significant migration of secretory granules in the gonadotroph to a 'marginal' zone close to the plasmalemma (Lewis, Morris and Fink, 1985a). It was therefore proposed that the priming effect of GnRH was associated with recruitment of LH from a non-releasable form stored near the centre of the gonadotrophs, to a readily-releasable form close to the plasmalemma (Pickering and Fink, 1976a, 1976b). This proposal mirrors the 'two pool' theory, proposed by Yen (1977) and described previously (section 1.3.3.).

Mini-pulses of GnRH (1-4 pg/ml) have been measured in the pituitary portal blood of sheep (Clarke and Cummins, 1982) and although they do not stimulate increased LH secretion, they may serve to prime the pituitary to further GnRH stimulation. The ability of this type of GnRH exposure, or exposure of the pituitary gland to a continuous infusion of a low concentration of GnRH have been shown to facilitate pituitary priming

and an increase in subsequent LH secretion in both rats (Fink *et al.*, 1976) and hypothalamo-pituitary disconnected ewes (Clarke *et al.*, 1984).

The concentration of pituitary GnRH-receptors is also influenced by prior exposure to GnRH, receptor numbers paralleling changes in the concentration of GnRH/LH (reviewed by Clayton and Catt, 1981). Therefore it was proposed that GnRH-receptors are controlled in an auto-regulatory fashion. This theory is supported by both *in vitro* and *in vivo* studies (Young *et al.*, 1983, Young, Naik and Clayton, 1985b; Detta, Naik, Charlton, Young and Clayton, 1984; dePaolo, King and Carrillo, 1987). GnRH is therefore one of the few hormones, along with prolactin and angiotensin-II, that are capable of inducing their own receptors. Exposure to physiological levels of GnRH causes an initial decrease, followed by a three-fold increase in GnRH-receptor number (Loumaye and Catt, 1982; Conn, Rogers and Seay, 1984; Young *et al.*, 1984). The induction of an increase in GnRH receptor numbers does not occur as a result of binding of GnRH to the surface receptor, but appears to follow GnRH-receptor activation. Therefore changes are not seen in GnRH-receptor number following the administration of GnRH antagonists, as antagonists merely bind to GnRH-receptors. However, techniques which mimic the intracellular events which occur following receptor activation, for example depolarization of the cell membrane, or stimulation of gonadotrophs with phorbol esters which act as activators of endogenous protein kinase C, do stimulate changes in GnRH-receptor number. Furthermore, when receptor activation is prevented by incubation of gonadotrophs in Ca^{2+} free media, or the blockade of Ca^{2+} channels or the events which occur after receptor activation, such as protein synthesis, RNA synthesis or microtubule function, are blocked with cycloheximide, actinomycin D and vinblastine respectively (Young *et al.*, 1985b; Young, Naik and Clayton, 1985c) changes in GnRH receptor number do not occur.

The priming effect is not mediated through the effects of steroids, but the magnitude of the effect does appears to be modulated by steroid concentrations (Mann and Barraclough, 1973; Aiyer *et al.*, 1974a; Meidan, Fink and Koch, 1981). The number of GnRH-receptors increases coincident with the concentration of oestradiol at the time of oestrus in the rat (Savoy-Moore, Schwartz, Duncan and Marshall, 1980; Clayton, Solano, Garcia-Vela, Dufau and Catt, 1980; Clayton and Catt, 1981; Clayton, Detta, Naik, Young and Charlton, 1985), the monkey (Adams, Norman and Spies, 1981) and the sheep (Crowder and Nett, 1984), and in ovariectomised animals following oestradiol treatment (sheep; Moss, Crowder and Nett, 1981: monkeys; Adams *et al.*, 1981: rats; Marian, Cooper and Conn, 1981). Exposure to oestradiol therefore stimulates the induction of increased numbers of GnRH-receptors and allows GnRH to stimulate LH release at lower than normal concentrations. The resulting cascade of LH secretion producing the preovulatory gonadotrophin surge (Clayton, 1989).

1.3.3.3. Gonadotrophin biosynthesis

LH and FSH are both comprised of two non-identical glycoprotein subunits (α and β) of approximately 15000 molecular weight. The two subunits are held together by non-covalent forces. The α subunit is common to other pituitary hormones, TSH and hCG, while the β subunit is specific to LH. Initially, *in vitro* studies on the effects of GnRH indicated that GnRH (Liu, Jackson and Gorski, 1976), and oestrogens (Liu and Jackson, 1977) had no effect on the synthesis of LH, but did significantly accelerate the glycosylation of stored LH. Following the development of more sophisticated study methods, exposure of rat gonadotrophs to GnRH, appeared to stimulate an increase in LH polypeptide biosynthesis; however, this increase occurred 1 to 2 hours after GnRH stimulation while LH release was immediate (Starzec, Jutisz and Counis, 1988, 1989). This data agrees with

the proposal of Liu and Jackson (1978), that the synthesis of LH subunit mRNA is not required for short term increases in LH secretion, but may be required for either a maximal response to GnRH or a period of sustained LH secretion.

The expression of genes encoding the LH subunits appears to be modified by the frequency and amplitude of GnRH pulses (Papavasiliou *et al.*, 1986a; Haisenleder, Khoury, Zmeilis, Papavasiliou, Ortolano, Dee, Duncan and Marshall, 1987). Differences in GnRH pulse frequency differentially stimulate LHβ and FSHβ subunit mRNA synthesis in male rats. Administration of GnRH pulses at a high frequency stimulates LHβ subunit synthesis, while slow frequency pulses stimulate FSHβ synthesis (Dalkin *et al.*, 1989). Similar studies using ovine pituitary cells however, have not been able to confirm this differential stimulation of LH and FSH subunit synthesis (Leung, Kaynard, Negrini, Kim, Maurer and Landerfeld, 1987). Haisenleder and colleagues (1988) found that in male rats, the optimum conditions for stimulating increased LH subunit synthesis was to administer 25ng pulses of GnRH every 30 mins for 48hrs. This regime produced GnRH pulses, in a pattern similar in both frequency (Steiner, Bremner and Clifton, 1982; Ellis, Desjardins and Fraser, 1983) and amplitude (Sarkar and Fink, 1980; Sarkar *et al.*, 1976) to those seen in castrated animals. It also induces changes in the number of GnRH receptors (Catt *et al.*, 1985) and the concentrations of α and LHβ subunit (Haisenleder *et al.*, 1987) similar to those seen in the castrate.

LHβ subunit mRNA synthesis is more responsive to GnRH stimulation than α subunit mRNA synthesis; therefore LHβ mRNA levels may be the limiting factor in GnRH action. This proposal is supported by the decrease in LHβ subunit mRNA, and the increase in α subunit mRNA in rats which have been desensitized by continous exposure to GnRH (Lalloz, Detta and Clayton, 1988b). This study also indicates that refractoriness to GnRH is

independent of LH synthesis and occurs at a translational or post-translational level (Haisenleder *et al.*, 1988). Alternative patterns of GnRH administration can however reverse this differential expression of LH subunit mRNA (Hubert, Simard, Gagne, Barden and Labrie, 1988).

1.3.4. Control of GnRH secretion and its effects

1.3.4.1. By steroids

The effects of steroids on GnRH release, and the effectiveness of GnRH in stimulating gonadotrophin secretion, can be seen following either the administration or the removal of gonadal steroids, and are well documented in a number of species. Studies where animals have been treated with exogenous oestradiol have demonstrated that the increases in peripheral steroid concentrations have a biphasic effect on gonadotrophin secretion. Firstly, a suppression of gonadotrophin secretion (Henderson, Baker and Fink, 1977; Vilchez-Martinez, Arimura, Debeljuk and Schally, 1974; Goodman, Legan, Ryan, Foster and Karsch, 1980; Karsch, Cummins Thomas and Clarke, 1987; Moenter *et al.*, 1990) due to inhibition of GnRH secretion from the hypothalamus, and secondly a period of increased gonadotrophin secretion (Libertun, Orias and McCann, 1974; Coppings and Malven, 1976; Henderson *et al.*, 1977; Caraty, Loatelli and Martin, 1989) due to an increase in the responsiveness of the gonadotrophs to GnRH stimulation (Aiyer *et al.*, 1974a; Wang, Lasley, Lein and Yen, 1976).

Removal of steroid negative feedback by gonadectomy is followed by a dramatic increase in LH pulse frequency, the final pulse frequency differing between species. In gonadectomised monkeys and humans, LH pulse frequency increases (Dierschke *et al.*, 1970; Knobil, 1974, 1980) to approximately one pulse per hour. In sheep, LH pulse frequency following gonadectomy is slightly greater, with an interpulse interval of between 40 and 60 minutes (Butler *et al.*, 1971, 1972; Reeves, O'Donnell and Denorsica,

1972; Diekman and Malven, 1973; Davis and Borger, 1974; Riggs and Malven, 1974; Schanbacher and D'Occhio, 1984; Caraty and Locatelli, 1988), while in gonadectomised rats LH pulses are seen at 30 minute intervals (Gay and Sheth, 1972). This increase in LH pulse frequency is presumably preceded by an increase in GnRH pulse frequency. However, this increase in GnRH synthesis and secretion has only been documented in the rat (Everett, 1969; Moguilevsky, Enero and Szwarcfarb, 1974; Ben-Jonathon, Mical and Porter, 1973).

The effects of steroids on GnRH and gonadotrophin secretion are therefore twofold, acting on both the hypothalamus and the pituitary gland. The steroidal regulation of LH secretion at the hypothalamus has been demonstrated in the rat (Orias, Negro-Vilar, Liberman and McCann, 1974; Kalra and McCann, 1975; Henderson *et al.*, 1977; Sakar and Fink, 1979, 1980; Cronin, Cheung, Weiner and Goldsmith, 1982; Melrose and Gross, 1987), the sheep (Cumming, Buckmaster, Cerini, Chamley, Findlay and Goding, 1972, Cumming, Brown, Cerini, Cerini, Chamley, Findlay and Goding, 1973; Clarke and Cummins, 1985b; Clarke, 1987, 1988; Herman and Adams, 1990; Moenter *et al.*, 1990) and the rhesus monkey (Neill *et al.*, 1977; Levine, Norman, Gleissman, Oyama, Bangsberg and Spies, 1985; Pau, Gleissman, Hess and Spies, 1988). Steroid induced reduction in LH secretion occurs due to a reduction in GnRH pulse frequency (Martin and Scaramuzzi, 1980; Wright, Geytenbeek, Clarke and Findlay, 1981; Goodman *et al.*, 1981a, Goodman, Bittman, Foster and Karsch, 1981b, 1981c, 1982; Steiner *et al.*, 1982; Martin, Scarammuzi and Henstride, 1983; Plant and Dubey, 1984: Dubey and Plant, 1985; reviewed by McCann and Rettori, 1987).

The responsiveness of the pituitary gland to GnRH stimulation has also been shown to be affected by the concentrations of steroids, both *in vivo* (rat; Arimura and Schally, 1970; Debeljuk, Arimura and Schally, 1972; Jackson, 1972, 1973; Aiyer *et al.*, 1974b; Bogdanove, Nolin and Campbell,

1975; Drouin and Labrie, 1976; Fink and Henderson, 1977; Hsueh, Erikson and Yen, 1978; Cronin *et al.*, 1982: the sheep; Reeves *et al.*, 1971a; Debeljuk *et al.*, 1972; Pant and Ward, 1973; Hopkinson *et al.*, 1974; Jackson, 1975; Coppings and Malven, 1976; Clarke and Cummins, 1984; Kaynard, Malpaux, Robinson, Wayne and Karsch, 1988; Gregg and Nett, 1989; Herman and Adams, 1990: the rhesus monkey; Knobil, 1974; Nakai, Plant, Hess, Keogh and Knobil, 1978; Plant, Nakai, Belchetz, Keogh and Knobil, 1978; Adams *et al.*, 1981) and *in vitro* (Huang and Miller, 1980; Miller and Huang, 1981, 1985). The results of the *in vitro* studies, which used ovine pituitary cells, indicated that steroids mediate their effects at the pituitary gland by altering the number of GnRH-receptors, and that different hormones have different effects, oestradiol and inhibin stimulating an increase in the number of GnRH-receptors (Gregg and Nett, 1989; Laws, Beggs, Webster and Miller, 1990a; Laws, Webster and Miller, 1990b), while progesterone stimulates a decrease in number of GnRH-receptors (Laws *et al.*, 1990a).

Following gonadectomy, the initial increase in the frequency of the LH pulses is followed by a gradual increase in the mean LH concentration over a period of several weeks in both the rat and the sheep (Gay and Midgley, 1969; Reeves *et al.*, 1972). This increase in mean gonadotrophin concentrations, occurs due to an increase in the frequency and amplitude of LH pulses (Butler *et al.*, 1972; Diekman and Malven, 1973) and reflects long term changes which occur in pituitary responsiveness. Gonadotrophin secretion does not respond immediately to acute changes in steroid concentrations, but long term changes affect both LH and FSH secretion, by altering pituitary responsiveness to GnRH. The effects are most noticeable in the first phase of LH release i.e. the release of LH from the readily-releasable pool of LH. Long-term castrated lambs initially release more LH than entire lambs following GnRH stimulation, but the magnitude of the second phase of LH release is similar between groups

(Bremner *et al.*, 1980). The changes in gonadotrophin secretion which occur following long-term alterations in steroid concentrations are accompanied by physical changes in the pituitary gland; hypertrophy of the gonadotrophs and changes in the chemical structure of both LH and FSH. These changes explain some of the physiological effects following long-term gonadectomy on gonadotrophin secretion. The high basal concentration of LH occurring due to leakage of LH from the hypertrophic gonadotrophs and an extended half-life for both LH and FSH have been reported in gonadectomised monkeys (Peckham, Yamaji, Dierschke and Knobil, 1973; Pekham and Knobil, 1976) sheep (Fry, Cahill, Cummins, Bindon, Piper and Clarke, 1987) and rats (Weick, 1977). Chemical analysis of the LH and FSH secreted by ovariectomised and entire monkeys (Peckham *et al.*, 1973; Pekham & Knobil, 1976) indicate that differences exist in the sialic acid content of the gonadotrophins secreted following gonadectomy and that these chemical differences may be responsible for the differences in their clearance rate from the peripheral circulation.

Long-term removal of gonadal steroid feedback, also results in an increase in the pituitary content of LH and FSH (Clayton and Catt, 1981) and implies that gonadotrophin biosynthesis is increased following gonadectomy. Further studies on the molecular aspects of gonadotrophin secretion in the rat (reviewed by Abbot, Docherty and Clayton, 1988a) have indicated that gonadotrophin subunit biosynthesis is also increased following gonadectomy. Studies on LH subunit mRNA concentrations in male and female rats have shown that following ovariectomy LH subunit mRNA synthesis is increased, the concentration of LHβ-mRNA increasing above the concentration of α-subunit (Abbot *et al.*, 1988a; Abbot, Docherty and Clayton, 1988b). This is supported by the finding that a 5'-flanking region on the LHβ gene has been identified which binds oestrogen

receptors and could provide a method by which oestrogen can modify the amount of gonadotrophin synthesised in response to GnRH stimulation.

The primary physiological effect of steroids in the control of reproduction is to regulate gonadotrophin secretion. This is achieved principally by the regulation of GnRH secretion by the hypothalamus. Studies have shown that steroids do not accumulate at the GnRH neurons and the number of oestrogen receptors associated with GnRH neurons is very low (Shivers, Harlan, Morell and Pfaff, 1983). Therefore it appears that the effects of steroids are mediated by indirect neuronal stimulation (Barraclough and Wise, 1982; Kalra and Kalra, 1983; Barraclough, Wise, and Selmanoff, 1984).

Adrenaline, noradrenaline, acetylcholine, morphine and dopamine are effectors in the CNS, and are known to affect gonadotrophin secretion. Noradrenaline and dopamine have a direct action on GnRH neurons (Rotsztejn, Charli, Pattou, Epelbaum and Kordon, 1976;, Rotsztejn, Charli, Pattou and Kordon, 1977; Rotsztejn, Drouva, Pattou and Kordon, 1978). However the other neurotransmitters exert their actions via the hypothalamic monoaminergic and opioid neurons which then interact with the GnRH neurons. Evidence exists that endogenous opioids inhibit LH secretion (Ebling and Lincoln, 1985; Brooks, Lamming, Lees and Haynes, 1986). However the proposed site of action of the opioid inhibition of LH secretion is not clear. Some reports suggest it occurs via uncoupling of the GnRH stimulus and secretion mechanism within the anterior pituitary (Clarke, Wood, Merrick and Lincoln, 1979) or by a central action at the hypothalamus (Drouva, Epelbaum, Tapia-Arancibia, Laplante and Kordon, 1981; Ebling, Schwartz and Foster, 1989). Therefore it was proposed that opioids are involved in the mediation of the effects of steroids on LH secretion (Bhanot and Wilkinson, 1983, 1984). However the similarity of the responses to exogenous opioid treatment in pre- and postpubertal animals

(Ebling *et al.*, 1989) suggest that some other pathway is also involved in the mediation of the steroidal effects on gonadotrophin secretion. Radiolabelled steroids have been found to accumulate on neurons associated with the neurotransmitter gamma-aminobutyric acid (GABA). These GABAergic neurons have therefore been suggested as possible mediators of the regulatory effects of steroids on gonadotrophin secretion (Flugge, Oertel and Wuttke, 1986). GABA is generally considered to be an inhibitory neurotransmitter and its secretion in response to steroid stimulation would inhibit the pulse generator. Steroid negative feedback acting through a GABAergic system would tonically inhibit the secretion of GnRH (Lamberts, Vijayan, Graf, Mansky and Wuttke, 1983).

1.3.4.2. By inhibin

The effects of inhibin on gonadotrophin secretion by the pituitary gland have mainly been conducted *in vitro* using cultured rat pituitary cells (Farnworth *et al.*, 1988a; Farnworth, Robertson, deKretser and Burger, 1988b; Castillo, 1989 cited by Muttukrishna and Knight, 1990). These studies indicate that inhibin primarily affected FSH secretion causing a reduction in basal and GnRH-stimulated FSH secretion, a reduction in the pituitary gland stores of FSH (Farnworth *et al.*, 1988b, Castillo, 1989 cited by Muttukrishna and Knight, 1990) and a reduction in the number of pituitary GnRH receptors (Wang, Farnworth, Findlay and Burger, 1988), with little or no effect on LH secretion. These results are in agreement with the available *in vivo* data on the effects of inhibin in the rat (DePaolo, Wise, Anderson, Barraclough and Channing, 1979; Lumpkin, Negro-Villar, Franchimont and McCann, 1981). Recent reports however have demonstrated that LH secretion is also affected by high concentrations of inhibin, which reduced LH secretion both *in vitro* and *in vivo* (Farnworth, *et al.*, 1988a, 1988b).

All of the above effects can be explained by the action of inhibin at the level of the pituitary gland. However injections of inhibin into the third ventricle of orchidectomised rats (Lumpkin *et al.*, 1981) will also result in the suppression of FSH secretion. Therefore the effects of inhibin on gonadotrophin secretion appear to be located at both the pituitary gland and a site located within the CNS.

Studies in sheep (Martin, *et al.*, 1987; Findlay *et al.*, 1985; Wrathall, McLeod, Glencross, Beard and Knight, 1990) and cultured ovine pituitary cells (Huang and Miller, 1984; Muttukrishna and Knight, 1990) have shown that the effects of inhibin may differ between species. *In vivo* studies in the sheep have shown that the effects of inhibin on FSH secretion are similar to those seen in the rat, but that inhibin appears to stimulate LH secretion. These effects on LH secretion are thought to occur due to the indirect action of inhibin, inhibin suppressing FSH secretion, arresting follicular growth and therefore oestradiol secretion (Martin *et al.*, 1987), and consequently preventing the oestradiol-induced sensitization of the gonadotrophs to GnRH stimulation (Muttukrishna and Knight, 1990).

1.3.4.4. By gonadotrophins

Gonadotrophins have also been shown to have an effect on GnRH secretion. Gonadotrophin implants in the mediobasal hypothalamus eliciting changes in GnRH/gonadotrophin secretion (Motta, Franschini and Martini, 1969). Melrose (1987) suggested that the short-loop feedback of gonadotrophins on GnRH secretion may in part, involve direct humoral interaction of the gonadotrophins with the GnRH neurons, as exposure of GnRH neurons to LH and FSH induced an increased in GnRH pulse frequency (LH) and amplitude (LH and FSH).

1.3.4.5. By GnRH inhibiting factor

A further degree of control of GnRH secretion comes from the discovery of a GnRH-inhibitory factor; a protein of approximately 12000 molecular weight, which has been purified from hypothalamic extracts and is proposed to inhibit the GnRH stimulated release of LH *in vitro* (Hwan and Freeman, 1987a) and in anaesthetized pro-oestrous rats (Hwan and Freeman, 1987b). Further studies however, must be undertaken to prove the existence of this hormone and to characterise its secretion and its effects (Clarke, 1989).

1.3.5. Mechanism of GnRH action

Following GnRH stimulation, multiple steps (at least seven) are involved in the stimulation of gonadotrophin secretion, gonadotrophin gene expression and GnRH-receptor regulation. Occupancy of 20% of pituitary GnRH binding sites is sufficient to stimulate 80% of maximal LH release (Naor, Clayton and Catt, 1980a), the receptor signal being amplified within the gonadotroph by the production of second messengers. The binding of GnRH to its receptor stimulates the activation of multiple second messenger systems including phosphoinositide (PI) turnover, calcium mobilization, activation of protein kinase C (PKC) and the induction of arachidonic acid (AA) release (Naor and Childs, 1986).

It is well established that calcium ions (Ca^{2+}) play a central role in the intracellular actions of GnRH stimulation and gonadotrophin release (Conn, McMillan and Rogers, 1980; Conn, Marian, McMillan, Stern , Rogers, Hamby, Penna and Grant, 1981a; Conn *et al.*, 1987a; Conn, 1989). Firstly, the GnRH-receptor is linked directly to Ca^{2+} channels in the cell membrane (Conn and Rogers, 1980; Chang, McCoy, Graeter, Tasaka and Catt, 1986b) and secondly, GnRH-stimulated LH release is dependent on the presence of extracellular calcium. Initially calcium was proposed to be the principal

second messenger system in the pituitary gonadotroph (Adams and Nett, 1979; Marian and Conn, 1979; Conn *et al.*, 1981a), as activation of the GnRH receptor is rapidly followed by a transient rise in the cytosolic Ca^{2+} concentration (Catt *et al.*, 1983; Clapper and Conn, 1985; Chang, Graeter and Catt, 1986a; Limor, Ayalon, Capponi, Childs and Naor, 1987). This rise in the intracellular Ca^{2+} concentration occurs independently of an influx of extracellular Ca^{2+}. *In vitro* studies using rat and chicken pituitary cells have shown that intracellular Ca^{2+} is mobilized following metabolism of membrane-bound phospholipids (Smith, Wakefield, King, Naor, Millar and Davidson, 1987). This probably results in the first rapid phase of LH release. The sustained release of LH occurs due to the influx of extracellular Ca^{2+} (Chang, McCoy, Graeter, Tasaka and Catt, 1986b; Conn, Staley, Yasumoto, Huckle and Janovick, 1987c) through calcium channels which open as a result of receptor activation (Williams, 1976). The importance of Ca^{2+} in gonadotrophin secretion is supported by data concerning the cellular utilization of calmodulin, a ubiquitous intracellular Ca^{2+} receptor. Administration of GnRH causes a redistribution of calmodulin in the cell from the cytoplasm to the plasma membrane (Conn, Chafouleas, Rogers and Means, 1981c) and the formation of Ca^{2+}-calmodulin complexes (Conn *et al.*, 1981c, 1987a; Conn, Rogers and Sheffield, 1981b; Rasmussen and Barrett, 1984) which regulate the activity of many Ca^{2+} dependent enzymes. Inhibitors of calmodulin inhibit GnRH stimulated LH release (Conn *et al.*, 1981b). Calmodulin therefore acts as an intracellular receptor mediating the effects of the increased concentrations of Ca^{2+}, mobilized in response to GnRH stimulation.

Stimulation of the receptor also initiates the hydrolysis of cellular phosphoinositides (PI) by a membrane-bound enzyme, phospholipase C, to form diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3) (Andrews and Conn, 1986; Morgan, Chang and Catt, 1987). The increase in the

intracellular concentration of IP_3 stimulates the mobilization of intracellular Ca^{2+} from the endoplasmic reticulum (Berridge and Irvine, 1984; Berridge, 1984). The increased concentration of Ca^{2+} interacts with calmodulin and activates phospholipase A2 which further metabolises PI and other phospholipids producing arachidonic acid and leukotrienes. These may also be involved in the further stimulation of LH release (Catt *et al.*, 1985; Chang, Graeter and Catt, 1987). The increase in the concentration of DAG results in the binding of the phosphorylating enzyme PKC to the inner surface of the plasma membrane (Hirota, Hirota, Aguilera and Catt, 1985; Naor, Jacob, Zakut and Hermon, 1985) and activation of the enzyme by formation of a Ca^{2+}-PKC complex (Yamanishi, Takai, Kaibuchi, Sano, Castagna and Nishizuka, 1983; Nishizuka, 1984; Hirota, *et al.*, 1985). Activation of PKC has been shown to stimulate LH release (Smith and Vale, 1980, Smith and Conn, 1984, Andrews and Conn, 1986) and to be involved in some of the other cellular effects of GnRH stimulation (reviewed by Conn, 1989) such as protein phosphorylation and cell proliferation (Berridge and Irvine, 1984; Nishizuka, Takai, Kishimoto, Kikkawa and Kaibuchi, 1984; Truneh, Albert, Golstein and Schmitt-Verhulst, 1985; Wolf, LeVine, May, Cuatrecasas and Sakyoun, 1985). Increased calcium concentrations and the activation of PI lead to the activation of the intracellular contractile element, actin, (Burn, Rotman, Meyer and Burger, 1985; Lassing and Lindberg, 1985) which is involved in the movement of secretory granules (Lewis *et al.*, 1985a) to the surface of the gonadotrophs where LH is released by exocytosis of the secretory granules. Neither administration of PKC nor depolarization of the plasma membrane of gonadotrophs result in maximal LH secretion (Adams and Nett, 1979). However administration of PKC with Ca^{2+} stimulates the release of similar quantities of LH to those seen following GnRH stimulation.

cAMP is involved in the intracellular transduction of stimulatory signals in a large number of secretory systems. However it has been demonstrated that cAMP is not involved in the stimulation of LH release following GnRH-receptor activation (Naor, 1982 cited by Clayton, 1989), but may play a role in the regulation of GnRH-receptor number and the glycosylation of LH (Liu, Wang and Jackson, 1981).

Therefore, it appears that the release of LH by endogenous GnRH is controlled by a number of secretory systems which act independently of each other. The two main mechanisms involve an increase in intracellular Ca^{2+} concentration and activation of PKC. These are the two principal second messenger systems involved in the stimulation of LH release (Naor *et al.*, 1984; Conn *et al.*, 1981a,; Catt, Loumaye, Wynn, Suarez-Quian, Kiesel, Iwashua, Hirota, Morgan, and Chang, 1984; Mason and Waring, 1985; Clayton, 1989).

1.3.6. Refractoriness

Refractoriness in cells following prior exposure to a ligand is a common phenomenon and is probably the means of preventing cell exhaustion (Catt, Harwood, Aguilera and Dufau, 1979). In the sheep, refractoriness to GnRH stimulation occurs progressively, reaching a maximum approximately 12 hours after the initial exposure to GnRH (Foster, 1978; Crighton and Foster, 1977) and lasts between 24 (Foster, 1978) and 72 hours (Rippel, Moyer, Johnson and Mauer, 1974a). The occurrence of a refractory period in ovariectomised ewes (Rippel *et al.*, 1974a) indicates that refractoriness to GnRH occurs independently of gonadal steroid feedback and is therefore primarily a pituitary phenomenon (Bremner *et al.*, 1980). Following GnRH stimulation there is a rapid clustering and clumping of GnRH-receptors and the receptor complexes become internalised into endocytotic vesicles (Catt *et al.*, 1985). The

resulting decrease in the number of GnRH receptors on the cell surface, however is not the cause of refractoriness (Gorospe and Conn, 1987). The population of receptors in refractory rats exposed to a continuous GnRH signal is only decreased by 40%, the remaining 60% of receptors being present on the cell surface and associated with GnRH molecules.

Gonadotrophs have also been shown to remain refractory to GnRH even when the concentration of pituitary receptors is elevated (Conn *et al.*, 1984, 1987a). The phenomenon of refractoriness has therefore been shown to be dependent upon receptor activation, as secretagogues such as phorbol esters, calcium ionophores, and KCl, which mimic the intracellular events that follow receptor activation, do not cause refractoriness (Clayton, 1989). However, the exposure of refractory cells to these agents stimulates LH secretion (deKoning *et al.*, 1978). The ability of agents such as calcium ionophores and phorbol esters, which stimulate the second messenger systems, and the observation that refractoriness can be induced in cells maintained in calcium-free media (Clayton, 1989), indicate that refractoriness is the result of an intracellular event and must occur at an intermediate stage between receptor activation and LH secretion. The ability of refractory cells to respond to stimulation with phorbol esters suggests that the effects of refractoriness are mediated before the activation of PKC and mobilization of Ca^{2+}. One intracellular event which occurs after receptor activation but before PKC activation, is signal transduction. This occurs via a GTP-binding protein (Andrews, Staley, Huckle and Conn, 1986). Therefore refractoriness could occur due to the uncoupling of the GnRH-receptor and the intracellular second messenger system at the level of the GTP-binding protien and signal transduction (Clayton, 1989). The concentration of LH is low in the refractory pituitary gland and it appears that refractoriness is accompanied by termination of β subunit mRNA synthesis (Lalloz *et al.*, 1988b). Refractoriness is therefore

accomplished by receptor down-regulation, uncoupling of the GnRH receptors from the effector signals and the inhibition of hormone synthesis.

1.3.7. Seasonal effects on hypothalamic function

As described previously, the direct measurement of GnRH secretion is difficult; therefore the effects of season on hypothalamic function have been studied indirectly as a function of gonadotrophin secretion. Seasonal changes in gonadotrophin secretion are proposed to occur following alterations in hypothalamic function in response to neural signals from the eyes. Robinson (1959) proposed that the effects of season were mediated by the hypothalamus becoming refractory to steroid positive feedback during anoestrus. The administration of oestradiol to a ewe during anoestrus stimulates the production of a surge of LH secretion similar to that observed following similar treatment during the breeding season (Gibson and Robinson, 1971; Symons *et al.*, 1973; Goodman *et al.*, 1981a). However the responsiveness of the hypothalamus to oestradiol positive feedback is reduced during anoestrus and a larger dose of oestradiol is required to stimulate an LH surge comparable in magnitude to that seen during the breeding season (Gibson and Robinson, 1971; Land *et al.*, 1976; Karsch *et al.*, 1980a; Goodman *et al.*, 1981a). The interval between oestradiol administration and the occurrence of the preovulatory surge is also greater during anoestrus than during the breeding season (Howland, Palmer, Sanford and Beaton, 1978; Karsch *et al.*, 1980a; Goodman *et al.*, 1981a).

Large seasonal differences are seen in the frequency of LH pulses between anoestrus and the breeding season. During anoestrus, the frequency of endogenous LH pulses is low in both rams and ewes (Yuthasatrakosol, Palmer and Howland, 1977; Jackson and Davis, 1979; Walton

et al., 1980; Lincoln, 1976a, 1976b; Schanbacher and Ford, 1976; Sanford, Palmer and Howard, 1977, Sanford, Beaton, Howard and Palmer, 1978; Pelletier, Garnier, deReviers, Terqui and Ortavant, 1982), with an interpulse interval of 1440 mins during anoestrus, compared with 120 min during the breeding season (Pelletier and Ortavant, 1975a; Lincoln and Short, 1980; Goodman and Karsch, 1981; Goodman *et al.*, 1982). Changes in gonadotrophin secretion occur rapidy following the transfer of rams (within 6 days) and ewes (within 40 days) from long to short days (Lincoln and Peet, 1977). It was proposed that the reduction of pulsatile gonadotrophin secretion during anoestrus occurs due to an increase in the sensitivity of the hypothalamo-hypophyseal system to the negative feedback effects of gonadal steroids (Pelletier and Ortavant, 1975a, 1975b; Scaramuzzi and Baird, 1977; Legan *et al.*, 1977; Goodman and Karsch, 1981. Goodman *et al.*, 1982; Martin *et al.*, 1983). Large variations are seen in LH pulse amplitude during the breeding season; these occur due to changes in the steroidal environment and the pattern of GnRH secretion, and reflect differences in GnRH secretion and pituitary responsiveness. During anoestrus, pituitary responsiveness remains relatively constant (Rippel *et al.*, 1974a), but is greatly reduced compared to the breeding season (Foster and Crighton, 1974; Lincoln, 1977; Evans and Robinson, 1980).

The hypothalamus and pituitary gland are both subject to seasonal changes in function which are co-ordinated by variations in photoperiod. However, as few neural connections exist between the hypothalamus and the pituitary gland, co-ordination of these changes in function are thought to be controlled via an independent endocrine system, itself regulated by changes in photoperiod. In the hamster, the photoperiodically-driven changes in the sensitivity of the hypothalamo-pituitary axis to testosterone negative feedback have been demonstrated to be mediated via the suprachiasmatic nucleus and the pineal gland (Turek, 1977, Turek

Jacobson and Gorski, 1980). The pineal gland also appears to be important in the seasonal control of gonadotrophin secretion in the ram (Lincoln, 1979b), as pinealectomy disrupts the seasonal changes in LH secretion (Munro, McNatty and Renshaw, 1980; Brown and Forbes, 1980; Kennaway, Obst, Dunstan and Friesen, 1981; Karsch, Bittman and Legan, 1981). The pineal gland secretes melatonin which is indirectly involved in the regulation of gonadotrophin secretion at the level of the CNS (Legan and Winans, 1981). Lesions around the suprachiasmatic nucleus, which contains a high concentration of GnRH neurons, prevent the antigonadotrophic effects of melatonin (Bittman, Goldman and Zucker, 1979; Rusak 1980) and stimulate reproductive activity during anoestrus (Domanski, Przekop and Polkowska, 1980; Przekopt and Domanski 1980; Pau, Kuehl and Jackson, 1982).

1.4. The reproductive biology of prolific breeds of sheep.

Hundreds of genetically distinct breeds of sheep have evolved throughout the world in response to a wide range of environmental pressures, both before and after their domestication. Great variation occurs between the different breeds in almost every physical characteristic, including size, shape, coat colour, coat type, milk yield and reproductive performance.

With regard to reproductive performance, a number of breeds exhibit above average prolificacy and these breeds have been studied extensively in order to characterise the physiological mechanism which governs their exceptional prolificacy. The mechanism of inheritance of this trait has meant that the breeds can be divided into two groups:- Group A), breeds which transmit their above average reproductive performance in an additive fashion, their increased prolificacy occurring due to the

effects of a large number of genes which have a positive effect on reproductive performance, eg. the Finnish Landrace or Finnsheep (Maijala, 1967), the Romanov (Desvignes, 1971) and the D'man (Bouix and Kadim, 1975) (Table 1.1.).

	ESTIMATES OF				
	OVULATION RATE		LITTER SIZE		
BREED	MEAN	RANGE	MEAN	RANGE	REFERENCE
Finnsheep	3.5	1-9	2.6	1-7	Hanrahan and Quirke, (1975a)
Romanov	3.4	1-7	2.6	1-5	Ricordeau, *et al.*, (1978)
D'man	2.8	1-8	2.5	1-7	Lahlou-Kassi and Marie (1985)

Table 1.1. Ovulation rate and litter size estimates for breeds of sheep of above average prolificacy, which transmit their above average prolificacy in an additive fashion.

and Group B), breeds which carry either a single gene, or a closely-linked group of genes, that exert a major effect on reproductive performance. These include the Booroola Merino (Turner, 1978), the Thoka (Jonmundsson and Adalsteinsson, 1985), the Javanese Fat tailed sheep (Bradford, Quirke, Sitorius, Inounu, Tiesnamurti, Bell, Fletcher, and Torell, 1986; Bradford, Inounu, Iniguez, Tiesnamurti, and Thomas, 1990), the Cambridge (Owens, 1976) (see Table 1.2.) and the Olkuska sheep (Martyniuk and Radomska, 1990). A number of other breeds are also under investigation as they are thought to carry a 'major gene' which influences reproductive performance. These include the Belclare (Hanrahan, 1990) and the Romney (Davis, Shackell and Kyle, 1990a; Davis, Shackell, Kyle, Farquhar, McEwan and Fennessy, 1990b).

