THE ROLE OF FOLLICLE STIMULATING HORMONE IN

THE CONTROL OF OVULATION RATE IN THE EWE

JACQUELINE MOIRA WALLACE

Thesis submitted for the degree of Doctor of Philosophy to the University of Edinburgh

To Mum and Dad
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DECLARATION

Except where acknowledgement is made by reference, the experiments described in this thesis were the unaided work of the author.

No part of this work has already been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

J.M. Wallace.

1985.
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Above all, I would like to thank Raymond Aitken not only for his skilled technical assistance during much of the animal work described in this thesis but also for his love and understanding throughout the completion of these studies.
The studies described in this thesis were designed to investigate the role of FSH in the control of ovarian follicular development. Plasma levels of FSH were suppressed for varying times during the luteal phase by injections of bovine follicular fluid and the consequences on follicle development, ovulation rate, corpus luteum function and levels of gonadotrophins and prolactin were assessed.

In the initial study twice daily administration of follicular fluid to prolific Damline ewes from Day 1 to 11 of the luteal phase resulted in a delay in the onset of oestrus behaviour and an increase in ovulation rate following cloprostenol-induced luteolysis on Day 12. Treatment for 10, 6 or 2 days before the induced luteolysis also resulted in an increase in ovulation rate which was reflected by a greater number of embryos surviving at Day 30-34 of gestation in the 6 and 10 day treatment groups (Chapter 4).

Detailed observations on the endocrine status of ewes treated with follicular fluid (Chapter 5) showed that during treatment plasma levels of FSH were suppressed for approximately 18h per day. This suppression of FSH was associated with a failure of follicle growth beyond 2.7mm diameter (Chapter 6) in ovaries removed on Day 10 of treatment and demonstrated that antral follicle development is FSH dependent. In contrast, basal LH levels were higher throughout treatment due to an increase in pulse amplitude and the maintainence of high pulse frequency characteristic of Day 1 of the normal cycle (Chapter 5). The total basal in vitto oestradiol production by follicles incubated from ewes treated with follicular fluid was significantly less than that of controls and suggests that the high
pulsatile LH secretion is due to the reduced negative feedback effects of oestradiol occurring as a result of the reduction in FSH. The high pulsatile LH secretion occurred in spite of normal or high progesterone secretion implying that oestradiol is a major negative feedback influence on both pulse frequency and amplitude during the luteal phase. After cloprostenol-induced luteal regression at the end of treatment, LH concentrations were initially higher than those of controls due to a rise in pulse amplitude but there was no differences in the amount of LH released immediately before or during the LH surge (Chapter 3 and 7). After the cessation of treatment a large rebound increase in FSH concentrations occurred. This endogenous hypersecretion of FSH is considered to be the most probable cause of the observed increase in ovulation rate.

In Chapter 8 the commercial implications of using follicular fluid treatment to increase ovulation rate in a low fecundity breed were examined. Treatment of Welsh Mountain ewes for 10 days was associated with the complete suppression of plasma FSH concentrations during treatment and an increase in ovulation rate from 1 to 2 after cloprostenol-induced luteolysis on Day 11. The possibility of manipulating the endogenous FSH secretion in ewes using only 1 or 2 injections of follicular fluid was investigated (Chapter 9). While a marginal increase in ovulation rate was observed a more effective injection regimen is required.

In Chapter 10 the relative contributions of LH and FSH to the induction of ovulation during seasonal anoestrus were examined by administering LH and FSH alone or in combination to prolific Damline and non-prolific Welsh Mountain ewes. The addition of FSH alone was ineffective while the administration of LH alone and in
combination with FSH induced ovulation in 18/19 ewes. This suggests that it is the infrequency of LH pulses which limits the development of preovulatory follicles during seasonal anoestrus. However the induced ovulation was frequently followed by inadequate luteal function in both breeds and probably reflects either defects in hormonal priming and/or inappropriate luteotrophic support after ovulation.
CHAPTER 1: REVIEW OF LITERATURE

INTRODUCTION

The sheep is seasonally polyoestrus exhibiting regular oestrous behaviour at 16-17 day intervals for several months of the year beginning in late summer in temperate latitudes (Marshall, 1903). The seasonal nature of breeding activity together with an inherently low ovulation rate in most breeds imposes a limit on the reproductive efficiency of the ewe. Removal of one or both of these limitations would therefore increase the levels of production. In order to successfully manipulate sheep production, knowledge of the hormonal mechanisms controlling the oestrous cycle and the endocrine causes of seasonal anoestrus is required.

ENDOCRINE CONTROL OF THE OESTROUS CYCLE

(a) Hypothalamic control of pituitary gonadotrophin secretion

The hormonal changes responsible for the control of the ovine oestrous cycle are governed principally by the hypothalamic-pituitary axis. Information about the state of the external environment (photoperiod, nutrition, sexual partners) and the state of the reproductive system (uterus, ovarian follicle and corpus luteum function, pregnancy and lactation) is collated and integrated in the brain which sends signals from the hypothalamus in the form of releasing factors to control the activity of the anterior pituitary gland. The pituitary in turn controls the ovaries. Communication between the brain - ovary is predominantly neuroendocrine and the control of the oestrous cycle is exerted primarily by the secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus (see review, Clarke, 1984). GnRH is synthesized by hypothalamic neurons and stored in the nerve
terminals of the median eminence. Following its release which is governed by complex neuronal and humoral inputs (see review, Kalra & Kalra, 1983), GnRH is transported in the hypothalamic-hypophysial portal vessels to the pituitary gonadotrophs. This decapptide hormone binds to specific plasma membrane receptors on the gonadotrophs and stimulates the synthesis and secretion of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Conn et al., 1981). The ovaries respond to this gonadotrophic stimulation by secreting steroid hormones (oestradiol, androgens and progesterone) and peptides e.g. inhibin, which in turn feedback information, by actions on the hypothalamus and pituitary to regulate further gonadotrophin release.

Studies using rodents suggested that there are two major hypothalamic centres controlling the secretion of GnRH (Halasz & Pupp, 1965), a 'tonic centre' in which GnRH pulses are generated and a 'phasic' or 'surge centre' responsible for promoting the preovulatory LH surge at oestrus. A similar two centre control has been proposed to exist in the ewe (see review, Domanski, Przekop & Polkowska, 1980) although the exact location of these hypothalamic centres is unknown (Clarke, 1984).

In recent years the focal point in our understanding of the endocrine events of the ovine oestrous cycle has been changes in pulsatile LH secretion. Evidence that the pulsatile pattern of LH secretion is a reflection of GnRH pulses has come from two sources. Firstly immunization of both ewes and rams against GnRH completely abolished LH pulses (Clarke, Fraser & McNeilly, 1978; Lincoln & Fraser, 1979) and, secondly, elegant techniques involving hypothalamo-hypophyseal portal blood sampling in conscious ewes have
shown that each pulse of LH is preceded by a pulse of GnRH in the portal blood (Clarke & Cummins, 1982). Similarly evidence that these GnRH pulses are a result of neural activity in the hypothalamus has been obtained by studying plasma LH levels during anaesthesia (Radford & Wallace, 1974; Clarke & Doughton, 1983) and following treatment with specific neural blocking agents (Jackson, 1975, 1977). While our understanding of the interactions between ovarian steroids, GnRH and the synthesis and release of LH is in a relatively mature state (see review, Savoy-Moore & Schwartz, 1980), the neuroendocrine factors which govern FSH synthesis and secretion are less well understood (Chappel, Ulloa-Aguirre & Coutifaris, 1983). A separate FSH-releasing factor has been postulated using the rat as an experimental model (McCann, Mizunumo, Samson & Lumpkin, 1983). However in the ewe active immunization against synthetic GnRH causes a decrease in pituitary and plasma concentrations of FSH as well as LH (Clarke et al, 1978; Jeffcoate, Foster & Crighton, 1978). Furthermore following surgical isolation of the pituitary gland from the hypothalamus FSH is no longer detectable in the peripheral circulation but can be restored by pulsatile GnRH replacement (Clarke, Cummins, Findlay, Burman & Doughton, 1984) confirming GnRH controls both gonadotrophins. Recently an additional peptide (GAP) derived from the GnRH prohormone has been found to stimulate both LH and FSH secretion in rat pituitary cell cultures (Nikolics, Mason, Szöjji, Ramachandran & Seeburg, 1985). GAP added alone showed a preference for stimulating FSH secretion but the presence of GnRH and GAP in equivalent amounts failed to show significant differences in LH and FSH secretion. The authors therefore speculate that a differential release of the two
Fig. 1.1. Hormone changes throughout the oestrous cycle of the ewe. From Baird & McNeilly (1981)
peptides from the hypothalamic neurons may change the ratio of circulating gonadotrophins observed in certain physiological states (see review, Knobil, 1980).

(b) Hormonal and follicular patterns throughout the oestrous cycle

The organisation of a single oestrous cycle is schematically illustrated in Fig.1.1. For convenience the 17 day oestrous cycle (Day 0 = oestrus) can be divided into the luteal phase (Day 2 to 13) and the periovulatory period (follicular phase) from Day 14 (3 days before oestrus) to Day 1.

1. Luteal Phase

Starting at Day 3 of the luteal phase the corpus luteum secretes increasing amounts of progesterone so that the peripheral plasma concentration reaches a plateau between Days 7 and 12 of the cycle (Edgar & Ronaldson, 1958; Short, McDonald & Rowson, 1963; Moore et al, 1969). The luteinized granulosa cells (large luteal cells) have widely been assumed to be the major source of luteal progesterone (see review, Rothchild, 1981). However recent evidence suggests that the small luteal cells may also be a major source of luteal progesterone production in the sheep (Rodgers, O'Shea & Findlay, 1983). The maintainence of the corpus luteum is achieved by a luteotrophic complex involving prolactin and small amounts of LH (Kaltenbach, Cook, Niswender & Nalbandov, 1967; Denamur, Martinet & Short, 1966, 1973). Progesterone has an important priming effect on the uterus which requires exposure to progesterone for 7-10 days before it will synthesise sufficient quantities of PGF-2α to induce luteolysis (Baird, 1978a). This feedback loop between the ovary and the uterus regulates the length of the luteal phase. In the presence of low basal levels of LH which exist by Day 12-13 the
corpus luteum becomes increasingly sensitive to the luteolytic effect of PGF-2α which is released in small quantities from the uterine endometrium into the uterine vein. By Day 13 sufficient PGF 2α has reached the ovary by a counter-current transfer between the utero-ovarian vein and the ovarian artery to effect a marked decline in progesterone secretion (McCracken, Baird & Goding, 1971; Baird, 1978a). This enhances further release of PGF-2α from the uterus until luteolysis is complete.

In recent years it has been shown that the ovine corpus luteum synthesizes oxytocin (Wathes & Swann, 1982; Flint & Sheldrick, 1982, 1983) and there is now considerable evidence that oxytocin is involved in the control of luteolysis (see review, Wathes, 1984). The importance of endogenous oxytocin in luteolysis is indicated by the prolongation of the luteal phase in ewes immunized against oxytocin (Sheldrick, Mitchell & Flint, 1980; Schams, Prokopp & Barth, 1983). Similarly the release of oxytocin from the ovary is stimulated by injection of PGF 2α (Sheldrick, et al, 1980) while oxytocin will stimulate the release of PGF 2α from the uterus (Baird, 1978a). Consequently, Flint & Sheldrick (1982, 1983) suggested that during luteolysis ovarian oxytocin and endometrial PGF-2α form a positive feedback loop leading to a more rapid decline of the corpus luteum. However, in the sheep the luteolytic action of PGF 2α is not completely dependent on oxytocin release as luteolysis can be induced in a corpus luteum prolonged by hysterectomy in which situation the luteal content of oxytocin is very low (Sheldrick & Flint, 1983).

During the luteal phase of the oestrous cycle LH pulses occur at a frequency of once every 3-10h (Baird, Swanston & Scaramuzzi,
Each LH pulse induces an increase in the secretion of oestradiol and androgens from the follicle as measured in ovarian vein plasma (Baird et al., 1976). However, the low frequency of LH pulses does not provide sufficient gonadotrophic support for the final stages of follicular development (see p. 11) hence ovulation is prevented. It is now widely accepted that the low pulse frequency observed in the luteal phase is maintained by a synergistic interaction between luteal progesterone and follicular oestradiol which feedback negatively on the hypothalamic-pituitary axis (Baird & Scaramuzzi, 1976; Goodman, Bittman, Foster & Karsch, 1981a; Martin, Scaramuzzi & Henstridge, 1983). However, the way in which LH pulse amplitude is regulated during the luteal phase is not so clear. It has generally been shown that pulse amplitude remains higher in the luteal phase relative to the follicular phase (Baird, 1978b; Martensz & Scaramuzzi, 1979). As peripheral oestradiol levels show the reverse of this trend and oestradiol has been shown to decrease pulse amplitude (Goodman & Karsh, 1980a; Wright, Geytenbeek, Clarke & Findlay, 1981) it is logical to conclude that oestradiol alone regulates pulse amplitude. However, progesterone apparently increases pulse amplitude in ovariectomised ewes in the absence of oestradiol (Goodman & Karsh, 1980a). It seems therefore that there is sufficient oestradiol present in the luteal phase to interact with progesterone to control pulse frequency but insufficient to reduce pulse amplitude to the levels seen in the follicular phase.

A greater understanding of the relationships between LH pulse frequency and amplitude has been gained from studies in
ovariectomised ewes whose pituitary gland had been surgically isolated from the hypothalamus (Clarke, Cummins & de Kretser, 1983). Administration of pulses of exogenous GnRH to mimic the transition from the luteal to follicular phase showed that LH pulse frequency determines pulse amplitude (Clarke et al, 1984). Similarly the amplitude of LH pulses is directly related to the amount of releasable LH in the pituitary gland so that as the frequency of GnRH pulses is decreased (as in the luteal phase) the amplitude of the LH response is increased in direct proportion to the size of the releasable LH pool (Clarke & Cummins, 1985).

The pattern of FSH secretion during the luteal phase is less well defined. There is no evidence that FSH secretion is pulsatile in the sheep but this may be due to the long half-life of the hormone (102 min: Akbar, Nett & Niswender, 1974). However FSH secretion is dependent upon GnRH as hypothalamo-pituitary disconnection results in the suppression of FSH secretion in the peripheral circulation and FSH levels can be restored by pulsatile GnRH replacement (Clarke et al, 1984). Several groups report wide variability both within and between ewes in the timing, frequency and amplitude of FSH fluctuations during the luteal phase (l'Hermite, Niswender, Reichert & Midgley, 1972; Salmons en et al, 1973; McNeilly, McNeilly, Walton & Cunningham, 1976; Pant, Hopkinson & Fitzpatrick, 1977). Conversely Goodman, Pickover & Karsch (1981b) reported that FSH levels did not vary significantly during the mid-luteal period. Attempts to define a rhythmical periodicity to these FSH fluctuations using auto-correlations have concluded that the waves of FSH secretion do not occur at fixed time intervals (Miller, Nordheim & Ginther, 1981a; Bister & Paquay, 1983; Wheaton,
Mullet & Cornelius, 1984). Within individual animals however an endogenous rhythm of FSH secretion occurring in periodic cycles of 4 to 6 day intervals was generally observed. Similarly, peaks of FSH occurring at about 4 day intervals were found when individual profiles in cattle were examined (Schams & Schallenberger, 1976; Schams, Shallenberger, Hoffman & Karg, 1977).

A high degree of follicle activity occurs during the luteal phase in the sheep with large antral-follicles being present throughout. This led to the suggestion that follicles are growing or regressing asynchronously at any given time during the luteal phase (Turnbull, Braden & Mattner, 1977) and does not support any wave theory of follicle growth. Conversely studies using ink labelling techniques at different times of the cycle, suggest that for large antral follicle populations 2-3 waves of growth occur during the cycle (Smeaton & Robertson, 1971; Brand & de Jong, 1973; Bherer, Matton & Dufour, 1977). Similarly as the secretion of oestrogen is limited to the largest one or two non-atretic developing follicles (Moor, 1973, Hay & Moor, 1978) the peaks of oestradiol-17β secretion observed on Days 3-4 (Cox, Mattner, Shutt & Thornburn, 1971; Holst, Braden & Mattner, 1972; Mattner & Braden, 1972; Baird & Scaramuzzi, 1976) and Days 6-9 and 11-15 of the cycle (Cox et al, 1971; Mattner & Braden, 1972) are presumably an indication of periodic antral follicle activity. However despite this apparent periodicity in follicle growth and oestradiol secretion it has not yet been shown that FSH secretion is directly linked to these changes. If follicle growth occurred at fixed time intervals during the luteal phase FSH would be expected to be low during the growth of a large follicle (due to the feedback effects
of oestradiol and inhibin) and high following atresia.

As indicated above, the tonic secretion of FSH in contrast to that of LH cannot be completely accounted for by the negative feedback effects of oestradiol and progesterone. Imitation of an artificial luteal phase in ovariectomised ewes using oestradiol and progesterone implants (alone or in combination) failed to depress FSH levels to equal those seen in cycling ewes (Goodman et al., 1981b). This provided evidence that the ovary secretes a hormone, probably inhibin, which acts throughout the cycle to selectively suppress secretion of FSH (see P. 41).

Plasma levels of prolactin do not change significantly throughout the luteal phase in the ewe (see review, McNeilly, 1985b). Nevertheless there is abundant evidence that prolactin is an essential component, together with LH, of the luteotrophic complex necessary for the maintenance of corpus luteum function and normal progesterone secretion (Denamur et al., 1966, 1973; Kann & Denamur, 1974). Hyperprolactinaemia may disrupt follicular development by interfering with the secretion of oestradiol by the developing follicles (McNeilly & Baird, 1983). It has therefore been suggested that during the normal oestrous cycle prolactin may play a minor but essential role in ovarian follicular development as well as in maintaining corpus luteum function (McNeilly, 1985c).

2. Periovulatory period

The endocrine events of the periovulatory period can be conveniently divided into three phases.

**Luteal regression to LH surge**

Following regression of the corpus luteum there is a marked
increase in the LH pulse frequency to around 1 pulse per hour as plasma progesterone levels fall (Baird, 1978b; Baird Swanston & McNeilly, 1981; Karsch et al, 1983). If the levels of progesterone are restored by a progesterone impregnated implant the concentration of LH and the secretion of oestradiol are suppressed to the levels found during the luteal phase confirming that progesterone is an important negative feedback regulator of LH secretion during the luteal phase (Baird & Scaramuzzi, 1976; Hauger et al, 1977).

Concurrent with the rise in LH secretion during the follicular phase there is an increase in the secretion of oestradiol from the follicles(s) destined to ovulate. The rise in androgens (testosterone and androstenedione) is less marked so that there is a progressive increase in the oestrogen/androgen ratio in ovarian venous plasma (Baird et al, 1981). A similar change in ratio is found in follicular fluid (Moor, Hay, Dott & Cran, 1978) suggesting that this reflects the increasing utilization of androgen precursor as it is aromatized to oestrogen by the preovulatory follicle. This increasing secretion of oestradiol from the preovulatory follicle has four important effects: 1) it stimulates further PGF 2α release from the uterus and thereby hastens luteolysis (Baird, 1978a), 2) it is the primary mediator of behavioural oestrus (Radford, 1967). 3) it suppresses FSH secretion (probably together with inhibin) so that FSH levels fall during the final 48h before the LH surge (Salamonsen et al, 1983; Goodman et al, 1981b), 4) rising oestradiol levels are responsible for the induction of the LH surge (see below).

The rise in LH secretion following prostaglandin-induced (Baird & Scaramuzzi, 1976) or natural luteolysis (Hauger, et al, 1977)
suggests that LH is the stimulus for the sustained rise in oestradiol secretion by the follicles destined to ovulate. This possibility is supported by several lines of evidence. Exogenous LH has been found to cause a marked increase in secretion of testosterone and androstenedione, the androgen precursors of oestradiol, when infused into the ovary (McCracken, Uno, Goding, Ichikawa & Baird, 1969). Similarly each endogenous spontaneous LH pulse is followed within 10 minutes by an increased secretion of oestradiol from the ovary (Baird, 1978b). Additional evidence from studies carried out during seasonal anoestrus highlight the importance of LH in stimulating the final maturation of the Graafian follicle. Follicular development and ovulation can successfully be induced by the infusion or repeated pulsing of LH (McNatty, Gibb, Dobson & Thurley, 1981; McNeilly, O'Connell & Baird, 1982) or by repeated hCG injections (Legan & Karsh, 1979), thus indicating that LH secretion is normally insufficient during seasonal anoestrus to stimulate oestradiol secretion to the threshold level required to induce a preovulatory LH surge.

Preovulatory LH surge

Approximately 50-60h after the initiation of luteal regression by prostaglandin injection, around the onset of behavioural oestrus, there is a marked rise in the concentration of both FSH and LH. Evidence in most species studied, including sheep suggests that the preovulatory surge of FSH and LH is stimulated by a rise in oestradiol secretion by the preovulatory follicle. This is supported by the observation that passive immunization against oestradiol prevents the induction of oestrus and the LH surge in progesterone primed ovariectomised ewes (Scaramuzzi, 1975). It is
unlikely however that a pulsatile oestrogen signal is important for
the induction of the surge as a single injection, multiple
injections or constant infusion of oestradiol appears to be equally
effective in eliciting the surge of LH (Goding et al, 1969; Goding,
Blockey, Brown, Catt & Cumming, 1970; Pant, 1977). The mechanism
triggering the LH surge is well documented (see review, Martin,
1984). Both an increased frequency of GnRH pulses and an increased
sensitivity of the pituitary gland to GnRH, induced by the high
oestradiol levels (Reeves, Arimura & Schally, 1971) result in a
rapid elevation of the LH levels as soon as the frequency of GnRH
discharges is high enough in relation to the half-life of LH to
permit summation to occur.

Passive immunization against GnRH has been a useful tool in
elucidating the role of GnRH in the control of pituitary function
during the periovulatory period (see review, Fraser, McNeilly &
Popkin, 1984). Passive immunization against GnRH in the
preovulatory period produced an immediate neutralization of GnRH as
evidenced by the abolition of pulsatile LH release (Fraser &
McNeilly, 1983) and results in the immediate cessation of pulsatile
secretion of ovarian oestradiol (McNeilly, Fraser & Baird, 1984).
Similarly, prevention of the preovulatory LH surge by administration
of GnRH antibodies, caused inhibition of ovulation as demonstrated
by the absence of a rise in serum progesterone (Fraser & McNeilly,

In response to the preovulatory LH surge the Graafian follicles
undergo a series of structural and functional changes (Hay & Moor,
1975) which culminate in ovulation some 24h later (Cumming et al,
1971a). Following the surge there is initially a stimulation
followed by a rapid decline in all ovarian steroids (Chamley et al., 1972, Baird et al., 1981). The secretion of oestradiol is inhibited before that of androstenedione and testosterone (Baird et al., 1981) and is compatible with the suggestion that LH in large amounts inhibits aromatase activity (Moor, 1974). While the LH surge prepares the dominant follicle(s) for ovulation there is evidence that it induces widespread atresia in the remaining antral follicles (>2mm diameter) which are already suffering from the preovulatory decline in FSH levels (Baird et al., 1981). Injection of human chorionic gonadotrophin (hCG) 48h after the administration of pregnant mares serum gonadotrophin (PMSG) resulted in most follicles becoming atretic (Turnbull et al., 1977). Similarly hCG treatment has been shown to induce widespread atresia in rats and is probably mediated by androgens (Payne & Runser, 1958; Louvet, Harman Schrieber & Ross, 1975). It is suggested that the theca cells of those follicles which have not developed to the stage at which the granulosa cells contain adequate amounts of LH receptors are unable to luteinize in response to LH or hCG and are stimulated to produce androgens in large quantities (Baird & McNeilly, 1981).

**LH surge to ovulation**

Ovulation has been shown to occur 21-26h after the preovulatory LH surge (Cumming et al., 1971a,1973). Steroid secretion from the dominant follicle declines rapidly as it prepares to expel the oocyte. The concentrations of LH and prolactin also decline at this time while the plasma FSH concentration shows a rise of longer duration and comparable peak height to that occurring 24h earlier (Salmonsen et al., 1973, Pant et al., 1977; Bindon, Blanc, Pelletier, Terqui & Thimonier, 1979).
This second FSH peak is a feature of the cycles of many other species with the exception of the primates. The factors causing this second peak are not fully understood. It is probable that it is related in some way to the events which follow the preovulatory LH surge (Baird & McNeilly, 1981). The infusion of exogenous LH on Day 10 of the cycle is followed within 24h by a second peak of FSH (Baird, McNeilly, O'Connell & Swanston, 1980). As oestrogen is inhibited following this infusion of LH, the rise in FSH secretion may be due to a reduction in negative oestrogen feedback. However the artificial maintainence of high oestradiol levels in rats did not block the second FSH surge (Chappel & Barraclough, 1977). Similarly administration of sodium pentobarbitone or GnRH antiserum after the concurrent LH-FSH surge but before the second FSH rise does not block the rise in the ewe (Dobson & Ward, 1977; Narayana & Dobson, 1979) or the rat (Blake & Kelsh, 1981; Hasegawa, Miyamoto, Yazaki & Igarashi, 1981). These studies indicate that GnRH is not significantly involved in initiating the second FSH rise.

Furthermore as the LH pulse frequency is low at the time of the second FSH surge and ovarian steroid secretion is at a minimum (Baird & McNeilly, 1981) it has been suggested that the rise in FSH may be related to the removal of both negative feedback influences by ovarian steroids and inhibin (Chappel & Barraclough, 1977; Hasegawa et al, 1981). Indirect support for these ideas come from studies in rats using pharmacological doses of inhibin (in the form of follicular fluid) which have been shown to abolish the second FSH peak (Marder, Channing & Schwartz, 1977; Schwartz & Channing, 1977).

The function of the second FSH surge in the ewe is not clear.
Several years ago it was proposed that in the rat and hamster, FSH released at the time of ovulation may programme the development of follicles destined to ovulate at the next oestrus (Schwartz & McCormack, 1972; Greenwald, 1973). This has since been investigated by several studies. In hamsters administration of antiserum to FSH to neutralise the FSH surge resulted in fewer follicles being available for ovulation at the following oestrus (Sheela Rani & Moudgal, 1977). A similar reduction in the number of ova ovulated was reported by Chappel & Selker (1979) using bovine follicular fluid (containing inhibin) to suppress the FSH surge. Furthermore the use of porcine follicular fluid in rats blocked the secondary FSH surge and follicle recruitment on the morning of oestrus (Hoak & Schwartz, 1980). Overall these studies confirm that in mammals with short reproductive cycles the second FSH surge probably has an important role in follicle recruitment for ovulation at the following oestrus presumably because it affects the follicles destined to ovulate 4 - 5 days later. However as yet in the sheep, only one study has proposed a similar model (Cahill et al, 1981). A direct correlation was found between the area under the second FSH peak and the number of follicles present 17 days later, leading to the suggestion that the rate follicles enter the antral phase may be under the control of FSH. However ovulation rate is unaffected when the corpus luteum is regressed prematurely at any stage after Day 4 (Bindon et al, 1979). Similarly, unilateral ovariectomy performed before Day 16 of the cycle does not affect the ovulation rate (Findlay & Cumming, 1977). Thus while it is feasible to suggest that the second FSH peak is involved in replenishing the stock of antral follicles after the wave of atresia following the LH peak it
seems unlikely that it determines how many follicles will ovulate at the following oestrus. It seems more probable rather that the large antral follicles present around Day 3 of the cycle are a result of the second FSH peak.

FOLLICULGENESIS

(a) Follicular growth

The ovary of the adult ewe contains a large pool of primordial follicles (12,000-86,000) and a smaller pool of between 100 and 400 follicles in various stages of growth, development and atresia (Cahill, Mariana & Mauleon, 1979; McNatty et al., 1982a). Data from histological studies suggest that in the sheep it takes 6 months for a primordial follicle to complete folliculogenesis and either become atretic or more rarely ovulate (Turnbull et al., 1977; Cahill & Mauleon, 1980). Most of this growing time (4.3 months) is spent in the pre-antral phase which is characterised by an increase in the size of the oocyte and multiplication of granulosa cells. In the sheep ovary there are twice as many pre-antral as antral follicles and the former can be influenced by several factors, including age, season, nutrition and gonadotrophin treatment by as yet unknown mechanisms (see Cahill, 1981). The slow growth rate and virtual absence of atresia in the pre-antral follicle population in the sheep, led to the suggestion (Cahill, 1981) that the role of the slow developing pre-antral follicle population may be to act as a short term reserve or buffer between the quiescent primordial follicles and rapidly growing antral follicles. Antrum formation starts in ovine follicles at a diameter of 0.2 - 0.3mm (Turnbull et al., 1977) and further increases in follicle size are due both to an increase in the rate of granulosa cell division and follicular...
fluid accumulation. Following antrum formation the follicle enters a rapid growth phase reaching a maximum at a diameter of 1mm. Thereafter granulosa cell multiplication decreases to reach zero in preovulatory follicles (7-8mm diameter) (Turnbull et al, 1977; Cahill & Mauleon, 1980). Turnbull et al (1977) concluded from histological studies that 3 – 4 follicles per day enter the antral growth phase taking between 25-35 days from antrum formation to become a mature preovulatory Graafian follicle.

(b) Hormonal control of folliculogenesis

As early as 1927, Smith and Engle showed that the initiation and maintainence of normal follicular function were dependent upon substances secreted by the adenohypophysis. Later work (Fevold, 1941; Greep, van Dyke & Chow, 1942) provided the first indication that FSH and LH may be working on separate cell types to stimulate oestrogen production and showed that only when FSH and LH were given simultaneously did all stages of normal follicular development occur. These findings were later confirmed by more recent studies (Lostrah & Johnson, 1966; Armstrong & Papkoff 1976).

In the ewe an interaction between thecal and granulosa cells is required for follicular oestrogen biosynthesis to occur. Consequently, the "two-cell type, two gonadotrophin" model to describe follicular steroidogenesis has evolved (see reviews, Armstrong, Goff & Dorrington, 1978; Hillier, 1981; Armstrong, Weiss, Selstam & Seamark, 1981). Briefly the thecal cells are the source of testosterone and androstenedione, the androgen precursors (Seamark, Moor & McIntosh, 1974; Fortune & Armstrong, 1977; Moor, 1977; Baird, 1977) which are converted to oestradiol-17β by an aromatase cytochrome P-450 enzyme system present in the granulosa
cells (Dorrington, Moon & Armstrong, 1975; Moon, Tsang, Simpson & Armstrong, 1978). In the sheep, testosterone is the preferred androgen substrate as the granulosa cells have been shown to be four times more effective at converting testosterone than androstenedione to oestradiol-17\(\beta\) (Hay & Moor, 1978). This suggests that in vivo androstenedione is probably converted to testosterone which is then aromatized to oestradiol-17\(\beta\). LH is the primary controller of androgen precursor synthesis by the thecal cells (Tsang, Moon, Simpson & Armstrong, 1979) interacting with its own receptor on the cell membrane. This results in activation of the adenyl cyclase enzyme and initiates the sequence of enzyme changes that result in the production of cyclic 3'5' adenosine monophosphate (cAMP) which converts cholesterol to pregnenolone (Marsh, 1976) the initial step in the steroid biosynthetic pathway. The induction or activation of the granulosa cell aromatase system is controlled by FSH (Dorrington et al, 1975; Erickson & Hsueh, 1978; Moon et al, 1978) and is probably mediated by an increase in intracellular cAMP levels. Evidence that the two-cell hypothesis of oestrogen synthesis is functional in the preovulatory ovine follicle in vivo comes from a study using auto-ovarian transplants (Baird, 1977). The infusion of antiserum to testosterone into the ovarian artery led to a marked reduction in oestrogen secretion. Assuming that the antiserum neutralized extracellular androgen it was suggested that 'androgens synthesized by the theca interna or stroma leave the cell and traverse the extra cellular space and basement membrane before being converted to oestrogens by the granulosa cells under the influence of FSH.'

The exact gonadotrophic requirements for folliculogenesis in
the sheep are not yet fully understood. Hypophysectomy on Day 10 of the oestrous cycle in the ewe reduced both the number of non-atretic pre-antral and antral follicles (Dufour, Cahill & Mauleon, 1979). However, although pre-antral follicles were found to be under the control of pituitary hormones the control mechanism was considered to be a slow process as the effects of follicle number were not evident until 70 days after hypophysectomy.

The stimulatory influence of gonadotrophins particularly FSH, on pre-antral follicle growth has been shown in an in vitro study in mice (Ryle, 1971) and an in vivo study in rats (de Reviers & Mauleon, 1972). Similarly autoradiographic studies have shown that exogenous FSH binds to specific receptors in the granulosa cells at all stages of growth and that, in the rat at least, FSH in the presence of oestradiol stimulates antrum formation and the acquisition of granulosa cell LH receptors (Midgley, 1973; Zeleznik, Midgley & Reichert, 1974; Richards & Midgley, 1976; Charlton, Parry, Halpin & Webb, 1982). LH binds primarily to the thecal cells at all stages of follicular development and to granulosa cells of the largest antral follicles with limited binding to the small antral and pre-antral follicles (mouse - Ryle, 1971; rat - Midgley, 1973; sheep - Carson, Findlay, Burger & Trounsen, 1979; Webb & England, 1982a). In the rat evidence suggests that the marked increase in granulosa cell LH receptors as antral follicles develop is stimulated by gonadotrophins (see reviews, Richards, 1979, 1980). Follicle development in the ewe follows a similar pattern during the follicular phase when peripheral oestradiol and LH levels increase (see previous discussion). It has therefore been suggested in the
ewe that the presence of LH/hCG receptors in the thecal and granulosa cells identifies the preovulatory follicle and that the significant increase in the concentration of thecal and granulosa cell receptors during the preovulatory period is a crucial part of the final follicle maturation (Webb & England, 1982a). In large follicles LH may promote the conversion of testosterone to oestradiol-17β (Moor, 1977) by stimulating the synthesis of the aromatase enzyme or by activating the enzymes present in the follicle (England, Webb & Dahmer, 1981b; McNeilly et al, 1984). In the absence of sufficient numbers of LH receptors on the granulosa cells of large antral follicles they do not luteinize and secrete progesterone in response to the preovulatory LH surge and become atretic. Following the LH surge there is a significant decrease in thecal and granulosa LH receptors (Webb & England, 1982b) and it is suggested that the fall in oestradiol secretion rate that occurs during the preovulatory surge may be due to a decrease in aromatase activity associated with the decrease in LH receptors. This sequence of events is confirmed in vivo by the observation that oestradiol secretion falls before that of testosterone and androstenedione (Baird et al, 1981). The high testosterone and low oestradiol concentrations in the ovulatory follicles destined to become atretic are probably due to an inactive aromatase system due to a lack of granulosa cell receptors (Webb & England, 1982b).

Similarly small follicles with LH receptors on the theca but not on the granulosa cells have a limited ability to secrete oestradiol. This is not because of a lack of androgen precursor as antral fluid testosterone levels are high (England, Dahmer & Webb, 1981a), as is the ratio of testosterone to oestradiol. A similarly high ratio of
androgen to oestrogen is found in atretic follicles (Moor et al., 1978) and suggests that whether or not a follicle becomes atretic may depend on the oestrogen/androgen ratio in its microenvironment (Baird & McNeilly, 1981). Thus as follicles grow they acquire granulosa cell LH receptors and an ability to convert large quantities of androgen to oestrogen (England et al., 1981a,b; Webb & England, 1982a,b; McNatty, 1982).

