Dynamics of Follicle Growth in the Sheep

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Declaration

Except where acknowledgement is made by reference the studies undertaken in this thesis were unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

C.J.H. de Souza
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Abstract

The terminal differentiation of ovarian follicles is acutely regulated by gonadotrophins, the secretion of which is carefully controlled by steroid and peptide hormones produced by the follicles in a short term feed-back system. The endocrine changes during the oestrous cycle and anoestrous season have been extensively documented in sheep, but very little is known about the growth dynamics of large antral follicles and its hormonal regulation. In many species, correlation of the dynamics of follicle growth as measured by serial ultrasound with the endocrine environment has increased the understanding of the factors regulating terminal stages of follicular development.

Using ewes with an ovarian autotransplant, the diameter of individual follicles and corpora lutea was measured by ultrasound scanning, to characterise the pattern of development of antral follicles over 2.5mm, in relation to gonadotrophin and ovarian hormone concentrations in jugular and ovarian venous blood. Studies were conducted to investigate the effects of season, age and the action of a major gene involved in increased prolificacy in sheep (FecB) on the dynamics of follicular growth and its hormonal regulation.

The results showed that the final stages of maturation of dominant follicles in sheep during the oestrous cycle and anoestrous occurs in a succession of waves of follicular growth. During the luteal phase 3 waves emerge on days 2, 7 and 11 post-LH surge. During the follicular phase, most of the follicles that ovulate after the LH surge originate from follicles above 5mm in diameter at the time of luteal regression, but additional smaller follicles can be recruited during the follicular phase. During the follicular and early luteal phase, when LH pulse frequency is elevated, follicles achieved dominance by secreting large amounts of oestradiol and inhibin A, depressing FSH concentrations below threshold levels. During the second and third waves of follicular development of the luteal phase a similar pattern of FSH secretion was evident but the relationship between the secretion of oestradiol and inhibin A was less clear. Despite these endocrine differences, the mechanism of follicular selection operated in each of these waves as the number of dominant follicles was similar to the ovulation rate. Similar investigations in ewes carrying the Booroola FecB gene showed that gene carrier ewes produce more follicles, that mature earlier at smaller size, without any differences in the concentration of FSH and the ovarian secretion of oestradiol, androstenedione and inhibin A compared to non-carrier animals. This suggests that the Booroola gene acts at an ovarian level modulating the gonadotrophin signal to increase the number of follicles selected. In order to study the effect of age in
pituitary-ovarian function, the dynamics of follicular diameter were studied in old ewes (12-13 years) with ovarian autotransplants and the results of gonadotrophin and ovarian hormone concentrations compared to those obtained from the same animals 6 years previously. Although the basal levels of FSH were raised in the older animals, the pattern of secretion of ovarian and pituitary hormones was similar to younger animals (6-7 years). The ovarian secretion of oestradiol was normal but there was a significant reduction in the secretion of inhibin A presumably reflecting the reduced population of antral follicles in the ovaries.

These results demonstrate the temporal relationship between the development of antral follicles beyond 2.5mm in diameter and the fluctuations in the concentration of FSH, which are regulated by the secretion of oestradiol and inhibin A by dominant follicles when under the influence of high LH pulse frequency. They also illustrate, in ewes carrying the FecB gene, how similar concentrations of gonadotrophins can be modulated by intraovarian factors to produce markedly different ovulation rates. Furthermore, they suggest that the secretion of inhibin A in sheep is related to the follicular population in the ovary, and its reduction in animals with advanced age is reflected by an increase in the levels of FSH in a similar fashion to that reported in peri-menopausal women.
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The development of a follicle to the pre-ovulatory stage in sheep has been estimated to be about 6 months, however the final stages of follicular maturation can be accomplished in as little as 2 days. The terminal differentiation of the follicle is acutely regulated by gonadotrophins, which are carefully controlled by steroid and peptide hormones produced by the follicles in a short term feed-back system.

Although the endocrine changes during the oestrus cycle and anoestrous season have been extensively documented in sheep, very little is known about the consequent growth dynamics of large antral follicles and its hormonal regulation. Ultrasound monitoring of follicle dynamics, has been applied successfully in several species and allows acquisition of longitudinal information of follicle growth that can be related to the endocrine environment, resulting in a better understanding of the regulation of terminal follicle development.

1.1 Folliculogenesis

The process of folliculogenesis in sheep begins early in fetal life, when around 30 days after conception most of the germ cells have migrated to the genital ridge and gonadal sex differentiation occurs (Mauleon, 1978). The germ cells proliferate through mitoses from day 23 to day 120 of gestation (McNatty et al., 1995). The maximum number of germ cells is observed on day 75, and from then to day 90 most (80%) of the germ cell population is lost by atresia resulting in a reduction in ovarian weight (McNatty et al., 1992b).

The arrangement of oocytes into a follicular structure begins on day 75 with the formation of the first primordial follicle, which consists of the oocyte surrounded by one layer of flat pre-granulosa cells. By day 100 most of the germ cell population, about 170,000 cells, are present as isolated oocytes (61%), followed by 28% arranged as primordial follicles, 8% as oogonia and 1% as primary follicles (with one layer of cuboidal granulosa cells). The germ cell differentiation started on day 55 is completed by day 120 when almost (98%) all have completed meiosis, with the majority being present as primordial follicles (51%), 48% as isolated oocytes and 1% as secondary follicles (up to three layers of granulosa cells). Around day 135, just prior to birth (term=145), almost all of the differentiated germ cells are contained within primordial follicles (91%), 4% as growing follicles (secondary and tertiary follicles up to 0.8 mm in diameter) and the remainder as isolated oocytes (McNatty et al., 1995).
In newborn ewes the ovaries are very active containing several pre-antral and antral follicles (Kennedy et al., 1974), this high level of ovarian activity result in a 10 fold increase in the ovarian mass from birth to 3 months of age, when the first antral follicles of preovulatory size (around 5 mm in diameter) are observed (McNatty et al., 1987b).

Table 1.1 Number of oocytes and follicles in the fetal sheep ovaries at different gestational stages. Adapted from McNatty et al., (1995).

<table>
<thead>
<tr>
<th>day of gestation</th>
<th>Germ cells number (x 10^3)</th>
<th>% of isolated oocytes</th>
<th>% primordial follicles</th>
<th>% growing follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>170</td>
<td>61</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>120</td>
<td>205</td>
<td>48</td>
<td>51</td>
<td>1</td>
</tr>
<tr>
<td>135</td>
<td>82</td>
<td>5</td>
<td>91</td>
<td>4</td>
</tr>
</tbody>
</table>

In young adult ewes (2 years of age) the reserve of follicles is between 12000 and 86000 primordial follicles with between 100 and 400 follicles with more than two layers of granulosa cells, at various stages of development (Cahill et al., 1979). The time for a follicle to evolve from the secondary to the preovulatory stage has been estimated to be approximately 6 months (Cahill and Mauleon, 1980) and can be divided on the basis of the functional stage of the follicles into 5 groups: primordial, committed, gonadotrophin-responsive, gonadotrophin-dependent and ovulatory follicles (Scaramuzzi et al., 1993).

1.1.1 Primordial Follicles

The primordial follicles are the stock of follicles formed during fetal life, that will begin development as soon as they are formed or most commonly several years later during the reproductive life of the ewe. These follicles are characterised by an oocyte arrested in the first meiotic prophase, devoid of zona pellucida, surrounded by a single layer of flat pre-granulosa cell contained by the basement membrane. At this stage the follicles do not have specific blood supply so the nutrients are delivered by diffusion (Hirshfield, 1991).

1.1.2 Committed Follicles

Once the follicles resume growth they cannot return to the quiescent state and are committed to grow. Normally these follicles will become atretic at some stage of their development but exceptionally few will complete development and ovulate. The
triggers for follicle development from the primordial pool are largely unknown, but is believed to occur independently of gonadotrophic input as it has been observed in hypophysectomised animals and anencephalic fetuses (McNatty et al., 1992b). Some of the putative factors potentially involved in recruiting primordial follicles into the growing pool have been recently reported, like the growth/differentiation factor-9 (GDF-9) a member of the transforming growth factor-beta superfamily expressed exclusively in oocytes at all stages of follicular development, except in primordial follicles, but disappearing by 1.5 days after fertilisation (Mcgrath et al., 1995). Another of those factors is basic fibroblast growth factor (bFGF, FGF2) which has been demonstrated by immunohistochemistry in the cytoplasm of oocytes from primordial and primary follicles and in most healthy antral follicles. It has been suggested that bFGF may activate follicle growth via stimulation of granulosa cell proliferation and follicular basement membrane synthesis (Vanwezel et al., 1995).

Recently, the importance of GDF-9 in folliculogenesis was demonstrated by the fact that follicular development beyond the stage of one layer of granulosa cell was blocked in knockout mice, leading to complete infertility. In these animals the growth of the oocyte and zona pellucida proceeded normally but oocyte differentiation was compromised (Dong et al., 1996).

The initial signs of growth resumption are RNA synthesis and proliferation of the surrounding squamous granulosa cells followed by an increase in oocyte size. The oocyte enlarges rapidly reaching full size early in follicular development long before the granulosa cells stop proliferating (Hirshfield, 1991). The granulosa cells, initially with a squashed shape become cuboidal as they develop and express mRNA for FSH receptor and βB-inhibin/activin subunit at the time that the second layer of cells emerges (Tisdall et al., 1995; Eckery et al., 1996). Subsequently the thecal cells evolve from the neighbouring stromal cells and when the follicles have more than 2-4 layers of granulosa cells they start to show expression of the α inhibin subunit and follistatin genes (Eckery et al., 1996).

In adult sheep at any given time there can be up to 400 committed or pre-antral follicles. They range in diameter from 0.03 mm to 0.1 mm and contain up to 5 thousand granulosa cells at the largest size (Cahill and Mauleon, 1980; Cahill, 1981).

1.1.3 Gonadotrophin-Responsive Follicles

The gonadotrophin-responsive follicles range between 0.1 to 2.5 mm and are also known as late pre-antral and small antral follicles. They have LH and FSH receptors on theca and granulosa cells, respectively, although gonadotrophin input is not essential for their development (Cahill, 1981; McNatty et al., 1990a). Nevertheless
granulosa cells from follicles beyond 0.1 mm in diameter can generate a second messenger (cAMP) response when stimulated by FSH in vitro (McNatty et al., 1992b).

As the follicles grow their responsiveness to gonadotrophins increases and they secrete progesterone and androgen when cultured in vitro (McNatty et al., 1986a). However, they do not secrete detectable amounts of oestradiol in vitro until they reach a diameter greater than 0.3 mm (McNatty et al., 1986a; McNatty et al., 1992b). Although follicles about the stage of antrum formation have the potential to synthesise all major ovarian steroids, they do so in very small amounts, and hence early follicular development is likely to occur independently of endogenous derived steroids (Eckery et al., 1996).

The formation of the fluid filled antral cavity occurs at a diameter of 0.2-0.4 mm in fixed ovaries (Turnbull et al., 1977; Cahill and Mauleon, 1980) and soon after its formation the follicles engage in a period of rapid growth marked by increased cell proliferation which is reflected by a high mitotic index between 0.7 and 1.5 mm declining at a diameter beyond 2.2 mm (Turnbull et al., 1977; Cahill and Mauleon, 1980). It has been estimated that, on average, a follicle takes about 5 days to grow from 0.5 to 2.2 mm in diameter (Turnbull et al., 1977).

In the adult sheep, the time for a follicle to evolve to the antral stage is thought to be 130 days, based on histological estimation (Cahill and Mauleon, 1980), an estimation recently confirmed in vivo in ewes with ovarian autograft (Gosden et al., 1994). After antrum formation the speed of growth increases and is estimated that 34-43 days are need to reach a size over 5 mm in diameter (Turnbull et al., 1977; Cahill and Mauleon, 1980). Early atresia is rarely observed in follicles smaller than 1 mm in diameter, whereas the greatest incidence of atresia is found in follicles ranging between 1.5 to 2.5 mm.

1.1.4 Gonadotrophin-Dependent Follicles

The requirement of gonadotrophins for follicular growth beyond 2.5 mm in diameter, has been demonstrated in several models such as hypophysectomy (Dufour et al., 1979), active immunisation against GnRH (McNeilly et al., 1986) or chronic GnRH-agonist infusion (McNeilly and Fraser, 1987). Of the gonadotrophins, FSH alone has been shown to be capable of stimulating pre-ovulatory follicle development, whereas LH alone cannot. While low amplitude LH has little effect on FSH-induced follicle development (Campbell et al., 1995), large amplitude LH pulses are able to inhibit FSH-stimulated follicle development by an undetermined mechanism (McNeilly et al., 1991).
The acute requirement of the gonadotrophin-dependent follicles for FSH is a characteristic that distinguish them from the gonadotrophin-responsive and ovulatory follicles and makes them particularly susceptible to atresia, perhaps accounting for the variable numbers of follicles in that stage (Scaramuzzi et al., 1993). During this stage the surviving follicles double their size in around 4 days. This rapid increase in volume is due almost exclusively to accumulation of follicular fluid as the rate of cell proliferation declines progressively to negligible amounts after a diameter of 3.5 mm (Turnbull et al., 1977; Cahill and Mauleon, 1980). As the mitotic rate declines, granulosa and theca cell differentiation occurs, which is reflected in an increase in the responsiveness to FSH measured as cAMP production by the granulosa cells, which increases 2-10 fold as the follicles progresses from 2.5 to 6 mm (Henderson et al., 1987).

The mechanisms that result in the selection of an ovulatory follicle from a numerous cohort are poorly understood but involve complex inter-relationship between pituitary gonadotrophins and intra-follicular factors that modulate the responsiveness of the follicular cells to gonadotrophins. These mechanisms will be dealt with in detail later in this chapter.

This phase of the development of the follicle is crucial since it is during this time that synchronisation of terminal follicular development with the oestrous cycle takes place to enable the ovulatory follicle to fulfil its purpose; that is to release a viable oocyte in a suitable environment at the right timing to maximise the chances of conception.

1.1.5 Ovulatory Follicles

The oestrogenic or activated follicles are also designated dominant follicles, for their ability to withstand atresia and get to final stages of maturation rather than inflict direct actions on contemporaneous follicles (Driancourt, 1994). These follicles have the potential to became ovulatory follicles if exposed to a suitable endocrine environment, especially a pattern of high LH pulse frequency, which will be discussed later in the review.

The main characteristic of this class of follicle is the expression of LH receptors in the granulosa cell layer (Carson et al., 1979; Webb and England, 1982a) and high concentrations of oestradiol in the follicular fluid (Webb and England, 1982b). These follicles secrete over 80% of the oestradiol and are also responsible for 55% of the immuno-reactive inhibin released in to the circulation (Campbell et al., 1991a). The changes in intracellular architecture of these follicles also reflect their high capability of hormone production, as they show a 3-fold increase in the area of smooth
endoplasmic reticulum, 5-fold increment in the golgi cisternae area and double the inner mitochondrial membrane area when compared to early antral follicles (McNatty et al., 1992b).

1.2 Gonadotrophins

The anterior pituitary secretes two gonadotrophins, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). Both are heterodimeric glycoproteins containing a common α-subunit and a hormone specific β-subunit (Gray, 1988). The synthesis and secretion of these hormones are under primary control of Gonadotrophin Releasing Hormone (GnRH) which is under the influence of central aminergic and neuromodulatory systems (Clarke, 1996). Additionally, gonadotrophin secretion can be modulated by direct action on the pituitary by gonadal peptides and steroids regulating the expression of GnRH receptors or transcription and translation of gonadotrophin genes (Haisenleder et al., 1994; Brooks and McNeilly, 1996). Although both gonadotrophins are made in the same cell they are secreted in a different manner; LH is secreted in a pulsatile fashion in response to an increase in intracellular Ca+ generated by the binding of GnRH to its receptor in the gonadotroph. FSH is mainly secreted in constitutive way with most of the hormone being secreted as it is produced but a minor portion can be stored and released along with LH in response to GnRH (Farnworth, 1995). Less than 10% of the pituitary content of LH is released during each 24h period compared with 60-80% of the FSH content during the same period (McNeilly, 1988).

The gonadotrophins act on their target cell by binding to hormone specific receptors on the cell surface. These receptors are characterised by an extracellular domain with the N-terminal region responsible for ligand binding and seven transmembrane domains that anchor the receptor to the membrane surface. The intracellular domain is coupled to a G-protein to activate intracellular signalling. The intracellular C-terminal region is not necessary for hormone binding and signalling but is involved in receptor recycling (Catt, 1996b). The binding of gonadotrophin to the receptor activates the associated G proteins by promoting the conversion of GDP to GTP, which binds to its α subunit, stimulating adenylate cyclase to generate the second messenger cAMP. Cyclic AMP in turn triggers a cascade of phosphorylation in the cyclic AMP dependent kinases (PKA), thus controlling multiple aspects of cell function through phosphorylation of protein substrates. Once the receptor-ligand interaction has taken place, the complex is internalized by endocytosis and degraded by lysosomes. The receptor is then recycled back to the membrane by exocytosis (Catt, 1996a).
1.2.1 Control of Gonadotrophin Secretion

In sheep, FSH secretion, although controlled by GnRH, appears to be continuous and not acutely responsive to GnRH, resulting in non-pulsatile secretion when measured in the peripheral circulation. Tonic FSH secretion is under the negative feedback control of both oestradiol and inhibin (Baird et al., 1991) suppressing mRNA transcription at the pituitary gland (Martin et al., 1988; Miller and Miller, 1996). FSH secretion exhibits cyclic variations during the oestrous cycle (Bister and Paquay, 1983; Campbell et al., 1991b; Ginther et al., 1995) which have been assumed to reflect the hormonal changes that occurs during the development and regression of large antral follicles. Direct evidence for this hypothesis, however, is lacking. The action of FSH may also be regulated by the pattern of secretion of the distinct isoforms of the hormone that appear to have different bioactivity (Phillips et al., 1994).

LH secretion is acutely regulated by the pulsatile release of hypothalamic GnRH into the portal circulation which results in the corresponding pulsatile release of LH by the pituitary (Clarke and Cummins, 1982). The ovarian feedback regulation of LH secretion is due to the action of steroids, namely progesterone and oestradiol (Martin et al., 1988). The feedback system varies in importance according to the season and phase of the oestrous cycle (for review see Goodman, 1994). Briefly, during the anoestrous season oestradiol alone is capable of reducing the LH pulse frequency due the interaction of low melatonin concentrations. In cycling ewes, during the follicular phase oestradiol in the absence of progesterone enhances LH pulse frequency and reduces pulse amplitude triggering the LH surge. In the early luteal phase progesterone acts synergistically with oestradiol in the control of LH pulse frequency, whilst during mid/late luteal phase the higher concentrations of progesterone are alone capable of regulating LH pulse frequency.

The release of GnRH is regulated by the gonadal steroid hormones, oestradiol and progesterone, which alter the characteristics of GnRH secretion during the oestrous and seasonal cycles. These steroids cannot exert their actions directly on the GnRH neurones as they do not possess steroid hormone receptors. Therefore, some other steroid-sensitive neuronal system must relay this information to the GnRH neurones. Gamma amino-butyric acid (GABA) neurones could fulfil this role as they contain steroid hormone receptors and synapse on GnRH neurones. GABA concentrations have been shown to fall when GnRH release is being stimulated by oestradiol during the pre-surge period, but to be increased when progesterone is depressing GnRH release. This inhibitory neurotransmitter may also be important in
mediating the seasonal switch in the negative feedback actions of oestradiol. During the anoestrous season, when oestradiol is a potent inhibitor of GnRH secretion, specific GABA receptor antagonists can stimulate neurohormone release, an action that is not observed in the breeding season when oestrogen is much less potent (Robinson, 1995).

1.3 The Oestrous Cycle

The sheep is a seasonal polyoestric animal, characterised by periods of sexual activity during the short days (autumn and winter) with regular interval oestrous cycles. During the long days (spring and summer) it is marked by sexual inactivity although follicular development is present throughout the year (Cahill and Mauleon, 1980). The oestrous cycle of the ewe lasts for around 17 days, with the day of onset of oestrous being designated day 0, with ovulation occurring on day 1. The cycle is normally divided into the luteal phase, that lasts from ovulation to luteolysis on day 14, and the follicular phase that comprises the 3 day periovulatory period from luteolysis to ovulation on day 1 (Baird and McNeilly, 1981).

1.3.1 The Luteal Phase

The luteal phase is characterised by an ovary bearing at least one corpus luteum (CL), which has evolved from the ovulatory follicle. As the luteal phase progresses the CL produces increasing amounts of progesterone, with the plasma concentrations reaching a plateau between days 6 and 12 (Pant et al., 1977). LH secretion between day 1 and 3 consists of low amplitude high-frequency pulses (1 pulse/h) (Campbell et al., 1990a) which increase in amplitude as the frequency decreases to a pulse every 2-4 h under the influence of increased production of progesterone by the CL. LH pulse frequency during the remainder of the of the luteal phase consists of low frequency (pulse/4h) high amplitude (4-6 ng/ml) pulses (Baird et al., 1976). FSH secretion is not affected by progesterone secretion (Martin et al., 1988; Mann et al., 1992) but is regulated by oestradiol and inhibin produced by the follicles that develop during this period of the cycle.

After the LH surge all large antral follicle that do not ovulate become atretic (Turnbull et al., 1977), and in consequence, the secretion of ovarian steroids is at its lowest levels on day 1 of the cycle (Baird and McNeilly, 1981). Immuno-reactive inhibin, however, reaches a peak at this stage of the cycle (Campbell et al., 1990b), a phenomenon which may be associated with the rupture of the ovulatory follicle and release of inhibin from the follicular fluid, as low levels of inhibin β-subunit gene expression are observed at this time (Engelhardt et al., 1993). The very low levels of
oestradiol are associated with a sharp rise in FSH concentration (the second FSH surge), which is followed by increasing amounts of oestradiol and inhibin secretion which in turn suppress the concentration of FSH (Baird et al., 1991).

On the basis of evidence from a number of techniques it is thought that the development and demise of large antral follicles occur several times throughout the luteal phase. The analysis of ovarian follicle population by histological methods demonstrated the occurrence of large antral follicular development during the luteal phase, but failed to agree on its pattern or regulation (Smeaton and Robertson, 1971; Brand and De Jong, 1973; Turnbull et al., 1977). These follicles secrete oestradiol and androstenedione in response to LH stimulation (Baird and Scaramuzzi, 1976; McNatty et al., 1981; Campbell et al., 1990b) and are responsible for asymmetric secretion of oestradiol from each ovary (Baird and Scaramuzzi, 1976), which has been assumed to be responsible for the cyclic variation in FSH concentration with a period of 4-5 days throughout the luteal phase (Bister and Paquay, 1983; Campbell et al., 1991b). Recently, more direct evidence has emerged from studies utilising transrectal ultrasonography, although the controversy on the regulation remains, with reports of random emergence of follicular growth (Schrick et al., 1993; Ravindra et al., 1994) or the emergence of 2-6 waves of follicular development associated with fluctuations in FSH (Ginther et al., 1995).

In sheep the CL secretes progestagens almost exclusively and unlike primates, does not secrete oestradiol (Baird et al., 1975) or inhibin (Mann et al., 1989; Engelhardt et al., 1993). The presence of high peripheral concentrations of progesterone during the luteal phase maintain low LH pulse frequency, reducing the secretion of androstenedione and consequently oestradiol by large antral follicles. As a result, while large antral follicles are present during this period, ovulation does not take place as there is insufficient oestradiol secretion to generate an LH surge. Progesterone also appears to have a direct role in inhibiting the positive action of oestradiol in generating the LH surge in ovariectomized ewes (Scaramuzzi et al., 1971). However the follicles that mature during the luteal phase are capable of ovulating, if the appropriate gonadotrophic (hCG) signal is given (Driancourt et al., 1990). In addition to controlling LH secretion, progesterone helps regulate the length of the luteal phase by down-regulating the expression of uterine epithelial oxytocin receptor, thus modulating oxytocin-induced PGF2α release (Wathes and Lamming, 1995). The rate of secretion of prostaglandin is also controlled by the action of oestradiol after day 12 of the cycle, stimulating the oxytocin mediated PGF2α release (Beard et al., 1994), an action that is probably due to the up-regulation of oxytocin receptors in the caruncular stroma and deep uterine glands (Wathes and Lamming,
Furthermore, progesterone also has a behavioural role in priming the animal to express oestrous behaviour in response to the rise in oestradiol during the follicular phase (Fabre-Nys and Martin, 1991).

1.3.2 Follicular Phase

The fall in plasma progesterone after luteolysis leaves oestradiol alone unable to inhibit LH pulse frequency (Goodman et al., 1981). By 24 hours after the decline in peripheral progesterone concentrations the interval between LH pulses is around 60-75 minutes and pulse amplitude around 1 ng/ml (Baird, 1978; Baird et al., 1981). This increase in pulse frequency, via GnRH secretion, is accompanied by a reduction in pulse amplitude which is mediated by an oestradiol-induced reduction in pituitary responsiveness to GnRH (Baird et al., 1981; Thiery and Martin, 1991). Despite the low LH pulse amplitude, the ovary responds to each of the LH pulses with a pulse of androstenedione and oestradiol secretion, which is thought to be primarily derived from the mature follicles present at the time (Baird, 1978; Campbell et al., 1990a). As LH pulse frequency increases there is an increase in LH mean concentrations and a corresponding rise in the secretion of the ovarian steroids (Baird et al., 1976; Baird et al., 1981; Campbell et al., 1990b). During this period the ratio of androgen to oestradiol decreases, presumably as a result of increased aromatization of androgens by the preovulatory follicles. The increase in oestradiol secretion during the follicular phase results in a fall in FSH concentration (Campbell et al., 1990b) possibly preventing the recruitment of further follicles. Eventually oestradiol secretion increases to a level sufficient to induce expression of oestrous behaviour (Scaramuzzi, 1975; Fabre-Nys and Martin, 1991) and trigger the LH surge. The LH surge is characterised by a rapid increase from basal concentrations to a peak of 20-100 ng/ml within 4-8 h, with concentrations returning to basal levels around 10 hours later, resulting in a surge of approximately 10-18 hours in duration (Land et al., 1973a; Thiéry and Martin, 1991). The LH surge occurs within 4-8 hours of the onset of oestrus, and ovulation occurs approximately 24 hours after the onset of the surge (Cumming et al., 1973) or around 32 hours from the onset of oestrus (Souza et al., 1994).

The so called positive feedback of oestradiol profoundly influences GnRH secretion during the follicular phase of the estrous cycle of the sheep. Oestradiol not only regulates the frequency and amplitude of GnRH pulses, but also produces qualitative changes in its pattern of release and induces a sustained GnRH surge during which discrete pulses are not readily evident. This consists of a progressive change from a strictly episodic pattern of GnRH release to one containing both episodic and non-episodic components followed by a period of extremely high values
during which individual episodic increases are no longer recognisable (Evans et al., 1995). The increase in GnRH secretion during the follicular phase is also accompanied by an increase in both mRNA expression and number of GnRH receptors. This increase is regulated primarily by oestradiol in the early follicular phase and by GnRH itself during the periovulatory period (Turzillo et al., 1995; Brooks and McNeilly, 1996). Generation of the GnRH surge seems to follow a characteristic progressive change in the pattern of GnRH in portal blood. High concentrations of oestradiol initially stimulate the secretion of GnRH between pulses; this is followed by augmentation of both pulsatile and interpulse GnRH release producing the rising limb of the surge. The GnRH surge is high in amplitude; the amount secreted increases on average more than 40 times above the pre-surge baseline value. The initial increment in GnRH secretion precedes or coincides with the onset of the LH surge but the GnRH surge lasts far longer than the preovulatory LH surge. A molecular variant of GnRH, which is less active biologically than native GnRH, is co-secreted at the time of the surge, but termination of the LH surge cannot be accounted for by a change in biological activity of the secreted GnRH (Caraty et al., 1995).

1.3.3 Anoestrus

The anoestrous season results from a change in oestradiol feedback, induced by photoperiod, so that oestradiol alone is capable of suppressing LH secretion. The changes in daylight are perceived by the retina and transduced to a neural signal transmitted to the pineal gland. The pineal responds with secretion of melatonin which increases shortly after the onset of darkness and remains elevated until the beginning of the light phase, creating a circadian rhythm of hormone in response to changes in photoperiod (Karsch et al., 1984). During short days (high melatonin), oestradiol restrain the amplitude of the LH pulses but has very little effect on pulse frequency. But on a low melatonin environment (long days) it is a potent suppresser of LH pulse frequency, an action that is exerted at the hypothalamus. Experiments involving sampling of hypophyseal portal blood have shown that during the breeding season, oestradiol had no effect on either the frequency or size of GnRH and LH pulses. During anoestrus, however, it produced a profound suppression of GnRH and LH pulses frequency, and an increase in GnRH pulse size (Karsch et al., 1993). LH secretion in the anoestrous season consists of low frequency pulses (every 4-16 hours) of high amplitude (5-7 ng/ml) (Scaramuzzi and Baird, 1977).

The control of FSH secretion during anoestrus appears to be similar to that observed during the follicular phase with oestradiol and inhibin acting synergistically at the level of the pituitary (Martin et al., 1988). Anoestrus FSH concentrations have
been reported to be lower (Findlay and Cumming, 1976), similar (Bister and Paquay, 1983) or higher (McNatty et al., 1984b) than luteal phase values. This variation is not fully understood but could be related to differences in breed and the depth of anoestrus.