As the different breeds have not been compared in a single environment, direct comparisons between the different breeds are not possible. Therefore the majority of studies have compared the prolific breeds with an appropriate low fecundity breed. A large amount of work has concentrated on the Booroola Merino, as it was one of the first breeds of sheep identified to have 'above average reproductive performance' due to the presence of a 'major gene' and therefore to be suitable for use in animal breeding programmes to rapidly increase reproductive performance in low prolificacy breeds of sheep.

	ESTIMATES OF				
	OVULATION RATE		LITTER SIZE		
BREED	MEAN	RANGE	MEAN	RANGE	REFERENCE
BOOROOLA-MERINO	4.2	1-11	2.5	1-7	Bindon and Piper, (1982a)
THOKA	-	-	2.27	1-6	Jonmundsson and Adalsteinsson (1985)
JAVANESE-FAT TAIL*	-	-	2.23	1-6	Bradford *et al.*,(1986)
CAMBRIDGE	4	2-13	>2.6	1-8	Hanrahan and Owens, (1985)

* A mixed population of carriers and non-carriers of the proposed gene.

Table 1.2. Ovulation rate and litter size estimates in breeds of sheep which differ in prolificacy and carry a 'major gene' for reproductive performance.

1.4.1. Group A

1.4.1.1. The Finnish Landrace.

The exceptional prolificacy of the Finnish Landrace was confirmed in Britain by Donald and Read (1967). Crossbreeding trials confirmed the additive inheritance of the Finn's reproductive performance, and therefore indicated it s potential use for increasing reproductive

performance in low fecundity breeds (Donald, Read and Russell, 1968; Land, Russell and Donald, 1974). Comparative studies with less prolific breeds indicated that the increased prolificacy of the Finn sheep was associated with a number of other reproductive characters such as the earlier attainment of puberty (Land *et al.*, 1974), an extended breeding season (Wheeler and Land, 1977) and increased ovarian activity post-partum (Land, 1971; Bindon and Piper, 1986a).

Due to the importance of the gonadotrophin surge with regard to ovulation, initial studies to find a physiological basis for the above average prolificacy of the Finnish Landrace concentrated on the characteristics of the preovulatory gonadotrophin surge and the subsequent events. The only characteristics which were found to be correlated with prolificacy were the duration of oestrus, which was longer in Finnish Landrace sheep (Land, 1970a, 1971) and the interval between the LH peak and behavioural oestrus, which was also longer in Finnish Landrace ewes (Quirke, Hanrahan and Gosling, 1979). Studies of gonadotrophin concentrations in female lambs indicated that a positive correlation existed between prolificacy and mean LH concentrations and the LH response to GnRH (Carr, Land and Sales, 1975). However, Echternkamp and Laster (1976) and Hanrahan, Quirke and Gosling (1975) were unable to confirm this relationship and a further study by Land, Carr and Thompson (1979), using older sheep, also found no correlation. The different ages of the sheep used in these trials and the transient nature of the correlation suggests that the difference in LH secretion may be due to developmental differences in pituitary responsiveness to GnRH or steroid feedback (Land and Carr, 1979). In the adult, intensive blood sampling indicated an inverse relationship between the mean LH concentration and ovulation rate (Land and Carr, 1979; Webb and England, 1982).

FSH concentrations were not found to differ significantly in entire ewes between the Finn and other breeds of sheep (Webb and England, 1982). However, in long term ovariectomised ewes, FSH secretion was significantly greater in Finnish Landrace ewes than ewes from low fecundity breeds (Webb, Preece, Baxter and Land, 1982), suggesting possible differences in the the regulation of FSH secretion by endogenous steroids in Finnish Landrace ewes.

Studies of mean steroid concentrations in Finnish Landrace ewes and ewes from other breeds indicated that mean progesterone concentrations are significantly higher in Finnish Landrace ewes (Quirke *et al.*, 1979), but failed to find any differences in mean oestradiol concentrations (Scaramuzzi and Land, 1976, 1978). However, other studies comparing oestradiol secretion in Finnish Landrace and Scottish Blackface ewes reported that the rate of oestradiol secretion into the ovarian vein was higher in Finnish Landrace ewes and that the Finnish Landrace ewes were less sensitive to the negative feedback effects of oestradiol (Wheeler, 1975 cited by Land and Carr, 1979). As well as the negative regulation of gonadotrophin secretion, oestradiol is also involved in the positive feedback of LH secretion, between breed studies have indicated that Finnish Landrace ewes are less sensitive to oestradiol positive feedback than ewes from less prolific breeds (Land, 1976; Land *et al.*, 1976).

Studies of ovarian follicle populations and follicular development in different breeds indicated that the Finnish Landrace had a greater proportion of developing oocytes (Land, 1971), and that the size of the preovulatory follicles and the proportion of follicles which become atretic were reduced in the Finn, when compared to other less prolific breeds (Webb and Gauld, 1984; Driancourt, Gauld, Terqui and Webb, 1986; Webb, Gauld and Driancourt, 1989). The period of selection of the ovulatory follicle also appears to differ in Finnish Landrace ewes, with a significantly

greater proportion of the ovulatory follicles being selected during the follicular phase (40% in Finn ewes compared to 6% in Merino x Scottish Blackface ewes) (Webb *et al.*, 1989).

In summary, ewes of the Finnish Landrace breed differ from other breeds in the concentrations of progesterone and possibly oestrogen in the peripheral circulation and therefore in the feedback control of gonadotrophin secretion. These differences in the level of, and the sensitivity to, gonadal negative feedback, possibly explain the increased ovulation rate in the Finnish Landrace by providing either a longer period for follicular selection or allowing a greater proportion of follicles to develop before the preovulatory gonadotrophin surge (Land *et al.*, 1976). A reduced sensitivity to gonadal feedback may also explain the increased length of the breeding season reported for the Finn (Land, 1976; Land *et al.*, 1976).

In the male, studies have indicated that the high prolificacy of the female is associated with an increased rate of testicular growth (Land and Sales, 1977) and a higher level of sexual activity (Land, 1970b) than that seen in other breeds. However, the physiology of the ram has received little attention. Sanford, Palmer and Howard, (1982) demonstrated a difference in the FSH concentration in prepubertal ram lambs from various breeds, Finnish Landrace lambs having higher mean FSH concentrations than lambs from breeds of lower prolificacy; however, this difference was not maintained in adult animals.

1.4.1.2. The Romanov

The Romanov breed originates from Russia and its exceptional prolificacy is well documented. Due to the additive nature of its above average reproductive performance, the Romanov can also be used to increase the prolificacy of less fecund breeds by crossbreeding (Land,

Pelletier, Thimonier and Mauleon, 1973). Puberty in the Romanov is consistently attained before the lambs are one year old and significantly earlier than comparative low fecundity breeds (Desvignes, 1971; Thimonier, 1975). This longer period of reproductive activity in the pubertal animal is maintained in the adults, where Romanov ewes exhibit a longer period of ovarian activity per year than low fecundity breeds (Thimonier and Mauleon, 1969; Land *et al.*, 1973; Bindon and Piper, 1986a).

The Romanov has a significantly longer period of oestrus than other low fecundity breeds (Desvignes, 1971; Land *et al.*, 1973), and within breed studies have indicated a relationship between the duration of oestrus and ovulation rate (Desvignes, 1971). The interval between the administration of PGF2α and oestrus is also shorter in the Romanov than in low fecundity breeds (Bindon, Blanc, Pelletier, Terqui and Thimonier, 1979) as is the interval between oestrogen administration and oestrus in prepubertal lambs (Land *et al.*, 1973). The characteristics of oestrus and the preovulatory LH surge have been studied (Land *et al.*, 1973; Bindon *et al.*, 1979; Cahill *et al.*, 1981), and indicated that the interval between the detection of oestrus and the start of the LH surge was longer in the Romanov ewes than in control ewes, but that no breed differences exist in the characteristics of the LH surge. When tonic LH secretion was compared between Romanov and control ewes, no differences were seen in the mean LH concentration over the whole of the oestrous cycle, but when analysed on a daily basis the Romanov ewes were found to have significantly higher mean LH concentrations on Days 1 and 8 of the oestrous cycle (Land *et al.*, 1973).

No differences were reported in the FSH concentration of the Romanov compared to other breeds (Bindon *et al.*, 1979). However when the FSH profiles were measured around the time of oestrus, and the profiles aligned with respect to the preovulatory LH discharge (Cahill *et al.*, 1981), a

positive relationship was observed between the magnitude of the second FSH peak and ovulation rate. The population of pre-antral and antral follicles is greater in the Romanov than in less fecund breeds (Cahill *et al.*, 1979; Driancourt *et al.*, 1986), and it was proposed that this increased follicle population in the Romanov is a result of the increased magnitude of the second FSH peak (Cahill *et al.*, 1981). Investigations of steroid concentrations have indicated that the Romanov has a higher mean progesterone concentration than comparative low fecundity breeds (Bindon *et al.*, 1979).

Physiological studies in the male indicate that a number of differences existed between the LH secretion profiles of Romanov lambs and ram lambs from low prolificacy breeds. The mean LH concentration in 5-week old Romanov lambs were twice as great as those measured in lambs from breeds of low prolificacy and the prepubertal peak of LH secretion occurred at an earlier age in Romanov ram lambs (Blanc, Courote, Pelletier and Thinonier, 1975).

1.4.1.3. The D'Man

The D'man breed originates from the oases south of the High Atlas mountains, in the valleys of Ziz Dades and Draa in Morocco. The breed was initially described by Bouix and Kadiri, (1975) and is renowned for its exceptional prolificacy and extended breeding season. The lambs of the D'man breed reach puberty at an early age, frequently before the animals are 6 months of age (Bouix and Kadiri, 1975) and 70-90% conceive before 1 year of age (Lahlou-Kassi and Marie, 1985). In the adult, there is no clearly defined breeding season, ewes being practically aseasonal (Bouix and Kadiri, 1975). Ovarian activity post-partum is resumed after a shorter interval in the D'man than in the Timahdite, a local breed of average prolificacy (Lahlou-Kassi and Marie, 1985). The early puberty and return

of ovarian activity post-partum of the D'man appear to be inherited in an additive fashion (Lahlou-Kassi and Marie, 1985).

Uterine capacity has been studied in the D'man, but does not appear to be greater than less prolific breeds. As embryo survival is also similar between breeds, the increased prolificacy appears to be the result of the above average ovulation rate of the breed (Lahlou-Kassi and Marie, 1985). Studies of the follicle populations in D'Man and the Timahdite ewes have indicated that the ovaries of the D'Man ewes contain a significantly greater number of growing follicles than the ovaries of the Timahdite ewes (Lahlou-Kassi and Mariana, 1984).

A limited number of studies have been reported regarding the physiological background of the high ovulation rate in the D'man. When the mean LH concentration was compared in D'man and Timahdite ewes during various stages of the oestrous cycle, the D'man ewes were found to have significantly lower mean LH concentrations than the low fecundity Timahdite (Lahlou-Kassi *et al.*, 1983). Between-breed differences have also been reported in the mean gonadotrophin concentrations, before and during oestrus. D'man ewes have a significantly greater FSH concentration in the periovulatory period, including the late follicular phase at the time of the preovulatory LH surge and at the time of the second FSH peak (Lahlou-Kassi *et al.*, 1983; Lahlou-Kassi and Marie, 1985) and a significantly lower concentration of LH (Lahlou-Kassi and Marie, 1985). Following ovariectomy however, the differences in the FSH concentrations of the two breeds were abolished (Lahlou-Kassi *et al.*, 1983). This suggests that in the D'man the increased ovulation rate could be the result of a difference in the mechanism that regulates the secretion of LH and FSH (Lahlou-Kassi and Marie, 1985).

1.4.2. Group B

1.4.2.1. The Booroola Merino

The Booroola Merino is a strain of medium woolled non-peppin Australian merino which was initially developed by the Seears brothers of "Booroola", Cooma, New South Wales by the selective breeding of animals with a history of multiple births. All selection was practiced on the dams, sires being bought with no reference to their past familial prolificacy (Turner, 1980). The exceptional prolificacy of the Booroola cannot be explained by an increase in the proportion of favourable alleles which exert a small effect on litter size, but can be explained by a single gene, or a very closely linked group of genes which exert a major effect on prolificacy, a 'major gene' (Piper and Bindon, 1982a, 1982b; Piper, Bindon and Davis, 1985b). Subsequent evidence, reviewed by Bindon and Piper (1986b) supported the existence of a major gene in the Booroola. This 'gene' was initially defined as the fertility or F gene, the possible genotypes being ++ or wild type, FF the homozygote, or +F the heterozygote. More recently, the fecundity gene present in the Booroola has been defined as the Fec^B gene (Piper and Bindon, 1990); however, in this thesis FF, F+ and ++ will be used when describing the three Booroola genotypes. Historical investigations (Turner, 1980) indicate that the F gene originated from the Cape or Bengal breeds of sheep that were imported to Australia in the late 18th century, with the Bengal sheep being the most likely source of the F gene. A large amount of the work on the Booroola has been done by the Commonwealth Scientific and Industrial Research Organisation (CSIRO), the selection and management programme of the CSIRO flock being documented by Turner (1978, 1980). The Booroola flock was established to complement previously established flocks selected for ('T' Merinos) and against twinning ('O' Merinos) (Turner, Hayman, Triffitt and Prunster, 1962). Crossbreeding studies indicate that the average effect of one copy of

the F gene is to increase ovulation rate by 1-1.5 eggs and to increase litter size by 0.8-1.2 lambs, the effects of the gene being additive (Piper and Bindon, 1987).

Puberty is attained earlier in the Booroola than in control Merinos (Bindon, Piper and Evans, 1980) and the sexual season of adult ewes is longer (Bindon, 1984), with 60% of ewes ovulating throughout the year (Bindon and Piper, 1976, 1982a). However oestrous cycle length (Bindon, Piper and Evans, 1982b), the duration of behavioural oestrus (Piper and Bindon, 1980; Bindon *et al.*, 1982b), fertilization rate (Piper *et al.*, 1982) and uterine capacity (Bindon, Piper, Cheers and Curtis, 1978c) do not differ significantly between Booroola and control Merinos.

The F gene appears to exert a number of effects on the ovary although disagreement exists between different groups as to the exact mechanisms involved. The ovaries of Booroola lambs are reported to contain a greater number of antral follicles than control Merinos (Tassell, Kennedy, Bindon and Piper, 1983; McNatty, Lun, Heath and O'Keeffe, 1987a; Braw-Tal and Goodwine, 1989) despite a reduction in ovarian weight due to a decrease in the amount of interstitial tissue (McNatty *et al.*, 1987a). In the adult, the total number of antral follicles is similar in both Booroola and control Merinos (Driancourt, Cahill and Bindon, 1985a; Driancourt, Yego, Cahill and Bindon, 1985b; Henderson, Keiboom, McNatty, Lun and Heath, 1985; McNatty, Henderson, Lun, Heath, Ball, Hudson, Fannin, Gibb, Keiboom and Smith, 1985a; McNatty, Lun, Heath, Ball, Smith, Hudson, McDiarmid, Gibb and Henderson, 1986a); however, the ovaries of Booroola ewes contain a greater proportion of large preantral and antral follicles than ewes from other low prolificacy breeds (Cahill, Mariana and Mauleon, 1979; Cahill, Loel, Turnbull, Piper, Bindon and Scaramuzzi, 1982). Despite this difference in the proportion of large antral follicles, the number of growing follicles in the Booroola is about half that seen in control Merinos (Scaramuzzi and

Turnbull, 1980) and the rate of follicular atresia is similar (McNatty *et al.*, 1985a) or slightly decreased (Driancourt *et al.*, 1985b). Differences in follicle recruitment appear to be an integral part of the high ovulation rate in the Booroola. In control Merinos, follicles ≥ 2mm in diameter are recruited between days 13 and 15 of the oestrous cycle and selection of the ovulatory follicles has occurred by day 15. In the Booroola however, follicles continue to be recruited during days 15-17. This extended phase of follicular recruitment and the ability of preovulatory follicles to be maintained until ovulation are important factors in the Booroola Merino's high ovulation rate (Driancourt *et al.*, 1985a, 1985b; Castonguay, Dufor, Minielle and Estrada, 1990). The follicles of ewes carrying the F gene appear to mature at a smaller size than follicles in control Merinos (Scaramuzzi and Turnbull, 1980; Scaramuzzi, Turnbull, Downing and Bindon, 1981; Baird, Ralph, Seamark, Amoto and Bindon, 1982; Henderson McNatty and Keiboom, 1984a; Driancourt *et al.*, 1985a, 1985b; McNatty, Keiboom, McDiarmid, Heath and Lun, 1986b), mature follicular diameter decreasing with increasing F gene copy number, FF 3.4 ± 0.3mm, F+ 4.1 ± 0.2mm, ++6.8 ± 0.3mm (McNatty *et al.*, 1986a). Due to the decrease in follicle size, the number of granulosa cells per follicle is also reduced (Scaramuzzi *et al.*, 1981; Baird *et al.*, 1982; Scaramuzzi and Radford, 1983; Driancourt *et al.*, 1985a; Henderson *et al.*, 1984a); however, as the number of mature follicles is increased, the mass of granulosa cells and the concentration of steroids produced on a per ewe basis is similar in Booroola and control Merinos (Henderson *et al.*, 1985; McNatty *et al.*, 1985a; McNatty, Hudson, Gibb, Ball, Henderson, Heath, Lun and Keiboom, 1986b). The ovarian follicles in the Booroola ewes may also be more responsive to gonadotrophins than control animals (Bindon, Thimonier and Piper, 1978; Bindon, Piper and Thimonier, 1984; Bindon and Piper, 1982a; McNatty *et al.*, 1986b), Booroola ewes having a higher ovulatory response to PMSG than control Merinos (Bindon *et al.*,

1971; Bindon and Piper, 1982a, 1982b; Piper *et al.*, 1982; Kelly, Owens, Crosbie, McNatty and Hudson, 1983; Piper, Bindon, Walher, Walkley and Phillips, 1985a; Davis and Johnstone, 1985). This difference in responsiveness is evident as early as 5 months of age (Oldham, Gray, Poidron and Bindon, 1984), and can be used to differentiate between FF, F+ and ++ ewe lambs (Davis and Johnstone, 1985).

Studies of peripheral gonadotrophin concentrations demonstrated similar LH profiles in Booroola and control merinos (Scaramuzzi and Radford, 1983; Bindon, 1984). However recently, McNatty *et al.*, (1987a) and McNatty, Hudson, Henderson, Gibb, Morrison, Ball and Smith (1987b) have reported a relationship between mean plasma LH concentration and F gene copy number which is maintained throughout the oestrous cycle and during anoestrus, LH pulse amplitude being increased in the Booroola ewes. This finding complements previous work which demonstrated an F gene specific increase in LH pulse amplitude in ewes during the follicular phase (McNatty *et al.*, 1985a, 1986a, 1986b; McNatty and Henderson, 1987).

Evidence for an increased concentration of FSH in Booroola Merinos comes from a number of sources. Findlay and Bindon (1976) noted an increased concentration of FSH in the peripheral circulation of female Booroola lambs at 30 days of age and this result was confirmed in further studies (Bindon, Findlay and Piper, 1985a; Braw-Tal and Gootwine, 1989; Montgomery, Scott, Littlejohn, Davis and Peterson, 1989). In the adult ewe the results have been variable. Initial reports indicated that there was no correlation between possession of the F gene and the concentration of FSH (Bindon, Findlay and Piper, 1982a; McNatty *et al.*, 1985b). However, subsequent studies have reported elevated plasma FSH concentration in ovariectomised ewes which carry the F gene (McNatty, Fisher, Collins, Hudson, Heath, Ball and Henderson, 1989), and entire Booroola ewes during the 24hrs preceding the LH surge (Bindon, Piper, Cummins, O'Shea, Hillard,

Findlay and Robertson, 1985b) and throughout the oestrous cycle and anoestrus (McNatty *et al.*, 1986a, 1987a, McNatty and Henderson, 1987). Studies of the FSH concentration around the time of the preovulatory surge indicate, as in other prolific breeds, that the Booroola ewe has a significantly larger second FSH peak after the preovulatory gonadotrophin surge. Ewes carrying the the F gene are also reported to have elevated concentrations of pituitary FSH (Bindon *et al.*, 1985b; Robertson *et al.*, 1984).

The effects of inhibin were studied in the Booroola after it was noted that the ovarian inhibin content was significantly lower in Booroola ewes compared to control Merinos (Cummins, O'Shea, Bindon, Lee and Findlay, 1983), while no difference was seen between 'T' and 'O' Merinos (Bindon *et al.*, 1985a). Bioactive and immunoactive inhibin concentrations were also measured in follicular fluid pools, collected from Booroola, control and 'T' Merinos, and no differences were observed between the different groups (McNatty, Henderson, Fleming, Clarke, Bindon, Piper, O'Shea, Hillard and Findlay, 1990). The reduced inhibin content in the Booroola is compatible with the altered follicle population described previously, in particular the reduced number of granulosa cells which are the main sources of inhibin in the female (Henderson and Franchimont, 1983). Administration of inhibin to ovariectomised Booroola and control ewes indicated that the Booroolas may have an increased sensitivity to inhibin feedback, the FSH depression occurring sooner in Booroola ewes than in controls (Cummins *et al.*, 1983). This difference was substantiated by the fact that control Merino ewes actively immunised against partially purified follicular fluid exhibit an increased ovulation rate (O'Shea, Cummins, Bindon and Findlay, 1982), and that puberty can be advanced following immunization of prepubertal lambs against inhibin (O'Shea *et al.*, 1982,; Henderson, Franchimont, Lecomte-Yerna, Hudson and Ball, 1984b; O'Shea, Al-Obaidi, Bindon, Hillard and Findlay, 1983; Al-Obaidi, O'Shea, Hillard and

Cheers, 1983). The regulatory control of ovarian function appears to be abolished in these immunised animals, in a way not unlike that in the Booroola (Bindon and Piper, 1986a). The increased ovulation rate in the Booroola may therefore occur via a reduction in ovarian inhibin.

No F gene specific differences were observed in the peripheral and ovarian vein concentrations of oestradiol, androstenedione, progesterone and testosterone (Scaramuzzi and Radford, 1983; McNatty *et al.*, 1985a, 1986a, 1986b; McNatty and Henderson, 1987). In addition the Booroola and wild type Merinos have also been shown to respond to steroid negative feedback in a similar manner (Cummins, 1983 cited by Bindon and Piper, 1986b).

In the ram, the F gene does not appear to be quantitatively expressed in the regulation of gonadal size or function (Bindon and Piper, 1976; Bindon *et al.*, 1980, 1982b; Purvis, Piper, Bindon, Edey, Curtis and Nethery, 1983, Purvis, Piper, Edey and Kilgour, 1988; Walker, Ponzoni, Walkley and Morbey, 1985), or in the endocrine characteristics of the adult ram (Bindon, 1984). However, in prepubertal ram lambs, differences are seen in the pattern of LH secretion in F+ and ++ lambs, LH pulse amplitude being reduced in animals carrying the F gene (Purvis, Ford and Martin, 1989). Differences have also been reported in LH pulse frequency in adult Booroola and Merino rams (Martin, Sutherland and Lindsay, 1987) but this relationship has been disputed by Price, Hudson and McNatty, (1990) who found no difference in LH pulse frequency, but reported a difference in the gonadotrophin response to GnRH, F gene carriers having a greater response than non-carriers. A gene-specific effect has also been noted in FSH secretion (Seck, Hochereau-de Reviers and Boormarov, 1988; Seck, Pisselet, Perreau, Cornu, Thimonier, Bodin, Elsen, Hochereau-de Reviers and Boormarov, 1990), but this relationship is also disputed by the studies of Purvis *et al.*, (1989), Purvis, Ford, Martin and McNeilly (1990) and Montgomery *et al.*, (1989). Significantly more testosterone and

progesterone are released by Booroola rams in response to an injection of PMSG (Oldham *et al.*, 1984) and, as in the females, this characteristic can be used to differentiate between FF and F+ sires (Davis and Johnstone, 1985; Piper *et al.*, 1985a).

The mechanism by which the F gene affects reproduction therefore appears to depend on an increased period of follicular recruitment and the ability of Booroola Merinos to 'hold follicles' of ovulatory size until ovulation. The physiological basis for these differences in follicle recruitment is not clear, as the results with regard to gonadotrophin and steroid concentrations differ between studies. Interestingly however, the studies of ovarian inhibin content revealed that the ovaries of the Booroola ewes contained significantly less inhibin than the ovaries from control Merinos and therefore provides a possible site for the effects of the F gene in the female.

1.4.2.2. The Borgarhoefn or 'THOKA' sheep

A strain of Icelandic sheep has been isolated from a number of flocks in the Borgarhoefn region which exhibits above average prolificacy (Jonmundsson and Adalsteinsson, 1985). Crossbreeding trials and pedigree records show that the increased prolificacy of the Thoka strain is inherited in a fashion which suggests the presence of a major gene. Analysis of historical records showed that all the sheep exhibiting the increased level of reproductive performance could be traced to a single ewe 'Thoka' whose production records and alleged family history indicate above average fertility. There are as yet, no physiological studies of the Borgarhoefn flock.

1.4.2.3. Javanese sheep.

The Javanese Thin tailed sheep (JTT) were reported to have above average prolificacy (Obst, Boyes and Chaniago, 1980; Mason, 1978; Fletcher,

Chaniago and Obst, 1982). While studies indicated a high degree of variability between sheep, within individual sheep the repeatability of reproductive traits was high (Bradford *et al.*, 1986). Therefore it was proposed that the JTT sheep carry a gene which has a large effect on ovulation rate and litter size (Bradford *et al.*, 1986, 1990). Further support for the existence of a major gene comes from the finding that considerable crossbreeding has occurred between JTT sheep and two other indigenous breeds, the Javanese Fat tail and Semarang sheep and that individuals in all three breeds could be found which displayed above average prolificacy with a high degree of repeatability. Analyses of progeny records also showed the apparent segregation of a major gene affecting prolificacy, which suggested that the high fertility dams were all heterozygous for the major gene. The heterozygous condition of the females was initially surprising and suggested that all males would be non-carriers. Further studies revealed that selection in the males was being practised against carriers as some strains of thin tailed sheep are used for ram fighting and the increased size of single born rams is therefore preferable. From the progeny data, it could be calculated that the presence of one copy of the 'gene' increased ovulation rate by 1.3. Physiological studies have characterised the preovulatory and ovulatory LH profiles, but these were not found to be correlated to the increased prolificacy of the Javanese Sheep.

1.4.2.4. The Cambridge

The Cambridge breed is an artificial breed that was formed in 1964 by selective breeding from ewes which had given birth to at least 9 lambs in 3 years. The foundation flock consisted mainly of Clun Forest and Finnish Landrace ewes but had a genetic contribution from at least 10 other breeds (Williams, Crees and Owens, 1984; Fell, 1987). The foundation

flock was mated to Finn rams and the proportion of Finn genes reduced to approximately 0.25 by backcrossing with the foundation ewes. Subsequent selection was based on litter size, which has been estimated to range between 1.73 ± 0.7 and 2.76 ± 0.96 depending on age (Owens, Crees, Williams and Davies, 1986; Fell, 1987). Estimates of ovulation rate range between 2-13 (Hanrahan and Owens, 1985). The Cambridge breed is reported to have a greater precocity than other comparable breeds (Williams *et al.*, 1984). Due to the large effects on reproductive performance and the repeatability of prolificacy, it was proposed that a major gene was present in the Cambridge (Hanrahan and Owens, 1985). Crossbreeding trials have indicated that the prolificacy of the Cambridge can be transferred to other breeds (Hanrahan, 1985).

1.4.3. A comparison of the different mechanisms which govern increased levels of reproductive performance.

In all of the prolific breeds reviewed the increase in prolificacy occurs due to an increase in ovulation rate. Further features which were common to all of the breeds studied were the early attainment of puberty and the lengthening of the breeding season. This extended period of reproductive activity was also accompanied in all breeds where information was available, except for the Booroola, by the earlier resumption of ovarian cyclicity post-partum, (Bindon *et al.*, 1982b).

Studies of follicle populations in the ovaries of the different breeds indicated that the Romanov (Cahill *et al.*, 1979) and D'man (Lahlou-Kassi and Mariana, 1984) have a greater number of growing follicles than low fecundity breeds, but again this trait does not hold across all breeds, the Booroola Merino (Driancourt *et al.*, 1985a, 1985b; McNatty *et al.*, 1985a) and the Finn (Webb *et al.*, 1989) having similar numbers of growing follicles to control breeds. The rates of follicular atresia are also not consistent

between the different breeds. The ovulatory follicle diameter appears to be related to prolificacy, ovulatory follicles being smaller in prolific breeds (Webb *et al.*, 1989), with differences occurring in follicular recruitment in Booroola and Finnish Landrace ewes.

Physiological studies of the different breeds did not reveal any common mechanism which could account for their increased prolificacy. For example, peripheral concentrations of LH varied between different breeds with periods of elevated LH secretion being reported in Romanov ewes, Booroola ewes and prepubertal Finnish Landrace lambs. In contrast however, in adult Finn and D'Man ewes an inverse relationship was reported between LH and prolificacy. Therefore it can be concluded that LH, and LH pulse patterns, are not the quantitative determinants of ovulation rate (Bindon and Piper, 1986a). In addition, there appears to be a greater interval between the preovulatory LH surge and the onset of oestrus in the highly prolific breeds, with the exception of the Booroola Merino (Bindon, Piper and Thimonier, 1984). However as follicle selection occurs 48-72 hours before the the LH surge (Cahill *et al.*, 1981), the timing of this peak relative to ovulation does not have any effect on prolificacy. More consistent reports exist for correlations between FSH and prolificacy (Cahill *et al.*, 1981; Lahlou-Kassi, Schams and Glatzel, 1984; Findlay and Bindon, 1976; Bindon *et al.*, 1985b; Ricordeau, Blanc, and Bodin, 1984). The maintenance of a higher FSH concentration during the follicular phase is postulated to encourage the growth of a larger pool of antral follicles with the appropriate complement of LH receptors to allow the maturational changes which are needed to complete development (Haresign, 1985). Again these correlations have not always been detected (Webb and England, 1982; Bindon *et al.*, 1982a). Inhibin concentrations have only been reported for the Booroola where ovarian inhibin is reduced and this may

account for the increases in plasma FSH concentrations (Cummins *et al.*, 1983).

No differences were seen in the concentrations of androgens and oestrogens in the peripheral circulation and ovarian veins of the prolific breeds when compared to comparative low fecundity breeds (McNatty *et al.*, 1985b; Bindon, 1984). Therefore if increased prolificacy is related to steroid concentration it must occur at the level of steroid feedback at the hypothalamus/pituitary gland. It has been postulated that prolific breeds achieve their higher ovulation rate by being less sensitive to the negative feedback effects of oestradiol and/or inhibin (Land, Atkins and Roberts, 1983; Webb, Land, Pathiraja and Morris, 1984) However this proposal is still to be proven.

1.5. The manipulation of reproductive performance.

Since domestication, man has selectively bred sheep to capitalise on, and maximize traits which are of economic usefulness. In the last 50 years, economic pressure has demanded that in commercial flocks, litter size should be increased. A variety of different methods have been studied to achieve this effect including 1) the artificial manipulation of the animals' own reproductive physiology by the supplementation of endogenous hormone and steroid concentrations, 2) the alteration of the animals' own hormone concentrations by active or passive immunisation and 3) the use of direct or indirect selection for increased reproductive performance. Artificial manipulation of reproductive performance (Methods 1 and 2) requires the repeated treatment of animals each breeding season, while changes achieved using animal breeding programmes are permanent and can be transmitted to subsequent generations. A large amount of work has concentrated on the ability of selective breeding to achieve increases in

reproductive performance. The different methods used, and the results achieved, are reviewed below.

1.5.1. Direct selection

Direct selection is the selection of animals based on measurements of the character to be changed e.g. litter size, number of lambs weaned or ovulation rate. These gross female characteristics have been used as the basis of improvement programmes as they are known to differ between breeds. A number of experimental selection programmes have been established to improve reproductive performance using direct selection, with varying degrees of success.

1.5.1.1. Direct selection based on litter size

Litter size in the sheep varies between breeds (Appendix 1), ranging between 1 and 3; however in some breeds such as the Booroola Merino and the Cambridge, litters of up to 7 lambs have been reported. Although there can be a two-fold difference in litter size between breeds (Appendix 1), caution must be exercised in assessing the amount of genetic variation, as the breeds were not compared in a single environment and differences in litter size may reflect differences in environmental conditions e.g. the two estimates of litter size in the Scottish Blackface (Gunn and Robinson, 1963; Weiner, 1967).

The heritability of a character is defined as the proportion of phenotypic variation which is due to the additive effect of the genes. Therefore, the success of any trait in a selection programme is dependent on the heritability of that character. The heritability of litter size has been estimated in a number of different breeds of sheep as shown in Table 1.3.

Breed	Heritability of litter size	Reference
Finn	-0.02 ± 0.07	Hanrahan and Quirke (1985)
Galway	0.08 ± 0.02	Hanrahan and Quirke (1985) Hanrahan (1984)
Merino	0.06 ± 0.05	Piper, Bindon, Atkins and Rogan (1984)
Many	0.10	Bradford (1985) (mean of 30 estimates)

Table 1.3. - Heritability estimates of litter size in 4 breeds of sheep.

Although the estimates of heritability are small, a number of within-breed selection experiments have been established using litter size as the selection criterion, the results of which were reviewed by Bradford (1985) and are summarised in Table 1.4.

	LITTER SIZE			
BREED	HIGH	LOW	CONTROL	REFERENCE
Romney (Ruakura)	1.62	1.13	1.22	Clarke (1972)
Merino	1.70	1.18		Turner (1978)
Merino (Trangre) *	1.52		1.32	Atkins (1980)
Galway *	1.67	1.44		Hanrahan (1982)
Targhee	1.39		1.24	Bradford, Torell Lasslo and Neira, (1981)
Merino	1.22		1.2	Mann, Taplin and Brady, (1978)

* No initial screening of base stock

Table 1.4. Mean litter sizes in experiments where attempts have been made to improve reproductive performance by selection for litter size.

The experiments varied in duration, selection intensity and the number of animals per line. Some of the selection lines were started by screening the base stock and the resultant response to selection in these experiments was increased. The small response seen in the Merinos in the studies of Mann *et al.*, (1978) was the result of the low selection intensity in this trial. In all experiments changes were seen in litter size. The magnitudes of the results are similar between studies and agree with the results predicted given the low heritability of litter size.

1.5.1.2. Direct selection based on ovulation rate.

Prolificacy embraces a number of characteristics including ovulation rate, conception rate and embryo survival. Hammond (1914) suggested that ovulation rate was the principle determinant of fecundity in the ewe and this view was echoed by Hanrahan (1979), who argued that there was a direct relationship between ovulation rate and litter size and that as the repeatability of ovulation rate was greater than that for litter size, selection for litter size should be based on ovulation rate. This proposal was supported by the demonstration that between-breed differences in litter size are accompanied by comparable or greater differences in ovulation rate (Haresign, 1985; Hanrahan, 1982) and that selection experiments based on litter size resulted in increases in ovulation rate (Hanrahan, 1982; Hanrahan and Piper, 1982). The between-breed studies also indicated that embryo mortality was increased in prolific breeds (Bindon and Piper, 1982a) and that a curvilinear relationship existed between litter size and ovulation rate (Hanrahan, 1982). A commercial disadvantage in the use of selection on ovulation rate to increase litter size, is that if an increase in ovulation rate is achieved then the variability in litter size is also increased (Bradford, 1985).