The importance of the appropriate ratio of FSH and LH in ensuring normal follicular development has long been recognised as discussed earlier in this section and is perhaps best illustrated by the clinical syndrome, polycystic ovarian disease. The condition is characterised by chronic anovulation and numerous antral follicles secreting large quantities of testosterone and androstenedione but very little oestradiol (Yen, 1980). Plasma LH levels are abnormally high and FSH levels suppressed so that the theca is stimulated to produce large quantities of androgen precursors which cannot be converted to oestrogen because the granulosa cells are deficient in aromatase enzyme activity (Short & London, 1961). The addition of FSH in vivo or in vitro causes a rapid increase in oestradiol-17β secretion and normal follicular development (Gemzell, Diczfalussy & Tillinger, 1958; Raj, Berger, Grimes & Taymor, 1977; Erickson, Hsueh, Quigley, Rebar & Yen, 1979).

(c) Follicle recruitment and selection

It is known that follicles are continuously growing and that most stages of growth are represented in the ovary at any one time (P. 17). Removal of the corpus luteum by enucleation (Lang, 1965), cautery (Smeaton & Robertson, 1971) or prostaglandin injection (Scaramuzzi & Baird, 1976; Acritopoulou, Haresign, Foster & Lamming,
1977) is followed by ovulation 48-72h later suggesting that mature follicles are present throughout the luteal phase which can ovulate if exposed to the correct gonadotrophin stimulus. Turnbull et al (1977) showed that in Merino ewes on average 3-4 follicles per day enter the antral growth phase but only one follicle normally ovulates per cycle in this breed. This implies that considerable selection processes are operating within the ovary to determine when and how many follicles will be selected to ovulate. Selection of the ovulatory follicle(s) is a two step process (di Zerega & Hodgen, 1981). Firstly, large antral follicles capable of ovulating are brought forward from the pool when exposed to sufficient gonadotrophin stimulation (recruitment) and secondly, amongst these follicles there is selection of the follicle(s) which is going to ovulate, the remaining follicles becoming atretic. In the ewe follicle recruitment is thought to occur soon after luteolysis with selection and the establishment of the dominant follicle(s) occurring later in the follicular phase. Evidence for this hypothesis comes from several sources. Unilateral ovariectomy of ewes before luteolysis on Day 14 of the cycle is fully compensated for by the remaining ovary 3 days later. If carried out on Day 16 however the time of ovulation was delayed (Land, 1973; Findlay & Cumming, 1977). Similarly follicle cautery of follicles 2-4mm or 4mm diameter (Tsonis, Cahill, Carson & Findlay, 1984) and x-radiation of 50% of the total follicle population (Driancourt & Mariana, 1982) did not alter the interval between prostaglandin-induced luteolysis and the onset of the LH surge. Smeaton & Robertson (1971) found that all large follicles marked with ink on Days 13-15 of the oestrous cycle subsequently regressed.
However, Bherer et al. (1977) reported that the largest follicle marked on Day 14 in 4 out of 5 ewes went on to ovulate but that when double ovulation occurs recruitment was from a smaller unmarked follicle suggesting that follicle recruitment occurred from a variety of size groups. Several approaches have been used to determine which specific follicle size group the ovulatory follicles come from. Growth-kinetic studies using ink-labelling showed that the follicle which ovulated was recruited from any size $\geq 2$mm at the time of both natural (Driancourt, Cahill & Bindon, 1985a) or prostaglandin-induced (Driancourt & Cahill, 1984) luteolysis. Alternatively, using electocautery, it was also shown (Tsonis et al., 1984) that the follicles capable of ovulating within the normal time for luteolysis to ovulation could come from follicles $2-4$mm or $\geq 4$mm diameter at luteolysis. When all follicles $\geq 2$mm were cauterised the onset of the LH surge was delayed by 24h. Conversely McNatty et al. (1982a) suggest that the preovulatory follicle emerges from a pool of small 'oestrogenic' follicles (1-3mm diameter) only 10h after prostaglandin injection. It has been suggested that this apparent contradiction may be due to breed differences (Driancourt & Cahill, 1984). Collectively these studies emphasise the great flexibility in follicle selection in the sheep ovary and the consensus of opinion suggests that only follicles $\geq 2$mm in diameter are recruited in the natural cycle. Confirmation that follicles of $< 2$mm diameter are not normally involved in the preovulatory follicular growth is suggested by the observation that when gonadotrophin levels are low as during seasonal anoestrus and following hypophysectomy (Smeaton & Robertson, 1971; McNatty et al., 1981; Dufour, et al. 1979) follicle growth can still proceed to 2mm.
The preceding data suggests that in the sheep the emergence of
the dominant follicle occurs late in the follicular phase. Ink
labelling techniques have shown that a significant decrease in the
number of growing follicles occurred at 48-54h after
prostaglandin-induced luteolysis in Corriedale ewes. As the LH
surge in this breed occurs approximately 60h after prostaglandin
injection, it was suggested that follicle selection is a relatively
late event in the follicular phase (Driancourt & Cahill, 1984). The
mechanism by which the dominant follicle is selected whilst the
others become atretic is often attributed to decreasing levels of
FSH (Baird, 1983; Driancourt, Gibson & Cahill, 1985b). It has been
suggested that in the sheep the rising levels of oestradiol-17β
secreted by the dominant follicle into the ovarian vein in response
to rising LH levels result in the suppression of FSH secretion so
that follicles at a slightly less advanced stage of development are
deprived of FSH and become atretic (Baird, 1983). Alternatively, it
has been hypothesised that the dominant follicle may produce a
selection factor which actively suppresses the concurrent growth of
the other follicles (Driancourt et al, 1985b). Such a factor
(protein) has been detected in human follicular fluid from the
dominant follicle (di Zerega, Marrs, Roche, Campeau & Kling, 1983)
and is postulated as being an important intraovarian regulator.
Once the dominant follicle has been selected atresia of the
remaining unselected follicles progresses and the percentage of
healthy antral follicles decreases. This decline in the healthy
follicle population can be bypassed by PMSG injection and lends
credibility to the suggestion that it is the lack of FSH which
increases the incidence of atresia during the late follicular phase
The number of eggs shed at ovulation sets the upper limit to production and there is no doubt that a low ovulation rate is one of the major factors currently limiting lambing percentages in ewe flocks throughout the world. Consequently, the factors affecting ovulation rate have been extensively investigated (see review, Scaramuzzi & Radford, 1983) in an attempt to understand the mechanisms involved so that ultimately sheep production levels can be improved.

FACTORS AFFECTING OVULATION RATE

Ovulation rate in sheep has been shown to be influenced by 6 main factors; genotype, season, age, social factors, nutrition and hormonal therapies, each of which will be briefly reviewed.

(a) Genotype

The majority of domestic sheep breeds have an ovulation rate of between 1-2. However several high fecundity breeds have been identified with an ovulation rate of 3 or more (e.g. Finnish Landrace, Romanov, Booroola Merino, D'man). Genetic improvement by the selection and crossing of these breeds with repeatedly high ovulation rates has led to the development of new highly prolific breeds, for example the Cambridge (Owen, 1971). Similarly, the introduction of highly prolific genotypes such as the Finnish Landrace (Quirke, 1978; 1979) and the Booroola Merino (Piper & Bindon, 1982) into traditional less prolific breeds has been shown to cause a significant improvement of the litter size of that flock. The heritability of ovulation rate varies with breed and possibly environment (Land, Atkins & Roberts, 1983) but can be increased by
genetic selection (Hanrahan, 1980a).

Comparison of the mechanisms involved in regulating the ovulation rate of the Romanov and the Booroola Merino indicate that their high ovulation rate may be achieved differently (Cahill, 1984). The ovulation rate of the Romanov (3.1) can be explained by a higher number of follicles (relative to Ile de France ewes, O.R.=1.5) at all stages of development (Cahill et al, 1979). Comparison of Booroola with control Merino ewes however failed to show any such differences in the number of growing follicles but indicated that there is additional growth into the recruitment pool of follicles (>2mm diameter) after luteolysis in the Booroola (Driancourt, 1984, as cited in Cahill, 1984).

(b) Season

In a seasonal breeder such as the ewe it is perhaps not surprising to find a changing pattern of ovulation rate within the breeding season. Generally, ovulation rate has been shown to be lowest at the beginning and end of the breeding season and highest in the middle (Radford, 1959; Hulet, Price & Foote, 1974; Davis, Kenny & Cumming, 1976). The mechanisms involved in this seasonal change in ovulation rate are unknown but it has been suggested that it is mediated by photoperiod (Scaramuzzi & Radford, 1983). Fewer large non-atretic follicles are present in ovaries at mid-anoestrus than during the breeding season (Kammerlade, Welsh, Nalbandov & Norton, 1952; Cahill & Mauleon, 1980) and similar seasonal variation in the incidence of atresia have been observed (Cahill, Oldham, Cognie, Ravault & Mauleon, 1984c). This suggests that differences in growth and atresia may exist within the breeding season and so account for the changes in ovulation rate.
(c) Age

The low ovulation rate of maiden ewes (1.5 years old) and its subsequent increase in mature ewes is well documented (McKenzie & Terrill, 1937). Ovulation rate reaches a plateau between 3 - 5 years and is maintained until at least 10 years old. A small study of Merino ewes showed an increase in number of pre-antral and antral follicles present in the ovaries of mature ewes (8 years old) compared with young ewes (2 years old). This change was mirrored by a similar increase in ovulation rate (Bindon, Piper & Evans, 1980) suggesting a direct relationship between age, follicle number and ovulation rate.

(d) Social factors

It has long been known that the introduction of rams into a ewe flock nearing the breeding season will stimulate the occurrence of a synchronised oestrus in a high proportion of the flock (Underwood, Shier, Davenport, 1944). This 'ram effect' has been demonstrated in many breeds of sheep around the world (see reviews, Martin & Scaramuzzi, 1983; Martin, 1984; Pearch & Oldham, 1984). Peripheral plasma LH levels increase within 10 minutes exposure to the ram at the start of the breeding season (Martin, Oldham & Lindsay, 1980). The subsequent preovulatory LH surge occurs within 24-30h of exposure and ovulation within 2-3 days (Oldham, Martin & Knight, 1978; Knight, Peterson & Payne, 1978). This induced ovulation results in an increase in ovulation rate (Oldham, 1980, Pearce, Gray, Oldham & Wilson, 1984). However, as the initial ovulation is normally a "silent" one (Schinckel, 1954) the increased ovulation rate is best commercially exploited by the use of progesterone priming to induce a concomitant behavioural oestrus (Martin &
Scaramuzzi, 1983).

(e) Nutrition

It is well documented that ovulation rate in ewes is enhanced by improved nutrition (Tassell, 1967; Lindsay, 1976; Gunn, 1983). The practice of giving ewes in moderate condition an improved diet for a few weeks before and during mating (flushing) increases their ovulation rate (Coop, 1966). This flushing response is influenced by both body condition at mating (the 'static' effect) and by the rate of change in body condition just before mating (the 'dynamic' effect) (Coop, 1966, Killeen, 1967; Edey, 1968; Fletcher, 1971). The relationship between liveweight at mating and ovulation rate is well established so that for every additional Kg a 2-2.5% increase in ovulation rate can be expected (Morley, White, Kenny & Davis, 1978). However the dynamic effect is less well defined mainly because of problems in the precise descriptions (in terms of energy and protein content) of the diet being offered (Smith, 1984).

Ewes in poor condition in Western Australia show an increase in ovulation rate when given a dietary supplement of lupin seed (Lightfoot & Marschall, 1976; Knight, Oldham & Lindsay, 1975; Lindsay, 1976; Fletcher, 1981). This effect has been ascribed to the high protein content of the grain and occurs without necessarily affecting body weight. The mechanism by which such a specific dietary supplement can increase ovulation rate is unknown. Responses to lupin feeding have been obtained in as little as 6-8 days (Knight, et al., 1975; Oldham & Lindsay, 1984) suggesting that lupins may be stimulating a short term ovarian response probably by altering follicle recruitment in the preovulatory phase (Cahill, 1984). In general the endocrine mechanism by which changes in
nutrition are translated into improved reproductive performance are
unknown. Several authors have been unable to find a relationship
between ovulation rate, liveweight or food intake and either the
absolute levels or the metabolic clearance rate of FSH (Cumming,
Findlay & Baxter, 1975; Findlay & Cumming, 1976). Similarly no
changes in either basal LH levels or the LH pulse frequency was
found in ewes with nutritionally induced differences in ovulation
rate (Scaramuzzi & Radford, 1983). However, recent studies using
Scottish Blackface ewes showed that ewes in a high body condition
had a higher mean ovulation rate and more large follicles than ewes
in low body condition and this was associated with higher FSH levels
during the luteal and follicular phase of the cycle (Rhind &
McNeilly, 1985). This indicates that the effects of body condition
(liveweight) on ovulation rate were primarily mediated by an
increase in the number of large follicles which developed under the
appropriate hormonal influences in the 2-3 days before ovulation.
Furthermore, Haresign (1981) showed that short-term 'flushing' of
ewes for one cycle increased the number of follicles > 2mm diameter
in the follicular phase, and the ovulation rate at the following
oestrus. He suggested that the increase in ovulation rate was due
to prevention of follicular atresia.

(f) Hormone therapy

1. Exogenous hormones

Ovulation rate can be increased by the exogenous administration
of gonadotrophin preparations. PMSG (Robinson, 1951, Moore &
Rowson, 1960; Gherardi & Lindsay, 1980), hCG (Braden, Lamond &
Radford, 1960; Radford, Avenell & Szell, 1984) and crude pituitary
gland extracts from horses (Moore & Shelton, 1964) are all effective
in inducing dose dependent increases in ovulation rate. PMSG given at various stages in the reproductive cycle results in a rise in oestradiol secretion by the ovary and an increase in the number of follicles producing this increased oestradiol (McCracken et al., 1971; Moor, Hay, McIntosh & Caldwell, 1973; Hay & Moor, 1978). The number of non-atretic follicles > 3mm diameter also increases suggesting that PMSG prevents follicular atresia (Hay & Moor, 1978, McNatty et al., 1981) and stimulates the growth rate of large follicles (Turnbull et al., 1977).

However as the above preparations contain both FSH and LH in varying quantities it is questionable that they are of any use in determining the mechanism involved in the ovulatory response. Administration of relatively pure FSH alone results in an an increase in ovulation rate in cows (Laster, 1973; Chupin & Procureur, 1983), goats (Bondioli & Wright, 1981) and sheep (Wright, Bondioli, Grammer, Kusan & Menino, 1981; Baird, McNeilly, Wallace & Webb, 1984; McNeilly, 1985a). In addition, ewes infused with ovine FSH from 24h before to 60h after prostaglandin-induced luteolysis had a higher mean ovulation rate (8.2 ova) when compared with ewes in which the infusion was started at luteolysis (3.0 ova) suggesting that there was a further increase in follicle recruitment when FSH levels were increased prior to luteolysis (Baird et al., 1984).

Infusion of ovine LH increased ovulation rate in the cyclic hamster and guinea pig by preventing atresia of the developing follicles (Garza, Shaban & Terranova, 1984). However the infusion of LH alone to hamsters following hypophysectomy was unable to completely prevent atresia suggesting that the superovulatory response to LH required the presence of the other pituitary gland
hormones. Conversely attempts to increase the plasma LH concentration in the follicular phase in the ewe, by repeated low dose GnRH injections did not affect ovulation rate or LH secretion (McLeod & Haresign, 1984a). In many of these studies in which ovulation rate is increased there is a highly variable response which probably reflects the wide variation in the number of follicles available to respond to gonadotrophin treatment at any one time (Webb & Gauld, 1985a).

Steroid hormones may also affect ovulation rate. Progesterones block ovulation and are widely used to synchronise oestrous behaviour although it is not known whether smaller doses would have any effect on ovulation rate. Oestrogens e.g. in the form of the phyto-oestrogen coumestral found in Lucerne grass (Smith, Jagusch, Brunswick & McGowan, 1980) and oestradiol benzoate (Land, 1976) will depress ovulation rate. These effects are probably mediated by an inhibition of tonic gonadotrophin secretion due to the oestrogen (Land, 1976; Kelly, Adams & Lindsay, 1976).

2. Immunization against steroids

Active immunization against androstenedione (Scaramuzzi, Davidson & Van Look, 1977) testosterone, oestrone and oestradiol-17β (Martensz, 1977; Scaramuzzi, Martensz & Van Look, 1980a; Scaramuzzi, Baird, Clarke, Martensz & Van Look, 1980b; Scaramuzzi, Baird, Martensz, Turnbull & Van Look, 1981) and progesterone (Hoskinson, Scaramuzzi, Downing, Hinks & Turnbull, 1982) have all been reported to increase ovulation rate in sheep. Similar increases in ovulation rate have been observed following passive immunization in the breeding season (Land, Morris, Baxter, Fordyce & Forster, 1982) and during anoestrous (Land, Fordyce, Gauld, Morris &
Webb, 1983). Ovulation rate is generally increased in a small and consistent way by steroid immunization making it an attractive commercial proposition for increasing lambing rates (Scaramuzzi & Radford, 1983). The mechanism(s) behind these ovulatory responses is not clear (see reviews, Scaramuzzi & Hoskinson, 1984; Webb, Land, Pathiraja & Morris, 1984). In general LH pulse frequency is higher in ewes immunized actively or passively against a range of steroids. FSH levels are increased in ewes immunized against oestrone (Martensz, Scaramuzzi & Van Look, 1979; Scaramuzzi et al, 1980a) while immunization against androgens produces either no change (testosterone) or a decrease (androstenedione) in FSH levels (Martensz & Scaramuzzi, 1979). As LH levels are high and FSH levels variable in these studies and all show an increase in ovulation rate it has been suggested that the response is due to an increase in LH with FSH having a permissive rather than an active role (Scaramuzzi, 1979).

Androgens have been shown to accelerate follicle atresia in rats (Payne & Runser, 1958) and the levels of androstenedione increase during follicular development and atresia in the ewe (Carson, Findlay, Clarke & Burger, 1980). Thus it is tempting to suggest that immunization against androgens in the sheep may be operating by increasing the number of large non-atretic follicles available for ovulation. However this does not explain why increases in ovulation rate occur following immunization against oestrogens (Scaramuzzi & Hoskinson, 1984).

3. Immunization against inhibin

The physiological role of inhibin in the normal regulation of FSH secretion and follicular maturation is largely unresolved and
will be discussed in a later section (see P. 41). In an attempt to elucidate the role of inhibin several groups have actively immunized animals against partially purified fractions from bovine follicular fluid, (sheep: O'Shea, Cummins, Bindon & Findlay, 1982; O'Shea et al, 1984; Henderson, Franchimont, Lecomte-Yerna, Hudson & Ball, 1984: cow, Price, Land, Morris, O'Shea & Webb, 1985). Ovulation was generally increased in the sheep studies but the number of ewes responding and the range of ovulation rates was very variable. None of the groups were able to detect any change in peripheral FSH concentrations following immunization. However the sampling frequencies used were probably inadequate in view of the wide day to day fluctuations in FSH concentrations observed during the normal cycle. Furthermore as the immunogen has not been characterised it is possible that the immunization procedures may have been against other intra-ovarian peptide(s) as well as inhibin.

HORMONAL CORRELATES OF OVULATION RATE

It has been suggested by the evidence reviewed in the previous section that changes in ovulation rate are mediated by the effects of the pituitary gonadotrophins on the growth, recruitment and maturation of the ovarian follicle population. Considerable effort has gone into attempts to determine the hormonal correlates of ovulation rate in the hope that this will lead to a better understanding and hence manipulation of the mechanisms involved. As gonadotrophins have been shown to be essential for follicular development, much of the work has concentrated on measuring peripheral gonadotrophin levels at various stages of the oestrous cycle, both within and between, prolific and non-prolific breeds.

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(a) Possible contribution of LH to ovulation rate

Changes in plasma levels of LH preceding the preovulatory surge are thought to play a major role in follicle maturation and subsequent corpus luteum function (Baird & McNeilly, 1981). However, the role of LH in determining how many follicles will ovulate is largely unresolved (Cahill, 1981; Scaramuzzi & Radford, 1983). Two studies have attempted to quantify the relationship between LH and ovulation rate by comparing the highly prolific Booroola genotype with control Merinos (Scaramuzzi & Radford, 1983; Bindon et al.; 1985). No differences in either the mean LH levels or mean pulse frequency (as measured at 15 and 20 minute intervals) were found on Day 16 of the oestrous cycle (Scaramuzzi & Radford, 1983) or during the 3 days before ovulation (Bindon et al., 1985) despite significant differences in ovulation rate. Conversely, the mean LH level has been compared in Finnish Landrace versus Suffolk ewes following progesterone implant removal (Webb & England, 1982a) and in D'man and Timahdite ewes in the natural cycle (Lahlou-Kassi, Schams & Glatzel, 1984). Although blood sampling was less frequent in both of these studies (1-2h and 3-6h respectively) the prolific breeds (Finn and D'man) were both found to have significantly lower LH concentrations during the preovulatory period when compared with the less prolific breeds (Suffolk and Timahdite). This may be a reflection of either 1) breed differences in sensitivity to the feedback effects of oestrogen or 2) differences in the amount of negative feedback.

Recent evidence suggests that LH levels during the luteal phase may influence ovulation rate. Merino ewes consistently having 2 ovulations had a higher frequency of LH pulses than flockmates having a single ovulation. Samples were withdrawn at 20 minute
intervals for 8h every second day from Days 2-12 of the luteal phase (Thomas, Oldham & Martin, 1984). Similarly, the frequency of LH pulses in the luteal phase was higher in Scottish blackface ewes with multiple ovulations than in ewes with a single ovulation (Rhind & McNeilly, 1985). In theory indirect evidence for a possible role of LH pulse frequency in determining ovulation rate should come from studies using exogenous LH (or GnRH) administration. However LH administration during an induced follicular phase in anoestrous (McNatty et al, 1981) and during the late luteal phase in the breeding season (Martin & Clapin, 1982) failed to significantly increase ovulation rate above that of controls despite a considerable elevation in LH levels. This further suggests that ovulation rate is independant of the amount and secretory pattern of LH during the cycle. However as the increase in ovulation rate following steroid immunization and the introduction of rams to seasonally anoestrous ewes both appear to be mediated by the increase in LH pulse frequency, it is difficult to accept that LH has no role to play in determining ovulation rate. Thomas et al (1984) suggests that 'the definitive experiment linking increased secretion of LH with increased ovulation rate has yet to be carried out'. Certainly the duration and sampling frequency in many of the studies reviewed is often inadequate especially during the preovulatory period when LH pulse frequency is known to be high (Baird & McNeilly, 1981) suggesting that significant changes in the amount and secretory pattern of LH may be being missed.

The LH parameter which has been most consistantly correlated with ovulation rate so far is the interval between the onset of oestrus and the preovulatory LH surge (Land, Pelletier, Thimonier &
However as the follicles destined to ovulate have already been differentiated by this time, this correlation is probably associated with, rather than a determinant of, ovulation rate (Cahill et al., 1979, 1981). Bindon et al. (1979) suggested that as the LH surge terminates preovulatory follicular activity the prolific ewe has a longer period available for preovulatory follicle development before this is brought to a halt by the LH surge. However Land et al. (1973) suggested that the correlation arises because of different sensitivities to oestrogen feedback on the hypothalamic centres responsible for the release of LH.

The majority of authors (Land et al., 1973; Bindon et al., 1978, 1979; Quirke et al., 1979; Haresign, 1981) have failed to observe any relationship between the magnitude and duration of the LH surge and ovulation rate. However Lahlou-Kassi et al. (1984) reported that the LH peak height and the area under the curve were significantly higher in the less prolific breed studied and suggested that these ewes were more sensitive to oestrogen feedback.

(b) Possible contribution of FSH to ovulation rate

The pattern of FSH release during the ovine oestrous cycle has previously been discussed in some detail (P. 8). It is generally agreed that during the preovulatory period there is a fall in FSH levels prior to the FSH discharge concurrent with the preovulatory LH surge with a second FSH peak occurring about 24h later. During the luteal phase however, individual FSH levels fluctuate in an
irregular wave pattern which is often obscured when the data for
groups of animals are combined. It is perhaps not surprising
therefore that numerous groups have failed to correlate mean FSH
levels with changes in ovulation rate (Findlay & Cumming, 1976;

Periovulatory FSH concentrations have been studied in some
detail in prolific compared with non-prolific breeds. Cahill et al
(1981) reported that the second FSH peak was higher in prolific
Romanov ewes when the data was aligned to the preovulatory LH surge.
A previous study (Bindon et al, 1979) failed to detect this
difference between the Romanov and other low fecundity breeds but
they analysed the FSH levels relative to prostaglandin-induced
luteolysis and oestrus which are perhaps less appropriate markers of
the periovulatory phase events. Breed differences in FSH levels
during the periovulatory period have also been reported by
Lahlou-Kassi et al, (1984). FSH concentrations were higher in the
prolific D'man than in Timahdite ewes throughout the periovulatory
period (pro-oestrus, preovulatory surge and area under the second
peak) in naturally cycling ewes. Similarly the Booroola Merino was
found to have higher FSH levels in the 24h preceding the
preovulatory surge and during the second FSH peak when compared with
control Merinos (Bindon et al, 1985). Added to these studies are
the observations that Booroola ewe lambs have higher FSH early in
development (Findlay & Bindon, 1976), that adult ewes have an
elevated pituitary FSH and FSH:LH ratio as determined on Day 3 of
the cycle (Robertson, Ellis, Foulds, Findlay & Bindon, 1984) and a
greater urinary FSH excretion during the follicular phase (Bindon et
al, 1985). Collectively these studies suggest that for the prolific
breeds at least, high FSH levels appear to be associated with high ovulation rates. Indirect evidence for this suggestion comes from studies using exogenous FSH to stimulate increased ovulation as discussed previously (P. 31). Although most authors have tended to look for differences in mean FSH concentrations between breeds known to have different ovulation rates this may be misleading as the mechanisms involved in follicle maturation and hence gonadotrophin requirement may be intrinsically different between breeds. In addition it has been suggested that a lower sensitivity of the hypothalamic/pituitary axis to the negative feedback effects of oestradiol may enable breeds with a high ovulation rate to tolerate higher concentrations of ovarian hormones before there is a reduction in the release of gonadotrophins (Land, 1976; Land, Wheeler & Carr, 1976). Hence differences in ovulation rate may occur without necessarily being reflected by differences in peripheral gonadotrophin levels. However FSH concentrations have been studied in Finnish Landrace and Scottish Blackface ewes following either ovariectomy or removal of an oestradiol implant from long-term ovariectomized ewes (Webb, Baxter, Preece, Land & Springbett, 1985). Long term ovariectomised Finn ewes have higher FSH than the Scottish Blackface ewes suggesting that even after removal of oestradiol negative feedback by ovariectomy an underlying breed difference in FSH secretion exists. Whether this difference in FSH level explains the difference in ovulation rate between the breeds is unknown. There is a lack of data attempting to correlate endogenous FSH levels with the normal variation in ovulation rate occurring within a breed. Rhind & McNeilly (1985) found that there was no difference in mean FSH concentrations between ewes with
single and multiple ovulations despite the relationships between body condition and ovulation rate and between condition and FSH concentrations. Conversely ewes with twin lambs have higher FSH concentrations from 8 to 3 days before ovulation than do those with singletons (Davis, Brien, Findlay & Cumming, 1981).

Several factors may be responsible for the lack of convincing evidence for a quantitative role of FSH in controlling ovulation rate. It may be that the present radioimmunoassay systems are too insensitive to detect subtle changes in concentration or that they do not reflect the biological activity of FSH (Scaramuzzi & Radford, 1983). Furthermore the sampling frequency and methods of analysis used have often failed to consider the wide individual fluctuations in FSH levels throughout the cycle. Similarly in the individual animal the differences in concentration of FSH which may be biologically significant in determining ovulation rate may be smaller than the differences between animals (Brown, 1978).

(c) Possible contribution of prolactin to ovulation rate

Prolactin has yet to be shown to vary directly with ovulation rate. The suppression of prolactin levels with ergocornine hydrogen maleinate or CB154 (2X-bromo-ergocryptine) in the cyclic ewe did not effect ovulation or luteal function (Louw, Lishman, Botha & Baumgartner, 1974; Niswender, 1974). However, Cahill et al (1981) found that prolactin concentrations were almost 2-fold greater during the two days before the preovulatory discharge of LH in prolific Romanov compared with the less prolific Ide-de-France ewes. Similarly the prolactin discharge preceeding the LH surge was correlated with the number of pre-antral follicles present in the ovary 17 days later suggesting that prolactin may be involved in
pre-antral follicle recruitment (Cahill et al., 1981). There was a significant correlation between the preovulatory peak prolactin values and ovulation rate in bromocryptine treated ewes after an induced oestrus during seasonal anoestrus. However no such relationship was found in control ewes (Rodway, Robinson & Phillippo, 1983).

The majority of studies reviewed in this section have attempted to correlate peripheral gonadotrophin concentrations with ovulation rate by examining the relationships within and between genotypes and the effects of environmental and or exogenous hormone treatments. While these approaches to understanding the control of ovulation rate are valuable they are often confounded in that the relative contributions of either LH or FSH cannot be dissected from each other. Similarly although the role of LH in the endocrine control of the oestrous cycle is well documented the role of FSH in both luteal and follicular phase events is largely unresolved. With this in mind much attention has recently focused on the use of follicular fluid containing 'inhibin' to selectively suppress plasma FSH concentrations. It is pertinent therefore to briefly review the nature and source of inhibin in relation to its proposed role in the female reproductive cycle.

**INHIBIN**

Mottram & Cramer (1923) described 'castration cells' in the adenohypophysis of male rats after selective testicular germ cell damage and first suggested that the seminiferous epithelium produced a specific factor, the absence of which resulted in the appearance of these castration cells. The name 'inhibin' was later coined by
McCullagh (1932) to describe a water soluble testicular hormone involved in the control of pituitary gland secretion of FSH. Inhibin activity has since been found in a variety of male gonadal secretions including testicular lymph, rete testis fluid, seminal plasma and Sertoli cell medium (see reviews, Davies, Main & Setchell, 1979; Franchimont, Demoulin, Verstraelen-Proyard, Hazee-Hagelstein & Turnbridge, 1979; Steinberger, 1979; Baker et al, 1983) and is considered to be an important component of the feedback control of the testis on FSH production by the pituitary.

Although originally postulated as a testicular hormone there is now conclusive evidence that a similar if not identical substance is found in the ovary. This 'ovarian FSH inhibitor' was first detected in bovine follicular fluid (de Jong & Sharpe, 1976) and ovarian extracts (Hopkinson et al, 1977) using castrated male or ovariectomised female rats as test animals. However despite having been recognised to exist in the male for over 50 years and in the female for almost a decade there is still a great deal of controversy about the nature and physiological role of inhibin mainly because it has not been completely purified or characterised (see reviews, Baker et al, 1976; Franchimont et al, 1979; de Jong, Jansen, Hermans & van der Molen, 1982; Baker et al, 1983; de Jong & Robertson, 1985).

The results of attempts to purify inhibin from testicular sources have largely been equivocal (Chari, Durawswami & Franchimont, 1978; Vaze, Thakur & Sheth, 1979; Ramasharma & Sairam, 1982). However there is better agreement between authors about the nature of inhibin using follicular fluid as a starting material and utilising in vitro pituitary cell bioassays to monitor inhibin
activity in the various purified fractions (Baker et al., 1983, Dobos, Burger, Hearn & Morgan, 1983; de Jong et al., 1982; de Jong, Van Dijk & Van der Molen, 1984; Sairam, Kato, Manjunath & Ramasharma, 1985). The range of molecular weights for inhibin reported by these authors varied from 10,000 to 65,000 daltons.

However, using bovine follicular fluid as the original source of inhibin, the most recent results following a 3,000 fold purification suggest that inhibin is a glycoprotein, molecular weight 56,000 composed of 2 disulphide linked polypeptide chains of molecular weight 44,000 and 14,000 daltons (Robertson et al., 1985). In addition there is general agreement that inhibin is moderately hydrophobic, acidic (pI 5-6) and inactivated by boiling and proteolytic enzyme digestion. Similarly, a loss of activity occurs when exposed to organic solvents, charcoal and oxidants (Baker et al., 1982, 1983; Tsonis et al., 1983).

In the female ovarian inhibin has been shown to be synthesised by the granulosa cells in the rat (Erickson & Hsueh, 1978) pig (Anderson, Shander, Channing & Barraclough, 1979) and cow (Henderson & Franchimont, 1981, 1983). In the ewe the individual inhibin content of follicles is related to follicle size and granulosa cell volume so that inhibin concentrations increase with size (Tsonis et al., 1983). Comparison of inhibin levels in follicular fluid from different sources and/or species is difficult however, because of the wide range of in-vitro and in-vivo bioassays in use and the absence of an international inhibin reference standard.

Evidence about the control of inhibin production in the female has largely come from studies using the PMSG-primed rat. Initial studies demonstrated that administration of PMSG caused a dose
related increase in ovarian and circulating peripheral inhibin levels (Lee, McMaster, Quigg, Findlay & Leversha, 1981). Furthermore this increase in inhibin was associated with a reduction in FSH levels providing evidence in the female of the inverse feedback relationship between inhibin and FSH. Similarly ovariectomy of PMSG-primed immature rats dramatically reduced circulating levels of inhibin and raised FSH concentrations confirming that the ovaries are the major source of inhibin secreted into the systemic circulation in the rat (Lee, 1983). In addition, FSH-inhibiting activity has been demonstrated in ovarian vein plasma of cyclic rats and varies inversely with peripheral plasma FSH concentrations (de Paolo, Shander, Wise, Barraclough & Channing, 1979b; Shander, Anderson & Barraclough, 1980a). In the monkey inhibin activity has been detected in the vein draining the ovary bearing the preovulatory follicle (Channing, Anderson & Hodgen, 1980) and similarly inhibin concentrations in follicular fluid from the rat (Fujii, Hoover & Channing, 1983), pig, (Lorenzen, Channing & Schwartz, 1978) cow (Grady, Charlesworth & Schwartz, 1982) hamster (Chappel, 1979) and human (Chappel, Holt & Spies, 1980) have been shown to vary with stage of cycle.

In the rat it is postulated that inhibin is involved in the feedback relationship between the total number of large follicles in the ovaries and FSH secretion as there is a negative correlation between FSH concentrations and the number of large follicles during the periovulatory period (Welchen, Hermans & de Jong, 1980). A similar negative feedback relationship between inhibin and FSH concentrations is hypothesised to exist in the ewe. Goodman et al (1981b) concluded that a factor other than oestradiol and
progesterone must contribute to the feedback regulation of FSH during the oestrous cycle as implants of these steroids alone could not suppress FSH to normal cycle levels. However using the bioassays currently available inhibin has not yet been measured in the ovarian vein or peripheral circulation of the cycling ewe. A preliminary study (Tsonis, 1984) has shown however that measurable quantities of inhibin are present in ovarian lymph during the ovine oestrous cycle with levels being highest during the preovulatory period. This suggests that inhibin can enter the peripheral circulation in the ewe via the lymphatic system. However, no concurrent changes in plasma FSH concentrations were detected in this study as measured in samples withdrawn at 4-6h intervals in one ewe.