In most sheep breeds FSH concentrations during anoestrus are sufficiently high to allow development of large non-atretic antral follicles (Smeaton and Robertson, 1971; Cahill and Mauleon, 1980; Webb and Gauld, 1985). As during the luteal phase FSH concentrations during anoestrus also exhibit cyclic variations that are believed to be related to the development of large oestrogenic follicles (Bister and Paquay, 1983). The total number of antral (McNatty et al., 1984b) or ovulatory-sized follicles (Smeaton and Robertson, 1971; Cahill, 1981; Noel et al., 1993) and the induced ovulation rate (Webb et al., 1992) are similar to that found in the breeding season, indicating that the mechanisms that control follicle selection continue to operate outside the breeding season. During anoestrus the follicular population is composed of both 'oestrogen-active' and 'inactive' follicles, with the former being capable of producing oestradiol at the same rate as equivalent follicles during the breeding season (McNatty et al., 1984b). The oestrogen active or dominant follicles contain more LH receptors than inactive ones (Webb et al., 1992), secrete oestradiol and androstenedione in acute response to LH pulses (Scaramuzzi and Baird, 1977; McNatty et al., 1984b; Webb et al., 1992) and can be induced to ovulate by pulsatile injection of LH (McNeilly et al., 1982; McNatty et al., 1984a) or GnRH (McLeod et al., 1982) or by a bolus injection of hCG (Webb et al., 1992). Additionally anoestrous ewes, especially from breeds of less marked seasonal reproduction such as Merino and its crosses, can be induced to ovulate by an endogenous increase in LH pulse frequency triggered by exposure to rams (Martin et al., 1986).

The anoestrous status can be reversed by pulsatile injection of GnRH, resulting in regular oestrous cycles in the treated ewes (McNatty et al., 1982a). Anoestrus does not result from defects in any other hormonal steps required to produce ovulation, since the ability of LH to stimulate oestradiol secretion (McNeilly et al., 1982) and oestradiol to induce an LH surge is not impaired in anoestrous ewes (Land et al., 1973b).

1.4 Gonadotrophin Induced Follicular Growth

Follicles start to express mRNA for FSH receptor in the granulosa cells of early preantral follicles with two cell layers and this pattern of expression persist throughout folliculogenesis into advanced stages of atresia (Tisdall et al., 1995). Studies examining the ability of ovarian tissue to bind labelled gonadotrophins in
follicles with diameter of 1 mm onward, showed binding of FSH in the granulosa and LH in the theca cells at similar rate in all follicle sizes and a reduction in binding with atresia. The presence of LH-binding in the granulosa cells was only found in non-atretic follicles greater than 4 mm in diameter (Carson et al., 1979). The follicles that have LH receptors on the granulosa cells also secrete large amounts of oestradiol in vivo and in vitro and presumably are at the stage where they are able to respond to an LH surge and ovulate (Webb and England, 1982b).

The action of gonadotrophins in the gonads could also be regulated by alternative splicing of mRNA, for the gonadotrophin receptors, which has been reported for both LH and FSH (Khan et al., 1993; Bacich et al., 1994; Tisdall et al., 1995). Several of the alternative splice variants encode for truncated forms of the receptor in which the intracellular domain is deleted, but the binding to the ligand is unaffected. Although the regulation of alternative splicing has not been demonstrated in vivo, they could potentially modulate the actions of gonadotrophins at the target organ.

The ovaries of ewes in which secretion of endogenous gonadotrophin has been suppressed by removal or inhibition of GnRH input, contain no follicles larger than 2.5 mm in diameter (McNeilly et al., 1986; McNeilly and Fraser, 1987; Picton et al., 1990). The development of follicles larger than 2.5 mm, in ewes chronically treated with gonadotrophin-releasing hormone agonist, can be induced by infusion of FSH in the absence of pulsatile LH secretion, causing a time-dependent stimulation of preovulatory follicle growth (Picton et al., 1990; Picton and McNeilly, 1991). The replacement of LH alone does not stimulate follicular growth, but its pulse frequency can modulate the action of FSH by increasing the threshold concentration needed to promote follicular growth in the presence of high amplitude LH pulses (McNeilly et al., 1992). Low amplitude pulses, however, have little effect on the ability of FSH to induce growth of large follicles (Campbell et al., 1995). Moreover, it has been shown that FSH-dependent follicles remain healthy when the FSH supply is withdrawn, if LH is injected in pulses of one per hour (Campbell et al., 1995).

The injection of supra physiological levels of gonadotrophins as eCG (FSH and LH-like activity) (McNatty et al., 1982b) or purified FSH preparations (Henderson et al., 1988) during the follicular phase increase the mean ovulation rate, although increasing the range of variation in the number of ovulations, indicating an active involvement of FSH in the process of selection. However, studies attempting to relate circulating FSH concentrations, particularly during the late luteal and early follicular phase, to ovulation rate have produced equivocal results. While some studies have demonstrated a higher concentration of FSH in ewes with multiple ovulations,
many other studies have found no such relation, see (Scaramuzzi and Campbell, 1990). A recent study investigated the relationship between the concentration of FSH during late-luteal and follicular phase and subsequent ovulation rates (OR) in ewes over 3 successive cycle (Fry and Driancourt, 1996). The data were grouped as to whether the ewes had the same OR at each oestrous cycle, different OR at each oestrous cycle or the same OR at two oestrous cycles and a different OR on one occasion. In the group with persistent OR the variation in FSH concentrations between cycles were up to 67%. The groups that changed OR there were no consistent association between increases in ovulation rate and proportional increases in FSH concentrations and the variation in the FSH concentration was similar to the group of animals where the OR did not vary. Hence, there is little evidence that FSH concentrations during the late-luteal and follicular phase are associated with changes in ovulation rate within individual ewes, suggesting that the large variability in the requirement for FSH between gonadotrophin-dependent follicles within a ewe, prevent the expression of any thresholds in the ovarian response to FSH (Fry and Driancourt, 1996).

Although FSH plays a key role in the development of gonadotrophin-dependent follicles, changes in its concentration are insufficient to fully explain why follicles sharing the same endocrine environment become atretic while others progress in their development. Follicles of similar size obtained from the same ewe, show markedly different responses to graded doses of FSH in vitro, measured by aromatase activity (Fry and Driancourt, 1996). Presumably the follicles within the ovary respond differentially to the action of the gonadotrophins due to locally produced factors.

1.5 Local Factors Influencing Terminal Folliculogenesis

Several compounds with the potential to regulate locally follicle development have been identified in rodents using in vitro systems or have been isolated from follicular fluid (Ackland et al., 1992; Jones and Clemmons, 1995; Billig et al., 1996). Among the most studied are the growth factors and the inhibin-like peptides. These factors are thought to regulate ovarian cell biology of follicular cells. Factors such as transforming growth factor α (TGF-α), epidermal growth factor (EGF), fibroblast growth factor (FGF) and IGF-binding proteins act by stimulating proliferation and/or inhibiting differentiation. In contrast, others such as insulin-like growth factor (IGF), TGF-β and activin are believed to work by increasing or inducing early differentiation of the cells, enhancing steroid and inhibin production and/or inducing hormone receptors (Campbell et al., 1995). This review will concentrate in factors that have been demonstrated to act at ovarian level, in sheep both in vivo and in vitro.
1.5.1 *In vivo*

Most reports of actions of local factors on the ovary *in vivo* have been studied by measuring the effect of ovarian arterial infusion, in ewes with ovarian autotransplants, on ovarian hormone secretion and changes in the follicle population. The arterial infusion of EGF during the follicular phase of the sheep, inhibited oestradiol and inhibin secretion but androstenedione secretion was unaffected. The rate of progesterone secretion increased in ewes receiving EGF, even in the absence of preovulatory LH surge. Concomitant increases in both LH and FSH secretion were associated with these effects of EGF on ovarian function. It was suggested that EGF acts directly on the granulosa cells of the follicle to inhibit aromatization and inhibit production by inducing atresia in medium to large antral follicles and may also be involved in the induction of functional luteinization (Murray *et al.*, 1993). The infusion of TGF-α during the follicular phase also results in acute inhibition of oestradiol, androstenedione and inhibin secretion compatible with induction of atresia in the large follicle population (Campbell *et al.*, 1994).

The infusion of EGF or basic FGF into the ovarian artery for 12 hours during the luteal phase, also suppresses the secretion of oestradiol and androstenedione during and for up to 30 h after the infusion. The secretion of progesterone is unaffected immediately after the infusion of both growth factors but subsequently increases in EGF-treated ewes. The infusion of either EGF or FGF has no detectable effect on the characteristics of pulsatile LH secretion. FSH concentrations increases steadily during the FGF infusion but decline rapidly to below pre-infusion concentrations after the end of the infusion. During the infusion of EGF the concentration of FSH remains unchanged, but increase after the end of the treatment probably as an indirect consequence of changing steroid secretion (Scaramuzzi and Downing, 1995). Recombinant human inhibin A or steroid-free bovine follicular fluid ovarian artery infusion during the follicular phase causes a acute inhibition of the ovarian secretion of oestradiol and androstenedione, delaying or in some cases inhibiting the LH surge and reducing progesterone concentrations during the subsequent luteal phase (Campbell and Scaramuzzi, 1996).

When ewes were injected daily with recombinant bovine somatotrophin (RBS) throughout the luteal phase there was no change in the total number of follicles greater than 1.0 mm in diameter. However, the population of follicles 2.1-4.0 mm in diameter increased whilst the number of follicles 1.0-2.0 mm in diameter was reduced. The treatment with RBS increased peripheral concentrations of GH, IGF-I, insulin and progesterone, but had no effect on the concentrations of FSH or LH. Both large and
small follicles from somatotrophin-treated ewes secreted in vitro more IGF-I than follicles from untreated animals. However, there was no effect on the secretion of oestradiol, testosterone and progesterone by either large or small follicles, suggesting that treatment of mature ewes with RBS can enhance the development of ovarian follicles to the gonadotrophin-dependent stages, through increased secretion of ovarian IGF-I, as well as increased peripheral concentrations of IGF-I and insulin (Gong et al., 1996).

IGF-I is actively produced in the liver and released in the circulation bound mainly to binding protein-3 (Giudice, 1992). The IGF-1 gene is also expressed in the ovary, although there is still some controversy as to the exact cellular location, with reports of absence of expression on follicles (Perks et al., 1995) or expression in both granulosa and theca cells of early antral follicles and in larger follicles thereafter (Leeuwenberg et al., 1995). The biological effects of IGFs are modulated by IGF-binding proteins (IGFBPs), whose levels in follicular fluid change markedly during folliculogenesis (Giudice, 1992). In sheep, there is an increase in the IGFBP-3/IGFBPs <40 kD (IGFBP-2, -4 and -5) ratio in follicular fluid as the healthy follicle get larger while during atresia a marked decrease in this ratio is observed (Monget and Monniaux, 1995). These variations result from alteration in expression of these IGFBPs by follicular cells and rate of binding protein migration from the plasma to the follicular fluid (Armstrong et al., 1996) and may also be associated with changes in local degradation by specific intrafollicular proteases (Bensard et al., 1996a). Such changes in IGFBP levels are thought to increase the bioavailability of IGF in growing healthy follicles while causing a decrease in atretic follicles, suggesting that intrafollicular IGFBPs play a key role in the regulation of follicular development by modulating IGFs and therefore gonadotrophin action (Monget and Monniaux, 1995; Bensard et al., 1996a). IGF type 1 receptors which exhibit high affinity binding for IGF-I are present in both granulosa and theca cells, with the highest concentration in follicles smaller than 2 mm, regardless of the state of health of the follicle (Perks et al., 1995). Ovarian arterial infusion of an IGF-I analogue that has reduced affinity for IGFBP for 12h prior to luteolysis, results in an increase in the number of gonadotrophin-dependent follicles and the secretion of oestradiol during the subsequent follicular phase (Campbell et al., 1995).

In summary, the infusion of putative local regulators of folliculogenesis such as EGF (Murray et al., 1993), TGF-α (Campbell et al., 1994), basic FGF (Scaramuzzi and Downing, 1995), recombinant human inhibin and steroid-free follicular fluid (Campbell and Scaramuzzi, 1996) all have inhibitory effects on ovarian function in vivo, reducing the secretion of steroids and inhibin or causing atresia of
large antral follicles. IGF-I has been the one factor reported to stimulate follicular development and ovarian steroid secretion (Campbell et al., 1995).

1.5.2 In vitro
The interpretation of results from experiments in vitro has been difficult because the majority of the culture systems do not reproduce all the physiological responses of the follicular cells which own in vivo. Granulosa cells luteinize spontaneously in culture resulting in a decrease in oestradiol and inhibin secretion accompanied by a marked rise in progesterone secretion (Hay and Moor, 1978; Campbell et al., 1996a). The same occurs with theca cells in culture when they almost cease to produce androgens while secreting large amounts of progesterone (Hay and Moor, 1978).

Recently serum-free systems have been described for ovarian follicular cells where the characteristic pattern of steroidogenesis and response to gonadotrophins is maintained (Campbell et al., 1996a). The granulosa cells in this culture system respond synergistically to insulin and IGF-1. Physiological levels of both compounds cause proliferation in undifferentiated cells from small follicles (< 3.5 mm) and maintenance in number of differentiated cells from large follicles (≥ 3.5 mm). But large doses of either compound result in no additional proliferation in undifferentiated granulosa cells and cause an inhibitory effect in cells from large follicles. The addition of EGF and TGF-α induce proliferation in cells from both small and large follicles accompanied by inhibition of oestradiol and inhibin secretion in a dose dependent manner (Campbell et al., 1996a).

The maintenance of androgen production by theca cells from small follicles (< 3 mm) in serum-free culture is influenced by plating density. Under optimal conditions they respond to LH stimulation by increasing androgen production, but high doses of gonadotrophin (> 1 ng/ml) inhibited androstenedione and increase progesterone production, suggesting induction of luteinization. Both insulin and IGF act synergistically stimulating androgen production in a dose dependent manner. In contrast basic FGF and EGF both inhibit androstenedione production (Campbell et al., 1996).

1.6 Follicular Steroidogenesis
Up to a diameter of around 3 mm ovine follicles contain low concentration of oestradiol and androstenedione compared to testosterone (Carson et al., 1981). In larger follicles oestradiol production increases rapidly while the concentration of testosterone falls. This shift in steroid production is due to an increase of 8-10 fold in aromatase activity (Tsonis et al., 1984b), which coincides with the appearance of LH
receptors in the granulosa cell layer (Carson et al., 1979). Atretic follicles show a pattern of steroid content similar to healthy follicles below 3 mm in diameter, but fail to show the increase in oestradiol production characteristic of healthy follicles above this diameter (Carson et al., 1981; Tsonis et al., 1984b). Around 90% of the oestradiol production is derived from large healthy follicles (> 4 mm) while androstenedione is produced by a wider range of follicles (Mann et al., 1992).

In the ewe the main ovarian androgens are androstenedione and testosterone, and as the expression of the enzyme cytochrome P450 17 alpha-hydroxylase (17α hydroxylase) in the ovary is confined to theca cells, these androgens are produced entirely by these cells (Conley et al., 1995). Androgen synthesis and release is stimulated by LH (Baird et al., 1976), via cAMP second messenger system (protein kinase A pathway), production of which is stimulated by the binding of LH to its receptors in the theca cells. The main role of LH in the theca cells is the stimulation of the enzyme cytochrome P450 side chain cleavage (CSCC) which is responsible by the conversion of cholesterol to pregnenolone in the mitochondria (Miller, 1988). The stimulation of this early step in the steroidogenic pathway results in an increase in the production of all steroids. More recently evidence has emerged that steroidogenesis is initially regulated by the transport of cholesterol from the cytoplasm to inner mitochondria membranes, mediated by steroidogenic acute regulatory protein (StAR) (Stocco and Clark, 1996). In sheep luteal cells the mRNA of StAR is highly expressed throughout the luteal phase. StAR expression is up-regulated by LH replacement in HPX animals and decreased after administration of PGF2α, an effect probably mediated by the protein kinase C pathway. Changes in StAR expression, however, are not responsible by the increase in progesterone secretion between day 4 and 10 of the cycle (Juengel et al., 1995).

Granulosa cells lack the enzymatic machinery for androgen production, but are nevertheless important in the conversion of thecal androgens to oestrogens, the so called two-cell two-gonadotrophin theory. This theory states that theca cells produce androgens under LH-drive and these androgens are converted to oestradiol in the granulosa cells by the enzyme P450 aromatase which is under the control of FSH (Hillier et al., 1994). Early reports in the sheep confirmed that theca cells cultured in vitro fail to secrete any oestradiol and the ability of granulosa cells are greatly improved by co-culture with theca cells or addition of androgen precursor to the culture media (Moor, 1977). Additionally, ovine granulosa cells are 4 times more effective at converting testosterone than androstenedione to oestradiol, suggesting that the former is the preferred substrate (Hay and Moor, 1978). Evidence for the 'two-cell' mechanism has also been provided in vivo, by a report of reduction of over 50%
in oestrogen secretion on ewes after passive immunisation against testosterone (Baird, 1977). Pulses of LH are followed by a rapid rise in the secretion of androstenedione and oestradiol, suggesting a quick transfer of androgen precursor from the theca to the granulosa layer (Baird and Scaramuzzi, 1976). It has also been suggested that oestradiol production takes place in the granulosa cells adjacent to the basement membrane close to the capillaries supplying the theca (Baird and McNeilly, 1981).

Small antral follicles have the ability to produce limited amounts of oestradiol. The aromatase activity per follicle increase with size up to a diameter of 3.5 mm and is maintained at constant levels in healthy follicles, while declining in atretic follicles (Tsonis et al., 1984b). FSH injection in ewes has been shown to increase the aromatase activity in large follicles (McNatty et al., 1985b). In granulosacells cultured in serum-free system FSH induces aromatase activity and oestradiol secretion in undifferentiated cells harvested from small follicles (< 3.5 mm). Under the same conditions cells from large follicles (≥ 3.5 mm) maintain oestradiol production in response to physiological doses of FSH (Campbell et al., 1996a).

1.7 Ovarian Inhibin-related Peptides

Inhibin and activin are glycoprotein dimers members of the TGF-β superfamily of peptides, both peptides are present in large amounts in the follicular fluid of several species including the sheep. Inhibin is a heterodimer composed of α and β subunits, each of them a product of a single gene, linked by disulphide bonds. Inhibin exists in two forms inhibin A (αβA) and inhibin B (αβB), both forms share an identical α subunit linked to a different β subunit. The activins are homodimers of the β subunit and exist as activin A (βAβA) and activin AB (βAβB) see (Baird and Smith, 1993). Follistatin is a protein structurally unrelated to inhibin and activin that was also isolated from follicular fluid and which binds to activin with high affinity (Ackland et al., 1992).

Inhibin was initially described by its selective suppression of FSH production while activin has the opposite effect. Follistatin also inhibits pituitary FSH release by inactivating activin in an irreversible bond. Follistatin can bind to two molecules of activin in an association of similar or greater affinity than activin to its receptor. It also binds to inhibin but with 1000-fold less affinity than activin, thus making it primarily an antagonist of activin (Giudice et al., 1996).

The messenger RNA for both α and β subunits and follistatin have been located to the granulosa cells of the sheep ovary (Engelhardt et al., 1993; Tisdall et al., 1994). The pattern of expression of the these genes seems to be developmentally regulated. Follistatin is expressed in the granulosa cells of most of preantral follicles.
followed by the expression of the α subunit and finally the β subunit which is only found in antral follicles. All three genes remain active in the healthy antral follicles (Braw-Tal, 1994; Engelhardt et al., 1995) but the evidence for α and βA subunit peptide is restricted to antral follicles (Engelhardt et al., 1995). During follicular atresia the expression of mRNA for all three genes is progressively decreased. In early atresia the expression of the βA subunit is restricted to the cells of the cumulus, whereas the α subunit and follistatin expression still present in the granulosa layer. As atresia progresses the message for the α subunit is lost, followed later by loss of follistatin mRNA expression (Braw-Tal, 1994).

The role of inhibin in regulating the production and secretion of FSH has been extensively documented in sheep, both in vivo (Martin et al., 1988; Mann et al., 1993; Tilbrook et al., 1993) and in vitro (Tsonis et al., 1988; Clarke et al., 1993; Muttukrishma and Knight, 1994). The pattern of secretion of inhibin in blood, however, is less clear, due to constraints in the assays employed for its measurement. Most of the radioimmunoassay available are based on antibodies against purified or synthetic fragments of the α subunit and measure 'so-called' immuno-reactive inhibin. They cross-react differentially to related proteins but all of then have a strong cross-reaction with non-bioactive forms of the α subunit. Although bioassays are able to measured the active forms of inhibin they can be potentially influenced by the presence of activin, follistatin and ovarian steroids in the sample (Baird and Smith, 1993).

A recently described immunoassay for dimeric inhibin was able to effectively monitor the changes in both inhibin A and B in humans during the menstrual cycle. The concentration of inhibin B is high during the early follicular phase and is associated with the presence of small follicles, decreasing as the dominant follicle mature, whereas the concentrations of inhibin A are low at the beginning of the follicular phase and increase prior to ovulation before increasing again to their highest levels during the luteal phase (Groome et al., 1994; Groome et al., 1996).

Besides their well recognised action in FSH production, an increasing body of evidence is emerging for a local action of inhibin, activin and follistatin in the ovary (Hillier and Miro, 1993; Findlay, 1994; Giudice et al., 1996). In the rat and human, activin inhibits androgen production of theca cells in vitro, conversely, inhibin increases androgen production and is able to reverse the inhibitory action of activin (Hillier and Miro, 1993). The action in granulosa cells indicates that activin increase aromatase activity, FSH receptor expression and inhibin production in vitro (Giudice et al., 1996). Activin also induces proliferation of granulosa cell from immature rats in vitro (Miro and Hillier, 1996).
1.8 Follicle Selection

Each of the several techniques which have been employed to determine the timing of follicle selection, has its limitation, accounting for the contradictory reports of when the selection of the preovulatory follicle occurs. Earlier studies which involved injecting carbon based dye into large follicles (4-5mm) indicated that the selected follicles are only present at or just before the onset of oestrus, and that selection is accomplished during the late follicular phase (Smeaton and Robertson, 1971). Studies involving chlorpromazine injection and unilateral ovariectomy, proposed that preovulatory selection takes place within the 3 days preceding the onset of oestrus (Land, 1973). The selection of the preovulatory follicle has also been suggested to occur during the luteal phase, based on analysis of histological sections from ovaries collected at different stages of the cycle (Brand and De Jong, 1973).

Studies involving ink labelling of individual follicles greater than 2 mm in diameter during the follicular phase suggested that selection of the preovulatory follicle occurs between 30 and 54 hours after luteolysis (Driancourt and Cahill, 1984). Other studies utilising follicle dissection and ovarian oestradiol secretion as marker for selection, concluded that the preovulatory follicle is selected from the pool of small follicles (2-4 mm) soon after luteolysis and emerge as a large oestrogenic follicle within 10 hours of luteal regression (McNatty et al., 1982b). However if the criteria for selection is taken as the presence of LH receptors in the granulosa cell layer and high concentrations of oestradiol in the follicular fluid, selected follicles have been identified in both the luteal and follicular phase (England et al., 1981; Webb and England, 1982a; Webb and England, 1982b).

The difference in the timing of selection can be best illustrated in a study using selective ablation of follicles at luteolysis, demonstrating that ovulatory follicles are derived from follicles greater than 2 mm at the time of luteal regression, over a wide range of size. This suggest that selection can occur in a flexible time frame, both prior to and after luteolysis, according to the follicular population at that time (Tsonis et al., 1984a).

The preovulatory follicle is identified as a large, highly vascularized follicle that produces large amounts of oestradiol and in which the granulosa cells contain LH receptors. The vascular theca layer is separated from the avascular granulosa layer by a basement membrane. One of the initial signs of variation between follicles is the blood flow, and consequent provision of nutrients and gonadotrophins to individual follicles. Evidence from histological observation indicate that number of blood vessels rise with follicular size and decrease with atresia (Hay et al., 1976). Morphological evidence of the state of health of the follicle can be inferred by the presence of
capillaries in the theca layer observed under low power magnification (McNatty et al., 1984b). Direct evidence shows that blood flow is higher in healthy than in atretic follicles, the difference increases progressively with atresia beginning with 2-fold in early atretic and can be up to 10-fold in follicles at later stages of atresia (Brown and Driancourt, 1989; Brown et al., 1991). In human the use of colour doppler ultrasound is capable of identifying the selected follicle on the basis of its higher levels of blood perfusion (Campbell et al., 1993). However, the question remains if the changes in blood flow are a cause or a consequence of the atretic process.

The selected follicles are those that take advantage of the hormonal environment and progress through the final stages of (gonadotrophin-dependent) and establish themselves as oestrogenic follicles, being able to generate trophic cAMP from both FSH and LH stimuli.

1.9 Prolific Sheep

The ewe with its wide range in litter size among the different breeds, is an ideal model for the study of follicular selection and its regulation. Prolificacy in sheep is determined mainly by the number of ova shed in each oestrous cycle or the ovulation rate (OR) (Hanrahan, 1980). The OR can be genetically regulated by several genes as in the Romanov and Finnish-Landrace breeds or alternatively by the action of a major gene or a small group of genes closely linked as observed in the Booroola and Inverdale ewes. Several putative major genes influencing the ovulation rate have been described in sheep, among them the Booroola gene which was the first to be identified and is the most studied gene (Bindon and Piper, 1986; Montgomery et al., 1992).

The Booroola gene is an autosomal mutation identified on the basis of segregational studies on litter size (Piper and Bindon, 1982) and OR (Davis et al., 1982). This gene has additive effects on OR and is dominant for litter size and was named Booroola, after the place where it was first isolated. The alleles were called F for the putative high prolificacy gene and + for the wild type. Later the nomenclature for major genes was standardised and the gene renamed Booroola or Fec and the alleles assigned FecB (B) for the favourable and Fec+ (++) for the wild type (COGNOSAG, 1989). Based on the segregation of the OR in Merino and Romney flocks, the genotypes in the ewes have been classified as homozygous non-carrier (++) with an OR of 2 or less, heterozygous carriers (B+) with OR of 3-4 and homozygous carriers (BB) with more than 5 ovulations per cycle (Davis et al., 1982). To date there is no characteristic phenotype for the different genotypes in the males, so their genotype is classified based on their pedigree and progeny (Hochereau-De Reviers and Seck, 1991). The classification of genotypes by OR is time consuming.
and can lead to wrong assignment, but despite the intense research the Booroola gene (or mutation, deletion, duplication) have not been identified (Montgomery et al., 1992; Nguyen et al., 1992; Crawford et al., 1993; Montgomery et al., 1993; Lanneluc et al., 1994). However, it has been mapped to sheep chromosome 6 in a region between genes for secreted phosphoprotein 1 (SPP1) and EGF. Interestingly, the Fec gene is not linked to many of the genes known to have major actions in reproduction, such as the gonadotrophins, many growth factors and the inhibin-related peptides (Montgomery et al., 1995).

1.9.1 Hypothalamus

The concentration of immunoreactive GnRH in hypothalamic and extra-hypothalamic areas of the brain from intact and ovariectomized (OVX) ewes is similar among the genotypes for the Fec gene (Gale et al., 1988). The secretory pattern and GnRH concentration in the portal blood in OVX ewes (McNatty et al., 1993) and GnRH-induced gonadotrophin secretion in OVX ewes in which the hypothalamic-pituitary stalk has been disconnected (HPD) are not influenced by genotype (McNatty et al., 1991b). Moreover, when HPD ewes primed with eCG are induced to ovulate by a pulsatile regime of GnRH, the difference in the OR between BB and ++ genotype is maintained (McNatty et al., 1993). All these studies suggest that the Booroola gene exerts its action 'downstream' from the hypothalamus.

1.9.2 Pituitary

The GnRH receptor in pituitary homogenates from intact and OVX ewes, have a single class binding site whose binding characteristics are not influenced by the Booroola genotype (Fleming et al., 1990). In pituitaries obtained from ++ and BB intact ewes during mid-luteal phase, OVX ewes and ovary-intact or OVX-HPD ewes given the same regimen of pulsatile GnRH, no Fec specific differences were detected in the number or size of mRNA transcripts for alpha gonadotrophin, FSH beta and LH beta genes. The genotypes also have similar relative amounts of mRNA encoding these genes and pituitary content of FSH or LH (Fleming et al., 1995). Studies using immunohistochemistry also shown no differences in total number of pituitary cells, pituitary volume, numbers or diameters of FSH beta- or LH beta-immunostaining cells (Heath et al., 1996).

There is no difference in the concentration of LH in blood or in the anterior pituitary between ewes carrying the Booroola gene and controls (Robertson et al., 1984; McNatty et al., 1987a). The data for FSH has been controversial, with reports of differences among genotypes in FSH concentration during various stages of the
oestrous cycle and anoestrus (Bindon, 1984; McNatty et al., 1987a; McNatty et al., 1989a) while others show no difference (McNatty et al., 1991a; Boulton et al., 1995). Nevertheless when hypophysectomized (HPX) ewes were induced to ovulate with standard doses of eCG and hCG the differences in the OR was maintained between genotypes (Fry et al., 1988). More recently a report using the GnRH antagonist model, showed that ewes infused with the same preparation of gonadotrophins, responded with different number of ovulation and maintained the difference in follicular/CL size characteristic of the genotypes, suggesting that the Booroola gene is likely to act at the ovary (Campbell et al., 1996b).