Selection experiments based on ovulation rate have been conducted in mice (Land and Falconer, 1969; Bradford, 1968, 1969) and pigs (Cunningham, England, Young and Zimmerman, 1979). Although the selection lines achieved their selective aim, by increasing ovulation rate, a concomitant increase in litter size did not occur. This is explained in both mice and pigs, by the maximum uterine capacity having been reached in these species and that any further increases in ovulation rate merely result in an increase in embryo mortality. In sheep and cattle, uterine capacity is not normally the limiting factor on reproductive performance; therefore selection based on ovulation rate would appear to be a viable prospect. In sheep, between-breed studies have shown that a large amount of variation also exists in ovulation rate (Appendix 2), mean ovulation rates ranging between 1.09 and 3.40 in most breeds.

As with litter size, above-average estimates for ovulation rate (up to 13) were reported for Booroola and Cambridge ewes (sections 1.4.2.1. and 1.4.2.4). Estimates of the heritability of ovulation rate are in general, greater than that of litter size (summarized in Table 1.5.), Therefore selection based on ovulation rate should be more successful than selection based on litter size.

Breed	Heritability of ovulation rate	Reference
Finn	0.55 ± 0.09	Hanrahan and Quirke, (1985)
Galway	0.32± 0.16	Hanrahan and Quirke, (1985) Hanrahan, (1984)
Merino	0.07 ± 0.03	Piper, Bindon, Atkins and Rogan, (1984)
	0.16 ± 0.07	Piper Bindon, Atkins and McGuirk, (1980)
Romanov	0.24	Ricordeau *et al.*,(1982)
	0.25	Hanrahan(1982)

Table 1.5. Heritability estimates for ovulation rate in 4 breeds of sheep.

A divergent selection line was set up in Ireland, selection being based on the sum of the ovulation rate at 2 consecutive oestrous cycles, in 18 months old Finnish Landrace ewes (Hanrahan and Quirke, 1982). The results are summarized in Table 1.6. and indicate that selection based on ovulation rate can achieve the selected aim.

	Ovulation rate			
Age(years)	High	Low	Control	Divergence
0.75	3.29	1.78	2.07	1.41± 0.23
1.5	4.53	2.25	3.00	2.18 ± 0.25
4.5	5.27	3.35	3.80	1.92 ± 0.41

(Taken from Hanrahan, 1987)

Table 1.6. Mean ovulation rate in ewes aged between 0.75 and 4.5 years, in High, Low and control lines following divergent selection for ovulation rate.

1.5.2. Indirect selection

The progress that can be made by direct selection to improve prolificacy in domestic animals is limited by a number of factors:- the small number of young produced per breeding season, the long generation interval of most domestic species, the low heritability of the traits in question (reviewed by Morris, 1990) and the sex-limited nature of the traits (ovulation rate and litter size are only measurable in the female). The sex-limited nature of reproductive traits is also compounded by the management systems employed with most domestic species, which rely on the maintenance of large numbers of females for the production of milk, and young animals for meat production. Therefore selection pressure on females is by necessity low and the relatively small number of males which are maintained are subjected to extreme selection pressure. Indirect selection is selection on an independent characteristic which is closely

correlated to the character to be changed. Therefore, to maximise the effectiveness of selection for reproductive performance selection programmes should be a combination of direct selection in the female and indirect selection in the male.

Indirect selection has been tried in a number of situations with varying degrees of success. Attempts to improve lamb growth rate by indirect selection were not successful, as the only selectable trait found to be correlated to growth rate was adult body weight and therefore the advantages of indirect selection were offset by the increased generation interval (Croston, Read, Jones, Steane and Smith, 1983). Indirect selection to alter body fat content was tried in broilers, selecting on the concentration of low- and very-low density lipoproteins in the plasma of the birds. This met with more success than the growth rate experiment in lambs, with significant differences being present in the fat content, but not the body weight of the birds (Griffin, Whitehead and Broadbent, 1982). The possibility of improving dairy merit in cattle by indirect selection on prepubertal male calves has also been discussed (Tilakaratne, Alliston, Carr, Land and Osmond, 1980; Osmond, Carr, Hinks and Land, 1981; Woolliams and Lovendahl, 1991).

Walkley and Smith (1980) investigated the possible advantages of using indirect selection to improve reproductive performance in sheep, considering variables such as heritability, the correlation between the traits involved, the use of traits in males and females singularly and the use of a combination of direct and indirect selection. Their analysis showed that indirect selection, in conjunction with direct selection for fertility, could achieve a doubling of the effects of a selection programme based solely on the direct measurement of the character in the female. It was also shown that indirect selection of a male trait could achieve the same effect as direct selection if the selected character was highly correlated to female

reproduction. Walkley and Smith (1980) concluded that the success of indirect selection was dependent on the indirect trait having a good genetic correlation with the trait to be changed.

Effectiveness of indirect selection = $r_A * h^2_i / h^2_D$

where:- r_A = Genetic correlation between the two traits.

h^2_i = Heritability of the indirect trait.

h^2_D = Heritability of the desired trait.

From the equation, it can be seen that the benefit of indirect selection increases as the heritability of the indirect trait increases, or when the correlation between the indirect trait and the desired trait increases. However, the effectiveness of indirect selection decreases as the heritability of the trait of interest increases (Haley, Cameron, Slee and Land, 1987). Indirect selection however, bears the disadvantage that there may be other unforeseen effects, which may confer advantages or disadvantages to the selected animals with respect to the selection programme or the overall commercial properties of the animal.

As described in section 1.5.1.2 ovulation rate is one of the the major factors in the determination of litter size in the sheep (Bradford, 1972; Packham and Triffit, 1966; Bindon, Ch'ang and Turner, 1971; Hanrahan, 1974a; Hanrahan, 1979). The cycles of ovarian activity which result in follicular growth and ovulation are controlled by the concentrations of gonadotrophins and steroids in the peripheral circulation and the sensitivity of the pituitary gland-hypothalamus to the negative feedback effects of gonadal steroids (section 1.2.). Differences in these control mechanisms may be responsible for some of the between-breed differences in ovulation rate and litter size. As reproductive function in the male is under similar control mechanisms to that of the female, and many of these characters although not functional as in the adult are present in the prepubertal lamb, Land (1973, 1974) proposed that these shared control

mechanisms could be used as criteria for indirect selection for female reproduction in the male.

In sheep, within- and between-breed studies have indicated a number of traits in both sexes which may be under similar physiological control and which could therefore be used as indirect selection criteria. In the female these include seasonality, or the number of hogget oestrous periods, which was reported to be correlated with litter size/ovulation rate (Ch'ang and Rae, 1972), although this relationship has been disputed by Baker, Clarke, Carter and Disprose (1981). However, most traits which have been identified are unsuitable for indirect selection as they are only measurable in adult animals. Traits suggested in the male include the intensity of sexual behaviour, (Land, 1970b; Land and Sales, 1977), the concentration of FSH in the peripheral circulation, testis growth (Land, 1973; Hanrahan, 1974b) and the LH response to GnRH (Land, 1973). Testis growth and the LH response to GnRH are suitable for indirect selection as they can both be measured in young male lambs.

1.5.2.2. Indirect selection based on Testis growth

Ovarian and testicular activity are both regulated by the concentration of gonadotrophins in the peripheral circulation. In the male, testis growth provides an easily measurable manifestation of the increased testicular activity which occurs following gonadotrophin stimulation. Therefore, it was proposed that ovarian activity, which is the result of gonadotrophin stimulation in the female, would be correlated to testis size. This theory was supported by the observation of a correlation between testis diameter and the mean peripheral concentration of LH, after correction for differences in body weight (Carr and Land, 1975). The relationship between testes size and ovulation rate was also positive in Finnish Landrace and Merino rams and their crosses (Land and Sales,

1977). As described previously, the success of indirect selection relies on the high heritability of the selected trait and a high correlation between the selected trait and the trait of interest. The estimates of the heritability of testes growth have been reviewed (Kilgour, Purvis, Piper and Atkins, 1985) and range between 0.13 and 0.60 in sheep and between 0.40 and 0.69 in cattle. The correlation between testis weight and ovulation rate was estimated in mice as being 0.97 (Land and Falconer, 1969), and in Finnish Landrace and Merino sheep as being 0.41 and 0.35 respectively (Hanrahan and Quirke, 1982; Purvis *et al.*, 1988). Therefore testis growth appeared to be a suitable character on which to base an indirect selection programme.

Divergent selection lines were therefore established by Land (1973). Finn Dorset ram lambs were selected on average testis diameter measured at 7, 10 and 14 weeks of age, after an adjustment for differences in body weight. After 8 generations of selection, a significant difference existed in testis diameter at 10 weeks of age which reflected the faster growth rate of the testes of the High line. The realized heritability of testis diameter was 0.4. No differences were seen in the size of the mature testes (McNeilly, Fordyce, Land, Lee and Webb, 1986). In the adult ewe, changes were seen in ovulation rate, the age of puberty, the median day of first oestrus in subsequent breeding seasons, litter size and mating efficiency (Lee and Land, 1985; Haley, Lee, Ritchie and Land, 1990).

Physiological studies indicated a positive relationship between the concentration of FSH and the line of origin in prepubertal lambs, and a pattern of LH secretion in the young High line lambs which was more similar to that normally seen in older Finn-Dorset ram lambs (McNeilly *et al.*, 1986). In the adult ewes, the mean LH concentrations were lower in the High line than in the Low line. Differences in the pattern of LH and FSH secretion in the ewes and lambs from the High and Low selection lines suggest that the two lines differ in their sensitivity to steroid negative

feedback and that this between-line difference may account for the differences in the reproductive performance of the adult ewes (McNeilly, Fordyce, Land, Martin, Springbett and Webb, 1988).

1.5.2.3. Indirect selection based on the LH response to a GnRH challenge

Mean LH secretion increases gradually following birth, reaching a peak in advance of puberty and thereafter declining. The age at which this prepubertal peak of LH secretion is attained differs between breeds and animals. This developmental difference in LH secretion, combined with the pulsatile nature of LH secretion, means that it is not possible to accurately assess the concentration of LH from a single blood sample. It was therefore proposed (Bindon and Turner, 1974) that selection based on the concentration of LH would require several blood samples, taken at intervals less than the half life of LH (30 minutes). An alternative method to measure the LH concentration was proposed, based on the ability of lambs to secrete LH in response to an exogenous dose of GnRH. This possibility was studied further and a significant genetic correlation was demonstrated between the LH response to GnRH and breed type/prolificacy in young lambs (Carr Land, and Sales, 1975), but not in adult ewes (Land *et al.*, 1979). Studies by other groups disputed the relationship between the LH response to GnRH and prolificacy (Bindon *et al.*, 1974; Hanrahan, Quirke and Gosling, 1981), with Hanrahan *et al.*, (1981) reporting an inverse relationship between the LH response to GnRH and prolificacy in female lambs. Double GnRH challenges have also been conducted on individually reared Finn x German-mutton Merino lambs, to investigate the effects of the environment and the correlation between the LH response and prolificacy (Gur-Arie, Rosenberg, Hillel, Folman and Eyal, 1986). The results of this study indicated that there was no correlation between basal LH levels and

prolificacy, but that a correlation was found between the LH response at 80 days of age and the age at which puberty was attained and that differences in the magnitude of the LH responses to GnRH were correlated with differences in prolificacy. The possibility of using GnRH challenges as indirect selection criteria was assessed by Bindon and Piper (1976), who concluded that the use of GnRH (or progesterone) challenges as a measure of endogenous LH would be too laborious to be a realistic option. This was disputed by Land (1982) who maintained that the intense selection pressure exerted on ram lambs would ensure that the number to be tested would not be excessive and a schedule was published indicating the number of lambs required.

The heritability of the LH response to GnRH was estimated (h^2 = 0.53), and found to be greater than that for the mean LH concentration (Lee and Land, 1985; Land, 1981). The approach of using a GnRH challenge was tested by Land and Carr (1979) to assess the effects of season and time of challenge on the LH responses of the lambs, but no effects were found.

A differential selection line was therefore set up in Edinburgh, selection being based on the LH response to a 5μg dose of GnRH, assessed as the mean LH response measured 30, 50 and 70 minutes after GnRH administration. By the fifth selected generation, a fourfold difference existed in the LH responses of the 10-week old High and Low line ram lambs to a 5μg GnRH challenge (Haley, Lee, Fordyce, Baxter, Land and Webb, 1989) and was maintained in 20-week old ram lambs. In the female lamb there was a threefold difference in the LH response at 10 weeks of age, however at 20-weeks of age the difference between the two lines decreased, the High line lambs only releasing 2.5-fold more LH than the Low line. In the adult ewe, several correlated changes have been recorded. Firstly ovulation rate has changed in response to selection in the first breeding season and at the beginning of the second season, but not at the time of mating in the second

season (2.42 v 2.04 , 2.49 v 2.15 and 2.51 v 2.65 on the three occasions respectively). Secondly the median day of first oestrus was significantly different in the first season (H 316 v L 324).

The results from the two indirect selection lines set up by Land and colleagues therefore did not completely fulfil their initial aim, to alter ovulation rate. However, the results demonstrated that indirect selection based on a physiological characteristic in young male lambs, can alter reproductive performance in related adult ewes. Therefore, it can be concluded that some aspects of the physiological control of reproductive function in the sheep are common to both sexes.

The physiological differences between the High and Low testis line animals have been studied in both prepubertal lambs and adult ewes. The results indicated that indirect selection on a reproductive characteristic can affect the reproductive physiology of related animals. Divergent selection has therefore created two lines of sheep which differ in the physiological regulation of gonadotrophin secretion (McNeilly *et al.*, 1986, 1988), possibly due to a difference in the sensitivity of the two lines to steroid negative feedback.

In the GnRH lines, selection was based directly on the measurement of a physiological character. After 8 generations of selection, the two lines had diverged so that High line ram lambs release approximately 5 times more LH in response to a 5μg dose of GnRH than Low line animals. Correlated changes have also been noted in the selected character in 20 week old male lambs and 10- and 20-week old female lambs. Small transient between-line differences have also been reported in the reproductive performance of related adult ewes (Haley *et al.*, 1989).

The remit of this project was to characterise the physiological effects of selection in both prepubertal and adult animals and thereby to locate the site of the between-line difference in the ability of the two lines

to respond to GnRH stimulation. It was also argued that this approach would provide some information on the genetic control of gonadotrophin secretion, and therefore when considered in relation to the between-line differences in reproductive performance, to provide information on the role of gonadotrophins in the regulation of ovine prolificacy.

CHAPTER 2. - Materials and methods

2.1 Animals

The sheep used in these studies were all of the Finn Dorset breed, a synthetic breed, formed by crossing the Finnish Landrace and the Dorset Horn (Land and McClelland, 1971). The sheep were maintained at the Institute's Blythbank farm in southern Scotland and managed on a traditional, upland, grassland sheep farming system. The ewes and lambs used in these experiments ~~are~~ were taken from two divergent selection lines. Ram lambs born in 1978, were challenged at 10 weeks of age with a 5µg dose of GnRH injected directly into the jugular vein, and the magnitude of the subsequent release of LH measured in three jugular blood samples, collected 30, 50 and 70 minutes post-injection. To normalise the variance and to linearize the decline in the LH concentrations between samples, the values were $\log_{10}$ transformed and on the basis of their mean $\log_{10}$ LH response, the lambs were allocated to either the High or Low lines. The rams from the two selection lines were mated in the autumn of 1978 to unselected females, born in 1977 and 1978. In 1979 and subsequent years, seven-month old ram lambs were selected in each line, with no more than 2 rams from each half sib group. Each ram was then mated to a total of forty 7-month old and forty 19-month old ewes from the same line. Males were only used for breeding for one year and females were used for two years (Haley *et al.*, 1989). Selection was maintained until 1986, where-after the lines were maintained by within-line mating.

2.2. Surgical procedures

2.2.1 Blood sampling.

Blood samples were collected from the jugular vein, by either vacutainer, syringe, or an indwelling jugular cannula into heparinised

glass tubes. The plasma was harvested by centrifugation at 3000rpm for 30 minutes at 4°C. Samples were stored at -20°C until required for assay.

If serial blood samples were to be collected via an indwelling jugular cannula, the animals were prepared at least 2 hours prior to use. The animals were restrained manually and the necks clipped and surgically prepared. The jugular vein was located and a 16-gauge cannula (B. Braun products; Dunlop Veterinary Suppliers) inserted and passed down the vein. Once in place, the cannula was fitted with either a membrane cap, or a manometer line and three-way tap, flushed with heparinised saline and secured in place with two sutures. The cannulae, manometer lines and taps were protected from damage by a tubular elastic bandage placed around the lamb's neck.

2.2.2. Castration

Animals were castrated either by the application of a tightly fitting rubber ring at the neck of the scrotum, or by open surgical castration on the day following birth. For surgical castration, the lambs were manually restrained, and the scrotum shaved and surgically prepared. Lignocaine (1ml) (Univet; Dunlop Veterinary Suppliers) was administered subcutaneously at the tip of the scrotum, 0.5 ml each side. An incision was made along the length of the scrotum including the most ventral part of the scrotum, so as to allow efficient drainage from the wound. Each testicle was externalised, until the spermatic cords were clearly visible. Two pairs of artery forceps were applied across the spermatic cords, proximal to the testis. The distal forceps were then rotated relative to the other forceps and the testis removed with traction, A transfixing ligature was applied proximal to the remaining artery forceps to ensure that the main vessels were sealed. The scrotal cavity was then dusted with Polybactrin (Welcome; Dunlop Veterinary Suppliers) and left open to allow drainage. The procedure was repeated for the remaining testis, and the scrotal sack sprayed with

Teramycin (Pfizer). The lambs were returned to their mothers, and remained indoors for a further 24hrs, to confirm that there were no mothering problems following treatment.

2.3. Hormone assays

2.3.1. Assay buffers

The assays used to measure gonadotrophin and steroid concentrations used two assay buffers, the bovine serum albumin- general assay diluent (BSA-GAD) which was used in the gonadotrophin assays and the phosphate-gelatin assay buffer (phosgel) which was used in the steroid assays. Both buffers are derivations of a 0.5M phosphate base which was prepared by dissolving 716g of disodium hydrogen orthophosphate into 4 litres of double distilled deionised water, and 78g of sodium dihydrogen orthophosphate into 1 litre of water. The sodium dihydrogen salt solution was then added to the disodium hydrogen salt solution until a solution of pH 7.5 was attained. The resultant buffer was stored at 4°C.

The 0.1% BSA-GAD buffer was prepared by the dilution of 200ml of the stock phosphate buffer with 1800 ml of water which contained 18g of sodium chloride, 2g of bovine serum albumin (fraction V; Sigma Ltd.) and 200mg of thimerosal. The phosgel was prepared by a similar method, but the bovine serum albumin was replaced by 2g of swine skin gelatin (300 bloom; Sigma Ltd.). The gelatin was dissolved in 400ml of buffer at 50°C and returned to the remainder of the buffer. After being well mixed, the buffer was filtered and stored at 4°C.

2.3.2. Luteinizing Hormone

Luteinizing hormone (LH) was measured by means of a direct radioimmunoassay routinely performed in our laboratory, based on the

assay of Martensz *et al.*, (1976) with modifications by Webb, Baxter, Preece, Land and Springbett (1985a).

Ovine LH (M4; 2.9 x NIDDK-LH-S1) was iodinated after the method of Greenwood, Hunter and Glover (1963). A 5μg aliquot of LH was dissolved in 5μl of H_2O in a reaction vial and made up to 30μl with sodium phosphate, pH 7.4. 1mCi of $Na^{125}I$ (Amersham International plc, Bucks.) and 20μl of chloramine-T (20μg in 0.05M sodium phosphate) were then added to the reaction vial, followed immediately by 100μl of sodium metabisulphite (2.4mg/ml in 0.05M phosphate buffer) and 200μl of potassium iodide (1% in 0.1% BSA-GAD). The labelled LH was then separated from the reaction mixture by column chromatography on Sephadex G50 and eluted with the assay buffer. The iodinated LH stock solution was diluted before use with 0.1% BSA-GAD to give a final concentration of 12000 counts/100μl/60 seconds.

The radioimmunoassay procedure was as follows. Standard curves were prepared, using a standard preparation of LH (NIH-LH-S18) diluted in 0.1% BSA-GAD to give LH concentrations ranging from 0.124 to 40.96 ng/ml. Quality control plasma samples of known LH concentration were also included in the standard curves to monitor between- and within-assay variation. For each assay the standard solutions, the quality control samples and the plasma samples were diluted to a final volume of 500μl with assay buffer (0.1%BSA-GAD) and dispensed into 12x75mm polystyrene assay tubes. The standard solutions and the quality control solutions were assayed in triplicate, whereas the unknown samples were measured in duplicate. The amount of sample that was diluted varied between experiments but normally ranged between 50 and 200μl. Following dilution, 200μl of R_3 anti-oLH antiserum, at an initial dilution of 1:200,000, was added to the standard curves and the unknown samples, and the tubes mixed thoroughly. The tubes were then incubated at 4°C for 48 hours. 100μl of ^{125}I-oLH(M4) was

then added to each tube and the tubes incubated for a further 48 hours at 4°C. The antibody-bound label, and free unbound label fractions, were separated by the addition of 100µl of 1:400 normal rabbit serum (NRS) and 200µl 1:45 donkey anti-rabbit serum (DARS) (Scottish Antibody Production Unit, Carluke; SAPU) containing a 1:10 dilution of 0.1M EDTA, The assay was then incubated overnight at 4°C, before the addition of 1ml 0.1% BSA-GAD and centrifugation at 2000g for 30 minutes at 4°C. The supernatant was then discarded and the bound activity counted on a Automatic gamma counter (LKB Ltd., Croydon).

2.3.3. Follicle-stimulating hormone

FSH was measured in a homologous radioimmunoassay supplied by NIDDK. Ovine FSH was iodinated by the lactoperoxidase method. 5µg of NIDDK oFSH-I-1 was dissolved in 5µl of water and made up to 30µl with 0.5M PO_4 buffer (pH 7.5) in a reaction vial. 1mCi of $Na^{125}I$ (Amersham International plc, Bucks) was then added to the reaction vial, followed by 10µl of lactoperoxidase (0.5mg/ml in 0.05M PBS pH7.5) and 10µl of H_2O_2 ($6x10^{-4}M$). The reaction mixture was incubated for two minutes and the reaction then terminated by the addition of 250µl of 0.1% BSA-GAD. The iodinated FSH was separated from the reaction mixture by column chromatography on Sephadex G50, eluting the column with assay buffer. The iodinated FSH preparation was diluted before use with assay buffer to give a final concentration of 12000 counts/100µl/60 seconds.

The assay procedure was as follows:- Standards (0.25-10ng/ml; NIDDK-oFSH-RP-1) and quality control plasma samples were diluted to 500µl with 0.1%BSA-GAD in 12 x 75mm polystyrene assay tubes, and incubated for 24 hours at 27°C in the presence of 200µl of anti-oFSH-1 (NIDDK) at an initial dilution of 1:20,000, and 100 µl of the diluted ^{125}I-FSH (NIDDK oFSH-I-1) solution. The bound and free fractions were separated by the addition of

100µl 1:400 of NRS and 200µl of DARS containing a 1:10 dilution of 1M EDTA. The tubes were then incubated for 24 hours at 4°C, before the addition of 1ml of assay buffer and centrifugation at 2000g, at 4°C for 30 minutes. The supernatant was discarded and the pellet counted on an automatic gamma counter (LKB Ltd).

2.3.4. Oestradiol-17β

Oestradiol concentrations were measured by means of a radioimmunoassay, following extraction of the plasma samples by affinity chromatography. The procedure for the preparation of the iodinated label and the antiserum has been reported previously by Webb, Baxter, McBride, Nordblom and Shaw (1985b).

Extraction procedure - Plasma samples were placed in screw-capped glass culture tubes, together with 100µl of CNBr-activated Sepharose 4B (Pharmacia) covalently linked to oestradiol antiserum, 10µl of 2,4,6,7,16,17-^{3}H-oestradiol-17β (1000 counts/100seconds; Amersham International plc) and 10 ml of water. The samples were mixed end-over-end, overnight at room temperature. Chromatography columns (10 x 120mm soda glass with glass sinter discs, porosity 1; Schott Glass, UK) were prepared by washing with 3ml 90% methanol, followed by three washes of 7ml water, the whole wash cycle being repeated at least twice. The culture tubes were emptied into individual columns, and washed with 7ml of water, the water being applied to the respective columns. The columns were then washed three times with 7mls of water, the water being expelled under positive pressure. The extracted oestradiol was recovered by washing the columns with 3ml of 90% methanol into 16 x 125mm glass tubes. The methanol was evaporated using a Buchler Vortex vacuum evaporator at 40°C and the oestradiol reconstituted into 1.8ml PBS-Gel, by mixing at 40°C for 40-50 minutes. The extraction efficiencies were estimated by counting the ^{3}H activity in a

500µl aliquot of each reconstituted sample. The 500µl sample was placed in a plastic minivial, mixed with 4ml of scintillant (Optiphase X; Fisons Scientific Apparatus) and counted on a Rackbeta counter (LKB Ltd) for 300 seconds. The counts were then compared to those obtained in samples where 10µl of ^{3}H-oestradiol was mixed with 500µl of PBS-gel and 4ml of scintillant, minus background activity which was estimated by counting vials containing only 500µl of PBS-gel and scintillant.

Radioimmunoassay- Standards for the oestradiol assay were prepared from a stock solution of oestradiol-17β (Sigma) in ethanol. Samples were assayed in duplicate. The assay procedure was as follows:- 500µl aliquots of each sample were dispensed into glass assay tubes and incubated with R48 oestradiol antiserum (200µl of an initial dilution of 1:40,000) and ^{125}I-oestradiol, diluted to approximately 12,000 counts per 60 seconds per 100µl, for 2 hours at room temperature. The bound and free fractions were separated by the addition of DARS (100µl, 1:40) and NRS (100µl, 1:400) and incubation at 4°C for 24 hrs. After centrifugation at 2000g for 30 minutes, the pellets were counted on an automatic gamma counter (LKB Ltd).

2.3.5. Progesterone

The concentration of progesterone in the samples was measured by radioimmunoassay following extraction of progesterone from the plasma samples by solvent extraction , according to the method of Corrie, Hunter and MacPherson (1981) and modified by Ashworth (1985).

Extraction Procedure -500µl of plasma, quality control or water were placed in screw-capped culture tubes with 10µl of 1, 2, 6, 7-^{3}H-progesterone approximately 1000 counts per 60 seconds (Amersham International plc). The tubes were mixed and left to equilibrate for 5 minutes, before the addition of 6ml of petroleum ether (40°C-60°C Distal grade; Fisons Scientific Apparatus Ltd). The tubes were then mixed vigorously for 20 minutes using

an SMI vortexer, and the aqueous and solvent layers allowed to separate. The tubes were then placed in a dry ice/methanol bath to a depth of approximately 2cm. When the aqueous layer was frozen the ether was decanted into glass tubes (16 x 125mm) and evaporated to dryness on a Buchler vortex evaporator under vacuum. The steroid was then reconstituted in 1.8ml of PBS-gel by mixing at 40°C for 40-50 minutes. The extraction recovery was estimated using a method similar to that described for oestradiol.

Radioimmunoassay - Duplicate 500µl aliquots of the reconstituted sample were taken for radioimmunoassay. These were placed in glass assay tubes (12 x 75mm) and 200µl of 1:8000 R31/8 rabbit anti-progesterone added, together with 100µl of ^{125}I-progesterone-11α-glucuronide-tyramine (prepared by the method of Corrie *et al.*, 1981; kindly provided by the MRC Immunoassay team) and diluted to give approximately 12,000 counts/60 seconds. The tubes were then incubated at room temperature for 2 hours. The bound and free fractions were separated by incubation overnight at 4°C with 100µl of 1:35 DARS and 100µl of 1:300 NRS. 1ml of PBS-gel was added to each tube and the tubes centrifuged at 2000g for 30 minutes at 4°C, the supernatant was discarded and the radioactivity of the pellet counted on an automatic gamma counter (LKB Ltd). The potency estimates were corrected for the percentage recovery and expressed as ng/ml.

2.3.6. Testosterone

Testosterone was measured in a radioimmunoassay (Webb *et al.*, 1985b) following extraction of the plasma samples using either of the two methods described above. The protocol for the radioimmunoassay was as follows. The testosterone standards (1 to 200 pg/tube) and the unknown samples were diluted in glass tubes, with PBS-gel to give a final volume of 500µl. 100µl of ^{125}I-testosterone-3-carboxymethyloxime-histamine (diluted

to 20,000 counts per 100 second), and 200µl of antiserum (S505) at an initial dilution of 1:700,000 were added to each tube and the assay incubated at room temperature for 2 hours. The bound and free fractions were separated by the addition of 100µl of NRS at an initial dilution of 1:400 and 100µl of DARS at an initial dilution of 1:25, using assay buffer containing 10% 0.1M EDTA, incubation overnight at 4°C, the addition of 2ml of ice-cold PBS-gel and centrifugation at 800g for 30 minutes at 4°C. The activity in the resultant pellets were counted on an automatic gamma counter (LKB Ltd.).

2.3.7. GnRH receptors

The concentration of GnRH receptors was assessed using the method of Bramley, Menzies and Baird (1985). Sections of pituitary were homogenised in approximately 10ml/ g of ice cold SET medium (0.3M Sucrose-1mM EDTA-10mM Tris-HCl, pH 7.4). All procedures, where possible were completed on ice, to minimize degradation and dissociation of the receptor-tracer complex. Triplicate aliquots of the pituitary homogenates were incubated for 4 h hours at 0°C in 1ml of 0.5% BSA-40mM Tris-HCl, pH 7.4 containing 100,000 counts per 60 seconds of radiolabelled GnRH agonist ($GnRH_A$). 0.5ml of ice-cold 0.5% bovine γ-globulin in 40mM Tris-HCl, pH 7.4 and 1ml of ice cold polyethylene glycol (8,000 mol wt; 25g/100ml 40mM Tris-HCl, pH 7.4) were then added to the tubes before vigorous mixing and centrifugation at 2,500g for 10 minutes at 4°C. The supernat nt was then discarded and the activity in the resulting pellet counted on a gamma counter. Nonspecific binding (NSB) was assessed in the presence of 10µg of unlabelled $GnRH_A$. Specific binding was calculated by subtracting the NSB value from the activity bound in the samples containing only labelled hormone but no unlabelled hormone. The protein content of the samples was determined using the Lowry protein assay (Lowry, Rosebrough, Farr and Randall, 1951)

and the results expressed as the amount (pg) of $GnRH_A$ specifically bound/mg of protein.

2.4. Pituitary cell culture

Pituitary cells were cultured following the method of Tsonis, McNeilly and Baird (1986), with modifications. Pituitary glands were recovered from sheep/lamb heads immediately after slaughter and transferred to sterile plastic universal containers (Sterilin, Feltham, Middx) containing approximately 10 ml of warm (37°C) Dulbecco's phosphate-buffered saline (DPBS; Imperial Laboratories) containing 7.5mM glucose (Sigma). The pituitary glands were removed from the DPBS, placed on a sterile siliconized glass plate and dissected free of dura mata and extra-pituitary tissue, using a sterile scalpel. The pituitary gland was weighed and chopped into 3-4mm blocks. The blocks of tissue were transferred to a fresh universal container and washed 6-8 times with DPBS containing 7.5mM glucose and 0.1% BSA (Fraction V; Sigma), to remove damaged cells from the tissue preparation. The pituitary blocks were then placed in 25ml spinner flasks (Bellco Biotechnology; Arnold R Horwell Ltd, Laboratory and Clinical supplies) with 10ml 0.5% trypsin (Type III; Sigma), made up in DPBS with 0.1% BSA, and stirred gently for 30 minutes at 37°C in a gased incubator (5% CO_2, 95% air). The enzyme solution was removed and the tissue was washed and replaced with 10ml of Dulbecco's Modified Eagles Medium with 20mM Hepes containing the following supplements (supplemented DMEM): 10% lamb serum, 2.5% fetal bovine serum (Flow, Laboratories), 10mM $NaHCO_3$, 2mM glutamine (Sigma), penicillin (50i.u./ml) and streptomycin (50µg/ml; Flow laboratories), and then stirred for a further 30 minutes. The supplemented DMEM was removed and replaced with DPBS without Ca^{2+} and Mg^{2+}(DPBS^{--}, Imperial Laboratories), containing 2mM EDTA (Sigma), but supplemented with 0.1% BSA and

stirred for a further 10 minutes. The tissue was then washed 3-4 times with DPBS^{--} and transferred to a sterile 20ml universal container. The remaining tissue was dispersed in 3-5ml of DPBS^{--} using three sterile siliconized, flame-treated Pasteur pipettes of progressively decreasing diameter (5 to 2 mm). The cell suspension was removed and transferred to a new sterile universal, supplemented DMEM was added to the remaining tissue and the dispersion procedure repeated until no further free cells were obtained. The cell suspension was made up to 20 ml with supplemented DMEM and centrifuged twice at 500g for 10 minutes. The supernatant was removed each time and stored at 37°C in the gased incubator, while the pellet was resuspended in supplemented DMEM. Following resuspension of the cell pellet, undispersed (collagenous) tissue, clumps of cells and any debris from damaged cells (DNA) which settled at unit gravity was removed and discarded. The supernatants were stored as they contain cells which can be harvested if cell numbers are low. Following the final resuspension an aliquot was removed for counting in a haemocytometer and viability estimated by a trypan blue (Sigma) exclusion test. The concentrations of the pituitary cell suspensions were adjusted to give a final concentration of approximately 200,000 cells/50µl.

550µl of supplemented DMEM was aliquoted into 24-well culture plates (Flow Laboratories Ltd, Irvine) and the plates stored in a gased incubator (37°C, 5% CO_2: 95% air). 50µl of the cell suspension was then added to each well. The plates were then incubated for 24 hours, before further treatment. During this twenty four hour period the cells sink to the bottom of the culture wells and attach to the surface of the plates. Thereafter the media was removed and replaced with supplemented DMEM which contained various secretogogues (see Chapter 9 for further details). The cells were then incubated for a further 3 hours, before removal and storage at -20°C, to await assay of LH.

CHAPTER 3. - Pituitary responsiveness to GnRH stimulation and peripheral gonadotrophin and steroid concentrations in adult ewes during the oestrous cycle.

3.1. Introduction

One of the premises of the initial selection programme was that peripheral tonic gonadotrophin concentrations, LH in particular, were positively correlated with adult female reproductive performance (Thimonier, Pelletier and Land, 1972; Carr and Land, 1975; section 1.5.3.2.). Studies of the reproductive performance of ewes from the High and Low GnRH selection lines, have indicated that High line females reach puberty significantly earlier than the Low line and that their ovulation rate during the first and at the start of the second breeding season was significantly greater than that measured in animals from the Low line. In general, litter size during the first breeding season was also greater in animals from the High line; however, the between-line difference in litter size was not statistically significant, and was not maintained during the second breeding season (Haley *et al.*, 1989). During the selection programme, no selection was practiced on the female lambs; however when challenged with a 5μg dose of GnRH, the mean peak LH response in the High line ewe lambs was significantly greater than the Low line lambs at both 10 (High, 31.11ng/ml; Low, 14.18ng/ml) and 20 (High, 46.32ng/ml; Low, 16.40ng/ml) weeks of age (Haley *et al.*, 1989). The size of the between-line difference however, was smaller in female lambs than in contemporary male lambs, the High to Low ratio for the female versus male lambs being, 2.19 v 3.06 and 2.83 v 3.53 at 10 and 20 weeks of age respectively.

The aims of this study were firstly, to determine if the between-line difference in the selected character is maintained in the adult ewes and secondly, to measure peripheral gonadotrophin and steroid concentrations in ewes from the High and Low selection lines during the luteal and follicular phases of the oestrous cycle, to determine whether selection has altered the regulation of endogenous gonadotrophin secretion.

3.2. Materials and methods

3.2.1. Experimental protocol

Thirty 1- to 2-year old ewes of proven prolificacy (15 from each line), were synchronised prior to the start of the experiment by treatment for 14 days with intravaginal progesterone impregnated sponges (Veramix, Intervet). Following sponge removal, the ewes were run with a raddled vasectomised ram to detect oestrus (Day 0), and studied during the middle of the following luteal phase (Day 9) and during an artificial follicular phase (Day -1/-2), induced on Day 12 by an intramuscular injection of PGF2α. On the day prior to study (luteal- Day 8; follicular- Day 12), the ewes were each fitted with an indwelling jugular cannula (section 2.2.1) and placed in individual metabolism crates. During the experimental periods, the ewes were fed standard concentrates and hay and had access to water ad libitum. At the end of each sampling period, the ewes were decannulated, treated with a long-acting antibiotic (Duphapen LA) and returned to the flock.