Indirect evidence for the involvement of inhibin in the control of FSH and hence follicle growth in the ewe comes from studies using the prolific Booroola Merino (Cummins, O'Shea, Bindon, Lee & Findlay, 1983). Booroola ewes with an ovulation rate of 2.8 had one third of the ovarian inhibin content of control ewes (ovulation rate 1.2) suggesting that low inhibin production may be responsible for the higher FSH concentrations seen in the plasma, urine and pituitary gland of the Booroola (see P. 38). This also suggests that the inhibin/FSH feedback in Booroola ewes may be set differently to that of control ewes. To date however there is no information about the inhibin content of ovaries of other prolific breeds with observed differences in FSH concentrations.

An alternative approach to understanding the physiology of inhibin in the sheep has been to actively immunize ewes against partially purified fractions of follicular fluid (see P. 33).
Table 1.1. The effect of follicular fluid (FF) administration on peripheral gonadotrophin levels in intact, cycling females of a variety of species.

<table>
<thead>
<tr>
<th>Experimental Animal</th>
<th>Follicular Fluid source</th>
<th>Reproductive stage</th>
<th>Effect on FSH</th>
<th>Effect on LH</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hamster</td>
<td>bovine</td>
<td>proestrus-oestrus</td>
<td>suppressed or abolished release at oestrus</td>
<td>none</td>
<td>dose-dependent fall in no. of ova shed at next cycle</td>
<td>Chappel &amp; Selker (1979)</td>
</tr>
<tr>
<td>rat</td>
<td>bovine</td>
<td>proestrus-oestrus</td>
<td>suppressed to basal levels</td>
<td>none</td>
<td>negative correlation between FSH levels and no. of large follicles</td>
<td>Welschen et al, (1980)</td>
</tr>
<tr>
<td>rat</td>
<td>bovine</td>
<td>dioestrus;HCG pre-treated</td>
<td>dose dependent suppression of surge</td>
<td>delayed surge; small rise</td>
<td>delayed FSH surge &amp; initiation of follicular maturation</td>
<td>Sasamoto et al, (1981)</td>
</tr>
<tr>
<td>rat</td>
<td>porcine</td>
<td>proestrus + 3h after LH Inject.</td>
<td>dose dependent suppression of surge</td>
<td>none</td>
<td>similar effect, following natural or artificially induced LH surge</td>
<td>Swartz &amp; Channing, (1977)</td>
</tr>
<tr>
<td>rat</td>
<td>porcine</td>
<td>proestrus-oestrus</td>
<td>decrease 2-5h after injection, abolished surge</td>
<td>none</td>
<td>FF pretreatment suppressed release of FSH in response to LH-RH</td>
<td>de Paolo et al, (1979c)</td>
</tr>
<tr>
<td>rat</td>
<td>porcine</td>
<td>proestrus-oestrus</td>
<td>suppression then rebound increase</td>
<td>none</td>
<td>no effect on ovulation rate despite both FSH surges being blocked</td>
<td>de Paolo et al, (1979a)</td>
</tr>
<tr>
<td>rat</td>
<td>porcine</td>
<td>early proestrus</td>
<td>High dose-blocked pre-ovulatory surge</td>
<td>normal LH surge</td>
<td>did not prevent ovulation or rise in P₄ &amp; fall in E₂</td>
<td>Hoffman et al, (1979)</td>
</tr>
<tr>
<td>Species</td>
<td>Species</td>
<td>Interval</td>
<td>Effect</td>
<td>Effect</td>
<td>Reference</td>
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<tr>
<td>Rat</td>
<td>Porcine</td>
<td>Early and late proestrus + E₂</td>
<td>Both surges suppressed</td>
<td>Surge blocked if given at early proestrus</td>
<td>E₀ administration did not affect FSH surges</td>
<td>Rush et al. (1982)</td>
</tr>
<tr>
<td>Monkey</td>
<td>Porcine</td>
<td>(a) Day 0-4 of menstrual cycle</td>
<td>Suppressed by 50-80%</td>
<td>None</td>
<td>Decrease in size of dominant follicles on Days 10-14</td>
<td>Channing et al. (1981)</td>
</tr>
<tr>
<td>Monkey</td>
<td>Porcine</td>
<td>(b) Midcycle</td>
<td>Postponement or abolition of spontaneous or E₂ induced LH/FSH surges</td>
<td>None</td>
<td>Failed to ovulate</td>
<td></td>
</tr>
<tr>
<td>Monkey</td>
<td>Porcine</td>
<td>Follicular phase; early or during midcycle surge</td>
<td>Suppressed FSH and blocked FSH surge</td>
<td>Normal surge</td>
<td>Delay in ovulation + corpus luteum insufficient</td>
<td>Stillman et al. (1983)</td>
</tr>
<tr>
<td>Monkey</td>
<td>Porcine</td>
<td>Mid-luteal or follicular phase</td>
<td>Suppressed at both stages of cycle</td>
<td>None</td>
<td>Lower progesterone during luteal phase</td>
<td>Stouffer et al. (1984)</td>
</tr>
<tr>
<td>Monkey</td>
<td>Porcine</td>
<td>Early or late follicular &amp; periovulatory</td>
<td>Suppressed within 24h, remained low until 1-2 days after treatment</td>
<td>None</td>
<td>Periovulatory treatment resulted in delay or failure of final follicle maturation</td>
<td>Di Zerga et al. (1981)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Bovine</td>
<td>(a) Early &amp; late follicular phase</td>
<td>Suppressed at all stages</td>
<td>Not measured in daily samples, normal surge</td>
<td>Delay in LH surge onset, magnitude of FSH rebound correlated with length of treatment</td>
<td>Miller et al. (1982)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Bovine</td>
<td>(b) Early luteal phase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>Bovine</td>
<td>Follicular phase</td>
<td>Suppressed during first 24h, rebound after treatment stopped</td>
<td>No effect on LH pulsatility</td>
<td>Delay in oestrus onset</td>
<td>McNeilly (1984)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Bovine</td>
<td>Follicular phase + FSH infusion</td>
<td>Suppressed 1.5-2h after injection</td>
<td>No effect on LH pulsatility</td>
<td>Delay in oestrus, counteracted by FSH infusion</td>
<td>McNeilly (1985a)</td>
</tr>
</tbody>
</table>
Table 1.2 The effect of follicular fluid (FF) administration on peripheral gonadotrophin levels after ovariectomy, in a variety of species

<table>
<thead>
<tr>
<th>Experimental Animal</th>
<th>Follicular Fluid source</th>
<th>Reproductive Stage (Time after Ovariectomy)</th>
<th>Effect on FSH</th>
<th>Effect on LH</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse</td>
<td>porcine</td>
<td>ovariectomised + E2 implant</td>
<td>50% suppression with FF alone, elevated in absence of E2</td>
<td>high LH not considered to be due to residual steroids in FF</td>
<td>Bronson &amp; Channing (1978)</td>
<td></td>
</tr>
<tr>
<td>rat</td>
<td>porcine</td>
<td>ovariectomised (7 days) + E2 implant</td>
<td>decreased 3h after injection for 5-14h</td>
<td>none</td>
<td>E2 implant did not completely lower FSH to baseline</td>
<td>Campbell &amp; Schwartz (1979)</td>
</tr>
<tr>
<td>rat</td>
<td>porcine</td>
<td>ovariectomised (same day)</td>
<td>dose dependent decrease</td>
<td>not measured</td>
<td>sensitive to FF than intact rats</td>
<td>Marder et al, (1977)</td>
</tr>
<tr>
<td>rat</td>
<td>porcine</td>
<td>ovariectomised (short &amp; long term)</td>
<td>suppression 48h after start of infusion - decrease in response after 20 days</td>
<td>none</td>
<td>after cessation of 10 or 30 day infusion, FSH rebounded to control levels within 2-4 days</td>
<td>Thomas &amp; Nikitovitch-Winer, (1984)</td>
</tr>
<tr>
<td>rat</td>
<td>porcine</td>
<td>ovariectomised (21 days) + E2 implant</td>
<td>decreased over a 7 day period</td>
<td>minimal</td>
<td>E2 operated additively to suppress FSH</td>
<td>Williams &amp; Lipner (1981)</td>
</tr>
<tr>
<td>rat</td>
<td>porcine</td>
<td>(a) ovariectomised (2 weeks)</td>
<td>dose dependent suppression-maximum 60% 10h after injection</td>
<td>elevated at low dose</td>
<td>effect on LH varied with state of GnRH stimulation</td>
<td>Charlesworth et al, (1984)</td>
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<tr>
<td></td>
<td></td>
<td>(b) ovariectomised + GnRH</td>
<td>&quot; &quot; variable &amp; inconsistent</td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
<td></td>
</tr>
<tr>
<td>rat</td>
<td>porcine/bovine</td>
<td>(a) unilateral-ovariectomised (same day)</td>
<td>dose dependent decrease 4h after treatment</td>
<td>none</td>
<td>porcine &amp; bovine follicular fluid equally effective</td>
<td>Welschen et al, (1977)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) ovariectomised (12 days)</td>
<td>&quot; &quot;</td>
<td>none</td>
<td>&quot; &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) unilateral-6 ovariectomised (same time)</td>
<td>totally prevents FSH rise</td>
<td>none</td>
<td>&quot; &quot;</td>
<td></td>
</tr>
<tr>
<td>Animal</td>
<td>Breed</td>
<td>Treatment</td>
<td>Suppression</td>
<td>Delay</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
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<td>-------</td>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>Horse</td>
<td>Equine</td>
<td>Ovariectomised (long term) + E&lt;sub&gt;2&lt;/sub&gt; implant</td>
<td>Dose dependent suppression 26-74%, 8-24h after injection</td>
<td>Whole FF-operates additively with FF to suppress FSH</td>
<td>No difference in effect during breeding season compared with anoestrus</td>
<td>Miller et al., 1979b, 1981b</td>
</tr>
<tr>
<td>Cow</td>
<td>Bovine</td>
<td>Ovariectomised (3 days)</td>
<td>Maximum suppression 66%, 10-12h after injection</td>
<td>None</td>
<td>-</td>
<td>Curato et al., 1984</td>
</tr>
<tr>
<td>Monkey</td>
<td>Porcine</td>
<td>Ovariectomised (same time)</td>
<td>Delay in postcastration rise until treatment stopped</td>
<td>Slight delay in postcastration rise but high before end of treatment</td>
<td>FF pretreatment prevented release of LH &amp; FSH in response to GnRH</td>
<td>Schenken et al., 1984</td>
</tr>
<tr>
<td>Monkey</td>
<td>Porcine</td>
<td>Ovariectomised (long term)</td>
<td>Decreased 15h after injection</td>
<td>None</td>
<td>-</td>
<td>Rettori et al., 1982</td>
</tr>
<tr>
<td>Monkey</td>
<td>Porcine</td>
<td>Ovariectomised (long term)</td>
<td>Dose dependent suppression, 23-80%</td>
<td>None</td>
<td>-</td>
<td>Channing et al., 1981</td>
</tr>
<tr>
<td>Sheep</td>
<td>Ovine</td>
<td>Ovariectomised (3 months)</td>
<td>Dose dependent suppression, 92% maximum</td>
<td>Marginal decrease at highest dose</td>
<td>Total dose 18,000 -72,000 units of inhibin</td>
<td>Cummins et al., 1983</td>
</tr>
<tr>
<td>Sheep</td>
<td>Ovine</td>
<td>Ovariectomised (6 weeks before first used)</td>
<td>Dose dependent suppression, 60-70%</td>
<td>Suppressed at highest dose, 50% maximum</td>
<td>None</td>
<td>Findlay et al., 1985</td>
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</table>
However although the ovulation rate has been increased by varying degrees using these techniques the serum of immunized ewes has not been shown to alter FSH concentrations (or ovulation rate) when administered to control ewes nor have the actively immunized ewes been shown to have higher FSH levels (Bindon et al, 1985). Thus in the ewe, at least, our knowledge of the negative feedback control of inhibin on FSH secretion is limited and will remain so until inhibin can be measured in the plasma throughout the oestrous cycle.

FOLLICULAR FLUID

(a) Effect on FSH secretion

In an attempt to understand the regulation and role of FSH in the ovarian cycle of a range of species a large number of investigators have used crude inhibin preparations in the form of follicular fluid (Tables 1.1. and 1.2). Ovine, bovine, porcine and equine follicular fluid preparations have all been shown to suppress FSH secretion when administered to a range of experimental animals (rodent, monkey, cow, horse and sheep) at various times of the reproductive cycle. In particular the administration of steroid-free follicular fluid to ovariectomised animals with or without steroid replacement (Table 1.2) has provided strong evidence for the involvement of a specific, non-steroidal ovarian inhibitor of FSH in the female. Furthermore as administration of follicular fluid has also been shown to effectively suppress FSH in the intact (de Jong & Sharpe, 1976) and castrated male (Thomas & Nikitovitch-Winer, 1984; Findlay, Gill & Doughton, 1985) there is little doubt that the active component is analogous to male inhibin. Thus while recognising that other proteins are present in follicular fluid (see P. 54), inhibin will be assumed to be the major active
component suppressing FSH in the work described in this thesis.

Since the reproductive state, frequency of follicular fluid administration and subsequent blood sampling varied widely between authors, it is difficult to generalise between studies. Similarly the majority of studies reviewed did not estimate the inhibin potencies of the follicular fluid preparations used. Nevertheless several generalisations can be made. The suppression of FSH levels lagged behind follicular fluid injection by a minimum of 2h and was dose dependent. Similarly the insertion of an oestradiol implant together with inhibin treatment in ovariectomised females had an additive effect of FSH suppression resulting in maintainence of or a return to baseline levels in the mouse (Bronson & Channing, 1978), rat (Williams & Lipner, 1981) and horse (Miller, Wesson & Ginther, 1981). As yet no such information is available in the sheep. However, if the hypothesis of Goodman et al (1981b) is correct a similar synergistic or additive action of inhibin and oestradiol should be operating to regulate FSH secretion in the ewe.

(b) Effects on LH secretion

Reports on the effects of follicular fluid on LH secretion are inconsistent. In a few isolated instances follicular fluid has been shown to enhance LH levels, e.g. in the ovariectomised mare (Miller, Wesson & Ginther, 1979b) and mouse (Bronson & Channing, 1978) coincident with FSH suppression, and in intact rats after the suppression of FSH had ceased (Hermans, Debets, van Leeuwen & de Jong, 1981). In addition variable increases in LH secretion which do not appear to be related to dose of follicular fluid have been reported in vitro (Charlesworth, Grady & Schwartz, 1983) but have low reproducibility. Conversely follicular fluid treatment on the
morning of proestrus abolished the preovulatory LH surge in rats (Rush et al., 1982) and decreased LH secretion in ovariectomised ewes when given at high doses (Cummins et al., 1983; Findlay et al., 1985). These conflicting results suggest that there may be other factor(s) in follicular fluid (see P. 54) which alter the secretion of LH independently of the effects of inhibin. However the majority of studies detailed in Tables 1.1 and 1.2 report no detectable effect of follicular fluid on LH secretion, although the sampling frequency in nearly all of these studies was inadequate to define possible differences in pulsatile LH frequency or amplitude. McNeilly (1984, 1985a) showed that injection of bovine follicular fluid to ewes in the preovulatory phase of the cycle did not effect pulsatile LH secretion. However LH pulse frequency is high at this stage of the cycle and was already maximum by the time sampling commenced.

(c) Site of action

The primary site of action of inhibin is probably the pituitary gonadotroph as steroid free follicular fluid (de Jong, Smith & Vander Molen, 1979; Shander, Anderson, Barraclough & Channing, 1980b; Huang & Miller, 1984) and granulosa cell culture medium (Massicotte, Lagace, Godbout & Labrie, 1984) both suppress the release of FSH from pituitary cells in vitro without affecting LH release. Similarly, follicular fluid inhibits the surge of FSH normally induced by GnRH infusion to phenobarbitol-blocked proestrus rats (de Paolo, Wise, Anderson, Barraclough & Channing, 1979b).

Conversely Inhibin-like material from spermatozoa and rete testis fluid suppressed FSH release in male rats when injected into the 3rd ventricle suggesting an additional hypothalamic site of action (Lumpkin, Negro-Vilar, Franchimont & McCann, 1981). However
a relatively purified inhibin fraction of bovine follicular fluid
did not change the hypothalamic content or release of GnRH into the
hypophysial portal blood (de Greef, de Jong, de Koning, Steenbergen
& van der Vaart, 1983) suggesting that, in the female at least,
fOLLICULAR fluid has a direct effect on the adenohypophysis
inhibiting the release of FSH.

(d) Effects on follicle growth

If the classical role of FSH stimulating the growth and
development of follicles is accepted, suppression of pituitary FSH
secretion by follicular fluid should theoretically affect follicle
growth.

Studies in monkeys (Channing, Anderson, Hoover, Gagliano &
Hodgen, 1981; di Zerega et al, 1981) and sheep (Miller, Critser,
Rowe & Ginther, 1979a) have observed a marked reduction in the size
of the largest follicle and a decrease in follicle number when
follicular fluid was administered repeatedly during the follicular
phase. These studies suggest that final follicular maturation is
FSH dependent. Similarly, suppression of the secondary FSH surge in
rats (Hoak & Schwartz, 1980) and both the preovulatory and second
FSH surge in hamsters, (Chappel & Selker, 1979) inhibited the growth
of follicles during the next cycle suggesting that plasma FSH levels
at pro-oestrus and oestrus are essential for folliculogenesis and
ovulation in the subsequent cycle at least in species with short
inter ovulatory intervals. In addition follicular fluid treatment
during the early follicular phase in monkeys is associated with low
progesterone levels in the subsequent luteal phase (Stouffer,
Hodgen, Ottobre & Christian, 1984). This suggests that dominant
follicle development was impaired during follicular fluid treatment,
as reflected by low FSH and oestradiol levels, leading to
dysfunction of the subsequent corpus luteum. In contrast, in the
ewe, luteal function is not affected by follicular fluid treatment
in the pre-ovulatory period as assessed by daily progesterone
concentrations (McNeilly, 1984, 1985a). This apparent contradiction
may merely be a reflection of the differences in the length of the
follicular phase and follicle selection processes in the two
species.

Although these studies using follicular fluid all seem to
suggest that normal follicle growth and subsequent luteal function
require adequate exposure to FSH the possibility of additional
inhibitory factor(s) in follicle fluid must be acknowledged.

(e) Other follicular fluid proteins

In addition to inhibin numerous compounds in follicular fluid
have been postulated to have a regulatory effect on follicle growth
(Cahill, 1984). Porcine follicular fluid has been shown to contain
(a) a luteinization inhibitor that prevents LH action on granulosa
cells of porcine follicles (Ledwitz-Rigby et al, 1977) and (b) an
oocyte maturation inhibitor which prevents meiosis within rat
follicles (Tsafari, Pomerantz & Channing, 1976; Tsafari, Channing,
Pomerantz & Linder, 1977). Reports about the effects of oocyte
maturation inhibitor in particular are controversial. Tsafari et
al, (1977) suggested that as porcine follicular fluid inhibited rat
oocyte maturation in vitro the inhibitor was not species specific.
Conversely another study (Fleming, Kahl & Armstrong, 1983) showed
that porcine follicular fluid was unable to inhibit the maturation
of rat oocytes in vitro. Similarly porcine follicular fluid does
not inhibit the maturation in vitro of cow or pig oocytes.
(Leibfried & First, 1980; Racowsky & McCaughey, 1982). The exact nature of the putative inhibitory factors has not yet been established beyond the fact that they are probably low molecular weight peptides and consequently cleared from the circulation before they can exert a significant effect when injected into test animals. More recently the existence of a potent follicle growth inhibitor has been demonstrated to exist in ovine follicular fluid (Cahill, Driancourt & Findlay, 1984b; Cahill, Clark, Cummins & Findlay, 1984a). These authors report that administration of ovine follicular fluid to hypophysectomised ewes whose follicles were either stimulated (chronic) or maintained (acute) by PMSG, caused a cessation of follicular growth, a decrease in mitotic division of granulosa cells and an increase in atresia. Similarly a protein secreted by the dominant follicle in women has been shown to inhibit the ovarian weight increase and oestrogen response in immature hypophysectomised rats challenged with human menopausal gonadotrophin (di Zerega, Goebelsmann & Nakamura, 1982; di Zerega et al, 1983). Although yet to be confirmed it seems that these various proteins may be important as inter or intra-ovarian regulators of follicle growth mediating the dominance of the large follicle(s) by the secretion of these factors which can locally suppress the growth of smaller follicles.
SEASONAL ANOESTRUS

In temperate latitudes most breeds of sheep have a clearly defined breeding and non-breeding season. Marshall (1903) described a period of 'anoestrus' during spring and summer in which the ewes did not display oestrus and their ovaries were devoid of corpora lutea. He later proposed (Marshall, 1937) that decreasing day length is the cue that induced breeding activity and this has subsequently been confirmed experimentally (Yeates, 1949, Hafez, 1952). The endocrine mechanism by which this environmental cue can govern ovarian function has been the subject of much research in the hope that an understanding of the endocrine causes of seasonal anoestrus may lead to more successful manipulation of this normally unproductive period.

(a) Role of LH

Failure to ovulate during seasonal anoestrus is not due to a lack of large follicles available for ovulation as the antral follicle population is generally reported to be similar to or greater than that found during the breeding season (Cole & Miller, 1935; Kammlade et al, 1952; Cahill, Oldham, Cognie, Ravault & Mauleon, 1984c; McNatty et al, 1984c). These potentially 'ovulatory' follicles are capable of producing significant amounts of oestradiol when stimulated by exogenous LH in vivo (McNeilly et al, 1982) or when incubated in vitro and have LH receptors in the theca and granulosa cells (Webb & Gauld, 1985b). They are, therefore, indistinguishable from the preovulatory follicles found in the ovary during the breeding season. The failure of these otherwise active follicles to ovulate is most probably due to an inadequate pattern of pulsatile LH secretion which has been shown to be essential to
stimulate the final phases of follicle development (Baird, 1978b). This hypothesis is supported by the observation that LH pulse frequency is lower during anoestrous than in the breeding season (Scaramuzzi & Martensz, 1975; Yuthasastrakasal, Palmer & Howland, 1977; Walton, Evins, Fitzgerald & Cunningham, 1980). Each LH pulse can elicit a pulse of oestradiol (Scaramuzzi & Baird, 1977) which is similar in magnitude to that observed in response to LH during the breeding season (Baird, 1978b). However the frequency of pulsatile LH secretion is too low to stimulate the sustained oestradiol rise required to evoke the preovulatory surge.

In the ewe, seasonal anoestrus is associated with a marked increase in sensitivity to the negative feedback effects of oestrogen (Legan, Karsch & Foster, 1977). This has been demonstrated in highly seasonal breeds such as the Welsh Mountain and Suffolk and in less seasonal breeds such as the Dorset Horn and Merino (Legan et al, 1977; Webster & Haresign, 1983; Martin et al, 1983) and is thought to be the basis of seasonal breeding in the ewe (Karsch, 1980; Goodman & Karsch, 1980b). As LH pulses are considered to be a good index of GnRH pulses, (Clarke & Cummins, 1982) it is suggested that the reduction in LH pulse frequency observed during anoestrus is due to an interaction between photoperiod and oestrogen feedback at the hypothalamic level (Karsch, 1980; Goodman & Karsch, 1981). As LH pulse amplitude is also higher during anoestrus an increase in responsiveness of the pituitary gland to GnRH in entire ewes is also postulated (Evans & Robinson, 1980). In addition to the increased sensitivity to the negative feedback actions of gonadal steroids there appears to be a steroid independent action of photoperiod on peripheral
gonadotrophin concentrations. LH pulse frequency was low and pulse amplitude high in untreated ovariectomised ewes during anoestrus (Goodman & Karsch, 1981; Goodman, Bittman, Foster & Karsch, 1982). Similarly a direct effect of photoperiod on LH pulse frequency has recently been reported in Ile-de-France ewes ovariectomised in either the breeding season or during seasonal anoestrus. (Montgomery, Martin & Pelletier, 1985). Recent work (Goodman & Meyer, 1984) using pentobarbital treated intact and ovariectomised ewes as experimental models suggested that during seasonal anoestrus LH pulse frequency is kept in check by a set of oestrogen sensitive inhibitory neurons. They further suggest that, as pulse frequency increases in both intact and ovariectomised ewes under pentobarbital anaesthesia during anoestrus that these neurons are activated by an inhibitory photoperiod which can account for both the steroid dependent and independent actions of photoperiod.

(b) Role of prolactin

Progesterone operates synergistically with oestradiol to regulate LH pulse frequency during the breeding season. However during seasonal anoestrus progesterone levels are undetectable due to the absence of the corpus luteum. It is tempting therefore to suggest that another hormone which shows a seasonal pattern of change may mediate the effects of photoperiod on the responsiveness of the hypothalamic-pituitary axis to the negative feedback effects of oestradiol. Prolactin is the primary candidate for such a role as the plasma levels of prolactin in the sheep show a marked seasonal change being highest during anoestrous (Walton, McNeilly, McNeilly & Cunningham, 1977; Thimonier, Ravault & Ortavant, 1978; Jackson & Davis, 1979). However the nature of the physiological
role of prolactin in the sheep is not known. Antagonadotrophic
effects of high prolactin concentrations have been demonstrated in
women, (see McNeilly, 1980) rats, (McNeilly, Sharpe & Fraser, 1983)
and birds, (Camper & Burker, 1977) and it has been suggested that
hyperprolactinaemia is the cause of lactational anoestrus in the ewe
(Kann & Martinet, 1975; Kann, Martiner & Schirar, 1977). However
during the breeding season in the ewe thyroid-releasing
hormone-induced hyperprolactinaemia in the preovulatory phase, was
associated with elevated LH and FSH levels and a reduction in
oestradiol secretion by the ovary suggesting that prolactin was
having a direct effect on the ovary (McNeilly & Baird, 1983). This
induced hyperprolactinanemia however did not affect pulsatile LH
secretion, ovulation or subsequent luteal function. Similarly in
the anoestrous ewe suppression of the high prolactin levels using
the dopamine agonist, bromocryptine had no effect on tonic LH levels
or the proportion of ewes ovulating in response to LHRH or oestrogen
(McNeilly & Land, 1979; Land, Carr, McNeilly & Preece, 1980). In
addition long term treatment with bromocryptine during anoestrus
suppressed ovulation rate after an induced oestrous and failed to
prevent ewes from returning to anoestrus after the induced oestrous
(Rodway et al, 1983).

Ovulation and normal luteal function can be induced by
appropriate gonadotrophin treatments during seasonal anoestrus
despite high prolactin levels in both Finn-Dorset (Rhind, Robinson,
Chesworth & Crofts, 1980a) and Finn-Merino ewes (McNeilly et al,
1982). Studies using Dorset Horn and Welsh Mountain ewes (long and
short breeding season respectively) under natural lighting
conditions showed that although the two breeds came into oestrus at
different times of the year the temporal changes in prolactin were identical (Webster & Haresign, 1983). This suggested that prolactin was not primarily involved in the control of seasonal changes in ovarian function. A similar observation was made (Worthy & Haresign, 1983) when these two breeds were studied under an artificial photoperiod in which half the ewes were switched abruptly from short to long days while the others remained on short days. The onset of seasonal anoestrus occurred in the ewes maintained on short days in the absence of high levels of prolactin. Furthermore there were no differences in the prolactin levels in either group between entire and ovariectomised ewes with an oestradiol implant. Both these studies using a very seasonal and less seasonal breed support the theory that prolactin is not a major determinant of the seasonal changes in responsiveness to negative oestrogen feedback in the ewe. It appears therefore that high prolactin levels during seasonal anoestrus are a coincident effect of the prevailing photoperiod rather than a cause of seasonal anoestrus.

(c) Role of FSH

There are few conclusive reports of seasonal changes in FSH secretion in the ewe. Some authors have reported higher levels during anoestrus coincident with an increase in antral follicle number (McNatty et al., 1984a) while others have found that FSH secretion is lowest during anoestrus (Findlay & Cumming, 1976). In contrast another group have reported little change in FSH secretion between seasons (Walton et al., 1977, 1980). In view of the wave-like pattern of FSH secretion observed during the cycle in the breeding season it is perhaps not surprising that observations on the seasonal changes in FSH secretion are contradictory. In
addition Oussaid (1982) observed significant differences in FSH secretion during seasonal anoestrus in the Ile-de-France ewes with levels being very much lower in mid compared with early anoestrus. If such a shift in FSH secretion within the anoestrus period is a feature of all breeds this would obviously confound breeding and non-breeding season comparisons.

(d) Induction of out-of-season breeding

Attempts to induce out-of-season breeding in the ewe by directly inducing a pre-ovulatory type LH surge using oestradiol (Hammond, 1944) or synthetic GnRH have largely been unsuccessful (Crighton, Foster, Haresign, Haynes & Lamming, 1973, Crighton, Foster, Haresign & Scott, 1975, Haresign, Foster, Haynes, Crighton & Lamming, 1975, Shareha, Ward & Birchall, 1976; McNeilly & Land, 1979). Although ovulation was induced in a proportion of ewes in each of these studies the resulting corpora lutea produced significantly less progesterone than the corpora lutea of the breeding season. Similarly these induced corpora lutea were shown to be smaller, have a reduced progesterone content and a lower ability to secrete progesterone in vitro (McNeilly, Hunter, Land & Fraser, 1981). Pretreatment of ewes with PMSG before a single large bolus injection of GnRH results in ovulation and normal luteal function (Haresign & Lamming, 1978) and has led to the suggestion that the failure of normal luteal function during seasonal anoestrus is probably due to inadequate gonadotrophin priming of the follicle before the preovulatory LH surge (McNeilly, 1980; McNeilly et al, 1981). Indeed as seasonal anoestrus is associated with a reduced frequency of pulsatile LH discharges relative to the breeding season it seems logical that manipulation of this component may induce
successful follicle development and ovulation. Several authors have tested this hypothesis and shown that either pulsatile or continuous infusion of GnRH will induce follicle development and ovulation in seasonally anoestrous ewes (Crighton et al, 1975; McNatty, Ball, Gibb, Hudson & Thurley, 1982b; McLeod, Haresign, Lamming, 1982a,b and 1983; McLeod & Haresign, 1984b). This effect has generally been attributed to an increase in LH secretion and any possible contribution of altered FSH secretion largely ignored even although it is known that GnRH stimulates both FSH and LH release (Pohl & Knobil, 1982). However pulsatile injection of relatively pure preparations of LH alone in an increasing frequency regime over a 72h period during anoestrus will induce ovulation in Finn-Merino (McNeilly et al, 1982) and progesterone primed Romney ewes (McNatty, et al, 1981). These studies support the hypothesis that it is the inadequate LH pulse frequency observed during seasonal anoestrus which normally prevents ovulation and that any alteration in FSH secretion is unimportant in the aetiology of seasonal anoestrus (McNeilly et al, 1982). In contrast however it has been shown that long term GnRH therapy administered once every 2 hours for 40 to 80 days will induce and maintain cyclic ovarian activity in anoestrous Romney ewes (McNatty et al, 1982b) while a similar study using LH alone did not induce and maintain cyclic ovarian activity beyond two consecutive cycles (McNatty et al, 1984b). These authors suggest that FSH supplementation as well as LH might be required for the long term maintainence of cyclic ovarian activity in seasonally anoestrous ewes (McNatty et al, 1984b).
For the experiments detailed in chapters 3, 4, 5 and 10, ovine plasma LH concentrations were measured in duplicate by a radioimmunoassay based on that described by Martensz, Baird, Scaramuzzi & Van Look (1976). Reference standard was NIH-LH-S18 supplied by NIAMDD, Bethesda, U.S.A. and the first antibody R3 1/1 was used at an initial dilution of 1:75,000. The lower limit of detection was 0.4ng/ml (Table 2.1). Intra-assay coefficient of variation (c.o.v.) for duplicates of two quality control plasmas run in 38 assays was 4.3%. Mean inter-assay c.o.v. for the two plasma pools was 11.6%. The assay protocol is shown in Table 2.2.

'New' LH assay

For the experiments detailed in chapters 6, 7 and 8 the assay was modified as described in McNeilly, Jonassen & Fraser (1985). This assay was based on a rabbit antiserum (R29) raised against ovine LH (NIH-OLH-S9) which showed minimal binding (<10% at a final dilution of 1:2000) of $^{125}$I labelled rat or ovine FSH. The reference standard was NIH-LH-S18 and the first antibody R29 was used at an initial dilution of 1:120,000. The lower limit of detection was 0.2ng/ml. There was no plasma interference assessed using plasma from hypophysectomized sheep (N=3) and goats (N=3) supplied by Dr. H. Buttle (The Animal and Grassland Research Institute, Reading, U.K.) and the cross reactions were <0.1% with ovine FSH (181-2, Dr. M. Jutisz; C.N.R.S., Gif-sur-Yvette, France), ovine LH alpha subunit (NIAMDD, oLH Alpha, WRR-1-Alp ha), prolactin...
Table 2.1 Details of protein radioimmunoassays

<table>
<thead>
<tr>
<th></th>
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<th>LH 'NEW'</th>
<th>FSH</th>
<th>PROLACTIN</th>
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<td>- Source</td>
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<td>NIH-LH-S18</td>
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<td>- initial dilution</td>
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<td>Jutisz Ovine LH4</td>
<td>Jutisz Ovine LH4</td>
<td>Jutisz Ovine FSH 181-2</td>
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<td>*Iodination procedure</td>
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<td>Chloramine T</td>
<td>Lactoperoxidase</td>
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<td>- specific activity</td>
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<td>70 - 100</td>
<td>78 - 92</td>
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<td>N</td>
<td>8</td>
<td>5</td>
<td>12</td>
<td>4</td>
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<td><strong>LIMIT OF DETECTION</strong></td>
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<td>4ng/ml</td>
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*Iodination procedures carried out by the author*
Table 2.2. Protein assay protocols

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<th>ASSAY VOLUMES ul</th>
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<td>50</td>
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</tr>
<tr>
<td>Tracer 125I (15,000c.p.m.)</td>
<td>Incubated 1 day at 4°C.</td>
<td>Incubated 1 day at 4°C.</td>
<td>Incubated 2 days at 4°C.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Donkey anti-rabbit serum (DARS)</td>
<td>200 (1:32)</td>
<td>100 (1:16)</td>
<td>100 (1:16)</td>
<td>100 (1:16)</td>
<td></td>
</tr>
<tr>
<td>Non-immune rabbit serum (NIRS)</td>
<td>100 (1:800)</td>
<td>100 (1:200)</td>
<td>100 (1:400)</td>
<td>100 (1:800)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binding % (range)</td>
<td>19</td>
<td>27</td>
<td>19</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Quality control (ng/ml)</td>
<td>13-25</td>
<td>22-35</td>
<td>12-27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- low</td>
<td>0.8-1.3</td>
<td>0.8-1.1</td>
<td>19-24</td>
<td>7.4-11</td>
<td></td>
</tr>
<tr>
<td>- medium</td>
<td>3.1-3.8</td>
<td>2.8-3.6</td>
<td>51-60</td>
<td>36-42</td>
<td></td>
</tr>
<tr>
<td>- high</td>
<td>10.1-12.1</td>
<td>17.0-20.0</td>
<td>328-365</td>
<td>80-104</td>
<td></td>
</tr>
<tr>
<td>Intra-assay c.o.v.</td>
<td>4.3</td>
<td>4.0</td>
<td>3.9</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>Inter-assay c.o.v.</td>
<td>11.6</td>
<td>11.9</td>
<td>9.5</td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>38</td>
<td>20</td>
<td>54</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2.1. Plasma levels of LH measured using R29 and R3 1/1 antisera in samples withdrawn at (a) 15 minute intervals on Day 10 of the luteal phase and (b) 10 minute intervals during the follicular phase of the oestrous cycle. In (a) r=0.969, y=0.791x+0.105. In (b) r=0.934, y=0.662x+1.045. r=correlation coefficient, where y = concentration of LH measured using R29 antiserum and x=concentration of LH measured using R3 1/1 antiserum.
Fig. 2.2. Standard curves for ovine LH-NIH-S18 in radioimmunoassays based on antisera R29 and R31/1. Each point is the mean of duplicate determinations. The vertical lines indicate the concentration range within which most pulses lie.
(NIAMDD-oPRL-15) and bovine TSH (Pierce). Intra-assay c.o.v. for the same two quality control pools used in the original assay was 4% for 20 assays. Mean inter-assay c.o.v. for the two plasma pools was 11.9%. The assay details are shown in Tables 2.1. and 2.2.