1.9.3 Ovary

In sexually mature ewes the total population of antral follicles and the proportion of non-atretic follicles is similar among genotypes, but the size that follicles mature and ovulate is significantly smaller in BB and B+ than ++ ewes (McNatty et al., 1985a; Driancourt et al., 1985; McNatty et al., 1986b; Souza et al., 1994). In addition, the non-atretic antral follicles of the carrier ewes have fewer granulosa cells when compared with follicles of similar sizes from ++ ewes (McNatty et al., 1986b). These features are consistent among different laboratories and were observed during the last trimester of fetal life (Smith et al., 1994), before puberty (McNatty et al., 1987b), during the oestrous cycle (Castonguay et al., 1990), anoestrus and also after long term HPX (McNatty et al., 1990a). Nevertheless, the total number of granulosa cells (~5.4 million) from oestrogenic follicles per animal are similar among genotypes (McNatty et al., 1986b) as were the secretion of ovarian steroid and immunoreactive inhibin obtained from single samples of anaesthetised animals (McNatty and Henderson, 1987; McNatty et al., 1992a). In ewes with ovarian autotransplants there was no difference between genotypes in the rate of secretion of oestradiol and progesterone on day 10 of the luteal phase and at various times during the follicular phase (Tsonis et al., 1988). Similar features are also observed regarding the CL population among the genotypes. Although BB ewes have individual CL with lighter mass and fewer cells per CL the total luteal weight, total number of CL cells and concentration of progesterone in the plasma is similar to the ++ ewes (Niswender et al., 1990). So despite significant differences in OR and ovarian morphology, the genotypes appear to secrete similar levels of the key components of the hormonal feedback systems that control the pituitary and hypothalamus (McNatty and Henderson, 1987).

The follicles in the carrier ewes mature at an earlier diameter than non-carrier counterparts, influencing the basal content of cAMP and gonadotrophin stimulated
cAMP response *in vitro* in follicles from the early antral stage onwards (McNatty *et al.*, 1986a). However, the difference in second messenger activity does not seem to be related to either FSH or LH receptor binding characteristics on granulosa or theca and luteal cells respectively (McNatty *et al.*, 1986c; McNatty *et al.*, 1989c). But LH receptors were found in granulosa cells from follicles of smaller diameter in carriers than non-carrier ewes (3-4.5 mm for BB/B+ and ≥ 5 mm for ++) (McNatty *et al.*, 1986c). *In vitro* culture of granulosa cells from follicles at similar stages of maturation, revealed no significant differences on cAMP synthesis after stimulation with FSH, LH, forskolin, cholera and pertussis toxin in the different genotypes and similar catabolism rate of cAMP (Henderson *et al.*, 1985; Henderson *et al.*, 1987; McNatty *et al.*, 1989b; McNatty *et al.*, 1990b), suggesting that the Booroola gene has no direct influence on gonadotrophin binding and second messenger generating systems.

The steroid content of follicles smaller than 1 mm is undetectable in freshly isolated tissue. However, when cultured *in vitro* in the presence of gonadotrophins, follicles around antral formation (0.1-0.2 mm) were able to synthesise progesterone and androstenedione while larger follicles (0.2-0.5 mm) produced testosterone and oestradiol, demonstrating that follicles over 0.5 mm in diameter are capable of producing the major ovarian steroids. When the steroidogenic capacity *in vitro* was compared among the genotypes for the Booroola gene, a significantly higher proportion of follicles were steroidogenic for any given size range in the carrier animals, showing that these follicles were more mature than follicles of similar diameter in the ++ ewes (McNatty *et al.*, 1986a).

In follicles larger than 1 mm, granulosa cells are the only source of oestradiol, and the capacity of these cells to aromatize androgens to oestradiol was at its highest level in follicles of different diameter according to the FecB genotype, 3-3.5 mm, 3-5.5 mm and ≥ 5 mm in diameter for BB, B+ and ++ ewes respectively (McNatty and Henderson, 1987). However, the steroid production of the theca cells, mainly androstenedione and to a lesser extent progesterone and testosterone, was not influenced by the Booroola genotype (McNatty *et al.*, 1986b; McNatty and Henderson, 1987). Short term culture (4 h) of small intact follicles, between 30 and 60% of the putative preovulatory size (2-4 mm in ++ and 1.5-3 mm in B+) with or without FSH showed that the granulosa cells of carrier ewes proliferate more in the presence or absence of gonadotrophin, while no differences were found in the theca cell layer. When large intact follicles, with diameter ≥ 80% of the preovulatory size, were cultured in a perifusion system for 24 h no genotype difference was found in the oestradiol production after FSH stimulation (Driancourt, 1991).
While there is wide agreement between laboratories showing similarity of the secretion of steroids among genotypes, the rate of production and secretion of inhibin, the other major product from granulosa cells, has been more controversial probably due to differences in the assay techniques employed. The total content of inhibin in whole ovarian homogenates measured by rat pituitary bioassay was reported to be lower in the Booroola gene carrier animals (Cummins et al., 1983). The production in vitro and the secretion in vivo of immunoreactive inhibin (Monash assay) was found to be similar in ewes of different genotypes (Henderson et al., 1991; McNatty et al., 1992a). A study on the ovarian secretion in vivo, on repeated samples using ewes with an ovarian autotransplant, showed similar rates during the luteal phase but gene-carriers had a transient but significant rise in bioactive inhibin secretion measured by sheep pituitary bioassay during the mid-late follicular phase (Tsonis et al., 1988). The levels of mRNA expression of α-inhibin and follistatin on pooled follicular tissue samples by northern blot analysis showed similar ratios in BB and ++ ewes. However, the rate of expression of the mRNA for βA-inhibin was higher in the BB samples, suggesting that the Booroola gene influences inhibin or activin secretion by increasing the stability or expression of the β-subunit (Fleming et al., 1992).

1.10 Ageing and Follicular Development

The ovary has a limited reserve of primordial follicles, which is continuously depleted by the commitment of follicles to growth throughout the life (Peters et al., 1975). The rate that primordial follicles join the growing pool seems to be regulated by the size of the reserve to primordial follicles. Studies in long term unilaterally ovariectomized mice have shown a 25% reduction in the number of expected oestrous cycles and compensatory growth of large antral follicles due to rescuing follicles that otherwise would undergo atresia (Gosden et al., 1989).

In the ewe lamb around 5 primordial follicles enter the growing pool every day (Sonjaya and Driancourt, 1987). The number of daily committed follicles falls to about 2-3 primordial follicles in the adult ewe (2 years old) (Driancourt et al., 1985). Nevertheless, by 8 years of age an ewe would have lost 60-80% of the initial reserve of primordial follicles (Driancourt et al., 1985). In women, the number of small follicles that start the growth phase also declines with age, and correspond to 31, 9 and 1 follicles per day at 29-30, 39-40 and 49-50 years of age respectively. However they show an increase in the percent of committed follicles relative to the number of remaining follicles in the primordial pool (Faddy and Gosden, 1995).

Most mammals do not experience the extinction of the follicular population, except the human, which out-lives the evolutionary determined follicle reserve
resulting in the menopause (Gosden and Faddy, 1994). The initial endocrine changes of the pituitary-ovarian axis in women are first become apparent around the age of 38 years, with a selective rise in serum FSH concentrations occurring in conjunction with a marked acceleration in the loss of primordial follicles from the ovary and increased atresia in the secondary follicle population (Gougeon et al., 1994; Faddy and Gosden, 1995). A recently described mathematical model predicts that the period of rapid loss of follicles starts when the primordial follicle reserve fall to around 25 thousand follicles and menopause occurs some 12 years later when the follicle reserve reaches 1 thousand follicles (Faddy and Gosden, 1996).

The monotrophic FSH rise in normally ovulating older women (age 40-45) is associated with decreased peripheral concentrations of inhibit B during the follicular phase, while oestradiol concentration is higher and inhibit A secretion is similar to a comparative younger cohort of subjects (age 20-25). The dominant follicle content of oestradiol and inhibit is similar in both group of women, suggesting that decreased inhibit B secretion reflects a diminished follicular pool in older women and may be an important regulator of the monotrophic FSH rise (Klein et al., 1996).

Menopause usually does not occur as a point transition, but during a variable period of menopausal transition. This is initiated when changes in cycle frequency or menstrual flow are first observed, with both gonadotrophins, oestradiol and immuno-reactive inhibit show a marked degree of variability with abrupt changes from typical post-menopausal patterns to those characteristic of the reproductive age group. Within 1-2 years of the final menstrual period or menopause; FSH concentrations are markedly elevated and LH levels moderately so, while oestradiol and immuno-reactive inhibit values are low or undetectable, reflecting the exhaustion of the follicle reserve (Burger, 1996).
Chapter 2
Materials and Methods

2.1 Experimental Animals
Two cross breeds of ewe were used in the experiments described in this thesis, Finnish Landrace x Merino and Scottish Blackface x Merino. All animals had undergone surgery to autotransplant the ovary and its vascular pedicle to a more accessible position in the neck (Goding et al., 1967). The jugular vein and carotid artery were placed in a surgically prepared skin loop 6-12 months before the transplant operation. At the transplantation the left ovary along with its vein and artery were dissected out and the ovarian vein and artery connected to the jugular vein and carotid artery respectively. At the same time the right ovary was removed, so that the animals were functionally hemi-ovariectomized. The layout of the ovary and its vasculature in this model is shown in Fig 2.1. The ovarian autotransplant model permits repeated collection of ovarian venous blood in conscious unstressed animals and because the ovary is fixed in an easily accessible location, high resolution ultrasound can be performed in two planes and the position of individual follicles determined accurately.

Figure 2.1 Diagram of the ovarian autotransplant preparation showing the location of the ovary in the neck and the arrangement of the blood vessels in a skin loop (based on Goding et al., 1967).
The Finn x Merino ewes used were mature 11-14 year old animals in which the transplant operation had been performed at least 8 years previously. The Blackface x Merino sheep were mature 2-6 year old ewes in which the ovarian autotransplant surgery had been performed at least 6 months prior to the experiments.

After the transplant the animals were permanently housed indoors in collective pens at the Marshall Building, Roslin, Mid Lothian, Edinburgh. They were provided with constant access to water and fed a maintenance diet consisting of ad libitum hay and 100 g of a pelleted ration. The ewes were transferred to metabolism crates during experimental procedures, which were carried out during the breeding season between November and January and in June during anoestrus. The breeding season lasts from September to March and October to February in the Finn and Blackface cross ewes, respectively.

2.2 Oestrous Synchronisation

Ewes with autotransplanted ovaries do not cycle spontaneously (Baird et al., 1976), because the relocation of the ovary to the neck results in persistence of the CL due to the absence of direct contact between the ovarian artery and the venous drainage of the adjacent uterine horn, thus preventing the migration of endogenous prostaglandin F2α to the ovarian arterial supply. Synchronisation of the oestrous cycle was therefore achieved with two injections of cloprostenol, a potent analogue of prostaglandin F2α (125 µg i.m. Estrumate, Cooper's Animal Health Ltd, Crewe, Cheshire, UK) given 17 days apart.

2.3 Jugular Venous Blood Sample Collection

The left jugular vein was cannulated under local anaesthesia (2 ml s.c. of Lignocaine 2%, Lignavet, Leyland, UK) with a 60 cm length of Silastic tubing (0.040 x 0.085 inches, internal and external diameter respectively; Osteotec Ltd., Christchurch, Dorset) that was inserted into the jugular vein through the bore of a suitably sized needle to a depth of 10 cm, the needle was removed, the tubing was stitched to the skin and connected to a flow switch (Secalon universal flowswitch, Ohmeda, Swindon, Wiltshire, UK). Following cannulation the animals received a broad spectrum long-acting antibiotic (3 ml i.m.; Clamoxil, SmithKline Beecham, Surrey, UK). This prophylactic antibiotic treatment was repeated every 3 days throughout the experimental procedures.

To minimise the effects of stress animals were cannulated several hours before the blood sampling was started. After each sample was collected the cannula was flushed with 5 ml of a solution of 250 000 IU of sodium heparin/l of isotonic saline,
so the animals received around 3 000 IU of heparin (Leo Laboratories, Aylesbury, Bucks., UK) every 12 hours to prevent clotting. After withdrawal by syringe (Plastipax; Becton Dickinson UK Ltd., Oxford) blood samples were placed in 5 ml or 7 ml plastic tubes (Sarstedt, Leicester, UK) containing heparinised quick-spin granules (Sarstedt, Leicester, UK) and centrifuged at 4 °C for 15 min at 2000g. The plasma was then decanted into 3 ml or 5 ml storage vials (Sarstedt, Leicester, UK) and frozen at -20 °C until assayed.

2.4 Ovarian Blood Sampling

In these ewes the left ovary and their vascular pedicle had been transplanted to a site in the neck (Fig. 2.1), as described in section 2.1. A 60 cm length of Silastic tubing (0.040 x 0.085 inches or 0.062 x 0.125, internal and external diameter respectively; Osteotec Ltd., Christchurch, Dorset) was inserted into the jugular vein at the cranial end of the vascular loop through the bore of a suitably sized needle, and passed down the vein until its tip lay opposite the site of anastomosis of the ovarian vein. To allow sample collection a pneumatic cuff (Disposa-cuff n° 3, Critikon, Johnson & Johnson) was placed around the upper limb of the skin loop and inflated to 100-120 mm Hg to temporarily stem jugular blood flow. Following manual occlusion of the jugular vein in the caudal limb of the loop, ovarian blood was collected by aspiration (Chapter 3, 4, 5) or by free fall (Chapter 6). This technique ensured that the blood flowing down the ovarian vein passed into the cannula with minimal dilution by jugular venous blood. To allow the ovarian blood flow rate to be calculated (Chapter 6), timed samples of 7.5 ml of free fall ovarian blood were collected. Ovarian blood flow measured by this technique is an over-estimate due to a contribution from the skin of the loop, but such skin contributions do not alter the calculated ovarian hormone secretion rate. A small quantity of each blood sample was used to determine the haematocrit. This was done by filling heparinised capillary tubes (Hawksley & Son, Lancing, Sussex, UK) with blood, heat-sealing one end of the tube, and then centrifuging for 10 min in a Minor™ centrifuge (MSE Crawley, Sussex, UK). The percentage of red blood cells was then determined using a Micro-Haematocrit reader (MSE Crawley, Sussex, UK). The actual secretion rate of a particular hormone was then calculated from the ovarian venous plasma hormone concentration, the flow rate and the haematocrit (Collett et al., 1973).

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\text{Secretion rate} = \left( \frac{\text{blood flow} \times \text{haematocrit}}{100} \right) \times \text{hormone concentration}
\]
2.5 Ultrasound

2.5.1 General Concepts

Ultrasound waves are generated by means of the piezoelectric effect which is the ability of a crystal to deform under high-voltage current and vibrate producing sound waves. The magnitude of the vibration is proportional to the voltage applied and results in the power of the ultrasound beam. The ultrasound used for diagnostic application is based in high frequency sounds waves, between 1 and 10 MHz. The frequency of emission and the wavelength are inversely proportional, at a frequency of 2 MHz the wavelength of the beam is around 0.8 mm, this relationship has consequences on the definition of the image produced and the penetration of the beam in the tissue. As the frequency increases the resolution is enhanced but the beam is more rapidly attenuated resulting in less penetration of the beam and smaller areas that can be examined. The beam source, which is called transducer or probe, can be arranged in different forms according to its application. In equipment with a linear array probe, the crystals are arranged in a longitudinal succession, and the ultrasound pulses are generated in sequence along the longitudinal axis (Goddard, 1995).

The principles of diagnostic ultrasound rely on the ability of distinct tissue to reflect the ultrasound beam. The beam is emitted by the probe and transmitted to the adjacent tissue and the proportion of the wave that is echoed by the different tissues is received by the transducer, converted into electric pulses and displayed as a grey-scale image. If the image is formed at a rate of at least 30 new frames per second it will generate a real-time effect. The ultrasound image is composed of thousands of pixels, each pixel represents the echo from a specific portion of the area scanned and is represented by a grade of 256 shades of grey ranging from white (0, minimum grey shade) to black (255, maximum grey shade). The less dense structures, especially liquids, do not reflect sound waves and are depicted in black on the view screen, while the highly dense structures appear in white resulting in a inverse relation between grey shade of the pixel and the density of the tissue (Pierson and Adams, 1995).

2.5.2 Scanning Procedure

The skin over the transplanted ovary was clipped and shaved at the beginning of each experiment and maintained free of wool throughout. Before each scan the area was covered with scanning gel (Siel Sound Gel; Siel Imaging Equipment Ltd, Aldermosten, Berkshire, UK). The ovary was scanned in both horizontal (dorso/ventral) and vertical (cranio/caudal) planes, using a 7.5 MHz linear transducer (Model UST-5512U-7.5; Aloka Inc., Japan) with a real time ultrasound scanner (SSD-500; Aloka Inc., Japan). All scans were recorded on video cassette tape for
subsequent analysis.

After the experiment was finished the tapes containing all the ultrasound exams were observed in chronological order and the evolution of all the follicles visible in the ovary were draw in a sequential map of the ovary.

The tapes were played in slow motion and the image of follicles greater than 2.5 mm frozen at the largest section of the antral cavity for each individual follicle, which was located within the ovary. The image of the ovary containing the ovarian structures in each plane was divided into 9 sectors (Fig. 2.2) so the spatial location of each ovarian structure could be determined. In experiment 1, each follicle was measured in the medio-lateral, dorso-ventral and cranio-caudal planes. The diameter of the follicles was determined as a mean of these three measurements. In the remaining experiments the selected image was digitised into pixels of 256 shades of grey ranging from 0 (white, maximum tissue density) to 255 (black, minimum tissue density) using an image grabber card (Falcon card, Scotsys, Edinburgh) in a Power Macintosh 6100/66 computer. The periphery of the follicle was identified in the digital image using the NIH Image software (http://rsb.info.nih.gov/NIH-Image/download.html) and measurements were taken for mean shade of grey of the pixels within the identified area and for the major and minor axes of the best fitted ellipsis for each follicle. The diameter of the follicles was determined as a mean of the two axes measured.

![Figure 2.2](image_url)

Figure 2.2 Picture of a typical digitised frame showing an ovary (O) with CLs and follicles (F) of various sizes. The dotted lines depict how the ovary was divided in 9 sectors, the grid marks in the left side indicate a distance of 10 mm and the skin (S).
2.6 Immunoassays
2.6.1 FSH Assay
FSH was measured in plasma samples using an assay previously described (Campbell et al., 1990) with rabbit anti-ovine FSH NIDDK-1 as first antibody at a final dilution of 1:96,000. This assay was run with a more highly purified standard, oFSH RP2, used in the range 0.2-30 ng/ml, with $^{125}$I-labelled NIAMDD oFSH-I-1 as tracer. The sensitivity of this assay was in the range 0.2-0.3 ng USDA oFSH SIAFP-RP2/ml and the intra and inter assay coefficients of variation for 3 quality control pools 7.2% and 10.1%. The protocol for this assay can be found in the appendix (Section 9.1.1).

2.6.2 LH Assay
LH was measured in plasma samples using the assay described by McNeilly and Fraser (1987) with rabbit anti-oLH R29 as first antibody at a final dilution of 1:600,000. The assay was run with NIH-oLH-S23 as standard used in the range 0.2-50 ng/ml, and with $^{125}$I-labelled oLH LER-1056-C2 as tracer. The sensitivity of this assay was 0.2 ng NIH, oLH, S23/ml and the intra and inter assay coefficients of variation for 3 quality control pools 9.4% and 12.3%. The protocol for this assay can be found in the appendix (Section 9.1.2).

2.6.3 Progesterone Assay
Progesterone was measured in plasma by a direct non-extraction RIA using a rabbit anti progesterone antibody (R31/8) at a final dilution of 1:50,000, $^{125}$I-labelled progesterone 11α glucuronide tyramine conjugate as tracer and pregn-4-ene-3,20 dione (Sigma, Poole, Dorset, UK) as standard in the range 0.05-10 ng/ml. The assay was modified to allow direct measurement in plasma by lowering the buffer pH to 6 and adding 8-anilino- 1- naphthalene sulphonic acid (100 μg/tube; Sigma) to displace binding of progesterone to carrier proteins (McNeilly and Fraser, 1987). A volume of plasma from an ovariectomized ewe equal to the volume of sample used was added to the NSB, Bo and standard tubes. The sensitivity of this assay was 380 pmol/l and the intra and inter assay coefficients of variation 8.4% and 13.2%. The protocol for this assay can be found in the appendix (Section 9.1.3).

2.6.4 Oestradiol Assay
Oestradiol was measured in plasma following extraction with diethyl-ether, the recovery rate after extraction was 92% ± 0.3 (mean ± SEM, n=20) and accordingly the results were not corrected for extraction losses. Samples of ovarian plasma were
assayed using sheep anti-oestradiol antibody (BW 26/9/82) at a final dilution of 1:9,000,000; \(^{125}\)I oestradiol-17\(\beta\) as tracer, and oestradiol-17\(\beta\) (Sigma, Poole, Dorset, UK) as standard in the range 30-10,000 pmol/l (Baird et al., 1981). The sensitivity of this assay was 50 pmol/l and the intra and inter assay coefficients of variation 10% and 12%. Another highly sensitive assay (Beard and Lamming, 1994) was used to measure the basal concentration of oestradiol during the anoestrus (Chapter 3). The assay was based in the oestradiol MAIA assay kit (Serono Diagnostics, Wokingham, Berks, UK). The first antibody supplied with the kit was diluted to the final dilution 1:42, the \(^{125}\)I oestradiol tracer provided with the kit and oestradiol-17\(\beta\) (Sigma, Poole, Dorset, UK) as standard in the range 0.23-58.8 pmol/l. The sensitivity of this plasma assay was 0.7 pmol/l and the intra and inter assay coefficients of variation 8.4% and 12.5%. The protocol for both assays can be found in the appendix (Section 9.1.4 and 9.1.5).

2.6.5 Androstenedione Assay

Androstenedione was measured in plasma by a previously described assay for use in sheep plasma (Campbell et al., 1990). The assay used an antiserum raised in a rabbit against androstenedione-3 carboxymethylxime-BSA (Rabbit C) (Thompson et al., 1989) at a final dilution of 1:112,000 with androstenedione-3-carboxymethylxime linked to \(^{125}\)I-histamine as tracer and 4-androstene-3,17 dione (Sigma, Poole, Dorset, UK) as standard in the range 14-873 pmol/l. Prior to assay plasma samples and standards were extracted using 10 volumes of a 4:1 hexane:ether mixture. The sensitivity of the assay was 175 pmol/l and the intra and inter assay coefficients of variation 7.1 and 13.2% . The protocol for this assay can be found in the appendix (Section 9.1.6).

2.6.6 Inhibin A Assay

Inhibin A was measured by enzyme-linked two-site immunoassay described for use in human plasma samples (Groome et al., 1994) and modified for use in sheep plasma (O'Brien et al., 1996). The immunoassay is based on the use of an immobilised monoclonal antibody (E4) to the \(\beta\)-A subunit as a capture antibody, a biotinylated alpha C specific monoclonal antibody (17329/H2) as detection antibody and immunopurified 32KD bovine inhibin in OVX sheep plasma as standard in the range 15.6-1000 pg/ml. Standards and samples are denatured and oxidised before the incubation of the assay in a 96 well dish. The sensitivity of the assay is 30 pg/ml and the intra and inter assay coefficient of variation 7.6 and 11.9%. The protocol for this ELISA can be found in the appendix (Section 9.1.7).
2.6.7 Assay Counting and Data Reduction

The assay tubes were counted using a 1261 Multigamma counter (Wallac, Turku, Finland) with well type aluminium-covered sodium iodide crystals and a minimum efficiency for $^{125}$I detection of 75%. The counts were transferred to an Apple Macintosh computer on which calculations were made using the Assayzap assay calculation programme (Elsevier Biosoft, Cambridge, UK). Assayzap plots a standard curve using a 4 parameter fit which uses the Bo and NSB values to estimate the upper and lower limits of the curve and then finds the best estimated fit through the standard points. The programme then adjusts this fit by weighting individual points according to how closely they agree with the estimated fit, effectively ignoring outlying points. The programme keeps a record of quality controls and provides a constantly updated measure of the inter-assay coefficient of variation, as well as calculating the intra-assay coefficient of variation for each of the quality controls.

The plates used in the ELISA for inhibin A were read using Labsystems Multiskan MCC/340 (Life Sci. Int. Ltd., Basinstoke, Hampshire, UK) plate reader at wave-length of 492 nm and the results were transferred to an Apple Macintosh computer on which calculations were made using the Assayzap software.

2.7 Statistical Analysis

Statistical analysis of data was carried out using an Apple Power Macintosh computer. Student's t-tests, repeated samples analysis of variance and multifactorial analysis of variance were performed using the Systat software (Systat Inc., Evanston, IL, USA). Pulse analysis was performed using the Munro pulse analysis programme (Zaristow Software, Haddington, East Lothian, UK).

The criteria used to identify a dominant follicle was that it had to have grown to a diameter of at least 5 mm and remained at that size or above for at least 2 observations. The emergence of the dominant follicle was defined as the first time a follicle was observed in the ultrasound scans with a diameter between 2.5-3 mm in diameter.

Detailed description of the statistical procedures regarding each experiment can be found in the methods section for each chapter.

2.8 Preliminary Trials

2.8.1 Ultrasound Exams Over the Oestrous Cycle

During the breeding season (January-February) of 1994 a trial was conducted to investigate the dynamics of follicular growth and the changes in the concentration of gonadotrophins and progesterone during the cycle. Nine Finn-Merino ewes (11-14
years old) with an ovarian auto-transplant, were housed indoors in 2 communal pens at the Marshall Building. The ewes in the luteal phase were injected with a luteolytic dose of cloprostenol, introduced to vasectomised rams with painted briskets for oestrus detection and checked for oestrus marks twice daily (day of the onset of oestrus = Day 0). On Day 15 of the induced cycle the ewes were again injected with cloprostenol and observed for oestrus. Throughout this period the animals were transferred once a day to metabolic cages and had an ovarian ultrasound scan and a sample of jugular venous blood taken, before being returned to the collective pens.

The ultrasound exams were performed in two planes (horizontal and vertical), recorded in video cassette tape and analysed subsequently on the day of recording. The tapes were played in slow motion and size of the ovary and the ovarian structures, after having been identified spatially within the ovary, were measured in the largest section with a digital calliper in the medio-lateral, dorso-ventral and cranio-caudal planes. The diameter of the ovary and the ovarian structures were determined as a mean of these three measurements.

The concentration of LH, FSH and progesterone in jugular venous plasma were determined using RIAs previously described in this section.

All ewes showed oestrus after both cloprostenol injections and the ovulation rate in the induced cycle was 2.1 ± 0.3 (mean ± SEM, n=9). The results of this preliminary trial revealed that there was a significant variation in the size of the ovary during the oestrous cycle (Fig 2.3). The size of the ovary was at its lowest values on the day of the expected ovulation, i.e. 24 hours after the onset of the oestrus (Day=0) and increased progressively until Day 4, remaining high until Day 15. Following induction of luteolysis with an injection of cloprostenol, ovarian size started to decrease returning to its lowest values at Day 1 of the subsequent cycle.

The analysis of the ovarian structures revealed follicles around 5 mm in diameter around the time of oestrus which grew to around 10 mm in diameter and persisted throughout the luteal phase. These structures were subsequently identified as CLs due to their relationship with progesterone secretion and the decrease in diameter after the second cloprostenol injection (Fig. 2.4). The evaluation of the dynamics of the follicle growth was compromised by the frequency of the ultrasound scans which made the development of individual follicles difficult to follow. As this was one of the main aims of the study, the methodology was improved in the subsequent studies to ensure the identification of the follicles. The frequency of the ultrasound exams and blood sampling was increased to at least twice a day and a system of mapping the follicles in the ovary over time was introduced facilitating the tracking of the individual follicles. The measurement system was also improved by digitising the images and the
use of the NIH image software, which was utilised throughout in the subsequent experiments.

Figure 2.3 Variation of the size of the ovary during the oestrous cycle depicted as mean diameter (A) and volume (B, calculated assuming that the ovary was spherical).
2.8.2 GnRH Challenge

During the breeding season (early February) 10 Blackface x Merino ewes with or without the Booroola prolificacy gene (3 Fec+ Fec+; 3 FecB Fec+ and 4 FecB FecB) were used to characterise the response to a GnRH challenge (250 ng i.v. in 2 ml of isotonic saline; Sigma, Poole, Dorset, UK) on day 15 of the oestrous cycle. The ovarian and jugular veins were cannulated as described earlier in this chapter, samples of ovarian venous blood were collected at 15 min starting 30 min before the challenge. Immediately after the second sampling the animals received the GnRH and samples were collected for a further 2 hours to evaluate the concentration of LH and oestradiol.
The effects of time and genotype on the hormonal dynamics after a GnRH challenge were analysed by repeated samples analysis of variance using Systat software. The results of this preliminary trial are presented in figure 2.5. All ewes, independently of genotype, showed increase in the LH and oestradiol concentrations after the injection of GnRH. The concentration of LH before the challenge was around 1 μg/l, it peaked immediately after the injection to around 5 μg/l and decrease gradually to reach basal levels an hour later. The concentration of oestradiol rose progressively from values around 150 pmol/l before the challenge and reach maximum values 30 min after, remaining high for a further hour before starting to decrease. The effect of time in the different genotypes was analysed by repeated samples analysis of variance and showed no genotype influence in the LH concentration in response to the challenge (P>0.05). The pattern of oestradiol was also similar among the genotypes, however, the concentrations of oestradiol in the heterozygous carrier ewes was significantly lower (P<0.05) than the homozygous carrier and non-carrier ewes.