To characterise the peripheral gonadotrophin and steroid concentrations in the adult ewes from the High and Low selection lines, the ewes from both lines were serially blood sampled (every 10 minutes for 6 hours) during the luteal and follicular phases of the oestrous cycle. The induction of an adequate follicular phase, following prostaglandin treatment, was confirmed following the follicular phase experimental

period, by 6-hourly checks for oestrous behaviour and the collection of 6-hourly blood samples, which were later assayed for progesterone.

To determine if the between-line difference in the LH response to a GnRH challenge was maintained in the adult ewes, the ewes from each line were allocated at random to three treatment groups:- Group 1 (n=6), GnRH challenge during the luteal phase (5μg of GnRH, made up to 2ml with physiological saline); Group 2 (n=6), GnRH challenge during the follicular phase; Group 3 (n=3) saline on both occasions. Following the serial bleeds during both the luteal and the follicular phases of the oestrous cycle, the ewes from each line received a bolus intravenous injection of either GnRH, or saline, depending on the treatment group. The ewes from Groups 1 and 2, which were not treated with GnRH, were treated as for Group 3; therefore, during each phase of the oestrous cycle, 6 ewes from each line received a GnRH challenge and 9 ewes from each line acted as saline-treated controls. Blood samples were collected 30, 50 and 70 minutes after each GnRH/saline challenge to characterise the gonadotrophin responses.

3.2.2. Hormone assays

The mean sensitivity of the LH assay over 10 assays was 0.06 ± 0.08 ng/ml; the inter- and intra-assay coefficients of variation were 10.58% and 4.39% respectively. The mean sensitivity of the FSH assay over 6 assays was 0.25ng/ml with inter- and intra-assay coefficients of variation of 12.84% and 6.5% respectively. Progesterone was measured in a single radioimmunoassay following solvent extraction of the samples. The mean extraction efficiency was 74%, the minimum detectable dose was 0.22ng/ml and the intra-assay coefficient of variation was 8.5%. The oestradiol concentrations were assessed in two assays with a mean extraction

efficiency of 71%, a minimum detectable dose of 0.75pg/ml and inter- and intra-assay coefficients of variation of 5.6% and 6.03% respectively.

3.2.3. Statistical analysis

No significant increases were seen in the peripheral LH and FSH concentrations in the control animals following the GnRH administration. Therefore the differences in the gonadotrophin responses, where appropriate, were compared by analysis of variance.

The LH concentrations during the luteal and follicular phases of the oestrous cycle were initially expressed as the mean LH concentration observed during the six hour sampling period. However as LH secretion is pulsatile, the LH secretion profiles were split into their three component parts:- 1) basal LH concentration, 2) LH pulse frequency and 3) LH pulse amplitude. LH pulses were defined as described by Webb *et al.*, (1985a). The basal LH concentrations in the animals from both lines exhibited similar degrees of variance and had approximately normal distributions; therefore analysis was conducted on the arithmetic scale. The LH characteristics however, were log transformed, to equalise within line variance and the two lines and the two phases of the oestrous cycle compared by analysis of variance and two sample 't' tests.

The mean peripheral FSH concentrations were measured in each animal during the luteal and follicular phases of the oestrous cycle as the average of 7× hourly samples. The mean FSH concentrations were calculated for each line, log transformed to equalise variance and compared between lines and between sampling periods, by analysis of variance and two sample 't' tests.

The mean oestradiol concentrations were compared between the two lines and the two sampling periods using Student's 't' tests. Similar analysis

was conducted on the mean progesterone concentrations, following log transformation of the data, to equalise within-line variation.

3.3. Results

3.3.1. LH response to 5μg of GnRH

Following the administration of exogenous GnRH, an increase was observed in the peripheral LH concentrations of all but one animal (Low line, follicular phase). The mean LH responses for each of the GnRH treated groups are presented in Table 3.1.

Table 3.1. The mean LH response (ng/ml ± s.e.m) in High and Low line ewes during the luteal and follicular phases of the oestrous cycle

	LUTEAL		FOLLICULAR	
TIME (mins)	High line (n=6)	Low line (n=6)	High line (n=6)	Low line (n=6)
+30	18.27 ± 9.29	5.44 ±0.79	14.28 ± 2.14[a]	7.13 ± 1.11[b]
+50	13.38 ± 6.48	6.28 ± 1.21	23.50 ± 3.73[a]	13.54 ± 4.93[b]
+70	9.31 ± 3.98	8.19 ± 2.29	39.86 ± 6.78[a]	16.09 ± 3.98[b]

Within rows, significant differences ($P < 0.05$) are indicated by different superscripts; a versus b.

Following GnRH treatment during the follicular phase, a gradual increase was seen in the peripheral LH concentration in all but one ewe (which exhibited no LH response), with the maximum LH concentration occurring in both lines in the +70 minute sample. This value was therefore taken as the peak LH response and compared between the two lines. Despite considerable within-line variation, the magnitude of the LH response differed significantly ($P < 0.05$) between the two lines, the LH response in

the High line animals was 2.5⁻ fold greater than the average response measured in the Low line animals.

Following the GnRH challenge in the luteal phase, the maximum LH concentration measured in all of the High line ewes occurred at T+30, the LH concentration decreasing over the remainder of the sampling period. In the Low line ewes, the pattern of LH release was not as consistent, with peripheral LH concentrations increasing over the sampling period in four of the ewes, whilst in the remaining two ewes, the pattern of LH secretion was similar to that seen in the High line, the peripheral LH concentration decreasing over the sampling period. Due to the difference in the LH profiles both within and between lines, it was not statistically correct to compare the magnitude of the LH responses between the two lines.

3.3.2. FSH response to 5μg of GnRH

GnRH stimulated the secretion of increased concentrations of FSH during both the luteal and follicular phases of the oestrous cycle; however the FSH responses were not as great as the LH responses, Table 3.2.

Table 3.2. The mean FSH response (ng/ml ± s.e.m) in High and Low line ewes during the luteal and follicular phases of the oestrous cycle

	LUTEAL		FOLLICULAR	
TIME (mins)	High line (n=6)	Low line (n=6)	High line (n=6)	Low line (n=6)
+30	3.78 ± 0.68	2.89 ± 0.54	1.83 ± 0.11	2.10 ± 0.51
+50	3.32 ± 0.66	3.54 ± 0.73	2.02 ± 0.66	2.30 ± 0.18
+70	3.38 ± 0.60	3.42 ± 0.68	2.61 ± 0.28	2.26 ± 0.41

The FSH response profiles in the High line ewes during both phases of the oestrous cycle were similar to the patterns of LH release. The concentration of FSH in the peripheral circulation decreased during the post-challenge sampling period in the luteal phase but increased between T+30 and T+70 during the follicular phase. In the Low line ewes, the FSH response profiles differed from the LH response profiles, during both the luteal and follicular phases of the oestrous cycle, the peripheral FSH concentration peaking in the T+50 sample on both occasions.

As the FSH response profiles differed between the two lines during the two phases of the oestrous cycle, comparisons of the magnitudes of the FSH responses are not statistically accurate. However, it would appear that the amount of FSH released during the first 70 minutes after the GnRH challenge was greater in both lines during the luteal phase than the follicular phase, and was similar between the two lines during both phases of the oestrous cycle.

3.3.3. Mean gonadotrophin concentrations

The mean gonadotrophin concentrations in the ewes from the High and Low selection lines, during the luteal and follicular phases of the oestrous cycle are presented in Table 3.3.

The mean concentrations of LH were significantly greater during the follicular phase, than the luteal phase in the ewes from both lines (High $P < 0.001$; Low $P < 0.005$). The mean FSH concentrations were significantly greater ($P < 0.05$) during the luteal phase than during the follicular phase in the ewes from both lines.

No significant between-line differences were seen in the mean gonadotrophin concentrations during either the luteal or the follicular phases of the oestrous cycle. However on all occasions, the mean

gonadotrophin concentrations in the High line ewes were greater than those measured in the Low line ewes.

Table 3.3. Mean LH and FSH (ng/ml ± s.e.m) concentrations in High and Low line ewes during the luteal and follicular phases of the oestrous cycle

	LH (ng/ml)		FSH (ng/ml)	
	Luteal	Follicular	Luteal	Follicular
High line (n=15)	0.74 ± 0.07^{a}	1.24 ± 0.08^{b}	2.17 ± 0.24^{e}	1.55 ± 0.19^{f}
Low line (n=15)	0.64 ± 0.08^{c}	1.09 ± 0.11^{d}	2.03 ± 0.27^{e}	1.33 ± 0.13^{f}

Within rows, significant differences are indicated by different superscripts: a versus b, $P < 0.001$; c versus d, $P < 0.005$; e versus f, $P < 0.05$.

3.3.4. Basal LH secretion

The mean LH concentration in the samples taken prior to the observation of pulses of LH secretion was calculated for each animal and meaned for each line to provide an estimate of the mean basal LH concentration (Table 3.4.).

Table 3.4. - The mean basal LH concentrations (ng/ml ± s.e.m.) in the High and Low selection lines, during the luteal and follicular phases of the oestrous cycle.

	LUTEAL	FOLLICULAR
High line (n=15)	0.57 ± 0.07^{a}	0.81 ± 0.08^{b}
Low line (n=15)	0.48 ± 0.07^{c}	0.75 ± 0.08^{d}

Within rows different superscripts indicate significant differences; a versus b, $P < 0.05$; c versus d, $P < 0.01$.

The mean basal LH concentration increased significantly between the luteal and follicular phases of the oestrous cycle, in both the High ($P < 0.05$) and Low line ewes ($P < 0.01$).

Although mean basal LH concentrations were consistently higher in the High line ewes, between-line comparisons failed to demonstrate a statistically significant difference between the two lines during either the luteal, or the follicular phases of the oestrous cycle.

3.3.5. LH pulse frequency

LH pulse frequency was defined as the number of pulses observed during each of the 6-hour sampling periods. In the ewes from both lines the LH pulse frequency was significantly greater ($P < 0.001$) during the follicular phase than the luteal phase (Table 3.5).

Table 3.5. Mean number of LH pulses per 6 hours (± s.e.m) in High and Low line ewes, during both the luteal and follicular phases of the oestrous cycle.

	LUTEAL PHASE	FOLLICULAR PHASE
High line (n=15)	1.67 ± 0.25^a	6.53 ± 0.26^b
Low line (n=15)	1.47 ± 0.13^a	6.33 ± 0.35^b

Within rows different superscripts indicate significant differences ($P < 0.001$).

The number of pulses observed during the luteal or follicular phases of the oestrous cycle did not differ significantly between the two selection lines, although the High line animals tended to have a slightly higher LH pulse frequency than the Low line animals, during both phases of the oestrous cycle.

3.3.6. LH pulse amplitude

The mean LH pulse amplitudes for the two selection lines during the luteal and follicular phases of the oestrous cycle, are presented in Table 3.6.

Table 3.6. Mean (± s.e.m) LH pulse amplitude (ng/ml), during the luteal and follicular phases of the oestrous cycle.

	LUTEAL PHASE	FOLLICULAR PHASE
High line (n=15)	1.26 ± 0.19	1.31 ± 0.12[a]
Low line (n=15)	1.31 ± 0.16[a]	0.88 ± 0.12[b]

Within rows and within columns different superscripts indicate significant differences ($P < 0.05$).

Differences were observed in mean LH pulse amplitude between the two phases of the oestrous cycle. The changes however were not the same in the ewes from the two selection lines. In the Low line ewes, LH pulse amplitude decreased significantly ($P < 0.05$) between the luteal and follicular phases of the oestrous cycle. However in the High line ewes, no significant difference existed between the mean LH pulse amplitude measured during the luteal and follicular phases of the oestrous cycle.

Statistical analysis of the mean LH pulse amplitudes of the High and Low line ewes during the luteal and follicular phases of the oestrous cycle demonstrated that although there was no significant difference between the two lines during the luteal phase, during the follicular phase the High line ewes secreted LH pulses of significantly greater amplitude ($P < 0.05$) than the ewes from the Low Line.

3.3.7. Steroids

The mean oestradiol and geometric mean progesterone concentrations were calculated for the two selection lines and the results are presented in Table 3.7.

Table 3.7. Mean (± s.e.m) steroid concentrations in High and Low line ewes during the luteal and follicular phases of the oestrous cycle.

	OESTRADIOL (pg/ml)		PROGESTERONE (ng/ml)	
	Luteal	Follicular	Luteal	Follicular
High line (n=15)	1.59 ± 0.45^{a}	3.92 ± 0.44^{b}	4.71 ± 0.63^{a}	0.96 ± 0.13^{b}
Low line (n=15)	1.68 ± 0.47^{c}	3.24 ± 0.56^{d}	4.64 ± 0.36^{a}	0.71 ± 0.05^{b}

Within rows different superscripts indicate significant differences; a versus b, $P < 0.001$; c versus d, $P < 0.05$.

The mean oestradiol concentration increased significantly between the luteal and follicular phases of the oestrous cycle in both selection lines (High, $P < 0.001$; Low, $P < 0.05$). However, the converse was true for the mean progesterone concentrations which were significantly higher ($P < 0.001$) in both lines, during the luteal phase than the follicular phase.

Between-line comparisons did not reveal any statistically significant between-line differences in the concentrations of either oestradiol, or progesterone, during either phases of the oestrous cycle.

3.4. Discussion

The main findings of this study were that , A), High line ewes released significantly more LH than Low line ewes, in response to a GnRH challenge during the follicular phase and that the between-line difference

was of similar magnitude to that measured in prepubertal female lambs (Haley *et al.*, 1989). During the luteal phase the LH response profiles following a GnRH challenge differed between the ewes from the two lines. B), The mean gonadotrophin and steroid concentrations did not differ significantly between the two selection lines during either the luteal or the follicular phases of the oestrous cycle and C), That during the follicular phase, the ewes from the High line secreted LH pulses of significantly greater amplitude than ewes from the Low line.

During the luteal phase, the LH response profiles following a GnRH challenge differed between the animals from the two lines. In the High line, the maximum LH concentration occurred either at or prior to the T+30 sample, whilst in the Low line the maximum LH concentration occurred either at or after the T+70 sample; therefore it was not statistically correct to compare the magnitudes of the LH responses measured. During the follicular phase the pattern of LH secretion following the administration of a 5μg dose of GnRH was similar in both lines. However the High line ewes released significantly more LH than the Low line ewes. Interestingly the magnitude of the between-line difference in the adult ewes (High:Low, 2.48) was similar to that observed in prepubertal female lambs (2.19 and 2.83, at 10 and 20 weeks of age respectively) (Haley *et al.*, 1989). Therefore the results of the GnRH challenge during the follicular phase demonstrate that the between-line difference in the selected character is maintained in adult ewes at a similar level to that seen in prepubertal ewe lambs.

The pattern of LH secretion following exposure to GnRH is affected by a number of physiological factors, including steroid negative feedback (Section 1.3.4.1.) and the immediate history of the pituitary gland with regard to GnRH exposure (Section 1.3.2.1.), both of which are known to vary between the luteal and follicular phases of the oestrous cycle. During

the luteal phase, due to the increased sensitivity of the hypothalamo/pituitary gland complex to steroid negative feedback and the high concentration of progesterone in the peripheral circulation, the frequency of GnRH pulses is low and peripheral LH concentrations are also low (Cumming *et al.*, 1972, 1973; Clarke and Cummins, 1984, 1985b; Clarke, 1987, 1988; Kaynard *et al.*, 1988; Gregg and Nett, 1989; Herman and Adams, 1990; Moenter *et al.*, 1990). During the follicular phase, due to a reduction in the sensitivity of the hypothalamus to steroid negative feedback (Karsch *et al.*, 1984) and a reduction in the peripheral progesterone concentration, GnRH and therefore LH pulse frequency increases (Baird *et al.*, 1976; Baird, 1978a; Wallace *et al.*, 1988).

The decrease in steroid negative feedback and the increased exposure of the pituitary gland to GnRH during the follicular phase stimulates a number of events at the level of the pituitary gonadotroph , which result in mobilization of intracellular stores of LH and an increase in the number of active GnRH - receptors on the exterior of the gonadotroph. These changes are collectively known as 'GnRH priming' and result in the secretion of increased amounts of LH in response to further GnRH stimulation. The priming of the pituitary gland during the follicular phase combined with the increase in GnRH secretion are important in the production of the surge of LH secretion which occurs prior to ovulation (Crighton and Foster, 1976, 1977; Stelmasiak and Galloway, 1977; section 1.3.3.2), and may provide an explanation for the differences in the LH response profiles following the GnRH challenges in the luteal and follicular phases of the oestrous cycle. During the follicular phase, due to the short endogenous interpulse interval, the pituitary glands of the ewes from both lines are 'primed' and therefore when exposed to a large dose of GnRH, the stimulated LH response profile is similar to that

observed during an LH surge. During the luteal phase, as the pituitary gland is in an 'unprimed state', exposure to exogenous GnRH should stimulate the secretion of a discrete pulse of LH, similar to that seen endogenously during the luteal phase or when prepubertal lambs (male or female) are challenged with GnRH. The High line ewes responded to the GnRH challenge as expected, the highest LH concentration occurring in the T+30 sample and decreasing thereafter. In the Low line ewes however, the LH response in the majority of the animals (4 of 6) increased over the duration of the sampling period. As neither the peripheral steroid concentrations nor the patterns of gonadotrophin secretion prior to the GnRH challenge differed significantly between the two lines, the results suggest that the pituitary glands of the Low line ewes are less responsive to GnRH stimulation than the pituitary glands of the High line ewes. Therefore when ewes from the Low line were challenged with a 5μg dose of GnRH, this was either sufficient to drive the pituitary gland into a surge type of LH release, or to activate sufficient GnRH receptors to release the readily-releasable and releasable pools of LH and to continually release newly-synthesised LH, thereby creating a gradual increase in the concentration of LH measurable in the peripheral circulation.

GnRH exposure stimulated the release of FSH from the ewes in both selection lines, during both the luteal and follicular phases of the oestrous cycle. However as the FSH response profiles differed between the two lines during both the luteal and follicular phases of the oestrous cycle, it was not possible to conclude if the between-line difference in the FSH response to a GnRH challenge observed in prepubertal lambs was maintained in the adult ewes.

The changes observed in the mean gonadotrophin and steroid concentrations between the luteal and follicular phases of the oestrous

cycle in the ewes in this study, were similar to those previously documented (L'Hermite *et al.*, 1972; Salamonsen *et al.*, 1973; Baird and Scarramuzzi, 1976; Hauger *et al.*, 1977; Pant *et al.*, 1977; Goodman *et al*, 1981c; Fraser *et al.*, 1981; Baird and McNeilly, 1981; McLeod *et al.*, 1982; Platt *et al.*, 1983; Clarke *et al.*, 1983; Karsch *et al.*, 1983; Wallace *et al.*, 1988; McNeilly *et al.*, 1988), the mean concentrations of FSH and progesterone decreasing between the luteal and follicular phases of the oestrous cycle, while the mean LH and oestradiol concentrations increased between the two phases of the oestrous cycle. No differences were seen between the mean gonadotrophin or steroid concentrations of the High and Low line ewes, during either phase of the oestrous cycle.

As LH secretion is pulsatile, the LH secretion profiles were divided into their component parts for analysis. As described earlier, LH pulse frequency is greatly influenced by the concentration of progesterone in the peripheral circulation. This relationship was also apparent in the present study, as a significant increase occurred in both the mean LH concentration and LH pulse frequency between the luteal and follicular phases of the oestrous cycle, coincident with a significant decrease in peripheral progesterone concentrations. In conjunction with the increase in LH pulse frequency, an increase also occurred in the basal LH concentration. This increase occurs due to the high frequency of LH pulses secreted during the follicular phase, the peripheral LH concentration following an LH pulse never decaying to the true LH baseline before the occurrence of the following pulse of LH secretion. As with the mean LH concentrations, although no significant between-line differences were seen in either the basal LH concentrations or LH pulse frequency during either the luteal or the follicular phases of the oestrous cycle, ewes from

the High Line had a consistently higher basal LH concentration and a consistently higher LH pulse frequency than ewes from the Low line.

During the luteal phase, the difference in the mean LH pulse amplitude between the ewes from the High and Low selection lines was not statistically significant. The slightly lower mean LH pulse amplitude in the High line ewes may possibly reflect the slightly increased LH pulse frequency seen in the High line ewes relative to the Low line during the luteal phase. This proposal is based on the inverse relationship between LH pulse amplitude and LH pulse frequency (Bindon and Turner, 1974; Foster *et al.*, 1975b) and assumes that the pool of LH available for release is similar in the ewes from both lines. As LH pulse amplitude and LH pulse frequency are inversely related, the increase in LH pulse frequency which occurs following the transition between the luteal and the follicular phase is normally accompanied by a decrease in LH pulse amplitude (Baird and Scaramuzzi, 1976; Karsch *et al.*, 1983, 1984). In this study, the animals from the Low line conformed to this pattern of LH secretion. However, in the High line, LH pulse amplitude did not change significantly between the luteal and follicular phases of the cycle, increasing slightly in the follicular phase relative to that measured in the luteal phase. When compared during the follicular phase, LH pulse amplitude in the High line ewes was significantly greater than that measured in the Low line ewes and indicates that as well as the difference in the ability of the two lines to respond to a GnRH challenge, between-line differences also exist in the regulation of endogenous LH secretion. As the mean steroid concentrations did not differ significantly between the ewes from the two selection lines during the follicular phase, the results suggest that the between-line difference in the regulation of endogenous gonadotrophin secretion occurs at the level of the hypothalamus/pituitary gland. However, as the

difference in LH pulse amplitude was only seen during the follicular phase, it would appear the effects of this difference are influenced by steroid negative feedback, the between-line difference in LH pulse amplitude being masked during periods when the effects of steroid negative feedback are high but observable during periods when steroid negative feedback is reduced i.e. the follicular phase.

The results of this study do not locate the exact site of the between-line difference in the regulatory control of LH pulse amplitude. However the between-line differences in the secretion patterns of LH and FSH could provide a possible mechanism at the pituitary gland itself which could result in the between-line difference in LH pulse amplitude. The mean concentrations of both LH and FSH were higher in the ewes from the High line than the Low line and a similar relationship was also seen with LH pulse frequency. As gonadotrophins, LH in particular, are secreted in response to pulses of GnRH secretion, these differences in the patterns of GnRH secretion may be sufficient to stimulate increased pituitary priming in the High line ewes and therefore to stimulate the production of LH pulses of greater amplitude in the High line ewes compared to those seen in the Low line. Alternatively, differences may exist in either the structure and function of the pituitary gland, the amount of GnRH secreted by the hypothalamus, or the responsiveness of the hypothalamo/pituitary gland complex to steroid feedback.

In summary, the between-line difference in the magnitude of the LH response to a 5μg GnRH challenge was maintained in the adult ewes during the follicular phase, and at a similar level to that seen in prepubertal female lambs. During the luteal phase, the gonadotrophin response profiles following the GnRH challenge differed between ewes from the High and Low selection lines, and therefore indicate that further between-line

differences may also be present in the regulation of gonadotrophin secretion in response to a bolus injection of GnRH.

Studies of the mean steroid and gonadotrophin concentrations during the luteal and follicular phases of the oestrous cycle did not reveal the presence of any significant between-line differences. However, when the LH secretion profiles were split and analysed in their separate components it was noted that during the follicular phase, the High line ewes secreted LH pulses of significantly greater amplitude than the Low line. Therefore in the adult ewes, as well as the between-line difference in the selected character, significant between-line differences also exist in the regulation of endogenous gonadotrophin secretion, and it would appear that these differences occur at the level of the hypothalamo/pituitary complex. However, the effects of this between-line difference can be masked by steroid negative feedback and are therefore only observed during the follicular phase of the oestrous cycle, when steroid feedback is reduced.

In conclusion, the effects of selection on the LH response to a GnRH challenge in 10-week old ram lambs are not limited by either sex or age, the selected character being altered in related adult ewes. In the adult ewes a between line difference also exists in the regulation of endogenous LH secretion, with High line ewes secreting LH pulses of significantly greater amplitude than Low line ewes during the follicular phase. This difference in LH pulse amplitude occurs in the absence of significant differences in the mean steroid concentrations and therefore appears to be located at the hypothalamo/pituitary gland complex; however its expression is influenced by steroid negative feedback.

CHAPTER 4. - LH and FSH secretion profiles in ram lambs from the two selection lines, between 2 and 10 weeks of age.

4.1. Introduction

Divergent selection based on the LH response to a 5μg dose of GnRH in 10-week old ram lambs has created two lines of sheep which differ physiologically in i) the ability of the 10 and 20 week old male and female lambs from the two selection lines, to release LH and FSH in response to a GnRH challenge (Haley *et al.*, 1989), ii) the ability of the adult ewes from the two lines to release LH in response to a GnRH challenge (Chapter 3) and iii) the regulation of endogenous LH secretion in adult ewes. Specifically, LH pulse amplitude during the follicular phase which was significantly ($P < 0.05$) greater in the High line ewes, than the Low line (Chapter 3).

LH and FSH are measurable in the peripheral circulation of ram lambs from at least 2 weeks of age (Lee *et al.*, 1976a) and age-related changes have been reported in the secretion patterns of both gonadotrophins between birth and puberty. These physiological changes regulate the physical changes which occur during the prepubertal period and are required for the attainment of puberty. In Spring-born ram lambs the mean concentrations of LH and FSH increase between birth and approximately 7-12 weeks of age, before decreasing transiently. Following this decrease, the peripheral LH concentration remains low until puberty is reached; however the peripheral FSH concentration increases gradually (Lee *et al.*, 1976a; Yarney and Sanford, 1985a, 1985b; Olster and Foster, 1986; Claypool and Foster, 1990). The changes which occur in the mean LH concentrations occur due to changes in the frequency and amplitude of the LH pulses secreted.

The aim of this study was to determine whether the difference in the ability of ram lambs from the two lines to release gonadotrophins in response to a GnRH challenge, as in the adult ewes, is accompanied by differences in the regulation of endogenous gonadotrophin secretion.

4.2. Materials and Methods

4.2.1. Experimental protocol

40 April born entire ram lambs, 20 from each line, were serially blood sampled at 2, 6 and 10 weeks of age. On the morning of blood sampling the animals were housed and cannulated as described in section 2.2.1. The animals were then allowed to settle for 2 hours before the start of the experiment. At each age, the lambs were serially blood sampled at 10 minute intervals for 6 hours. Following each sampling period the animals were decannulated, treated with a long-acting antibiotic (Duphapen LA; Duphar Veterinary Limited, Southampton) and returned to the flock.

4.2.2. Hormone assays

The FSH concentrations were measured in two assays, with a mean detection limit of 1.24ng/ml and inter- and intra-assay coefficients of variation of 10.1 and 6.5% respectively.

LH concentrations were measured over 16 assays, with an average minimum sensitivity of 0.42 ± 0.04ng/ml and inter- and intra-assay coefficients of variation of 8.3% and 6.8% respectively.

4.3. Results

4.3.1. FSH

As FSH secretion is not pulsatile, the FSH concentration was measured at hourly intervals in each animal during each sampling period

(6 samples/animal/age). The FSH concentrations were then meaned per animal and the mean concentration per line per age calculated. The data was log-transformed to equalise between-line variance and all subsequent analyses were conducted on the geometric scale. The results for each line are presented graphically in Figure 4.1. as geometric mean FSH concentrations with approximate standard errors calculated on the arithmetic scale.

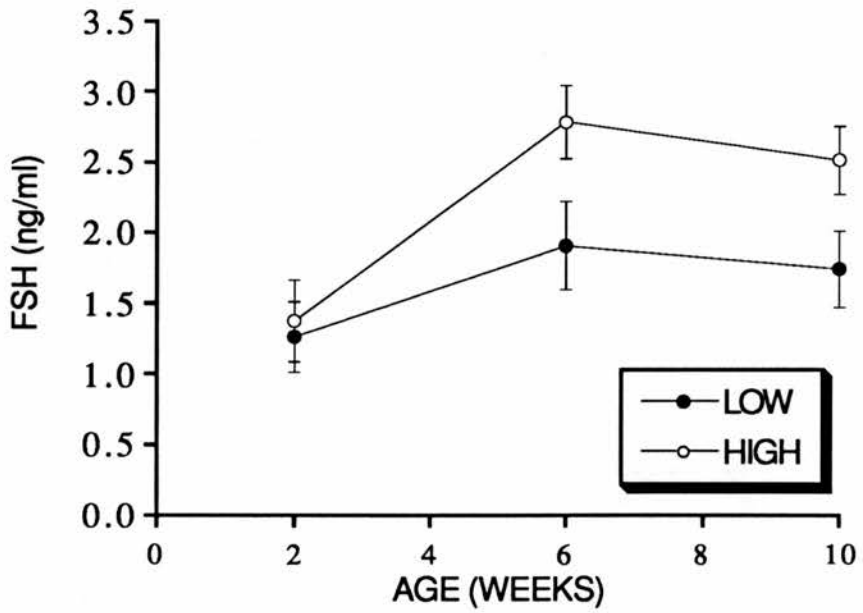

Figure 4.1. The geometric mean FSH concentrations (ng/ml ± s.e.m) in entire ram lambs from the High and Low GnRH selection lines, at 2, 6 and 10 weeks of age.

A significant (Low, $P < 0.05$; High, $P < 0.001$) increase occurred in the mean FSH concentrations of the lambs from both selection lines between 2 and 6 weeks of age. Between 6 and 10 weeks of age, the peripheral FSH

concentrations declined in both lines; however the changes observed were not statistically significant in either line.

The mean peripheral FSH concentrations were higher in the High line lambs than the Low line lambs at all of the ages tested; however these differences were only statistically significant at 6 and 10 weeks of age ($P < 0.05$). The proportional difference between the two lines at 6 and 10 weeks of age was relatively constant, the High line secreting 1.45 fold more FSH than the Low line.

4.3.2. LH

The concentration of LH was measured in all samples and the LH data analysed as the mean LH concentration over the whole of the sampling period, mean LH pulse frequency and mean LH pulse amplitude.

4.3.2.1. Mean LH concentrations

The LH concentrations were measured in all of the samples collected from each animal, at each age. The LH concentrations were averaged for each animal at each age and the mean LH concentrations, per line, per age calculated. The mean LH concentrations were log-transformed to equalise the between-line variance and all subsequent analysis was conducted on the geometric scale. The geometric mean LH concentrations, with approximate standard errors calculated on the arithmetic scale, are presented in Figure 4.2.

The mean LH concentration increased significantly ($P < 0.001$) in both High and Low line lambs between 2 and 6 weeks of age. Between 6 and 10 weeks of age, a further increase was seen in the peripheral LH concentrations; however this increase was not statistically significant in either line.

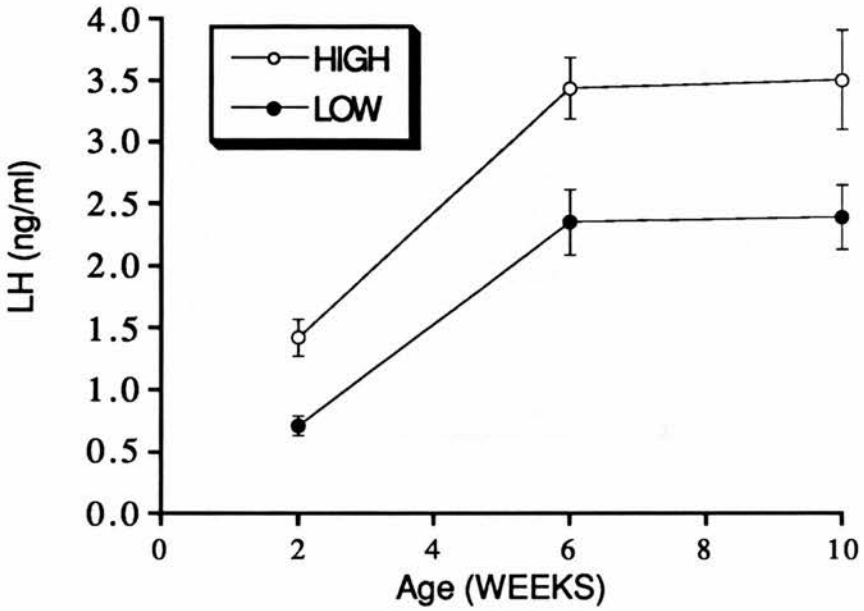

Figure 4.2. The geometric mean LH concentrations (ng/ml ± s.e.m) in High and Low line lambs at 2, 6 and 10 weeks of age.

When compared between lines, the High line lambs secreted significantly more LH than the low line, at all ages tested (2 and 10 weeks, $P < 0.001$; 6 weeks, $P < 0.05$). At two weeks of age, the mean LH concentration in the High line lambs was double that seen in the Low line; however the High:Low $^{LH}_{\lambda}$ ratio decreased at 6 and 10 weeks of age, to 1.46 and 1.58 respectively.

4.3.2.2. LH pulse frequency

LH pulses were identified using the Munro Hormone pulse profile analysis programme (Zaristow Software, Haddington, East Lothian). LH pulse frequency was defined as the number of pulses observed during each 6 hour sampling period. The data was analysed on the arithmetic scale and the mean LH pulse frequencies are presented in Table 4.1.

Table 4.1. Mean LH pulse frequency (Nº pulses per 6hrs ± s.e.m) in High and Low line ram lambs at 2, 6, and 10 weeks of age.

AGE (weeks)	HIGH LINE (n=20)	LOW LINE (n=20)
2	2.61 ± 0.47	2.42 ± 0.47
6	3.50 ± 0.37a	2.74 ± 0.38
10	2.35 ± 0.19b	2.37 ± 0.18

Different subscripts indicate statistically significant differences ($P < 0.05$).

LH pulses were not seen in all of the animals during the experimental sampling periods. The number of animals which did not exhibit an LH pulse decreased with age; 6 animals at 2 weeks of age, 2 animals at 6 weeks of age and none at 10 weeks of age. A greater proportion of the animals which did not exhibit an LH pulse were from the Low line, 5/6 at 2 weeks of age, 2/2 at 6 weeks of age.

In both lines, the frequency of LH pulses varied between the different ages, increasing between 2 and 6 weeks of age and decreasing between 6 and 10 weeks of age, the resultant LH pulse frequency at 10 weeks of age being lower than that measured at either 2 or 6 weeks of age. Due to the small magnitude of the changes in LH pulse frequency, the only statistically significant change occurred in the High line lambs between 6 and 10 weeks of age ($P < 0.05$).

The mean LH pulse frequency was higher in the High line animals than the Low line animals at 2 and 6 weeks of age, and lower at 10 weeks of age. However at all ages the between-line differences were small and were not statistically significant.

4.3.2.3. LH pulse amplitude.

The mean LH pulse amplitude was calculated for each animal and averaged for each line (Table 4.2.). The mean LH pulse amplitudes were compared between ages and between lines by analysis of variance.

Table 4.2. Mean LH pulse amplitude (ng/ml ± s.e.m) at 2, 6 and 10 weeks of age in ram lambs from the High and Low selection lines.

AGE (weeks)	HIGH LINE (n=20)	LOW LINE (n=20)
2	5.63 ± 1.29ay	1.22 ± 0.28az
6	8.48 ± 0.81b	7.85 ± 1.21b
10	15.61 ± 2.76y	8.28 ± 1.39z

Within columns different subscripts indicate significant differences (a versus b, $P < 0.05$). Within rows different subscripts indicate significant differences (y versus z, $P < 0.05$).

LH pulse amplitude increased in both selection lines throughout the experimental period. The mean LH pulse amplitude increased significantly ($P < 0.05$) between 2 and 6 weeks of age in both selection lines. The increase in LH pulse amplitude between 6 and 10 weeks of age was not as great in either line and therefore was not statistically significant in the Low line and only bordered on statistical significance in the High line ($P = 0.06$).

The mean LH pulse amplitude in the High line lambs was greater than that seen in the Low line throughout the experimental period. These differences between the two lines were statistically significant ($P < 0.05$) at 2 and 10 weeks of age, but not at 6 weeks of age.