Comparison of the two radioimmunoassays for ovine LH

To assess the relative efficiencies of the two radioimmunoassays for ovine LH based on antisera R29 and R3 1/1 measuring pulsatile secretion of LH, plasma samples withdrawn at frequent intervals during different studies were selected at random from 2 control Damline ewes and measured using both assay systems. Fig. 2.1. shows the high correlation (overall, $r=0.952$, $N=64$) between plasma levels of LH measured using the R29 antiserum compared with the R3 1/1 antiserum. There was no difference in the number of LH pulses measured using both antisera. However the R29 system was favoured because of its lower limit of detection together with a more suitable B/BO over the LH concentration range of 1 - 11 ng/ml, within which most pulses lie (Fig. 2.2). The LH radioimmunoassay based on antiserum R 29 has only recently been developed and hence was not used in the original studies described.

LH bioassay.

To determine whether the radioimmunoassays used were measuring biologically active LH a further 14 samples were measured by M. Abbot using a highly sensitive mouse leydig cell bioassay as described by Abbot (1985). The assay utilizes the steroidogenic capacity of the mouse leydig cell to determine biologically active LH levels in unextracted plasma with testosterone production as
Fig. 2.3. Plasma levels of LH measured using an LH bioassay compared with radioimmunoassays using R29 and R3 1/1 antisera. LH bioassay compared with R29 assay system. $r=0.952$, $y=0.847x + 0.462$. LH bioassay compared with R3 1/1 assay system. $r=0.937$, $y=1.044x + 0.533$. 
measured by radioimmunoassay (³H-Testosterone, Corker & Davidson, 1978) as the end point. The mice used were a cross-bred strain DBA-2/C57 BL-6 used at approximately 5 - 8 weeks old. Plasma samples were measured in triplicate at 2 dilutions (100ul of 1:20 and 1:40). Reference standard was NIH-LH-S18 (NIAMDD, Bethesda, U.S.A.) as used in the radioimmunoassays, and the lower limit of detection was 0.024 ng/ml for samples diluted 1:20. Intra- and interassay c.o.v. for quality control pools for a range of intact and ovariectomised ewe plasmas were 7% and 9% respectively (N=5). The LH concentrations measured using the two radioimmunoassays were both highly correlated with the LH bioassay as shown in Fig. 2.3. 

LH pulse definition.

Using the original R3 1/1 LH assay an LH rise was considered to be a pulse if the value of two consecutive samples ("peak" value) was greater than the mean of the two previous samples ("basal" value) and exceeded that mean by more than 3 times the intra-assay coefficient of variation of the assay (based on Backstrom, McNeilly, Leask & Baird, 1982). For the new assay, using the R29 antiserum, 4 times the intra-assay c.o.v. was used, as the lower limit of detection of this assay was considered to be more reliable.

To estimate how many, if any false positives would be identified using the R29 assay system a plasma pool of known LH concentration (0.8 - 1.1 ng/ml, equivalent to the low quality control pool) was treated as 75 samples and measured in duplicate. The resulting LH concentrations were analysed both by hand using the above definition and using a computer program written by
Fig. 2.4. (a) the LH concentration of the low plasma pool when treated as 75 samples in duplicate compared with (b) 75 samples from a control ewe (IJ078) sampled at 10 minute intervals during the follicular phase when LH pulse frequency is high and pulses most difficult to resolve and (c) 75 samples from a control ewe (IJ042) during the same time period where LH concentrations were very low and largely undetectable for most of the follicular phase. Significant pulses are indicated by arrows. Results grouped around time of cloprostenol injection (Pg).
Dr. F.J.P. Ebling, for the Commodore 4032 microcomputer. The latter utilises a moving c.o.v. which was calculated using the duplicates of each successive group of 4 values that was tested for the existence of a significant pulse. No false positives were identified using either method (see Fig. 2.4) using a minimum pulse amplitude of 0.3 ng/ml. LH pulse amplitude was calculated as the difference between the higher of the two peak values and the mean of the two baseline values. Identification of LH pulses was by hand, in all experiments.

Radioimmunoassay of FSH.

Plasma FSH concentrations were measured in duplicate using the assay described by McNeilly, et al, (1976). Reference standard was NIH-FSH-59 (NIAMDD, Bethesda, U.S.A.) and the antibody M94 was used at an initial dilution of 1:2000. The lower limit of detection was 4 ng/ml (Table 2.1.) Intra-assay c.o.v. for duplicates of 3 quality control plasmas run in a total of 54 assays was 3.9%. Mean inter-assay c.o.v. for the 3 plasma pools was 9.5%. The assay protocol is shown in Table 2.2.

Radioimmunoassay of prolactin

Plasma prolactin concentrations were determined in duplicate using the radioimmunoassay developed by McNeilly & Andrews (1974). Reference standard was NIH-OPRL-S13 and the antibody R2532 was used at an initial dilution of 1:200,000. The lower limit of detection was 0.4ng/ml (Table 2.1.). Intra- and inter-assay c.o.v. for 3 quality control pools were 4.1 and 10.7% respectively. The assay protocol is shown in Table 2.2.
Steroid Hormones

Radioimmunoassay of Progesterone

All progesterone concentrations were determined by radioimmunoassay after extraction from the plasma. Samples were extracted and then assayed in duplicate, with an individual recovery measured for each extract. Using glass tubes throughout 20 ul of recovery trace made up in PGBS buffer (phosphate gelatin buffered saline) (^H-Progesterone, 1000-1,200 cpm/20ul) was added to 150ul of sample (75x15mm tubes). Two buffer and two solvent blank tubes were added to check for buffer and/or solvent interference in each assay. At the same time 20ul of recovery trace was added to 4 scintillation vials (total count vials). Tubes were vortexed and allowed to stand on the bench for a minimum of 30 minutes to equilibrate. 2ml of freshly distilled petroleum ether (40° to 60° boiling range) which had previously been passed through an aluminium oxide column (to remove peroxides) was added to all tubes and extracted for 5 minutes on a Multi-tube vortexer (SMI, Model 2601). The tubes were then immersed in a dry ice/ethanol bath to freeze the aqueous layer, whilst allowing the organic phase to be decanted into fresh glass tubes (75 x 12mm). The progesterone containing organic layer was then dried down under nitrogen on a dry-block set at 50°C. Tubes were allowed to cool and the residue reconstituted in 300ul of buffer and vortexed vigorously. The reconstituted extract was left on the bench overnight. To the total count vials 30ul of buffer was added to give a total volume of 50 ul. To determine the individual extraction efficiency 50 ul of the reconstituted sample was then added to 3 mls of scintillation fluid.
Table 2.3 Details of steroid radioimmunoassays

<table>
<thead>
<tr>
<th></th>
<th>PROGESTERONE</th>
<th>TESTOSTERONE</th>
<th>OESTRADIOL-17B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STANDARD</strong></td>
<td>Pregn-4ene-3,20 dione</td>
<td>17B Hydroxyandrost-4en-3one</td>
<td>Oestra-1:3:5(10)-triene-3:-17B-diol</td>
</tr>
<tr>
<td><strong>Source</strong></td>
<td>Sigma Chemical Co. Ltd.</td>
<td>Sigma Chemical Co. Ltd</td>
<td>Sigma Chemical Co. Ltd</td>
</tr>
<tr>
<td><strong>Concentration Range</strong></td>
<td>640 - 5pg / .1ml</td>
<td>640 - 2.5pg/ .1ml</td>
<td>2000 - 3.1 pg/.1ml</td>
</tr>
<tr>
<td><strong>FIRST ANTIBODY</strong></td>
<td>Sheep 361</td>
<td>Sheep 505</td>
<td>Rabbit 48</td>
</tr>
<tr>
<td><strong>Initial Dilution</strong></td>
<td>1:12,000</td>
<td>1:350,000</td>
<td>1:50,000</td>
</tr>
<tr>
<td><strong>MATERIAL FOR IODINATION</strong></td>
<td>Progesterone 11α-gluc-</td>
<td>Testosterone-3-caroxymethyl-</td>
<td>Oestradiol-17B-11B tyrosine methyl ester</td>
</tr>
<tr>
<td></td>
<td>uromide-tyramine conjugate</td>
<td>oxime</td>
<td></td>
</tr>
<tr>
<td><strong>Iodination procedure</strong></td>
<td>Chloramine T</td>
<td>Chloramine T</td>
<td>Chloramine T</td>
</tr>
<tr>
<td><strong>LIMIT OF DETECTION</strong></td>
<td>0.2ng/ml</td>
<td>5pg/tube</td>
<td>5pg/tube</td>
</tr>
</tbody>
</table>
Table 2.4 Steroid assay protocols

<table>
<thead>
<tr>
<th></th>
<th>Assay volumes ul</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Progesterone</td>
</tr>
<tr>
<td>Sample</td>
<td>100</td>
</tr>
<tr>
<td>Buffer (PGBS)</td>
<td>-</td>
</tr>
<tr>
<td>First antibody</td>
<td>100</td>
</tr>
<tr>
<td>Tracer 125I</td>
<td>100</td>
</tr>
<tr>
<td>(15,000cpm)</td>
<td>Incubated 3h at room temp.</td>
</tr>
<tr>
<td>Donkey anti-sheep serum</td>
<td>100(1:20)</td>
</tr>
<tr>
<td>Normal sheep serum</td>
<td>100(1:700)</td>
</tr>
<tr>
<td>Separation</td>
<td>Incubated overnight at 4°C. Add 1ml of 0.9% saline + 0.2% Triton x, spin for 30 minutes at 1000g, tip off supernatant, dry tubes and count precipitate.</td>
</tr>
<tr>
<td>Binding (range)</td>
<td>27</td>
</tr>
<tr>
<td>Quality Control</td>
<td>Plasma Follicular Fluid (pg/ml)</td>
</tr>
<tr>
<td>-low</td>
<td>undetectable</td>
</tr>
<tr>
<td>-medium</td>
<td>2.8-3.5</td>
</tr>
<tr>
<td>-high</td>
<td>8-10</td>
</tr>
<tr>
<td>Intra-assay c.o.v</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>21</td>
</tr>
</tbody>
</table>
(RIA LUMA, LKB Instruments Ltd., Croyden, Surrey) and counted for 5 minutes on a rack-beta counter (LKB Instruments Ltd.). Thus individual recovery values were obtained and the necessary adjustments made to the subsequent progesterone concentration estimate. Extraction efficiency varied considerably both within and between assays. Mean recovery of progesterone (± sem) added to 150 ul plasma was 73 ± 3.2% as measured in 7 consecutive assays (Total N= 1786, range 55-95%). Because of the high variability in recoveries individual estimates of recovery were carried out on every sample. Progesterone concentrations were assayed using the method described previously (Djahanbahkch, Swanston, Corrie & McNeilly, 1981) using 125I-labelled progesterone as tracer and second antibody separation. The reference standard was Pregn-4ene-3,20-dione (Sigma Chemical Co. Ltd., Poole, Dorset) and the first antibody (Sheep 361) was used at an initial dilution of 1:12,000. The lower limit of detection was 0.2 ng/ml (Table 2.3.) Three quality control pools were extracted in duplicate for each assay and subsequently assayed in duplicate. The low quality control was a pool of plasma from ovariectomised ewes and was undetectable. The mean intra-assay c.o.v. for quadruplicates of the medium and high quality control plasmas obtained from early and late luteal phase ewes was 8.7% for 21 assays. Mean inter-assay c.o.v. was 15%. The assay protocol is shown in Table 2.4. Where possible samples from one experiment were assayed within the same assay. Progesterone concentrations in follicle incubation media were assayed using the same assay but no extraction was necessary. Ovine follicular fluid diluted 1:100 and 1:1000 was used as 2 quality control pools. The intra- and inter- assay c.o.v.
Table 2.5 Showing the % cross reactivities of the steroid assays with a range of other steroids

<table>
<thead>
<tr>
<th></th>
<th>Progesterone</th>
<th>Testosterone</th>
<th>Oestradiol-17β</th>
</tr>
</thead>
<tbody>
<tr>
<td>andrenosterone</td>
<td>–</td>
<td>0.001</td>
<td>–</td>
</tr>
<tr>
<td>aldosterone</td>
<td>–</td>
<td>0.005</td>
<td>–</td>
</tr>
<tr>
<td>androstenedione</td>
<td>0.1</td>
<td>1.6</td>
<td>0.001</td>
</tr>
<tr>
<td>11β androstenedione</td>
<td>–</td>
<td>–</td>
<td>0.001</td>
</tr>
<tr>
<td>colesteral</td>
<td>–</td>
<td>0.001</td>
<td>–</td>
</tr>
<tr>
<td>cortisol</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>1.1</td>
<td>0.005</td>
<td>–</td>
</tr>
<tr>
<td>de-oxy cortisol</td>
<td>–</td>
<td>0.001</td>
<td>–</td>
</tr>
<tr>
<td>desoxycorticosterone</td>
<td>25.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>di-hydrotestosterone</td>
<td>–</td>
<td>23.0</td>
<td>–</td>
</tr>
<tr>
<td>17α hydroxyprogesterone</td>
<td>–</td>
<td>0.001</td>
<td>–</td>
</tr>
<tr>
<td>oestradiol-17β</td>
<td>0.001</td>
<td>0.01</td>
<td>100</td>
</tr>
<tr>
<td>oestradiol-17α</td>
<td>–</td>
<td>0.01</td>
<td>–</td>
</tr>
<tr>
<td>oestriol</td>
<td>–</td>
<td>0.001</td>
<td>0.016</td>
</tr>
<tr>
<td>oestrone</td>
<td>–</td>
<td>3.1</td>
<td>0.024</td>
</tr>
<tr>
<td>pregnenolone</td>
<td>0.1</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>progesterone</td>
<td>100</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>5α-OH progesterone</td>
<td>8.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>20α-OH progesterone</td>
<td>12.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11α-OH-progesterone</td>
<td>35.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11-B-OH-progesterone</td>
<td>6.6</td>
<td>–</td>
<td>0.001</td>
</tr>
<tr>
<td>17-α-OH-progesterone</td>
<td>9.6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11-keto-progesterone</td>
<td>15.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>testosterone</td>
<td>0.1</td>
<td>100</td>
<td>0.016</td>
</tr>
<tr>
<td>17α-testosterone</td>
<td>–</td>
<td>0.02</td>
<td>–</td>
</tr>
</tbody>
</table>
for these 2 quality control pools was 9.7 and 15.5% respectively. Cross-reactivities with other physiological steroids are shown in Table 2.5.

Radioimmunoassay of Testosterone

Testosterone concentrations in follicle incubation medium were assayed as described by Sharpe & Bartlett, (1985). Reference standard was 17β Hydroxyandrost-4-en-3-one (Sigma Chemical Co. Ltd) and the first antibody sheep No. 505 was used at initial dilution of 1:350,000 (Table 2.3). The lower limit of detection was 5 pg per tube and the assay protocol is shown in Table 2.4. Intra- and inter-assay c.o.v. for 2 ovine follicular fluid quality control pools was 9.2 and 14.5% respectively. Cross-reactivities with other physiological steroids are shown in Table 2.5.

Radioimmunoassay of Oestradiol-17β

Oestradiol-17β concentrations in follicle incubation medium were measured in a double-antibody radioimmunoassay using 125I-labelled oestradiol-17β-11β tyrosine methyl ester as tracer and an antiserum (R48) raised in rabbits against oestradiol-17β-11α tyrosine methyl ester. Reference standard was oestra-1:3:5(10)-triene-3:17β-diol (Sigma Chemical Co. Ltd.) and the first antibody R48 was used at an initial dilution of 1:50,000 (Table 2.3). The lower limit of detection was 5 pg per tube and the assay protocol is shown in Table 2.4. Intra- and inter-assay c.o.v. for 2 ovine follicular fluid quality control pools was 9.8 and 15.3% respectively. Cross-reactivities with other physiological steroids are shown in Table 2.5.

Calculation of radioimmunoassay results

Two methods of counting and calculation were employed following separation of bound and free tracer. Protein assays were generally
counted in a LKB Wallac nuclear counter (1280 Ultrogamma, LKB Instruments Ltd.). Raw counts were processed by the LKB RIA 02 program, run on a 1222 Databox microprocessor linked to the gamma counter. The program employs log-logit transformation of the binding data for the standards and linear interpolation between the standard points so that the curve is assumed to be made up of straight lines joining successive standard points. The concentration of unknowns is found by determining between which 2 standards the counting response lies and then by interpolation along the line joining the standard points.

Steroid assays were all counted in a NE 1600 gamma counter (Nuclear Enterprises, Edinburgh). Raw counts were processed by the RIA G.T. program (N.E. 1982) run on a Commodore 4320 microcomputer directly linked to the gamma counter. The program employs non linear transformation of the binding data for the standards allowing calculation of unknown samples.

**Bovine Follicular Fluid.**

The bovine follicular fluid used during the course of this study was collected from cow ovaries obtained from the local abbatoir on a daily basis over a period of two years. The follicular fluid was aspirated from follicles (>2mm in diameter) within 4 h of obtaining the ovaries and stored in 20 ml universal containers (Sterilin Ltd., Middlesex) at -20°C until charcoal extraction to remove steroids.
Table 2.6 The percentage of $^3$H-labelled progesterone and oestradiol-17B remaining after charcoal treatment of bovine follicular fluid.

<table>
<thead>
<tr>
<th></th>
<th>Charcoal</th>
<th>Concentration</th>
<th>mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>$^3$H-oestradiol-17B</td>
<td>1.4</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>$^3$H-progesterone</td>
<td>1.6</td>
<td>1.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Charcoal extraction.

Access to an *in-vitro* inhibin bioassay was not available during the course of the initial studies. However using the intact ewe as an *in-vivo* bioassay, follicular fluid from the same source, treated in an identical manner had been shown to be biologically active in that FSH levels were suppressed relative to controls (McNeilly, 1984). Studies by Tsonis et al. (1983) had shown that at least 10-fold more inhibin activity was measured in both ovine and bovine follicular fluid after charcoal treatment of 1 mg/ml compared with 10 mg/ml. As the *in-vitro* inhibin bioassay had not been established in Edinburgh at the start of this study it was aimed to achieve a balance between removal of steroids from the follicular fluid while minimising the possible effects on inhibin potency.

To assess the concentration of charcoal required to remove steroids from the follicular fluid 50,000 cpm of $^3$H labelled progesterone was added to aliquants of follicular fluid which were subsequently treated with 0, 5, 20 and 50 mg/ml of charcoal respectively (see below). Volumes of 0.1, 0.5 and 1.0 ml of treated follicular fluid were added to scintillation vials, scintillant added and counted for 5 minutes on an LKB Rackbeta counter. The counts were expressed as a % of label remaining in the corresponding volumes of untreated follicular fluid. The procedure was repeated using $^3$H labelled oestradiol-17β and the results are summarized in Table 2.6. As there was very little difference between the effectiveness of steroid removal at the various charcoal concentrations, a concentration of 5 mg/ml was used throughout the study in an attempt to minimise the loss of inhibin activity.
Table 2.7 Concentration of steroids (ng/ml) in bovine follicular fluid (BFF) before and after charcoal treatment.

<table>
<thead>
<tr>
<th>BFF Batch number</th>
<th>Total volume treated (L)</th>
<th>Oestradiol-17B (ng/ml)</th>
<th>Progesterone (ng/ml)</th>
<th>Percentage steroid removal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>before charcoal</td>
<td>after charcoal</td>
<td>before charcoal</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>85.4</td>
<td>0.53</td>
<td>157.0</td>
</tr>
<tr>
<td>2</td>
<td>3.8</td>
<td>80.9</td>
<td>0.87</td>
<td>170.6</td>
</tr>
<tr>
<td>3</td>
<td>1.3</td>
<td>96.8</td>
<td>0.94</td>
<td>130.3</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>96.5</td>
<td>1.25</td>
<td>110.2</td>
</tr>
<tr>
<td>5</td>
<td>3.9</td>
<td>123.0</td>
<td>2.13</td>
<td>161.4</td>
</tr>
<tr>
<td>6</td>
<td>2.3</td>
<td>123.5</td>
<td>2.84</td>
<td>208.7</td>
</tr>
</tbody>
</table>
Table 2.8  Concentration of LH and FSH (ng/ml) in bovine follicular fluid (BFF) before and after charcoal treatment.

<table>
<thead>
<tr>
<th>BFF</th>
<th>LH (ng/ml) before</th>
<th>LH (ng/ml) after</th>
<th>FSH (ng/ml) before</th>
<th>FSH (ng/ml) after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.9</td>
<td>0.6</td>
<td>21.3</td>
<td>20.9</td>
</tr>
<tr>
<td>5</td>
<td>0.6</td>
<td>0.7</td>
<td>30.6</td>
<td>28.7</td>
</tr>
<tr>
<td>6</td>
<td>1.2</td>
<td>1.1</td>
<td>25.7</td>
<td>26.8</td>
</tr>
</tbody>
</table>

Table 2.9  Inhibin activity U/ml in batches of bovine follicular fluid treated with 5mg/ml charcoal.

<table>
<thead>
<tr>
<th>BFF</th>
<th>Inhibin potency U/ml</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8,729</td>
<td>6,531 - 11,613</td>
</tr>
<tr>
<td>2</td>
<td>11,002</td>
<td>9,010 - 15,007</td>
</tr>
<tr>
<td>3</td>
<td>5,798</td>
<td>4,308 - 7,708</td>
</tr>
<tr>
<td>4</td>
<td>10,107</td>
<td>8,511 - 13,532</td>
</tr>
<tr>
<td>5</td>
<td>9,830</td>
<td>7,424 - 11,917</td>
</tr>
<tr>
<td>6</td>
<td>9,509</td>
<td>6,985 - 12,429</td>
</tr>
</tbody>
</table>

Index of precision (\( \bar{\lambda} \)) = -0.153
A known volume of follicular fluid sufficient for all injections within an experiment was thawed and mixed in a sterile beaker with washed charcoal added at a concentration of 5 mg/ml follicular fluid. This was stirred at room temperature for 90 minutes followed by centrifugation at 4°C. for 30 minutes at 1500g. The fluid was then decanted and stored at -20°C. in 20 ml volumes. A total of six batches of bovine follicular fluid were used during the course of this study. A 5 ml aliquot was taken before and after charcoal treatment to determine residual steroid levels as shown in Table 2.7. Concentrations of LH and FSH were determined before and after charcoal treatment in batches 4, 5 and 6 (Table 2.8.) and did not differ from those previously reported for bovine serum (Schams et al, 1977).

**Inhibin bioassay**

An aliquot of follicular fluid from each batch was retrospectively assayed to determine inhibin potency by Dr. C.G. Tsonis using the method as described in Tsonis et al (1983). Inhibin activity was measured by an in-vitro pituitary cell culture system using FSH release into medium as the end point. The inhibin activity of the unknowns were expressed relative to the potency of a standard reference preparation of inhibin derived from ovine testicular lymph which has been designated an arbitrary potency of 1 U/mg (Eddie, Baker, Higginson & Hudson, 1979). The standard curve in quadruplicate and charcoal treated (5 mg/ml) unknowns in triplicate were assayed at 5 dilutions (1:250, 1:750, 1:2250, 1:6750 and 1:20250) to generate FSH inhibition curves. The follicular fluid samples had no significant effects on the LH release and had no toxic effects on the cultured cells. Relative potencies
were calculated for those samples showing no significant departure from parallelism to the standard curve and were analysed by regression analysis and parallel line assay statistics (Borth, 1976). The inhibin potencies for the 6 batches of bovine follicular fluid are shown in Table 2.9.

Bovine serum

Bovine serum which is reported as having a similar protein composition to bovine follicular fluid (Caravaglios & Cilotti, 1957) was used for injection as a control fluid in Experiments 2-7. Whole cow blood was obtained on two occasions from the local abbatoir, ringed and allowed to clot overnight. The serum was then decanted and centrifuged at 4°C. for 20 minutes at 1500g. The serum was charcoal extracted exactly as described for follicular fluid. Residual progesterone levels were < 0.2 ng/ml.

Bovine follicular fluid: route of administration

Background

Experiments carried out in this laboratory prior to commencing these studies had used an intravenous route of follicular fluid administration (McNeilly, 1984). This was shown to be effective in suppressing plasma levels of FSH without affecting LH secretion. However a subcutaneous route of administration of ovine follicular fluid was shown to have a greater inhibitory effect on ovulation in intact ewes than intravenous injection of equivalent doses (O'Shea, Cummins, Lutjen & Bindon, 1980). Plasma FSH levels were not measured in this latter study. It was therefore hypothesised that injection of exogenous inhibin in the form of follicular fluid by
Fig. 2.5. Changes in plasma FSH concentrations (expressed as % of time zero) in anoestrous ewes treated with a single 10ml injection (▼) of bovine follicular fluid administered i.v. or s.c. Controls received bovine serum. Values are mean ± s.e.m.
these two routes may result in differential rates and degrees of FSH suppression.

Materials and methods

The effectiveness of bovine follicular fluid at suppressing FSH concentrations when administered by a subcutaneous compared with an intravenous route was determined in mid-seasonal anaestrus (late May). Nine Damline ewes were randomised for weight into 3 groups of 3 and housed indoors in metabolism crates (0.5 x 1m) under natural lighting conditions. Ewes were cannulated (P. 90) on the afternoon before the day of the experiment. The following morning ewes were either (a) injected with 10ml charcoal-treated bovine follicular fluid (Batch 1) via the jugular cannula (i.v. N=3); (b) injected at 2 subcutaneous sites on the neck (2 x 5 ml BFF) or (c) injected subcutaneously (N=2) or intravenously (N=1) with charcoal treated bovine serum to serve as controls. Blood samples were withdrawn at 15 minute intervals for 1 h before and 1 h after injection (time zero=09:30 h), and thereafter at 30 minute intervals for 90 minutes, then hourly intervals until 12.5 h after injection. All plasma samples were assayed in duplicate for FSH as described previously (P. 72).

Results.

Plasma FSH concentrations as measured in the 4 samples withdrawn at 15 minute intervals during the pre-injection hour were not significantly different from each other within all 9 animals and were meaned to give the FSH concentration at time zero (100%). The results following injection were expressed as a % of time zero as shown in Fig.2.5. Plasma FSH levels in the 3 control ewes were variable but did not alter significantly during the sampling period.
Table 2.10 Genetic composition and desirable traits of Damline ewes.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Desirable Traits</th>
<th>Contribution %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finnish Landrace</td>
<td>litter size, early sexual maturity</td>
<td>47</td>
</tr>
<tr>
<td>East Friesland</td>
<td>milk production, body size</td>
<td>24</td>
</tr>
<tr>
<td>Border Leicester</td>
<td>body size</td>
<td>17</td>
</tr>
<tr>
<td>Dorset Horn</td>
<td>conformation and extended breeding season</td>
<td>12</td>
</tr>
</tbody>
</table>
In ewes injected with follicular fluid intravenously mean FSH levels fell between 150 and 210 minutes after injection and plateaued around 7.5 - 8.5 h after injection, FSH levels having been suppressed to approximately 30% of their original level. The fall in FSH levels in response to subcutaneous injection of follicular fluid as determined by the 50% point, appeared to lag behind that of i.v. injection by between 90 - 120 minutes reaching a plateau 8.5 - 9.5 h after injection. However with only 3 animals per group the FSH concentrations of i.v. compared with s.c. injected ewes were not significantly different from each other at any of the time points studied. It was therefore decided to use intravenous administration of follicular fluid in the subsequent experiments.

Experimental animals.

The animals used in Chapters 3 to 7, 9 and 10 were Damline ewes purchased from the flocks of the Animal Breeding Research Organisations (A.B.R.O.) farms at Blythbank and Skedsbush and held at the A.B.R.O. Dryden Field Station, Roslin, Midlothian, U.K. (55°N). The breed was developed by A.B.R.O. to produce a new synthetic line for crossing with hill sheep to produce crossbred ewes for lowland lamb production. The genetic composition of the Damline and the desirable traits of each contributing breed are shown in Table 2.10. The high input of the prolific Finnish Landrace breed results in a mean ovulation rate of between 2.25 and 2.5. The breeding season extends from early September until mid April for adult ewes (based on flock records at Skedsbush farm). The animals used in Chapters 8 and 10 were Welsh Mountain ewes purchased from the flocks at the A.B.R.O. Dryden Field Station,
Roslin. In its natural environment the Welsh Mountain is a hill breed with an ovulation rate of 1 – 1.25. The breeding season extends from late October until early March. During oestrus synchronisation procedures ewes of both breeds were at pasture, their diet being supplemented with hay and turnips during the winter. Housing indoors during the subsequent experiments was largely in individual pens under natural lighting conditions unless otherwise stated. Ewes were offered a complete ruminant diet (A66 sheepnuts, East of Scotland College of Agriculture) twice daily and had ad libitum access to water.

Oestrus synchronisation.

In experiments carried out during the breeding season initial synchrony was achieved by withdrawing synthetic progestagen impregnated intravaginal pessaries (Intervet Lab. Ltd., Cambridge, U.K.) 12 days after their insertion. Generally all ewes displayed behavioural oestrus within 48 h (range 32 – 56 h) as determined by raddled vasectomized Finnish Landrace rams. Luteolysis was then induced on Day 10 of the second cycle via an i.m. injection of a potent analogue of prostaglandin F-2α (100ug cloprostenol; I.C.I., Cheshire, U.K.) Oestrus was detected as before and the ewes moved indoors prior to the start of treatment.

Blood sampling.

Blood samples were withdrawn on a once or twice daily basis using 7 ml heparanized vacutainers (Becton Dickinson U.K. Ltd., Oxford, U.K.). For more frequent sampling, ewes were cannulated on the day before sampling commenced to minimize any possible effects of stress. The cannulae (Braunula, 14G, Dunlop Vet. Supplies,
Dumfries) were placed in the jugular vein, and stitched securely to the neck with sutures through the skin of the neck. The cannulae were connected to 3-way taps (Vygon - VCl, Vygon U.K. Ltd.) by 60 cm polythene tubes (Portex Ltd. Kent) and kept patent with sterile saline (9gNaCl/L) containing heparin (20 i.u./ml; Weddel Pharm. Ltd. London, U.K.). Samples were withdrawn using 5 or 10 ml plastic syringes (Plastipak) and immediately placed in 7 ml glass tubes containing heparin (100 i.u./ml) and spun at 1000g for 20 minutes. The resulting plasma was aspirated into stoppered plastic vials (5ml; Sardstedt) and stored at -20°C until assayed.

Laparoscopy.

Ovulation rate was determined by direct observation of the ovaries using a laparoscope. The number of corpora lutea and corpora albicantia together with an estimate of the number of large follicles (>5mm diameter) were determined between Day 4 and 13 of the oestrous cycle.

Animals were starved of food and water on the evening before the operation. Prior to laparoscopy general anaesthesia was induced with an intravenous injection of sodium intraval on a volume /Kg bodyweight basis (May & Baker Ltd., Dagenham), an endotracheal tube inserted and anaesthesia maintained with an oxygen, fluorathane mixture. The ewe was positioned on a trolley (tilted 20°) on its back and the ventral abdominal area sheared and disinfected (Hibitane, I.C.I.). Two incisions (1cm on left and 2cm on right) were made in the skin either side of the mid-line about 15cm anterior to the mammary glands and within 4 inches of the midline. A large trochar (8mm diameter) and cannula were inserted via the 2cm incision into the abdominal cavity medially and then
Plate 1:
Left ovary of Ewe OJ249 viewed using a laparoscope. Three corpora lutea were observed on Day 7 of the oestrous cycle.
posteriorally towards the pelvic canal. The trochar was withdrawn and a fibre-optic light laparoscope (R. Wolf, U.K. Ltd., Surrey) inserted through the cannula. After determining that the tip of the cannula was clear of subcutaneous or omental fat a small trochar and cannula (3mm) were inserted in the smaller skin incision, the trochar withdrawn and the manipulator introduced through the small cannula. Carbon dioxide was passed through the cannula until the abdomen was firm to pressure and both ovaries viewed and manipulated as necessary (see Plate 1). After examination of the ovaries the instruments were removed and the abdomen deflated. The incisions were treated with antibiotic powder (Optocol, Willingtons) and closed with Michel clips. All ewes were further treated i.m. with 4 ml of antibiotic (Streptopen, Glaxovet Ltd.). Miss Marjorie Fordyce carried out all laparoscopies without knowledge of the animals previous treatment and was assisted by the author.
CHAPTER 3: EXPERIMENT 1

Introduction

There is general agreement in the literature reviewed in the previous section that peripheral FSH levels fluctuate in an asynchronous wave pattern during the luteal phase of the oestrous cycle. Similarly there appears to be a rhythmical periodicity in follicle growth and oestrogen secretion, however changes in FSH secretion have not been shown to directly reflect these patterns. It has been suggested therefore that FSH secretion is sufficient to ensure the development of large antral follicles (4-6mm diameter) throughout the luteal phase and has a permissive rather than an active role at this stage (Baird, 1983). The use of exogenous FSH and gonadotrophin preparations with FSH-like activity (Wright, Bondioli et al, 1981; Betteridge, 1981) to stimulate additional ovulations has led to the suggestion that short term regulation of FSH may be important in the final control of ovulation. Numerous studies have failed to demonstrate consistent differences in the levels of FSH both within and between breeds of sheep with widely different ovulation rates (Findlay & Cumming, 1976; Bindon et al, 1979; Webb & England, 1982a). However periovulatory FSH concentrations (in particular the height and duration of the second FSH surge) have been found to be higher in several prolific breeds (Cahill et al, 1981; Lahlou-Kassi et al, 1984; Bindon et al, 1985). Furthermore it has been suggested that this second FSH surge is involved in the recruitment of small antral follicles for ovulation 17 days later. In animals with short reproductive cycles e.g. rat and hamster, the second FSH surge does appear to be involved in...
follicle recruitment because the use of follicular fluid (Chappel & Selker, 1979; Hoak & Schwartz, 1980) or antiserum to FSH (Sheela Rani & Moudgal, 1977) to suppress the surge results in fewer follicles ovulating at the next oestrus.

Previous studies in this laboratory (McNeilly, 1984) showed that twice daily injections of bovine follicular fluid for 3 days following prostaglandin induced luteolysis caused a significant delay in the onset of oestrus which was associated with a specific suppression of FSH levels while pulsatile LH secretion remained unaffected. Furthermore this suppression of FSH during the preovulatory period did not affect ovulation rate or subsequent luteal function (McNeilly, 1985a).

Experiment 1 was carried out to investigate the role of FSH in follicular development throughout the luteal phase of the oestrous cycle by suppressing plasma levels of FSH with bovine follicular fluid from the time of the expected second FSH surge until prostaglandin induced luteolysis on Day 11 of the cycle.