Figure 2.5 Pattern of LH secretion in jugular venous blood and oestradiol secretion in ovarian venous blood after an GnRH challenge (250 ng i.v.) in ewes of different genotypes of the Booroola gene on day 15 of the oestrous cycle.
3.1 Introduction

The development of large antral follicles in sheep occurs throughout adult reproductive life but, until recently the absence of suitable techniques has made the elucidation of the pattern of follicle development difficult. The advent of ultrasonography as a non-invasive and repetitive method of monitoring development of individual follicles has enabled a more comprehensive understanding of follicular dynamics in a number of species (Hackeloer et al., 1979; Griffin and Ginther, 1992). The use of this technique in cattle has led to the discovery of wave-like cycles of selection, dominance and regression of large antral follicles throughout the luteal phase (for review see Fortune, 1994). In sheep, however, the presence of follicular waves is still uncertain.

Although early evidence from histological (Smeaton and Robertson, 1971; Brand and De Jong, 1973) and endocrinological (Cox et al., 1971; Mattner and Braden, 1972; Miller et al., 1981; Bister and Paquay, 1983) studies proposed that dominant follicle development in sheep did occur as a series of waves. More recent studies using transrectal ultrasonography have shown a random emergence of ovulatory sized follicle, i.e. greater than 5 mm in diameter, during the luteal phase (Schrick et al., 1993; Ravindra et al., 1994) or 2-6 waves of follicular development per cycle (Ginther et al., 1995). Transrectal ultrasound studies in sheep, are more difficult to perform and interpret than in cattle due to the problems of anatomical access and the smaller size difference between dominant and subordinate follicles. In addition, it is difficult to relate steroid production to the ovarian follicular population due to the low concentrations in the peripheral circulation in this species.

During periods of anoestrus in most breeds of sheep the pattern of follicle development is similar to that found in the breeding season with no change in the total number of antral (McNatty et al., 1984b) or ovulatory-sized follicles (Smeaton and Robertson, 1971; Cahill, 1981; Noel et al., 1993). This follicular population is composed of both oestrogen active and inactive follicles, where the former are capable of producing oestradiol at the same rate of equivalent follicle in the breeding season (McNatty et al., 1984b). These oestrogen active or dominant follicles contain more LH receptors than inactive ones (Webb et al., 1992), secrete oestradiol and androstenedione in acute response to LH pulses (Scaramuzzi and Baird, 1977) and can be induced to ovulate by pulsatile injection of LH (McNeilly et al., 1982;
McNatty et al., 1984a) or GnRH (McLeod et al., 1982a) or by a bolus injection of hCG (Webb et al., 1992).

In the present study we have used sheep in which the left ovary has been autotransplanted to a site under the skin in the neck (Goding et al., 1967) to examine the pattern of follicle development in anoestrous sheep. Unlike transrectal ultrasonography, this model has the advantages of (i) allowing easy access to an ovary which is fixed in position so that scans can be performed in two planes and the spatial location of individual follicles determined and (ii) allowing repeated collection of ovarian venous blood so that the secretory status of the ovarian follicle population can be determined. Anoestrous animals were chosen for this study in order to avoid perturbations in cycles of follicle development induced by the preovulatory LH surge (Baird and McNeilly, 1981).

3.2 Material and Methods
3.2.1 Experimental Animals

The experiment was performed during the non-breeding season (June) at the Marshall Building. The seasonal anoestrous in the Finn-Merino cross utilised in this study last from early April to late September. During which time they present an mean LH pulse of 6 ng/ml in amplitude at 5 hourly intervals with maximum oestradiol secretion in response to the LH pulse being observed after 25 minutes (Scaramuzzi and Baird, 1977).

The animals were housed indoors, under natural lighting and received a maintenance diet consisting of hay and a pelleted ration. Nine ewes with an ovarian autotransplant (Goding et al., 1967) were studied for 10 days. The animals received Cloprostenol, a potent PGF2α analogue, (125 μg i.m., Estrumate, Cooper’s Animal Health Ltd, Crewe, Cheshire, UK) 15 days before the start of the experiment and to ensure they were anoestrous the concentration of progesterone in jugular venous plasma was determined in a sample collected 10 days after prostaglandin injection.

On the day before the start of blood sampling the animals received both ovarian and jugular venous cannulae under local anaesthesia, as previously described (Chapter 2).

3.2.2 Blood Sampling

Over the 10 day experimental period two sets of samples of both ovarian (5 ml) and jugular (3 ml) venous blood were collected at 12 hourly intervals, one at basal conditions and the other 30 min after a GnRH challenge (250 ng in 2 ml sterile saline i.v.; Sigma, Poole, Dorset, UK). This dose of GnRH has been shown to induce
a LH pulse of 4-6 ng/ml in amplitude (McLeod et al., 1982b; Chapter 2) and was
given so that unstimulated and LH-stimulated concentration of steroid hormones
could be determined. After sampling each cannula was flushed with 5 ml of a
solution of 250 iu of sodium heparin ml⁻¹ of isotonic saline, so the animals received
3000 iu of heparin every 12 hours. The blood was centrifuged at 4 °C for 15 min at
2000 g, the plasma separated and stored at -20 °C until assayed.

3.2.3 Scanning Procedure and Radioimmunoassay

The ovary was scanned daily in both horizontal and vertical planes, using a
7.5 MHz linear transducer with a real-time ultrasound scanner (Chapter 2). All
exams were recorded in video cassette tape and subsequently analysed at the same
day of recording.

The tapes were played in slow motion and the image of follicles greater than
2.5 mm frozen at the largest section of the antral cavity for each individual follicle,
which was locate within the ovary and measured with a digital calliper in the medio-
lateral, dorso-ventral and cranio-caudal planes. The diameter of the follicles was
determined as a mean of these three measurements.

Gonadotrophin and steroid plasma concentrations were measured in duplicate
using previously described (Chapter 2) double-antibody RIA for FSH, LH and
progesterone which were determined in unextracted jugular samples. Androstenedione, GnRH-stimulated oestradiol, and unstimulated oestradiol were
measured in ovarian venous plasma samples after solvent extraction.

3.2.4 Statistical Analysis

Although cycles of development and regression of large antral follicles were
evident in profiles from individual animals these cycles were not synchronised in
different animals. The data were grouped from all animals by identifying dominant
follicles using three parameters: 1. Achieve a diameter of 5 mm; 2. maintenance of a
diameter ≥ 5 mm for 2 days; 3. at least one measurement having been made before it
achieved a diameter of 5mm.

Using these parameters, designed to ensure that the population analysed was
composed of growing healthy follicles that achieved dominance, 12 dominant
follicles were identified, as some ewes had more than one wave during the
observation period. The size of the largest follicle in the ovary and the hormone
concentrations were aligned according to the day the dominant follicle achieved a
diameter of 5 mm (Day 0) and the data incorporated from 1 day before until 3 days
after Day 0 (Day -1 to 3).
The effect of time on the dominant and largest follicle diameter and the concentration of hormones were analysed by repeated samples ANOVA on untransformed data using the general linear means model procedure of Systat software (Systat Inc., Evanston, IL, USA).

3.3 Results

All animals but one remained in anoestrus during the experimental period and the ewe that ovulated was withdrawn from the analysis.

3.3.1 Pattern of Follicle Development and Steroid Secretion

Over the 10 day observation period the ovaries of the experimental ewes contained on average $2.1 \pm 0.4$ follicles that remained between 3-5 mm (medium) and $4.2 \pm 0.4$ follicles that achieved a diameter greater than 5 mm (large). In individual animals there were clear cycles of development and regression of large follicles with a period between 5 and 10 days (Fig. 3.1; Table 3.1).

Table 3.1 Number and duration of the dominant follicular waves per animal. The wave length was defined as the time that the dominant follicle remained over 3.5 mm in diameter.

<table>
<thead>
<tr>
<th>Ewe id.</th>
<th>Number of waves</th>
<th>Length in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2</td>
<td>6; 7</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>9; 6</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>9; 7</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>58</td>
<td>2</td>
<td>5; 7</td>
</tr>
</tbody>
</table>

Total (mean) 12 7.1 ± 0.2

Measurements were available for at least 4 days during the growth phase of only five follicles. The mean size of the dominant follicles on day -3 was $3.3 \pm 0.2$ mm (mean ± SE, n=5) and they grew in a linear fashion at a rate of 0.64 mm/day until they achieve a diameter of 5mm on Day 0 (Fig. 3.2).

LH and progesterone remained at basal concentrations throughout the sampling period and did not vary with time (profiles not shown). The overall concentrations (mean ± SE, n=8) were $1.91 \mu g \text{ LH/l} \pm 0.27$ and $0.52 \pm 0.1 \text{ nmol progesterone/l}$. 

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In addition to these follicular waves, there were also wave-like changes in the level of GnRH-stimulated oestradiol and androstenedione secretion. In individual animals the follicular and secretory waves were positively related during the growth phase of a large antral follicle but unrelated thereafter. Jugular venous FSH concentrations remained relatively stable throughout the experimental period and there was no clear relationship with the pattern of follicular enlargement or steroid secretion (Fig. 3.1).

![Figure 3.1](image)

**Figure 3.1** Profiles from an individual animal showing follicular dynamics in the top panel (dominant follicles are highlighted), in the lower panels the concentration of FSH (□) in jugular venous plasma and the concentration of oestradiol (○), androstenedione (●) in ovarian venous plasma of blood samples collected following a GnRH-challenge (250 ng i.v.). Values are means ± SEM, n=8.
Figure 3.2 Photographs of sequential daily scans (from A to D) showing the enlargement of the second dominant follicle (days 4-7) presented in Figure 3.1. The large arrowhead indicates the dominant follicle while subordinate follicles are indicated by small arrowheads, S indicates the skin and O the margin of the ovary. The grid marks visible at the top of each scan indicate a distance of 10 mm.

3.3.2 Relationship Between Development of Dominant Follicle and Hormone Secretion

In order to clarify the relationship between follicular waves and the ovarian hormone concentration, dominant follicles were identified using the criteria presented in the analysis section and data was grouped around the time each dominant follicle reached 5 mm in diameter (Figure 3.3). Neither the size of the largest follicle nor unstimulated oestradiol secretion changed ($P>0.05$) during the period of growth or regression of a dominant follicle. In contrast, as the dominant follicle grew between Day -1 and 0 the secretion of stimulated oestradiol increased to a peak on Day 1 ($P<0.05$) and then declined ($P<0.05$) between Days 1 and 2, while the size of the dominant follicle remained constant, demonstrating a close association between stimulated oestradiol secretion and follicular growth during the ascending part of the wave. In addition to oestradiol, stimulated androstenedione secretion was also positively related to follicular development and exhibited a similar profile to stimulated oestradiol with an increase during the growing phase of a dominant follicle and a decline preceding any decrease in follicular size. Unlike oestradiol, however, androstenedione secretion increased again on Day 3 ($P<0.05$).

Jugular venous FSH concentrations did not show a clear association with the pattern of follicular enlargement or stimulated oestradiol, but did show a small but
significant change with time, decreasing between Day -1 to -0.5 and increasing from -0.5 to 0 ($P<0.05$).

**Figure 3.3** Relationship among the means of diameter of the largest follicle in the ovary (□), diameter of the dominant follicle (■), concentration of androstenedione (◇), oestradiol in ovarian plasma collected prior to (○) and after (●) a GnRH (250 ng i.v.) challenge and FSH in jugular venous plasma (♦) in relation to the day the dominant achieved a diameter of 5 mm (Day 0). Values are means ± SEM, $n=8$. 

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3.4 Discussion

In this experiment, by using the ovarian autotransplant model, we have been able to demonstrate the existence of follicular waves in sheep and show that size and steroidogenic capacity of large antral follicles in sheep are only positively related during periods of follicle growth thus defining stages of both functional and morphological dominance in sheep during anoestrus.

The observation that a follicle identified as dominant on the basis of its morphology (size) may not be functionally dominant (steroidogenic) has also been made in cattle (Fortune, 1994). However, in that mono-ovulatory species ultrasound alone can be reliably used to identify the stage of the follicular wave due to the fact that the single dominant follicle is substantially larger than subordinate follicles and that the duration of the follicular wave is relatively long and well defined. By contrast, in sheep the small size difference between dominant and subordinate follicles and the short and variable period of the waves makes identification of the dominant follicle by size alone difficult. It is therefore perhaps not surprising that studies which have attempted to characterise follicle dynamics in sheep solely on the basis of size have variously concluded that follicular growth is either continuous and independent of the stage of the oestrous cycle (Turnbull et al., 1977; Lahlou-Kassi and Mariana, 1984; Ravindra et al., 1994) or consists of 2 (Brand and De Jong, 1973), 3 (Smeaton and Robertson, 1971; Noel et al., 1993) or 2-6 (Ginther et al., 1995) waves per cycle. Although we have used the term 'dominant follicle' to describe a large oestrogenic follicle it is clear from Figure 1 that follicle development in sheep is a very dynamic process, and this could explain the report of lack of follicular dominance in sheep (Driancourt et al., 1991; Driancourt, 1994). However, it is important to emphasise that stimulated oestradiol secretion is probably a marker for functional dominance and not its cause, since dominance is likely to be exerted and/or modulated at a local level (Campbell et al., 1995). Nevertheless, dominance in sheep seems to be less pronounced than observed in the cow (Sirois and Fortune, 1990; Savio et al., 1993) and could contribute to the higher incidence of multiple ovulations in this species.

The necessity for FSH to drive the growth of follicles over 2.5mm (gonadotrophin dependent) has been demonstrated in several models such as chronic hypophysectomy (Dufour et al., 1979), active immunisation against GnRH (McNeilly et al., 1986) or chronic GnRH-agonist infusion (McNeilly and Fraser, 1987). In the present study we did not observe cyclic variation in the FSH profiles as reported in non-prolific breeds (Bister and Paquay, 1983; Campbell et al., 1991b) nor any direct link with FSH secretion and the emergence of the follicle waves as
proposed by (Ginther et al., 1995) during the breeding season. One possible explanation for this observation is that during anoestrus LH pulse frequency is so low that oestradiol secretion by the ovary is insufficient to modulate cyclical changes in FSH secretion by the pituitary. This idea is supported by the fact that non-stimulated oestradiol secretion showed no relationship with follicular waves in the present experiment. Alternatively, the presence of follicular waves in the absence of FSH waves suggests that the presence of follicular waves may be inherent due to locally produced factors and that FSH fluctuations merely reflect changes in the steroidogenicity of the ovarian follicle population rather than being the driving force behind them (Campbell et al., 1995).

The results from androstenedione and oestradiol secretion showed that stimulated oestradiol secretion can be used as a marker of follicular steroidogenic capacity thus allowing an assessment of the health status of the follicle. The fact that the fall in oestradiol secretion did not precede the fall in androstenedione production support the hypothesis that aromatase activity is not the first sign of follicular atresia, as suggested by data generated from studies on the expression of steroidogenic enzymes in cows during the first follicular wave (Xu et al., 1995). The generally higher levels of androstenedione secretion and the rise in androstenedione on day 3 when oestradiol remained at basal levels can be attributed to the fact that oestradiol is mainly secreted by the dominant follicle whereas ovarian androstenedione secretion receives a significant contribution from smaller follicles (Baird et al., 1976; Campbell et al., 1991a). Thus the day 3 increase in androstenedione was probably due to evolving follicles from the next wave.

We conclude that follicular growth and ovarian steroid secretion in sheep occurs in a wave like form with the ascending and static part of both waves being synchronous but with a decline in steroid secretion that precedes any changes in follicular diameter. Therefore, in sheep follicular size alone is not a good parameter to assign dominance and the secretory status of the follicle at any given time must be taken into account when studying the dynamics of follicular growth.
Chapter 4
Follicular Dynamics and Ovarian Hormone Secretion in Sheep during the Follicular and Early Luteal Phases of the Oestrous Cycle

4.1 Introduction

Folliculogenesis in sheep occurs from puberty throughout adult life, when from a pool of several thousand follicles only a few will grow to an ovulatory size and fewer still will ovulate. This process is thought to take around six months with most of this time being devoted to the growth of primary follicles to a diameter of 2.5 mm. During this time little selection takes place (Turnbull et al., 1977; Cahill and Mauleon, 1980), in a process that is seemingly independent of gonadotrophin input (Dufour et al., 1979) and involves no significant secretion of oestradiol (Carson et al., 1981; McNatty et al., 1982; Webb and England, 1982). However, while growth of follicles from 2.5 to 5 mm occurs in a few days this is the critical step in the selection of a follicle to a 'dominant' or 'oestrogenic' stage, depending on the hormonal environment (McNeilly et al., 1986; McNeilly and Fraser, 1987; Scaramuzzi et al., 1993).

The endocrine changes in the secretion of pituitary and ovarian hormones during the follicular and early luteal phase of the oestrous cycle are well characterised in intact ewes and in the autotransplanted model (Baird et al., 1976a; Hauger et al., 1977; Baird, 1978; Baird et al., 1981; Baird and McNeilly, 1981; McNeilly et al., 1991; Baird et al., 1991). Following the fall in the concentration of progesterone during luteal regression, there is a rise in the secretion of oestradiol stimulated by an increase in the frequency of LH pulses. In contrast, the concentration of FSH declines due to suppression by the increasing secretion of oestradiol and inhibin from the preovulatory follicle (Baird et al., 1991). Following the LH surge and ovulation while the levels of LH and ovarian steroids remain low, there is a marked rise in the concentration of FSH on Day 1 followed by an increase in the secretion of oestradiol by a new cohort of antral follicles. However, the exact temporal relationships between the endocrine changes and the dynamics of follicle growth of individual follicles is not clear.

Previous attempts that have been made to determine the timing of the selection of ovulatory follicles have yielded conflicting results. Earlier studies found that selection occurred before or at luteolysis (Smeaton and Robertson, 1971; Brand and De Jong, 1973; Land, 1973). Driancourt and Cahill (1984), using ink labelling of large follicles after luteolysis, suggested that final selection does not take place until the late follicular phase (48-54 hours after injection of prostaglandin F2α). Cross-sectional data derived from follicle dissection and oestradiol secretion suggested that
the preovulatory follicle established itself as a large 'oestrogenic' follicle within 10 hours of luteolysis from a pool of antral follicles (2-4 mm in diameter) present at that time (McNatty et al., 1982). Tsonis et al. (1984a), using selective ablation of follicles at luteolysis, demonstrated that ovulatory follicles were derived from follicles greater than 2 mm at the time of luteal regression. However, smaller follicles were also able to ovulate, suggesting that selection can occur in a flexible time frame, both prior to and after luteolysis, according to the follicular population at that time. The use of ultrasonography as a non-invasive and repetitive method of monitoring development of individual follicles in humans (Hackeloer et al., 1979; Kerin et al., 1981) and cattle (Sirois and Fortune, 1988; Ginther et al., 1989), has enabled a more comprehensive understanding of follicular dynamics. This technique in cattle has revealed wave-like cycles of selection, dominance and regression of large antral follicles during the oestrous cycle (Fortune, 1994).

In sheep, however, the use of transrectal ultrasonography has proved more difficult to perform and interpret than in cattle due to problems of anatomical access and the smaller size difference between dominant and subordinate follicles. Recent studies using this technique reported a random emergence of ovulatory sized follicles, i.e. greater than 5 mm in diameter, during the luteal phase (Schrick et al., 1993; Ravindra et al., 1994). However, another study observed waves of follicular development and suggested a relationship with the fluctuation of FSH during the cycle, although steroid secretion was not studied (Ginther et al., 1995).

The ovarian autotransplanted model in which the left ovary is relocated to a site under the skin of the neck, permits repeated collection of ovarian venous blood in the conscious unstressed animal (Goding et al., 1967). Moreover, because it is fixed in an easily accessible location, individual follicles can be identified early by scanning in two planes with high resolution ultrasound. In this study we have investigated the dynamics of follicular development by serial ultrasound measurements, using ovarian steroid secretion and inhibin A as markers of functional dominance, during the follicular and early luteal phase of the oestrous cycle in ewes with autotransplanted ovaries.

4.2 Material and Methods

4.2.1 Experimental Animals.

Six Finn-Merino cross ewes with an ovarian autotransplant (Goding et al., 1967) were studied during the breeding season (November). The animals were housed indoors at the Marshall Building, Roslin, Midlothian, Edinburgh, under natural lighting and received a maintenance diet consisting of hay and a pelleted ration. The
left ovary had been autotransplanted at least five years previously by anastomosing the ovarian artery and utero ovarian vein to the carotid artery and jugular vein respectively. The right ovary was removed at the time of autotransplantation and, hence, the total ovarian secretion of steroids can be measured by cannulating the ovarian vein (Goding et al., 1967). Because these ewes do not cycle spontaneously due to maintenance of the corpus luteum, synchronisation of the estrous cycle was achieved with two injections of cloprostenol a potent analogue of prostaglandin F$_{2\alpha}$ (125 µg i.m. Estrumate, Cooper's Animal Health Ltd, Crewe, Cheshire, UK) given 17 days apart. The day prior to the start of blood sampling both ovarian and jugular veins were cannulated under local anaesthesia as previously described (Chapter 2) and the ewes were then placed in metabolism crates.

4.2.2 Blood Sampling
Samples of ovarian (5 ml) and jugular (3 ml) venous blood were collected at 12 hourly intervals on the day prior to the second injection of cloprostenol (Day=0) and 6 hourly thereafter until Day 8, when the sampling interval returned to 12 hourly until the end of the experiment on Day 10. After sampling each cannula was flushed with 5 ml of a solution of 250 IU of sodium heparin/ml of isotonic saline. The blood was centrifuged at 4°C, the plasma separated and stored at -20°C until assay.

4.2.3 Scanning Procedure and Immunoassay
The ultrasound exams were performed after each blood sample, as described earlier (Chapter 2).

Gonadotrophin and steroid plasma concentrations were measured in duplicate using previously described double-antibody RIA and the concentration of inhibin A in ovarian venous plasma was measured by two site ELISA, as described in Chapter 2.

4.2.4 Statistical Analysis
The data was normalised with respect to two time periods of physiological significance. The first was the time of cloprostenol injection (± 24 hours) and the second related to the onset of the LH surge, defined as the nadir point before LH concentrations exceed 10 ng/ml (Day = 0, from day -1.5 to day 6). For analysis of the relationship between diameter of the follicles that developed during the early luteal phase and ovarian hormone secretion, the data was aligned to the time of emergence of the dominant follicles (first time the follicle was observed with a diameter between 2.5-3 mm). The effects of time on follicular diameter and hormone concentrations were analysed by repeated samples ANOVA on untransformed data using the general
linear means model procedure of SYSTAT software (SYSTAT Inc., Evanston, IL, USA).

4.3 Results

4.3.1 Pattern of Hormonal Secretion

The pattern of gonadotrophin and ovarian hormone secretion in relation to injection of cloprostenol and the LH surge are presented in Figure 4.1. The concentration of progesterone in jugular venous blood declined in all animals after the injection of cloprostenol \((P<0.01)\) and remained at basal levels until Day 4 (Figure 4.1).

The concentrations of LH increased \((P<0.05)\) within 12 hours of cloprostenol injection and remained stable at around 3 ng/ml until the onset of the LH surge 59 ± 4.7 hours later (mean ± SEM). The peak values of the LH surge were observed at 70 ± 5.9 hours. After the surge, the values remained around 5 ng/ml until Day 5 when they decreased \((P<0.05)\). The concentration of FSH in jugular venous blood decreased after injection of cloprostenol \((P<0.05)\) and remained around 1 ng/ml until the time of the LH surge when a synchronous FSH peak occurred \((P<0.05)\). No discrete second FSH peak was apparent in the mean data but they were evident in some profiles from individual animals (Figure 4.2). By Day 1, FSH levels had fallen to their lowest value of the early luteal phase \((P<0.05)\). The concentration of FSH then sharply increased \((P<0.01)\) and remained constant from Day 2 until the end of the experiment.

Androstenedione and oestradiol concentrations in ovarian venous blood increased progressively after injection of cloprostenol \((P<0.05)\) and reached a maximum at the time of LH surge \((P<0.01)\). By Day 1, the concentration of these steroids had fallen to the lowest values observed during the cycle \((P<0.01)\), but subsequently started to increase on Day 2 \((P<0.05)\). The level of androstenedione reached its maximum 3.5 days after the LH surge and decreased thereafter. Oestradiol concentration, however, remained stable from Day 3 until Day 5 before starting to decline \((P<0.01)\).

The concentration of dimeric inhibin A increased progressively during the follicular phase \((P<0.05)\), and reached the maximum values at the onset of the LH surge. After the surge the values of inhibin A steadily decreased and achieved lowest concentrations on Day 2. It then increased again \((P<0.05)\), reaching highest values on Day 3.5 and started to decrease after Day 4.
Figure 4.1. Mean (± SEM) of concentration of progesterone, LH and FSH in jugular and oestradiol, androstenedione and inhibin A in ovarian venous blood. Data has been grouped around the time of injection of cloprostenol (first dotted line) and time of the beginning of the LH surge (second dotted line). Note the break in the scale.
Figure 4.2.
Dynamics of (triangles) ovulatory follicles/CL, (diamonds) follicles from the first wave and second wave of follicular development during the luteal phase (top panel); and the concentrations of FSH in jugular venous blood and oestradiol and inhibin A in ovarian blood from two representative animals (lower panels). The dotted line indicates the time of the onset of the LH surge.
4.3.2 Relationship Between Follicular Development and Hormonal Secretion

The pattern of follicular growth and hormonal profiles are illustrated in Figure 4.3. The follicles that ovulated at the end of the follicular phase arose mainly from large antral follicles present at the time of luteolysis, although smaller follicles could be recruited during the follicular phase (Figure 4.2). Ovulatory follicles persisted following the LH surge and continued to increase in diameter during the luteal phase. The mean diameter of the ovulatory follicle at the time of injection of cloprostenol was 5.1 ± 0.4 mm (mean ± SEM), 5.8 ± 0.3 mm at the onset of LH surge and 6.8 ± 0.5 on Day 1 at the time of estimated ovulation. Between Day 0 and Day 3 the ovulatory follicle/corpus luteum doubled in size, remaining around 10 mm in diameter during the luteal phase.

The ovulatory follicles did not collapse after the LH surge, perhaps because the transplanted ovary is encased in a capsule of connective tissue, by Day 4 changes in the echogenicity of these structures could be noticed as the luteal tissue colonised the antral cavity. These changes were synchronous with the increase in jugular venous progesterone concentrations.

In the early luteal phase of the cycle all animals developed at least one large follicle which grew in a linear fashion at a rate of 1 mm/day until they achieved a diameter of 5 mm on Day 3. No further significant changes in follicle diameter were observed until Day 5.5 when the follicles started to regress (P<0.05). The concentration of oestradiol in ovarian venous blood did not increase until Day 2, when the follicles from the first wave had a mean diameter of 4.0 ± 0.4 mm (P<0.05), and continued to rise until Day 3, then remained unchanged until Day 4.5 when it fell rapidly (P<0.01). The concentration of inhibin A also started to increase from Day 2, continued to increase until Day 3.5 and by Day 4 it started to decrease. In four of the six animals the emergence of a second wave of follicles was evident during the observation period although the levels of ovarian steroids were not of the same magnitude as those from the first wave.

4.3.3 Dominance in the First Wave of Follicular Development in the Luteal Phase

The relationship between follicular development during the first wave of follicles in the luteal phase and pattern of ovarian hormones, is presented in detail in Figure 4.4, with the data being aligned to the time of emergence of the dominant follicles.
Figure 4.3 Relationship between dynamics of ovulatory follicles/CL (triangles), follicles (diamonds) from the first wave and second wave of follicular development during the luteal phase (top panel); and the concentrations of FSH in jugular and oestradiol and inhibin A in ovarian venous blood. Values are mean ± SEM; n=6. Data has been grouped around time of cloprostenol injection (first dotted line) and time of the LH surge (second dotted line). Note the break in the scale.
The follicles grew progressively from the time of emergence (2.9 ± 0.1 mm) until 2.5 days later \((P<0.01)\) and reached a maximum mean diameter of 5.5 ± 0.2 mm. A similar pattern was observed in the concentration of ovarian steroids, except that the concentration of androstenedione and oestradiol did not start to rise until one day after emergence when the follicles were around 4 mm in diameter \((P<0.01)\). There was a progressive decline \((P<0.01)\) in the concentration of oestradiol and androstenedione starting on third day after the emergence of the dominant follicle (Figure 4.4). The concentrations of inhibin A also increased in association with the growth of the dominant follicles, however, the magnitude of these changes in concentration were lower than for the steroids. The first significant decline in follicle diameter was not observed until a day later and, thereafter, the follicle persisted for some days after the steroid secretion had declined to basal levels. Thus, the follicle remains as a recognisable structure long after it has become atretic and has ceased to be a significant source of steroid secretion.
Figure 4.4 Mean (± SEM) diameter of the dominant follicle and the concentration of FSH in jugular and androstenedione, oestradiol and inhibin A in ovarian venous blood of six Finn ewes with ovarian autotransplants. Data has been aligned in relation to the time of the emergence of dominant follicles from the first wave.
4.4 Discussion

By using the ovarian autotransplant model in this experiment, we have been able to monitor the development of individual ovulatory follicles from the late luteal phase, through the follicular and subsequent luteal phase and relate the pattern of follicle development to changes in the concentration of ovarian steroids and pituitary gonadotrophins. We observed the occurrence of follicular waves during the luteal phase and confirmed that the size and steroidogenic capacity of large antral follicles varies during specific periods of follicle growth, therefore, characterising stages of functional and morphological dominance in sheep (Chapter 3).