4.4. Discussion

The results of this experiment demonstrate firstly, that the age-related changes in gonadotrophin secretion seen in ram lambs in this study were similar to those reported previously for spring-born ram lambs. Secondly, that the mean LH concentrations at 2, 6 and 10 weeks of age and the mean FSH concentrations at 6 and 10 weeks of age were significantly greater in lambs from the High line than in lambs from the Low line. The higher mean LH concentrations in the High line lambs, at 2 and 10 weeks of age were primarily due to secretion of LH pulses of a significantly greater amplitude, and at 6 weeks of age, due to a combination of both increased LH pulse amplitude and LH pulse frequency.

The LH concentration in the peripheral circulation of lambs from both lines increased between 2 and 10 weeks of age and was similar to that reported previously for spring-born ram lambs (Lee, *et al.*, 1976a; Yarney and Sanford, 1985a, 1985b; Olster and Foster, 1986; Claypool and Foster, 1990). The changes observed in the peripheral FSH concentrations were the same in the lambs from both selection lines and similar to the maturational changes described by Lee *et al.*, (1976a) and Olster and Foster (1986), peripheral FSH concentrations increasing between 2 and 6 weeks of age and decreasing between 6 and 10 weeks of age. However the results of the present study differ from those reported by Yarney and Sanford (1985b) where the decrease in the peripheral FSH concentration occurred at an older age (12 weeks).

The increase in peripheral gonadotrophin concentrations during the early prepubertal period are proposed to occur due to developmental changes at the level of the hypothalamus and/or pituitary gland, which result in an increase in either GnRH secretion and/or the responsiveness of the pituitary gland to GnRH stimulation. This proposal is supported by

the results of the present study, where increases were seen in both LH pulse amplitude and LH pulse frequency between 2 and 6 weeks of age independently of a reduction in steroid negative feedback, and the demonstration that the amount of LH and FSH secreted in response to an infusion of GnRH (Lee *et al.*, 1976b) or a single dose of GnRH (Chapter 5) increases with age. In the present study the concentration of FSH in the peripheral circulation decreased in both lines between 6 and 10 weeks of age. The results of extended studies of gonadotrophin concentrations during the prepubertal period (Lee *et al.*, 1976a; Yarney and Sanford, 1985; Olster and Foster, 1986) indicate that a decrease also occurs in the peripheral LH concentration prior to puberty; however this normally occurs after the decrease in FSH and was therefore not seen in the present study.

The decreases in gonadotrophin secretion observed during the late prepubertal period are proposed to occur due to the increasing influence of the gonads on the regulation of gonadotrophin secretion. During the prepubertal period gonadotrophin secretion is acutely sensitive to gonadal negative feedback, as evidenced by the dramatic increases which occur in the concentrations of both LH and FSH following the removal of gonadal negative feedback by castration. The gonads produce a number of hormones and proteins which affect gonadotrophin secretion, such as steroids and inhibin. The role played by steroids in the gonadal regulation of gonadotrophin secretion has been demonstrated by their ability to prevent the post-castration increase in gonadotrophin secretion and their ability to suppress gonadotrophin secretion in entire lambs. As testosterone is the predominant steroid secreted by the testes, it was proposed to be the principal steroid involved in the gonadal regulation of gonadotrophin secretion. However data from adult rams (Schanbacher and Ford, 1976) and

ram lambs (Claypool and Foster, 1990) have indicated that oestradiol may also play an important role in the regulation of gonadotrophin secretion.

Inhibin is a gonadal protein which is secreted by the Sertoli cells of the testis (de Jong, 1987) and has been shown to have a significant effect on gonadotrophin secretion in both adult ewes (Martin *et al.*, 1987; Martin *et al.*, 1988; Knight and Castillo, 1988; Wrathall *et al.*, 1990) and rams (Lincoln and McNeilly, 1989), preferentially suppressing the secretion of FSH but having little or no effect on LH secretion. As the decrease in peripheral LH concentration during the late prepubertal period occurs coincident with a significant increase in the concentration of testosterone in the peripheral circulation (Lee *et al.*, 1976a; Yarney and Sanford, 1985a, 1985b; Olster and Foster, 1986), but is not correlated to any significant changes in the concentration of oestradiol (Olster and Foster, 1986, Chapter 5), it has been proposed that LH secretion during the late prepubertal period is principally controlled by testosterone. The earlier suppression of peripheral FSH concentrations occurs either because FSH is more sensitive to testosterone negative feedback or because FSH secretion is influenced by some other gonadal factor, such as inhibin.

The LH secretion profile was divided into LH pulse amplitude and LH pulse frequency to characterise the developmental changes in LH secretion and to determine if there were any differences in the pattern of LH secretion between the two lines. The results indicated that both LH pulse amplitude and LH pulse frequency changed in the ram lambs from both lines with age. Changes in the frequency of pulsatile LH secretion are important in the female for the induction of puberty, as evidenced by the increase in endogenous LH pulse frequency prior to the attainment of puberty (Huffman *et al.*, 1987) and the induction of puberty by the supplementation of endogenous LH secretion by the administration of

either exogenous LH (Foster, Ryan and Papkoff, 1984; Keisler *et al.*, 1985) or GnRH (Pirl and Adams, 1987). In the male, no significant changes occur in either LH pulse frequency or LH pulse amplitude at the time of puberty; however, the increase which occurs in the mean LH concentration during the early prepubertal period, coincident with the increase in the peripheral FSH concentration, is important for the initiation of spermatogenesis (Olster and Foster, 1976). The results of this study compare with previous studies and indicate that this increase in the mean LH concentration between 2 and 6 weeks of age occurs primarily due to an increase in LH pulse frequency. This demonstrates that an increase in LH pulse frequency is also required for the attainment of puberty in the male.

Changes in LH pulse amplitude during the prepubertal period have not been extensively characterised in the ram lamb. Yarney and Sanford (1985a, 1985b) reported that pulse amplitude increased between 4 and 8.5 weeks of age and decreased thereafter. In the present study, LH pulse amplitude was measured at 2, 6 and 10 weeks of age and the results confirm and extend previously reported work (Yarney and Sanford, 1985a, 1985b), by demonstrating that LH pulse amplitude in prepubertal ram lambs increases between 2 and 10 weeks of age.

When LH pulse amplitude and LH pulse frequency are considered together, the results of the present experiment suggest that changes which occur in mean LH concentration between 2 and 10 weeks of age are primarily related to changes in LH pulse amplitude and are not caused by changes in LH pulse frequency. As the concentration of steroids in the peripheral circulation and the sensitivity of the hypothalamo/pituitary gland complex to steroids are both low during this period, the differences in pulse amplitude may be a reflection of the maturational changes which occur in the hypothalamo-pituitary gland complex. The results of more

extended studies indicate that during the late prepubertal period (8-20 weeks of age), when the hypothalamo-pituitary gland complex is more sensitive to gonadal negative feedback, the changes in mean LH concentrations are principally controlled by changes in LH pulse frequency.

Between line comparisons at each age revealed that the High line lambs had significantly greater mean LH and FSH concentrations at all of the experimental ages, except FSH at 2 weeks of age, where although the mean FSH concentration was higher in the High line animals the difference between the two lines was not statistically significant. Therefore the results indicate that as in the adult ewes (Chapter 3), the between-line differences in the ability of the two lines to respond to a GnRH challenge is accompanied by differences in the regulation of endogenous gonadotrophin secretion. As the between line difference was always present in the mean LH concentrations, but not present in some of the mean FSH concentrations, the results are in agreement with the proposal that the between-line difference is associated with differences in the regulation of gonadotrophin secretion by GnRH, which has a greater short-term effect on LH secretion than FSH secretion. At 2 and 10 weeks of age, the difference between the mean LH concentrations in the two lines appears to be due to a significant between-line difference in LH pulse amplitude. However at 6-weeks of age, the difference in the mean LH concentration in the High and Low line lambs is not caused by a significant between-line difference in any particular aspect of LH secretion, but occurs due to a combination of the increased LH pulse amplitude and frequency seen in the High line lambs. Differences in LH pulse amplitude and frequency could occur due to differences in the gonadal regulation of gonadotrophin secretion. However, due to the presence of significant

differences in LH secretion at 2 weeks of age when the effect of steroid feedback on gonadotrophin secretion is presumably minimal, the results suggest that the between-line difference in the regulation of LH secretion is not caused by differences in the levels of, or the effects of, steroid feedback. An alternative site for the selected difference is the control of gonadotrophin secretion within the hypothalamo-pituitary gland complex itself. This proposal is supported by the larger between-line differences in LH rather than FSH secretion (Haley *et al.*, 1989, Chapter 3), the differences in LH pulse amplitude in the lambs in this study and the reported between-line differences in gonadotrophin secretion in the adult ewes (Chapter 3).

In conclusion, the age-related changes in gonadotrophin secretion in the lambs from both selection lines were similar to those reported previously for spring-born ram lambs. However the High line lambs secreted more LH than the Low line lambs throughout the whole of the experimental period and more FSH than the Low line during the 6 and 10 week sampling periods. This indicates that, as in the adult ewes, the between-line difference in the LH response to a 5μg GnRH challenge in prepubertal ram lambs is accompanied by significant between-line differences in the regulation of endogenous gonadotrophin secretion. The between-line differences in the mean LH concentrations, at 2 and 10 weeks of age, is due to a significantly greater LH pulse amplitude in the High line lambs. The differences seen in LH pulse amplitude throughout the experimental period together with the differences in the regulation of LH and FSH secretion at 2 weeks of age, suggest that the selected difference in the regulation of endogenous gonadotrophin secretion is not caused by differences in the gonadal regulation of gonadotrophin secretion, but is related to differences in the control of gonadotrophin secretion at the level of the hypothalamo-pituitary gland complex.

CHAPTER 5 - The effect of gonadal negative feedback on the between-line difference in the gonadotrophin response to GnRH.

5.1. Introduction

Selection has produced two lines of sheep which differ in their LH response to a GnRH challenge in both male and female lambs at 10- and 20-weeks of age (Haley *et al*, 1989) and in adult ewes during both the luteal and follicular phases of the oestrous cycle (Chapter 3). Significant between-line differences have also been demonstrated in endogenous gonadotrophin secretion patterns in prepubertal ram lambs and adult ewes, the High line ram lambs having significantly higher mean LH and FSH concentrations than the Low line between 2- and 10-weeks of age, due to the secretion of LH pulses of significantly greater amplitude (Chapter 4). Furthermore in the adult ewes, the High line secreted significantly larger LH pulses than the Low line, during the follicular phase (Chapter 3).

The concentrations of LH and FSH in the peripheral circulation are controlled by a series of interrelated feedback systems (Karsch *et al.*, 1984; Martin, 1984: McCann and Rettori, 1987; McNeilly *et al.*, 1988). LH and FSH are released from the pituitary gland in response to stimulation of the pituitary gonadotrophs by GnRH, which is secreted from the hypothalamus. The amount of GnRH released from the hypothalamus is regulated by steroid feedback (Section 1.3.4.; Schanbacher and D'Occhio, 1984; Legan, I'Anson, Fitzgerald, and Fitzovich, 1985; Karsch *et al.*, 1987) and the responsiveness of the pituitary gland to this GnRH stimulation is regulated by the peripheral concentrations of gonadotrophins (Motta, 1969; Motta *et al.*, 1969) and steroids (Section 1.3.4.; Reeves *et. al.*, 1971a; Clarke and Cummins, 1984; Goodman and Karsch, 1980). The aim of this study was to

investigate whether the difference in the ability of the two lines to release LH in response to stimulation with GnRH was due to differences in the control of LH secretion at the hypothalamus/pituitary gland or due to differences at the level of the gonad, via differences in gonadal negative feedback.

5.2. Materials and Methods

5.2.1. Experimental design

The High and Low line lambs were born between the 3rd and the 25th of April as the result of within-line matings. Ram lambs were allocated at birth to one of two treatment groups (Entire or Castrated), thereby producing four groups in all; Group 1- Intact High line (n=21); Group 2- Intact Low line (n=14); Group 3- Castrated High line (n=22); Group 4- Castrated Low line (n=13). Castrates were produced by open surgical castration on the day following birth (Section 2.2.2.). The LH response to GnRH was assessed at 2, 6, 10 and 20 weeks of age (± 3 days). The GnRH (HRF, Ayerst Laboratories Ltd.) was administered intravenously as a 2ml bolus injection at Time Zero. Two blood samples were collected at twenty minute intervals before the administration of GnRH and then blood samples were collected 30, 50 and 70 minutes after the administration of GnRH. At 2, 6 and 20 weeks of age, blood samples were collected by jugular venipuncture, while at 10 weeks of age, the blood samples were collected via an indwelling jugular cannula. At 10 weeks of age, the sampling period post-GnRH injection was extended, with blood samples being collected at 20 minute intervals for a further 60 minutes and again at 190 and 250 minutes after GnRH treatment. At 20 weeks of age, the sampling schedule was the same as for weeks 2 and 6, with one further sample being collected 120 minutes after the GnRH challenge. Following the last blood sample the animals were

decannulated, and treated with a long-acting antibiotic (Duphapen LA; Duphar Veterinary Products Ltd).

5.2.2. Hormone Assays

The limits of detection for the LH and FSH assays were 0.092ng NIH-LH-S18/ml and 0.130ng NIDDK-oFSH-RP1/ml. The inter- and intra-assay coefficients of variation were 10.35% and 8.36% for the LH assays and 10.80% and 6.48% for the FSH assays respectively.

The testosterone concentrations were measured in three radioimmunoassays following extraction of the samples by affinity chromatography (mean extraction efficiency 63%). The minimum detectable concentration of testosterone in the radioimmunoassay was 2.64pg/ml and the inter- and intra-assay coefficients of variation were 2.1% and 4.43% respectively. The oestradiol concentrations were measured in a single radioimmunoassay following extraction of the samples by affinity chromatography (mean extraction efficiency 71%). The minimum detectable dose of oestradiol in this assay was 0.04pg/ml and the intra-assay coefficient of variation was 1.38%.

5.2.3. Statistical Analyses

The concentration of FSH and LH exhibited a skewed distribution, which was approximately log-normal; therefore all analyses were performed on the log scale. The data is presented as geometric means with approximate standard errors calculated on the arithmetic scale. The results were compared between lines and between ages by analysis of variance. The decay rate of FSH in the peripheral circulation was calculated on each sampling occasion by fitting a linear model which included the effects of line, gonadal status (castrate/entire), age and sampling time. The

concentrations of testosterone and oestradiol in the two lines were compared on the normal scale using Student's 't' test.

5.3. Results

5.3.1. Testosterone

Testosterone was measured in the entire and castrated lambs from both lines in the samples taken before GnRH administration (Fig 5.1.).

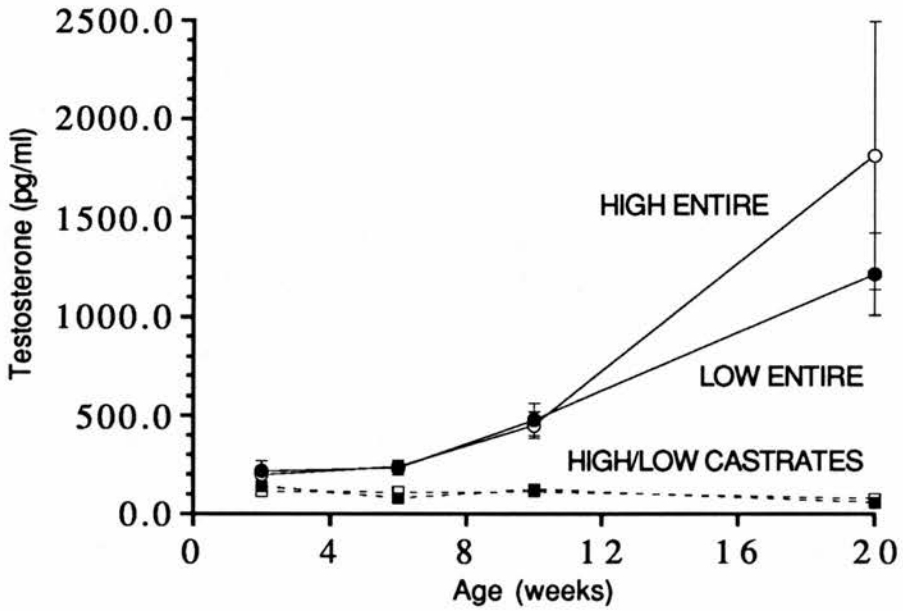

Figure 5.1. Mean testosterone concentrations (± s.e.m) measured before the administration of GnRH in entire (solid lines:- High n=21; Low n=14) and castrated (dotted lines:- High n=22; Low n=13) lambs from both the High (o,□) and Low (•,■) selection lines.

The concentration of testosterone in the castrated animals from both lines was low on all sampling occasions. No significant changes occurred with age in either line. In the entire lambs from both selection lines, the

concentration of testosterone in the peripheral circulation increased with age, with a statistically significant ($P < 0.001$) increase occurring between 10 and 20 weeks of age. Due to the pulsatile nature of testosterone secretion, there was considerable within-line variation in the mean testosterone concentrations on all sampling occasions and hence no significant difference was observed between the two lines during any of the sampling periods.

5.3.2. Oestradiol

The concentration of oestradiol was measurable in all animals and at all of the ages tested. The mean oestradiol concentrations are presented in Table 5.1. No statistically significant differences were seen in the mean oestradiol concentrations either between lines, between treatments, or between ages.

Table 5.1. The mean oestradiol concentrations (pg/ml ± s.e.m) in entire and castrated lambs from the High and Low selection lines between 2 and 20 weeks of age.

	ENTIRE		CASTRATE	
AGE (weeks)	High line (n=21)	Low line (n=14)	High line (n=22)	Low line (n=13)
2	4.48 ± 0.39	4.25 ± 0.36	4.35 ± 0.57	4.31 ± 0.52
6	3.87 ± 0.32	3.10 ± 0.30	3.50 ± 0.25	3.50 ± 0.48
10	7.90 ± 1.77	5.15 ± 1.56	7.83 ± 3.95	10.31 ± 5.24
20	4.11 ± 0.98	1.57 ± 0.56	2.47 ± 0.55	6.05 ± 3.99

5.3.3. Basal gonadotrophin concentrations

Samples were taken before the GnRH challenge to provide a measure of the basal concentrations of LH and FSH.

5.3.3.1. LH

Baseline LH concentrations were estimated in those animals in which the concentration of LH remained relatively constant in both samples taken before the GnRH challenge. The LH concentrations were meaned per animal and the geometric mean LH concentrations calculated for each group (Table 5.2.).

Table 5.2. - Mean baseline LH concentrations (ng/ml ± s.e.m) in entire and castrated lambs from the High and Low GnRH selection lines between 2 and 20 weeks of age.

	ENTIRE		CASTRATE	
AGE (weeks)	High line (n=21)	Low line (n=14)	High line (n=22)	Low line (n=13)
2	0.60 ± 0.06	0.43 ± 0.05	0.88 ± 0.10	0.74 ± 0.10
6	1.16 ± 0.09	1.01 ± 0.10	7.32 ± 0.59	3.92 ± 0.35
10	1.26 ± 0.09	1.06 ± 0.08	8.18 ± 0.59	5.72 ± 0.41
20	0.93 ± 0.14	0.47 ± 0.07	11.94 ± 2.00	9.29 ± 1.20

The basal LH concentrations increased in the entire animals from both lines between 2 and 10 weeks of age and decreased between 10 and 20 weeks of age. In the castrated animals a different pattern of basal LH secretion was observed, the LH concentration increasing throughout the experimental period in both lines.

Analysis of the results showed that there was no interaction between line of origin of the lambs and the experimental treatment; hence the two characters were analysed separately. Within each line, the LH concentration in the castrated lambs was significantly greater than that measured in the entire animals, even at 2 weeks of age (2 weeks, $P < 0.01$; 6, 10 and 20 weeks, $P < 0.001$). The effects of line of origin were compared within each treatment group. In the castrates, the High line animals had a significantly higher basal LH concentration than the Low line at 2, 6, 10 and 20 weeks of age ($P < 0.001$). In the entire animals, the difference between basal LH concentration approached statistical significance at 2 weeks of age and differed significantly between the two lines at 6 weeks of age ($P < 0.05$), but the increase in the within-line variation at 10 and 20 weeks of age resulted in no significant difference between the two lines.

5.3.3.2. FSH

The concentration of FSH in the two samples taken before the GnRH challenge showed very little within-lamb variation. The results for each animal were therefore meaned and the geometric mean baseline concentration calculated per group, (Table 5.3).

A significant interaction ($P < 0.01$) was noted between the effects of age and gonadal status (entire/castrate), as seen by the changing magnitude of the between-line difference in the mean baseline FSH concentrations in the entire and castrated animals with age. The mean basal FSH concentration in entire animals from both lines increased significantly ($P < 0.05$) between 2 and 6 week of age, but declined between 6 and 20 weeks of age. The pattern of basal FSH secretion in the castrated animals was similar to that seen for LH, increasing significantly ($P < 0.05$) in both lines over the whole of the experimental period. Basal FSH

concentrations differed significantly ($P < 0.01$) between the entire and the castrated groups in both lines at all of the ages tested. Significant differences ($P < 0.01$) were also observed throughout the experimental period between the basal FSH concentrations of the lambs from the High and Low selection lines for both the entire and the castrated treatment groups.

Table 5.3. Mean baseline FSH concentrations (ng/ml ± s.e.m) in entire and castrated lambs from the High and Low GnRH selection lines between 2 and 20 weeks of age.

	ENTIRE		CASTRATE	
AGE (weeks)	High line (n=21)	Low line (n=14)	High line (n=22)	Low line (n=13)
2	0.46 ± 0.24	0.42 ± 0.26	5.73 ± 1.58	2.84 ± 1.14
6	2.01 ± 0.35	1.64 ± 0.45	11.67 ± 1.8	8.77 ± 2.06
10	1.90 ± 0.27	1.10 ± 0.46	17.5 ± 4.19	13.94 ± 3.05
20	0.86 ± 0.56	0.73 ± 0.26	21.08 ± 6.43	17.80 ± 8.0

5.3.4. Gonadotrophin responses to 5μg of GnRH

5.3.4.1. LH

The LH response to GnRH was defined as the maximum LH concentration seen on any of the three sampling occasions following the administration of GnRH, minus the mean basal LH concentration for that group. In all cases the maximum LH response was measured in the first sample following the GnRH challenge. The geometric mean LH response for each group was calculated and the results plotted against age (Figure 5.2., a and b).

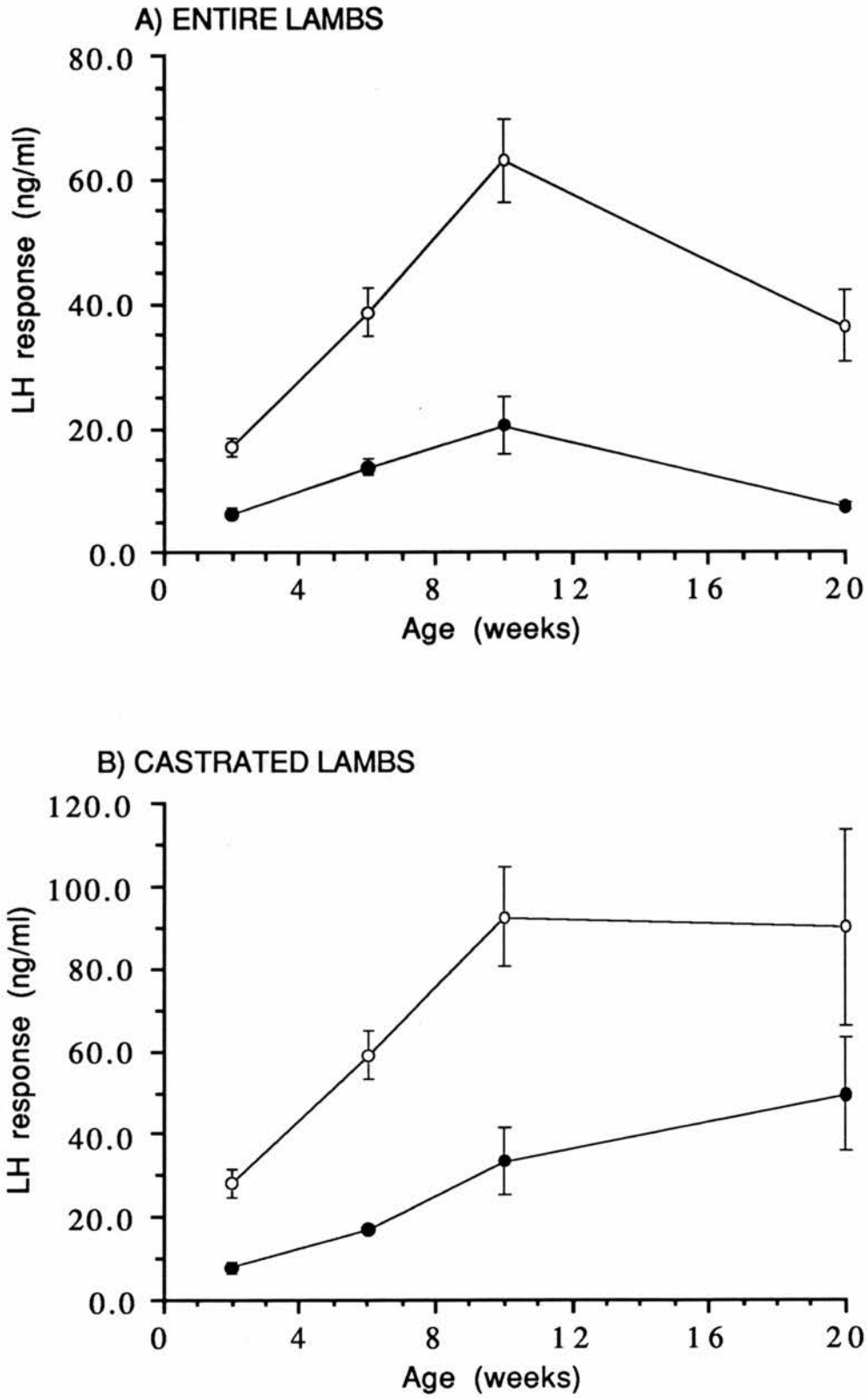

Figure. 5.2. The geometric mean of the peak LH response (ng/ml ± s.e.m) to a 5μg GnRH challenge in A) entire, and B) castrated lambs from the High (o) and Low (•) GnRH selection lines

The magnitude of the LH response in entire animals from both lines increased significantly ($P < 0.001$) between 2 and 10 weeks and then decreased between 10 and 20 weeks of age. This pattern of change is similar to that seen in the basal LH concentrations of entire lambs. In the castrated animals, the magnitude of the LH response to GnRH increased with age in both lines. The LH response in the High line animals reached a plateau at 10 weeks of age, with no significant increase occurring between 10 and 20 weeks of age.

As with the basal LH concentrations there was no significant interaction between the LH response to GnRH stimulation and either the line of origin of the lambs or their gonadal status. Therefore the effects of the line and gonadal status were analysed independently.

Significant differences (2 and 10 weeks, $P < 0.05$; 6 and 20 weeks, $P < 0.001$) were present between the entire and the castrated lambs of each line throughout the experimental period. The mean LH responses being greater in the castrated animals than the entire animals at each age. Similarly, significant ($P < 0.001$) differences were also seen throughout this period between the two selection lines, the High line releasing more LH than the Low line in both treatment groups. The magnitude of the between-line difference in the LH responses of the High and Low selection lines did not change with age, the High line animals having approximately a 3-fold greater response than the Low line animals on all occasions. In the castrated animals, a 3-fold difference was also present in favour of the High line animals at the 2, 6, and 10 week sampling occasions. However at 20 weeks of age, the ratio of the responses of the two lines decreased. It should be noted that the pattern of the LH response in the castrated animals from the two lines diverged at this point, the Low line response increasing in

magnitude, while the High line had reached an apparent plateau at 10 weeks of age.

5.3.4.2. FSH

The concentration of FSH in the peripheral circulation did not increase greatly above baseline following the GnRH challenges in any of the groups, at any of the ages tested. The differences between the FSH concentrations at the different ages were influenced more by changes in the basal FSH concentrations. The concentrations of FSH in the peripheral circulation after the administration of GnRH are presented in Figure 5.3.

The FSH response was defined as the maximum FSH concentration measured, minus the FSH baseline. Castrated animals had significantly larger FSH responses than entire animals within each line, on all of the sampling periods ($P < 0.001$). Similarly at each age, within each treatment group (entire/castrated), the High line animals had a significantly ($P < 0.01$) larger FSH response than the Low line animals. The FSH response to GnRH stimulation was examined in terms of the rate of decay of FSH in the peripheral circulation. At 2 and 6 weeks of age, the decay rate of FSH was the same in all four treatment groups, although as previously described the magnitude of the response differed. At 10 weeks of age there were significant differences in the FSH decay rate between the entire (-3.3 ± 0.3pg/ml/min) and castrated lambs (-2.0 ± 0.3pg/ml/min). At 20 weeks of age the FSH decay rate differed significantly between the High and Low line animals, the High line animals (-4.8 ± 1.1pg/ml/min) having a faster rate of decay than the Low line (-0.9 ± 1.0pg/ml/min).

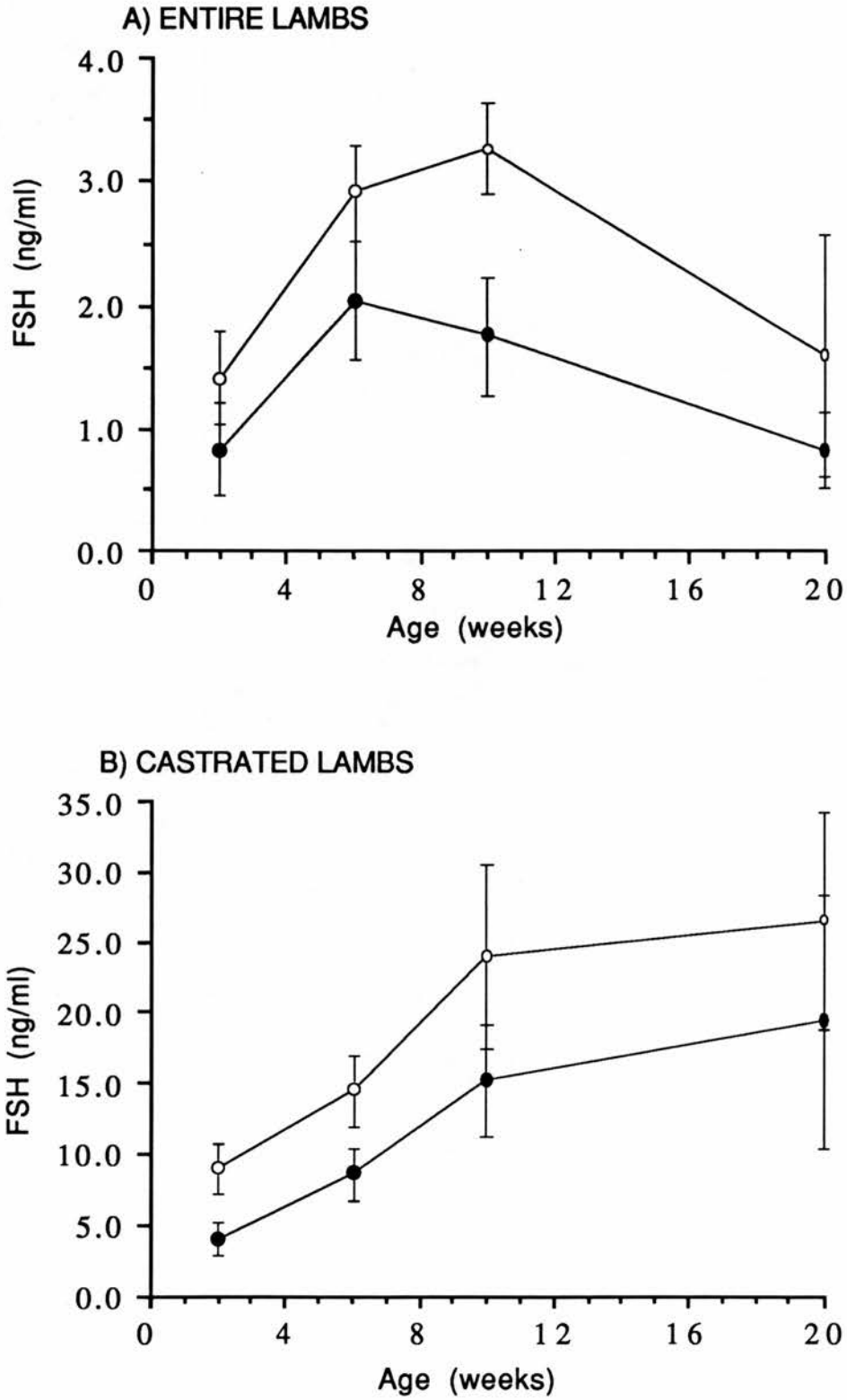

Figure 5.3. The mean FSH concentrations (ng/ml ± s.e.m) measured in the peripheral circulation of A) entire and B) castrated lambs from the High(o) and Low (•) selection lines following administration of GnRH, at 2, 6, 10 and 20 weeks of age.

5.4. Discussion

The maturational changes in the patterns of basal gonadotrophin secretion observed in the entire lambs in this study confirm and extend the results presented in Chapter 4. Between 10 and 20 weeks of age the mean basal concentrations of LH and FSH decreased in both selection lines. These findings agree with those previously reported by other groups working with spring-born ram lambs (Lee *et al.*, 1976a; Sanford *et al.*, 1982; Yarney and Sanford, 1985a; Olster and Foster, 1986; Echternkamp and Lunstra, 1984; Claypool and Foster, 1990). The results of this study confirm that the increase in gonadotrophin secretion observed during the early prepubertal period (2 to 6-10 weeks of age) occurs due to a combination of the increased competence of the hypothalamo-pituitary complex to secrete GnRH, as evidenced by the increase in basal gonadotrophin concentrations (Tables 5.1 and 5.2), and an increase in the ability of the pituitary glands to respond to GnRH stimulation (Fig 5.2 and 5.3). It would appear that the decreases in the basal concentrations of FSH (between 6 and 20 weeks of age) and LH (between 10 and 20 weeks of age), occur due to an increase in the effect of gonadal negative feedback on gonadotrophin secretion (section 4.4.). The results of this study therefore confirm the reported effects of gonadal negative feedback on gonadotrophin secretion (Riggs and Malven, 1974; Crim and Geschwind, 1972; Olster and Foster, 1986) and extend these studies by demonstrating that gonadotrophin secretion is negatively regulated by gonadal feedback in ram lambs, from at least two weeks of age.

The concentrations of both testosterone and oestradiol were measured in the entire lambs in this study and confirm the previously reported age-related patterns of steroid secretion in spring-born ram lambs (Lee *et al.*, 1976a; Echternkamp and Lunstra, 1984; Yarney and Sanford,

1985a, 1985b; Olster and Foster, 1986). The feedback effects of the gonad appear to be independent of the effects of oestradiol, as the concentration of oestradiol in the peripheral circulation did not change significantly with age in either line or in either treatment group (entire/castrate). The concentration of testosterone increased in the entire lambs from both lines between 2 and 20 weeks of age, a significant increase occurring between 10 and 20 weeks of age coincident with a decrease in both endogenous and exogenously-stimulated LH release. Therefore it appears that testosterone is the principal gonadal factor in the negative regulation of basal LH secretion. The same does not apply for basal FSH secretion however, which decreased from at least 6 weeks of age. Due to the similarities in the secretion patterns of basal FSH concentrations and the mean FSH concentrations described in Chapter 4, it can be assumed that basal FSH concentration is regulated by the same mechanism discussed in the previous chapter (section 4.4).