Materials and Methods

Experimental design

Sixteen Damline ewes were studied during the breeding season in February 1983. The ewes were 2 or 3 years old and weighed 46.4 ± 1.4 kg. They had been exhibiting regular ovarian and oestrous cycles before the start of the experiment as determined by laparoscopy and detection of oestrus records. Oestrus was synchronized as described earlier (P. 90). The 8 ewes in the treatment group received 10ml bovine follicular fluid (i.v.) (Batch 1) at 09:00 and 17:00h on Day 1 (oestrus + 24h) to Day 11 of the cycle inclusive. The 8 control
ewes remained untreated. Luteal regression was induced at 08:00h on Day 12 of the treatment cycle by using cloprostenol. The subsequent onset of oestrus was assessed using a vasectomised ram at 12h intervals (08:00 and 20:00h) between 36 and 120h after cloprostenol injection. Ovulation rate was determined by laparoscopy between days 5 and 10.

**Blood sampling schedule**

Daily blood samples were taken before the 09:00h injection of follicular fluid throughout the luteal phase of the treatment cycle. On Day 8 of the cycle the ewes were cannulated and samples collected every 15 minutes between 10:00 and 16:00h. After luteal regression samples were taken at 4h intervals until 122h after cloprostenol injection or until 36h after oestrus was first detected, whichever was the shortest period. In addition, frequent 15 minute samples were withdrawn between 24 and 30h after cloprostenol injection. During the subsequent cycle blood samples were taken every 2nd or 3rd day until oestrus was detected. All samples were assayed for LH, FSH and prolactin and the daily samples of the treatment and subsequent cycles were assayed for progesterone (see Chapter 2). Plasma LH concentrations were determined using the assay based on the R3 1/1 antiseraum.

**Definitions**

Onset of oestrus was considered to be the time when a ewe first stood to allow a vasectomised ram to mount/mate her minus 6h because of the 12h intervals between heat detection. Similarly, 2h was subtracted from the onset times of the preovulatory LH surge (4h sampling) which was considered to have occurred when LH levels exceeded 15ng/ml.
Table 3.1. Effect of treatment of ewes with bovine follicular fluid on time to onset of oestrus from cloprostenol-induced luteal regression, characteristics of the preovulatory LH surge and ovulation rate.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Treated</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=8</td>
<td>N=8</td>
<td></td>
</tr>
<tr>
<td><strong>Onset of oestrus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time from cloprostenol (h)</td>
<td>41.1 ± 2.3</td>
<td>89.0 ± 8.5</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td><strong>LH surge</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Onset-time from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cloprostenol (h)</td>
<td>55.4 ± 3.7</td>
<td>99.0 ± 7.0</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>(b) Oestrus to surge (h)</td>
<td>14.3 ± 2.4</td>
<td>8.5 ± 1.1</td>
<td>N.S</td>
</tr>
<tr>
<td>(c) Maximum peak height(ng/ml)</td>
<td>90.5 ± 9.1</td>
<td>79.5 ± 19.2</td>
<td>N.S</td>
</tr>
<tr>
<td><strong>Ovulation rate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(range)</td>
<td>2.3 ± 0.3</td>
<td>3.4 ± 0.3</td>
<td>P&lt;0.02</td>
</tr>
<tr>
<td>(1-3)</td>
<td>(2-5)</td>
<td>(2-5)</td>
<td></td>
</tr>
<tr>
<td><strong>No. of large follicles</strong> (&gt;5 mm diam)</td>
<td>1.9 ± 0.5</td>
<td>2.3 ± 0.6</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
Statistics

The Mann Whitney 'U' test was used to test the effect of treatment on ovulation rate. Student's 't' test was used to test the effects of treatment on onset of oestrus and duration of the subsequent oestrous cycle. It was also used to analyse the characteristics of the pulsatile secretion of LH and the preovulatory LH surge. The differences in hormone concentrations between the two groups were analysed by two-way analysis of variance after log transformation of the data to remove skewness. Daily FSH concentrations were analysed using linear contrast analysis to determine when significant changes occurred during the cycle. An F test of significance of regression analysis was applied to changes in FSH during the period of rapid blood sampling in the luteal phase to determine whether the slopes were significantly different from time zero.

Results

Oestrus and ovulation rate

Twice daily injections of bovine follicular fluid throughout the luteal phase of the oestrous cycle resulted in a significant delay (P<0.001) in onset of oestrus compared to that of control animals 89.0 ± 8.5(s.e.m.) N=8, compared with 41.1 ± 2.3h, N=7, in controls (see Table 3.1). Follicular fluid treatment also resulted in a significantly (P<0.02) higher ovulation rate compared to that of controls as assessed by the number of corpora lutea observed at laparoscopy (3.4 ± 0.3, N=8, compared with 2.3 ± 0.3, N=8, in controls). No correlation existed between the delay in onset of oestrus and the subsequent ovulation rate. The number of large follicles (>5mm diam) was not significantly different
Fig. 3.1. Daily changes in the concentrations of FSH and LH during the luteal phase of control ewes and animals treated with bovine follicular fluid. Blood samples were collected daily just prior to the morning injection. Values are mean ± s.e.m.
Fig. 3.2. Changes in the concentrations of FSH in blood samples collected at 15 minute intervals from control ewes and after the i.v. injection of 10 ml bovine follicular fluid on Day 8 of the luteal phase. Values are mean ± s.e.m.
Fig. 3.3. Changes in the concentration of FSH in blood samples collected at 15 minute intervals in a control ewe (○) and a ewe injected with 10ml bovine follicular fluid (●) on Day 8 of the luteal phase.
Table 3.2. Effect of treatment of ewes with bovine follicular fluid on pulsatile secretion of LH on Day 8 during the luteal phase and 24-30h after the injection of cloprostenol on Day 11.

<table>
<thead>
<tr>
<th></th>
<th>Control (N=8)</th>
<th>Treated (N=8)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luteal Phase: Day 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal LH (ng/ml)</td>
<td>1.2 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>LH pulses / 6h</td>
<td>1.8 ± 0.2</td>
<td>2.9 ± 0.4</td>
<td>P &lt; 0.02</td>
</tr>
<tr>
<td>LH pulse amplitude/6h (ng/ml)</td>
<td>1.6 ± 0.4</td>
<td>2.9 ± 0.5</td>
<td>P &lt; 0.02</td>
</tr>
<tr>
<td>Follicular Phase 24-30h after PG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal LH (ng/ml)</td>
<td>1.9 ± 0.2</td>
<td>2.1 ± 0.3</td>
<td>N.S.</td>
</tr>
<tr>
<td>LH pulses / 6h</td>
<td>2.9 ± 1.5</td>
<td>3.6 ± 0.9</td>
<td>N.S.</td>
</tr>
<tr>
<td>LH pulse amplitude (ng/ml)</td>
<td>1.4 ± 0.6</td>
<td>2.7 ± 1.4</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
Fig. 3.4. Short term changes in the concentration of LH in a control ewe (o) and a ewe injected with 10ml bovine follicular fluid (●) on Day 8 of the luteal phase.
between treatments (2.3 ± 0.3 and 1.9 ± 0.5 in controls) and there was no effect of treatment on the subsequent cycle length (17.4 ± 0.7 days and 18.2 ± 1.3 days in controls).

Hormone concentrations during treatment

**FSH.** Over the first 7 days of treatment plasma levels of FSH were significantly lower (P<0.001) in follicular fluid treated ewes than in controls (Fig.3.1a). From Day 8 of the cycle onwards no significant differences in FSH concentrations as determined by daily sampling were evident. In spite of this, injection of bovine follicular fluid at least on Day 8, caused a suppression of FSH levels starting about 2h after injection (Fig.3.2). Linear regression analysis of all individual slopes showed that 7/8 treated ewes had a significantly negative regression with time from injection, contrasting with a significantly positive regression with time in 3/8 control ewes and no change in the remaining 5 control ewes. (see Fig.3.3.)

**LH.** Daily LH concentrations were significantly higher in treated ewes (P<0.05) than in controls throughout the luteal phase (Fig.3.1.b). Detailed analysis of the short term changes in LH on Day 8 of the cycle further indicate that basal LH concentrations, pulse frequency and amplitude were all significantly greater in the treated group (P<0.02) than in the control ewes (Table 3.2, and Fig.3.4.).

**Prolactin.** Plasma levels of prolactin in control and treated ewes throughout the luteal phase were not significantly different (data not shown).

**Progesterone.** There were no significant difference in plasma
Fig. 3.5 Changes in the concentrations of FSH and LH after the injection of cloprostenol (100 ug i.m.) on Day 11 of the cycle in control ewes and ewes injected daily with bovine follicular fluid throughout the luteal phase. The results have been grouped around the time of the cloprostenol injection (PG) or the LH peak. Blood samples were collected at 4h intervals. Values are mean ± s.e.m.
progesterone concentrations between groups. Initially, plasma progesterone values were all $< 0.2$ ng/ml, characteristic of oestrus. Concentrations began to rise on Days 3-4 of the cycle and reached peak values of $5.3 \pm 1.6$ and $5.9 \pm 1.7$ng/ml for the control and treated ewes respectively on Day 12.

**Hormone concentrations after luteal regression**

**FSH.** The changes in plasma levels of FSH after cloprostenol-induced luteal regression are illustrated in Fig. 3.5a. Mean values increased after luteolysis, reaching a peak of $164 \pm 21.8$ ng/ml (range 120-224 ng/ml) 14h after cloprostenol injection. This represents a 4-fold increase over control group levels of $35.3 \pm 5.1$ ng/ml (24-68 ng/ml) which did not alter significantly after luteolysis. FSH levels in treated ewes were significantly greater ($P<0.005$) than in controls during the period +2 to +30h after cloprostenol injection. During the 24h period before the preovulatory LH surge, FSH concentrations were still higher in the treated group compared with controls but this was not significant. Within this 24h period, FSH concentrations declined slightly in the treated ewes before the FSH surge which occurred coincident with the preovulatory LH surge in both groups. There was no significant difference in the magnitude of this surge between the two groups. After the preovulatory surge, FSH levels declined and then began to rise again 12-24h later (second FSH peak) to a similar extent in control and treated animals.

**LH.** The changes in plasma LH concentrations after luteolysis are shown in Fig.3.5b. The follicular fluid treated ewes had significantly higher ($P<0.05$) plasma LH levels in the period 2-30h
after cloprostenol injection. These differences were diminished towards the end of this period as highlighted by detailed analysis of 15 minute samples taken 24-30h after cloprostenol. At this time no differences in basal LH concentrations or pulse frequency were found although pulse amplitude was significantly greater (P<0.05) in the treated ewes (Table 3.2.). There was no significant difference in basal LH concentrations measured in 4-hourly samples collected in the 24h before or after the preovulatory surge or in the magnitude or duration of the preovulatory LH surge between control and treatment groups (Table 3.1.).

The onset of the preovulatory LH surge was significantly later relative to cloprostenol injection in the treatment than control group (Table 3.1.). However, the interval from oestrus to the LH surge did not differ.

Subsequent oestrous cycle

There was no significant effect of treatment on plasma concentrations of FSH, LH or prolactin during the subsequent oestrous cycle. Plasma progesterone levels were characteristic of a normal cycle in ewes of both groups, no differences being found in basal or mid-luteal values in spite of the observed differences in ovulation rate between groups.

Discussion

Injections of bovine follicular fluid to ewes in the luteal phase resulted in a significant decrease in plasma FSH concentrations. During the second half of the treatment period FSH levels were no longer suppressed as measured by a daily blood sample
collected immediately before the injection of follicular fluid at 09:00 h. A similar observation was made by Miller, Critser & Ginther (1982) during an 8-day period of treatment with bovine follicular fluid and they concluded that this was due to a decrease in endogenous inhibin production by the ovary. The bovine follicular fluid used in the present study was, however, still effectively suppressing FSH secretion on Day 8 of treatment. FSH levels already appear to be decreasing at the start of the frequent sampling period 1h after administration of follicular fluid and continue to fall throughout the 6h bleed. It seems probable that because of the uneven time interval between consecutive follicular fluid injections the effects of the 17:00h injection may be diminished and FSH levels returned to normal by the following morning. This suggests that sampling at 09:00h probably gave an inaccurate picture of the degree of FSH suppression achieved by the treatment.

A gradual onset of refractoriness to the FSH-suppressing effects of follicular fluid has been observed following repeated administration of porcine follicular fluid to monkeys (Channing, Tanabe, Turner & Hodgen, 1982) and rats (Thomas & Nikitovitch-Winer, 1984). This was attributed to foreign proteins in the follicular fluid inducing an immunological response capable of inhibiting the FSH suppressing activity of the fluid. Whilst the possible immunogenic properties of bovine follicular fluid cannot be ignored in the sheep it is doubtful because of the short duration of treatment and its continuing effectiveness, that this is the cause of the apparently higher FSH concentrations in the second part of the treatment cycle in this study.
After the cessation of follicular fluid treatment at the induced luteolysis, FSH levels showed a significant rebound to reach levels 3-4 fold higher than those of controls. The magnitude of this rebound appears to be correlated with both the length of treatment and the degree of FSH inhibition during treatment (Miller et al., 1982; McNeilly, 1985a). This rebound may reflect a release of an accumulation of FSH stores or substances involved in its synthesis in the pituitary which is released once the effects of follicular fluid have ceased.

The hypersecretion of FSH observed in this and other studies (Hermans et al., 1981; de Paolo et al., 1979a) may equally be the result of low endogenous inhibin and steroid(s) production due to insufficient FSH stimulation of follicular growth during the period of treatment with follicular fluid. Then, when the treatment ends, the pituitary releases its stores of FSH and follicular development progresses. Once the growing follicles secrete sufficient inhibin and steroid(s) into the circulation the rebound ceases.

Reports of the effects of follicular fluid on LH secretion in vivo and in vitro are inconsistent (Charlesworth, et al., 1983). Enhanced plasma LH concentrations after follicular fluid treatment have been observed in the ovariectomized mare (Miller et al., 1979) and mouse (Bronson & Channing, 1979) coincident with the FSH suppression, and in intact rats after the depression of FSH had ceased (Hermans, et al. 1981). Conversely, a decrease in LH secretion has been reported when high doses of follicular fluid were given to ovariectomized ewes (Cummins et al., 1983; Findlay et al.,

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1985). However, the majority of studies report no detectable effect on LH secretion (Campbell & Schwartz, 1979; de Paolo et al., 1979a; Thomas & Nikitovitch-Winer, 1984) although the sampling frequency in all of these studies was inadequate to define possible difference in pulsatile LH frequency or amplitude. McNeilly (1985a) has shown that injection of bovine follicular fluid to ewes in the preovulatory phase of the cycle did not affect the normal increase in pulsatile secretion. However, normal pulsatile LH secretion is near maximum at this time and the 15 minute sampling frequency used may have been inadequate to detect subtle changes in secretion. In contrast, in the present study, follicular fluid injections in the luteal phase were associated with an increase in pulsatile LH secretion at a time when endogenous pulsatile secretion is normally at a minimum. Since increased pulsatile LH secretion in intact ewes has been observed after immunization against androstenedione and oestradiol-17β (Martensz et al., 1976; Martensz & Scaramuzzi, 1979) and is thought to arise because of a lack of negative feedback by oestradiol on LH secretion, it seems likely in the present study that the low FSH levels produced by the follicular fluid treatment resulted in less oestradiol being secreted from the follicles, with a consequent loss of negative feedback by oestradiol and hence an increase in pulsatile LH release. Conversely it is possible that some other as yet unknown factor in follicular fluid may be stimulating pulsatile LH secretion. The residual steroids in the follicular fluid would be expected to decrease rather than promote LH secretion.
The injection of bovine follicular fluid throughout the luteal phase resulted in a significant delay in the onset of oestrus. Since behavioural oestrus is an oestrogen dependent event (Robinson, 1959) the delay is probably a result of insufficient oestradiol secretion by the developing follicle population immediately after luteal regression. Studies on monkeys (Channing et al, 1981) and sheep (Miller et al, 1979a) indicate that suppression of FSH by follicular fluid resulted in significant follicular inhibition. The delay in oestrus in the follicular fluid treated animals of the present experiment suggests that at luteal regression follicles which are destined to ovulate are less well developed than those of control ewes, presumably as a result of the reduction in plasma levels of FSH throughout the luteal phase. Consequently, these follicles take longer to respond to the increase in LH/FSH to become preovulatory. Conversely the delay in oestrus may be the result of another component of bovine follicular fluid acting directly on the ovary. It has recently been shown that administration of ovine follicular fluid to hypophysectomized ewes whose follicles were maintained with PMSG resulted in a cessation of follicle growth (Cahill et al, 1984a,b, and see discussion Chapter 6). However this is not supported by the observation that infusion of FSH coincident with bovine follicular fluid administration during the preovulatory per iod counteracts the delay in oestrus observed when follicular fluid is given alone (McNeilly, 1985a).

Treatment with bovine follicular fluid did not alter the magnitude and duration of the preovulatory LH or coincident FSH surges and their timing relative to behavioural oestrus. This
supports the concept that the events resulting in the onset of oestrus were simply delayed due to the lack of follicular development rather than being altered in any way.

It has been suggested that ovulation rate in the sheep is related to the amount of FSH released prior to and during the preovulatory (Lahlou-Kassi et al., 1984; Bindon et al., 1985) and second surge (Cahill et al., 1981; Lahlou-Kassi et al., 1984) of FSH. However, the present study was initiated to suppress the second FSH surge and was associated with an increase in ovulation rate. Thus while the second FSH surge may be responsible for replenishing the stock of antral follicles after the wave of atresia following the LH surge it seems highly unlikely that it is responsible for the ovulation rate observed 15-16 days later.

The most probable reason for the observed increase in ovulation rate in this experiment is the hypersecretion of FSH during the follicular phase following cessation of follicular fluid treatment at luteolysis, since administration of exogenous FSH (Wright, Bondioli, et al. 1981; Baird et al., 1984) or of preparations with FSH-like activity, such as PMSG, after luteal regression (Robinson, 1951; Gordon, 1958; Cherardi & Lindsay, 1980) have been shown to increase ovulation rates. The possible contribution of altered LH secretion relative to observed changes in ovulation rate in this study cannot be ignored. The frequent sampling period during the follicular phase did not reveal any major differences in pulsatile LH secretion between the two groups but the samples were withdrawn at a totally different time relative to the onset of oestrus. Since
LH pulse frequency increases and amplitude decreases during the follicular phase until the onset of oestrus (Baird, 1978b), the sampling window in this study cannot accurately be used for comparative purposes. LH levels were significantly higher in the follicular fluid treated ewes in the 24h following cloprostenol injection, but as samples were taken at 4 hourly intervals it is not possible to determine whether this is a reflection of high pulse frequency, pulse amplitude or both. However previous studies also failed to detect any differences in pulsatile LH secretion during the preovulatory phase in Merino ewes with nutritionally induced differences in ovulation rate (Scaramuzzi & Radford, 1983) and in Booroola compared with control Merino ewes with genetically determined differences in ovulation rate (Scaramuzzi & Radford, 1983; Bindon et al., 1985). Furthermore, administration of exogenous pulses of GnRH in the preovulatory phase to increase LH secretion did not affect ovulation rate but also failed to affect LH secretion (McLeod & Haresign, 1984).

In contrast, the increase in LH pulse frequency and amplitude measured in the luteal phase of this study is consistent with that observed following immunization of ewes against both androgens and oestrogens (Martensz et al., 1976: Martensz & Scaramuzzi, 1979). The increase in ovulation rate observed after immunization occurs in spite of variable or low FSH concentrations. Whether the follicular population in the present study is sufficiently developed to benefit from such an increase in pulsatile LH is unknown.

In summary twice daily injections of bovine follicular fluid
throughout the luteal phase of the oestrous cycle resulted in a considerable suppression of plasma levels of FSH and enhanced pulsatile secretion of LH. Following prostaglandin induced luteolysis FSH levels increased four fold compared with controls while LH levels were marginally higher. The onset of oestrus behaviour was delayed and the subsequent ovulation rate significantly higher. Whether this increase in ovulation rate was entirely related to the altered pattern of FSH secretion, the increase in LH secretion or a combination of the two remains to be clarified.
The success of the sheep industry in many parts of the world is hampered by a low reproductive rate. In Scotland the average estimated level of fecundity over the last decade was 1.09 lambs/breeding animal/year (Economic Report on Scottish Agriculture, 1983) and is well below the uterine capacity of 5 lambs per pregnancy (Wilson, 1968). The number of eggs shed by the ovaries sets the upper limit to production and there is no doubt that a low ovulation rate is one of the major factors currently limiting lambing percentages throughout the world. Experiment 1 showed that twice daily injections of bovine follicular fluid resulted in a moderate but significant increase in ovulation rate at the following oestrus. The production of relatively pure inhibin preparations is now imminent (de Jong et al, 1984; Robertson et al, 1985) and therefore the possible practical significance of such a treatment is important. As with all other available non-genetic methods of increasing ovulation rate such as immunization against steroids (Van Look, Clarke, Davidson & Scaramuzzi, 1978; Martín, Scaramuzzi, Cox & Gheradi, 1979), administration of PMSG (Robinson, 1951; Gheradi & Lindsay, 1980), short term (Davis et al, 1981) and long term (Gunn, Doney & Russel, 1969) nutritional treatments, it is vital to determine whether an increase in ovulation rate is reflected by increased lambing percentages.

Experiment 2 had three main objectives:

(a) to determine the length of treatment with follicular fluid required to produce an increase in ovulation rate.

(b) to determine whether the increase in ovulation rate was
FIG. 4.1

EXPERIMENTAL DESIGN: LUTEAL PHASE

PG

BFF OR SERUM INJECTIONS AT 09:00 & 17:00h

GROUP

FF10

FF6

FF2

CONTROL

1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11

DAY OF CYCLE
reflected by a greater number of embryos surviving at Day 30-34 of gestation. (c) to assess whether follicular fluid treatment resulted in any visible embryo abnormalities.

Materials and Methods

Experimental design

Forty Damline ewes were studied during the breeding season in October - December, 1983. The ewes were between 1½ and 2½ years of age and had never been used previously for experimental purposes. Following oestrus synchronisation the ewes were moved indoors to pens of 3 - 4 animals each. Ewes were weighed, ranked and allocated to one of 4 treatments so that weights were equivalent between treatments before the start of the study. Ewes were weighed again at mating and prior to slaughter. The experimental design is summarized in Fig. 4.1. Ewes in Group FF10 (N=10) received 9ml bovine follicular fluid i.v. at 09:00 and 17:00h on Days 1 - 10 inclusive of the treatment cycle. Ewes in Group FF6 (N=10) received 9ml charcoal-extracted bovine serum (acting as a control fluid) for the first 4 days of the cycle followed by 9ml bovine follicular fluid twice daily for the next 6 days of the cycle. Ewes in Group FF2 (N=10) received bovine serum for 8 days followed by follicular fluid twice daily for 2 days. Ewes in Group C (N=10) received bovine serum at 09:00 and 17:00h for the entire 10 days and acted as controls. Follicular fluid Batch 2 was used throughout. Luteal regression was induced in all ewes at 10:00h on Day 11 of the treatment cycle using cloprostenol. The onset of behavioural oestrus was assessed using a ram at 12h intervals (08:00 and 20:00h) between 22 and 142h after cloprostenol injection. All ewes were hand mated with a fertile ram at the first recorded onset of
oestrous behaviour and again 12h later, different rams being used on both occasions. Ovulation rate was assessed by laparoscopy between Days 5 and 10 of the cycle. Two raddled vasectomised rams were run with the ewes for 24 days after mating and those ewes that did not again demonstrate oestrous behaviour were considered to be pregnant.

Blood samples were taken twice daily throughout the treatment before follicular fluid or serum injection and at +6, +24, +36, +48 and +60h after cloprostenol-induced luteolysis until oestrus was detected. Thereafter samples were withdrawn on every 2nd or 3rd day until Day 20 or 21. All samples were assayed for LH, FSH and progesterone as described previously.

Slaughter procedures

All pregnant ewes were slaughtered between Days 31 and 34 of gestation and the intact gravid uterus and ovaries were removed immediately and placed on ice. The ovaries were dissected to determine the number and weight of the corpora lutea present. The gravid uterus was opened by a dorsal mid-line incision, taking care not to rupture the placental tissue. The foetal cotyledons were separated from the cotyledonary burrs on the uterine epithelium by gentle traction. The position, number and macroscopic appearance of the foetuses were recorded for each dissected uterus. After ligation of the umbilical cords, the foetuses were detached from their placentae, measured and weighed. Due to the small size of the foetuses at this early stage of gestation it was only feasible to measure the straight crown-rump and head lengths.

Progesterone content of corpora lutea

The progesterone content per mg of wet tissue was determined in all corpora lutea by D. Stirling. The tissue was homogenised in
Table 4.1. Effect of treatment with bovine follicular fluid on onset of oestrus, ovulation, conception and pregnancy rates in ewes

<table>
<thead>
<tr>
<th>Group</th>
<th>Total no. of ewes showing oestrus</th>
<th>Mean ± s.e.m. oestrus (h)</th>
<th>Mean ± s.e.m. ovulation rate (range)</th>
<th>Mean ± s.e.m. ovulation rate (range)</th>
<th>Mean ± s.e.m. ovulation rate (range)</th>
<th>Mean ± s.e.m. ovulation rate (range)</th>
<th>Conceiving (non-return rate)</th>
<th>Pregnant at slaughter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>9</td>
<td>62.8 ± 9.9</td>
<td>2.6 ± 0.2(^a) (N=9)</td>
<td>3.1 ± 0.2(^e) (N=8)</td>
<td>3.1 ± 0.2(^e) (N=8)</td>
<td>3.1 ± 0.2(^e) (N=8)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Group FF2</td>
<td>10</td>
<td>86.7 ± 10.8</td>
<td>3.4 ± 0.2(^b) (N=10)</td>
<td>3.5 ± 0.2(^f) (N=8)</td>
<td>3.5 ± 0.2(^f) (N=8)</td>
<td>3.5 ± 0.2(^f) (N=8)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Group FF6</td>
<td>10</td>
<td>78.4 ± 8.7</td>
<td>3.4 ± 0.3(^c) (N=10)</td>
<td>3.6 ± 0.5(^g) (N=9)</td>
<td>3.6 ± 0.5(^g) (N=9)</td>
<td>3.6 ± 0.5(^g) (N=9)</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Group FF10</td>
<td>9</td>
<td>66.7 ± 7.1</td>
<td>4.7 ± 0.2(^d) (N=9)</td>
<td>5.0 ± 0.3(^h) (N=9)</td>
<td>5.0 ± 0.3(^h) (N=9)</td>
<td>5.0 ± 0.3(^h) (N=9)</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

ab, ac, cd, gh, P<0.05; bd, fh, P<0.01; ad, eh, P<0.001.
100 mg/ml of ice cold 0.3 M sucrose/1 mM EDTA/10 mM Tris-HCl, pH 7.4 with 8-10 complete strokes of a loose fitting Dounce homogenizer. Any visible surface membranes were removed and the homogenate stored at -20°C until extracted and assayed for progesterone as described previously for plasma samples.

Statistics

The effect of treatment on onset of oestrus, ovulation rate and embryo survival was determined using analysis of variance followed by Duncan's New Multiple Range Test where appropriate. The differences in hormone concentrations between the groups were also analysed by analysis of variance.

The correlation coefficients and regression lines were calculated for several parameters using linear regression analysis.

Results

Reproductive performance

Onset of oestrus

Oestrus behaviour was observed in 38 of the 40 ewes originally allocated to the experiment. The remaining 2 ewes were subsequently found to be acyclic and excluded from the experiment. Although the onset of oestrus was slightly earlier in Group C ewes relative to those in Groups FF2, FF6 and FF10, the differences were not significant (Table 4.1).

Conception rate

Whether or not the ewes had conceived was initially determined from the non-return rate, i.e., those ewes that did not return to oestrus as determined from markings by a raddled vasectomised ram during the 12-24 days after mating. Two ewes (one in Group C and one in Group FF6) returned to oestrus within this period. At
Fig. 4.2 (a) Joint frequency distribution of total numbers of corpora lutea and foetuses at slaughter in ewes treated with bovine serum (○) for 10 days or bovine follicular fluid for 2 (■), 6(▲) or 10 (●) days prior to cloprostenol-induced luteolysis.

(b) Distribution of ovulation rate at slaughter for the 4 groups.
slaughter a further 2 ewes both from Group FF2 were not pregnant but were still cycling. It seemed unlikely from examination of the uteri that either of them had originally conceived. Conception rate between groups was not significantly different, giving an overall conception rate of 89% for ewes mated.

**Ovulation rate**

The design of the experiment allowed a comparison to be made between the number of corpora lutea observed at laparoscopy with that found on dissection of the ovaries at slaughter (Table 4.1). A total of 8 'additional' corpora lutea were found at slaughter. They were all without exception very small relative to the other corpora lutea on the ovary and tended to be buried between them. These 'additional' corpora lutea were randomly spread throughout the four groups but did alter the mean ovulation rate and significance levels as shown in Table 4.1. At laparoscopy the ewes in Groups FF2, FF6 and FF10 had a significantly higher mean ovulation rate than did those in Group C ($P<0.05, P<0.05, P<0.001$ respectively). However, of the ewes pregnant at slaughter, only the mean ovulation rate of ewes in Group FF10 was significantly different from that of controls ($P<0.001$). In addition, the ewes in Group FF10 had a significantly higher mean ovulation rate than did those in Group FF6 and Group FF2 ($P<0.05$ and $P<0.01$ respectively). This highlights a degree of error (6%) in using the laparoscope as the sole experimental tool when measuring ovulation rate, particularly when multiple corpora lutea may be involved. The distribution of the corpora lutea at slaughter within the 4 groups of ewes is shown in Fig. 4.2.b. The majority of ewes ovulated from both ovaries but 6 ewes (16%) failed to ovulate from the right and 6 from the left.
Fig. 4.3 Relationship between ovulation rate and the mean corpus luteum weight per ewe. A significantly negative correlation, $r = -0.658$, $P < 0.001$, $y = 0.470 \times + 0.470$ was calculated irrespective of treatment group.
Fig. 4.4 Range of corpus luteum weights within individual ewes irrespective of treatment group. Each symbol represents an individual ewe within each ovulation rate group. The 8 corpora lutea below the horizontal line were the additional corpora lutea found at slaughter.
Table 4.2. Effect of treatment with bovine follicular fluid on the number of viable foetuses embryo survival and projected lambing percentages in Damline ewes.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of ewes</th>
<th>Total no. of CL at slaughter</th>
<th>Total no. of foetuses</th>
<th>Mean ± s.e.m. no. of foetuses (range)</th>
<th>Projected lambing %</th>
<th>% embryonic survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>8</td>
<td>25</td>
<td>17</td>
<td>2.12 ± 0.22 (1-3)</td>
<td>212</td>
<td>68a</td>
</tr>
<tr>
<td>Group FF2</td>
<td>8</td>
<td>28</td>
<td>17</td>
<td>2.12 ± 0.35 (1-3)</td>
<td>212</td>
<td>61b</td>
</tr>
<tr>
<td>Group FF6</td>
<td>9</td>
<td>32</td>
<td>21</td>
<td>2.33 ± 0.33 (1-4)</td>
<td>233</td>
<td>66c</td>
</tr>
<tr>
<td>Group FF10</td>
<td>9</td>
<td>45</td>
<td>26</td>
<td>2.89 ± 0.39 (1-4)</td>
<td>289</td>
<td>58d</td>
</tr>
</tbody>
</table>

ab, ac, ad, N.S.

Table 4.3. The relationship between ovulation rate and the proportion of viable foetuses at slaughter, irrespective of treatment group

<table>
<thead>
<tr>
<th>Ovulation rate</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of ewes</td>
<td>2</td>
<td>14</td>
<td>11</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Viable foetuses at slaughter (%)</td>
<td>75.0</td>
<td>81.0</td>
<td>56.8</td>
<td>50.00</td>
<td>66.6</td>
<td>36.0</td>
</tr>
</tbody>
</table>
ovary; these ewes occurred randomly in the groups.

Irrespective of treatment group; as the ovulation rate increased the mean corpus luteum weight per ewe decreased as shown in Fig.4.3. The 8 'additional' buried corpora lutea had a mean weight of 0.101g (range 0.035-0.135g) which is well below the lowest mean corpus luteum weight per ewe in all 4 treatment groups.

Within ewes the variability in corpus luteum weight was significantly lower (overall mean, 8.5%) than that of the population examined as a whole (34%) and was not related to treatment or ovulation rate (Fig 4.4).

There was no significant differences in the progesterone content per mg of corpus luteum tissue in corpora lutea from control ewes compared with any of the follicular fluid treated groups. The mean progesterone content was 1.2 µg/mg ± 0.2 (s.e.m.) in control ewes compared with 1.5 µg/mg ± 0.2 in Group FF10 ewes.

**Embryo survival**

The number of viable foetuses at slaughter varied from 1 to 4 with an overall mean (+ s.e.m.) of 2.4 ± 0.2. The distribution between treatment groups is shown in Fig. 4.2a. The increase in the number of ova shed after follicular fluid treatment was not proportionally reflected by the number of embryos surviving at Day 31 -34 of pregnancy as is shown in Table 4.2. However without estimating for foetal losses after Day 34 the projected lambing percentages show that an overall increase of 21 and 77 lambs born per 100 ewes in Groups FF6 and FF10, respectively could be expected on the basis of this study. Although the ewes in Group FF10 had a higher number of embryos surviving when compared with controls, the percentage embryonic survival in this group was 10% less than that
Plate 2:

Conceptus of Ewe 1J095 (Group FF6) on Day 34 of gestation. All 4 ova shed were represented by a viable foetus.

Plate 3:

Conceptus of Ewe 1J375 (Group FF10) on Day 34 of gestation. A total of 5 ova were shed only one of which was viable at Day 34 of gestation. At least a further 2 ova had been fertilized but subsequently died. Although difficult to resolve because of the twisted membranes, both were visible as being at different stages of resorption by Day 34.
of controls. The values for ewes in Groups FF6 and FF2 were intermediate between the two.

Only 32% of the ewes had all their corpora lutea represented by viable foetuses at slaughter. This was not influenced by follicular fluid treatment in that ewes with maximum (100%) embryonic survival were found in all 4 groups. However it was affected by ovulation rate as 91% of these ewes had an ovulation rate of 3 or 4.

Plate 2 shows the conceptus of a Group FF6 ewe in which all 4 ova shed are represented by a viable foetus at slaughter on Day 34 of gestation. Irrespective of treatment no ewe with an ovulation rate of >4 had a full complement of embryos at slaughter. The optimum ovulation rate in the present study was 3, above which embryonic survival decreased markedly (Table 4.3).

Intra-uterine embryo migration had occurred in at least 9 ewes and was not related to treatment group.