In the present study the ovulatory follicles were derived mainly from large antral follicles around 5 mm in diameter, present at the time of luteal regression, although a wider range of follicle sizes could be selected to ovulation. The contradictory findings of previous studies (Smeaton and Robertson, 1971; Brand and De Jong, 1973; Land, 1973; McNatty et al., 1982; Driancourt and Cahill, 1984) regarding the timing of selection, can be explained by the fact that ovulatory follicles can be selected from follicles of a wide size range. Our results corroborate those of Tsonis et al. (1984a) showing that all follicles bigger than 2 mm are capable of being promoted into the ovulatory pool. Although most of the ovulatory follicles came from large follicles that were presumably oestrogenic, little oestradiol secretion prior to luteal regression was observed in most animals. This apparent anomaly can be accounted for by the fact that the secretion of oestradiol by the dominant follicle is acutely responsive to LH pulses that are infrequent during the late luteal phase (Baird et al., 1976). A similar situation is present during anestrus when the steroidogenic potential of the follicle can be revealed by providing an LH pulse by injection of gonadotrophin-releasing hormone (GnRH) (Chapter 3).

The observation of waves of follicular development during the early luteal phase support histological observations (Smeaton and Robertson, 1971; Brand and De Jong, 1973) and recent ultrasound studies from both cycling (Ginther et al., 1995) and anestrous ewes (Chapter 3). The fact that the secretion of ovarian steroids declined prior to any significant change in the diameter of the follicle is in agreement with the findings from follicle dissection which identified the loss of aromatase activity as one of the initial signs of atresia preceding morphological changes (Carson et al., 1981; Tsonis et al., 1984b). However, the decline in androstenedione secretion coincident with the decline of oestradiol, suggests that the decrease in oestradiol secretion may be due to lack of androgen precursor. This view is supported by recent studies in cattle which found that 17α-hydroxylase gene expression decreases prior to any chance in the expression of the aromatase gene (Xu et al., 1995). Alternatively, the decline in
steroid secretion could be due to the decrease in LH pulse frequency after Day 3 of the luteal phase (Baird and Scaramuzzi, 1976; Hauger et al., 1977; Campbell et al., 1990b). The periods of functional and morphologic dominance observed in this experiment, are similar to those of the first wave of follicle development in cattle (Sirois and Fortune, 1990; Savio et al., 1993). However, the pattern in sheep appears more dynamic and less pronounced than in the cow, which could account for reports of lack of dominance in sheep (Driancourt et al., 1991; Driancourt, 1994) and explain the higher ovulation rate in this species.

Previous data on immuno-reactive inhibin (α subunit RIA) show a modest increase during the follicular phase and a peak after the LH surge (Campbell et al., 1990a), but the assay used in these studies cross-reacts with non-bioactive forms of inhibin even when calculation of ovarian secretion rates are subtracted from the peripheral concentrations to avoid interference from free α-chain secreted by the adrenal gland. The present data show similar patterns of secretion of inhibin A during the follicular phase, which is less marked than the increase in oestradiol indicating that inhibin A is derived from a wider range of antral follicles while oestradiol is almost exclusively secreted by the dominant follicles (Campbell et al., 1991; Mann et al., 1992; Tisdall et al., 1994). The sharp decrease in inhibin A concentration after the LH surge, in opposition to the peak observed in data from the α subunit RIA, is indicative of the widespread atresia of non-ovulatory follicles caused by the LH surge and is supported by the report of lack of mRNA expression for both inhibin subunits in large antral follicles at the time of the surge (Engelhardt et al., 1993). The increase in immuno-reactive inhibin after the surge is probably due by the release of non-bioactive forms of inhibin in the follicular fluid at the time of ovulation (Engelhardt et al., 1993).

Although there is a significant decline in FSH during the follicular phase as the dominant follicle secretes increasing amounts of oestradiol and inhibin (Baird and McNeilly, 1981; Baird et al., 1991), the relationship between follicle development and FSH during the early luteal phase was less apparent. In some individual animals, e.g. Figure 2, the post ovulatory peak of FSH preceded the emergence of an estrogenic follicle and FSH levels declined in the early luteal phase as the follicle secreted increasing amounts of oestradiol. However, the time of emergence of the first wave was variable between animals and, hence, overall there was no consistent relationship in the mean data. Moreover, the emergence of the second wave of follicles in the luteal phase that was observed in some animals appeared to bear no relationship to fluctuations in the level of FSH or LH. Although the requirement for FSH to promote follicular growth beyond a diameter of 2.5 mm is well documented in different models
(Dufour et al., 1979; McNeilly et al., 1986; McNeilly and Fraser, 1987), the lack of relationship between FSH and the emergence of the follicles from the second wave suggests that the presence of follicular waves may be due to locally produced factors and that FSH fluctuations merely reflect changes in the steroidogenicity of the ovarian follicle population (Campbell et al., 1995).

The presence of the cavity observed in the corpus luteum during the luteal phase, could be an artefact of the transplant procedure as the ovary is subcutaneous and covered in connective tissue, which may prevent the release of follicular fluid at ovulation. However, progesterone concentrations and profile of secretion in ewes with ovarian autotransplants are normal (Collett et al., 1973; Baird et al., 1976b; Campbell et al., 1990a) and morphologically normal corpora lutea have been described in autotransplanted ovaries (Goding et al., 1967). Alternatively, the luteal cavity could be attributed to treatment with prostaglandin as Schrick et al. (1993) reported occurrence of a cavity in over 65% of corpora lutea induced but this had no effect on the profile of progesterone secretion or number of embryos. The luteal cavity has also been reported to have no effect on pregnancy rate or progesterone secretion in heifers (Kastelic et al., 1990; Sprecher et al., 1989).

We conclude that (i) the preovulatory follicles are derived usually from the large follicle population present at the time of luteal regression but the sheep has the ability to promote smaller follicles if required; and (ii) the second peak of FSH stimulates the development of large oestrogenic follicles during the early luteal phase, but the period of functional dominance is shorter than the period of morphological dominance.
Chapter 5
Follicular Waves and Ovarian Hormone Secretion in Sheep during the Luteal Phase of the Oestrous Cycle

5.1 Introduction

The luteal phase is characterised by an ovary bearing at least one corpus luteum (CL), which has evolved from the ovulatory follicle. The dynamics of the endocrine changes that occur during this phase of the cycle are well documented in sheep (for review see (Goodman, 1994). The secretion of progesterone increases progressively from ovulation to mid-cycle and reduces the frequency of LH pulses to values incompatible with ovulation (McNeilly et al., 1991). FSH secretion is not affected by progesterone but is regulated by the oestradiol and inhibin produced by the follicles that develop during this period of the cycle (Baird et al., 1991).

On the basis of evidence from a number of techniques it is thought that the development and demise of large antral follicles occur several times throughout the luteal phase. Analysis of the ovarian follicle population by histological methods demonstrated the occurrence of the development of large antral follicles during this period of the cycle, but failed to agree on its pattern or regulation (Smeaton and Robertson, 1971; Brand and De Jong, 1973; Turnbull et al., 1977). These follicles secrete oestradiol and androstenedione in response to LH stimulation (Baird and Scaramuzzi, 1976; McNatty et al., 1981; Campbell et al., 1990a), which has been assumed to be responsible for the cyclic variation in FSH concentration with a period of 4-5 days throughout the luteal phase (Bister and Paquay, 1983; Campbell et al., 1991b). Studies utilising transrectal ultrasonography have produced direct evidence of follicular growth during the luteal phase, although without further insights into the regulation of follicular development, with reports of random emergence of follicular growth (Schrick et al., 1993; Ravindra et al., 1994) or the emergence of 2-6 waves of follicular development associated with fluctuations in FSH (Ginther et al., 1995).

Recently, studies utilising ewes with an ovarian autotransplant during the follicular and early luteal phase have shown development of waves of follicle growth are associated with increased secretion of oestradiol during periods of high LH pulse frequency (Chapter 4). During anoestrus, when the frequency of endogenous LH pulses is low, the same effect was observed in samples collected after a GnRH challenge (Chapter 3). Both these studies report a period of functional dominance, characterised by high oestradiol secretion, shorter than the period of morphological dominance when the follicle was still visible in the ovary. The aim of this study was to investigate the patterns of development of large antral follicles in relation to the
secretion of ovarian steroids and peripheral gonadotrophins throughout the luteal phase.

5.2 Material and Methods
5.2.1 Experimental Animals

Six Scottish Blackface-Merino (Blackface) and five Finn-Merino (Finn) cross ewes with an ovarian autotransplant were studied during the breeding season (November). The animals were housed indoors at the Marshall Building, Roslin, Midlothian, Edinburgh, under natural lighting and received a maintenance diet consisting of hay and pelleted ration. The ovarian autotransplant preparation (Goding et al., 1967) facilitates the collection of ovarian venous blood and determination of the ovarian follicle population by ultrasound. Because ewes with autotransplanted ovaries do not cycle spontaneously (Baird et al., 1976) synchronisation of the oestrous cycle was achieved with two injections of cloprostenol, a potent analogue of prostaglandin F2\alpha (125 μg i.m. Estrumate, Cooper’s Animal Health Ltd, Crewe, Cheshire, UK) given 17 days apart.

The day prior to the start of blood sampling the left jugular vein was cannulated under local anaesthesia as previously described (Chapter 2) and the ewes placed in metabolism crates. Three days after the second cloprostenol injection, at the expected time of ovulation, a second cannula was inserted into the contralateral jugular vein (transplant side) to enable collection of ovarian venous blood (Chapter 2). The animals received a prophylactic treatment of broad spectrum long-acting antibiotic (3 ml i.m.; Clamoxil, SmithKline Beecham, Surrey, UK) every three days throughout the experiment.

5.2.2 Blood Sampling

Samples of jugular (3 ml) venous blood were collected at 12 hourly intervals from the day prior to the second injection of cloprostenol. In addition two sets of ovarian venous blood (5 ml) samples were collected every 12 hours, starting 3 days after injection of cloprostenol. One sample of ovarian venous blood was collected under basal conditions and the other 30 minutes after a GnRH challenge (250 ng in 2 ml sterile saline i.v.; Sigma, Poole, Dorset, UK). This dose of GnRH has been shown to induce a LH pulse of amplitude similar to that occurring spontaneously (McLeod et al., 1982; Chapter 2) and was given to allow the measurement of basal and LH-stimulated levels of steroid hormone secretion. After sampling each cannula was flushed with 5 ml of a solution of 250 IU of sodium heparin/ml in isotonic saline.
The blood was centrifuged at 4 °C, the plasma separated and stored at -20 °C until assayed.

5.2.3 Scanning Procedure and Hormone Immunoassays

The ultrasound exams were performed after each blood sample when the ovary was scanned in two planes and were recorded on video-cassette tape for subsequent analysis, as previously described (Chapter 2).

Gonadotrophin and steroid plasma concentrations were measured in duplicate using previously described (Chapter 2) double-antibody RIAs. The concentration of inhibin A in ovarian venous plasma was measured by two site ELISA (Chapter 2).

5.2.4 Statistical Analysis

Because the two group of animals used in this experiment differ both in age and breed background, no attempt to compare the groups statistically was made, so the data will be presented based on the results of the Blackface group (young cohort) and only obvious differences between the Blackface and the Finn group will be mentioned.

The hormone data was normalised to the onset of the LH surge, defined as the nadir point before LH concentrations exceed 10 μg/l (Day = 0). For analysis of the relationship between diameter of the dominant follicles (diameter of at least 5mm) that developed during the luteal phase and hormone secretion, follicles were assigned to classes according to the day of the cycle each follicle emerged (when it was first observed with a diameter between 2.5 and 3 mm). The follicles that emerged on days 1-5, 6-10 and 11-15 were assigned as follicle waves 1 (W1), 2 (W2) and 3 (W3), respectively. Within each group the data was analysed from the time of emergence of the dominant follicle to 5 days after. The effects of time on follicular diameter and hormone concentrations were analysed by repeated samples ANOVA on log transformed data using the general linear means model procedure of SYSTAT software (SYSTAT Inc., Evanston, IL, USA). Comparison of the number of follicles per ewe in each wave and the ovulation rate was performed by paired t-test using the same software.

5.3 Results

5.3.1 Progesterone Secretion and Ovulatory Follicles/Cl Development

Luteolysis was induced in all animals after the injection of cloprostenol (PG). However, two ewes (one from each group) did not have an LH surge and were excluded from the analysis. In the remaining animals the LH surge occurred 52.8 ± 2.9 hours (mean ± SEM, n=5) and 54.0 ± 3.5 hours (mean ± SEM, n=4) after the
prostaglandin treatment in Blackface and Finn ewes, respectively. The diameter of the follicles which eventually ovulated increased from a diameter below 5 mm at the time of cloprostenol injection, to a mean diameter of above 5 mm at the time of LH surge in both breed groups and remained at similar size until the expected time of ovulation 24 hours later (Table 5.1). Follicle sizes at all stages tended to be smaller in the Finn ewes.

Table 5.1 Mean (± SEM) diameter of the ovulatory follicles at various times of the oestrous cycle in the ewes of the two groups.

<table>
<thead>
<tr>
<th>Stage of the cycle</th>
<th>Blackface ewes</th>
<th>Finn ewes</th>
</tr>
</thead>
<tbody>
<tr>
<td>cloprostenol injection</td>
<td>4.4 ± 0.4 mm</td>
<td>3.9 ± 0.4 mm</td>
</tr>
<tr>
<td>onset of the LH surge</td>
<td>5.9 ± 0.3 mm</td>
<td>5.1 ± 0.4 mm</td>
</tr>
<tr>
<td>ovulation (LH+24h)</td>
<td>5.8 ± 0.2 mm</td>
<td>5.2 ± 0.3 mm</td>
</tr>
</tbody>
</table>

The ovulation rate in the induced cycle was 2.4 ± 0.2 (mean ± SEM, n=5) and 2.7 ± 0.5 (mean ± SEM, n=4) in the Blackface and Finn ewes, respectively. The CL progressively increased in diameter between day 2 and 5, to around 12 mm in Blackface ewes and 10 mm in the Finn ewes, and remained at that size for the rest of the luteal phase (Figure 5.1). CL diameter was smaller in Finn than Blackface ewes.

In both groups of ewes the mean concentration of progesterone in the peripheral blood remained low during the follicular phase and started to rise on Day 4, progressively increasing until day 7 ($P<0.05$), when it stabilised around 20 nmol/l, remaining at this concentration for the rest of the luteal phase. An increase in CL density, reflected by a decrease in pixel density, preceded the rise in progesterone production by one day, beginning at day 3 and continued to increase until day 5 ($P<0.05$) and remaining steady thereafter.
Figure 5.1 Relationship between concentration of progesterone (circle) in jugular plasma and diameter (triangle) and pixel density (square, 255 = minimal tissue density) of the CL throughout the luteal phase. Values are means ± SEM for Blackface (filled symbols, n=5) and Finn (open symbols, n=4) ewes.
5.3.2 Secretion of Ovarian Hormones During the Luteal Phase

The concentration of oestradiol and androstenedione in the samples collected before and after the GnRH challenge were similar in the interval between Day 1 and 3.5 after LH surge (P>0.05), but thereafter the LH-stimulated steroid values were consistently higher (P<0.05) than unstimulated values (Fig. 5.2 A and B).

The oestradiol and androstenedione concentrations in basal samples increased progressively from Day 1 until Day 3.5 (P<0.05) and then decreased by Day 7 (P<0.05) to low values similar to those observed at the onset of the luteal phase. Oestradiol concentration remained low for the rest of the luteal phase, apart from some isolated episodes of high secretion in different animals. Androstenedione concentration followed the same pattern of oestradiol until Day 7, but showed consistently higher concentrations during the rest of the luteal phase (Fig 5.2 A). Finn ewes had similar patterns of stimulated and unstimulated oestradiol and androstenedione secretion (Fig 5.2 B), with the exception that stimulated androstenedione was 2-fold lower than in the Blackface ewes.

The concentrations of inhibin A throughout the luteal phase were 603 ± 65 and 421 ± 60 ng/l in the Blackface and Finn ewes, respectively. The pattern of secretion in the Blackface ewes started with low values on Day 1 (around 0.3 μg/l), which rapidly increased by Day 2 to concentrations above 0.7 μg/l, where they remained until Day 4 when they reduced to around 0.6 μg/l, remaining at this concentration for the rest of the luteal phase. The pattern of inhibin A secretion in the Finn ewes was similar, however, the increase between Day 1-4 was less acute and mean concentrations during the luteal phase were lower in Finn ewes than in the Blackface (Fig 5.2 B).

Mean concentrations of FSH during the luteal phase in the Blackface ewes were characterised by 3 distinct peaks (P<0.05). The first peak, which occurred on Day 1-2 of the cycle, was the most distinct and occurred at the time of minimal secretion of oestradiol and androstenedione. The second and third peaks, which occurred between Day 5-8 and 10-12 respectively, were broader and of lower magnitude and bore no clear relationship with mean basal or LH-stimulated steroid secretion. The pattern of FSH secretion in the Finn ewes was distinct, it started with higher base line on Day 1 (2 μg/l) and increased to values above 3.5 μg/l on Day 2 and returned to values of 2.5 μg/l by Day 5 and remained around 3 μg/l thereafter. As
Figure 5.2
Concentration of FSH in jugular venous plasma and concentration of oestradiol, androstenedione and inhibin A in ovarian venous plasma during the luteal phase (filled symbols showing basal steroid concentration and open symbol steroid concentrations following a GnRH-challenge, 250 ng i.v.). Values are means ± SEM, for Blackface (A, n=5) and Finn (B, n=4) ewes.

Values are means ± SEM, for Blackface (A, n=5) and Finn (B, n=4) ewes. As shown by the large error bars there was large variation in FSH concentration between the aged Finn ewes. On Day 2 and returned to values of 2.5 μg/l by Day 5 and remained around 3 μg/l thereafter.
5.3.3 Relationship Between Follicular Development and Hormonal Secretion

The pattern of follicular growth and hormone secretion in two individual animals throughout the luteal phase are illustrated in Figure 5.3. In all animals at least one dominant follicle emerged in each wave during the luteal phase. In the Blackface ewes these wave of follicular development were preceded by a rise in the concentration of FSH (Fig 5.3 A) but in the Finn ewes FSH remained high throughout the luteal phase and did not have a clear association with the emergence of the dominant follicle in all animals (Fig 5.3 B). The presence of follicles over 4 mm in diameter was synchronous with episodes of high oestradiol secretion after an GnRH challenge. For further analysis of the relationship between follicular waves and the hormone concentration, dominant follicles were divided according to the day of the cycle as presented in the analysis section and data were grouped around the time of emergence of each follicle.

In individual animals follicle emergence for each of the 3 waves of follicular development was synchronised with dominant follicles from each wave emerging within 36 hours (Fig. 5.3). Due to variation in the wave interval, day of emergence was less synchronised between animals with differences over 48 hours. Nevertheless there was a higher incidence of follicle emergence around days 2, 7 and 11 in the Blackface ewes (Fig. 5.4 A) and days 2, 4-5 and 9 in the Finn ewes (Fig. 5.4 B). No follicles emerged on Days 4, 5 and 9 in the Blackface ewes, days when the concentration of FSH was at its lowest. In the Finn ewes there was no follicle emergence on days 3, 6-8 and 12, despite the mean FSH concentration being above 2 µg/l throughout.

A total of 29 dominant follicles were identified from 5 Blackface ewes during the luteal phase. Ten, 11 and 8 follicles were allocated to the groups W1, W2 and W3, respectively. The mean number of follicles per ewe was 2.0 ± 0.3 in the group W1, 2.2 ± 0.6 follicles for group W2 and 1.6 ± 0.2 in the remaining group. The number of dominant follicles per ewe in each wave was similar (P>0.05) and closely resembled the ovulation rate in the induced cycle (P>0.05).
Figure 5.3
Dynamics of ovulatory follicles/CL (triangles), follicles (diamonds) from the waves of follicular development during the luteal phase (top panel); and the concentrations of FSH in jugular venous blood and oestradiol and inhibin A in ovarian blood (lower panels) from a representative Blackface ewe (A) and a Finn ewe (B).
Figure 5.4 Relationship between emergence of dominant follicles (solid bars) and mean concentration of FSH (O) in jugular venous blood during the luteal phase in Blackface (n=5, A) and Finn ewes (n=4, B).
The dominant follicles in all three waves grew steadily for 2 days from emergence, at a rate of around 1 millimetre per day. The FSH concentrations during the growing phase of each wave were at their highest values at the time of emergence and decreased \( P<0.05 \) as the follicle grew to 5 mm in diameter. The relationship between the hormone concentrations and follicular development were detailed in the groups W1 and W2, where the full evolution of the follicular wave was observed (Fig. 5.5). The dominant follicles in both waves grew at a similar rate from emergence until 2 days later when they were around 5 mm in diameter. They remained over 5 mm in diameter for at least 2 days before decreasing in size, but the follicles from the second wave start to decrease in diameter 4.5 days after emergence \( P<0.05 \) in contrast to the follicles from the first wave which remained at a similar diameter. The ascending limb of the follicular wave was accompanied by a progressive increase in the concentration of unstimulated and LH-stimulated oestradiol in group W1 \( P<0.05 \), while in group W2 the secretion of oestradiol remained constant throughout the development and demise of the dominant follicles.

The concentration of inhibin A increased during the first day of W1 \( P<0.05 \) but did not changed significantly during the remainder of both waves and remained around 0.6 µg/l.

The concentrations of FSH in both waves were high at the emergence of the dominant follicles and decreased as the follicles grew \( P<0.05 \) and remained low while the follicles from W1 were actively secreting oestradiol. In W2 a similar reduction in FSH \( P<0.05 \) was observed although in the absence of significant changes in both basal and LH-stimulated secretion of oestradiol. However, the reduction in the concentration of FSH was greater \( P<0.05 \) in W1 between 2 and 4 days after emergence when oestradiol was actively secreted by the dominant follicles.

In the Finn ewes there were also three waves of follicular development but only the W1 was similar to the OR \( P>0.05 \), the number of dominant follicles was lower in the W2 and W3 \( P<0.05 \). The follicles of W1 and W2 grew from emergence until 2.5 days later but reached smaller maximal diameter. The concentration of oestradiol before and after a GnRH challenge did not reach values above 1 nmol/l and did not vary during both waves. The mean concentration of FSH remained above 2.5 µg/l in both waves and was higher than 3.5 µg/l during the first half of W1. The mean concentration of inhibin A increased progressively during the W1 and reached values around 0.5 µg/l at the end of it, during W2 inhibin A concentrations remained steady (0.5 µg/l) at similar levels of those at the end of W1.
Figure 5.5
Relationship between diameter of the dominant follicle, ovarian secretion of basal and LH-stimulated oestradiol and inhibin A; and concentration of FSH in jugular venous plasma during the first (filled symbols) and second (open symbols) wave of follicular development of the luteal phase. Values are means ± SEM for BlackFace (A; n=5) and Finn ewes (B; n=4). Data has been aligned in relation to the emergence of the dominant follicles (first time the follicle was observed with a diameter of 2.5-3 mm).
5.4 Discussion

This experiment demonstrates the existence of three follicular waves with similar rates of growth in the luteal phase of the sheep. In Blackface ewes, each of these waves was preceded by a rise in FSH concentration which decrease as the dominant follicle progressively increase in size. However, significant changes in oestradiol secretion were observed only during the first follicular wave suggesting that the concentration of FSH during the luteal phase is differentially regulated.

The observation of waves of follicular development in the sheep during the luteal phase is in agreement with previous reports using ultrasound to access the follicular population (Ginther et al., 1995; Chapter 4) and supports the hypothesis that in sheep, in contrast to primates, the concentrations of FSH during the luteal phase are high enough to allow development of dominant follicles (Baird et al., 1975). These results support the hypothesis proposed by Brown (1978) that a threshold concentration of FSH is required for development of dominant follicles, and that an increase in the concentrations of FSH of as little as 10% is able to trigger follicular development in hypogonadal subjects. Similarly, in hypogonadotrophic sheep the infusion of FSH equivalent to the peak levels during the mid-late luteal phase induce growth of preovulatory follicles (Picton and McNeilly, 1991). However, the concentration of FSH necessary to keep large antral follicles growing is modulated by the LH pulse frequency (McNeilly et al., 1992; Campbell et al., 1995) and by intraovarian factors (Cahill et al., 1985; Findlay, 1994; Campbell et al., 1995).

The relatively high secretion of oestradiol and androstenedione during the first wave (Cox et al., 1971; Campbell et al., 1990a; Schrick et al., 1993; Chapter 4) is almost certainly due to the fact that the frequency of LH pulses at this time is higher than that found late in the luteal phase (Campbell et al., 1990b). Steroid secretion starts to decline 2 days after emergence and several days before any decline in follicular diameter. The fact that there is a marked increase in the secretion of steroids after the injection of GnRH strongly suggests that the decline in oestradiol secretion during the first wave is due to a relative lack of LH-stimulated androgen precursor. In contrast, during the second wave the follicles are incapable of secreting increased amounts of oestradiol even after a LH pulse which stimulates an increased secretion of androstenedione. These results suggest that a relatively high frequency of LH pulses is required during the growth of the follicles from 2.5-4 mm if is to acquire the capacity to secrete oestradiol in normal amounts. This hypothesis is supported by the observation that large antral follicles obtained during the mid and late luteal phase produce less oestradiol in short term culture compared to follicles from the early luteal
phase (McNeilly et al., 1992). Thus, follicular oestradiol secretion may be influenced in the long term by the previous pattern of LH pulse frequency.

The secretion of LH-stimulated oestradiol is a marker for the health status of the dominant follicle as it shows the amount of steroid the follicles are capable of producing (Chapter 3) but does not necessarily reflect the levels of steroid in the peripheral circulation. The basal concentration of oestradiol in the ovarian blood is a product of the steroidogenic capacity of the dominant follicles and the degree of stimulation by LH pulses and is indicative of the changes in oestradiol concentration that regulate the secretion of FSH by the pituitary. The decrease in the basal secretion of oestradiol before the reduction in stimulated oestradiol during the first wave of follicular development is due to the normal decrease in LH pulse frequency. The difference between stimulated and basal oestradiol after Day 3 emphasises the way that ovarian oestradiol secretion, and hence FSH concentration, is regulated by the pattern of LH stimulation. Further, the fact that stimulated oestradiol increased for several days after basal oestradiol secretion falls suggest that the reduction in basal oestradiol secretion is not due to atresia of the dominant follicle. Thus LH may be supporting the dominant follicle during this period of low FSH and the lack of LH stimulation may leads to its demise.

The pattern of secretion of dimeric inhibin A during the luteal phase, low at Day 1 and increasing with the emergence of the first wave but remaining constant during the rest of the cycle, is in agreement with the data from Chapter 4 that showed a sharp decrease in the secretion of inhibin after LH surge which is likely to be the result of widespread atresia of non-ovulatory antral follicles. This pattern of secretion also corroborates the hypothesis that inhibin A is secreted by a wider range of antral follicles and not exclusively by the dominant follicles, as is the case for oestradiol (Campbell et al., 1991a; Mann et al., 1992; Engelhardt et al., 1993).

The slower rate of increase of the inhibin secretion after the LH surge and an overall lower concentration during the luteal phase in the older Finn ewes is likely to be the result of a reduced population of antral follicles (see Chapter 7). This hypothesis is reinforced by the fact that the secretion of androstenedione was also lower in the old animals and this steroid is also secreted by other antral follicles in addition to the dominant ones (Baird and Scaramuzzi, 1976; Campbell et al., 1991a; Mann et al., 1992).

The secretion of FSH is regulated by oestradiol and inhibin (Martin et al., 1988; Baird et al., 1991; Mann et al., 1992) but as the secretion of oestradiol by the dominant follicles is reduced during the mid-late luteal phase (second and third waves) by the low levels of LH, inhibin may take a more prominent role in the feed-back
system controlling the production of FSH. Nevertheless, the number of follicles in each wave during the luteal phase was similar to the ovulation rate, supporting the view that follicular selection occurs during the luteal phase (England et al., 1981). Thus despite the distinct patterns of gonadotrophins and ovarian steroids during the follicular phase and the different waves of the luteal phase, the mechanism of follicle selection still remains functional. The OR of the Blackface ewes in this study was somewhat higher than expected (1.5-2), this can be attributed to the good body condition of the animals under experiment. It is, however, interesting, that in Finn ewes, which were expected to have a higher OR, the relationship between FSH fluctuation and follicle waves were much less marked than for the Blackface ewes, suggesting differences in the mechanism of selection in these animals. Unfortunately, as these effects are confounded by age (see Chapter 7), definitive conclusions on this subject must await further experimentation where the effects of age have been controlled for.

Although follicular waves during the luteal phase have been reported earlier (Ginther et al., 1995) our data differ in the number of waves. This difference could be attributed to the great variability in cycle length reported in this previous study. The overall pattern of follicle turnover in sheep during the luteal phase seems to be similar to that observed in the cow which have 2 or 3 waves of follicle development (Sirois and Fortune, 1988; Ginther et al., 1989). However, there are clear differences between the species. In sheep, the wave interval is just 4-5 days, perhaps reflecting the smaller diameter of the dominant follicle, so that the ovulatory wave in sheep is likely to be the third or the fourth, not the second or the third as in the cow. Furthermore, cows only have one dominant follicle per wave (Fortune, 1993), whereas the sheep can have more than one dominant follicle per wave, depending on the ovulation rate of the breed.

In ewes with an ovarian autotransplant the follicles that ovulate do not collapse after the LH surge (Chapter 4). The increase in the CL density prior to the increase in progesterone secretion is likely to be a reflection of the differentiation of the luteal cells leading to the colonisation of the luteal cavity (Niswender et al., 1994).