The magnitude of the gonadotrophin response to the GnRH challenge also changed with age. The pattern of change was consistent in both lines and similar to that reported for both basal (Table 5.2. and 5.3.) and mean (Chapter 4) gonadotrophin concentrations. Therefore, it is probable that the magnitude of the gonadotrophin response to GnRH is regulated by the same control mechanisms which regulate endogenous gonadotrophin secretion and were discussed in Chapter 4. The changes in the magnitude of the LH response with age were similar in both the entire and the castrated lambs from each line to the age-related changes in the basal LH concentration. However in the High line castrated lambs, the magnitude of the LH response to the GnRH challenge appeared to plateau at 10 weeks of age. This contrasts with the Low line animals where a significant increase was seen in the LH response to GnRH between each of

the ages tested. This plateau in the LH response to GnRH in the High line castrated lambs could be due to the exhaustion of the pituitary stores of LH at both 10 and 20 weeks of age and/or the maximal occupancy of GnRH receptors.

Apart from at 20 weeks of age, when the LH response in the High line castrated lambs had plateaued, the magnitude of the between–line difference in the LH response to GnRH was the same, irrespective of the presence or absence of gonadal negative feedback. Therefore it can be concluded that the between-line difference in the LH response to GnRH is independent of the effects of gonadal negative feedback.

The FSH response to the GnRH challenge was small in both the castrated and entire lambs from both selection lines throughout the experimental period. However significant between-line differences were present within each treatment group (entire/castrated) at all of the ages tested (Figure 5.3.). Following the GnRH challenges significant differences were seen in the rate of decay of FSH in the peripheral circulation in the various groups and at the different ages. FSH secreted by the castrated lambs at 10 weeks of age and the Low line lambs at 20 weeks of age had an extended half-life relative to the entire and High line lambs at the two ages respectively. This effect is similar to that reported for the FSH decay rate in adult ewes (Fry *et al.*, 1987) and rhesus monkeys (Peckham *et al.*, 1973) following gonadectomy, where the decay rate of FSH was found to be faster in entire animals than in ovariectomised animals. A similar effect has also been reported in the rate of decay of LH in gonadectomised monkeys (Pekham and Knobil, 1976). Analysis of the chemical structure of the LH and FSH, secreted by the ovariectomised and entire rhesus monkeys (Peckham *et al.*, 1973; Peckham and Knobil, 1976), indicated that the sialic acid content was increased following gonadectomy and provides a possible

explanation for the extended half-lives of the two gonadotrophins in the gonadectomised animals. Therefore, with regard to the pattern of FSH secretion following the GnRH challenge, significant between-line differences also exist, and as for LH, these differences do not appear to be caused by differences in gonadal negative feedback, but may be related to differences in the chemical structure of FSH between the two lines.

Comparison of the basal gonadotrophin concentrations between the two lines across the experimental period indicated that persistent differences were present in the basal FSH concentrations irrespective of gonadal status (Table 5.3). However, the between-line difference in the basal LH concentrations differed between the entire and castrated lambs. In the entire lambs, the between-line difference in the basal LH concentration decreased with age, being significantly different at the two earlier ages but not at 10 and 20 weeks of age. In the castrated lambs basal LH concentrations were greater than those seen in the entire lambs and the between-line difference in the basal LH concentration was maintained at all ages. The results therefore support the concept of differential regulation of LH and FSH secretion (Clarke *et al.*, 1986) and demonstrate that despite the lack of a significant effect of gonadal negative feedback on the LH response to a GnRH challenge between the animals from the two lines, as with the adult females, gonadal negative feedback does affect the between-line difference in endogenous LH secretion, by masking the inherent between-line difference in basal LH secretion at 10 and 20 weeks of age.

In summary, the results of this study extend those of Haley *et. al.* (1989) by demonstrating that the between-line difference in the LH response to a GnRH challenge in ram lambs is present from at least 2 weeks of age and furthermore that this difference is present following the

removal of gonadal negative feedback by castration. Therefore the results demonstrate that the between-line difference in the regulation of gonadotrophin secretion occurs at the level of the hypothalamus/pituitary gland. Furthermore significant between-line differences also exist in basal LH and FSH secretion, the High line animals secreting more LH and FSH than the Low line. However despite the demonstration that gonadal negative feedback does not have a significant effect on the between-line difference in the gonadotrophin response to GnRH, the increase in gonadal negative feedback at 10 and 20 weeks of age appears to reduce the between-line difference in basal gonadotrophin secretion, thereby masking the between-line difference in basal LH secretion seen at the earlier ages.

CHAPTER 6. - The measurement of pituitary sensitivity to GnRH and an indirect assessment of endogenous GnRH secretion, in 10 week old ram lambs from the two selection lines.

6.1. Introduction

The results of Chapter 5 demonstrated that the between-line difference in pituitary responsiveness to GnRH stimulation does not occur due to differences in steroid negative feedback, but due to differences in the regulation of LH secretion at the level of the hypothalamo-pituitary gland complex. LH is secreted by the pituitary gland in a pulsatile fashion, pulses of LH being observed in the peripheral circulation in response to discrete pulses of GnRH released from the hypothalamus into the hypophyseal portal blood stream (Clarke and Cummins, 1982; Levine *et al.*, 1982). As the amount of LH released is dependent on the size of the stimulatory signal and the sensitivity of the pituitary gland to GnRH stimulation, the between-line difference in the regulation of LH secretion in the two selection lines could be due to differences in either the sensitivity of the pituitary glands to GnRH stimulation and/or differences in the amount of GnRH secreted by the hypothalamus.

Gonadotrophin secretion is intimately connected with the central nervous system. Administration of barbiturates such as sodium pentobarbitone and sodium phentobarbitone, which act by reducing neuronal activity, affect both gonadotrophin and steroid secretion in a number of species including rats (Blake, 1974a, 1974b; Aiyer *et al.*, 1974a) and sheep (Radford and Wallace, 1974; Ellicott, Benoit, Borth, Stickler and Woolever, 1975; Dobson and Ward, 1977; Radford, Nancarrow and Findlay, 1978; Webb *et al.*, 1981; Goodman and Mayer, 1984). However the effects of

barbiturate anaesthesia in sheep are profoundly influenced by the season of study and the endocrinological state of the animals. General anaesthesia of ovariectomised (Goodman and Mayer, 1984) and entire ewes during the breeding season (Radford and Wallace, 1974; Dobson and Ward, 1977; Radford *et al.*, 1978; Webb *et al.*, 1981; Goodman and Meyer, 1984) completely blocks endogenous pulsatile LH secretion, whilst anaesthesia of seasonally anoestrous ewes causes an increase in LH pulse frequency (Goodman and Meyer, 1984). The pituitary gland is not directly affected by anaesthesia, as administration of exogenous GnRH to ewes in which endogenous LH secretion has been blocked by barbiturates, results in the stimulation of LH secretion (Radford and Wallace, 1974; Blake, 1974a, 1974b; Webb *et al.*, 1981).

The aim of this study was to develop a model system which allowed GnRH dose response curves to be constructed for each line in the absence of endogenous GnRH secretion, thereby allowing 1) the estimation of pituitary sensitivity; pituitary sensitivity being defined as the minimum dose of GnRH needed to stimulate LH release from the pituitary gland which could be measured as an LH pulse in the peripheral circulation, and 2) the indirect estimation of endogenous GnRH pulse amplitude.

6.2. Materials and Methods

6.2.1. Animals

The ewes were mated within lines and the experimental lambs born indoors, during the first two weeks of April. Castrated ram lambs were produced by the application of a rubber ring at the base of the scrotum soon after birth (section 2.2.2.). Ewes and lambs were turned out the day following birth and were maintained at pasture under normal husbandry conditions. All lambs were housed during the experimental periods.

6.2.2. Hormone assays

The LH concentration was assessed in all of the samples from each animal, samples from each animal being measured in duplicate, in a single assay to reduce inter-assay variation. The detection limit for the LH assay was 0.17ng NIH-LH-S18/ml and the inter- and intra-assay coefficients of variation were 9.52% and 11.59% respectively. Testosterone was measured in two radioimmunoassays following solvent extraction of the samples (mean extraction efficiency 83%). The mean detection limit of the assay was 5.8 $\pm$ 1.2 pg/ml, and the inter- and intra-assay coefficient of variation were 0.54% and 6.25% respectively.

6.2.3. Experimental Protocols

6.2.3.1. Experiment 1

The aim of this experiment was to determine both the minimum dose of GnRH required to stimulate the release of LH from the pituitary gland and the doses of GnRH required to stimulate LH pulses whose amplitudes would fall in the physiological range of 10-week old ram lambs.

Five entire, 6-week old ram lambs (±3 days) (High n=2, Low n=3), were fitted with indwelling jugular cannulae (16G) approximately 2 hours before the start of the experimental. The lambs received all 5 doses of GnRH (25ng, 50ng, 100ng, 250ng, 500ng) (HRF, Ayerst Laboratories Ltd) at 90 minute intervals, in a random order. Blood samples were collected at 15 minute intervals, starting from the time of injection, centrifuged at 3000rpm for 20 minutes and the resulting plasma frozen at -20^{o}C until assayed for LH and testosterone.

6.2.3.2. Experiment 2

The aim of this experiment was to confirm that general anaesthesia using sodium pentobarbitone would abolish endogenous GnRH/LH pulses in prepubertal ram lambs, thereby allowing the development of an animal model in which the LH responses of the lambs from both lines could be studied in the absence of endogenous GnRH. Castrated lambs were used as the removal of steroid negative feedback allows increased hypothalamic activity with a resultant increase in LH pulse frequency (Crim and Geschwind, 1972; Riggs and Malven, 1974). Two 6 week old castrated ram lambs, one from each selection line, were fitted with an indwelling jugular cannula and anaesthetised for 3.5 hours using sodium pentobarbitone (Sagatal, May & Baker). The lambs were blood sampled at 10 minute intervals during the period of anaesthesia, and the samples were processed as described in section 6.2.3.1.

6.2.3.3. Experiment 3

Eighteen, 10-week old ram lambs (± 3 days) from the High (n=7) and Low (n=11) GnRH selection lines were fitted with bilateral indwelling jugular cannulae; one cannula was used for the collection of blood samples while the other was used for the administration of GnRH and sodium pentobarbitone. On Day 1, all animals were serially blood sampled at 15 minute intervals for three hours to estimate endogenous LH pulse amplitude. On Day 2, general anaesthesia was induced with sodium pentobarbitone at a dose rate of approximately 30mg/kg, injected in two stages: half the volume was injected rapidly and the remainder was given slowly to effect. Following induction, the animals were intubated and maintained under light anaesthesia by incremental doses of sodium pentobarbitone. The lambs were positioned in sternal recumbency with

head elevated to prevent reflux and inhalation of oesophageal fluid. The animals were turned at regular intervals and their condition and temperature monitored closely for the duration of anaesthesia. Supplementary oxygen was available.

Previous results from Experiment 1 indicated that 50, 100, 250 and 500ng of GnRH (administered as 2ml bolus injections) would enable the construction of an LH dose response curve which should cover the physiological range of LH pulse amplitudes. The results of Experiment 2 indicated that the animals should be anaesthetised for two hours before the initiation of GnRH treatment.

After the induction of anaesthesia the animals were blood sampled every 15 minutes for two hours (Period A). Each animal then received each of the four doses of GnRH in a random order, at 90 minute intervals. After each GnRH injection, blood samples were collected at five minute intervals for 30 minutes and every ten minutes thereafter until the next GnRH injection (Period B). At the end of the experiment the animals regained consciousness, the cannulae were removed and the lambs were treated with a long-acting antibiotic.

6.2.4. Statistics

The LH results from Experiment 3 were analysed by fitting separate exponential models for each animal assuming an LH peak at the third sample following GnRH treatment. The model gives estimates for the pulse amplitude and the rates of rise and fall of LH for each dose of GnRH. The fitted equation for the post-injection rise in the LH concentration is:-

$$y = \alpha \exp\{(\chi - 3)/ \beta \}$$

Where α is pulse amplitude, β is the rate of rise and χ is the sample number. The equation for the LH decay after the peak is:-

$$y = \alpha \exp\{(\chi - 3)/ \gamma \}$$

Where α and χ are as previously defined and γ is the rate of decay. At the peak, when $\chi = 3$, both equations have the same value, namely α, the pulse amplitude. Thus the peak LH concentration for each animal and each dose of GnRH can be characterised by the three parameters (α, β, γ) and the values of these parameters for different animals and different doses can be evaluated. The testosterone concentrations were compared between lines using Student's 't' test.

6.3. Results

6.3.1. Experiment 1

During the experimental period a number of endogenous LH pulses were observed; however these pulses did not coincide with the GnRH challenges.

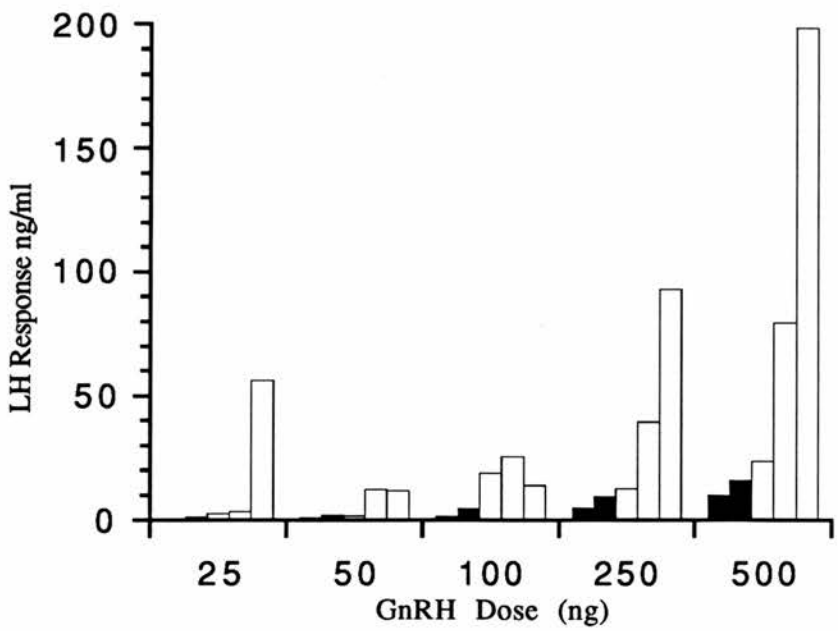

Figure 6.1 The LH responses of animals from the High □ (n=3) and Low ■ (n=2) selection lines to the five doses of GnRH.

Irrespective of line, where LH secretion was stimulated the concentration of LH measured in the peripheral circulation was maximal in the first sample taken after the administration of GnRH (T+15 minutes). The peripheral LH concentration thereafter decreased exponentially. The LH responses to the five doses of GnRH are shown in Figure 6.1.

The 25 and 50ng doses of GnRH failed to induce a significant LH response in the Low line animals. The High line animals all released a measurable LH pulse in response to the 50ng dose of GnRH, but as with the Low line, little or no LH was released in response to a 25ng dose of GnRH. Therefore it was concluded that the lowest dose of GnRH to be used in the dose response study (Experiment 3) should be 50ng. All animals responded to the higher doses of GnRH. The two lines differed in the magnitude of their responses. Within-line variation was large even with the small sample numbers used. The LH response to the 500ng dose of GnRH varied between 9ng/ml and 24ng/ml in the Low line and between 79ng/ml and 200ng/ml in the High line animals.

6.3.2. Experiment 2

Figure 6.2. shows the LH profiles of the two castrated animals during the 3.5 hours of general anaesthesia, and the following hour during which the animals regained consciousness. Initially, anaesthesia appeared to stimulate the release of LH; however in both lines LH concentrations decreased to 'baseline' within 120 minute and no further increases in LH secretion were seen during the period of anaesthesia. The amount of LH released differed between the two lines, with the High line animals releasing approximately four times more LH than the Low line animals.

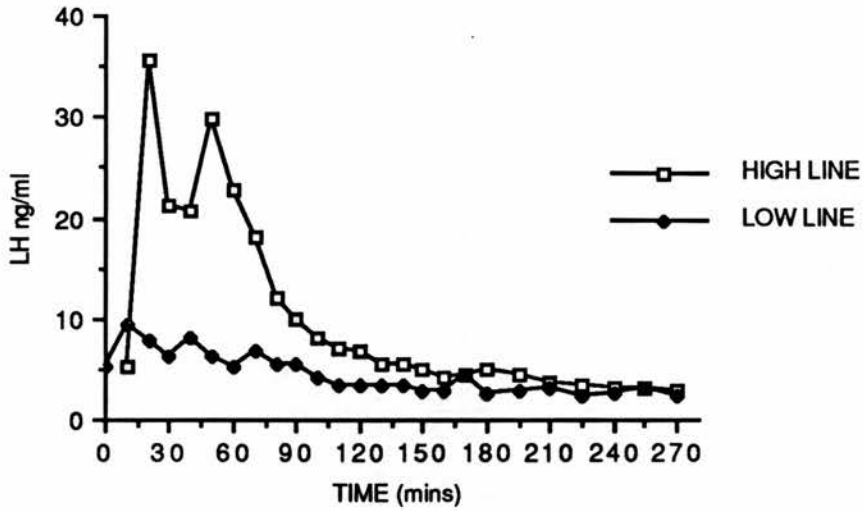

Figure 6.2. Effect of sodium pentobarbital anaesthesia on the peripheral LH concentration of animals from both the High and Low selection lines.

Therefore the results of Experiment 2 demonstrated that sodium pentobarbital anaesthesia inhibits endogenous GnRH in 6-week old ram lambs and confirms the suitability of barbiturate anaesthesia for the construction of a model system to study pituitary sensitivity to GnRH and to indirectly measure endogenous GnRH secretion.

6.3.3. Experiment 3

Figure 6.3 a) and b) shows the LH profiles of representative lambs from the High and Low selection lines during the period of anaesthesia.

6.3.3.1. Period A

During the 2 hour pre-GnRH treatment period, two of the eighteen animals exhibited elevated plasma LH concentrations at the start of anaesthesia, as in Experiment 2. In both of these animals the LH concentration decayed to baseline before the administration of GnRH.

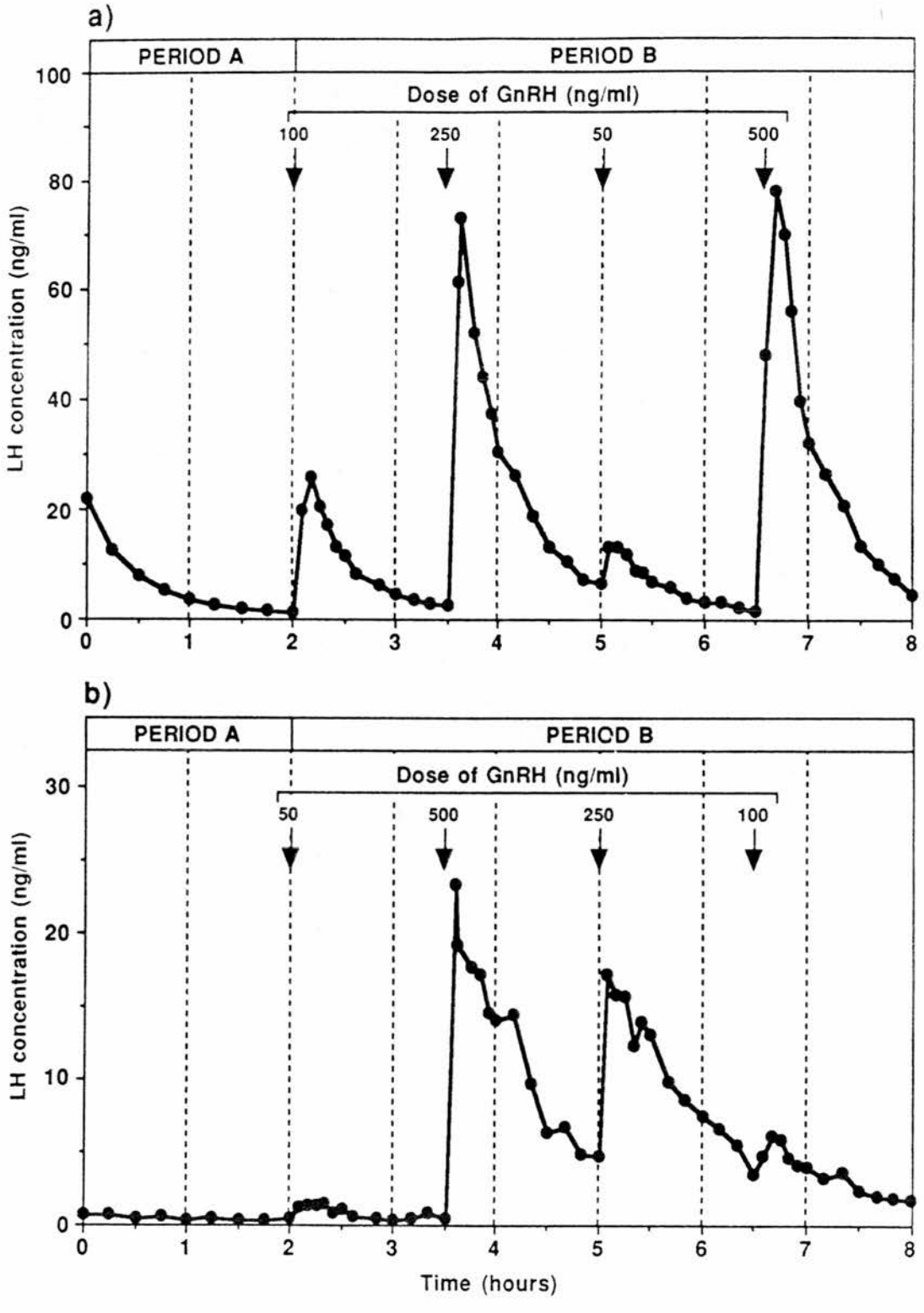

Figure 6.3. The LH profiles from representative lambs from both the a) High and b) Low selection lines.

LH pulses were completely abolished by anaesthesia in all but one animal, which released a single LH pulse during this period.

6.3.3.3. Period B

Throughout Period B (6 hours), endogenous LH pulses were abolished in all animals. An immediate release of LH was detected in the peripheral plasma in the majority of lambs in response to the injections of GnRH. A normal exponential decay was seen after each LH pulse. As the LH concentrations following the larger GnRH challenges did not always return to baseline before the next GnRH injection, the results were analysed by fitting an exponential model which calculated the actual LH response to each GnRH challenge, and compensated for the decay in the LH concentration resulting from the previous GnRH injection. The meaned LH responses of the High- and Low-line animals to the different doses of GnRH, are given in Table 6.1.

Table 6.1. Mean LH responses (ng/ml ± s.e.m) of the High and Low GnRH selection lines after the administration of various doses of GnRH.

	LH CONCENTRATION (ng/ml)	
GnRH dose	High line (n=7)	Low line (n=11)
50ng	10.1 ± 2.8	1.8 ± 0.8
100ng	27.6 ± 2.9	5.1 ± 0.7
250ng	65.9 ± 3.2	13.9 ± 0.8
500ng	113.9 ± 3.1	24.6 ± 0.8

In both lines the magnitude of the LH responses measured were related to the GnRH doses received. The LH responses increased significantly in accordance with the increasing doses of GnRH in both

lines. The relationship between the dose of GnRH given and the LH response was linear at the low doses of GnRH however at the higher doses the LH response plateaued.

When individual animals from both lines were studied, increased concentrations of LH were detected in the peripheral circulation of all animals in response to the 100, 250 and 500ng doses of GnRH. The 50ng dose of GnRH produced a significant release of LH in all of the High line animals (10.1 $\pm$ 2.8ng/ml), but only 4 of the Low line animals responded, these animals releasing significantly ($P < 0.05$) less LH (1.8 $\pm$ 0.8ng) than the High line.

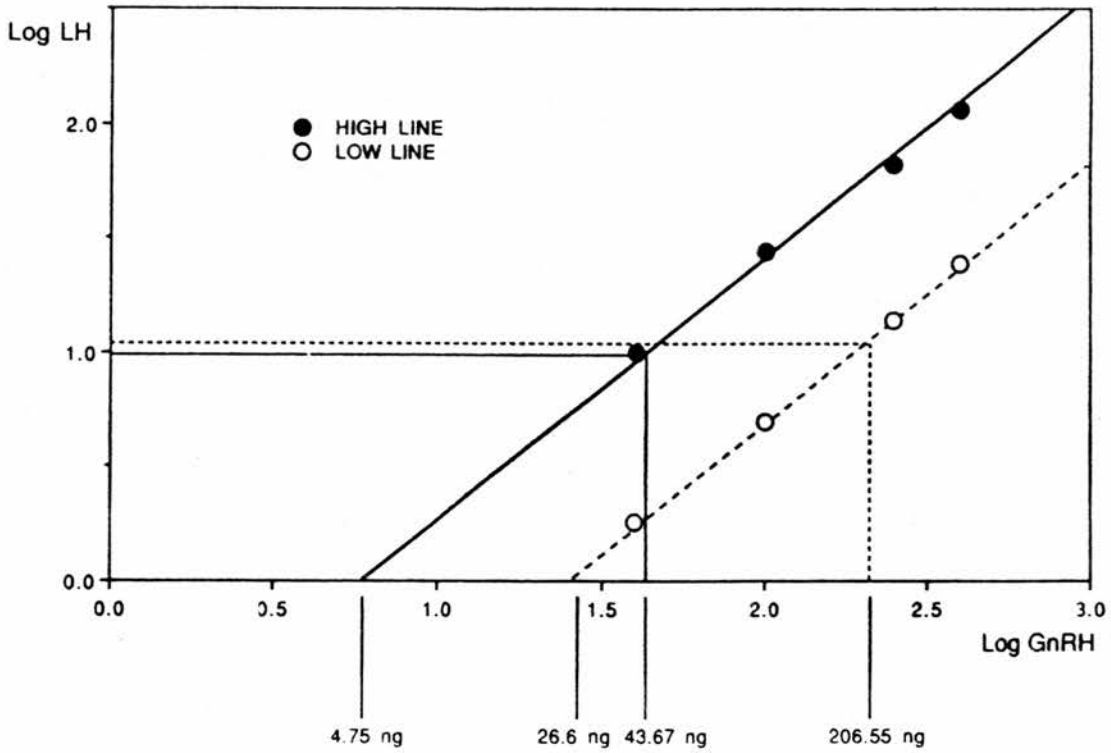

Figure 6.4. Regression analysis for the log GnRH dose response curves for lambs from the High (n=7) and Low (n=11) selection lines. Pituitary gland sensitivity was estimated for the two selection lines as the dose of GnRH where the regression lines cross the x axis. Indirect measurements of the amplitudes of the endogenous GnRH pulses were also obtained, for the High (solid lines, 43.67ng) and Low (dotted lines, 206.55ng) line animals.

A statistically significant interaction existed between the dose of GnRH and the line of origin of the lambs. This effect was removed by transformation of the data onto the log scale and regression lines were fitted (Figure 6.4.). The points where the regression lines intercept the x axis indicate the theoretical doses of GnRH that must be exceeded for an LH response to be measurable in the peripheral circulation and therefore provides a measure of pituitary sensitivity for both selection lines. The regression lines intercepted the x axis at the equivalent of 26.6ng and 4.75ng of GnRH for the Low and High lines respectively, thus demonstrating a 5.5-fold difference in the sensitivities of the pituitary glands of the two lines to GnRH stimulation.

The results of the window bleed on the first day of Experiment 3 allowed the endogenous LH pulse amplitudes of both selection lines to be estimated. Due to the relatively low LH pulse frequency in lambs of this age, not all of the lambs secreted endogenous LH pulses during the 3 hour sampling period. The amplitude of the LH pulses observed varied greatly within each line. The mean LH pulse amplitude in the High line lambs (10.13ng/ml) was slightly higher than the Low line animals (9.99ng/ml); however this difference was not statistically significant. The mean endogenous LH pulse amplitudes were transposed onto the dose response graphs in order to get an indirect measurement of the quantity of GnRH required to reproduce an endogenous LH pulse. The doses of GnRH required to induce endogenous LH pulses were estimated to be 43.67ng and 206.55ng for the High and Low lines respectively.

The mean testosterone concentrations were assessed for each lamb in 5 samples taken during the serial bleed on Day 1 of Experiment 3. The mean testosterone concentrations per line were then calculated and were 74.17 $\pm$ 16.0pg/ml and 58.36 $\pm$ 12.33pg/ml for the High and Low lines

respectively. Due to the large amount of both within- and between-lamb variation the difference between the two lines was not statistically significant.

6.4. Discussion

The results of this study demonstrated firstly, that general anaesthesia using sodium pentobarbitone in entire prepubertal Finn Dorset ram lambs completely blocked endogenous GnRH secretion, thereby providing a suitable model with which to study pituitary gland sensitivity to GnRH stimulation in the absence of endogenous GnRH. Secondly, that the High line animals are 5-fold more sensitive to GnRH than the Low line animals. Thirdly, that the High line lambs required 5-times less GnRH than the Low line to stimulate the secretion of LH pulses of similar amplitude.

The effects of barbiturate anaesthesia on gonadotrophin secretion have been studied in the adult ewe in a wide variety of physiological conditions (Radford, 1966; Dobson and Ward, 1977; Radford *et al.*, 1978; Webb *et al.*, 1981; Goodman and Mayer, 1984), and the effects found to differ depending on the season of study and the endocrinological state of the animals. Goodman and Mayer (1984) explained the differential effects of barbiturate anaesthesia on gonadotrophin secretion in adult ewes by proposing that GnRH secretion was under the control of two neuronal systems; a stimulatory system, the 'GnRH pulse generator' and an inhibitory oestradiol-sensitive system. They argued that the GnRH pulse generator was the dominant neuronal system during the breeding season, while the oestradiol-sensitive neuronal system assumes dominance during seasonal anoestrus. Therefore, the effects of barbiturate anaesthesia, which reduces neuronal activity (Cross and Dryer, 1971; Barker and Ransom, 1978)

are dependent upon which of the two neuronal control systems is dominant, and therefore varies with season.

As far as we are aware, the effects of sodium pentobarbitone anaesthesia have not been previously reported in prepubertal ram lambs. The results of Experiments 2 and 3 demonstrate that endogenous pulsatile LH secretion is abolished in both castrated and entire ram lambs during sodium pentobarbitone anaesthesia. The results of this study therefore appear to agree with Goodman and Meyer's (1984) theory, as in 10-week old Finn Dorset ram lambs the GnRH pulse generator is the dominant neuronal system, steroid negative feedback not having a significant effect on LH secretion, in Finn Dorset ram lambs, until at least 10 weeks of age (Chapter 4).

In the castrated lambs in Experiment 2, the mean basal LH concentrations reached at the end of the period of anaesthesia (High, 2.98ng/ml; Low, 2.28ng/ml) are lower than those observed in 6-week old castrated lambs (High, 7.32ng/ml; Low, 3.92ng/ml) and are similar to those seen in 6-week old entire ram lambs (High, 1.16ng/ml; Low, 1.01ng/ml) (Chapter 5). It would therefore appear that sodium pentobarbitone anaesthesia, as well as suppressing pulsatile LH secretion, is also suppressing basal LH secretion. This suppressive effect of barbiturate anaesthesia after gonadectomy has previously been noted in adult sheep (Lewis, Millar and Bolt, 1985b) and in rats (Ajika, Kalra, Fawcett, Krulich and McCann, 1972).

The effects of general anaesthesia on LH secretion are not due to the direct effects of barbiturates on pituitary function, as in rats and adult ewes in which endogenous GnRH is blocked by barbiturate anaesthesia, the administration of an appropriate pattern of exogenous GnRH is sufficient to restore a normal pattern of LH secretion (Radford and Wallace, 1974; Blake,

1974a, 1974b, 1976; Webb *et al.*, 1981). The results of this trial demonstrate that a similar effect is seen in the prepubertal ram lamb, because although endogenous GnRH secretion is blocked by anaesthesia, the ability of the pituitary gland to respond to stimulation with exogenous GnRH is not compromised.

The GnRH dose response curves obtained in this experiment (Figure 6.4.) were extrapolated in order to obtain a measure of the sensitivity of the pituitary glands of the two lines to GnRH stimulation. The results demonstrated that the mean sensitivity of the pituitary glands of the ram lambs from the two selection lines to GnRH stimulation differed by a factor of five. This study therefore provides the first documented evidence of two different strains of a single breed of sheep, which differ in the sensitivity of their pituitary glands to GnRH stimulation. This between-line difference in the magnitude of the LH response to GnRH was maintained over all of the doses of GnRH used in this study, the High line lambs releasing five-fold more LH than the Low line. The magnitude of the between-line difference in the LH responses to these physiological doses of GnRH is of similar magnitude to that seen when lambs from the two lines were challenged with a pharmacological dose (5μg) of GnRH (Haley *et al.*, 1989; Chapter 5).

The mean endogenous LH pulse amplitudes were calculated for the two lines from the results of the serial bleed on Day 1 of Experiment 3. The results demonstrated that the mean LH pulse amplitude did not differ significantly between the two selection lines, although it was slightly higher in the High line lambs than the Low line lambs. The doses of GnRH required to stimulate pulses of LH secretion of similar magnitude to those seen endogenously were calculated for each line using the GnRH dose response curves and were 43.67ng and 206.55ng, for the High and Low lines respectively. Therefore the Low line animals appear to require

approximately 5-fold more GnRH in order to produce an LH pulse of similar amplitude to those seen in High line animals. Clarke *et al.*, (1984) monitored the concentration of GnRH present in the hypophyseal portal blood after the administration of exogenous GnRH and found that the peak concentrations of GnRH measured in the hypophyseal portal blood ranged between 0.01 and 0.029% of the dose given. If the same realised concentration of GnRH was to hold for the lambs in this study, the concentration of GnRH required to stimulate LH pulses similar to those produced endogenously in the High and Low line lambs would range between 4.37 to 12.66pg and 20.66 to 59.90pg respectively. The endogenous GnRH pulse amplitude has been measured directly in both ovariectomised (Clarke and Cummins, 1982; Levine *et al*, 1982), and cyclic ewes (Clarke *et al.*, 1987) and the validity of the GnRH pulses checked by the measurement of LH pulses in the peripheral circulation. Despite the fact that the size of the endogenous GnRH pulses was estimated indirectly in this study, the estimated amplitudes of the GnRH pulses are of a similar magnitude to those measured in the studies of Clarke *et al.*, (1982, 1987) and Levine *et al.*, (1982).

In conclusion the results of this study demonstrated that the model system constructed in this experiment provides a suitable approach with which to study the regulation of LH secretion by the hypothalamo-pituitary gland complex and demonstrated that the pituitary glands of the High line lambs are 5-fold more sensitive to GnRH stimulation than the Low line. However, pituitary sensitivity, as defined here is a comprehensive term and encompasses variables such as the number of gonadotrophs in the pituitary gland, the number of available GnRH receptors, the amount of LH stored in the pituitary gland and the rate that the pituitary gland is able to transcribe and translate the appropriate DNA message into active

protein. As the endogenous LH pulses measured in this experiment were similar between the two lines the results of this experiment also suggest that differences exist in the amount of GnRH secreted from the hypothalami of the two lines, the High line lambs secreting 5-fold less GnRH than the Low line lambs.

Chapter 7. - Pituitary gland weight and GnRH-receptor number in 20-week old ram lambs from the two selection lines.

7.1. Introduction

Differences have been identified in both male and female animals from the High and Low GnRH selection lines, at a range of ages in 1) their ability to release LH in response to both pharmacological (Haley *et al.*, 1989; Chapters 3, 5) and physiological doses (Chapter 6) of GnRH and 2) in some aspects of endogenous gonadotrophin secretion, e.g. prepubertal High line ram lambs have significantly greater mean (Chapter 4) and basal (Chapter 5) LH and FSH concentrations, and LH pulses of significantly greater amplitude than the Low line (Chapter 4). In the adult ewe, during the follicular phase, the High line also secreted LH pulses of significantly greater magnitude (Chapters 3).

The results of Chapter 5 demonstrated that the between line difference in the LH response to a GnRH challenge was not caused by differences in gonadal negative feedback and indicated that the selection lines differ in the regulation of gonadotrophin secretion at the level of the hypothalamo-pituitary gland complex. The results of Chapter 6 demonstrated that the pituitary glands of the High line lambs are 5-fold more sensitive to GnRH stimulation than the Low line and that differences may also exist in the magnitude of the endogenous pulses of GnRH secreted by the hypothalami of the two lines.