**Ovum wastage and embryo loss**

Overall ovum wastage was 36.7% of the total number of corpora lutea and, as suggested above, was related to ovulation rate, being highest in ewes ovulating 4 or more eggs. In many cases the fate of the lost eggs is unknown but in several instances eggs had been successfully fertilised and death of the foetus occurred prior to implantation. In these cases, the degenerating embryos still enclosed in their amniotic sacs were observed at varying stages of resorption (Plate 3). The incidence of detectable embryonic death was too rare however to test for any correlation with ovulation rate or follicular fluid treatment. A total of 3 ewes had visible signs of embryo resorption (1 in Group FF6, 1 in Group FF10 and 1 control).
Table 4.4. Distribution of gestational stage at slaughter

<table>
<thead>
<tr>
<th>Group</th>
<th>31</th>
<th>32</th>
<th>33</th>
<th>34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Group FF2</td>
<td>-</td>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Group FF6</td>
<td>-</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Group FF10</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 4.5 Positive correlation between the individual foetus weights and the day of gestation on which they were slaughtered.

\[ r = 0.843, \, P < 0.001, \, y = 0.254 \times + 0.565. \]
Fig. 4.6 Changes in the plasma concentrations of FSH in blood samples withdrawn before injection of bovine follicular fluid (▼) at 09:00h (●) and 17:00h (○) for (a) 0 (group C), (b) 2 (Group FF2), (c) 6 (Group FF6), or (d) 10 (Group FF10) days during the luteal phase of the cycle. After cloprostenol-induced luteolysis samples were withdrawn at 6, 24, 36, 48 and 60h. Values are mean ± s.e.m.
Foetal weight and size

The ewes were slaughtered over a 4 day period because of the spread in original mating dates and the number of ewes involved. Consequently the embryos were recovered between Days 31-34 of gestation, the distribution of which relative to treatment group is shown in Table 4.4. In view of the fact that (a) the embryos are rapidly growing at this time and (b) there was an uneven distribution of the 4 groups killed on each of the 4 days, statistical comparison of embryo weight and size between treatment groups and/or litter sizes was not undertaken. Fig. 4.5 emphasises the positive correlation between the stage of gestation and embryo weight irrespective of litter size.

Maternal weight changes

Ewes were randomised for weight at the start of the experiment and initially weighed 42.6 ± 3.8Kg (mean ± s.e.m.). There were no significant differences in weight changes between treatment groups during the study. All ewes gained weight with a mean increase of 5.7 ± 0.3Kg while indoors for the duration of follicular fluid treatment and mating procedures. In contrast a very marginal increase of 1.7 ± 0.2Kg was measured during the first 30 days of gestation. There was no correlation between ovulation rate and weight at mating or between embryo survival and weight at either mating or slaughter.

Hormonal data

Plasma concentrations of FSH and LH

The changes in plasma FSH values throughout the treatment cycle are illustrated in Fig. 4.6. The first two samples taken on Day 1 from ewes in Groups C, FF2 and FF6 reflect the high plasma FSH concentrations normally seen during the period of the second FSH
surge. The first follicular fluid injection (09:00h) in Group FF10 ewes suppressed this high concentration in the other 3 groups by over 50% by the 17:00h sample, i.e. 8h after injection.

In the ewes receiving bovine serum (Groups C, FF2 and FF6), plasma FSH levels were low during Days 2 and 3 and began to rise on Day 4 of the luteal phase, reaching a plateau during Day 6 in Groups C and FF2 only. This rise was abolished by the start of follicular fluid administration in Group FF6. A significant depression in plasma concentrations of FSH occurred after the first follicular fluid injection on Days 5 and 9 in Groups FF6 and FF2 respectively. In all ewes in Groups FF2, FF6 and FF10, irrespective of stage of cycle, plasma FSH levels were lower at the 17:00h sample (8h from injection) than at the 09:00h sample (16h from last injection). It appears that following the 17:00h sample FSH levels are suppressed for an unknown length of time overnight, and then rebound before the 09:00h injection the following morning.

After cloprostenol induced luteolysis on Day 11 of the cycle, plasma levels of FSH did not alter significantly in control ewes. In contrast, mean FSH levels in all ewes in Groups FF2, FF6 and FF10 increased after luteolysis, reaching a peak 6 - 24h after cloprostenol injection, 41h after the final follicular fluid injection.

The individual FSH concentrations at 6 and 24h after cloprostenol injection were combined and the average increase in plasma concentrations of FSH were found to be significantly correlated (r=0.772; P<0.001) with the length of follicular fluid treatment in a dose response manner. The magnitude of this rebound in FSH concentrations was not significantly correlated with
Fig. 4.7 Changes in plasma concentrations of LH on Days 1–11 of the cycle before injection of bovine serum (○) or bovine follicular fluid (●) at 09:00h and 17:00h. Values are mean ± s.e.m. The profiles of ewes in Groups FF2 and FF6 were not significantly different from those of Group C ewes and are excluded for clarity.
Fig. 4.8 Changes in plasma concentrations of progesterone on Days 1-11 of the cycle prior to injection of bovine serum or bovine follicular fluid for 2, 6 or 10 days during the luteal phase. The range is shown by the dotted lines.
ovulation rate.

Basal concentrations of LH in twice daily samples were significantly higher (P<0.001) in Group FF10 ewes than in those in Group C throughout the luteal phase of the cycle from Day 2 onwards (Fig 4.7). In contrast, levels of LH in ewes in Groups FF2 and FF6 were not significantly different from those of Group C ewes irrespective of stage of cycle or treatment. In addition, there were no significant differences in the plasma level of LH measured at 09:00h compared with 17:00h in any of the control or treated ewes.

There were no significant effect of treatment on plasma concentrations of FSH or LH during the subsequent oestrous cycle or early pregnancy.

Plasma concentrations of progesterone

There were no significant differences in plasma progesterone concentrations between groups during the treatment cycle as measured in twice daily samples (Fig. 4.8). Initially on Days 1 and 2 plasma progesterone levels were < 0.2 ng/ml, characteristic of oestrus. Levels began to rise on Days 3-4 of the cycle reaching a plateau by Day 7. Thereafter, the mean progesterone concentrations in ewes in Group FF2 was slightly lower than that of controls, although this difference was not significant.

The ewes that returned to oestrus 18 days after mating had progesterone concentrations typical of those measured during a normal oestrous cycle. Similarly, the progesterone levels of 2 ewes found not to be pregnant at slaughter returned to basal levels (< 0.2ng/ml) characteristic of oestrus, between Days 17 and 19, although they did not show behavioural oestrus as detected by a
Fig. 4.9 Changes in plasma concentrations of progesterone during the first 20 days of gestation analysed according to (a) treatment group (b) ovulation rate and (c) number of viable embryos irrespective of treatment group. Values are mean ± s.e.m.
vasectomised ram.

As samples were withdrawn every 2nd or 3rd day during the first 20 days of gestation, the data were analysed as the mean progesterone concentration per 3-day block, behavioural oestrus representing Day 0. Analysis of the progesterone concentrations of the 34 pregnant ewes showed that there were no significant differences between treatment groups during the first 20 days of gestation (Fig 4.9a). Plasma progesterone levels began to rise on Days 3–5 of pregnancy, reaching a plateau by Days 9–11. When the individual progesterone profiles were grouped and analysed according to ovulation rate (irrespective of previous treatment), the mean (+ s.e.m.) progesterone concentration on Days 9–20 of gestation was higher in ewes with 4 or more corpora lutea (4.9 ± 0.2ng/ml; N=18) compared with those having 2 or 3 corpora lutea (4.1 ± ng/ml; N=16), as shown in Fig. 4.9b. However this difference failed to reach significance. Similarly, there were no significant differences in the mean progesterone concentrations when analysed according to the number of embryos surviving at slaughter (Fig. 4.9c).

Discussion

The results of Experiment 2 show that administration of bovine follicular fluid during the luteal phase of the cycle for various lengths of time (2, 6 or 10 days) before the induction of luteolysis with cloprostenol increased ovulation rate. This confirms and extends the previous observations made in Experiment 1 using an 11-day treatment regime. A wide range in ovarian response was observed within treatment groups, and the increase in ovulation rate was most consistent in Group FF10. However contrary to the previous observations following administration of bovine follicular fluid in
both the luteal phase (see Chapter 3) and follicular phase (Miller et al., 1979; McNeilly 1984, 1985a) of the cycle the onset of behavioural oestrus was not significantly delayed in the present study. The interval between cloprostenol-induced luteal regression and the onset of oestrus in the control group was 18-24h later than that previously observed for Damline ewes (see P. 97). It is possible that other environmental factors may have masked any differences between groups in that the ewes were studied in randomised groups early in the breeding season in the continued presence of rams. In contrast in Experiment 1 the ewes were housed in individual pens at the end of the breeding season and removed to an adjacent room for oestrus-detection when necessary. One or more of these variables may be responsible for the differences in the time of onset of behavioural oestrus in the two studies. Furthermore the potency estimate of the follicular fluid (Batch 2) used in this study was similar to Batch 1 used in the initial study in which significant delays in onset of oestrus relative to controls were recorded. Thus differences in follicular fluid potency do not appear to be the cause of the discrepancy.

The conception rate in the present study was high and was not affected by follicular fluid treatment or ovulation rate. This is in agreement with Experiment 1 which showed that, although the onset of oestrus and the preovulatory LH surge had been delayed after treatment with follicular fluid in the previous luteal phase, the interval from oestrus to the preovulatory LH surge was not altered.

As fertilisation is normally an 'all or nothing' event in the sheep (Restall, Brown, Blockey, Cahill & Kearins, 1976) and
conception rate was high, it seems unlikely that partial failure of fertilisation was responsible for any of the embryo loss observed in the study. The majority of embryo loss occurs during the first month of pregnancy in the ewe (Robinson, 1951; Quinlivan, Martin, Taylor and Cairney, 1966), therefore by slaughtering ewes at Day 30-34 of gestation a reasonably accurate projection of litter size at term is assumed. Estimates of normal embryo loss during this early stage of pregnancy range from 20 to 30% (Edey, 1976). Thus, the overall embryo loss irrespective of treatment, in the present study (37%) was higher than expected. However, the study was completed early in the breeding season using 1½ - 2½ year old ewes and both factors are known to contribute substantially to embryonic loss (Hulet, Voigtlander, Pope & Casida, 1956; Cumming, Blockey, Winfield, Parr & Williams, 1975; Restall et al, 1976). The possible effects of age are confounded, however, by the low body weight of young ewes, as used in this study, because body weight at mating was 70% of that normally recorded for cycling adult Damline ewes from the same flock. Although bodyweight at mating is known to influence both the number of ovulations and embryo survival (Morley et al, 1978) a direct correlation was not evident in this relatively small study. However, this is not surprising as the Finnish-Landrace cross used in this experiment show less variation in ovulation rate as body condition and weight changes (MLC, 1981).

While the factors discussed above are no doubt important in influencing embryo survival in prolific or superovulated ewes it is believed that in the present study the higher ovulation rate per se was the major cause of the embryo loss observed, particularly in the ewes treated with bovine follicular fluid. Previous studies using
the prolific Finnish Landrace X Dorset Horn sheep treated with PMSG (Rhind, Robinson, Fraser & McHattie, 1980b) have shown that with each increase in ovulation rate above 5, there was a reduction in the % of ova represented by viable foetuses at the time of slaughter. Similarly, data from embryo transfer experiments and information on litter size as a function of the number of corpora lutea at mating, show a negative linear relationship between the number of embryos entering the uterus and the probability of survival of the individual embryo (Hanrahan, 1980a,b).

The optimum ovulation rate in the present study was found to be 3 ova. It is perhaps not surprising therefore that in the ewes treated with follicular fluid for 10 days (and to a lesser extent in those treated for 2 and 6 days) mean embryonic survival decreased as the ovulation rate exceeded this optimum. Consequently, the higher ovulation rate was not proportionately reflected by an increase in viable embryos, although the mean number of embryos in the 10 and 6 day treatment group was higher.

The possible reasons for the relatively low optimum ovulation rate are not clear. As ovulation extends over a longer period of time in multiple ovulators (Whyman, Johnson, Knight & Moore, 1979) there will theoretically be a wider range of developmental ages between the embryos and hence a greater chance of embryo loss because of asynchrony between a ewe and one or more of her embryos (see review, Wilmut, Sales & Ashworth, 1985). This range in time of ovulation is presumably a reflection of follicles at different developmental stages and size groups being recruited to ovulate. However in the present study (with the exception of the small 'additional' corpora lutea) there was very little variation in the
size of corpora lutea within ewes indirectly suggesting that the follicles recruited to ovulate were from a similar size group (see Chapter 6). Furthermore the functional capacity of these corpora lutea in vitro was not related to follicular fluid treatment but to ovulation rate (D. Stirling, personal communication). This suggests that, the follicles recruited to ovulate after follicular fluid treatment were fully competent at ovulation and supports the theory that follicular fluid treatment per se was not the cause of the relatively high embryo mortality observed in this study. In addition, the small incidence of detectable embryonic death was not attributed to follicular fluid treatment as it occurred in only 3 ewes, of which 1 was a control. The existence of the 8 'additional' corpora lutea at slaughter remains an enigma. On the basis of the in vitro studies it seems highly unlikely that these corpora lutea would have been capable of supporting a pregnancy. Furthermore it is difficult to resolve how these follicles could have ovulated when buried within the ovary.

**Hormonal data:** As a result of follicular fluid treatment, this study provided an opportunity to study luteal phase plasma progesterone levels in early pregnancy in ewes with a range of ovulation rates. Previous studies (Quirke et al, 1979) have shown that progesterone concentrations were significantly higher in prolific Finnish Landrace ewes than in less prolific breeds during the luteal phase of the cycle (Days 10-13) and that each successive increment in ovulation rate was accompanied by a disproportionately small increase in progesterone level. In the present study there was no relationship between follicular fluid treatment and progesterone concentrations in the subsequent cycle. Furthermore,
although the mean progesterone levels (irrespective of treatment) during the luteal phase of early pregnancy were higher for ewes with 4 or more corpora lutea the differences were not significant. Similarly plasma progesterone concentrations in Booroola Merino ewes do not increase with increasing ovulation rates (Bindon, Cummins, Piper & O'Shea, 1981) and may partly be due to reduced weights of individual corpora lutea. Moreover there were no abnormalities in the progesterone profiles of ewes suffering varying degrees of embryo loss (20-86%) when compared to ewes suffering no loss suggesting that inadequate progesterone production was not related to embryo loss in the present study.

The results give further support to the suggestion made in Chapter 3, that the hypersecretion or rebound of FSH during the follicular phase after termination of follicular fluid treatment at luteolysis allows more follicles to mature and ovulate. There was no direct correlation between the magnitude of the rebound and ovulation rate on an individual basis irrespective of treatment but this is probably a reflection of the inadequate sampling frequency and comparatively small size of the study. The magnitude, and possibly duration, of this rebound was positively correlated with the length of follicular fluid treatment and is in agreement with the work of Miller et al, (1982). Thus it seems possible that ewes in the 10 day treatment group had more FSH accumulated in their pituitary stores due to the longer period of low FSH release produced by the follicular fluid injections. The early morning elevations in FSH levels as detected by the 09:00h sample (Day 2 onwards) is thought to reflect the start of release from the pituitary stores which is always terminated by the subsequent injection. It is only at luteolysis and the cessation of
follicular fluid treatment that the full rebound and release of FSH from the pituitary gonadotrophs proceeds. A similar overnight rebound in FSH levels was observed following follicular fluid treatment in the follicular phase of the cycle (McNeilly, 1985a). The rebound was inversely related to the dose of follicular fluid injected and resulted in an intermittent pattern of FSH suppression and release. Despite this, ovulation rate and subsequent luteal function were not affected by follicular fluid treatment. Active immunization of sheep with inhibin partly purified from bovine follicular fluid (Henderson et al., 1984) also resulted in an increase in ovulation rate. However immunization had no significant effect on plasma FSH or LH concentrations as assayed in thrice weekly samples and ovulation rate was increased at laparoscopy in the first cycle following immunization (approximately 7 days later). In view of this rapid response and the initial lack of detectable antibodies the authors acknowledged that the inhibin preparation itself may have had a direct action on the pituitary.

The possible contribution of higher LH levels to the additional increase in ovulation rate observed in the 10 day treatment group is uncertain as discussed in Chapter 3. However as LH concentrations in the 2 and 6 day treatment groups were not different during treatment relative to those in controls, the higher LH values in the ewes treated for 10 days may simply be a reflection of the more prolonged FSH suppression resulting in low oestradiol production by the follicles, and consequently a lack of negative feedback from the ovary on pulsatile LH release as suggested previously.

It has been shown that within the scope of this small trial that the increase in ovulation rate observed after treatment of
Damline ewes with follicular fluid in the luteal phase of the cycle can be expected to yield increased lambing percentages. However the use of follicular fluid treatments in less prolific breeds may potentially be more commercially important (See Chapter 8).
Chapter 5: EXPERIMENT 3

Introduction

It is largely agreed in the literature reviewed in Chapter 1 that follicular fluid specifically suppresses FSH secretion in a variety of species with little or no effect on LH secretion. Studies in the ovariectomised ewe have shown that bovine follicular fluid can only marginally suppress LH at very high doses of follicular fluid (72,000 units of inhibin; Cummins et al, 1983; Findlay et al, 1985). Similarly, McNeilly (1984, 1985a) has shown that the injection of bovine follicular fluid to ewes in the preovulatory phase of the cycle did not affect the normal increase in pulsatile LH secretion which is approaching maximum at this time. However the results of Experiment 1 showed that when follicular fluid is administered during the luteal phase at a time when LH pulse frequency is normally low, there is a significant increase in both LH pulse frequency and amplitude, measured during 6h on Day 8. In addition the twice daily plasma LH concentrations in Experiment 2 were higher in ewes treated with follicular fluid for 10 days than in ewes treated for 6 or 2 days prior to cloprostenol induced luteolysis, as well as in controls.

Using the same experimental protocol as described in Chapter 3 the present study was undertaken to confirm and extend these observations by measuring pulsatile LH secretion for 24h after the initial follicular fluid injections on Day 1 and again for 25h on Days 6 and 10 of treatment.

Similarly, blood samples taken once or twice daily in Experiments 1 and 2 indicated that plasma FSH concentrations were not completely suppressed in all animals between successive
follicular fluid injections. Furthermore it was observed in these and other studies (Miller et al, 1982) that the effectiveness of follicular fluid at suppressing FSH levels was diminished towards the end of treatment. One of the objectives of this study was to precisely determine the time course and degree of FSH suppression achieved by the 10 day regimen of follicular fluid injections on Days 1, 6 and 10 of the luteal phase treatment. In addition a 2 day treatment group was included to determine the pattern of gonadotrophin secretion produced by short term follicular fluid administration.

Materials and Methods

Experimental animals

Fifteen Damline ewes were studied during the breeding season in February 1984. The ewes were 3 or 4 years old and weighed 62.5 + 2.0Kg at the start of the experiment. Oestrous behaviour was synchronised as described previously and the ewes moved inside into individual pens before the start of treatment on Day 1 (oestrus + 24h).

Experimental design

Ewes were weighed, ranked and allocated to one of three treatments prior to the start of the study. Ewes in Group FF10 (N=5) received 10ml of bovine follicular fluid i.v. (Batch 3) at 09:00 and 17:00h on Days 1-10 of the cycle inclusive. Ewes in Group FF2 (N=5) received 10ml bovine serum twice daily for the first 8 days of the cycle followed by 10ml follicular fluid twice daily for 2 days. Ewes in Group C (N=5) received 10ml of bovine serum at 09:00h and 17:00h for the entire 10 days and acted as controls.
Luteal regression was induced in all ewes at 09:00h on Day 11 of the treatment cycle using cloprostenol. After cloprostenol injection the ewes were moved into a large pen with 2 vasectomised rams in an adjacent pen. In an attempt to resolve the anomalies in oestrus onset times recorded in Experiments 1 and 2, the onset of behavioural oestrus was assessed at 4h intervals between 23 and 107h after cloprostenol injection by unobtrusively observing each ewe with a ram for a minimum of 5 minutes through a glass partition. Ovulation rate was assessed by laparoscopy between Days 7 and 10 of the cycle.

Blood sampling

Ewes in Group C and FF10 were cannulated on Day 0. Blood samples (3ml) were withdrawn at 15 minute intervals from 08:00h on Day 1 to 09:00h on Day 2 and again from 08:00h on Day 6 to 09:00h on Day 7. Similarly samples were taken at 15 minute intervals from all 3 groups from 08:00h on Day 10 to 09:00h on Day 11 (=time of cloprostenol injection). In addition blood samples were taken twice daily before follicular fluid or bovine serum injection throughout the luteal phase. After cloprostenol-induced luteolysis daily blood samples were taken until Day 13 of the subsequent cycle. All samples were assayed for LH, FSH and prolactin. Daily samples taken during the treatment and subsequent cycle were assayed for progesterone. The LH assay used was based on the R31/1 antiserum.

Statistics

The effect of treatment on onset of oestrus and ovulation rate was determined using analysis of variance. Similarly the differences in hormone concentrations between the groups were
Table 5.1. Effect of treatment with bovine follicular fluid on onset of oestrus, ovulation rate and the number of large follicles in Damline ewes.

<table>
<thead>
<tr>
<th></th>
<th>Time to onset of oestrus (h)</th>
<th>Ovulation rate (range)</th>
<th>Number of large follicles (&gt;5mm diam) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C (N=4)</td>
<td>39.0 ± 5.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.25 ± 0.25</td>
<td>1.25 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>(2-3)</td>
<td>(0-3)</td>
<td></td>
</tr>
<tr>
<td>Group FF2 (N=5)</td>
<td>57.4 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.0 ± 0.31</td>
<td>1.0 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>(2-4)</td>
<td>(0-3)</td>
<td></td>
</tr>
<tr>
<td>Group FF10 (N=5)</td>
<td>56.6 ± 2.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.0 ± 0.44</td>
<td>1.4 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>(2-4)</td>
<td>(1-3)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

ab, ac, P<0.05
Fig. 5.1 Changes in the plasma concentrations of FSH in blood samples withdrawn before injection of bovine follicular fluid (▼) at 09:00h (●) and 17:00h (○) for 0 (Group C); 2 (Group FF2) or 10 (Group FF10) days during the luteal phase of the cycle. Values are mean ± s.e.m.
analysed by analysis of variance followed by Duncan's New Multiple Range Test where appropriate. Within treatment group effects were determined using analysis of variance with repeated measures.

Results

Oestrus and ovulation rate

Twice daily injections of bovine follicular fluid for 10 or 2 days of the luteal phase prior to cloprostenol-induced luteolysis resulted in a significant delay (P<0.05) in oestrus onset compared to that of controls (Table 5.1). There was no significant difference between Groups FF2 and FF10. One control ewe did not display behavioural oestrus within the allotted time and was subsequently found to have had a preovulatory LH surge on Day 10 of the treatment cycle and hence excluded from further analysis. The mean ovulation rate after follicular fluid treatment was higher than that of controls but failed to reach significance (Table 5.1). Two ewes in Group FF10 had a large preovulatory follicle and 2 ewes in Group FF2 had a 'corpus luteum like' structure which were not classified as being 'true' corpora lutea because no ovulation point was visible. There was no significant difference in the number of large follicles (>5mm diameter) in any of the 3 groups, and no correlation between the delay in oestrus onset and ovulation rate.

Hormone concentrations during treatment

FSH: The changes in plasma levels of FSH measured twice daily throughout the treatment cycle are illustrated in Fig. 5.1. and are similar to those described in Experiment 2. In ewes receiving bovine serum (Group C and FF2) FSH levels were initially high reflecting the concentrations normally seen during the period of the second FSH surge. Plasma FSH levels then fell to lowest levels
Fig. 5.2. Mean changes in the plasma concentrations of FSH in blood samples collected at 15 minute intervals for 25h starting 1h before the 09:00h injection (jured bovine serum or bovine follicular fluid on Days 1, 6 and 10 in Group C (○) and Group FF10 (●), and on Day 10 of the luteal phase in Group FF2 (□).
during Days 2 and 3 and began to rise on Day 4 reaching a peak on Days 5 and 6. Thereafter FSH levels fell slightly and appeared to be increasing again on Day 10 in Group C ewes only. This further rise was abolished by the administration of follicular fluid to Group FF2 on Day 9. The pattern of FSH concentrations in Group FF10 ewes throughout the luteal phase was also similar to that observed in Experiment 2 with levels being very low at the 17:00h sample (8h from injection) and higher or similar to that of controls at the 09:00h sample (16h from last injection). The exact nature and time course of this apparent suppression then increase in FSH concentrations was clarified by the intensive sampling periods.

The changes in plasma levels of FSH measured in samples withdrawn at 15 minute intervals for 25h starting at 08:00h on Days 1, 6 and 10 of treatment are illustrated in Fig. 5.2. On Day 1 of the luteal phase, FSH concentrations in Group FF10 ewes were rising at the start of the sampling period between 08:00 and 09:00h prior to follicular fluid injection. This probably reflects the ascending limb of the secondary FSH surge which is subsequently prevented by administration of follicular fluid. The decline in FSH concentrations began 2-3h after the first follicular fluid injection, reaching 26% of pretreatment levels by the next injection at 17:00h that afternoon. The half-life of the decrease in FSH response to the first follicular fluid injection was calculated from the first time levels decreased from the plateau concentrations (approximately 2 hours after follicular fluid injection) to the point where basal levels were first recorded. The resulting mean half-life of the decrease in FSH after the first follicular fluid injection was 130 minutes (range 105–165; N=5). After the 17:00h
injection plasma levels of FSH continued to decrease to reach a minimum of 13% of pretreatment values by approximately 22:00h (i.e. 13h from initial injection and 5h from the second follicular fluid injection). In 4/5 Group FF10 ewes the FSH concentrations were at or below the sensitivity of the radioimmunoassay in many of the samples withdrawn from 22:00h onwards, hence a minimum FSH level of 4ng/ml was allocated to these samples. Plasma FSH concentrations remained totally suppressed for a further 8h until between 06:00 and 07:00h the following day (13-14h after last follicular fluid injection) when FSH levels began to rise to reach similar levels to those of control ewes by 09:00h on Day 2. Overall during the 24h period following the first follicular fluid injection FSH concentrations were significantly suppressed relative to controls for 18h from 14:00 - 07:00h (P<0.02 or P<0.01 at all hourly sample points, see Fig. 5.2a) The rise in plasma FSH levels following almost total suppression continued until the next follicular fluid injection became effective as indicated by the mean FSH profiles on Days 6 and 10 (Fig.5.2. b and c). On both days the plasma FSH concentrations increased from 08:00h until approximately 11:00h and then began to decline in response to the 09:00h injection. The degree of suppression achieved by follicular fluid injection on both Day 6 and 10 was marginally less than that following the initial follicular fluid injections on Day 1. Nevertheless FSH concentrations were significantly lower than those of controls for 15h on Day 6 (P<0.05 - P<0.001 at all hourly sample points) and 16h on Day 10 (P<0.05 - P<0.001). On both of these days plasma FSH levels began to rise around 06:00h (13h from last injection) as observed on Day 1 of treatment.
The pattern of FSH release and suppression for Group FF2 in response to the 3rd and 4th injections of follicular fluid on Day 10 was not significantly different from that of Group FF10. FSH concentrations were significantly suppressed relative to controls for 17/25h (P < 0.02 - P < 0.001) between 15:00 and 07:00h.

On Day 1 of the luteal phase plasma FSH concentrations in control ewes were initially high and similar to those of Group FF10 ewes at 08:00 – 12:00h. FSH concentrations remained constant during most of the sampling period and began to decrease towards the end of the sampling period on Day 2, probably reflecting the descending limb of the secondary FSH surge. On Days 6 and 10 of the luteal phase plasma FSH concentrations were marginally lower than on Day 1 and did not fluctuate throughout the 25h sampling periods. There was no evidence of pulsatile FSH secretion in either control or follicular fluid treated ewes at any of the time points studied.

At 24h after cloprostenol-induced luteal regression on Day 11 of the cycle plasma FSH levels were significantly correlated with the length of follicular fluid treatment (r=0.682; P < 0.001, N=14) but not with the subsequent ovulation rate.

LH: Plasma concentrations of LH measured in twice daily samples were significantly higher (P < 0.05) in Group FF10 ewes than in those in Groups C and FF2 throughout the luteal phase of the cycle from Day 2 onwards (data not shown). The levels of LH in groups C and FF2 were not significantly different from each other. Furthermore there were no significant differences in the plasma levels of LH measured at 09:00h compared with 17:00h in any of the control or treated ewes.

Detailed analysis of the pulsatile LH secretion of samples
Table 5.2. Effect of treatment of ewes with bovine follicular fluid on the pulsatile secretion of LH on Days 1, 6 and 10 of the luteal phase.

<table>
<thead>
<tr>
<th>Day</th>
<th>Group C N=4</th>
<th>Group FF10 N=5</th>
<th>Group FF2 N=5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pulses/25h</td>
<td>Pulse amplitude/25h (ng/ml)</td>
<td>Mean level/25h (ng/ml)</td>
</tr>
<tr>
<td>Day 1</td>
<td>16.3 ± 2.8a</td>
<td>1.3 ± 0.1c</td>
<td>1.8 ± 0.4e</td>
</tr>
<tr>
<td></td>
<td>19.4 ± 0.6b</td>
<td>1.5 ± 0.1d</td>
<td>2.0 ± 0.2f</td>
</tr>
<tr>
<td>Day 6</td>
<td>13.3 ± 2.99</td>
<td>1.4 ± 0.1l</td>
<td>1.5 ± 0.2k</td>
</tr>
<tr>
<td></td>
<td>17.8 ± 1.8h</td>
<td>2.5 ± 0.2j</td>
<td>2.6 ± 0.4l</td>
</tr>
<tr>
<td>Day 10</td>
<td>11.0 ± 1.1m</td>
<td>1.5 ± 0.1p</td>
<td>1.3 ± 0.2s</td>
</tr>
<tr>
<td></td>
<td>18.0 ± 2.5n</td>
<td>3.3 ± 0.2q</td>
<td>2.9 ± 0.5t</td>
</tr>
<tr>
<td></td>
<td>9.4 ± 0.9o</td>
<td>1.8 ± 0.2r</td>
<td>1.3 ± 0.3u</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

ab, cd, ef, gh, mo, pr, su  N.S.
ij, kl, mn, st, no, qr, tu,  P<0.05
pq, dq,  P<0.01
CONTROL

LUTEAL PHASE DAY 1 = OESTRUS + 24h

Fig. 5.3. Changes in the pulsatile secretion of LH in a control ewe in blood samples collected at 15 minute intervals for 25h, from 1h before the 09:00h injection of bovine serum on Day 1 (oestrus + 24h), 6 and 10 of the luteal phase.
Fig. 5.4. Changes in the pulsatile secretion of LH in a ewe treated with follicular fluid in blood samples collected at 15 minute intervals for 25h, from 1h before the 09:00h injection of bovine follicular fluid on Day 1 (oestrus + 24h), 6 and 10 of the luteal phase.
Fig. 5.5. Changes in the pulsatile secretion of LH in a control ewe and a ewe treated with follicular fluid in blood samples collected at 15 minute intervals for 25h on Day 10 of the luteal phase.
collected on Days 1, 6 and 10 of the luteal phase are summarised in Table 5.2. The data obtained on Day 1 of the cycle were difficult to quantify in that the initial samples in profiles for 3 of the ewes (1 in Group C and 2 in Group FF10) encompassed the end of the preovulatory LH surge (see Fig. 5.4). Nevertheless LH pulse frequency was high and pulse amplitude low in control ewes on Day 1 of the luteal phase when progesterone levels were undetectable. By Day 6 pulse frequency was decreasing in control ewes, reaching a minimum by Day 10 of the luteal phase. Conversely LH pulse amplitude showed a slight but non-significant increase during the cycle in control ewes. LH pulse frequency in Group FF10 did not change significantly during the luteal phase, remaining high throughout Days 1, 6 and 10. Mean LH pulse frequency was higher but just failed to reach significance compared with controls on Day 6 but was significantly higher ($P < 0.05$) than controls by Day 10 of the cycle. Mean LH pulse amplitude in Group FF10 ewes increased between Days 1 and 6 (not significant) and again between Days 6 and 10 ($P < 0.01$). Similarly, pulse amplitude in Group FF10 was significantly higher than control ewes on Day 6 ($P < 0.05$) and Day 10 ($P < 0.01$) of the cycle. Figures 5.3 and 5.4 emphasise the changes in pattern of LH secretion observed throughout the luteal phase in an individual control and follicular fluid treated ewe respectively. Similarly Fig. 5.5 emphasises the dramatic differences in the pattern of pulsatile secretion of LH on Day 10 of the luteal phase when data from control and treated ewe are plotted on the same scale. In contrast LH pulse frequency and amplitude in Group FF2 ewes were not significantly different from that of controls on Day 10 of the cycle. Within each individual 25h sampling period
Fig. 5.6. Changes in the plasma concentrations of prolactin in control ewes in blood samples collected at 15 minute intervals for 25h starting at 08:00h on Days 1, 6 and 10 of the luteal phase. Values are mean ± s.e.m. Values marked by a vertical line indicate the times during each 25h sampling period when at least 3/4 control ewes had prolactin levels above the daily mean of each individual.
Fig. 5.7 Changes in the plasma concentrations of prolactin in ewes treated with follicular fluid in blood samples collected at 15 minute intervals for 25h starting at 08:00h on Days 1, 6 and 10 of the luteal phase. Values are mean ± s.e.m. Values marked by a vertical line indicate the times during each 25h sampling period when at least 4/5 follicular fluid treated ewes had prolactin levels above the daily mean of each individual.
Table 5.3. Effect of treatment of ewes with bovine follicular fluid on plasma concentrations of prolactin during 25h on Days 1, 6 and 10 of the luteal phase

<table>
<thead>
<tr>
<th></th>
<th>Plasma Prolactin (ng/ml)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
<td>Day 6</td>
<td>Day 10</td>
</tr>
<tr>
<td>Group C</td>
<td>10.3 ± 1.7\textsuperscript{a}</td>
<td>16.1 ± 1.2\textsuperscript{c}</td>
<td>18.4 ± 2.9\textsuperscript{e}</td>
<td></td>
</tr>
<tr>
<td>Group FF10</td>
<td>7.6 ± 0.7\textsuperscript{b}</td>
<td>15.2 ± 3.0\textsuperscript{d}</td>
<td>22.0 ± 2.0\textsuperscript{f}</td>
<td></td>
</tr>
<tr>
<td>Combined mean</td>
<td>8.8 ± 0.9\textsuperscript{g}</td>
<td>15.6 ± 1.7\textsuperscript{h}</td>
<td>20.4 ± 1.7\textsuperscript{i}</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

ab, cd, ef, hi N.S.
ac, ae, bf \( P < 0.05 \)
gh \( P < 0.01 \)
gi \( P < 0.001 \)
irrespective of stage of cycle there did not appear to be any direct
effect or immediate change in LH secretion after injection of
follicular fluid or bovine serum.

**Prolactin:** The changes in plasma prolactin concentrations for
control and Group FF10 ewes on Days 1, 6 and 10 are shown in Fig.
5.6. and 5.7. Values marked by a vertical line indicate the times
during each 25h sampling period when at least 3/4 controls and 4/5
follicular fluid treated ewes had prolactin levels above the daily
mean of each individual. This method of analysis has previously
been validated in the ram (Lincoln, McNeilly & Cameron, 1978).
There were no significant differences in plasma prolactin
concentrations between Group C and FF10 on any of the 3 days studied
(Table 5.3.). Mean prolactin levels were low on Day 1 and increased
throughout the luteal phase to reach significantly higher
concentrations (relative to Day 1) by Day 10 in both groups (P<
0.05). The mean prolactin level for Group FF2 ewes on Day 10 was
15.9 ± 0.9 (s.e.m.) ng/ml and was not significantly different from
either Group C or FF10.