In conclusion, during the luteal phase there are three waves of follicular development, the emergence of which is preceded by a rise in the concentration of FSH in Blackface animals. The secretion of oestradiol and androstenedione in the unstimulated and LH-stimulated samples were similar during the first 3 days of the luteal phase but differed thereafter, with the LH-stimulated being significantly higher than the basal concentrations, reflecting the endogenous low LH pulse frequency. In the first wave of follicular development the changes in follicular size were
accompanied by an increase in ovarian steroid secretion. During the second follicle wave, although changes in follicle diameter were similar to the first wave, the secretion of ovarian steroids remained unchanged throughout the period of emergence and demise of the dominant follicles. These results confirm that the development of large antral follicles during the luteal phase of the sheep occurs in successive waves and suggest that the concentration of FSH during the luteal phase is differentially regulated according to the stage of the cycle.
Chapter 6
Effect of the Booroola Gene ($F_{ec}^B$) on Follicular Dynamics and Ovarian Hormone Secretion During Follicular and Early Luteal Phases of the Oestrous Cycle

6.1 Introduction

Ewes carrying the Booroola autosomal mutation ($F_{ec}^B$) have increased ovulation rate and litter size (Piper and Bindon, 1982; Davis et al., 1982). In sexually mature ewes the total population of antral follicles and the proportion of non-atretic follicles is similar among genotypes, but the size that follicles mature and ovulate is significantly smaller in ewes carrying the Booroola gene (McNatty et al., 1985; Driancourt et al., 1985; McNatty et al., 1986b; Souza et al., 1994). The non-atretic antral follicles of gene-carrier ewes have fewer granulosa cells when compared with follicles of similar sizes from non-carrier ewes (McNatty et al., 1986b). However, granulosa cells from Booroola gene-carrier ewes show higher mitotic index (Driancourt et al., 1985), cAMP response after FSH or LH stimulation and aromatase activity (Henderson et al., 1987) than cells originating from follicles of non-carrier ewes of a similar diameter.

Despite the differences in follicle number, the total number of granulosa cells from oestrogenic follicles per animal are similar among genotypes (McNatty et al., 1986b) as are the secretion of ovarian steroid and immunoreactive inhibin, estimated from single samples of anaesthetised animals (McNatty and Henderson, 1987; McNatty et al., 1992). Similarly, in Merino ewes with an ovarian autotransplant there was no difference between genotypes in the rate of secretion of oestradiol and progesterone on day 10 of the luteal phase and at various times (24, 36 and 48 hours after cloprostenol induced luteolysis) during the follicular phase (Tsonis et al., 1988).

There is no difference in the concentration of LH in blood or in the anterior pituitary between entire ewes carrying the Booroola gene and controls (Scaramuzzi and Radford, 1983; Robertson et al., 1984; McNatty et al., 1987). The data for FSH has been controversial, with reports of differences among genotypes in FSH concentration during various stages of the oestrous cycle and anoestrus (Bindon, 1984; McNatty et al., 1987; McNatty et al., 1989a) while others reported no difference (Tsonis et al., 1988; McNatty et al., 1991; Driancourt et al., 1991; Wheaton et al., 1996). A previous report from the same flock used in this study showed that FSH concentrations during the anoestrous season were similar among genotypes but during the oestrous cycle and after ovariectomy homozygous carrier of the Booroola gene had...
higher FSH concentrations than heterozygous carriers and non-carrier ewes (Boulton et al., 1995).

Booroola ewes that have been hypophysectomized and induced to ovulate with standard doses of eCG and hCG maintained the differences in the OR between genotypes (Fry et al., 1988). Furthermore, a study using animals made hypogonadotrophic with GnRH antagonist, has shown that ewes treated with physiological doses of FSH and LH in a pattern designed to mimic the normal follicular phase maintained the difference in the number and size characteristics of the genotypes in terms of both follicles and CL (Campbell et al., 1996). Together, these observations suggest that the Booroola gene is likely to act at the ovary.

Despite intensive study, the temporal relationship between follicular development and changes in the concentration of gonadotrophins and ovarian hormone secretion are not well characterised in ewes carrying the FecB gene. In this study we investigated the effect of the FecB gene on the dynamics of follicular development and the secretion of pituitary and ovarian hormones during the follicular and early luteal phase of the oestrous cycle in ewes with an ovarian autotransplant.

6.2 Material and Methods
6.2.1 Experimental Animals

Thirteen Scottish Blackface Merino mature ewes (4-6 years old) with ovarian autotransplants, 7 carrying the FecB gene (4 FecB FecB and 3 FecB Fec+) and 6 non-carriers, were studied during the breeding season (January). The animals were housed indoors at the Marshall Building, under natural lighting and received a maintenance diet of hay and pelleted ration. The ovarian autotransplant operation (Goding et al., 1967) was performed at least six months prior to the study when the right ovary was removed and the left ovary relocated to a site under the skin of the neck. Because ewes with autotransplanted ovaries do not cycle spontaneously (Baird et al., 1976) synchronisation of the oestrous cycle was achieved with two injections of cloprostenol, a potent analogue of prostaglandin F2α (125 µg i.m. Estrumate, Cooper's Animal Health Ltd, Crewe, Cheshire, UK) given 17 days apart.

The day prior to the start of blood sampling the jugular and ovarian vein were cannulated under local anaesthesia as previously described (Chapter 2) and the ewes were placed in metabolism crates.

6.2.2 Blood Sampling

Samples of ovarian (7 ml) and jugular (3 ml) venous blood were collected at 6 hourly intervals for 10 days starting on the day before the second cloprostenol
injection. The ovarian blood flow on every sample was measured by timing the collection of ovarian venous blood and hormone secretion rates were calculated after correcting for the haematocrit (Collett et al., 1973). The blood was centrifuged at 4°C, the plasma separated and stored at -20°C until assay.

In addition, 3 periods of intensive blood sampling were carried out at the following times: (a) late luteal phase, before the second injection of cloprostenol (15 min intervals for 3 h) to evaluate the ovarian response to a GnRH challenge (250 ng in 2 ml sterile saline i.v.; Sigma, Poole, Dorset, UK); (b) early follicular phase, 12 h after the cloprostenol treatment (10 min intervals for 4 h); (c) late follicular phase, 36 h after the second cloprostenol injection (10 min intervals for 4 h). Ovarian blood flow was measured hourly during periods of intensive blood sampling with the intervening samples being withdrawn by syringe.

6.2.3 Scanning Procedure and Immunoassays

The ovaries were scanned at 12 hourly intervals in two planes, using a 7.5 MHz linear transducer with a real time ultrasound scanner. All ultrasound exams were recorded on video cassette tape for subsequent analysis, as previously described (Chapter 2).

Gonadotrophin and ovarian hormone plasma concentrations were measured in duplicate using previously described immunoassay (Chapter 2).

6.2.4 Statistical Analysis

The data was normalised with respect to two time periods of physiological significance. The first was the time of cloprostenol injection (± 12 hours) and the second related to the onset of the LH surge, defined as the nadir point before LH concentrations exceed 10 ng/ml (Day = 0). For analysis of the relationship between diameter of the dominant follicles (diameter of at least 4.5 mm) that developed during the luteal phase and hormone secretion, follicles were aligned by emergence (first time of a follicle was observed in the scans with a diameter 2.5-3 mm). The effects of time on follicular diameter and hormone concentrations were analysed by repeated samples ANOVA using the Systat software (Systat Inc., Evanston, IL, USA). Comparison of the interval luteolysis-onset LH surge and the ovulation rate between the genotypes was performed by independent t-test using the same software.

The pattern of pulsatile hormone secretion during the intensive blood sampling were determined using the Munro pulse analysis software (Zaristow Software, Haddington, East Lothian, UK). The effect of genotype on the number of pulses, amplitude, nadir and mean concentration during the periods of intensive blood
sampling was analysed by repeated samples ANOVA using the Systat statistical package.

6.3 Results

Luteolysis occurred in all animals after the injection of cloprostenol, however, 2 ewes were excluded from subsequent analysis (1 from each group). One non-carrier ewe showed signs that it had not completely recovered from the transplant procedure as it had an ovary half the size compared with other animals in the group and FSH concentration 3 times higher than normal values (Fig. 6.1a). The other carrier ewe showed luteolysis, an LH surge and progesterone secretion similar to the remaining ewes in the group, but had an ovarian cyst (maximum diameter of 7.8 mm) during the first wave, a structure that was significantly bigger than the follicles from the other ewes which did not secrete oestradiol or inhibin A (Fig. 6.1b).

6.3.1 Pattern of Hormonal Secretion

The pattern of gonadotrophin and ovarian secretion in relation to luteal regression and the LH surge in both genotype groups are presented in Figure 6.2. In all ewes, disregarding genotype, the concentration of progesterone in jugular venous blood declined ($P<0.05$) after cloprostenol treatment and remained at basal levels until 4 days after the LH surge when it started to increase ($P<0.05$) and continued to rise until the end of the sampling period.

The timing of onset of the LH surge after cloprostenol injection was not influenced by the genotype ($P>0.05$) and was $48 \pm 1.9$ h (means ± SEM, n=5) and $49 \pm 2.4$ h (means ± SEM, n=6), for the non-carrier and carrier ewes respectively. However, the ovulation rate was significantly higher ($P<0.05$) in gene carrier ewes ($4.0 \pm 0.5$, means ± SEM, n=6) than the non-carrier animals ($2.6 \pm 0.2$, means ± SEM, n=5).

There was no difference in the concentration or profiles of FSH between the genotypes (Fig. 6.2). The concentration of FSH in jugular venous blood, decreased after PG ($P<0.05$) and remained around 1 μg/l until the time of the LH surge when a synchronous FSH peak occurred ($P<0.05$). Due to slight differences in timing, no discrete second FSH peak was apparent in the mean data but were evident in profiles from individual animals (Fig. 6.3). On Day 1, FSH levels fell to their lowest value of the luteal phase ($P<0.05$) and then sharply increased ($P<0.01$) and doubled its concentration on Day 2, remaining around 2 μg/l for a day before starting to decrease.

As with FSH, there were no significant difference between the genotypes in the rate of secretion of oestradiol and androstenedione and both hormones had similar
Figure 6.1 Dynamics of ovulatory follicles/CL (triangles) and follicles from the first wave of follicular development (diamonds) during the luteal phase (top graph); ovarian secretion of oestradiol and inhibin A, and concentration of FSH in jugular venous blood from two atypical animals showing an ewe with reduced ovarian size, low inhibin A and high FSH concentration (a); and an ewe with an ovarian cyst in the first wave which was associated with low oestradiol and inhibin A secretion (b). The dotted line indicates the onset of the LH surge.
Figure 6.2 Mean (± SEM) concentration of LH, progesterone and FSH in jugular venous blood; and mean ovarian secretion of androstenedione, oestradiol and dimeric inhibin A in ewes carrying the Fec^B gene (open symbols, n=6) and non-carrier animals (filled symbols, n=5). Data have been grouped around the time of injection of cloprostenol (first dotted line) and time of the beginning of the LH surge (second dotted line). Note break in the scale.
Figure 6.3
Dynamics of ovulatory follicles/CL (triangles) and follicles from the first wave of follicular development (diamonds) during the luteal phase (top graph); ovarian secretion of oestradiol and inhibin A and concentration of FSH in jugular venous blood in two representative animals. Panel A show typical patterns of a non-gene carrier ewe and FecB gene-carrier ewe in panel B. Dotted line indicates the time of the onset of the LH surge.
patterns of secretion. The secretion of androstenedione and oestradiol increased progressively after PG injection ($P<0.05$) and reached a maximum at the time of the LH surge ($P<0.05$). On Day 1 the rate of ovarian steroid secretion had fallen to its lowest values observed during the cycle ($P<0.05$), subsequently starting to increase on Day 2 ($P<0.05$) to plateau on Day 3.5 before starting to decline on Day 4.5 ($P<0.05$).

The secretion of inhibin A was not different between the genotypes and, in contrast to the ovarian steroids, remained relatively stable during the follicular phase. Inhibin A secretion decreased ($P<0.05$) after the LH surge in both genotypes and increased after Day 1, reaching concentrations around 0.6 ng/ml on Day 3.

**6.3.2 Pattern of Episodic Hormone Secretion**

Mean data for both genotypes during the luteal and early and late follicular phase on the characteristics of pulsatile LH release and oestradiol and androstenedione secretion are shown in Figure 6.4. During the luteal phase the amplitude of GnRH-induced LH pulses was similar between the genotypes but non-carrier ewes had higher nadir concentrations of LH ($P<0.05$) resulting in increased mean concentrations of LH in non-carrier ewes. However, the characteristic of the pulsatile steroid responses to this LH stimulus were similar between the genotypes ($P>0.05$).

The pattern of pulsatile hormone secretion was similar between the genotypes ($P>0.05$) during the transition from the luteal to the follicular phase, with an increase ($P<0.05$) in the pulse frequency of LH, oestradiol and androstenedione, and a reduction in amplitude of the LH pulses. No change in the amplitude of steroid pulsatile secretion was apparent over this transition period.

During the follicular phase the pulse frequency progressively increased for all the 3 hormones but there was no effect on the amplitude of the pulses. Neither frequency nor amplitude of pulses were influenced by the Booroola gene ($P>0.05$). However, the pulse nadir and mean concentration of LH, oestradiol and androstenedione were higher ($P<0.05$) in the non-carrier ewes at 12 hours after cloprostenol-induced luteolysis. At 36 hours after luteolysis the pulse nadir and mean concentration of androstenedione remained higher in the non-carrier ewes ($P<0.05$) but the values for LH and oestradiol were not different between the genotypes ($P>0.05$).

**6.3.3 Relationship Between Follicular Development and Hormonal Secretion**

The pattern of follicular growth and hormonal profiles are illustrated in Figure 6.3 and 6.5. The ovulatory follicles in gene carrier ewes were significantly smaller
than non-carrier animals and also gave rise to corpora lutea of smaller diameter ($P<0.05$). The mean diameter of the ovulatory follicles at the time of injection of cloprostenol was 4.8 ± 0.3 mm (means ± SEM, n=5) and 4.0 ± 0.3 mm (means ± SEM, n=6), 6.0 ± 0.3 and 5.4 ± 0.3 mm at the onset of LH surge and 5.9 ± 0.3 and 5.6 ± 0.3 mm at the time of estimated ovulation on Day 1, in the non-carrier and carrier ewes respectively. Ovulatory follicles persisted following the LH surge and continued to increase in diameter during the luteal phase. Between Day 0 and Day 4 the ovulatory follicle/CL doubled in size and were 12.4 ± 0.6 and 10.4 ± 0.4 mm in the non-carrier and carrier ewes respectively. The CL remained around these diameters during the remainder of the luteal phase.

In the early luteal phase of the cycle all ewes developed at least one large follicle. However, the number of dominant follicles was significantly higher ($P<0.05$) in gene carrier ewes (3.0 ± 0.4, means ± SEM, n=6) than the non-carrier animals (1.8 ± 0.2, means ± SEM, n=5). The dominant follicles grew in a linear fashion at a rate of 1 mm/day until it achieved a diameter of around 5 mm on Day 3. No further significant changes in follicle diameter were observed until Day 5.5 when the follicles started to regress ($P<0.05$). The concentration of oestradiol, androstenedione and inhibin A in ovarian venous blood increased in parallel with growth of the dominant follicles in both genotypes. However the secretion of steroids decreased ($P<0.05$) before any reduction in the dominant follicles.

### 6.3.4 Dominance in the First Wave of Follicular Development in the Luteal Phase

The relationship between follicular development during the first wave of follicles in the luteal phase and pattern of gonadotrophin and ovarian hormones in both genotypes, is presented in detail in Figure 6.6, with the data being aligned to the time of emergence of the dominant follicles.

The dominant follicles in both genotypes grew for two days from emergence at a similar rate but the follicles from non-carrier ewes continued growing for a further day resulting in a higher diameter ($P<0.05$). The maximum diameter that the follicles achieved 3 days after emergence was influenced by the Booroola gene ($P<0.05$) and was 6.0 ± 0.3 and 5.1 ± 0.3 mm in the non-carrier and carrier ewes, respectively.

The pattern of ovarian hormones was not influenced by the Booroola gene. The secretion of ovarian hormone increased as the dominant follicles grew, except that the concentration of oestradiol did not start to rise until one day after emergence when the follicles were above 3.5 mm in diameter ($P<0.05$). There was a progressive decline ($P<0.05$) in the secretion of oestradiol starting on 3.5 days after the follicle
Figure 6.4 Pattern of pulsatile hormone secretion during the late luteal phase (Day 15) and early (PG+12) and late (PG+36) follicular phase non-carrier ewes (filled bars) and ewes carrying the FecB gene (hatched bars). Different letters show statistical difference over time ($P<0.05$) and stars differences between the genotypes ($P<0.05$).
Figure 6.5 Dynamics of ovulatory follicles/CL (triangle) and dominant follicles (diamonds) from the first wave of follicular development during the luteal phase (top panel); ovarian secretion of oestradiol and inhibin A and concentration of FSH in jugular venous plasma. Values are mean ± SEM for ewes carrying the FecB gene (open symbols, n=6) and non-carrier animals (filled symbols, n=5). Data have been grouped around the time of injection of cloprostenol (first dotted line) and time of the beginning of the LH surge (second dotted line). Note break in the scale.
Figure 6.6 Relationship between diameter of the dominant follicle, ovarian secretion of oestradiol and inhibin A; and concentration of FSH in jugular venous plasma during the first wave of follicular development of the luteal phase. Values are means ± SEM for ewes carrying the FecB gene (open symbols, n=6) and non-carrier animals (filled symbols, n=5). Data has been aligned in relation to the emergence of the dominant follicles (first time each follicle was observed with a diameter of 2.5-3 mm).
emergence (Fig. 6.5). The secretion of inhibin A also started to decrease at similar time but the reduction was of a lesser magnitude. The first significant decline in dominant follicle diameter was not observed until a day later, therefore, the follicle persists as a recognisable structure long after it had become atretic and had ceased to be a significant source of steroid secretion.

The concentrations of FSH in both genotypes were high at the emergence of the dominant follicles and decreased as the follicles grew \((P<0.05)\) and remained low while the follicles were secreting oestradiol.

6.4 Discussion

The results of this experiment confirm and extend observations made by other workers that ewes carrying the Fec\(^B\) gene have more dominant follicles that mature at a smaller diameter. The use of ultrasound allowed us to show that the dynamics of follicle development in ewes with and without the Fec\(^B\) gene are similar during the follicular and early luteal phase. In addition, the use of the transplant model has enabled a very clear demonstration that, despite having more follicles, gene-carrier animals secrete similar quantities of oestradiol, androstenedione and inhibin A, in exactly the same pattern, as non-carrier ewes. The similarity in ovarian oestradiol and inhibin A secretion explains the observation that FSH concentrations in the two groups were very similar, an observation that contrasts with the often repeated assertion that FSH concentrations are higher in gene-carriers and one that strongly suggests that the Fec\(^B\) acts at the level of the ovary to modulate gonadotrophic signals.

The ovulatory follicles in the gene-carrier ewes were present in higher numbers and smaller diameter than those from the non-carrier animals (McNatty et al., 1985; Driancourt et al., 1985; McNatty et al., 1986b; Souza et al., 1994) and are the most consistent characteristics of the Booroola gene action. The dominant follicles in the first wave of follicular development of the luteal phase were also in greater number in the gene-carrier ewes and matured at smaller size than those in the non-carrier animals, an observation which is consistent with what has been previously demonstrated in vitro that in ewes carrying the Booroola gene the peak in granulosa cell proliferation (measured by mitotic index) occurs in follicles of smaller diameter (Driancourt et al., 1985). Further, follicles from gene-carrier ewes also exhibit precocious differentiation of granulosa cells, with LH-receptor (McNatty et al., 1986c), gonadotrophin stimulated cAMP production (Henderson et al., 1985; Henderson et al., 1987; McNatty and Henderson, 1987) and aromatase activity (McNatty et al., 1986a; Henderson et al., 1987) occurring at a smaller size than follicles from non-carrier ewes.
The overall pulsatile pattern of LH, androstenedione and oestradiol during the luteal and follicular phases were not influenced by the presence of the Booroola gene, with frequency of pulses increasing progressively during the follicular phase in a similar pattern to that which has been observed in other breeds of sheep (Campbell et al., 1990a). However, there were differences between genotypes in some characteristics of the pulsatile secretion of LH and steroids. The most consistent difference were lower LH and steroid pulse nadir which led to lower overall levels of LH and steroid secretion in the carrier ewes, particularly during the luteal and early follicular phase. These results differ from earlier studies that reported no effect of the Booroola gene on the mean concentration of LH or pulse frequency (Scaramuzzi and Radford, 1983; Bindon, 1984) or consistently higher LH pulse amplitude and concentration in ewes homozygous carrier of the Booroola gene throughout the follicular phase (McNatty et al., 1987). We have no explanation for this difference but the fact that the difference in the OR is maintained between the genotypes and the response after GnRH stimulation is similar indicate that the gene action is unlikely to be due to LH.

In this study the secretion of ovarian hormones was not influenced by the Booroola gene, a result which support previous findings that the secretion of oestradiol is similar among the genotypes in ovarian venous blood obtained from anaesthetised animals (McNatty et al., 1986a; McNatty et al., 1986b; McNatty and Henderson, 1987) and during short-term in vitro cultures of granulosa cells (Henderson et al., 1987; Driancourt, 1991; Henderson et al., 1991; Webb et al., 1995). Tsonis et al. (1988), reported that the pattern of steroid secretion in ewes with ovarian autotransplant at selected intervals during the follicular phase was similar between genotypes of the Booroola gene. The present results extend this observation on steroid secretion, showing that the pattern of ovarian steroid secretion during the follicular and early luteal phase of the oestrous cycle is not different between the Booroola genotypes and similar to other breeds of sheep (Campbell et al., 1990b; Chapter 4). The pattern of secretion of dimeric inhibin A was also similar to what observed in other breed of sheep (Chapter 4). The secretion rate of inhibin A was not different between the genotypes of the Booroola gene during the luteal or follicular phases, which is in agreement with others reports of the secretion of bioactive inhibin (Tsonis et al., 1988) and immunoreactive inhibin (McNatty et al., 1992). These findings are supported by the fact that the FecB gene does not affect the FSH response of ovariectomized ewes to treatment with oestradiol or follicular fluid (McNatty et al., 1989b). The response of FSH concentration to passive immunisation against an
inhibin peptide is similar between the genotypes of the Booroola gene (Wheaton et al., 1996).

The fact that FSH concentrations were similar between the genotype groups, is contradictory to a number of studies (Bindon, 1984; McNatty et al., 1987; McNatty et al., 1989a) but is in agreement with others (Tsonis et al., 1988; Driancourt et al., 1991; McNatty et al., 1991; Wheaton et al., 1996) and is consistent with the exclusion of linkage with the FecB gene in chromosome 6 and the FSHβ gene located in chromosome 15 (Montgomery et al., 1992; Montgomery et al., 1994). Further, the number and size transcripts of the FSHβ gene in pituitary mRNA preparations from different genotypes has found not to be different (Montgomery et al., 1992) and there is no genotype difference in the pituitary volume or number of cells and number and characteristics of gonadotrophs immunostained for FSHβ (Heath et al., 1996). The FecB gene also does not affect the median charge of FSH or its half-live (Fry et al., 1987; Phillips et al., 1995).

The similarity between genotypes in the concentrations of FSH observed in this study is perhaps not surprising as the secretion of FSH in sheep is regulated by oestradiol and inhibin (Baird et al., 1991), and the secretion of these ovarian hormones were similar between the genotypes. Higher FSH concentrations do not seems to be essential for the action of the Booroola gene, since the characteristic differences in ovulation rates are maintained when hypophysectomized ewes of different Booroola genotypes are induced to ovulate with standard doses of eCG and hCG (Fry et al., 1988). Further, ewes with or without the FecB gene, made hypogonadotrophic by GnRH-antagonist treatment, the infusion of the same regime of gonadotrophins (FSH and LH) results in different number of ovulations and maintained the difference in follicular/CL size characteristic of the genotypes (Campbell et al., 1996), suggesting that the Booroola gene is likely to act at the level of ovary.

We conclude that the ovulatory follicles and corpora lutea are smaller but in greater number in ewes carrying the FecB gene, resulting in similar secretion of androstenedione, oestradiol and dimeric inhibin A and identical concentrations of FSH. The dominant follicles that emerge in the first wave of follicular development of the luteal phase also mature at a smaller diameter suggesting that the Booroola gene acts at an ovarian level modulating gonadotrophic signals during both the follicular and early luteal phases of the cycle.
Chapter 7
FSH Concentration in Peri-Menopausal Sheep

7.1 Introduction

The follicle population in the ovary of mammalian species is established before the onset of puberty, but this limited reserve is continuously depleted by the commitment of primordial follicles during adult reproductive life (Hirshfield, 1991). However, humans are unique among other mammalian species and normally experience the extinction of the ovarian follicle population resulting in a reproductive status known as menopause (Gosden and Faddy, 1994). Menopause generally does not occur as a point transition, but during a variable period of menopausal transition. This is initiated when changes in cycle frequency or menstrual flow are first observed, with both gonadotrophins, oestradiol and immuno-reactive inhibin showing a marked degree of variability with shifts from typical post-menopausal patterns to those characteristic of reproductive age. Within 1-2 years after the final menstrual period or menopause; FSH concentrations are markedly elevated and LH levels moderately so, while oestradiol and immuno-reactive inhibin values are low or undetectable, reflecting the exhaustion of the follicle reserve (for review see Burger, 1996).

The initial endocrine changes of the pituitary-ovarian axis in women first become apparent around the age of 38 years, with a selective rise in serum FSH concentrations occurring about the same time as a marked acceleration in the loss of primordial follicles from the ovary and increased atresia in the secondary follicles population (Gougeon et al., 1994; Faddy and Gosden, 1995). The monotrophic FSH rise in normally ovulating older women (age 40-45) is associated with decreased peripheral concentrations of inhibin B during the follicular phase, while oestradiol concentration is higher and inhibin A secretion is similar to a comparative younger cohort of subjects (age 20-25), suggesting that decreased inhibin B secretion reflects a diminished follicular pool in older women and may be an important regulator of the monotrophic FSH rise (Klein et al., 1996). However, to date there is no reliable hormonal marker for the size of the primordial follicular pool to predict the reproductive life span and the lack of animal experimental models to relate follicular population in the ovary and ovarian hormone secretion has meant that the development in this area has been slow.

The results from experiment 3 (Chapter 5) where the older sheep have higher concentrations of FSH suggest that a similar increase in FSH concentration with ageing occurs in sheep and consequently may be associated with the loss of follicles from the primordial pool. To evaluate the effect of age in the ovarian-pituitary axis, the
concentration of FSH, oestradiol and inhibin A in ovarian venous blood was measured in ewes 12-13 years old and compared with values of these hormones in samples from the same animals collected 6 years previously.

7.2 Material and Methods
7.2.1 Animals and Samples
During the breeding season, a group of five Finn-Merino ewes with an ovarian autotransplant (12-13 years old), had their oestrous cycle synchronised by 2 injections of cloprostenol given 17 days apart. The availability of animals with this unusually advanced age, twice the life-span of ordinary sheep in farming conditions, was possible because these sheep have always been kept in doors and fed a hay and concentrated ration which led to the maintenance of the dentition in good condition. Teeth loss and consequent poor body condition is the most frequent cause of culling of ewes from breeding flocks.

Samples of ovarian venous plasma were taken during the luteal (Day 15) and follicular phases (24 and 48 hours after cloprostenol injection) and samples at equivalent stages of the cycle collected from the same animals 6 years previously (Campbell et al., 1990; Campbell et al., 1991b) were used to measure the concentration of FSH, oestradiol and inhibin A within the same assay.

7.2.2 Immunoassay and Statistical Analysis
Gonadotrophin and ovarian hormones concentrations were measured in duplicate using previously described immunoassay (Chapter 2).

The effect of age on the hormone concentration was measured by paired t-test using the Systat software (Systat Inc., Evanston, IL, USA).

7.3 Results
The concentration of the hormones at both ages are presented in the Figure 7.1. The concentrations of FSH were significant higher ($P<0.05$) in the samples taken at older age with this increase being particularly marked during the luteal phase. Increasing age was also associated with a decrease in the concentration of inhibin A ($P<0.05$) but no change in oestradiol concentrations. The magnitude of the difference in FSH concentration decreased during the follicular phase but remained distinct between the ages ($P<0.05$). The secretion of inhibin A and oestradiol in the ovarian vein increased progressively during the follicular phase in both age groups.
Figure 7.1 Mean (± SEM; n=5) concentration of FSH in jugular venous blood; and concentration of dimeric inhibin A and oestradiol in ovarian venous blood during the luteal and follicular phases of the cycle in ewes at different ages (* denotes P<0.05).
7.4 Discussion

The concentration of FSH was higher in the samples collected at an older age. The increase in FSH values was associated with a decrease in the concentration of dimeric inhibin A but unrelated to changes in the concentration of oestradiol.

The observation of increased concentrations of FSH at advanced age is consistent with previous reports in rats and women (Meredith and Butcher, 1985; Ebbiary et al., 1994). In women, this selective increase in FSH seems to be unrelated to the concentration of oestradiol in a similar fashion to observed in this study, however, in the human the increase in gonadotrophin secretion is associated with a decrease in the concentration of inhibin B (Klein et al., 1996) but not inhibin A, which seems to be the likely cause of the FSH increase in sheep.