The events which occur following exposure of the pituitary gland to GnRH and which culminate in the release of gonadotrophins are initiated by binding of GnRH to a specific protein moiety or receptor located on the exterior of the gonadotroph (Clayton, Shakespear and Marshall, 1978;

Marian and Conn, 1983). Binding of GnRH to its receptor 'activates' the receptor and stimulates a series of extra- and intra-cellular events including receptor aggregation (Hopkins and Gregory, 1977) and a cascade of intra-cellular enzyme systems (Clayton, 1989), which culminate in the release of gonadotrophins into the peripheral circulation. Following receptor activation the GnRH receptors are internalised and subsequently degraded (Hazum, Cuatrecasas, Marian and Conn, 1980). The concentration of GnRH receptors in the pituitary gland is regulated physiologically by two systems; auto-regulation, whereby GnRH regulates the number of its own receptors (Clayton and Catt, 1981; Young *et al.*, 1983, 1985b; Detta *et al.*, 1984) and gonadal feedback, where peripheral steroid concentrations affect GnRH receptor numbers (Reeves *et al.*, 1971b; Debeljuk *et al.*, 1972; Hopkinson *et al.*, 1974; Jackson, 1975; Coppings and Malven, 1976; Clarke and Cummins, 1984; Kaynard *et al.*, 1988; Gregg and Nett, 1989; Herman and Adams, 1990).

The amount of LH released in response to a given dose of GnRH could therefore be influenced by the number and availability of GnRH receptors in the pituitary gland. If differences exist in the GnRH receptor populations of the two selection lines it would provide an explanation for the between-line differences observed in pituitary gland sensitivity to GnRH stimulation. Therefore the aim of this experiment was to determine if the between-line difference in pituitary sensitivity to GnRH stimulation (Chapter 6) is related to a difference in the number of GnRH receptors present in the pituitary glands of the animals from the two lines

7.2. Materials and methods.

7.2.1. Experimental protocol.

Twenty eight, 20-week old ram lambs, 14 from each line, were sacrificed by means of a barbiturate overdose. The top of the cranium was cut away and the brain removed to expose the pituitary gland. The pituitary gland was extracted from the hypophyseal fossa and dissected free of extra-pituitary tissue. The weights of the individual pituitary glands were recorded and the pituitary glands were chopped into 3-4mm blocks using a sterile scalpel. A sample of pituitary tissue was removed, snap frozen in liquid nitrogen and stored at -80°C until assayed for GnRH receptor content. The GnRH receptor and protein concentrations in the tissue samples were measured, by Dr T. A. Bramley, at the Centre for Reproductive Biology, Chalmers Street, Edinburgh.

7.2.2. Statistics

The mean pituitary gland weights for the two lines were compared on the arithmetic scale, using Student's 't' tests. The GnRH receptor concentrations were log-transformed to equalise within-line variance and compared on the geometric scale by one way analysis of variance.

7.3. Results

7.3.1. Pituitary gland weight

Pituitary gland weight was recorded in all animals, and the mean calculated for each line. The mean weights of the pituitary glands were 0.382 ± 0.017g and 0.301 ± 0.022g for the High and Low line lambs respectively. Statistical analysis of the results indicated that the difference between the two lines approached, but did not reach, statistical significance at the 5% level (P = 0.07).

7.3.2. GnRH - receptor number

The number of receptors in the tissue samples was expressed as the weight of GnRH agonist (pg $GnRH_A$) specifically bound per mg of protein in the pituitary homogenates. Therefore differences in receptor number could reflect differences in GnRH receptor binding affinity. However the large concentration of GnRH agonist used in the assay ensures that the equilibrium is weighted so as to measure receptor number and not to measure differences in receptor binding efficiency. GnRH - receptor binding affinity was also measured as the association constant, and no differences were seen between the two lines, the K_a s ranging between 0.65 and 0.7 x 10^{10} $M.^{-1}$

The estimated concentration of GnRH receptors varied greatly within each line, more variation being seen in the High line animals than the Low line animals. The geometric mean GnRH receptor concentrations, with approximate standard errors, calculated on the arithmetic scale and the range of GnRH receptor concentrations for each line are presented in Table 7.1.

Table 7.1. The mean concentration and range of GnRH receptors (pg $GnRH_A$ bound/mg protein) in homogenised pituitary gland tissue from the High and Low selection lines

	MEAN CONCENTRATION OF	
LINE	GnRH RECEPTORS(pg/mg protein)	RANGE
HIGH (n=14)	34.2 ± 3.3^a	18.5 - 60.6
LOW (n=14)	11.0 ± 1.1^b	6.0 - 17.1

Different superscripts indicate significant differences ($P < 0.001$).

The mean concentration of GnRH receptors differed significantly ($P < 0.001$) between the two lines, the pituitary tissue from the High line containing more GnRH receptors than the tissue derived from the Low line lambs.

7.4 Discussion

The results of this experiment demonstrate that the pituitary glands from the High line lambs contain significantly more GnRH-receptors/mg of protein than the pituitary glands from the Low line lambs and indicate that differences may also be present in the size of the pituitary glands, the High line animals having larger pituitary glands than the Low line animals.

The High and Low GnRH selection lines have been shown to differ in both their responsiveness and their sensitivity to GnRH stimulation. The results of this study indicate that these differences are accompanied by differences in the number of GnRH receptors in the pituitary glands of the two lines. Transient differences have been reported in pituitary responsiveness to GnRH stimulation in a variety of endocrinological situations and these differences are reported to occur due to changes in pituitary GnRH-receptor numbers (Marian *et al.*, 1981; Clayton and Catt, 1981; Conn *et al.*, 1987a), GnRH receptor binding affinity remaining constant (Loumaye and Catt, 1982). The importance of changes in the number of GnRH-receptors in relation to these temporary changes in pituitary responsiveness is unclear. The largest endogenous change in pituitary responsiveness to GnRH occurs at the time of the preovulatory gonadotrophin surge (Aiyer *et al.*, 1974b; Wang *et al.*, 1976; Fink and Pickering, 1980). This increase in pituitary responsiveness is accompanied by an increase in the number of GnRH-receptors and occurs in a variety of

species including rats (Savoy-Moore *et al.*, 1980; Clayton *et al.*, 1980, 1985; Clayton and Catt, 1981), monkeys (Adams *et al.*, 1981) and sheep (Crowder and Nett, 1984). Although an increase in the concentration of GnRH receptors in the pituitary gland is sufficient to explain the initial rise in pituitary responsiveness observed prior to the preovulatory gonadotrophin surge, other changes must also occur in the regulation of gonadotrophin secretion in order to maintain the sustained increase in pituitary responsiveness which is seen during this period.

A large increase is also seen in pituitary responsiveness to GnRH stimulation following gonadectomy. Studies in the rat have shown that this increase is closely accompanied by an increase in pituitary GnRH receptor content (Clayton, Naik, Young and Charlton, 1984). However, this association is not maintained in all species. In mice, gonadectomy is followed by an increase in the responsiveness of the pituitary gland to GnRH stimulation despite a 50% decrease in GnRH receptor content (Clayton, Young, Naik, Detta and Abbot, 1986). Therefore the transient differences in pituitary responsiveness to GnRH stimulation which occur prior to the preovulatory gonadotrophin surge and following gonadectomy, as with the difference between the High and Low selection lines, appear to be associated with changes in the GnRH receptor content of the pituitary glands (Conn *et al.*, 1987b). However, it should be noted that the difference in pituitary responsiveness between the High and Low selection lines is maintained at a relatively constant level over a wide range of physiological situations; in prepubertal females at 10 and 20 weeks of age (Haley *et al.*, 1989), in adult females (Chapter 3) and in male lambs from 2 to 20 weeks of age (Haley *et al.*, 1989; Chapters 4, 5), while the changes in pituitary responsiveness and GnRH receptor content described above are transient.

The number of GnRH receptors in the pituitary gland is influenced by both peripheral gonadotrophin and GnRH concentrations. The relationship between the concentration of LH and the number of pituitary gland GnRH-receptors has been studied in the rat and reviewed extensively (Clayton and Catt, 1981; Clayton, 1989). Changes in the concentrations of gonadotrophin in the peripheral circulation occur in association with changes in the number of GnRH-receptors in the pituitary gland. Studies have indicated that similar quantitative changes occur in species other than the rat, including primates, and sheep (Crowder, Gilles, Tamanini, Moss and Nett, 1982; Crowder and Nett, 1984; Wise, Neiman, Stewart and Nett, 1984). It is well documented that following exposure to GnRH, the gonadotrophs become "primed" and are hence more responsive to further GnRH stimulation (section 1.3.3.2). The GnRH induced priming of the gonadotrophs occurs due to the mobilisation of intracellular stores of LH, stimulation of LH synthesis and an increase in the numbers of GnRH-receptors (Pickering and Fink, 1976a; Clayton *et al.*, 1980; Bourne and Baldwin, 1980). The results of Chapter 4 demonstrated that the High line lambs secreted endogenous LH pulses of greater amplitude than the Low line, but that the High line lambs required 5-fold less GnRH than the Low line lambs to stimulate LH pulses of equal amplitude (Chapter 6). Therefore these results suggest that the pituitary glands of the High line lambs are exposed to less GnRH than the pituitary glands of the Low line lambs and therefore, that the increased number of GnRH-receptors present in the High line animals is probably not due to differences in GnRH priming of the pituitary gland.

Studies have been carried out using cultured gonadotrophes to try to elucidate the exact relationship between GnRH-receptor concentrations and cellular responsiveness in the absence of GnRH (Clayton, 1989). In these

studies, steroids were found to be involved in the regulation of GnRH-receptor number. Oestradiol enhancement of the culture media induced an increase in both cellular responsiveness and GnRH-receptor number (Tang, Martellock and Horiuchi, 1982), while testosterone treatment induced a decrease in both characters (Giguere, Lefebvre and Labrie, 1981). However, as no significant differences were seen in the steroid concentrations of the High and Low selection line lambs (Chapter 4), unless there are differences in the sensitivity of the two lines to steroid negative feedback, it appears unlikely that the differences in GnRH-receptor number described above are due to differences in the steroidal regulation of GnRH receptor numbers. As with the studies of the physiological changes which occur during the preovulatory period and following gonadectomy, it is not possible to determine if the between-line difference in GnRH-receptor number described in this study are the cause of the increased gonadotrophin concentrations or the effect of exposure of the pituitary gland to elevated concentrations of GnRH.

Differences have been reported in the weight of pituitary glands between entire and gonadectomised animals (Bogdanove, 1963; McDonald and Clegg, 1966). Pituitary weight and gonadotrophin secretion are increased in gonadectomised animals. The increase in the size and the weight of the pituitary gland following gonadectomy occurs due to the hypertrophy of the pituitary gonadotrophs. The results of this study showed that the weight of the pituitary glands of the High line animals were increased relative to those of the Low line. Body weights at the time of slaughter were not recorded for these animals; however 25 of the animals (High, 12; Low, 13) were weighed at 16 weeks of age. No significant difference was found between the two lines at this age (High, 25.42 ± 1.11kg, Low, 23.77 ± 0.90kg), which agrees with previously reported data (Haley *et*

al., 1989), which stated that the mean body weight did not differ between the two selection lines. Therefore the differences in pituitary gland weight are not a function of differences in body weight.

As far as we are aware the GnRH selection lines therefore provide the only documented example of two strains of sheep, from within a single breed, which consistently differ in pituitary responsiveness to GnRH stimulation over a wide range of physiological conditions. The results of this study indicate that the between-line difference in pituitary responsiveness is accompanied by differences in pituitary gland size and a statistically significant difference in the concentration of GnRH-receptors. However, as between-line differences have been observed in both basal gonadotrophin concentrations and the pattern of LH/GnRH release, it is not possible to conclude from this data whether the difference in GnRH-receptor number is the cause or the effect of the differences in pituitary responsiveness/gonadotrophin secretion.

CHAPTER 8. - LH responses of cultured pituitary cells from the two selection lines following either GnRH-receptor activation or the independent activation of the Ca^{2+} and PKC intracellular second messenger systems.

8.1. Introduction

The results of Chapter 6 demonstrated that the between-line difference in the ability of animals to release LH in response to GnRH stimulation is associated with a difference in the sensitivity of the pituitary glands to GnRH stimulation. However the measure of pituitary sensitivity used encompasses a large number of variables such as the number of gonadotrophs present in the pituitary gland, the number of GnRH-receptors, the DNA/RNA transcription and translation rates, the amount of stored LH, etc. In Chapter 7, one aspect of pituitary sensitivity, the number of GnRH-receptors, was investigated further, the results demonstrating that the pituitary glands of the High line lambs contain significantly greater numbers of GnRH-receptors and are larger in size than the pituitary glands from Low line lambs. However, the results did not prove whether the differences in pituitary gland morphology were the cause, or an effect of the between-line differences in pituitary gland function.

When GnRH binds to its receptor it stimulates a series of intracellular events which regulate GnRH-receptor number, the movement and secretion of stored gonadotrophins, and the synthesis of both LH and FSH (reviewed by Conn *et al.*, 1987a; Clayton, 1989; Naor, 1990; Section 1.3.5.). To summarise, following GnRH-receptor binding the two main intracellular second messenger systems in the gonadotrophs are the Ca^{2+}-calmodulin system, which is activated by an increase in the cytosolic Ca^{2+}

concentration (Conn *et al.*, 1981a; Catt *et al.*, 1985) and the PKC system, which is activated by changes in the intracellular concentrations of DAG (Naor and Eli, 1985; Harris, Stanley and Conn, 1985; Conn *et al.*, 1987b; Clayton, 1989; Das, Fahmy and Bourne, 1989; Naor, 1990).

In vitro studies have demonstrated that the two second messenger systems can be activated independently of each other by the treatment of gonadotrophs with either calcium ionophores or DAG/phorbol esters. Treatment of gonadotrophs with calcium ionophores, such as A23187, result in an influx of extracellular Ca^{2+} and activation of the Ca^{2+}-calmodulin system (Reed and Lardy, 1972; Pressman, 1976; Hopkins and Walker, 1978; Conn, Rogers and Sandhu, 1979; Conn *et al.*, 1980; Pickering and Fink, 1979b; Kamel and Krey, 1983; Rassmussen and Barrett, 1984; Woodge and Conn, 1987; Beggs and Miller, 1989). The PKC second messenger system can be activated by administration of exogenous DAG's to gonadotrophs in culture, but this also stimulates IP_3 production (Huckle and Conn, 1987), the release of intracellular Ca^{2+} and activation of the Ca^{2+}-calmodulin second messenger system. However the PKC second messenger system can be stimulated independently of the Ca^{2+}-calmodulin system, by treatment of gonadotrophs with phorbol esters. Phorbol esters sensitize PKC to Ca^{2+} and therefore stimulate PKC activity in the absence of changes in cytosolic Ca^{2+} concentrations (Castagna, Takai, Kaibuchi, Sano, Kikkawa and Nishizuka, 1982; Yamanishi *et al.*, 1983; Nishizuka, 1984; Rasmussen and Barrett, 1984). Therefore phorbol esters have been used to study Ca^{2+} independent LH release in the rat (Hopkins and Walker, 1978; Naor *et al.*, 1980b; Smith and Vale, 1980; Martin and Kowalchyk, 1984a, 1984b; Smith and Conn, 1984; Keisel and Catt, 1984; Harris *et al.*, 1985; Naor and Eli, 1985; Catt *et al.*, 1985; Conn, Ganong, Ebling, Staley, Neidel and Bell, 1985; Andrews and Conn, 1986; Turgeon and Waring, 1986; Das *et al.*, 1989)

and the sheep (Castagna *et al.*, 1982; Beggs and Miller, 1989). The study of Beggs and Miller (1989), using ovine pituitary cells, indicated that the stimulatory effects of the phorbol ester, phorbol 12-myristate-13-acetate (PMA), and GnRH were additive, suggesting that LH release following GnRH stimulation in the sheep was not intrinsically linked to PKC activation. Similar results were also reported in the rat (McArdle, Huckle and Conn, 1989), but when considered in the context of the other work, it was concluded that both the Ca^{2+} and PKC second messenger systems acted independently and that stimulation of both systems is required to simulate the effects of GnRH stimulation (Martin and Kowalchyk, 1984a, 1984b; Harris *et al.*, 1985; Naor and Eli, 1985; Das *et al.*, 1989, Naor, 1990).

The studies using ovine (Beggs and Miller, 1989) and porcine (Walker and Hopkins, 1978; Hopkins and Walker, 1978) gonadotrophs demonstrated that the intracellular events which occur following GnRH stimulation in the sheep and the pig are similar to those described in the rat. However the effectiveness of different secretagogues in stimulating LH release have been shown to differ between ovine and rat gonadotrophs, GnRH stimulating the release of 15-20% of the stored LH in ovine gonadotrophs (Beggs and Miller, 1989) and 50% of the stored LH in rat gonadotrophs (McArdle *et al.*, 1987). The converse was true for the phorbol ester, PMA, which stimulated the release of 80% (Beggs and Miller, 1989) and 20% (McArdle *et al.*, 1987) of the stored LH from the ovine and rat gonadotrophes respectively. Species differences were also seen in the ability of gonadotrophs to synthesise gonadotrophins, rat gonadotrophs synthesising gonadotrophin *in vitro*, while ovine gonadotrophs were unable to do so (Beggs and Miller, 1989). Therefore, in general, it can be assumed that the same second messenger systems are operative in the sheep as in the rat and that secretagogues will have the same effects.

The aim of this trial was to determine if the between-line differences in pituitary sensitivity/GnRH receptor number are caused by between-line differences in the transduction of the signal between receptor activation and gonadotrophin secretion. Therefore LH release was stimulated from pituitary gonadotrophs from both lines maintained *in vitro*, by treatment of the cells with either GnRH, which activates the full endogenous secretory mechanism, or two secretagogues which activate the two principal second messenger systems independently of each other, the calcium ionophore A23187, which selectively activates the Ca^{2+}-calmodulin second messenger system, and the phorbol ester, phorbol 12, 13 dibutyrate (PDB), which selectively activates the PKC second messenger system.

8.2. Materials and methods.

8.2.1. Experimental protocol.

Following removal of a sample of tissue for the assessment of GnRH-receptor content (see Chapter 7, section 7.2.2.3.), the remaining pituitary tissue from eighteen of the lambs, 9 from each line, was dispersed by trypsin digestion as described in section 2.4. A sample of the cell suspension was snap frozen in liquid nitrogen and stored at -80°C, until assayed for GnRH-receptor content. The remainder of the cell suspension was dispensed into 24-well culture plates containing 'supplemented' DMEM, at a concentration of approximately 2 x 10^5 cells per well, with a maximum of 12 plates per animal. The cells were then placed in a humidified incubator (37°C, 5% CO_2, 95% air). After 24 hours the media was removed and replaced with media containing one of the three secretogogues. A preliminary study using 4 culled Welsh mountain ewes was carried out to ascertain the dose levels to be used in the main trial. It was decided that each secretagogue should be used at 4 concentrations:-

GnRH- 8.5x 10^{-9}M, 8.5x 10^{-10}M, 8.5x 10^{-11}M, 8.5x 10^{-13}M

A23187- 1x10^{-4}M, 1x10^{-5}M, 1x10^{-6}M, 1x10^{-7}M.

PDB- 1x10^{-6}M, 1x10^{-8}M, 1x10^{-10}M, 1x10^{-12}M.

This produced 12 treatment groups. In animals where sufficient cells were recovered to set up 12 plates, each plate contained a separate treatment, twenty wells receiving secretagogue – supplemented media (treatment wells), while 4 wells received media which was not supplemented with secretagogues (control wells). In animals where insufficient cells were recovered for the production of 12 plates, the number of treatment wells was reduced, but four control wells were always run with each treatment. The cells were incubated in the presence of the secretagogue – supplemented media or control media for a further three hours. The media was then removed and stored at -20°C until assayed for LH.

8.2.2. Statistical analyses

All of the media samples (treatment and control wells) were assayed for LH content. The data was initially expressed as the average LH concentration per treatment, per animal and the mean LH concentrations per treatment, per line were also calculated. When the LH concentrations in the control wells were studied, it was seen that a considerable amount of variation existed in both the mean control LH concentrations per animal, and in the mean LH concentrations in the control wells run per treatment. Therefore in order to obtain a more accurate estimation of the average amount of LH released per treatment, the results for each animal were expressed as the average LH concentration measured in the treatment wells minus the mean LH concentration measured in the control wells run with that particular treatment. The results were then meaned per line, and due

to the large amount of variation between the different treatment groups, compared both between- and within-lines using two sample 't' tests.

8.2.3. Assays

8.2.3.1. LH assays

The LH concentrations in the media samples from each animal were measured in duplicate in a single assay, to reduce inter-assay variation. The mean detection limit over the 16 LH assays was 0.55ng NIH-LH-S18/ml, and the inter- and intra-assay coefficients of variation were 17.56% and 11.63% respectively.

8.2.3.2. GnRH receptors

The concentration of GnRH receptors in the cell suspensions was measured as described in section 2.3.6.

8.3. Results

8.3.1. GnRH receptor concentrations

The concentration of GnRH receptors measured in the cell suspensions ranged between 0 and 3.38pg of $GnRH_A$ bound per mg of protein. The mean GnRH receptor concentration in the cell suspensions from the High line lambs (2.08 ± 0.72pg $GnRH_A$/mg of protein) was greater than that seen in the Low line (1.60 ± 0.88pg $GnRH_A$/mg of protein), but due to the large amount of within-line variation the between-line difference was not statistically significant. The concentration of GnRH receptors measured in the cell suspensions was compared within each animal to those measured in the samples of fresh tissue previously presented in Chapter 7. The percentage of GnRH receptors remaining in the cell suspensions following trypsin digestion was 9.67 ± 3.39 and 13.17 ± 7.92% in

the samples from the High and Low line lambs respectively. The severity of the dispersion process can therefore be seen to be similar between both lines, with approximately 11% of receptors remaining immediately following trypsin digestion.

8.3.2. LH release in response to secretogogue stimulation.

8.3.2.1. LH response to GnRH stimulation.

The LH responses of the cultured pituitary cells from the two selection lines to the four doses of GnRH are presented in Figure 8.1.

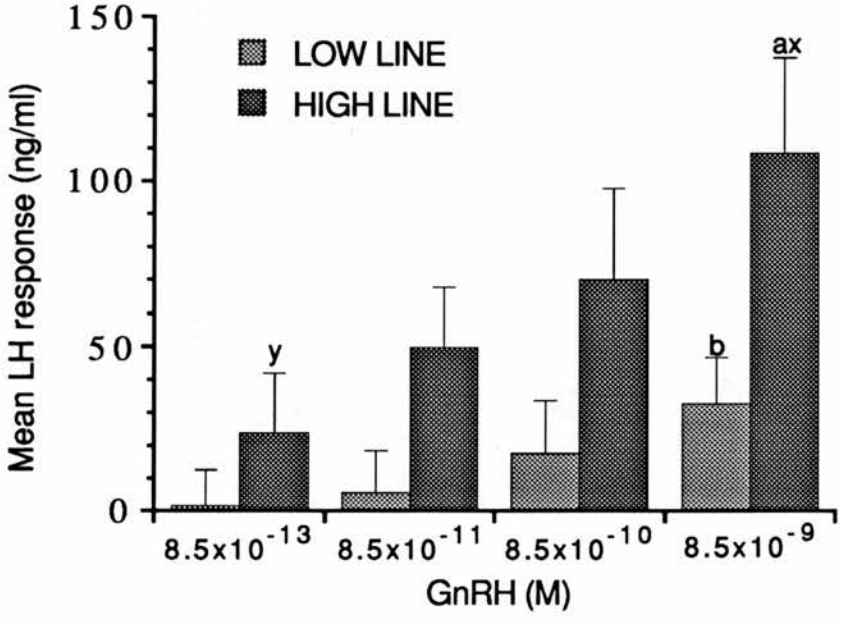

Figure 8.1. Mean LH responses (ng/ml ± s.e.m.) of pituitary cells exposed for three hours to four doses of GnRH. Different superscripts indicate significant ($P < 0.01$) between-(a versus b) and within-(x versus y) line differences.

A significant ($P < 0.05$) LH response was seen in the High line cells following treatment with all of the doses of GnRH and in the Low line

following treatment of the cells with the two highest doses of GnRH. The mean LH responses of the cells from both selection lines increased with the increased doses of GnRH. In the Low line, the magnitude of the LH responses did not differ statistically between the different doses of GnRH. However in the High line a statistically significant difference ($P < 0.05$) was seen in the magnitudes of the LH responses to the two extreme doses of GnRH.

In response to the two highest doses of GnRH (8.5×10^{-9}M and 8.5×10^{-10}M), the cells from both lines released significantly ($P < 0.05$) more LH than the control wells. Therefore the magnitude of the LH responses to these doses of GnRH were compared between the two lines. The cells from the High line secreted 3.13- and 3.89-fold more LH than the Low line in response to the 8.5×10^{-9}M and 8.5×10^{-10}M doses of GnRH respectively. However, due to the large amount of within-line variation, a statistically significant ($P < 0.05$) difference was only seen between the two lines in the magnitude of the LH response to the 8.5×10^{-9}M dose of GnRH.

8.3.2.2. LH response to A23187.

The responses of the cells from the High and Low line animals following stimulation with A23187, are presented in Figure 8.2. The concentration of LH released in response to the three lowest doses of A23187 did not differ significantly from the controls in either line. However, a significant ($P < 0.05$) LH response was seen in the cells from both the High and Low lines to the 1×10^{-4}M dose of A23187. The cells from the High line released 3.19-fold more LH than the cells from the Low line. As with the LH responses to GnRH, there was a large amount of variation between animals within each line, therefore the between-line difference was not statistically significant.

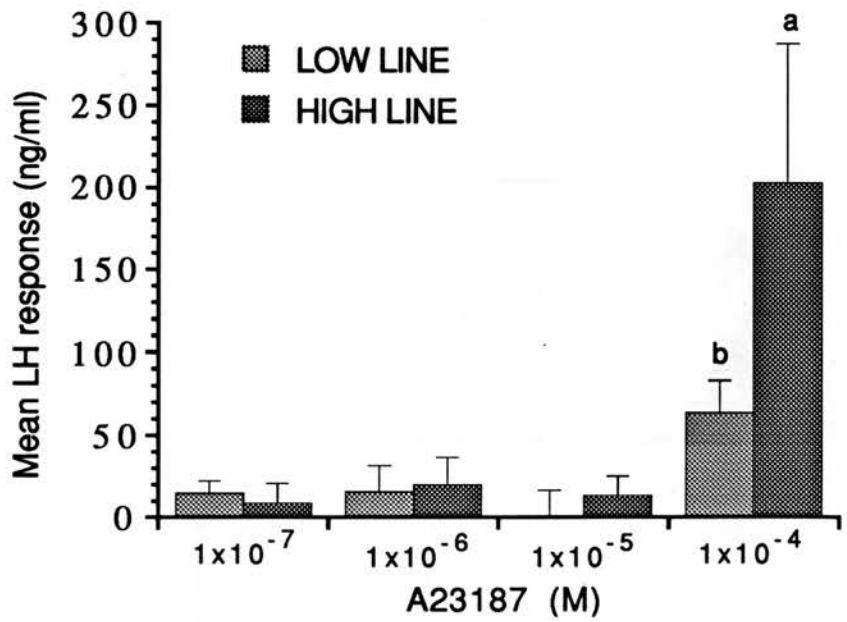

Figure 8.2. Mean LH response (ng/ml ± s.e.m.) of pituitary cells exposed to four doses of the calcium ionophore, A23187, for three hours. Different superscripts indicate statistically significant between-line differences (a versus b, $P < 0.05$).

8.3.2.3. LH response to PDB.

Figure 8.3. shows the LH response of the cells from the High and Low lines following stimulation with PDB. In general an increase was seen in the LH response of the cells from both lines in response to the increasing doses of PDB. Within the High line the LH response to the $1x10^{-6}M$ was significantly greater than the LH response to the $1 \times 10^{-10}M$ ($P < 0.01$) and $1 \times 10^{-12}M$ ($P < 0.05$) doses of PDB, and the LH response to the $1 \times 10^{-8}M$ dose of PDB was significantly greater ($P < 0.05$) than that produced in response to the $1 \times 10^{-10}M$ dose of PDB. In the Low line however, the only statistically significant ($P < 0.05$) difference in the LH responses following exposure to PDB occurred between the $1 \times 10^{-6}M$ and the $1 \times 10^{-12}M$ doses of PDB.

In response to the 1 x 10^{-6}M, and 1 x 10^{-8}M doses of PDB a significant LH response was seen in the cells from both lines. The High line secreted 2.23- and 4.38-fold more LH than the Low line in response to the 1x10^{-6}M and 1 x 10^{-8}M doses of PDB respectively. The between-line difference in the magnitude of the LH response to the 1 x 10^{-8}M dose of PDB was statistically significant ($P < 0.05$).

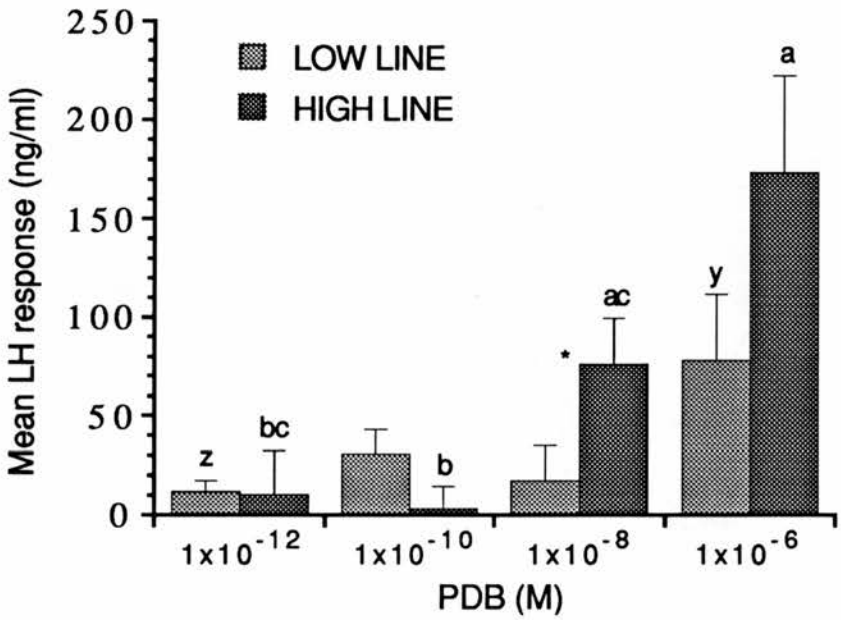

Figure 8.3. Mean LH responses (ng/ml ± s.e.m.) of pituitary cells from the two selection lines following PDB exposure. Within the lines different superscripts indicate significant differences (High line: a versus b, $P < 0.001$; b versus c, $P < 0.05$: Low line: y versus z, $P < 0.05$). Between lines significant differences are indicated by * ($P < 0.05$).

8.4. Discussion

The results of this study demonstrate firstly, that ovine pituitary cells in culture are able to respond to GnRH, A23187 and PDB by releasing

significant quantities of LH and secondly, that the cells from the High line responded to each of the three secretogogues by releasing more LH than the cells from the Low line.

Incubation of pituitary cells from both selection lines with GnRH resulted in an increase in the concentration of LH in the culture media from the treatment wells above that seen in the control wells. The amount of LH released increased in an approximately linear fashion, with the increasing dose of GnRH. Previously GnRH has been used to stimulate LH release in cultured ovine (Beggs and Miller, 1989) and porcine gonadotrophs (Hopkins and Walker, 1978). However in both species the size of the LH response increased in a sigmoidal fashion with the increasing doses of GnRH. The difference in the relationship between the GnRH dose and the LH response in the present study and the previous study using ovine gonadotrophs (Beggs and Miller, 1989) can however be explained by the range of doses of GnRH, since in the present study the doses of PDB (8.5×10^{-9}M - 8.5×10^{-13} M) correspond to the lower doses used by Beggs and Miller (1989) (1×10^{-7}M - 1×10^{-12} M) and were therefore on the more linear part of their dose response curve. Interestingly, the mean LH response measured following stimulation of the cells from the High line by GnRH was approximately 3-fold greater than that measured in the cells from the Low line (3.13 and 3.98, for the 8.5×10^{-9}M and 8.5×10^{-10}M doses of GnRH respectively), and therefore of similar magnitude to the between-line difference in GnRH-receptor number (3.11) reported in Chapter 7. The dispersion method used in this study involves digestion of the pituitary gland with trypsin, a non-specific protease, and therefore during the dispersion process a large number of GnRH-receptors are removed. Comparison of GnRH-receptor numbers prior to (see Chapter 7) and after trypsin digestion demonstrated that approximately 89% of the GnRH

receptors were removed from the pituitary tissue from the animals of both lines. As the pituitary glands of the High line lambs initially contained a significantly greater concentration of GnRH receptors (Chapter 7), and the proportion of GnRH receptors removed was similar in both lines, the gonadotrophs from the High line should theoretically have more GnRH receptors than those from the Low line. Therefore the difference in the LH responses of the cultured pituitary cells derived from the animals from the High and Low selection lines following GnRH exposure may reflect differences in the number of GnRH receptors present on the cells, or differences in the intracellular regulation of GnRH secretion.

A23187 has been used to stimulate LH release using cultured pituitary cells in a number of species including the pig (Hopkins and Walker, 1978) and the rat (Conn *et al.*, 1979; Kamel and Kray, 1983; Woodge and Conn, 1987). As with GnRH, the relationship between the dose of A23187 administered and the LH response appeared to be sigmoidal. Studies in sheep using A23187 as a secretogogue, are limited. Beggs and Miller (1989) reported using A23187 to stimulate LH release from cultured ovine pituitary cells, However only two doses were used; $1x10^{-5}M$ which had no significant effect on LH release and $1 \times 10^{-4}M$ which stimulated a significant release of LH. The results of the present study indicated that a similar relationship existed between the dose of A23187 and the LH response in prepubertal ram lambs, as described for the adult ewes (Beggs and Miller, 1989), the three lowest doses of A23187 ($1x10^{-5}M$, $1 \times 10^{-6}M$ $1 \times 10^{-7}M$) having no significant effect on LH release in the animals from either line. However in response to the $1x10^{-4}M$ dose of A23187 both lines responded by releasing significantly more LH than the controls. As only two doses of A23187 stimulated a significant release of LH we were unable to construct dose

response curves for the two selection lines. However, it is of interest to note that, as with GnRH stimulation, the cells from the High line lambs released approximately 3-fold more LH than the cells from the Low Line. The maintenance of the between-line difference in the amount of LH released following stimulation of the cells from the two lines with A23187, indicates that the between line difference in the ability of the pituitary glands of the two selection lines to release LH in response to GnRH stimulation appears to be related to differences in the Ca^{2+}-regulated release of LH from the pituitary gonadotrophs.

PDB has also been used for the construction of LH dose response curves in two previous studies, one study using ovine pituitary cells (Beggs and Miller, 1989) and one study using rat pituitary cells (Smith and Conn, 1984). As with the GnRH and A23187 dose response studies, the dose response curves obtained in both species indicated a sigmoidal relationship between the stimulatory doses of PDB and the amount of LH released. Since the cells from the two selection lines only responded significantly to the two highest doses of PDB ($1x10^{-4}$ and $1x10^{-5}$), it was not possible to construct dose response curves for the two selection lines in this study. The previous study using ovine gonadotrophs (Beggs and Miller, 1989), differed significantly from the present study in that the pituitary cells used were derived from adult ewes and the experimental protocol was also different (different dispersion process, longer pre-challenge period, longer period of secretogogue exposure); hence a direct comparison of the results is not possible. However in general, the cells from adult ewes appear to be more responsive to PDB stimulation than the pituitary cells from the prepubertal ram lambs and may indicate that maturational changes may occur in the LH release mechanism at the level of the pituitary gland between the prepubertal and the adult animal which are associated with the changes in

the PKC second messenger system. Obviously there may also be an effect of sex.

At both the 1 x 10^{-6}M and the 1 x 10^{-8}M doses of PDB, the mean LH responses in the cells from the High line lambs were significantly greater than the responses from the Low line cells, the High line releasing on average 3.31-fold more LH than the Low line. Therefore it would appear that the two selection lines also differ in the amount of LH released after the stimulation of the PKC second messenger system.