Although ewes were bled under artificial light at night, a
night-time peak in prolactin levels was generally recorded in all
groups on each sampling day between midnight and 04:00h (see Fig.5.6
and 5.7.). In addition to the 'night-time' peak there was a
distinct rise in prolactin levels between mid-day and 14:00h in both
groups on Days 6 and 10. All other peaks in prolactin secretion for
example between 09:00 and 10:00h on Day 1 in the control group, were
usually due to one animal and were most likely stress induced.
Plasma prolactin concentrations did not appear to be correlated with
changes in FSH or LH secretion.
Fig. 5.8  

(a) Changes in plasma concentrations of progesterone on Days 1-11 of the cycle prior to injection of bovine serum (0) or bovine follicular fluid for 2 (■) or 10 (●) days during the luteal phase.

(b) Changes in plasma concentrations of progesterone on Days 1-13 of the subsequent oestrus cycle.

The range is shown by dotted lines.
There were no significant effects of follicular fluid treatment on FSH, LH or prolactin levels during the subsequent cycle.

**Progesterone:** There were no significant differences in plasma progesterone levels between the 3 groups at any stage of the luteal phase during treatment. Initially on Days 1 and 2 of the luteal phase progesterone levels were all < 0.2 ng/ml. Concentrations began to rise on Days 3-4 of the cycle reaching a plateau by Days 7-8. The changes in plasma progesterone concentrations during the subsequent cycle as measured on Days 1-13 were also characteristic of a normal cycle and identical to those described during the treatment cycle (Fig. 5.8).

**Discussion**

The present study has confirmed that injections of bovine follicular fluid during the luteal phase in the ewe, for either 2 or 10 days prior to cloprostenol-induced luteolysis, results in a significant decrease in plasma FSH concentrations and an increase in pulsatile LH secretion. This was associated with a delay in the onset of oestrus and a marginal but non-significant increase in ovulation rate.

The decrease in FSH levels began 2-3h after the first follicular fluid injection and is in agreement with other studies in intact (McNeilly, 1984, 1985a) and ovariectomised ewes (Tsonis, 1984). A similar time-lag of FSH suppression has been observed after injection of bovine or porcine follicular fluid into female rats (Hermans et al., 1981; Campbell & Schwartz, 1979; de Paolo et al., 1979a) and after injection of equine follicular fluid in mares (Miller et al., 1979b). This suggests that the inhibin activity in different sources of follicular fluid operates to suppress FSH in a
similar manner between species. The half-time of the decrease in FSH levels in this study, in response to the first follicular fluid injection, was 130 minutes and is similar to previously recorded half-time clearance rates for FSH of 120 minutes (Akbar et al., 1974) and 159 minutes (McNeilly, 1985a) respectively. Similarly, it has been shown following a bolus injection of follicular fluid (40,000 or 80,000 units of inhibin) in ovariectomised ewes, that there was a lag of between 30 and 60 minutes before inhibin activity decreased (Tsonis, 1984). This suggests that inhibin is not rapidly cleared from the circulation. The half-time of clearance of inhibin following this lag phase was estimated to be between 45 and 50 minutes (Tsonis, 1984). The similarity in the time course of these events argues strongly for the fact that inhibin is the active component of bovine follicular fluid inhibiting the release of FSH from the pituitary.

The twice daily injection regimen (09:00 and 17:00h) used in this and other studies described in this thesis maintained FSH suppression for between 16h and 18h/day. In the majority of ewes FSH levels began to increase to reach or exceed comparable control levels between 06:00 and 07:00h i.e. 13-14h after the last follicular fluid injection. Hence sampling at 09:00h did give a misleading picture of the degree of FSH suppression achieved by follicular fluid administration as suggested in Chapter 3. This small 'day to day' rebound in FSH levels probably reflects the accumulation of stores of releasable FSH in the pituitary gonadotrophs which are released once the suppressive effects of follicular fluid have dissipated. This release of FSH is terminated by the subsequent follicular fluid injection.
Previous studies have indicated that the degree of FSH suppression is reduced when follicular fluid was administered over a period of several days (Miller et al, 1982; McNeilly, 1984). In the present study however, follicular fluid was still effectively suppressing FSH concentrations on both Days 6 and 10 of treatment, although a marginal decrease in the degree and duration of suppression was noted. A similar trend was observed in the twice daily blood samples taken in this and earlier studies. Furthermore the degree of FSH suppression on Day 10 of the luteal phase in Group FF2 ewes was slightly higher than that of Group FF10 ewes confirming that there is a gradual loss of effectiveness with time. This gradual increase in FSH levels towards the end of treatment may be a reflection of the progressive reduction in ovarian oestradiol and/or endogenous inhibin production by the follicles because of insufficient FSH stimulation of follicular growth during follicular fluid treatment.

The present study confirmed and extended the previous observation that injections of follicular fluid throughout the luteal phase resulted in the maintainance of high LH pulse frequency and amplitude on Days 6 and 10 of treatment, at a time when pulsatile LH secretion is normally suppressed. As this experiment has confirmed that FSH secretion was suppressed for most of the time during treatment it seems probable that less oestradiol was secreted from the follicles of follicular fluid treated ewes, resulting in a loss of negative feedback and hence an increase in pulsatile LH release (see Chapter 6). If this hypothesis is correct, the loss of oestradiol negative feedback must occur over several days of follicular fluid treatment as there was no difference in the
pulsatile LH secretion of Group FF2 ewes (24-48h after the first follicular fluid injection) compared with controls on Day 10. The lack of effect of follicular fluid treatment on pulsatile LH secretion in Group FF2 ewes would also argue against the existence of another factor in follicular fluid which stimulates LH secretion. Furthermore if the increase in pulsatile LH secretion is in response to a decrease in oestrogen feedback this would explain why administration of follicular fluid did not markedly affect LH secretion in long-term ovariectomised ewes (Cummins et al, 1983; Findlay et al, 1985).

In the control ewes in the present study LH pulse frequency was initially high on Day 1 of the luteal phase at a time when progesterone levels were still undetectable. The LH frequency was decreasing by Day 6 and was at its lowest by Day 10 of the luteal phase when progesterone concentrations were maximal. It was because of similar observations of the inverse relationship between progesterone and LH, that progesterone was originally suggested as being the major negative feedback hormone involved in regulating LH pulse frequency during the oestrous cycle (Hauger et al, 1977; Goodman & Karsch, 1980a). However, studies in ovariectomised ewes showed that progesterone and oestradiol were both required before a potent inhibitory effect on LH pulse frequency could be observed during the breeding season (Goodman et al, 1981a; Martin et al, 1983). It is now generally accepted that a low frequency of LH pulses is maintained by an interaction of luteal progesterone and follicular oestradiol (see Karsch et al, 1980b; Martin, 1984). However, in the present study, the LH pulse frequency in follicular fluid treated ewes remained high throughout the cycle despite a
normal rise in plasma progesterone concentrations which was identical to that of control ewes. Similarly, active or passive immunization of ewes against oestradiol will increase the frequency of LH pulses even when normal progesterone levels are maintained (Scaramuzzi et al., 1980a; Pathiraja, 1982) supporting the hypothesis that the normal corpus luteum is refractory to additional LH stimulation (Collett, Land & Baird, 1973).

Collectively, the absence of an effect of high LH pulse frequency on plasma progesterone concentrations in these studies suggests that it is oestradiol secretion by the ovarian follicles that is of paramount importance in determining the frequency of LH pulses. Furthermore, it seems probable that the inhibitory action of progesterone on LH pulsatility can only operate when there is sufficient oestradiol present to sensitize the hypothalamic pulse generator to progesterone (Goodman et al., 1981a). Thus during the normal luteal phase progesterone appears to determine the overall pattern of LH release while oestradiol determines the upper and lower limits within which LH pulse frequency can fluctuate. Thus when the normal oestradiol negative feedback breaks down as during follicular fluid treatment the upper limit of LH pulse frequency is exceeded and resembles that observed following ovariectomy of cyclic ewes (Montgomery et al., 1985).

Previous studies have also indicated that the amplitude of LH pulses is highest when peripheral levels of oestradiol are low (Hauger et al., 1977; Goodman & Karsch, 1980a) and oestradiol treatment has been shown to reduce the amplitude of LH pulses in ovariectomised cyclic and post partum ewes (Goodman & Karsch, 1980a; Wright et al., 1981). These authors suggest that oestradiol acts at
the pituitary to inhibit its response to each GnRH discharge, thereby decreasing LH pulse amplitude. Similarly, when progesterone and oestradiol are present in ovariectomised ewes, progesterone appears to have no effect of the amplitude of LH pulses (Goodman et al, 1981a). The results of the present study are in agreement with these observations. In control ewes LH pulse amplitude did not change throughout the luteal phase despite rising progesterone levels. In contrast the LH pulse amplitude in follicular fluid treated ewes increased dramatically throughout the cycle and is most probably a direct reflection of low oestradiol secretion by the follicles as a result of the low FSH levels.

The individual LH profiles obtained in this study serve to illustrate the importance of adequate sampling periods when determining mean LH pulse frequency particularly during the luteal phase when frequency is known to be low. For example if the profile obtained for a control ewe on Day 6 and 10 of the luteal phase illustrated in Fig. 5.3. is divided into 4, 6h blocks the pulse frequency would be 2, 3, 4 or 2 on Day 6 and 3, 1, 4 or 3 on Day 10 depending on which 6h 'window' was used. Moreover this supports the suggestion (Martin, 1984) that pulse frequency is probably constantly changing within a particular stage of the cycle with periods of high frequency followed by periods of low frequency as steroid secretion and hence negative feedback increases. A similar relationship between LH pulses and testosterone secretion has been demonstrated in the male rat (Ellis & Desjardin, 1982).

Studies in the ovariectomised mare have shown that steroid-free follicular fluid was as effective as whole follicular fluid at suppressing plasma levels of FSH (Miller et al, 1979b). Similarly
de Jong & Sharpe (1976) reported no differences in FSH suppressing activity in castrated rats between whole, ether or charcoal extracted or ultrafilter retained (MW 10,000) follicular fluid, suggesting that the FSH suppressing activity of follicular fluid was not due to residual steroids. Conversely, a slight increase in effectiveness of steroid-free follicular fluid when combined with oestradiol has been reported for the ovariectomised mouse (Bronson & Channing, 1978) and rat (Campbell & Schwartz, 1979). The possible role of residual steroids remaining in the charcoal treated follicular fluid used in the present studies cannot be completely ruled out. However, indirect evidence against this possibility comes from studies in which the delay in oestrus onset was found to be identical in ewes treated with whole or charcoal treated follicular fluid (Miller et al 1979a; McNeilly, 1984). Whole bovine follicular fluid or steroids alone have been shown to decrease LH in castrated rats (de Jong & Sharpe, 1976). However, in view of the marked increase in pulsatile LH secretion observed during follicular fluid treatment it seems highly unlikely that the levels of residual steroids were similarly effective in the present study.

The increase in overall prolactin levels throughout the luteal phase in both control and follicular fluid treatment animals has not been reported previously. However this increase in prolactin secretion may be due to the change in photoperiod which is particularly marked at the end of the breeding season. Alternatively plasma prolactin levels are known to increase in response to housing (F.J.P. Ebling, personal communication).

The present study has shown that the injection of bovine follicular fluid throughout the luteal phase results in a marked
suppression of plasma levels of FSH. This is associated with a significant increase in pulsatile LH secretion despite normal progesterone levels, and suggests that oestradiol is a major negative feedback hormone involved in regulating LH pulse frequency and amplitude during the luteal phase.
CHAPTER 6: EXPERIMENT 4

Introduction

It has long been recognised that the initiation and maintainence of normal follicular development is dependent on adequate gonadotrophin support (Smith & Engle, 1927). In the ewe FSH secretion is normally sufficient to ensure the development of large antral follicles (4-6mm diameter) throughout the luteal phase of the cycle and is considered to have a permissive role (Baird, 1983).

Previous studies in monkeys (Channing et al, 1981; di Zerega et al, 1981) and sheep (Miller et al, 1979) showed that suppression of FSH release by follicular fluid during the follicular phase of the cycle resulted in a reduction in the size of the largest follicle and a decrease in follicle number. In previous chapters administration of follicular fluid throughout the luteal phase of the cycle resulted in the suppression of plasma FSH concentration, an increase in pulsatile LH secretion and was associated with a delay in the onset of oestrus. It seemed reasonable to suggest that at luteal regression the follicles were less well developed due to the reduction in FSH concentrations and that the increase in pulsatile LH secretion occurred because of insufficient oestradiol feedback from the follicle population. Consequently these follicles take longer to produce sufficient oestrogen to induce behavioural oestrus.

To test this hypothesis an experiment was undertaken to determine the effects of follicular fluid treatment throughout the luteal phase on ovarian follicular development on Day 10. The effect of FSH withdrawal on the functional capacity of individual
follicles was assessed by measuring in vitro oestradiol and testosterone production basally and after stimulation with ovine LH. As treatment with follicular fluid throughout the luteal phase has previously resulted in an increase in ovulation rate at the following oestrus one of the objectives of this study was to determine the size of follicles available prior to the onset of luteolysis which are capable of being recruited as the ovulatory follicle(s).

**Materials and Methods**

**Experimental animals**

Twenty Damline ewes were studied during the breeding season in October 1984. The ewes were 3.5 or 4.5 years old and weighed 64.1 ± 1.7Kg at the start of the experiment. Oestrous behaviour was synchronised as described previously (P. 90) and the ewes moved inside into individual pens before the start of treatment on Day 1 (oestrus + 24h). To facilitate the follicle dissection, the study was carried out using 4 groups of 5 ewes each, which were staggered over the course of two weeks. All results were initially analysed separately, but, as no differences were found, the data were combined for analysis.

**Experimental design**

Ewes were weighed, ranked and allocated to one of two treatment groups prior to the start of the study. The 10 ewes in the treatment group received 10ml of bovine follicular fluid (Batch 5) i.v. at 09:00 and 17:00h on Day 1 (oestrus + 24h) to Day 9 of the cycle inclusive, and the final injection was staggered between 06:00 and 08:00h on Day 10. The 10 remaining ewes received bovine serum at an identical injection frequency and acted as controls.
The ovaries were removed on Day 10 between 10:00 and 12:00h, exactly 4h after the final follicular fluid injection. Half of the ewes, 5 treated and 5 controls were ovariectomised by mid-ventral laparotomy under anaesthesia induced by intravenous injection of sodium intraval on a volume/Kg bodyweight basis* (May & Baker Ltd., Dagenham) and maintained with an oxygen, fluothane mixture. The remaining 10 ewes were killed by lethal injection (20ml) of sodium pentobarbitone (Euthatal, 200mg/ml; May & Baker Ltd.) and the ovaries removed within two minutes. On removal, the ovaries were placed in medium (Medium 199 buffered with 20mM Hepes [N-2-hydroxyethylpiperazine-N\(^-\)2-ethanesulphonic acid] and containing Hank's salts and 2mM glutamine; Flow Laboratories, Irvine, U.K.) and placed in an incubator at 37°C until dissected. All ovaries were dissected within 30 minutes of being removed from the ewe. The ovaries were trimmed with fine scissors and fine forceps were used to isolate all individual follicles \(>1\)mm and as many follicles as possible \(<1\)mm. The diameter of each follicle was measured to the nearest 0.1mm using an ocular graticule and the mean of the two perpendicular diameters was used. After dissection, individual follicles were incubated in 1ml medium for 2h at 37°C after which the medium was removed and replaced with medium containing 10ug ovine LH (NIH-LH-S23) and incubation continued for a further 2h. At the end of this second incubation period, medium was removed and stored at -20°C until assayed for oestradiol, testosterone and progesterone as described previously.

The number and weight of the corpora lutea from each pair of ovaries were recorded.

*12ml 5%w/v
Fig. 6.1. Changes in the plasma concentrations of FSH in blood samples withdrawn before injection of bovine serum (○) or bovine follicular fluid (•) at 09:00h and 17:00h (Y) on Days 1 to 10 of the luteal phase. The insert panel (a) shows the changes in FSH concentrations during 15 minute sampling on Day 9 of the luteal phase and at 4h intervals from 18:00h to just before the ovaries were removed on Day 10. Values are mean ± s.e.m.
Blood sampling schedule

Blood samples were taken twice daily prior to bovine serum or follicular fluid injection throughout the luteal phase treatment. Ewes were cannulated on Day 8 of the luteal phase and blood samples withdrawn at 15 minute intervals from 10:00 to 16:00h on Day 9. Thereafter, samples were taken at 4h intervals from 18:00h until the ovaries were removed 4h after the final follicular fluid/serum injection the following morning. All samples were assayed for LH and FSH, and the daily samples taken during the treatment cycle for progesterone. The LH assay used was based on the R29 antiserum.

Statistics

The differences in hormone concentrations between the groups were analysed using analysis of variance. Differences in pulsatile LH secretion, follicle size distribution and steroid production by follicles during the first and second incubations were compared by Student's 't' test (paired and unpaired). Regression analysis was used to determine the relationships between follicle diameter and basal steroid production. The steroid data was log transformed where necessary and all tables are of untransformed data.

Results

Hormone concentrations during treatment

FSH: The changes in plasma levels of FSH, measured throughout the treatment cycle are illustrated in Fig. 6.1., and are similar to those described in previous experiments.

In ewes receiving bovine serum FSH levels were initially high on Day 1 of the luteal phase and then fell during Day 2 to reach a low by Days 3 and 4. Thereafter FSH levels began to rise to reach a peak on Days 5 and 6 after which they fell slightly to a plateau
Table 6.1. Effect of treatment with bovine follicular fluid on pulsatile LH secretion on Day 9 of the luteal phase.

<table>
<thead>
<tr>
<th></th>
<th>Control ewes N=10</th>
<th>Treated ewes N=10</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 9: Luteal Phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal LH (ng/ml)</td>
<td>1.4 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>$P&lt;0.01$</td>
</tr>
<tr>
<td>LH pulses /6h</td>
<td>1.6 ± 0.4</td>
<td>2.9 ± 0.3</td>
<td>$P&lt;0.01$</td>
</tr>
<tr>
<td>LH pulse amplitude /6h</td>
<td>2.9 ± 0.5</td>
<td>6.8 ± 0.5</td>
<td>$P&lt;0.001$</td>
</tr>
<tr>
<td>Mean LH level/6h (ng/ml)</td>
<td>1.8 ± 0.2</td>
<td>3.5 ± 0.5</td>
<td>$P&lt;0.01$</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
during Days 8 to 10. Plasma FSH concentrations in control ewes did not vary significantly during the frequent sampling period on Day 9 nor in the samples withdrawn at 4h intervals from 18:00h on Day 9 until the ovaries were removed on Day 10. The first follicular fluid injection at 09:00h on Day 1 in the treated ewes suppressed the initially high FSH levels by approximately 75% by the 17:00h blood sample taken 8h later. Throughout the cycle FSH concentrations were higher at the 09:00h sample (16h from injection) than at the 17:00h sample (8h from last injection) reflecting the early morning rebound described in Chapter 5. This is also reflected in the samples withdrawn at frequent intervals on Day 9. Plasma FSH concentrations initially increased and then began to fall 2-3h after the 09:00h follicular fluid injection. The 17:00h injection maintained the suppression of plasma FSH levels overnight and levels began to rise by 06:00h on Day 10. Plasma FSH levels in the sample taken immediately before the ovaries were removed were lower in the follicular fluid treated group than in controls, but this difference failed to reach significance.

LH: Twice daily LH concentrations were significantly higher in treated ewes (P < 0.05) than in controls from Day 2 to 10 of the luteal phase (data not shown). Detailed analysis of the short term changes in LH secretion on Day 9 of the cycle confirm previous observations in Chapters 3 and 5, that basal LH concentrations, pulse frequency and amplitude were all significantly higher in the treated group (P < 0.01) than in control ewes (Table 6.1).

Progesterone: There were no significant differences between groups in the initial basal levels of progesterone on Days 1 and 2, nor in the rise in progesterone levels between Days 3 and 7 of the
Fig. 6.2. Changes in plasma concentrations of progesterone on Days 1-10 of the cycle prior to injection of bovine serum (○) or bovine follicular fluid (●). Values are mean ± s.e.m.
Fig. 6.3. Distribution of follicle diameter in follicles dissected from control and bovine follicular fluid treated ewes removed on Day 10 of the luteal phase.
luteal phase. Progesterone concentrations reached a peak and plateaued in both groups by Day 8 of the cycle. However levels were significantly higher in the follicular fluid treated ewes ($P < 0.05$) than in controls during Days 8-10 (Fig. 6.2).

**Corpora lutea:** The mean number of corpora lutea per ewe was identical in both groups during the treatment cycle as assessed by dissecting the ovaries on Day 10 of the luteal phase ($3.1 \pm 0.2$ (s.e.m.), range 2-4 in controls, compared with $3.1 \pm 0.5$, range 2-7 in follicular fluid treated ewes, N=10 ewes per group). However the corpora lutea of follicular fluid treated ewes were significantly heavier ($P < 0.01$) than those of controls ($391 \pm 23$mg, range 158-612mg compared with $289 \pm 26$mg, range 71-557mg in controls). The total mass of corpora lutea per ewe was correlated ($r=0.460$) with the *in vivo* progesterone production, calculated as the mean progesterone level on Days 8-10 inclusive.

**Ovarian follicular development**

The size distribution of follicles dissected from ovaries of control and bovine follicular fluid treated ewes, removed on Day 10 of the luteal phase is shown in Fig. 6.3. There was a greater number of follicles dissected from the ovaries of control ewes compared with those of follicular fluid treated ewes but this difference was not significant due to the large individual variation in follicle number between animals within each group ($50.7 \pm 7.7$ (s.e.m.) follicles, range 28-96 per ewe in controls; $39.5 \pm 5.1$ follicles, range 22-72 in treated ewes; N=10 ewes/group). Similarly there were no significant differences in the number of follicles < 1mm or between 1.0 and 2.7 mm in diameter in control compared with follicular fluid treated ewes ($5.7 \pm 0.6$ versus $6.6 \pm 4.4$ follicles
Fig. 6.4. Relationship between basal in vitro oestradiol production (pg/ml/h) and follicle diameter in follicles dissected from control (○) and follicular fluid treated (●) ewes on Day 10 of the luteal phase.
Table 6.2: Steroid production in vitro by ovarian follicles from control (N=10) and follicular fluid treated ewes (N=10). Follicles were incubated for 2h at 37°C (basal steroid production), medium changed and incubated for a further 2h at 37°C with medium containing 10μg ovine LH.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of follicles</th>
<th>Basal steroid production (pg/h/follicle)</th>
<th>LH stimulated steroid production (pg/h/follicle)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Oestradiol</td>
<td>Testosterone</td>
</tr>
<tr>
<td>Follicles ≤ 2.7mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>399</td>
<td>56.7 ± 7.2a</td>
<td>26.3 ± 1.8b</td>
</tr>
<tr>
<td>Follicular Fluid Treated</td>
<td>395</td>
<td>26.0 ± 2.0e</td>
<td>29.3 ± 2.0f</td>
</tr>
<tr>
<td>Follicles &gt; 2.7mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>108</td>
<td>517.6 ± 68.21a</td>
<td>70.5 ± 9.6j</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

*a, b, c, d, e, f, g, h, i, j, k, l, P<0.01; a, e, a, b, f, b, j, f, h, j, l, b, j, P<0.001
1mm and 34.2 ± 5.7 versus 32.9 ± 4.4 follicles ≥ 1mm and ≤ 2.7mm. There were 0/395 follicles dissected from ovaries of follicular fluid treated ewes with diameters ≥ 2.7mm in contrast with 108/507 follicles in control ewes (range 8-21 per ewe). The maximum diameter of follicles from control ewes varied between 5.1 and 6.4mm (5.8 ± 0.1mm(s.e.m.) with 1-4 follicles ≥ 5mm being present in each ewe (2.1 ± 0.3). In contrast the maximum diameter of follicles from treated ewes was significantly lower (P<0.001) and varied from 1.7-2.7mm (2.3 ± 0.1mm). In the control group, and, to a lesser extent, the treatment group, there appeared to be 2 distinct peaks of follicle distribution, of a similar magnitude, at 1.5 and approximately 2.0mm diameter respectively.

Steroid production

(a) Follicles ≤ 2.7mm diameter

The basal oestradiol secretion measured during the first 2h incubation period varied widely between individual follicles of the same size both within and between ewes (Fig. 6.4). There was a significant positive correlation between basal oestradiol production and follicle diameter across all follicles from control ewes (overall r=0.583, P<0.001, N=507). However there was no significant relationship between follicle size and oestradiol production in follicles ≤ 2.7mm diameter from either control or follicular fluid treated ewes. The oestradiol and testosterone production of all follicles within this size group from control and follicular fluid treated animals were compared (Table 6.2). Overall, the basal oestradiol production during the first 2h incubation period was significantly higher (P<0.001) from the follicles of control ewes compared with follicles from treated ewes.
Table 6.3 Steroid production in vitro by ovarian follicles ≤ 2.7 mm diameter from control (N=10) and follicular fluid treated ewes (N=10) in which (a) the LH stimulated oestradiol production exceeded basal production by > 2.5 pg/ml/h (b) the LH stimulated oestradiol production decreased or did not change relative to the basal production.

<table>
<thead>
<tr>
<th>No. of follicles</th>
<th>Basal steroid production (pg/h/follicle)</th>
<th>LH stimulated steroid production (pg/h/follicle)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestradiol</td>
<td>Testosterone</td>
</tr>
<tr>
<td>(a) Increase in E₂ production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>288</td>
<td>49.9 ± 4.4a</td>
</tr>
<tr>
<td>Follicular Fluid Treated</td>
<td>311</td>
<td>24.8 ± 2.0e</td>
</tr>
<tr>
<td>(b) Decrease or no change in E₂ production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>111</td>
<td>57.1 ± 16.5i</td>
</tr>
<tr>
<td>Follicular Fluid Treated</td>
<td>84</td>
<td>35.4 ± 7.6m</td>
</tr>
</tbody>
</table>

ik P<0.05; bj,fn,P<0.01; ac,eg,bd,fr,ae,cg,fl,np,dl, hp. P<0.001

Values are mean ± s.e.m.
The basal production of testosterone was not significantly different between groups and there was no correlation between basal testosterone and follicle size.

The follicles were classified into 2 broad categories according to the change in oestradiol production after LH stimulation (Table 6.3). Thus, following the addition of LH, 72% of follicles from control ewes and 79% from follicular fluid treated ewes showed an increase in oestradiol production above basal secretion of > 2.5 pg/ml/h. The oestradiol production by the remaining follicles was below or equivalent to that measured basally and was not significantly different between treatment groups. Furthermore these follicles were scattered throughout the diameter size range ≤ 2.7mm.

Of the follicles which responded positively to LH stimulation the oestradiol production during the second incubation period was significantly higher (P<0.001) than that secreted basally in both control and follicular fluid treated animals. The level of oestradiol production remained significantly higher (P<0.001) in the control relative to the follicular fluid treatment group during the second incubation period. However the overall increment above basal production expressed as the ratio of LH stimulated oestradiol production: basal oestradiol production was significantly less (P<0.01) in follicles from control (4.09 ± 0.3) compared with follicular fluid treated ewes (5.87 ± 0.6). As the number of follicles responding positively to LH stimulation was not significantly different between groups, this suggests that the greater overall increment above basal production in the follicular fluid treated group represents a higher individual responsiveness.
The addition of LH resulted in a highly significant increase (P<0.001) in testosterone production by follicles from control and follicular fluid treated animals. Both the basal and LH stimulated testosterone production was significantly higher (P<0.01 and P<0.001 respectively) from those follicles which showed an increase in oestradiol production after LH stimulation compared with those follicles from which the oestradiol production decreased or remained the same. Within each of these classifications, there was no significant difference in testosterone production between treatment groups basally or after LH stimulation. Similarly there was no significant difference in the overall increment in testosterone secretion which was similar in all 4 groups.

The basal progesterone production was low and not significantly different between treatment groups; 8.9 ± 0.8 pg/ml/h by follicles from control ewes (N=399) and 7.5 ± 0.5 pg/ml/h by follicles from follicular fluid treated ewes (N=395). Similarly there was no significant change in progesterone production after LH stimulation in either the control (11.3 ± 1.5 pg/ml/h) or follicular fluid treated group (7.6 ± 1.0 pg/ml/h).

The concentrations of oestrogen and androgen in the antral fluid are often expressed as a ratio and used as an index of follicular atresia i.e. the lower the ratio the more atretic the follicle (Carson et al, 1981; Hillier, Reichert & van Hall, 1981). Similarly, in the present study the in vitro oestradiol to testosterone production was expressed as a ratio for all individual follicles where the basal production of testosterone was > 2.5pg/ml/h. Follicles were classified according to whether or not the in vitro oestradiol:testosterone ratio increased, decreased or
Table 6.4. Change in ratio of oestradiol:testosterone production by ovarian follicles in vitro from control and follicular fluid treated ewes after incubation for 2h at 37°C with medium containing 10ug ovine LH.

<table>
<thead>
<tr>
<th>Follicles ≤2.7mm</th>
<th>E:T Ratio</th>
<th>Group</th>
<th>N</th>
<th>Basal E:T Ratio</th>
<th>LH stimulated E:T Ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Increase</td>
<td>Control</td>
<td>60</td>
<td></td>
<td>0.97 ± 0.16</td>
<td>7.46 ± 1.80</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>120</td>
<td></td>
<td>0.92 ± 0.18</td>
<td>4.57 ± 0.97</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>(ii) Decrease</td>
<td>Control</td>
<td>253</td>
<td></td>
<td>2.86 ± 0.24</td>
<td>0.77 ± 0.09</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>165</td>
<td></td>
<td>1.71 ± 0.20</td>
<td>0.55 ± 0.08</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>(iii) No change</td>
<td>Control</td>
<td>8</td>
<td></td>
<td>0.55 ± 0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>19</td>
<td></td>
<td>0.29 ± 0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Follicles ≥2.7mm</th>
<th>E:T Ratio</th>
<th>Group</th>
<th>N</th>
<th>Basal E:T Ratio</th>
<th>LH stimulated E:T Ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Increase</td>
<td>Control</td>
<td>20</td>
<td></td>
<td>1.33 ± 0.35</td>
<td>45.98 ± 20.59</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>(ii) Decrease</td>
<td>Control</td>
<td>83</td>
<td></td>
<td>20.95 ± 4.05</td>
<td>5.96 ± 1.98</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Follicles producing &gt;1000 E2pg/ml/h</th>
<th>E:T Ratio</th>
<th>Group</th>
<th>N</th>
<th>Basal E:T Ratio</th>
<th>LH stimulated E:T Ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Increase</td>
<td>Control</td>
<td>0</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(ii) Decrease</td>
<td>Control</td>
<td>22</td>
<td></td>
<td>56.43 ± 13.02</td>
<td>8.31 ± 3.76</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
remained the same after LH stimulation (Table 6.4). Significantly more follicles from follicular fluid treated ewes showed an increase in the oestradiol:testosterone ratio following the addition of LH than in controls (39.5% and 18.7% respectively). The basal oestradiol:testosterone ratio in this category was low (<1) and increased significantly (P<0.01) after LH stimulation in a similar manner in both groups. Correspondingly significantly more follicles (P<0.001) from control ewes showed a decrease in oestradiol:testosterone ratio after LH stimulation (78.8% compared with 54.3% in the follicular fluid treated group). Within this category the basal oestradiol:testosterone ratio was significantly higher (P<0.01) from follicles from control ewes than in those from follicular fluid treated ewes and there was a significant decrease (P<0.001) in the oestradiol:testosterone ratio after LH stimulation in both groups. The ratio of oestradiol:testosterone production during the first incubation period in this category was significantly higher (P<0.01) in both treatment groups compared with that observed basally by follicles which subsequently showed an increase in the oestradiol:testosterone ratio.

(b) Follicles > 2.7mm diameter

The basal oestradiol secretion measured during the first incubation period varied widely in follicles > 2.7mm diameter dissected from control ewes (Fig.6.4). Nevertheless, there was a positive correlation between oestradiol production and follicle diameter in these follicles (r=0.239, P<0.001, N=108). In contrast there was no relationship between testosterone production and follicle size. Basal oestradiol and testosterone production by these large follicles was significantly higher (P<0.001) than in
Table 6.5 Steroid production in vitro by ovarian follicles from control ewes (a) > 2.7mm diameter and/or (b) producing > 1000 pg oestradiol/ml/h. Follicles were further classified according to whether or not the LH stimulated oestradiol production exceeded basal production by > 2.5pg/ml/h.

<table>
<thead>
<tr>
<th>(a) All Follicles</th>
<th>Basal steroid production (pg/h/follicle)</th>
<th>LH stimulated steroid production (pg/h/follicle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of follicles</td>
<td>Oestradiol</td>
<td>Testosterone</td>
</tr>
<tr>
<td>Increase in E2</td>
<td>65</td>
<td>349.1 ± 58.6а</td>
</tr>
<tr>
<td>Decrease or no change in E2</td>
<td>43</td>
<td>776.3 ± 139.9е</td>
</tr>
<tr>
<td>(b) Follicles producing &gt; E2 1000 pg/ml/h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increase in E2</td>
<td>8</td>
<td>1414.0 ± 119.4и</td>
</tr>
<tr>
<td>Decrease or no change in E2</td>
<td>15</td>
<td>1863.4 ± 187.6μ</td>
</tr>
</tbody>
</table>

*eg, ko, P<0.01; ac, bd, fh, ik, mo, jl, np, P<0.001

Values are mean ± s.e.m.
follicles ≤ 2.7mm diameter from control ewes (Table 6.2).

Follicles were again classified according to the change in oestradiol production after LH stimulation and 60% of follicles showed a significant (P < 0.001) increase in oestradiol production above basal secretion of ≥ 2.5 pg/ml/h (Table 6.5).

The addition of LH to the incubation medium resulted in a significant increase (P < 0.001) in testosterone production irrespective of whether oestradiol production increased or not. There were no significant differences between categories in either the basal or LH stimulated testosterone production.

Progesterone production by follicles ≥ 2.7mm did not change significantly between the first and second incubation periods (29.3 ± 7.8 versus 26.0 ± 2.7 pg/ml/h). However the progesterone production by these large follicles was significantly higher (P < 0.01) compared with that of follicles ≤ 2.7mm diameter after both the basal and LH stimulated incubation periods.

The oestradiol:testosterone ratio was initially high and fell significantly (P < 0.01) in 81% of follicles ≥ 2.7mm after LH stimulation (Table 6.4). The remaining 19% of follicles had a basal oestradiol:testosterone ratio similar to that of follicles ≤ 2.7mm diameter which increased significantly (P < 0.001) after LH stimulation.

Potentially 'ovulatory' follicles

Follicles were classified as potentially 'ovulatory' when the basal oestradiol secretion exceeded 1000 pg/ml/h and the in vitro oestradiol versus testosterone ratio was greater than 1 (Webb & Gauld, 1985a). Using this classification the number of 'ovulatory' follicles dissected from the ovaries of control ewes varied from 0-6
per ewe (2.3 ± 0.6 follicles) with 1 ewe having no 'ovulatory'
follies. These follicles had a mean diameter of 4.2 ± 0.3mm, 
range 2.1 to 6.4mm and a mean basal oestradiol production of 1707 ± 
135pg/ml/h. Only 2 potentially 'ovulatory' follicles had a diameter 
of ≤ 2.7mm. There were no 'ovulatory' follicles dissected from the 
ovaries of follicular fluid treated ewes as the maximum basal 
oestradiol production measured was 248pg/h/follicle in this group.