The pattern of dimeric inhibin secretion during the follicular phase of the menstrual cycle is well defined with predominance of the inhibin B form associated with undifferentiated follicles at the beginning of the follicular phase and a shift to the secretion of inhibin A as the follicles differentiate and the dominant follicle is established during the late follicular phase (Groome et al., 1996). In the rat, the concentration of inhibin B is higher during meteostrous (recruitment) while inhibin A is predominant during the proestrous (follicle selection), however the values of both forms of dimeric inhibin were measurable at all stages of the oestrous cycle (Woodruff et al., 1996). Nevertheless, in species that have continuous turnover of waves of follicular growth like the sheep, the pattern of inhibin A could be more important in regulating basal concentration of FSH, since inhibin A is 100-fold more potent than the B form in suppressing the release of FSH from ovine pituitary cells cultured in-vitro (Robertson et al., 1996). Further, the same preparation of inhibin A assayed for biopotency in rat pituitary cells was only 5-fold more efficient than inhibin B in suppressing FSH release in vitro, suggesting species differences in FSH regulation (Robertson et al., 1996).

The regulation of the basal concentration of FSH by inhibin has been suggested to operate in the sheep while changes in oestradiol secretion are thought to be responsible for fluctuations during the cycle (Baird et al., 1991). The pattern of inhibin A secretion during the oestrous cycle (Chapters 4, 5 and 6) is stable throughout the luteal phase, but increases above the base line during periods of high frequency of LH pulses and is associated with the presence of large oestrogenic follicles during the follicular phase and early luteal phase (first wave). Inhibin A secretion is greatly reduced after the LH surge and this pattern of secretion is consistent with the lack of mRNA expression for both α and β subunits of inhibin A in large antral follicles during and after the LH surge (Engelhardt et al., 1993). The
pattern of dimeric inhibin B secretion in sheep is less understood. Previous reports on the secretion of immuno-reactive inhibin have shown that at least half of the Inhibin secreted by the ovary is originated from follicles other than the large oestrogenic follicle (dominant) (Campbell et al., 1991a; Mann et al., 1992). This observation is congruous with the pattern of expression of mRNA and protein for α, βA and βB subunits of inhibin which are located in the granulosa cells of the majority of non-atretic antral follicles (Engelhardt et al., 1993; Braw-Tal, 1994; Engelhardt et al., 1995).

We conclude that FSH concentration are elevated in ewes older than 12 years of age, which is associated with decreased ovarian secretion of inhibin A but similar levels of oestradiol. This suggests a relationship between the secretion of inhibin A and the total follicle population in the ovary. The reduction of which may result in an increase in the concentrations of FSH in a similar fashion to that observed in perimenopausal women. Thus the sheep could be a useful animal model to study the variations in the ovarian follicle population and hormone secretion in order to obtain hormonal predictors of the onset of menopause and its pathological expression, premature ovarian failure.
8.1 Follicular Waves in the Sheep

Although the presence of large antral follicles in sheep ovaries during the luteal phase and anoestrus has been known since the sixties (Hutchinson and Robertson, 1966), the pattern of terminal follicular development in sheep has been a controversial issue for the last three decades. The presence of follicular waves was first suggested on the basis of histological studies (Smeaton and Robertson, 1971) but direct confirmation remained elusive despite descriptions of cyclical variation in the concentrations of peripheral oestradiol (Cox et al., 1971; Mattner and Braden, 1972) and FSH (Bister and Paquay, 1983; Campbell et al., 1991b). Only recently has ultrasound equipment become readily available (portability and cost) to allow direct studies in vivo to be undertaken.

Studies utilising transrectal ultrasonography have produced direct evidence of follicular growth during the luteal phase, although without further insights into the regulation of follicular development, with reports of random emergence of follicular growth (Schrick et al., 1993; Ravindra et al., 1994) or the emergence of 2-6 waves of follicular development (Ginther et al., 1995). Transrectal ultrasound in sheep, is difficult to perform and interpret due to the problems of anatomical access, the smaller size difference between dominant and subordinate follicles plus the possibility of more than one dominant follicle makes the identification of each follicle paramount for the interpretation of the scan. In addition, it is difficult to relate steroid production to the ovarian follicular population due to the low concentrations in the peripheral circulation in this species.

The studies described in this thesis, utilising ewes with an ovarian autotransplant, clearly demonstrate that in sheep the growth of antral follicles beyond 2.5 mm in diameter occurs as a succession of waves during anoestrus (Chapter 3) and during the luteal phase of the oestrous cycle (Chapters 4, 5 and 6). During the follicular phase the ovulatory follicles are mainly derived from large dominant follicles present at the time of luteolysis but smaller follicles can be promoted to the ovulatory pool (Chapter 4). The development of the dominant follicles during anoestrus and during the luteal phase is associated with an increase in the secretion of ovarian oestradiol, androstenedione and dimeric inhibin A but hormone secretion decreases before any significant changes in follicular diameter, thus characterising periods of morphological and functional dominance (Chapters 3-6). The number of dominant follicles in each wave during the luteal phase is similar to the OR in the same cycle.
(Chapters 5 and 6) suggesting that the mechanisms of selection operate throughout the luteal phase. The Booroola gene does not change the overall pattern of follicular development, but as the follicles in gene-carrier animals mature at smaller size, the ovulation rate is increased without changes in the concentration of FSH or the ovarian secretion of oestradiol, androstenedione or dimeric inhibin A (Chapter 6).

Although the studies described in this thesis are essentially descriptive, some insights into the control of follicle selection can be gained from the temporal relationship between the concentration of gonadotrophins and secretion of ovarian steroids and inhibin. The major question, however, is how these waves are regulated into synchronised wave-like patterns.

8.2 Control of Follicle Development

It has been demonstrated in sheep that FSH is essential for follicular development beyond a diameter of 2.5 mm (Dufour et al., 1979; McNeilly et al., 1986; Driancourt et al., 1987; McNeilly and Fraser, 1987) and can increase the rate of growth of antral follicles with a diameter up to 2 mm (Dufour et al., 1979). Physiological doses of FSH in vitro induce proliferation and oestradiol secretion in granulosa cells from follicles smaller than 3.5 mm and maintain oestradiol secretion in cells from larger follicles (Campbell et al., 1996). The relationship between increases in FSH concentration above a threshold level and terminal follicle development has been demonstrated in humans (Brown, 1978), cows (Adams et al., 1992) and sheep (Picton and McNeilly, 1991).

There are interesting differences in the behaviour of the follicular waves among these three species. In women, follicular cells do not lose their ability to synthesise oestradiol and inhibin A after luteinization (Baird et al., 1975; Groome et al., 1994) resulting in concentrations of FSH during the luteal phase which are below the levels required to stimulate follicular development. The levels of FSH only rise above threshold after luteolysis resulting in the emergence of a new cohort of follicles at the beginning of the follicular phase from which the dominant follicle is selected.

In ruminants, the CL does not secrete either oestradiol or inhibin (Mann et al., 1989; Campbell et al., 1990) so the major regulators of FSH secretion are exclusive products of the follicles allowing follicular development throughout the luteal phase. The overall pattern of follicle turnover in sheep during the luteal phase seems to be similar to that observed in the cow which have 2 or 3 waves of follicle development (Sirois and Fortune, 1988; Ginther et al., 1989). However, there are differences between these species. In sheep, the wave interval is about 4-5 days, perhaps reflecting the smaller diameter of the dominant follicle, so that the ovulatory wave in
sheep is likely to be the third or the fourth, not the second wave as is usual in the cow. Furthermore, cows only have one dominant follicle per wave (Fortune, 1993), whereas the sheep can have more than one dominant follicle per wave, depending on the ovulation rate of the breed. Another peculiarity of the sheep is the ability to promote additional ovulatory follicles during the follicular phase, even in the presence of large antral follicles (Chapters 4, 5 and 6). The late promotion of ovulatory follicles in combination with the very dynamic pattern of the waves could be the reason for the repeated assertion that the mechanism of dominance does not operate in sheep (Driancourt et al., 1991; Driancourt, 1994). Nevertheless, dominance in sheep seems to be less pronounced than observed in the cow (Sirois and Fortune, 1990; Savio et al., 1993) and could contribute to the higher incidence of multiple ovulations in this species.

FSH is clearly involved in the emergence of the dominant follicles, so to maintain the pattern of follicular waves the concentration of FSH must fluctuate in a carefully controlled manner during the cycle. Two ovarian hormones (oestradiol and inhibin) are known to regulate the secretion of FSH (Baird et al., 1991) and it has long been hypothesised that the secretion of these hormones by the dominant follicle directs the development of the follicular waves. This hypothesis states that the demise of a dominant follicle will lead to a fall in oestradiol and inhibin secretion which in turn leads to an increase in FSH that promotes the recruitment of a new cohort of follicles. The selection of the dominant follicle leads to an increase in oestradiol and inhibin secretion and a fall in FSH concentration, thereby inducing atresia in subordinate follicles. This hypothesis would therefore predicts that the sheep cycle is characterised by waves of oestradiol and inhibin secretion that would readily follow the development and demise of dominant follicles with FSH concentration exhibiting reciprocal fluctuations. Examination of the data presented in this thesis suggests that this hypothesis is over simplistic and the model needs to be modified to account for the level of LH stimulation for oestradiol secretion by the dominant follicle and the population of subordinate follicles in the ovary, which make a significant contribution to ovarian inhibin secretion.

The emergence of the first wave of the luteal phase in sheep is fairly well synchronised between animals but as the luteal phase progresses they become less synchronous. The follicular growth is probably synchronised by the wide spread atresia of the non-ovulatory follicles after the LH surge. During the oestrous cycle the highest rate of atresia of follicles over 1 mm in diameter is found on Day 1. During the luteal phase the same effect is also found in ewes primed with eCG for 48 hours and challenged with hCG, all follicles greater than 3.5 mm in diameter are luteinized and
two-thirds of the follicles between 1-3.5 mm show signs of atresia in ovaries collected 18 hours after hCG (Turnbull et al., 1977). The class of antral follicles between 1.5 and 2.5 mm in diameter shows the greatest incidence of atresia during the oestrous cycle (Turnbull et al., 1977), so this population could work as a buffer zone between continuous recruitment and the wave pattern of development beyond 2.5 mm.

The emergence of the wave from the pool of follicles below 2.5 mm in diameter occurs synchronous with a rise in FSH concentrations which was particularly evident when young Blackface animals were used (Chapters 5 and 6). In the results from the older Finn ewes this relationship was less clear (Chapters 4 and 5) but although the effect of breed cannot be discarded, it seems more likely to be an effect of ageing as discussed earlier (Chapter 7).

During the first wave of follicular development in the luteal phase, when the secretion of oestradiol is relatively high under frequent LH pulse stimulation, there is an inverse relationship between oestradiol and FSH concentration. The fact that the dominant follicles secrete oestradiol in response to LH-induced stimulation suggests that the demise of these follicles from the first wave is due to a fall in LH drive (Chapter 5). In the following waves of follicular development during the remainder of the luteal phase, when the progesterone concentration increases, this relationship is less clear even when oestradiol ovarian secretion is measured after an induced LH-pulse (Chapter 5). The secretion of ovarian inhibin A also tended to be more pronounced during the first wave, but this tendency fades in the following waves. However, in contrast to the secretion of oestradiol the dominant follicle is not the only source of inhibin (Campbell et al., 1991a; Mann et al., 1992; Engelhardt et al., 1993). So oestradiol is the ideal regulator of acute changes in the FSH concentration while inhibin A could be responsible for long-term regulation of FSH (base line levels). This hypothesis explains the result from the first wave but does not invalidate the possibility of inhibin A playing a more prominent role in FSH control in periods that LH pulse frequency and hence oestradiol secretion are low (Chapter 5). Another possibility is that the levels measured in ovarian blood do not reflect the same pattern in peripheral blood during the mid-late luteal phase. The secretion of inhibin B in sheep is not known and could also be involved in the regulation of tonic FSH release, but results in vitro indicate that the action of the B form is much less potent than inhibin A in suppressing FSH release in the sheep (Robertson et al., 1996). A further possibility is that FSH fluctuations do not control follicles waves, but simply reflect intra-ovarian mechanisms that result in cyclic variation in ovarian oestradiol and/or inhibin secretion (Campbell et al., 1995). These possibilities are amenable to further experimental investigation.
The majority of ovulatory follicles in sheep are large antral follicles at the time of luteal regression, but smaller follicles can be promoted to the ovulatory pool during the follicular phase. This occurred in around 10% of the cycles observed in these experiments and could be due to a reduction in the FSH threshold when associated with high LH pulse stimulation, as suggested by McNeilly et al., (1992).

The results on the effect of the Booroola gene on follicular dynamics (Chapter 6) also illustrate that the action of FSH can be modulated at an ovarian level. The secretion of ovarian oestradiol and inhibin A are not affect by the Booroola gene resulting in similar concentrations of FSH despite significant differences in OR and diameter of the follicles. The genic product of the Booroola gene remains unknown but is likely to be paracrine factor. Several growth factors have been shown to modulate gonadotrophin action in sheep. Factors such as TGF-α, EGF, FGF and IGF-binding proteins act by stimulating proliferation and/or inhibiting differentiation of follicular cells. In contrast, others such as IGF, TGF-β and activin are believed to work by increasing or inducing early differentiation of this cells, enhancing steroid and inhibin production and/or inducing hormone receptors (Campbell et al., 1995).

8.3 Conclusions

The studies described in this thesis show that in sheep the growth of antral follicles beyond 2.5 mm in diameter occurs as a succession of waves during anoestrus and during the oestrous cycle. These results also demonstrate the temporal relationship between the development of antral follicles beyond 2.5 mm in diameter and the fluctuations in the concentration of FSH, which are regulated by the secretion of ovarian oestradiol and inhibin A produced by the mature follicles. However, similar concentration of gonadotrophins can be modulated by intra-ovarian factors to produce markedly different ovulation rates e.g. the Booroola gene. In addition these data show that the secretion of inhibin A in the sheep is related to the follicular population in the ovary, and a reduction in inhibin A concentrations with advanced age is reflected by an increase in the levels of FSH in a similar fashion to that observed in peri-menopausal women, suggesting that sheep could be a useful animal model to study the effects of ageing on the dynamics of the ovarian follicle population and hormone secretion.
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### Appendix

**Assay Protocols**

**Assay Buffers**

#### 0.01M phosphate buffered saline (PBS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tr>
<td>Na$_2$HPO$_4$.2H$_2$O</td>
<td>1.75 g</td>
<td>0.35 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$ (anhydrous)</td>
<td>5.90 g</td>
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<tr>
<td>NaCl</td>
<td>45.0 g</td>
<td>9.00 g</td>
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<tr>
<td>Distilled water</td>
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**Phosphate Citrate Buffer (PCB)**

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<td>Na$_2$HPO$_4$</td>
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<td>Citric acid powder</td>
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<td>Thiomerosalate</td>
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#### 0.1M Carbonate Buffer

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<td>Na bicarbonate</td>
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pH to 9.6

**Blocking Buffer**

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<td>NaCl</td>
<td>0.9 g</td>
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<tr>
<td>BSA (A 3294)</td>
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<td>Deionised Water</td>
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**6% SDS**

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<td>SDS</td>
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**6% H$_2$O$_2$**

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<td>30% v/v H$_2$O$_2$</td>
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**Wash Buffer (25x)**

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<td>Tris</td>
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<td>NaCl</td>
<td>225 g</td>
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<tr>
<td>Tween-20</td>
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pH to 7.5

| Deionised water | 11 |

122
Tris-azide Assay Buffer

0.1 M Tris 2.423 g
0.15 M NaCl 1.753 g
5% triton x-100 10 ml
10% BSA (A3294) 20 g
0.1% Na azide 0.2 g
Deionised water to 200 ml

Stop Solution (0.4M HCl)
1M HCl 40 ml
Deionised water 60 ml

FSH Assay

Buffer - PBS + 1% BSA, pH 7.4

Day 1

<table>
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<tbody>
<tr>
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<td>-</td>
</tr>
<tr>
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<td>-</td>
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<td>50 μl</td>
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<tr>
<td>BO’s</td>
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<tr>
<td>samples</td>
<td>-</td>
<td>150 μl</td>
<td>150 μl</td>
<td>50 μl</td>
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</tbody>
</table>

Antiserum (NIADDK anti-oFSH -1, rabbit)
Add 1 ml of distilled water to lyophilised powder. Dilute neat antiserum into aliquots of 1:100 with 1% BSA/PBS and freeze as stock solution. Batch (AFP C5288113) to be used at a working dilution of 1:12,000 (8.3 μl/ml buffer). Mix tubes and incubate for 24 hours at 4°C.

Day 2
Add 50 μl tracer to all tubes (approx. 15,000 cpm), mix tubes and incubate overnight at 4°C.

Day 3
Add 100 μl DARS (7144X 1:32) and 100 μl NRS (7375Y 1:800) to all tubes except total counts, mix tubes and incubate overnight at 4°C.

Day 4
Add 1 ml 0.9% saline to all tubes (except total counts), spin 3000 rpm for 25 minutes, at 4°C. Discard supernatant and count pellets on gamma-counter for 60 seconds.
**LH Assay**

Buffer- PBS + 0.1% BSA, pH 7.5

**Day 1**

<table>
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<tr>
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<th>Ab</th>
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<tr>
<td>samples</td>
<td>-</td>
<td>200 µl</td>
<td>100 µl</td>
<td>100 µl</td>
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</table>

Antiserum (in house R29)

Dilute neat antiserum into aliquots of 1:100 with 0.1% BSA/PBS and freeze (stock solution). Working dilution is currently 1:120,000 (0.83 µl/ml buffer).

Mix tubes and incubate for 24 hours at 4° C.

**Day 2**

Add 100 µl tracer to all tubes (approx. 15,000 cpm), mix tubes and incubate overnight at 4° C.

**Day 3**

Add 100 µl DARS (5651P 1:32) and 100 µl NRS (5731R 1:1600) to all tubes except total counts, mix tubes and incubate overnight at 4° C.

**Day 4**

Add 1 ml 0.9% saline to all tubes (except total counts), spin 3000 rpm for 25 minutes, at 4° C. Discard supernatant and count pellets on gamma-counter for 60 seconds.
Progesterone Assay
Buffer PCB+ 0.1% gelatine, pH 6
Day 1

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<td>NSB's</td>
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</tr>
<tr>
<td>BO's</td>
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<tr>
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<tr>
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<tr>
<td>sample</td>
<td>-</td>
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<td>50 µl</td>
<td>100 µl</td>
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</table>

Dilute neat antiserum (R 31/8) into aliquots of 1:100 with PCB without gelatine and freeze (stock solution). Working dilution is currently 1:20,000.
Add 100 µl of 125I P4 tracer, diluted in PCB without gelatine with 10 mg/10 ml ANSA (8-Anilino-1 Naphthalene Sulphonic Acid), to all tubes. Mix tubes and incubate for 3 hours at room temperature.
Add second antibody 100 µl of DARS diluted in assay buffer to 1:35 and 100 µl NRS 1:300. Mix tubes and incubate overnight at 4°C.
Day 2
Add 1 ml of 0.9% saline to all tubes (except total counts), spin 2500 rpm for 30 minutes at 4°C. Decant supernatant and count pellets on gamma-counter for 60 seconds.

5 Oestradiol Assay
Buffer PBS + 0.1% gelatine
Extraction - 100 µl of plasma + 1 ml of diethyl ether (double distilled) to 75 x 12 glass tubes. Vortex for 1 min. Freeze in dry ice/ethanol bath. Decant ether extract into 75 x 10 mm glass tubes. Dry down in heating block (40°C).
Day 1
Reconstitute the extract in 100 µl of assay buffer. Add 100 µl of antibody (BW anti E2 at 1:3000,000) and 100 µl of tracer (15,000 cpm) incubate for 2 hours at room temperature. Add the second antibody 100 µl of DAS diluted in assay buffer to 1:8 and 100 µl of NSS 1:400. Mix the tubes and incubate overnight at 4°C.
Day 2
Add 1 ml of 0.9% saline to all tubes (except total counts), spin 3000 rpm for 30 minutes at 4°C. Decant supernatant and count pellets on gamma-counter for 60 seconds.
**MAIA Kit Oestradiol Assay**

Buffer PBS + 0.1% gelatine + 0.3% EDTA, pH 7.6

Extraction - Similar to the previous assay

Day 1

Reconstitute the extract in 250 µl of assay buffer. Add 50 µl of first antibody (supplied with the kit) diluted in assay buffer to 1:6 and 50 µl of oestradiol tracer (10,000 cpm). Incubate for 2 hours at room temperature and then add 100 µl of the assay kit separation reagent. Incubate for a further 10 min and then add 1 ml of PBS. Spin 2500 rpm for 10 minutes at 4° C. Aspirate the supernatant and count pellets on gamma-counter for 60 seconds.

**Androstenedione Assay**

Buffer PBS + 0.25% BSA, pH 7.2

Extraction - 200 µl of plasma + 2 ml of 4:1 hexane:diethyl ether to 75 x 12 glass tubes. Vortex for 2 x 60 seconds. Freeze in dry ice/ethanol bath. Decant ether extract into 75 x 10 mm glass tubes. Dry down in heating block (50-60° C) under nitrogen.

Day 1

<table>
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<tr>
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<tr>
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<td>-</td>
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<td>200 µl</td>
</tr>
<tr>
<td>standards</td>
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<td>-</td>
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<tr>
<td>QC's</td>
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<td>-</td>
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<td>200 µl</td>
</tr>
<tr>
<td>sample</td>
<td>200 µl</td>
<td>2 ml</td>
<td>-</td>
<td>200 µl</td>
<td>200 µl</td>
</tr>
</tbody>
</table>

Antiserum (Ab C) stored 1:90 dilution stored frozen. Working dilution 1:56,250 (80 µl in 50 ml buffer). Tracer 125I-A4 (collected from Royal Infirmary Glasgow), shelf life is approximately 8 weeks. Working solution approximately 12,000 cpm / 200 µl.

Mix tubes and incubate overnight at 4° C.

Day 2

Add 100 µl of DARS (1:20) and 100 µl NRS (1:200) to all tubes except total counts and incubate overnight at 4° C.

Day 3

Spin at 3000 rpm for 45 minutes, at 4° C. Aspirate the supernatant and count pellets on multigamma counter for 60 seconds.
Inhibin A Assay

Day 1

Coat Plates

E4 @ 2 mg/ml dilute to 10 μg/ml (1:200 dilution in carbonate buffer pH 9.6).

Add 50 μl/well, incubate at room temperature for 4 hours in a sealed box.

Wash the plate twice with Tris wash buffer (pH 7.5) no tween.

Add 150 μl of blocking buffer and incubate for a minimum of 2 hours at room temperature.

Samples

100 μl sample/standard + 50 μl 6% SDS, boil for 3 minutes, cool.

Add 100 μl assay buffer and add 50 μl 6% H₂O₂.

Oxidise at room temperature for 30 minutes.

Wash E4 coated and blocked plate 10x and bang dry.

Add samples/standards @ 100 μl/well. Cover the plate and incubate overnight at room temperature.

Day 2

Dilute the α-subunit specific biotinylated monoclonal antibody (17329/H2) 1:330 tris-azide assay buffer (15 μl/5 mls assay buffer), giving a working concentration of 1 μg/ml. Wash the plate 10x, add antibody @ 50 μl / well. Incubate at room temperature on a shaker for 1 hour.

Dilute the streptavidin alkaline phosphatase @ 1:200 (25 μl / 5 mls tris-azide assay buffer). Wash plate thoroughly (5x incubate with wash buffer for 5 minutes, wash 5x) and add strep. adv. alk. p. @ 50 μl / well. Incubate at room temperature on a shaker for 1 hour.

Wash plate thoroughly (5x incubate with wash buffer for 5 minutes, wash 5x) and add detection system 50 μl / well substrate, incubate on a shaker for 2 hours at room temperature. Add amplifier @ 50 μl / well.

When colour develops (20 -3 0 minutes) stop plate with 50 μl / well 0.4M HCl. Read plate at 490 nm, top std should read approx. 2.000.

Standards

32k Bovine stds double diluted in OVX plasma from 1000 pg/ml to 15.6 pg/ml

Plates

Nunc-Immuno Plate, Maxisorp flat bottom Cat No: 4-42404A from Life Technologies

Amplification System

Elisa amplification Kit Cat No: 195 89019 from Gibco BRL, Life Technologies
Published Papers

Part of the data presented in this thesis have been published in two articles to the Journal of Reproduction and Fertility and Biology of Reproduction. These articles have been reproduced with permission of the joint authors and journal publishers.


Follicular dynamics and ovarian steroid secretion in sheep during anoestrus

C. J. H. Souza, B. K. Campbell and D. T. Baird

Department of Obstetrics and Gynaecology, Centre for Reproductive Biology, University of Edinburgh, 37 Chalmers St, Edinburgh EH1 9EW, UK

The dynamics of ovarian follicular development and its relationship to ovarian and pituitary hormones during seasonal anoestrus were investigated for 10 days in nine ewes with autotransplanted ovaries in a longitudinal study. The size and position in the ovary of individual follicles over 2.5 mm in diameter were recorded by daily ultrasonography. Samples of ovarian and jugular venous blood were collected at intervals of 12 h, before and after a GnRH challenge (250 ng GnRH, i.v.) so that basal and LH-stimulated ovarian steroid secretion could be determined. Throughout the experimental period, all animals developed at least one large antral follicle > 5 mm, which secreted increased (P < 0.05) amounts of oestradiol and androstenedione in response to an LH challenge as the diameter of the follicle increased. However, a decrease (P < 0.05) in ovarian steroid secretion preceded any significant change in follicular diameter, indicating a dissociation between morphological and functional stages of dominance in sheep. We conclude that follicular growth and ovarian steroid secretion in sheep occur in wave-like forms, with the ascending and static part of both waves being synchronous but with a decline in steroid secretion preceding any changes in follicular diameter. Therefore, in sheep, follicular size alone is not an adequate parameter to assign dominance, and the secretory status of the follicle at any given time must be taken into account when studying the dynamics of follicular growth.

Introduction

The development of large antral follicles in sheep occurs throughout adult reproductive life but until recently the absence of suitable techniques has made the elucidation of the pattern of follicle development difficult. The advent of ultrasonography as a noninvasive and repetitive method of monitoring development of individual follicles has enabled a more comprehensive understanding of follicular dynamics in a number of species (Hackett et al. 1979; Griffin and Ginther, 1992). The use of this technique in cattle has led to the discovery of wave-like cycles of selection, dominance and regression of large antral follicles throughout the luteal phase (for review see Fortune, 1994). In sheep, however, the presence of follicular waves is still uncertain.

Although early evidence from histological (Brand and De Jong, 1973; Turnbull et al., 1977) and endocrinological (Cox et al., 1971; Mattner and Beaden, 1972; Miller et al., 1981; Bister and Paquay, 1983) studies proposed that dominant follicle development in sheep did occur as a series of waves, more recent studies using transrectal ultrasonography have shown a random emergence of ovulatory-sized follicles (> 5 mm diameter) during the luteal phase (Schirke et al., 1993; Ravindra et al., 1994). Transrectal ultrasound studies in sheep are more difficult to perform and interpret than in cattle owing to problems of anatomical access and the smaller size difference between dominant and subordinate follicles. In addition, it is difficult to relate steroid production to the ovarian follicular population owing to the low concentrations of steroids in the peripheral blood in this species.

During periods of anoestrus in most breeds of sheep the pattern of follicle development is similar to that found in the breeding season, with no change in the total number of antral (McNatty et al., 1984a) or ovulatory-sized follicles (Smith and Robertson, 1971; Cahill, 1981; Noel et al., 1993). This follicular population is composed of both oestrogen active and inactive follicles, where the former are capable of producing oestradiol at the same rate as equivalent follicles in the breeding season (McNatty et al., 1984a). These oestrogen active or dominant follicles contain more LH receptors than do inactive ones (Webb et al., 1992), secrete oestradiol and androstenedione in acute response to LH pulses (Saramuzzii and Baird, 1977) and can be induced to ovulate by pulsatile injection of LH (McNatty et al., 1982, McNatty et al., 1984b) or GnRH (McLeod et al., 1984c) or by a bolus injection of hCG (Webb et al., 1992).

In the present study we used sheep in which the left ovary had been autotransplanted to a site under the skin in the neck (Godin et al., 1967) to examine the pattern of follicle development in anoestrous sheep. Unlike transrectal ultrasonography, this model has the advantages of (i) allowing easy access to an ovary which is fixed in position so that scans can

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be performed in two planes and the spatial location of individual follicles can be determined, and (ii) allowing repeated collection of ovarian venous blood so that the secretory status of the ovarian follicle population can be determined. Anestrous animals were chosen for this study to avoid perturbations in cycles of follicle development caused by the widespread atresia of nonovulatory follicles induced by the prevoluntary LH surge (Baird and McNeilly, 1981).

Materials and Methods

Experimental animals

The experiment was performed during the nonbreeding season (June) at the Marshall Building, Roslin, Mid Lothian, Edinburgh. The anestrous season in the Finn-Merino cross used in this study lasts from early April to late September. During anestrous the ewes have, on average, an LH pulse of 6 μg l⁻¹ amplitude at 5 h intervals with the maximum oestradiol secretion in response to an LH pulse being observed after 25 min (Scaramuzzi and Baird, 1977).

The animals were housed indoors, under natural lighting and received a maintenance diet consisting of hay and pelleted ration. Nine ewes with ovarian autotransplants were studied for 10 days (Goding et al., 1987). The animals received cloprostenol, a potent PGF₂α analogue, (125 μg i.m.; Estrumate, Cooper’s Animal Health Ltd, Crawie) 15 days before the start of the experiment and, to confirm they were anestrous, the concentrations of progesterone in jugular venous plasma was determined in a sample collected 10 days after prostaglandin injection.