Following stimulation of cultured pituitary cells with GnRH, the cells from the High line released approximately 3-fold more LH than the cells from the Low line. As the results of Chapter 7 indicated that the pituitary glands of the High line lambs contained approximately 3-fold more GnRH receptors than the Low line, the results initially indicate that the between-line difference in the LH responses of the two selection lines occurred due to differences in GnRH receptor number. However, as the between-line difference in LH release was maintained at approximately the same level following both receptor activation (GnRH) and the stimulation of LH release independently of receptor activation (A23187 and PDB), it would appear that the difference in the sensitivity of the pituitary glands of the two lines to GnRH may not be due to differences in GnRH receptor number, but due to intracellular differences in the regulation of LH secretion. The differences in GnRH receptor number may therefore augment or alternatively occur as an effect of the intracellular differences in the regulation of LH secretion. As the between-line difference was maintained at a relatively constant level following the activation of both intracellular second messenger systems, if the between-line difference must occur at a step which is common to both the Ca^{2+} and PKC second messenger systems, but distal to the stimulatory sites of action of A23187 and PDB.

In conclusion the results of this study demonstrate firstly, that exposure of cultured ovine pituitary cells to GnRH, A23187 and PDB, result in the stimulation of LH secretion, demonstrating that both the Ca^{2+} and PKC second messenger systems are involved in LH release from ovine pituitary cells. Secondly, that the between-line difference in the regulation of LH secretion is not caused by a specific difference in either of the two second messenger systems, but appears to occur at a step which is common to both pathways, but distal to the stimulatory sites of action of A23187 and PDB, i.e. either in the enzyme reactions activated by Ca^{2+}-calmodulin complexes and PKC, or in the amount of LH stored in the pituitary glands of the two lines.

CHAPTER 9. - Measurement of the quantity of releasable LH stored in the pituitary glands of the two selection lines.

9.1. Introduction.

The results of Chapter 8 demonstrated that pituitary cells from the High line animals, when challenged *in vitro* with either GnRH, A23187 or PDB, released approximately 3 times more LH than cells from the Low line. While the between-line difference in the LH response to GnRH could be the result of differences in the GnRH-receptor number (Chapter 7), the differences in the LH responses to A23187 and PDB suggest that differences also occur in the intracellular regulation of LH secretion, at a site common to both the Ca^{2+}-calmodulin and PKC second messenger systems, but distal to the sites of action of A23187 and PDB. One possible site of the intracellular difference could be the amount of releasable LH stored in the pituitary gland of the two lines.

The magnitude of LH response to a 5μg GnRH challenge has been studied in both adult females and male lambs between 2 and 20 weeks of age, from the two selection lines (Haley *et al.*, 1989, Chapter 3, Chapter 5). In the majority of these studies the maximum LH concentration was observed in the first blood sample (30mins) and then declined during the subsequent sampling period. LH is released from the pituitary gland almost immediately following GnRH stimulation and elevated peripheral LH concentrations can be detected within minutes of the administration of exogenous GnRH (15 minutes - Rippel *et al.*, 1974a; Bremner *et al.*, 1976 Foster and Crighton, 1976; Fink *et al.*, 1976; Crighton and Foster, 1977: 4 minutes- Goodman and Karsch, 1980: 2 minutes - Kanchev *et al.*, 1984). The pattern of LH release is dependent on the dose of GnRH given and the

method of administration (Hopkinson *et al.*, 1974; Rippel *et al.*, 1974a; Chapter 6), with small doses of GnRH stimulating a monophasic LH release, while large doses, or continuous infusions of GnRH, stimulate a biphasic pattern of LH release (Hopkinson *et al.*, 1974; Bremner *et al.*, 1976, 1980; Kanchev *et al.*, 1984). In the case of the biphasic pattern of LH release, the first phase occurs between 5 and 30 minutes after the administration of GnRH or the start of the infusion and the second more prolonged phase of LH secretion peaks between 40 and 130 minutes after the administration of GnRH (Hopkinson *et al.*, 1974; Bremner *et al.*, 1976, 1980; Kanchev *et al.*, 1984). This biphasic release of LH has been proposed to occur due to the storage of LH in 'two pools' in the pituitary gland (Yen, Vandenburg and Silver, 1974; Bremner and Paulsen, 1974). The initial phase of LH secretion is thought to occur due to the release of LH from a 'readily-releasable pool' of LH, which contains fully processed LH stored near the cell membrane. The second phase of the LH response has been proposed to occur due to the release of LH from the 'releasable pool' of LH stored in secretory granules towards the interior of the gonadotroph and which therefore require transportation to the cell membrane before release.

The aim of this experiment was firstly to measure the total amount of releasable LH stored in the pituitary glands of the two lines, and secondly to characterise the LH response profiles of the two lines following two GnRH challenges to determine if a biphasic pattern of LH release occurs and to characterise the distribution of the stored LH with respect to the 'two pools' hypothesis.

9.2. Materials and Methods.

9.2.1. Experimental protocol.

Twenty one, 10- week old (± 2/3 days) ram lambs from each line were allocated at random to three groups (n=7). Each animal was challenged twice with GnRH (GnRH-1 and GnRH-2); Group 1 received 0.5μg (low dose); Group 2 received 5μg of GnRH (medium dose) and Group 3 received 50μg (high dose). All doses of GnRH were made up in 2ml of physiological saline. The interval between the two injections differed between each treatment group and was calculated so that the second GnRH challenge would be administered before the LH concentration in the peripheral circulation had returned to baseline. Intervals between the two GnRH challenges of 3, 2 and 1.5 hours were used for the high, medium and low doses of GnRH respectively.

The animals were cannulated as described in section 2.2.1. At the start of the experimental period the animals received an intravenous injection of GnRH (GnRH-1) and all animals were intensively blood sampled, samples being collected at 1 minute intervals for the first 10 minutes, 5 minute intervals for the following 20 minutes and thereafter at 15 minutes intervals until the second GnRH challenge (GnRH-2) when the blood sampling procedure was repeated. Blood samples were centrifuged at 3000 rpm for 20 minutes and the resulting plasma frozen at -20°C until assayed for LH

9.2.2. LH assays

All samples from each animal were measured in duplicate in a single assay to reduce inter-assay variation. The mean detection limit over 12 assays was 0.07ng NIH-LH-S18/ml, and the inter- and intra-assay coefficients of variation were 9.31% and 5.45% respectively.

9.2.3. Statistical analyses

The total amount of LH released during the whole of the experimental period was calculated for each animal and for each dose of GnRH, by integration of the area under the LH response curves, and the mean quantities of LH released calculated per dose and per line. In order to characterise the pituitary gland stores of GnRH the LH secretion profiles were then analysed as follows. Initially a statistical approach was attempted where a two-peak exponential model, similar to that described in section 6.2.4. was fitted to the data. However, due to variation in the timing of the two phases of LH release and the extended duration of the second phase of LH release, this proved inappropriate in a significant number of animals. Therefore the LH responses to GnRH-1 and GnRH-2 were analysed manually, periods of increased LH secretion being identified using the parameters defined by Webb *et al.*, (1985a). In response to both the GnRH challenges with the low dose of GnRH a single period of increased LH secretion was seen in the animals from both lines; however, in response to the medium and high doses, the majority of animals responded to GnRH-1 with a biphasic LH response (phases A and B). Therefore the LH responses to the 0.5μg dose of GnRH were analysed separately. The total quantity of LH released and the peak LH concentrations produced in response to the three doses of GnRH in response to GnRH-1(A and B) and GnRH-2 were compared between- and within-lines, by analysis of variance after transformation onto the log scale.

9.3. Results

9.3.1. Total LH released following GnRH stimulation.

The total quantity of LH released following both individual GnRH challenges, and the total from both challenges were calculated for each animal and the means calculated per line (Table 9.1).

Table 9.1. The geometric mean quantity of LH (ng ± s.e.m) released in response to the three doses of GnRH.

	TOTAL QUANTITY OF LH RELEASED (ng/ml ± sem)		
LINE	GnRH-1	GnRH-2	TOTAL
	LOW (0.5µg) dose of GnRH		
High (n=7)	1739 ± 376^{ay}	1758 ± 266^{cw}	2998 ± 587^{euy}
Low (n=7)	607 ± 85^{by}	640 ± 84^{dy}	1370 ± 185^{fyw}
	MEDIUM (5µg) dose of GnRH		
High (n=7)	5792 ± 684^{az}	5505 ± 856^{cx}	11351 ± 1476^{az}
Low (n=6)	1976 ± 403^{bz}	1460 ± 313^{dz}	3185 ± 633^{bx}
	HIGH (50µg) dose of GnRH		
High (n=7)	6964 ± 1050^{az}	5382 ± 662^{ax}	8957 ± 2290^{ev}
Low (n=7)	2367 ± 348^{bz}	1725 ± 166^{bz}	4154 ± 400^{fz}

Within each dose different superscripts indicate significant differences between each line, (a versus b, $P < 0.001$; c versus d, $P < 0.005$; e versus f, $P < 0.05$). Within line, between doses, different superscripts indicate significant differences, (u versus v, $P < 0.05$; w versus x, $P < 0.005$; y versus z, $P < 0.001$).

The magnitude of the LH responses between GnRH-1 and GnRH-2 did not differ significantly for any of the three doses of GnRH. However, small non-significant changes were seen between the LH responses to the two challenges which appeared to be related to the dose of GnRH. The LH responses in both lines increased between GnRH-1 and GnRH-2 following

the administration of 0.5μg of GnRH, but decreased between GnRH-1 and GnRH-2 following the administration of 50μg of GnRH.

The amount of LH released in response to the lowest dose (0.5μg) of GnRH was significantly less ($P < 0.001$) than that released in response to the medium and high doses of GnRH, which did not differ significantly between each other.

In response to each injection of GnRH the High line released significantly (at least $P < 0.005$, see figure legend) more LH than the Low line. The magnitude of the between-line difference in responses to all three doses of GnRH was slightly greater following GnRH-2 than GnRH-1.

The mean total quantity of LH released during the experimental period exhibited similar differences between lines and between doses to those observed with the individual challenges. The High line released significantly more LH than the Low line in response to all 3 doses of GnRH. Also the mean quantity of LH released within each line differed significantly ($P < 0.05$) between the low and medium dose and the low and high dose, with no significant difference between the medium and high doses of GnRH.

9.3.2. The LH response profiles following challenges with the low (0.5μg) dose of GnRH.

In response to the two GnRH challenges with the low dose of GnRH, monophasic LH responses were observed in the animals from both lines. Peak LH concentrations were reached approximately 8 minutes after the administration of GnRH-1 and 9 minutes after GnRH-2.

The mean peak LH responses per dose for the High and Low selection lines are shown in Table 9.2. No significant differences were seen between the LH responses to the two GnRH challenges in either selection line.

However, on both occasions the peak LH response in the High line was significantly (GnRH-1, $P < 0.005$; GnRH-2, $P < 0.05$) greater than the Low line.

Table 9.2. Geometric mean peak LH concentrations (ng/ml ± s.e.m) in response to two GnRH challenges with 0.5μg of GnRH.

	PEAK LH CONCENTRATIONS (ng/ml ± s.e.m)	
LINE	GnRH-1	GnRH-2
High (n=7)	51.40 ± 12.82^a	55.44 ± 15.68^c
Low (n=7)	19.21 ± 3.51^b	20.16 ± 2.99^d

Between lines different superscripts indicate significant differences, (a versus b, $P < 0.005$; c versus d, $P < 0.05$).

9.3.3. The LH response profiles following GnRH challenges with the medium (5μg) and high (50μg) doses of GnRH.

In the majority of animals, the LH response profiles produced in response to GnRH-1 with both the 5μg (High 5/7; Low 4/6) and 50μg (High 6/7; Low 6/7) doses of GnRH, were biphasic. However, in response to both doses of GnRH, the LH response profiles following GnRH-2 were monophasic. The first peak of LH secretion following GnRH-1 occurred approximately 6 minutes after the administration of GnRH and the second peak occurred approximately 10 to 40 minutes later. Following GnRH-2 the peak LH concentration was reached in both lines at a time intermediate to the two peaks of LH secretion seen following GnRH-1, approximately 11 minutes after the administration of GnRH. The mean peak LH concentrations (GnRH-1: A and B and GnRH-2) are presented in Table 9.3.

Within each line no significant differences were seen in the magnitude of the LH peaks released in response to the two doses of GnRH, or in the peak LH concentrations measured in response to the two GnRH

challenges (GnRH-1 and GnRH-2). Between lines, in response to the 5 and 50μg doses of GnRH, all three mean peak LH concentrations were significantly higher in the High line than the Low line.

Table 9.3. The geometric mean peak LH concentrations produced in response to GnRH-1 (biphasic, A and B) and GnRH-2 (monophasic).

	PEAK LH CONCENTRATIONS (ng/ml ± s.e.m)		
LINE	GnRH-1 Peak A	GnRH-1 Peak B	GnRH-2
Medium dose (5μg)			
HIGH (n=7)	73.34 ± 6.18^{a}	85.41 ± 4.95^{c}	114.56 ± 21.64^{c}
LOW (n=6)	39.47 ± 16.06^{b}	37.74 ± 12.29^{d}	40.63 ± 7.67^{d}
High dose (50μg)			
High (n=7)	59.47 ± 14.54^{a}	90.45 ± 11.95^{a}	74.78 ± 9.63^{a}
Low (n=7)	32.16 ± 6.64^{b}	34.23 ± 2.86^{b}	28.95 ± 2.78^{b}

Between lines different superscripts indicate significant differences, (a versus b, $P < 0.05$; c versus d, $P < 0.001$).

9.4. Discussion

The main findings of this study were firstly, that the 5μg dose of GnRH, the dose used in the initial selection programme, stimulates the maximal release of LH from the animals in both selection lines. Secondly, that the amount of releasable LH stored in the pituitary glands of the High line ram lambs at 10 weeks of age was significantly greater than that stored in the pituitary glands of the Low line. Thirdly, that despite a consistent difference in the magnitude of the LH responses, no differences were seen in the pattern of LH release between the two lines.

Within each line, there was no significant difference in the amount of LH released in response to the two GnRH challenges, irrespective of the dose of GnRH. However, trends were seen in the magnitude of the LH

response to the low and high doses of GnRH which are consistent with the known sensitizing (Crighton *et al.*, 1975; Crighton and Foster, 1977; Clarke and Cummins, 1982, Clarke *et al.*, 1984) and down regulating effects of GnRH stimulation (Rippel Johnson and White, 1974b; Crighton and Foster, 1977; Foster, 1978; Bremner *et al.*, 1980; Kanchev *et al.*, 1984). Small doses of GnRH, such as the 0.5μg dose of GnRH used in the present study, have been shown to increase the number of GnRH receptors in the pituitary gland and to induce a number of intracellular effects (see section 1.3.3.2.) which result in enhanced pituitary responsiveness to further GnRH stimulation and hence a larger LH response following a second GnRH challenge. In contrast, large doses of GnRH, such as the 50μg dose used in the present study, decrease pituitary responsiveness due to a reduction in GnRH receptor number, uncoupling of the GnRH receptor from the effector signal and inhibition of hormone synthesis (see section 1.3.6.).

The amounts of LH released in response to the 0.5μg GnRH challenges were significantly smaller in both lines than those produced in response to either the 5 or 50μg doses of GnRH. Therefore it can be concluded that the magnitude of the LH response to the low dose of GnRH was not maximal and was a function of both pituitary sensitivity and the dose of GnRH administered. As no significant differences were seen between the LH responses produced by the 5 and 50μg GnRH challenges the results demonstrate that a maximal LH response was achieved in response to both doses of GnRH and therefore provides a measure of the amount of releasable LH stored in the pituitary glands of the two lines. As the amount of LH released in response to the medium and high doses of GnRH following both GnRH-1 and GnRH-2 ($P < 0.001$) and during the whole of the experimental period (5μg, $P < 0.001$; 50μg, $P < 0.05$) were significantly greater in the High line than the Low line, the results demonstrate that the

pituitary glands of the High line contain significantly more stored releasable LH than the Low line.

It has been proposed that LH is stored in the pituitary gland in two pools, a 'readily-releasable' pool, which can be released immediately upon exposure of the pituitary gland to GnRH and a 'releasable pool' from which LH is released following a large or more prolonged exposure to GnRH. A biphasic pattern of LH release has been demonstrated in rats (Vilchez-Martinez, Arimura and Schally, 1976; Pickering and Fink, 1976a, 1979a, Baldwin, Ramey and Wilfinger, 1983), humans (Bremner and Paulsen, 1974; Yen *et al.*, 1974) and sheep (Bremner *et al.*, 1976) and has been proposed to occur due to the release of LH from these two pools, the first phase corresponding to the release of LH from the 'readily-releasable pool' and the second phase occurring due to the release of LH from the 'releasable pool'. Analysis of the LH profiles produced in response to GnRH-1 could be explained by the storage of releasable LH in 'two pools' within the pituitary gland. As in previous studies (Hopkinson *et al.*, 1974; Rippel *et al.*, 1974a; Chapter 6) exposure to a small discrete dose of GnRH (0.5μg) was followed, in both selection lines, by a monophasic pattern LH release, as the dose of GnRH did not stimulate the release of more LH than was stored in the readily-releasable pool. However, administration of the medium (5μg) and high (50μg) doses of GnRH, as with experiments using continuous infusion or large doses of GnRH (Bremner and Paulsen, 1974; Yen *et al.*, 1974; Vilchez-Martinez *et al.*, 1976; Bremner *et al.*, 1976), stimulated a biphasic LH response. The first phase of the LH response consisted of a peak of LH secretion which occurred within 10 minutes of the administration of GnRH, and was followed by a more extended period during which peripheral LH concentrations were elevated, the second phase.

According to the two pool proposal of Yen *et al.*, (1974) and Bremner and Paulsen (1974), the amount of LH released in response to a low dose of GnRH comes only from the LH stored in the readily-releasable pool and is therefore a function of the dose of GnRH. If the dose of GnRH stimulates the release of more LH than is present in the readily-releasable pool, then LH is discharged from the releasable pool, the magnitude of the second phase of the LH response increasing with the dose of GnRH, until a maximal LH response is achieved with the liberation of all the releasable LH. This proposal was substantiated in the sheep by the work of Kanchev *et al.*, (1984), who demonstrated that following exposure to two different doses of GnRH (1 and 50μg) the first phase of the LH response was of similar magnitude, but that the peak LH concentrations (second phase) differed in a dose dependent manner.

In the present study the two doses of GnRH which stimulated a biphasic pattern of LH release were both maximal, Therefore it was not possible to determine if the first phase of the LH response produced in response to the two doses of GnRH was of fixed magnitude or if the peak LH concentration reached during the second phase was controlled by the dose of GnRH.

In response to GnRH-2, irrespective of the dose of GnRH used, a monophasic LH response pattern was observed in both lines. The two pool theory would have predicted this result for the low dose of GnRH where the stimulated LH response did not exceed the magnitude of the 'readily releasable' pool of LH. However, in response to the two higher doses of GnRH a biphasic response would be expected, LH release occurring from both the 'readily-releasable' and 'releasable' pools of LH. Although the reason for the monophasic LH response following the second GnRH challenge is unknown, the phenomenon could be explained in a number of

ways. The relatively short time interval between the administration of two maximally stimulating doses of GnRH could mean that the pituitary glands have insufficient time to replenish their 'readily-releasable pools' of LH and therefore the second LH response consists of LH released from the releasable stores. If this were the case however, the time of the peak LH concentration following GnRH-2 would be expected to be similar to that for GnRH-1 B. However this was not the case, as peak LH concentration was in fact closer to the time of the first peak of LH secretion (GnRH-1 A). Alternatively, the pituitary glands may not have fully restored the 'readily-releasable pool' of LH and therefore its release in response to GnRH-2 is only seen as an accelerated increase following the release of LH from the releasable pool. The most plausible explanation however, comes from studies which indicate that newly synthesized hormones, including LH, may be released in preference to previously synthesized and stored hormones (Liu and Jackson, 1979; Halban, 1982; Morin, Rosenbaum and Tixer-Vidal, 1984; Ramay, Wilfinger, Highsmith and Baldwin, 1984; Ramey, Highsmith, and Baldwin, 1985) and the demonstration that GnRH stimulates gonadotrophin biosynthesis (Apfelbaum and Taleisnik, 1976; Khar, Debeljuk and Jutisz 1978; Liu and Jackson, 1978; Ramey *et al.*, 1984, 1985; Baldwin, Highsmith, Ramey and Krummen, 1986; Starzec *et al.*, 1988, 1989). Therefore following the stimulation of the pituitary gland with large doses of GnRH, LH biosynthesis is stimulated and following exposure to a second GnRH challenge it is this newly synthesised LH which is preferentially released, irrespective of the presence or absence of 'readily-releasable' and 'releasable' pools of stored LH. If this is the case then the two lines may differ in their ability to synthesize LH in response to stimulation with GnRH. This proposal is supported by studies of mRNA concentrations in the two lines (J. R. McNeilly, J. Clarke, N. P. Evans and R. Webb, unpublished

observations), which indicated that the concentrations of LHβ and FSHβ subunit mRNA were higher in the pituitary glands obtained from the High line than the Low line.

In conclusion, it would appear that the LH responses produced following the administration of 5 and 50μg of GnRH were maximal in both selection lines, and represent the amount of releasable LH stored within the pituitary glands of the two lines. Unknowingly therefore the selection programme was based on the amount of releasable LH stored in the pituitary glands of the two lines and has created approximately a 3-fold between-line difference in this character. Analysis of LH secretion profiles of the two lines, following the GnRH challenges with the three doses of GnRH, indicated that this releasable LH could be stored in 'two pools' within the pituitary glands of the two lines, and demonstrated that although the LH responses differed in magnitude no differences were seen in the pattern of LH release between the two lines.

CHAPTER 10. - General Discussion

Divergent selection, based on the LH response to a 5μg GnRH challenge in 10-week old ram lambs, has created two lines of sheep which differ in the selected character, in male (Haley *et al.*, 1989; Chapters 5, 9) and female lambs at 10 and 20 weeks of age (Haley *et al.*, 1989) and adult ewes (Chapter 3). Significant correlated between-line differences are also seen in both male and female lambs in the peripheral FSH concentrations following a GnRH challenge, and a number of aspects of female reproductive performance including the age of puberty in the ewe lambs and the ovulation rate during the first and at the start of the second breeding season (Haley *et al.*, 1989).

The work presented in this thesis has demonstrated that the between-line difference in the ability of the lambs from the two lines to secrete gonadotrophins, following a GnRH challenge, is present from at least 2 weeks of age and is maintained at a relatively constant level until the lambs are at least 20 weeks of age (Chapter 5). In the adult ewes, the between-line difference was present when the ewes where challenged during the follicular phase of the oestrous cycle but could not be confirmed during the luteal phase, due to differences in the timing of LH release between the two lines. Correlated between-line differences were also seen in the regulation of endogenous gonadotrophin secretion in the adult ewes (Chapter 3) and the prepubertal ram lambs (Chapters 4, 5). In the adult ewes, mean peripheral gonadotrophin and steroid concentrations were higher in the High line than the Low line, during both phases of the oestrous cycle, However the differences were not statistically significant. When LH pulse amplitude and frequency were studied, it was seen that during the follicular phase of the oestrous cycle the High line secreted LH pulses of

significantly greater amplitude than the Low line. In the prepubertal ram lambs no significant differences were seen in the mean steroid concentrations (Chapter 5), However the mean gonadotrophin concentrations were significantly greater in the High line than the Low line at all of the ages tested, except for FSH at 2 weeks of age. Detailed analysis of the patterns of LH secretion in the lambs at 2, 6, 10 and 20 weeks of age indicated that basal LH concentrations were significantly higher in the High line than the Low line during the early prepubertal period, but that this difference decreased with age, coincident with the increasing effects of gonadal negative feedback (Chapter 5). Significant differences were also noted in LH pulse amplitude between the two lines, which appeared to be the principal factor in the determination of mean LH concentrations during the early prepubertal period.

More specifically, the results of Chapter 5 demonstrated that the between-line differences were not caused by differences in the regulation of LH secretion by the gonads via gonadal negative feedback, but were principally the result of differences at the level of the hypothalamo-pituitary gland complex. However the expression of the between-line differences in endogenous LH secretion were affected by gonadal negative feedback, and therefore were only observed in the adult ewes during the follicular phase when progesterone negative feedback was reduced, and decreased in the ram lambs during the prepubertal period, with the increasing effects of age and gonadal negative feedback.

Two aspects of the regulation of LH secretion by the hypothalamo-pituitary gland complex were studied in Chapter 6; endogenous GnRH secretion, and the sensitivity of the pituitary gland to GnRH stimulation. As it was not possible to measure GnRH directly, a model system was developed in which the amplitude of endogenous GnRH pulses

were estimated using GnRH dose response curves and the known amplitudes of endogenous LH pulses. The estimated concentrations, when corrected for the dilution of GnRH in the peripheral circulation (as measured by Clarke *et al.*, (1984)), were similar to those obtained in sheep where hypophyseal portal blood was collected and the GnRH concentration assayed directly (Clarke and Cummins, 1982; Levine *et al.*, 1982; Clarke *et al.*, 1987). The model system therefore appears to provide an indirect method of estimating endogenous GnRH secretion. The results of this study indicated that the High line secreted significantly less GnRH than the Low line and that pituitary sensitivity, defined as the minimum dose of GnRH which must be exceeded for a pulse of LH secretion to be observed in the peripheral circulation, was significantly increased in the High line.

Pituitary sensitivity is a broad term encompassing a large number of variables which individually, or collectively, could create the observed between-line difference in pituitary sensitivity. Some of these variables were studied in Chapters 7, 8, and 9. Gonadotroph number/size was estimated indirectly as pituitary gland weight and found to be greater in the High line than the Low line. However the difference between the two lines was not statistically significant. A subsequent study (T. A. Bramley, N. P. Evans, J. R. McNeilly and R. Webb, unpublished observations) has demonstrated that this between-line difference in pituitary gland weight is increased in adult ewes, where the pituitary glands of the High line (0.86 ± 0.06g) were significantly ($P < 0.005$) heavier than the Low line (0.61 ± 0.04). The results of Chapter 7 also demonstrated that the pituitary glands of the High line ram lambs contained significantly more GnRH-receptors than the Low line, with a similar difference in the adult ewes (High, 13.49 ± 1.22pg/mg of protein; Low, 6.22 ± 1.65pg/mg of protein: $P < 0.005$).

Signal transduction was studied in cultured pituitary cells from the two lines as the amount of LH released in response to various stimulants: i) GnRH, which activates the GnRH receptor and the full endogenous intracellular secretory cascade, ii) a Ca^{2+} ionophore, A23187, which independently stimulates the Ca^{2+}-calmodulin second messenger system and iii) a phorbol ester, PDB, which independently stimulates the PKC second messenger system. A between-line difference was seen following stimulation of the cells with GnRH which could be explained by differences in GnRH receptor number, However the demonstration that the between-line difference was maintained at a similar level when LH secretion was stimulated independently of GnRH receptor activation (with A23187 and PDB) suggests that the two lines differ in the intracellular regulation of LH secretion at a step common to both second messenger systems and irrespective of GnRH receptor number. The differences in GnRH receptor number therefore could augment or alternatively occur as a result of the between line differences in the intracellular regulation of gonadotrophin secretion.

Studies of the releasable stores of LH (Chapter 9) demonstrated that the patterns of LH secretion following GnRH stimulation were the same in both selection lines and that the pituitary glands of the High line stored significantly more releasable LH than the Low line. The results of Chapter 9 also raised the possibility that the two lines may differ in their ability to synthesize LH, a possibility which is supported by studies of gonadotrophin subunit mRNA concentrations (J. R. McNeilly, J. Clarke, N. P. Evans, and R. Webb, unpublished observations) in which a trend existed for the concentrations of LHβ and FSHβ subunit mRNA to be higher in the pituitary glands obtained from the High line than the Low line

As 5μg of GnRH stimulates a maximal LH response in the 10 week old ram lambs from both selection lines (Chapter 9), it would appear that the initial selection programme was in effect selecting for differences in the size of the releasable pool of LH. The between-line differences observed in the LH responses following the administration of pharmacological doses of GnRH (Haley *et al.*, 1989; Chapters 3, 5, 9) indicate that the selection programme was successful in altering this character, and that the between-line difference is maintained irrespective of age or sex. The results presented in Chapters 6 and 9 indicate that in addition to the differences in the pituitary stores of LH, between-line differences also exist in the ability of the pituitary glands of the two lines to respond to physiological doses of GnRH (50, 100, 250 and 500ng). At these doses of GnRH the LH response is not primarily a function of the amount of stored releasable LH, but represents a combination of i) the dose of GnRH administered and ii) the ability of the pituitary gland to recognise the stimulatory signal and to respond and subsequently secrete LH. These between-line differences also appear to be present in the endogenous pattern of LH secretion in prepubertal ram lambs and adult females (Chapters 3 and 4), where LH pulse amplitude is increased in the High line at certain times.

Pituitary gland function, as described in section 1.3, can be regulated both positively and negatively by alterations in the pattern of GnRH secretion by the hypothalamus. The available information on the effects of GnRH on pituitary function, including the control of GnRH-receptor number, pituitary sensitivity, gonadotroph number/size and LH synthesis, indicate that the differences observed between the two lines in these characters could occur if endogenous GnRH secretion was elevated in the High Line relative to the Low line. However the results from Chapters 6 and 8 provide two lines of evidence in opposition to this proposal. Firstly, in

Chapter 6 GnRH secretion was measured indirectly and found to be reduced in the animals from the High line relative to the Low line. Secondly the *in vitro* LH responses observed in Chapter 8 differed between the two lines despite a 24 hour pre-incubation period, during which the cells from the two lines were exposed to identical concentrations of GnRH. To conclusively prove whether the between-line differences in pituitary gland function are due to differences at the pituitary gland or differences in the hypothalamic control of pituitary function, the direct concurrent measurement of GnRH and LH concentrations, in portal and peripheral blood samples would be necessary.

At the level of the pituitary gland, the alteration of a number of structural variables such as, gonadotroph number/size or the proportion of gonadotrophs relative to other pituitary cell types, could explain the observed effects of selection on pituitary function. These characters have not been measured in the two selection line, however it was noted in Chapter 7 that the pituitary glands of the High line were larger than the Low line and it was proposed that this difference in pituitary gland size could be related to differences in gonadotroph number or morphology. Between-line differences in these characters however, could not account for all of the differences observed. For example a difference in gonadotroph number could explain the between-line differences in the amount of stored LH, but could not explain the between-line differences in pituitary sensitivity to GnRH, the *in vitro* LH responses observed in Chapter 9 and the observed differences in GnRH receptor number (Chapter 7) unless the proportion of gonadotrophs relative to other pituitary cell types is increased. A difference in gonadotroph morphology, specifically gonadotroph size, could also explain the between-line differences in the ability of the pituitary glands to store LH and in the sensitivity of the

pituitary glands to GnRH, if this is caused by differences in GnRH receptor number. However it does not necessarily provide an explanation for the between-line differences in the *in vitro* responses to A23187 and PDB (unless these are due to differences in the amount of stored LH) or the possible between-line differences in the rate of LH synthesis. Therefore it would seem unlikely that the between-line differences in pituitary gland function are the result of differences in pituitary gonadotroph number or size. This conclusion is further supported by the relatively small difference between the lines in pituitary gland weight (1.26 fold) compared to the large differences in pituitary gland sensitivity and responsiveness to GnRH and GnRH receptor number (3- and 5- fold). The only remaining variable which could explain the observed differences in pituitary function is if the proportion of gonadotrophs within the pituitary gland differs between the two lines. However to confirm this, a detailed histological study of the pituitary glands of the two lines is required.

Although the primary effect of selection was to alter pituitary function by a direct effect at the level of the pituitary gland, selection also appears to have had an indirect effect on the hypothalamus. The estimated amplitude of the endogenous GnRH pulses secreted by the High line appears to be significantly less than the Low line. Therefore selection appears to have affected two aspects of LH secretion with opposing effects, the up-regulation of LH secretion by increasing pituitary sensitivity/responsiveness and the down-regulation of LH secretion by decreased GnRH secretion in the High line relative to the Low line. This apparent 'balance' in the regulatory control of endogenous LH secretion, if a true effect, could explain why the largest between-line differences are observed when the animals are challenged with pharmacological doses of GnRH whereas the differences observed in endogenous gonadotrophin

secretion are transient, and are only observed during periods when the regulatory control of gonadotrophin secretion is subject to change. These periods include the prepubertal period, when gonadal feedback increases dramatically and the hypothalamo-pituitary complex becomes increasingly sensitive to gonadal feedback and in the adult ewe during the follicular phase when progesterone negative feedback is removed and the hypothalamo-pituitary gland complex becomes increasingly sensitive to oestradiol positive feedback. This balance in the control of LH secretion may also explain why the between-line differences observed in reproductive performance are also transient, occurring at puberty and at the start of the breeding season, when the regulatory control of gonadotrophin secretion is subject to change, and why the changes in the reproductive characteristics are small relative to the change in the selected character.

In conclusion, the effects of indirect selection on the LH response to a 5μg dose of GnRH are not limited by either age or sex, and are not limited to the selected character. Selection based on the amount of releasable LH stored in the pituitary glands of the two lines has created between-line differences in the selected character, which would be expected, but significant between-line differences are also present in the sensitivity of the pituitary glands of the two lines to stimulation with GnRH. Specifically, selection has affected pituitary gland weight, GnRH-receptor number, the ability of the pituitary glands to release LH following activation of the Ca^{2+}-calmodulin and PKC intracellular second messenger systems, the amount of stored LH and possibly the rate of gonadotrophin synthesis. These differences in pituitary structure and function appear to occur due to the effects of selection on the pituitary gland itself, However the exact site of the selected difference/s remains to be elucidated. The

characterisation of the between-line difference in pituitary gland function has demonstrated that selection has created a unique model with which to study the regulation of gonadotrophin synthesis and secretion in the sheep.

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APPENDIX 1

Estimations of litter size in a range of different breeds of sheep.

Breed	Litter Size	Reference
Finnish Landrace	3.0	Donald and Read, (1967)
	3.0	Land, Russell and Donald, (1974)
	3.0	Timon, (1969)
	2.6	Harrahan and Quirke, (1985)
	2.55	Young, Dickerson and Fogarthy, (1985)
Romanov	2.38	Smirnov, (1935)
	2.83	Desvignes and Lefevre, (1970) cited by Bradford (1985)
	2.6	Ricordeau, Razungles and Lajous, (1982)
Scottish Blackface	1.85	Wiener, (1967)
	1.29	Gunn and Robinson, (1963)
Lincoln	1.56	Wiener, (1967)
Welsh Mountain	1.40	Wiener, (1967)
Tasmanian Merino	1.0	Land et al., (1974)
D'Man	2.1	Lahlou-Kassi and Marie, (1985)
Rambouillet	1.58	Young et al., (1985)
Suffolk	1.62	Young et al., (1985)
Targhee	1.51	Young et al., (1985)

APPENDIX 2

Estimations of ovulation rate in a range of different breeds of sheep.

Breed	Ovulation Rate	Reference
Welsh mountain	1.47	Bradford, (1972)
Border Leicester	2.00	Bradford, (1972)
	2.25	Moore, (1968)
Merino	1.27	Moore, (1968)
	1.06	Land et al., (1974)
	2.68	Bindon et al., (1978)
	1.18	Bradford (1972 citing McGwirk, 1970)
Finn	3.40	Bradford, (1972)
	3.31	Bradford, Quirke and Hart, (1971)
	2.96	Land et al., (1974
	4.11	Hanrahan, (1974a)
	3.50	Hanrahan and Quirke, (1985)
Berrichon	1.13	Land, Pelletier, Thimonier, and Mauleon, (1973)
Romanov	2.57	Land et al., (1973)
	2.86	Bindon, Blanc, Pelletier, Terqui and Thimonier, (1979)
	3.40	Ricordeau et al., (1982)
Solognote	1.13	Land et al., (1973)
Scottish Blackface	1.33	Land, (1970a)
D'Man	2.85	Lahlou-Kassi and Marie, (1981)
Timahdite	1.09	Lahlou-Kassi and Marie, (1985)
Galway	1.57	Hanrahan (1974b)
Hu Yang	3.11	Guo,Ding, Shi, Zong, Jiang, Wang, Xu and Zhu, (1981)