On the day before the start of blood sampling both ovarian and jugular veins were cannulated under local anaesthesia (2 ml s.c. of Lignocaine 2%; Lignavet, Leyland) and the animals were placed in metabolism crates. The cannulae consisted of a 60 cm length Silastic tube (0.8 mm x 1.7 mm, internal and external diameter, respectively; 002-285, Sanitech, Hants). The ovarian venous cannula was introduced into the jugular vein of the loop anterior to the ovarian and jugular anastomosis and the tip was advanced until it was adjacent to the anastomosis, to allow collection of ovarian venous drainage. The jugular venous cannula was inserted into the contralateral jugular vein to a depth of 10 cm. After cannulation the animals received a bolus i.v. injection of 5000 μl of sodium heparin to prevent venous clot formation (Leo Laboratories Ltd, Bucks) and a broad spectrum long-acting antibiotic (3 ml i.m. Clamoxyl, SmithKline Beecham, Surrey). The prophylactic antibiotic treatment was repeated every 3 days throughout the experiment.

Blood sampling

Over the 10 day experimental period two sets of samples of both ovarian (5 ml) and jugular (5 ml) venous blood were collected at 12 h intervals, one under basal conditions and the other 30 min after a GnRH challenge (250 μg in 2 ml sterile saline i.v.; Sigma, Poole). This dose of GnRH has been shown to induce an LH pulse of 4–6 μg l⁻¹ in amplitude (McLeod et al., 1992b) and was given so that the unstimulated and LH-stimulated concentration of steroid hormones could be determined in ovarian venous plasma. After sampling, each cannula was flushed with 5 ml of a solution of 250 000 μl of sodium heparin l⁻¹ in isotonic saline, so the animals received 3000 μl of heparin every 12 h. The blood was centrifuged at 4°C for 15 min at 2000 g, the plasma separated and stored at −20°C until assayed.

Scanning procedure

The skin over the transplanted ovary was clipped and shaved at the beginning of the experiment, and the latter procedure repeated every 2 days during the course of the experiment. Before each examination the area was covered with scanning gel. The ovary was scanned daily in both horizontal and vertical planes, using a 7.5 MHz linear transducer (Model UST-5512U-7.5; Aloka Co. Ltd, Tokyo) with a real time ultrasound scanner (Aloka SSD-500; Aloka Co. Ltd). All examinations were recorded on video cassette tape and stored for subsequent analysis of the follicular diameter.

The tapes were played in slow motion and the image of follicles >2.5 mm frozen at the largest section of the antral cavity for each individual follicle, which was located within the ovary and measured in the medio-lateral, dorso-ventral and crano-caudal planes. The diameter of the follicles was determined as a mean of these three measurements.

Radioimmunoassay

Gonadotrophin and steroid plasma concentrations were measured in duplicate using previously described double antibody radioimmunoassays (RIA) for FSH (Campbell et al., 1990), LH (McNeilly and Fraser, 1987) and progesterone which were determined in unextracted jugular samples (Campbell et al., 1990). Androstenedione (Campbell et al., 1990), stimulated oestradiol (Baird et al., 1981), and unstimulated oestradiol (Baird and Lamming, 1994) were measured in ovarian venous plasma samples after solvent extraction by established RIA. Both oestradiol assays were performed using 100 μl of ovarian plasma extracted with 1 ml diethyl ether. The recovery rate after extraction was 92% ± 0.3 (mean ± SEM, n = 20) and hence the results were not corrected for extraction losses.

The sensitivities of the assays for FSH, LH, progesterone, androstenedione and LH-stimulated oestradiol and unstimulated oestradiol were 0.3 μg l⁻¹ (NIADDK, oFSH, SIAFP-RP2), 0.2 μg l⁻¹ (NIADDK, oLH, SIAFP-RP2), 380 pmol l⁻¹, 175 pmol l⁻¹, 50 pmol l⁻¹ and 0.5 pmol l⁻¹, respectively. The intra- and interassay coefficients of variation were < 15% in the ED20–80 range.

Statistical analysis

Although cycles of development and regression of large antral follicles were evident in profiles from individual animals, these cycles were not synchronized in different animals. The data were grouped from all animals by identifying dominant follicles using three parameters: (1) achievement of a diameter of 5 mm; (2) maintenance of a diameter ≥5 mm for 2 days; (3) at least one measurement having been made before it achieved a diameter of 5 mm.
Using these parameters, designed to ensure that the population analysed was composed of growing healthy follicles that achieved dominance. 12 dominant follicles were identified, as some ewes had more than one wave during the observation period. The size of the largest follicle and the hormone concentrations were aligned according to the day the dominant follicle achieved a diameter of 5 mm (day 0) and the data incorporated from 1 day before until 3 days after day 0 (days −1 to 3).

The effect of time on the dominant and largest follicle diameter and the concentration of hormones were analysed by repeated samples ANOVA using the general linear means model procedure of SYSTAT software (SYSTAT Inc., Evanston, IL).

Results

All animals but one remained in anoestrous during the experimental period and the ewe that ovoluted was withdrawn from the analysis.

Pattern of follicle development and hormone secretion

Over the 10 day observation period the ovaries of the experimental ewes contained on average 2.1 ± 0.39 follicles that remained between 3 mm and 5 mm (medium) and 4.25 ± 0.36 follicles that achieved diameter > 5 mm (large). In individual animals there were clear cycles of development and regression of large dominant follicles with a period of between 5 and 10 days (Fig. 1; Table 1).

Measurements were available for at least 4 days during the growth phase of only five follicles. The mean size of the dominant follicles on day −3 was 3.3 ± 0.2 mm (mean ± SEM, n = 5) and they grew in a linear fashion at a rate of 0.64 mm day⁻¹ until they achieved a diameter of 5 mm on day 0 (Fig. 2).

LH and progesterone remained at basal concentrations throughout the sampling period and did not vary with time (profiles not shown). The overall concentrations (mean ± SEM, n = 8) were 1.91 ± 0.27 μg LH L⁻¹ and 0.52 ± 0.1 nmol progesterone L⁻¹.

In addition to the follicular waves, there were also wave-like changes in the amount of GnRH-stimulated oestradiol and androstenedione secretion. In individual animals the follicular and secretory waves were positively related during the growth phase of a large antral follicle but unrelated thereafter. Jugular venous FSH concentrations remained relatively stable throughout the experimental period and there was no clear relationship with the pattern of follicular enlargement or steroid secretion (Fig. 1).

Relationship between development of dominant follicle and steroid secretion

In order to clarify the relationship between follicular waves and ovarian hormone secretion, dominant follicles were identified using the criteria presented in the analysis section and data were grouped around the time of emergence of each dominant follicle (Fig. 3). Neither the size of the largest follicle nor unstimulated oestradiol secretion changed (P > 0.05) during the period of emergence or regression of a dominant follicle. In contrast, as the dominant follicle grew between day −1 and day 0, the secretion of stimulated oestradiol increased to a peak on day 1 (P < 0.05) and then declined (P < 0.05) between day 1 and day 2, while the size of the dominant follicle remained constant, demonstrating a close association between stimulated oestradiol secretion and follicular growth during the ascending part of the wave. In addition to oestradiol secretion, stimulated androstenedione secretion was also positively related to follicular development and exhibited a similar profile to stimulated oestradiol with an increase during the growing phase of a dominant follicle and a decline preceding any decrease in follicular size. Unlike oestradiol, however, androstenedione secretion increased again at day 3 (P < 0.05).

Jugular venous FSH concentrations did not show a clear association with the pattern of follicular enlargement or stimulated oestradiol, but did show a small but significant
Table 1. Number and duration of dominant follicular waves per animal

<table>
<thead>
<tr>
<th>Ewe id.</th>
<th>Number of waves</th>
<th>Length in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>2</td>
<td>6.7</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>6.2</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>6.7</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>3.7</td>
</tr>
<tr>
<td>Total (mean)</td>
<td>12</td>
<td>(7.1 ± 0.2)</td>
</tr>
</tbody>
</table>

The wave length was defined as the time that the dominant follicle remained over 3.5 mm in diameter.

change with time, decreasing between day −1 and day −0.5 and increasing from day −0.5 to day 0 (P < 0.05).

Discussion

In this experiment, by using the ovarian autotransplant model, we have been able to demonstrate the existence of follicular waves in sheep and show that size and steroidogenic capacity of large antral follicles in sheep are only positively related during periods of follicle growth, thus defining stages of both functional and morphological dominance in sheep during anoestrous.

The observation that a follicle, identified as dominant on the basis of its size, may not be functionally dominant (steroidogenic) has also been made in cattle (Fortune, 1994). However, in that mono-ovulatory species, ultrasound alone can be reliably used to identify the stage of the follicular wave owing to the fact that the single dominant follicle is substantially larger than subordinate follicles and that the duration of the follicular wave is relatively long and well defined. In contrast, in sheep, the small size difference between dominant and subordinate follicles and the short and variable period of the waves makes identification of the dominant follicle by size alone difficult. It is therefore not surprising that studies which have attempted to characterize follicle dynamics in sheep solely on the basis of size have variously concluded that follicular growth is either continuous and independent of the stage of the oestrous cycle (Turnbull et al., 1977; Lahlou-Kassi and Mariama, 1984; Schrick et al., 1993) or consists of two (Brand and De Jong, 1973; three (Smeaton and Robertson, 1971; Noel et al., 1993) or 1-6 (Ginther et al., 1993) waves per cycle. Although we have used the term ‘dominant follicle’ in this paper to describe a large oestrus-stage follicle it is clear that follicle development in sheep is a very dynamic process, and this could explain the reported lack of follicular dominance in sheep (Driancourt et al., 1994; Driancourt, 1994). However, it is important to emphasize that stimulated oestradiol secretion is probably a marker for functional dominance and not its cause, since dominance is likely to be exerted and modulated at a local level (Campbell et al., 1995).

Fig. 2. Photographs of sequential daily scans (a-d) showing the enlargement of the second dominant follicle (days 4–7) presented in Figure 1. The large arrowhead indicates the dominant follicle while subordinate follicles are indicated by small arrowheads. S, skin; O, margin of the ovary. The grid marks visible at the top of each scan indicate a distance of 10 mm.

The necessity for FSH to drive the growth of follicles > 2.5 mm (gonadotrophin dependent; Scaramuzzi et al., 1993) has been demonstrated in several models such as chronic hypophysectomy (Dufour et al., 1979), active immunization against GnRH (McNeilly et al., 1980) or chronic GnRH-agonist infusion (McNeilly and Fraser, 1987). In the present study we did not observe cyclic fluctuations in the concentration of FSH, as reported in nonprolific breeds (Bister and Paquay, 1983; Campbell et al., 1991a), nor any direct link with FSH secretion.
activity is associated with the rise in androstenedione on day 3. The concentration of androstenedione was significantly higher on day 3 compared to previous days.

The authors conclude that androstenedione secretion and ovarian steroid secretion in sheep occur in wave-like forms with the ascending and static part of both waves being synchronous but with a decline in steroid secretion preceding any changes in follicular diameter. Therefore, in sheep, follicular size alone is not a good parameter to assign dominance, and the secretory status of the follicle, at any given time, must be taken into account when studying the dynamics of follicular growth.

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Fig. 3. Relationship among the means of diameter of the largest follicle in the ovary (•), the diameter of the dominant follicle (○), the concentration of androstenedione (♦), and the concentration of oestradiol in ovarian plasma collected before (■) and after (♦) a GnRH (250 ng i.v.) challenge and the concentration of FSH in jugular venous plasma (♦) in relation to the day the dominant achieved a diameter of 5 mm (day 0). Values are means ± SEM, n = 8.


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Follicular Dynamics and Ovarian Steroid Secretion in Sheep during the Follicular and Early Luteal Phases of the Estrous Cycle

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ABSTRACT

In this study, we investigated follicular dynamics and ovarian steroid secretion during the follicular and early luteal phases of the estrous cycle in sheep. Six Finn-Merino ewes with ovarian autotransplanted ovaries were monitored for 16 days during the follicular phase and subsequent early luteal phase after luteal regression was induced with cloprostenol (a potent analogue of prostaglandin F2α). Over this period, follicular diameter was measured by serial ultrasound scans, and the concentration of gonadotropins and steroids in ovarian venous blood was measured at intervals of 6-12 h. All animals had an LH surge (Day 0) 29-41 h after injection of cloprostenol. The ovulatory follicles were derived mainly from large antral follicles present at the time of injection of cloprostenol (5.1 ± 0.4 mm; mean ± SEM, n = 6), although in some animals recruitment of additional small follicles was observed after luteolysis. The concentration of FSH decreased during the follicular phase and peaked synchronously with the LH surge, while estradiol and androstenedione concentrations in ovarian venous plasma increased progressively from luteal regression to a maximum at the LH surge. The rise in concentration of FSH on Day 1 was followed by the growth of a new cohort of follicles. Follicular size and ovarian steroid secretion increased in a linear fashion from Day 1 to Day 3, with ovarian steroid secretion reaching a maximum when the first wave of luteal phase follicles achieved a diameter of 5 mm or more. On Day 4, steroid secretion began to decline without significant changes in follicular diameter, and a second wave of follicles emerged. We conclude that 1) the preovulatory follicles are usually derived from the large follicle population present at the time of luteal regression, but the sheep has the ability to promote smaller follicles if required; and 2) the second peak of FSH stimulates the development of large estrogenic follicles during the early luteal phase, but the period of functional dominance is shorter than the period of morphological dominance.

INTRODUCTION

Folliculogenesis in sheep occurs from puberty throughout adult life; during these years, only a few follicles from a pool of several million will grow to an ovarian size, and fewer still will ovulate. The process of folliculogenesis is thought to take around 6 mo, with most of this time being devoted to the growth of primary follicles to a diameter of 2.5 mm. During this time, little selection takes place [1, 2], and the growth is seemingly independent of gonadotropin input [3] and involves no significant secretion of estradiol [4–6]. However, the growth of follicles from 2.5 to 5 mm occurs in just a few days, and this is the critical step in the selection of a follicle to a "dominant" or estrogenic stage, depending on the hormonal environment [7–9].

The endocrine changes in the secretion of pituitary and ovarian hormones during the follicular and early luteal phase of the estrous cycle are well characterized in intact ewes and in the autotransplanted model [10–16]. After the concentration of progesterone falls during luteal regression, there is a rise in the secretion of estradiol stimulated by an increase in the frequency of LH pulses. In contrast, the concentration of FSH declines, suppressed by the increasing secretion of estradiol and inhibin from the preovulatory follicle [14]. After the LH surge (Day 0) and ovulation, while levels of LH and ovarian steroids are low, there is a marked rise in the concentration of FSH on Day 1 followed by an increase in the secretion of estradiol by a new cohort of antral follicles. However, the exact temporal relationships between the endocrine changes and the dynamics of follicle growth of individual follicles is not clear.

Previous attempts to determine the timing of the selection of ovulatory follicles have yielded conflicting results. Earlier studies found that selection occurred before or at luteolysis [17–19]. Driancourt and Cahill [20], using ink labeling of large follicles after luteolysis, suggested that final selection does not take place until the late follicular phase (48–54 h after injection of prostaglandin [PG] F2α). Cross-sectional data derived from follicle dissection and estradiol secretion suggested that the preovulatory follicle established itself as a large "estrogenic" follicle within 10 h of luteolysis from a pool of antral follicles (2–4 mm in diameter) present at that time [6]. Tsonis et al. [21], using selective ablation of follicles at luteolysis, demonstrated that ovulatory follicles were derived from follicles more than 2 mm at the time of luteal regression. However, smaller follicles were also able to ovulate, suggesting that selection can occur in a flexible time frame, both before and after luteolysis, according to the follicular population at that time. The use of ultrasonography as a noninvasive and repetitive method of monitoring development of individual follicles in humans [22, 23] and cattle [24, 25], has enabled a more comprehensive understanding of follicular dynamics. This technique in cattle has revealed wave-like cycles of selection, dominance, and regression of large antral follicles during the estrous cycle [26].

In sheep, however, the use of transrectal ultrasonography has proved more difficult to perform and interpret than in cattle because of problems of anatomical access and the smaller size difference between dominant and subordinate follicles. Recent studies using this technique reported a random emergence of ovulatory-sized follicles, i.e., more than 5 mm in diameter, during the luteal phase [27, 28]. However, another study observed waves of follicular development and suggested a relationship with the fluctuation of FSH during the cycle, although steroid secretion was not studied [29].

The ovarian autotransplanted model, in which the left
ovary is relocated to a site under the skin of the neck, permits repeated collection of ovarian venous blood in the conscious unanesthetized animal [30]. Moreover, because the ovary is fixed in an easily accessible location, individual follicles can be identified early by scanning in two planes with high resolution ultrasound. In this study, we have investigated the dynamics of follicular development by serial ultrasound measurements, using ovarian steroid secretion as a marker of functional dominance, during the follicular and early luteal phase of the estrous cycle in ewes with autotransplanted ovaries.

MATERIALS AND METHODS

Experimental Animals

Six Finn-Merino cross ewes with an ovarian autotransplant [30] were studied during the breeding season (November). The animals were housed indoors at the Marshall Building, Roslin, Midlothian, Edinburgh, under natural lighting and received a maintenance diet consisting of hay and a pelleted ration. The left ovary had been autotransplanted at least five years previously by Anastomosing the ovarian artery and utero ovarian vein to the carotid artery and jugular vein, respectively. The right ovary was removed at the time of autotransplantation and, hence, the total ovarian secretion of steroids could be measured by cannulating the ovarian vein [30]. Because these ewes do not cycle spontaneously due to maintenance of the corpus luteum, synchronization of the estrous cycle was achieved with two injections of cloprostenol, a potent analogue of PGF2α ([125 μg i.m. Estrumate; Cooper's Animal Health Ltd., Crewe, Cheshire, UK]) given 17 days apart.

The day before the start of blood sampling, both ovarian and jugular veins were cannulated under local anesthesia as previously described [31], and the ewes were then placed in metabolism crates.

After cannulation, the animals received a bolus i.v. injection of sodium heparin (5000 IU; Leo Laboratories Ltd., Bucks, UK) and were treated prophylactically with a broad-spectrum long-acting antibiotic (3 ml i.m., Clamoxil; SmithKline Beecham, Surrey, UK), which was repeated every 3 days.

Blood Sampling

Samples of ovarian (5 ml) and jugular (3 ml) venous blood were collected at 12-h intervals on the day before the second injection of cloprostenol and every 6 h thereafter until 8 days after the injection, at which time the sampling interval returned to every 12 h until the end of the experiment two days later. After sampling, each cannula was flushed with 5 ml of a solution of 250 IU of sodium heparin per milliliter of isotonic saline. The blood was centrifuged at 4°C, and the plasma was separated and stored at −20°C until assay.

Scanning Procedure

The skin over the transplanted ovary was clipped and shaved at the beginning of the experiment and was maintained free of wool throughout. Before each examination, the area was covered with scanning gel (Siel Sound Gel; Siel Imaging Equipment Ltd., Aldermosten, Berkshire, UK). The ultrasound exams were performed after each blood sample: the ovary was scanned in both horizontal (dorsal/ventral) and vertical (cranio/caudal) planes, using a 7.5-MHz linear transducer (Model UST-5512LI-7.5; Aloka Inc., Tokyo, Japan) with a real-time ultrasound scanner (SSD-500; Aloka Inc.). All exams were recorded on video cassette tape for subsequent analysis. The tapes were played in slow motion, and the image was frozen at the largest section of the antral cavity for each individual follicle greater than 2.5 mm in diameter. The image was digitized and measured with the NIH Image software on the basis of the major and minor axes of the best-fitted ellipse for each follicle. The diameter of each follicle was determined as a mean of these measurements.

RIA

Gonadotropin and steroid plasma levels were measured in duplicate using previously described double-antibody RIAs. FSH [16], LH [8], and progesterone were determined in unextracted jugular venous samples [16]. Androstenedione [16] and estradiol [13] were measured in ovarian venous plasma samples after solvent extraction. The sensitivities of the assays for FSH, LH, progesterone, androstenedione, and estradiol were 0.3 ng/ml (USDA, ovine (o) FSH, SIAFP-RP2), 0.2 ng/ml (NIADDK, oLH, S2D), 58.0, 175 pm, and 50 pm, respectively. The intra- and interassay variations were less than 15% in the ED20–80 range.

Statistical Analysis

Data were normalized with respect to two time periods of physiological significance. The first was the time of cloprostenol injection (±24 h), and the second was related to the onset of the LH surge, defined as the nadir point before LH concentrations exceed 10 ng/ml (from Day −1.5 to Day 6). For analysis of the relationship between diameter of the follicles that developed during the early luteal phase and steroid secretion, the data were aligned to the time that the dominant follicle reached a diameter of 5 mm, from days −2 to 3 (corresponding to Days 1–4 of the estrous cycle). The effects of time on follicular diameter and hormone concentrations were analyzed by repeated-samples ANOVA on untransformed data using the general linear means model procedure of SYSTAT software (SYSTAT Inc., Evanston, IL).

RESULTS

Pattern of Hormonal Secretion

The pattern of gonadotropin and ovarian steroid secretion in relation to injection of cloprostenol and the LH surge are presented in Figure 1. The concentration of progesterone in jugular venous blood declined in all animals after the injection of cloprostenol (p < 0.01) and remained at basal levels until Day 4 (Fig. 1).

The concentrations of LH increased (p < 0.05) within 12 h of cloprostenol injection and remained stable at around 3 ng/ml until the onset of the LH surge 59 ± 4.7 h later (mean ± SEM). The peak values of the LH surge were observed at 70 ± 5.9 h. After the surge, the values remained around 5 ng/ml until Day 5, when they decreased (p < 0.05). The concentration of FSH in jugular venous blood decreased after injection of cloprostenol (p < 0.05) and remained around 1 ng/ml until the time of the LH surge, when a synchronous FSH peak occurred (p < 0.05). No discrete second FSH peak was apparent in the mean data, but second FSH peaks were evident in some profiles from individual animals (Fig. 2). By Day 1, FSH levels had fallen to their lowest value of the early luteal phase (p < 0.05). The concentration of FSH then sharply increased (p
The concentrations of ovarian venous blood increased progressively after injection of cloprostenol ($p < 0.05$) and reached a maximum at the time of LH surge ($p < 0.01$). By Day 1, the concentrations of estradiol had fallen to the lowest values observed during the cycle ($p < 0.01$), but subsequently they started to increase on Day 2 ($p < 0.05$). The level of estradiol concentrations, however, remained stable from Day 3 until Day 5 before starting to decline ($p < 0.01$).

**Relationship between Follicular Development and Hormonal Secretion**

The pattern of follicular growth and hormonal profiles are illustrated in Figure 3. The follicles that ovulated at the end of the follicular phase arose mainly from large antral follicles present at the time of luteolysis, although smaller follicles could be recruited during the follicular phase (see Fig. 2). Ovarian follicles persisted after the LH surge and continued to increase in diameter during the luteal phase. The mean diameter of the ovulatory follicle was 5.1 ± 0.4 mm (mean ± SEM) at the time of injection of cloprostenol, 5.8 ± 0.3 mm at the onset of LH surge, and 6.8 ± 0.5 mm on Day 1 at the time of estimated ovulation. Between Day 0 and Day 3, the ovulatory follicle/corpus luteum doubled in size, remaining around 10 mm in diameter during the luteal phase.

The ovulatory follicles did not collapse after the LH surge, perhaps because the transplanted ovary is encased in a capsule of connective tissue; by Day 4, changes in the echogenicity of these structures could be noticed as the luteal tissue colonized the antral cavity. These changes were synchronous with the increase in jugular venous progesterone concentrations.

In the early luteal phase of the cycle, all animals developed at least one large follicle that grew in a linear fashion at a rate of 1 mm/day until it achieved a diameter of 3 mm on Day 3. No further significant changes in follicle diameter were observed until Day 5-5, when the follicles started to regress ($p < 0.05$). The concentration of estradiol in ovarian venous blood did not increase until Day 2, when the follicles from the first wave had a mean diameter of 4.0 ± 0.4 mm ($p < 0.05$), and it continued to rise until Day 3 and then remained unchanged until Day 4.5, when it fell rapidly ($p < 0.01$). In four of the six animals, the emergence of a second wave of follicles was evident during the observation period although the levels of ovarian steroids were not of the same magnitude as those from the first wave.

**Dominance in the First Wave of Follicular Development in the Luteal Phase**

The relationship between the follicular development during the first wave of follicles in the luteal phase and the pattern of ovarian steroids is presented in detail in Figure 4, with the data aligned to the time the dominant follicle achieved a diameter of 5 mm. The follicles grew progressively from days -2 to 1 (corresponding to Days 1–4 of the estrous cycle) to a maximum mean diameter of 5.6 ± 0.2 mm ($p < 0.001$). A similar pattern was observed in the concentration of ovarian steroids, except that the concentrations of androstenedione and estradiol did not start to rise until Day -1, when the follicles were around 4 mm in diameter ($p < 0.01$). There was a progressive decline ($p < 0.01$) in the concentration of estradiol and androstenedione starting on Day 1 after the follicle reached a diameter of 5 mm. The first significant decline in follicle diameter was not observed until Day 2.5, and thereafter, the follicle persisted for some days after the steroid secretion had declined to basal levels. Thus, the follicle persists as a recognizable structure long after it has become atretic and has ceased to be a significant source of steroid secretion.

**DISCUSSION**

By using the ovarian autotransplant model in this experiment, we have been able to monitor the development of individual ovulatory follicles from the late luteal phase through the follicular and subsequent luteal phase, and relate the pattern of follicle development to changes in the concentration of ovarian steroids and pituitary gonadotropins. We observed the occurrence of follicular waves during the luteal phase and confirmed that the size and steroidogenic capacity of large antral follicles varies during specific periods of follicle growth, thus characterizing the stages of functional and morphological dominance in sheep [31].
In the present study, the ovulatory follicles were derived mainly from large antral follicles around 5 mm in diameter, present at the time of luteal regression, although a wider range of follicle sizes could be selected to ovulate. The contradictory findings of previous studies [6, 17–20] regarding the timing of selection can be explained by the fact that ovulatory follicles can be selected from follicles of a wide size range. Our results corroborate those of Tsonis et al. [21], showing that all follicles larger than 2 mm are capable of being promoted into the ovulatory pool. Although most of the ovulatory follicles came from large follicles that were presumably estrogenic, little estradiol secretion before luteal regression was observed in most animals. This apparent anomaly can be accounted for by the fact that the secretion of estradiol by the dominant follicle is acutely responsive to LH pulses that are infrequent during the late luteal phase [32]. A similar situation is present during anestrus when the steroidogenic potential of the follicle can be revealed by providing an LH pulse through injection of GnRH [31].

The observation of waves of follicular development during the early luteal phase supports histological observations [17, 18] and recent ultrasound studies from both cycling ewes [29] and anestrous ewes [31]. The fact that the secretion of ovarian steroids declined before any significant change in the diameter of the follicle is in agreement with findings from follicle dissection that identified the loss of aromatase activity as one of the initial signs of atresia preceding morphological changes [4, 33]. However, the decline in androstenedione secretion coincident with the decline of estradiol suggests that the decrease in estradiol secretion may be due to lack of androgen precursor. This view is supported by recent studies in cattle reporting that 17α-hydroxylase gene expression decreases before any reduction in the expression of the aromatase gene [34]. Alternatively, the decline in steroid secretion could be due to the decrease in LH pulse frequency after Day 3 of the luteal phase [12, 35, 36]. The periods of functional and morphologic dominance observed in this experiment are similar to those of the first wave of follicle development in cattle [37, 38]. However, the pattern in sheep appears to be more dynamic and less pronounced than in the cow, which could account for reports of lack of dominance in sheep [39, 40] and explain the higher ovulation rate in this species.

Although there is a significant decline in FSH during the follicular phase as the dominant follicle secretes increasing amounts of estradiol and inhibin [14, 41], the relationship between follicle development and FSH during the early luteal phase was less apparent. In some individual animals (see, e.g., Fig. 2), the postovulatory peak of FSH preceded...
the emergence of an estrogenic follicle and FSH levels declined in the early luteal phase as the follicle secreted increasing amounts of estradiol. However, the time of emergence of the first wave was variable among animals, and thus, overall, there was no consistent relationship in the mean data. Moreover, the emergence of the second wave of follicles in the luteal phase that was observed in some animals appeared to bear no relationship to fluctuations in the level of FSH or LH. Although the requirement for FSH to promote follicular growth beyond a diameter of 2.5 mm is well documented in different models [3, 7, 8], the lack of relationship between FSH and the emergence of the follicles from the second wave suggests that the presence of follicular waves may be due to locally produced factors and that FSH fluctuations merely reflect changes in the steroidogenicity of the ovarian follicle population [42].

The presence of the cavity observed in the corpus luteum during the luteal phase could be an artifact of the transplant procedure, since the ovary is subcutaneous and covered in connective tissue, which may prevent the release of follicular fluid at ovulation. However, progesterone concentrations and the profile of secretion in ewes with ovarian autotransplants are normal [16, 43, 44], and morphologically normal corpora lutea have been described in autotransplanted ovaries [30]. Alternatively, the luteal cavity could be attributed to treatment with PG: Schrick et al. [27] reported occurrence of a cavity in over 65% of corpora lutea induced, but this had no effect on the profile of progesterone secretion or number of embryos. The luteal cavity has also been reported to have no effect on pregnancy rate or progesterone secretion in heifers [45, 46].

We conclude that 1) the preovulatory follicles are usually derived from the large follicle population present at the time of luteal regression, but the sheep has the ability to promote smaller follicles if required; and 2) the second peak of FSH stimulates the development of large estrogenic follicles during the early luteal phase, but the period of functional dominance is shorter than the period of morphological dominance.

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