The 23 potentially 'ovulatory' follicles were also classified 
according to whether or not they responded to LH stimulation (Table 
6.5). There was a significant increase (P<0.001) in oestradiol 
production above that secreted basally in 8 follicles while the 
remaining 15 follicles showed a significant decrease (P<0.001) in 
oestradiol production after LH stimulation. It was interesting to 
note that the level of oestradiol production during the second 
incubation period in the group that responded positively to LH 
stimulation was equivalent to that being produced basally by the 
follies which did not respond. The basal and LH stimulated 
testosterone production was not related to the responsiveness of the 
follies and there was a significant increase in testosterone 
production between the first and second incubation periods in both 
categories.

The basal oestradiol:testosterone ratio was initially very high 
(Table 6.4) and decreased significantly (P<0.001) in all follicles 
after LH stimulation.

**Total ovarian steroid production**

The total basal in vitro oestradiol production per pair of 
ovaries per ewe was calculated as an estimation of the amount of 
oestradiol normally leaving the ovary to feedback on the
hypothalamic-pituitary axis. The total oestradiol production was significantly higher ($P<0.001$) in follicles from control compared with follicular fluid treated ewes ($8.51 \pm 1.18 \text{ng/ml/h}$ versus $0.99 \pm 0.27 \text{ ng/ml/h}$ respectively). In addition, there was a significantly negative correlation ($r=-0.583$, $P<0.001$, $N=20$) between the total in vitro oestradiol production per ewe in ovaries removed on Day 10 of the luteal phase and the mean LH level measured during 6h of 15 minute sampling on Day 9 of the luteal phase ($1.8 \pm 0.2 \text{ng/ml}$ versus $3.5 \pm 0.5 \text{ng/ml}$ in control and follicular fluid treated ewes respectively).

**Discussion**

Administration of bovine follicular fluid throughout the luteal phase of the cycle was associated with a reduction in plasma FSH concentrations and an increase in pulsatile LH secretion as reported previously (Chapters 3 and 5). The suppression of plasma FSH concentrations by follicular fluid prevented normal follicle growth with no follicles $>2.7 \text{mm}$ diameter being found. These results are in agreement with those from previous studies in monkeys (Channing et al, 1981; di Zerega et al, 1981) and sheep (Miller et al, 1979a) in which the size of the largest follicle was markedly reduced following follicular fluid treatment in the follicular phase. Similarly, the increase in size of large follicles in prepuberal heifers after unilateral ovariectomy was blocked by bovine follicular fluid administration (Johnson, Smith & Elmore, 1985).

Antral follicles $>2 \text{mm}$ diameter have been shown to be almost totally dependant on gonadotrophins in that all such follicles become atretic within 4 days of hypophysectomy (Dufour et al, 1979). Similarly in LHRH immunized ewes with significantly suppressed
plasma LH and FSH levels no follicles $\geq 2.5\text{mm}$ were found 30 months after primary immunization (McNeilly et al, 1985). Based on the results of Experiment 3 (Chapter 5) plasma FSH concentrations in the present study were suppressed throughout the 10 day luteal phase treatment period for approximately 18h/day. The short daily exposure (6h) to normal FSH concentrations probably accounts for the limited follicle growth up to 2.7mm in some ewes. In addition it is evident that follicle growth beyond 2.7mm in the present study was dependent primarily on FSH rather than LH stimulation in that follicles from follicular fluid treated ewes had been exposed to normal or high pulsatile LH secretion throughout the luteal phase of the cycle.

There was no difference in the number of follicles $< 2.7\text{mm}$ diameter in follicular fluid treated ewes compared with controls which argues against there being a build-up of follicles failing to grow beyond this point and suggests that the growth and recruitment of follicles into the antral follicle population up to 2.7mm and their subsequent regression was largely unaffected by the lower FSH concentrations. However such conclusions must be made tentatively as the number of follicles/ovary/ewe varied widely within both treatment groups and the size distribution of the follicle populations in individual ewes before and after follicular fluid treatment was unknown. Similarly it is not possible to determine whether the follicles $< 2.7\text{mm}$ dissected on Day 10 of the luteal phase from follicular fluid treated ewes had been present in the antral follicle population for the same time as the equivalent follicles from control ewes.

There were large variations in the in vitro oestradiol and
testosterone production by follicles of the same size both within and between ewes in both groups. This confirms previous observations on the steroid content of antral fluid from follicles of equivalent size and stage of atresia (Moor, 1973; Moor et al, 1978; Carson et al, 1981) and emphasises the large variations which exist in the microenvironment of individual follicles. In small follicles (≤ 2.7mm) from both groups there was no relationship between oestradiol production and follicle size. However in large follicles (> 2.7mm) from control ewes oestradiol production increased with follicular diameter in agreement with previous studies of antral fluid oestradiol content in large follicles of sheep (Carson et al, 1981; England et al, 1981a) and women (McNatty & Baird, 1978). The in vitro oestradiol production by follicles from follicular fluid treated ewes was significantly lower than that of controls (≤ 2.7mm diameter). Thus, as FSH stimulates the induction or activation of the granulosa cell aromatase enzyme system (Dorrington et al, 1975; Erickson & Hsueh, 1978, Moon et al, 1978) it seems reasonable to suggest that the suppression of FSH in follicular fluid treated ewes resulted in a reduction in the aromatization of androgen to oestrogen. Although androstenedione was not measured in the present study basal testosterone production by follicles ≤ 2.7mm was very similar in both groups suggesting that the availability of androgen substrate was not limiting the conversion of androgen to oestradiol. As LH is the primary controller of androgen precursor synthesis (Tsang et al, 1979) the follicles from follicular fluid treated ewes which were exposed to high pulsatile LH secretion may be expected to secrete more androgen in vivo. However this was not reflected by basal testosterone
production in vitro and suggests that 'down-regulation' of the number and effectiveness of thecal LH receptors may be occurring in the follicular fluid treated ewes.

The addition of ovine LH during the second incubation period resulted in a significant increase in testosterone production by the theca of follicles ∝ 2.7mm from both groups and is consistent with previous observations that hCG stimulates androgen secretion from 1-3mm follicles (Weiss & Armstrong, 1979; Armstrong et al, 1981). In the present study in both treatment groups the basal testosterone production was significantly lower in the follicles which did not respond positively to LH stimulation by increasing oestradiol production. This suggests that in these follicles, a shortage of androgen substrate may have been limiting oestradiol production and hence these follicles were destined to become atretic.

The lower basal oestradiol:testosterone ratio in follicle incubates from follicular fluid treated ewes suggests that these follicles are more androgenic than the follicles of a similar size from control ewes. As follicles grow they are known to pass through an androgen dominated phase (2-3mm diameter) when they have a limited capacity to convert androgens to oestrogens (Moor et al, 1978; Carson et al, 1981; England et al, 1981a,b). Thus it seems probable that the lack of FSH stimulation in follicular fluid treated ewes may result in the follicles being 'held' in this androgen dominated phase for a longer period of time than normal. One of the characteristic features of steroid production in atretic follicles is a high level of androgen production with a decrease in production of oestradiol (Moor et al, 1978; Carson et al, 1981). However it seems unlikely that the generally lower basal
oestradiol:testosterone ratio in follicles from follicular fluid treated ewes was due to a higher incidence of atresia compared with controls because the addition of LH caused an increase in both testosterone and oestradiol production by these follicles. Furthermore a higher proportion of follicles from follicular fluid treated ewes showed an increase in oestradiol:testosterone production after LH stimulation. In addition the increment in oestradiol production was greater than in follicles from control ewes suggesting that follicles which had limited exposure to FSH were more sensitive to LH stimulation. If the receptors on these follicles from follicular fluid treated ewes were in fact 'down regulated' in vivo as suggested previously the addition of 10ug ovine LH may be sufficient to overcome this 'down regulation' and may have a role in the activation of the aromatase enzyme. Although the follicles ≤ 2.7mm from control ewes responded to LH stimulation the ratio of oestradiol:testosterone decreased in a higher proportion of follicles than in the follicular fluid-treated group, suggesting an increase in atresia after LH stimulation in the control group. This agrees with the observation that follicular atresia is associated with a reduction in the binding of 125I labelled hCG (Carson et al., 1979). In follicles > 2.7mm from control ewes the basal oestradiol to testosterone ratio was generally high and characteristic of 'oestrogenic' follicles. The addition of LH resulted in a reduction of the oestradiol:testosterone ratio in the majority of these follicles and suggests that many of these follicles were becoming atretic.

The number of follicles dissected from control ewes on Day 10 of the luteal phase (2.3) and defined as being potentially
ovulatory, using the criteria of Webb & Gauld (1985a) was similar to the ovulation rate of the Damline breed (2.25-2.5). A close correlation between the number of mature follicles and ovulation rate has previously been reported in a variety of breeds during the follicular and luteal phase of the cycle and during seasonal anoestrus (England et al., 1981b; Webb & Gauld, 1985b). In the luteal phase large Graafian follicles are capable of ovulating if exposed to the correct gonadotrophin stimulus as demonstrated after enucleation of the corpus luteum or administration of luteolytic agents (Smeaton & Robertson, 1971; Acritopoulou et al., 1977).

Whether or not these potentially 'ovulatory' follicles from control ewes would have ovulated in the present study if luteolysis had been induced on Day 10 of the cycle is unknown. The decrease in oestradiol to testosterone ratio in all of these follicles after the addition of LH in vitro suggests that many of these follicles would have become atretic if they were exposed to the normal increase in pulsatile LH secretion observed during the follicular phase in vivo. Alternatively the similarity between the level of oestradiol production during the second incubation from those follicles which respond to LH compared with the basal production from those follicles which do not respond suggests that oestradiol production was already at maximum in the latter group. Hence the addition of 10µg/oLH/ml may have been sufficient to inhibit oestradiol secretion by these large follicles in a manner similar to that observed in the dominant follicle after the preovulatory LH surge (Moor, 1974; Baird & McNeilly, 1981). Nevertheless it seems probable that the follicles which would have been recruited to ovulate in the present study would not necessarily have been the same large oestrogenic
follicles dissected on Day 10 of the cycle. Indeed, numerous studies have shown that there is a large variability in the size of follicles that can be recruited at the time of natural or prostaglandin-induced luteolysis (Smeaton & Robertson, 1971; McNatty et al, 1982; Driancourt & Cahill, 1984; Driancourt et al, 1985a) and there is general agreement that recruitment occurs from follicles of all sizes $\geq 2$ mm diameter. In the present study no follicles $\geq 2.7$ mm were dissected from follicular fluid treated ewes on Day 10 of the cycle. Thus had luteolysis been induced follicle recruitment would have been from a less variable follicle size range than in controls. Using electocautery it has been shown that follicles ovulating within the normal time from luteolysis to ovulation can come from follicles of 2-4 or $\geq 4$ mm diameter at luteolysis but when only follicles $< 2$ mm remain at luteolysis there is a 24h delay in the LH surge (Tsonis et al, 1984). This is consistent with the observation in previous chapters that the onset of oestrus and hence the LH surge is delayed in follicular fluid treated ewes for 12-48h.

It seems reasonable to suggest that if luteolysis had been induced in the follicular fluid treated ewes in the present study the onset of oestrus/LH surge would have been delayed because of the time taken for these small follicles ($< 2.7$ mm) to grow and mature. Similarly the results of the present study confirm the suggestion made in Chapter 3 that the follicle population of the follicular fluid treated ewes at the time of luteolysis were producing insufficient oestradiol to induce behavioural oestrus within the normal time.

As these follicles are of a similar size and steroidogenic capacity at luteolysis the hypersection of FSH following the
cessation of follicular fluid treatment presumably allows more follicles to be recruited and become oestrogenic before FSH levels fall and the remaining follicles become atretic. This is consistent with the observation that in prolific Booroola ewes the follicle recruitment period is extended relative to control Merino ewes (Driancourt et al., 1985a) and the suggestion that this may be linked to the high FSH levels which have been found in Booroola ewes during the follicular phase (Bindon et al., 1985).

A highly significant correlation has been shown between the in vivo oestradiol production from the ovary and the in vitro oestradiol production by follicles removed from the same ovary (Webb & Gauld, 1985b). Thus in the present study the total in vitro oestradiol production by all follicles from each ewe was assumed to be a reasonable estimate of the amount of oestradiol normally leaving the ovary to feedback on the hypothalamic-pituitary axis, and was significantly lower in follicular fluid treated ewes. This confirms the suggestion made in previous chapters that the low FSH secretion in response to follicular fluid administration results in a reduction in oestradiol production by the follicle and hence a loss of oestradiol negative feedback on pulsatile LH secretion. As FSH interacts with oestradiol to stimulate the development of LH receptors on the granulosa cells of the maturing follicles it seems unlikely that the follicles from follicular fluid treated ewes which are deficient in FSH stimulation and oestradiol would be sufficiently developed to benefit from this increase in LH secretion.

In contrast to earlier studies described in Chapters 3-5 mid-luteal plasma progesterone concentrations were higher in
follicular fluid treated ewes than in controls. Similarly the total mass of corpora lutea per ewe was also higher in treated ewes. An increase in weight of the corpora lutea removed during the mid-luteal phase has also been observed in steroid immunized ewes (Scaramuzzi & Hoskinson, 1984) and it has been suggested that this may be due to more luteal cells and/or larger luteal cells. As pulsatile LH secretion is high in both follicular fluid treated and steroid immunized ewes and LH is known to stimulate luteal progesterone production it seems likely that LH may be regulating the increase in corpus luteum mass in these studies. Further evidence to support this suggestion comes from the observation that pulses of GnRH administered over a 72h period increase both the weight and progesterone content of the corpus luteum at the end of the treatment period (Adams, Kinder, Chakraborty, Estergreen & Reeves, 1975).

A recent study showed that administration of ovine follicular fluid to hypophysectomised ewes whose follicles were maintained with PMSG resulted in a cessation of follicle growth, a decrease in the mitotic division of granulosa cells and an increase in atresia (Cahill et al, 1984a,b). The possible effects of such a growth inhibiting factor present in the follicular fluid used in the present studies cannot be ignored. However when FSH was infused coincident with bovine follicular fluid administration during the follicular phase the normal delay in oestrus observed after follicular fluid treatment is abolished (McNeilly, 1985a) which is consistent with the suggestion that it is the lack of FSH which prevents normal follicle growth in the present study. Similarly, in earlier studies described in this thesis the increase in secretion
of endogenous FSH after follicular fluid treatment has ceased results in an increase in ovulation rate and normal luteal function. If such a potent follicle growth inhibitor was functional in these studies it may reasonably be expected that 10 days of treatment using 20ml of follicular fluid per day would result in the complete suppression of the follicle population of these ovaries which would therefore not be able to respond to the hypersecretion of FSH in the follicular phase.

The present study has shown that suppression of plasma FSH concentrations by follicular fluid treatment throughout the luteal phase resulted in a failure of follicle development beyond 2.7mm. This was associated with a decrease in oestradiol production and consequently a reduction of the negative feedback on pulsatile LH secretion.
CHAPTER 7: EXPERIMENT 5

Introduction

In previous chapters administration of follicular fluid throughout the luteal phase was associated with (a) a rebound in plasma FSH concentrations after the cessation of treatment at cloprostenol-induced luteolysis (b) a delay in the onset of oestrus behaviour and (c) an increase in ovulation rate at the subsequent oestrus. Furthermore no follicle development beyond 2.7mm diameter was observed on Day 10 of the luteal phase in ewes treated with follicular fluid.

Using the same experimental protocol described earlier the present study was undertaken to determine the nature of the gonadotrophin signal which these small androgenic follicles are exposed to after the cessation of follicular fluid treatment at cloprostenol-induced luteolysis. The hypothesis under test was that differences in gonadotrophin secretion during the follicular phase may be responsible for the increase in ovulation rate normally observed in ewes treated with follicular fluid. However, to date, there is a lack of data on the detailed changes in FSH and pulsatile LH secretion which normally occur in untreated ewes at this time. The increase in pulsatile secretion of LH during the follicular phase is considered to be essential for the final stages of follicular development while the role of FSH at this time is not clear (Baird & McNeilly, 1981). The current model suggests that when the corpus luteum regresses the subsequent increase in LH pulse frequency promotes the sustained increase in oestradiol production by the preovulatory follicle(s) required to induce oestrous behaviour and the LH surge (Baird & Scaramuzzi, 1976; Hauger et al.,
1977; Karsch et al, 1979; Baird et al, 1981). This is supported by reports that repeated injection of LH or GnRH at frequent intervals will induce ovulation and luteal function in prepubertal lambs (Ryan & Foster, 1980) and in anoestrous ewes (McNeilly et al, 1982; McLeod et al, 1982,a,b). However studies in anoestrous ewes have also shown that ovulation can be induced by the non-pulsatile infusion of LH (Karsch et al, 1980a; McNatty et al, 1981), GnRH (McLeod et al, 1983) or hCG (Karsch et al, 1979). Both pulsatile and non-pulsatile exogenous hormone treatments have been associated with variable luteal function. It is not clear, therefore, which component of the LH signal during the follicular phase is required by the follicles to ensure successful maturation, ovulation and normal luteal function.

Therefore one of the aims of the present study was to determine the changes in pulsatile secretion of LH, and FSH secretion after cloprostenol-induced luteolysis in normal cyclic ewes to establish a firm base for subsequent studies on the induction of ovulation by exogenous hormone treatments in anoestrous ewes.

**Materials and Methods**

**Experimental design**

Fourteen Damline ewes were studied during the breeding season in November, 1984. The ewes were 3 to 6 years old and weighed 59.1±1.2Kg. Oestrus was synchronised as described earlier and the ewes weighed, ranked and allocated to one of two treatment groups so that weights were equivalent between treatments at the start of the study. The 7 ewes in the treatment group received 10ml bovine follicular fluid (i.v.) (Batch 5) at 09:00 and 17:00h on Day 1 (Oestrus + 24h) to Day 10 of the cycle inclusive. The 7 remaining ewes received bovine serum and acted as controls. Luteal regression
was induced at 09:00h on Day 11 of the treatment cycle using cloprostenol. The subsequent onset of oestrus was assessed by using a vasectomised ram at 8h intervals (09:00, 17:00 and 01:00h) between 32 and 88h after cloprostenol injection. Ovulation rate was determined by laparoscopy between Days 5 and 7 of the subsequent cycle.

**Blood sampling schedule**

Blood samples were taken twice daily throughout the treatment cycle immediately before follicular fluid or serum injection. On Day 6 of the cycle the ewes were cannulated and samples collected every 10 minutes between 10:00 and 16:00h. The cannulae were removed after the 17:00h sample that day and ewes were cannulated again on the afternoon of Day 10. On Day 11 blood samples were taken at 10 minute intervals for 1h before cloprostenol injection and thereafter for a maximum of 72h. Blood samples were then withdrawn at 2h intervals for up to 24h to determine the onset of the preovulatory LH surge. Within these maximum sampling limits, blood was sampled at 10 minute intervals for 12h after the first positive test of the onset of behavioural oestrus and thereafter at 2h intervals for 24h. During the subsequent oestrous cycle blood samples were taken daily at 09:00h until Day 12.

Ewes were maintained on a 8h light 16h dark photoperiod throughout the intensive blood sampling period. Lights were on between 08:00 and 16:00h. During the dark phase ewes were bled under red light. Lights were on during oestrus detection for approximately 45 minutes.

All blood samples withdrawn during the luteal and follicular phases of the cycle were assayed for LH using the assay based on the
R29 antiserum, and 4 times the intra-assay co-efficient of variation was used to determine significant pulses. All samples taken twice daily during the luteal phase and those withdrawn at 30 minute intervals on Day 6 and throughout the follicular phase were assayed for FSH. Plasma progesterone concentrations were measured in daily samples taken during the treatment and subsequent cycle. In addition samples taken at 1h intervals for 10h from cloprostenol-induced luteolysis were also assayed for progesterone.

Definitions

Onset of oestrus was considered to be the time when a ewe first stood to allow a vasectomised ram to mount/mate her minus 4h because of the 8h intervals between heat detection. The onset of the preovulatory LH surge was considered to be the time when levels exceeded 20ng/ml for the first time.

Statistics

The Mann-Whitney "U" test was used to examine the effect of treatment on ovulation rate and the number of large follicles at laparoscopy. Students 't' test was used to determine the effect of treatment on onset of oestrus and the characteristics of the preovulatory LH surge. The differences in gonadotrophin secretion between the two groups were analysed by analysis of variance. One way analysis of variance with repeated measures was used to analyse the changes in the characteristics of pulsatile LH secretion during the follicular phase in conjunction with Duncan's Multiple Range Test where appropriate. Linear regression analysis was applied to changes in FSH and progesterone secretion as indicated in the text to determine whether the slopes were significantly different (a) from time zero or (b) between treatment groups. Split-plot
Table 7.1  Effect of treatment of ewes with bovine follicular fluid on time to onset of oestrus from cloprostenol-induced luteal regression, characteristics of the preovulatory LH surge and ovulation rate.

<table>
<thead>
<tr>
<th></th>
<th>Control N = 7</th>
<th>Treated N = 7</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Onset of oestrus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time from cloprostenol (h)</td>
<td>45.0 ± 2.5</td>
<td>59.8 ± 3.7</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td><strong>LH Surge</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Onset-time from cloprostenol (h)</td>
<td>55.1 ± 2.3</td>
<td>77.6 ± 4.1</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>(b) Oestrus to surge (h)</td>
<td>10.1 ± 2.2</td>
<td>17.7 ± 2.1</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>(c) Maximum peak height (ng/ml)</td>
<td>140.1 ± 24.6</td>
<td>111.3 ± 18.1</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Ovulation rate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(range)</td>
<td>3.3 ± 0.3</td>
<td>3.7 ± 0.3</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>No. of large follicles (&gt; 5mm diam)</strong></td>
<td>2.0 ± 0.4</td>
<td>3.8 ± 0.7</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>(range)</td>
<td>(1 - 4)</td>
<td>(2 - 7)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
Fig. 7.1 Changes in the plasma concentrations of FSH in blood samples withdrawn before injection of (a) bovine serum or (b) bovine follicular fluid (▼) at 09:00h (●) and 17:00h (○) during the luteal phase of the cycle. Values are mean ± s.e.m.
analysis of variance was used to assess the differences between
groups and the change in progesterone concentrations with time.

**Results**

**Oestrus and ovulation rate**

Twice daily injections of bovine follicular fluid for 10 days
of the luteal phase prior to cloprostenol-induced luteolysis
resulted in a significant delay \((P < 0.01)\) in onset of oestrus
compared to that of control animals (Table 7.1). Ovulation rate was
not significantly different between groups but there were
significantly more \((P < 0.05)\) large follicles \((>5\text{mm diam})\) as assessed
at laparoscopy in the follicular fluid treatment group. The delay
in onset of oestrus was not correlated with the subsequent ovulation
rate or the number of large follicles.

**Hormone concentrations during treatment**

**FSH**: The changes in plasma levels of FSH measured twice daily
throughout the luteal phase are illustrated in Fig.7.1. and are
consistent with those described in Chapters 4, 5 and 6. In control
ewes receiving bovine serum FSH, levels were initially high at
09:00h on Day 1 and then fell during Day 2 to reach a nadir on Days
3 and 4. Thereafter FSH levels began to increase to reach a peak on
Days 5 and 6 after which they decreased slightly to a plateau on
Days 7 to 11. The first injection of follicular fluid at 09:00h on
Day 1 suppressed the initial plasma FSH levels by approximately 60%
by the 17:00h blood sample. Throughout the luteal phase there was a
'day to day' rebound in FSH levels in most treated ewes as described
previously with concentrations being higher at the 09:00h sample
(16h from injection) than at the 17:00h sample (8h from injection).
Table 7.2 Effect of treatment with bovine follicular fluid on pulsatile LH secretion on Day 6 of the luteal phase. Samples were withdrawn at 10 minute intervals.

<table>
<thead>
<tr>
<th></th>
<th>Control ( N = 6 )</th>
<th>Treated ( N = 7 )</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6: Luteal Phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal LH (ng/ml)</td>
<td>1.3 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>LH pulses/6h</td>
<td>2.3 ± 0.5</td>
<td>3.7 ± 0.3</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>LH pulse amplitude/6h (ng/ml)</td>
<td>2.0 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Mean LH level/6h (ng/ml)</td>
<td>1.9 ± 0.2</td>
<td>2.4 ± 0.1</td>
<td>( P &lt; 0.05 )</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
Fig. 7.2 (a) Changes in the plasma concentrations of progesterone in blood samples withdrawn before the morning injection of bovine serum (O) or bovine follicular fluid (•) during the luteal phase of the cycle. Values are mean ± s.e.m.

(b) Changes in the plasma concentrations of progesterone in blood samples withdrawn at 1h intervals after the injection of cloprostenol (100ug i.m.) on Day 11 of the cycle in control ewes (O) and ewes injected with bovine follicular fluid (•) throughout the luteal phase. Values are mean ± s.e.m.
Similarly during the frequent sampling period on Day 6 of the luteal phase (data not shown) the plasma FSH concentrations were low throughout the 6h in 2/7 ewes. In the remaining 5 ewes FSH levels began to decrease approximately 2h after the 09:00h follicular fluid injection. In contrast FSH concentrations in the control group did not fluctuate throughout the 6h period and the mean FSH level measured in samples withdrawn at 30 minute intervals was significantly higher than in ewes treated with follicular fluid (28.8 ± 9.2 ng/ml versus 5.8 ± 3.5 ng/ml; P<0.02).

LH: Twice daily LH concentrations were marginally higher in follicular fluid treated ewes than in controls throughout the luteal phase but this difference did not reach significance. Analysis of the short term changes in LH secretion on Day 6 of the cycle are in agreement with previous observations that basal LH concentrations, pulse frequency and pulse amplitude were all significantly higher in the treated group (P<0.05) than in controls (Table 7.2). One ewe in the control group (1J586) was excluded from the analysis as examination of her plasma progesterone profile showed that the first rise above baseline progesterone concentrations was on Day 6. It was therefore estimated that she was only at Day 3 of the cycle during the frequent sampling period which would explain her high pulse frequency and amplitude normally characteristic of the early luteal phase (see Chapter 5).

Progesterone: The changes in plasma progesterone concentrations measured daily throughout the luteal phase are illustrated in Fig.7.2.a. Progesterone concentrations were significantly higher (P<0.005) in ewes treated with follicular fluid than in controls between Days 3 and 11 of the luteal phase. Similarly
Changes in the concentrations of FSH after the injection of cloprostenol on Day 11 of the cycle in control ewes (○) and ewes injected twice daily with bovine follicular fluid (●) throughout the luteal phase. The results have been grouped around the time of (a) cloprostenol injection (PG), samples withdrawn at 30 minute intervals, or, (b) the LH peak, samples withdrawn at 2h intervals. Values are mean ± s.e.m.
linear regression analysis of the individual slopes showed that the rate of increase in progesterone concentrations was significantly higher ($P < 0.05$) in treated ewes.

**Hormone concentrations after luteal regression**

**FSH:** The changes in plasma FSH concentrations after cloprostenol-induced luteal regression are illustrated in Fig. 7.3. FSH levels in ewes treated with follicular fluid increased after luteolysis to reach a maximum of $164 \pm 7.7$ ng/ml (range 138-190 ng/ml) between 8 and 14h after cloprostenol injection ($11.4 \pm 1.0$ h). This represents a 3-4 fold increase over control group levels which decreased after cloprostenol injection to reach a nadir +16 to +18h later. Linear regression analysis of the individual slopes of the control group ewes showed that there was a significantly negative regression with time between zero to +16h after cloprostenol in all 7 ewes (range $r = -0.579$ to $-0.959$; $P < 0.001$). This represents an overall decrease in FSH concentrations of approximately 56%.

Plasma FSH concentrations in treated ewes were significantly greater ($P < 0.001$) than in controls throughout the period 0 to +24h after cloprostenol injection. This was also reflected by the mean FSH concentrations during the 24h period; $32.2 \pm 3.2$ ng/ml in control ewes and $95.4 \pm 4.1$ ng/ml in treated ewes. Thereafter during the period from +24 to +48h after cloprostenol there were no significant differences between treatments. Similarly there were no significant differences between treatment groups in the FSH levels during the 24h before the preovulatory FSH surge, nor in the magnitude of the surge which coincided with the preovulatory LH surge in both groups (Fig. 7.3.b). Although the sampling period did not encompass the secondary FSH surge in all ewes in both groups the
data indicate that there was no significant difference between treatment groups in the ascending limb of the second FSH surge and further suggest that the magnitude of the secondary FSH surge was considerably greater than that of the preovulatory surge in both groups.

There was no relationship between FSH concentrations during the follicular phase and ovulation rate in either treatment group or for all animals irrespective of treatment group. However, there was a significant positive correlation between the mean FSH concentrations during the first 24h after cloprostenol injection and the number of large follicles observed at laparoscopy 5–6 days after ovulation within the follicular fluid treatment group ($r=0.534$, $P<0.05$, $N=7$). No such relationship was evident within the control group.

**Progesterone:** The decline in plasma progesterone concentrations after cloprostenol-induced luteolysis are illustrated in Fig. 7.2.b. Progesterone levels had decreased by 1h after cloprostenol injection in both groups and were significantly lower relative to time zero by +1h in the control group ($P<0.05$) and by +2h in the treated group ($P<0.05$). Linear regression analysis of the individual slopes showed that progesterone levels fell significantly with time in all ewes ($P<0.05$) and that the overall rate of decline during the first 10h after cloprostenol was greater in follicular fluid treated ewes than in controls ($P<0.02$). Progesterone levels were at or below basal concentrations ($<0.3\text{ng/ml}$) by 24h after cloprostenol injection in both groups.

**Pulsatile LH secretion; data analysis**

The LH pulse frequency data is presented as changes in inter-pulse interval which was calculated as the time (multiples of
Changes in LH inter-pulse interval (multiples of 10 minutes) after the injection of cloprostenol (PG) on Day 11 of the cycle in control ewes, and ewes injected with bovine follicular fluid throughout the luteal phase. Values are mean ± s.e.m.

Fig. 7.4
Fig. 7.5 Changes in LH pulse frequency per 8h block after the injection of cloprostenol (PG) on Day 11 of the cycle in control ewes, and ewes injected with bovine follicular fluid throughout the luteal phase. Values are mean ± s.e.m.
Fig. 7.6. Changes in LH pulse amplitude after the injection of cloprostenol (PG) on Day 11 of the cycle in control ewes, and ewes injected with bovine follicular fluid throughout the luteal phase. Values are mean ± s.e.m.
Fig. 7.7 Changes in mean LH pulse amplitude per 8h block after the injection of cloprostenol (PG) on Day 11 of the cycle in control ewes and ewes injected with bovine follicular fluid throughout the luteal phase. Values are mean ± s.e.m.
10 minutes) between the peak of interest and the preceding LH pulse (Fig. 7.4). The inter-pulse interval is easier to visualise than fractions of pulses per hour and conveys the dynamic nature of the change in pulse frequency during the follicular phase. For the purposes of statistical analysis within treatment groups the number of pulses and mean pulse amplitude were calculated for each 8h block after cloprostenol-induced luteolysis (Fig. 7.6 and 7.7). Due to the spread in time of onset of behavioural oestrus and hence the LH surge within both groups it was only possible to compare the changes in pulsatile LH secretion until +40h and +64h after cloprostenol injection in control and follicular fluid treated ewes respectively. Similarly the 10 minute sampling schedule did not extend to the onset of the preovulatory LH surge in all ewes in either treatment group. Thus the data could not be analysed relative to the LH surge.

One ewe in the control group (1J482) was excluded from the pulsatile LH secretion analysis. Although regular oscillations above basal concentrations were observed in this ewe throughout the follicular phase the amplitude of these 'pulses' was too low to determine whether or not they represented significant pulses using the criterion defined previously. A selection of samples from this ewe were measured using the LH bioassay but this did not clarify the 'pulses' further.

Changes in pulse frequency

Control ewes: The inter-pulse interval was initially long in control ewes (118 ± 6 minutes) and decreased rapidly to reach a plateau of approximately 1 pulse/60 minutes within 6h after cloprostenol injection in all ewes (Fig 7.4 ). Thereafter a more gradual decrease in inter-pulse interval with time was apparent
Fig. 7.8 Changes in mean LH level per 8h block after the injection of cloprostenol (PG) on Day 11 of the cycle in control ewes and ewes injected with bovine follicular fluid throughout the luteal phase. Values are mean ± s.e.m.
Fig. 7.9. Changes in the pulsatile secretion of LH in a representative control ewe (OJ312). Blood samples were withdrawn at 10-minute intervals for 6 h on Day 6 of the luteal phase and from 2 h before to +6 h after injection of cloprostenol on Day 11 of the cycle. Each peak represents a significant pulse. The hatched area indicates when lights were off.
Fig. 7.10 Changes in the pulsatile secretion of LH in a ewe treated with follicular fluid (OJ404). Blood samples were withdrawn at 10 minute intervals for 6h on Day 6 of the luteal phase, and from 2h before to +56h after injection of cloprostenol on Day 11 of the cycle. Each \( \uparrow \) represents a significant pulse. The hatched area indicates when lights were off.
until a minimum pulse interval of approximately 50 minutes was reached by 40h after cloprostenol injection. Correspondingly this increase in pulse frequency during the follicular phase was significant ($P < 0.001$) when analysed in 8h blocks from zero to +60h after cloprostenol (Fig.7.5). In the profiles of the 3 ewes for which the 10 minute sampling extended to the onset of the preovulatory LH surge there was no further detectable change in pulse frequency (Fig.7.9). A direct statistical comparison of the change in pulse frequency in the luteal compared with the follicular phase is difficult because of the dynamic nature of pulse frequency especially during the follicular phase and also because of the comparatively short sampling period during the luteal phase. Furthermore it was shown in Chapter 5 that pulsatile LH secretion changes between Days 6 and 10 of the cycle in both control and follicular fluid treated ewes. As a result of these observations the pulse frequency during the follicular phase in the present study was compared with both the 6h profile obtained for the same animals during Day 6 of the luteal phase and the 25h profile for a different group of animals on Day 10 of the luteal phase (as detailed in Chapter 5). The general trends were similar in both cases. The mean pulse frequency during the luteal phase (1 pulse/2.6h and 1 pulse/2.3h on Day 6 and 10 respectively) was markedly lower than that observed during the follicular phase (1 pulse/h).

**Follicular fluid treated ewes:** In these ewes the inter-pulse interval was also initially high (120 ± 11 minutes) and decreased rapidly to reach a plateau (1pulse/60 minutes) identical to that of controls within 6h after cloprostenol injection (Fig 7.4 ). The inter-pulse interval remained constant for a longer time period than
in controls before declining in a similar manner to reach a minimum inter-pulse interval of approximately 50 minutes. Pulse frequency showed a significant increase \( (P < 0.001) \) with time during the follicular phase when analysed in 8h blocks between zero and +56h after cloprostenol (Fig.7.5). A complete follicular phase profile in which the frequent sampling encompassed the onset of the preovulatory LH surge was obtained for 2 ewes treated with follicular fluid and showed that there was no further detectable change in LH pulse frequency. (Fig 7.10).

Comparison of the luteal compared with the follicular phase of the cycle showed that the mean pulse frequency during the luteal phase in ewes treated with follicular fluid \( (1 \text{ pulse/1.6h} \text{ and } 1 \text{ pulse/1.4h on Day 6 and 10 respectively}) \) was also distinctly lower than that observed during the follicular phase \( (1 \text{ pulse/h}) \). However the magnitude of the increase in pulse frequency in the follicular compared with the luteal phase was considerably less in ewes treated with follicular fluid than in controls.

Changes in pulse amplitude

Immediately after cloprostenol injection there was a significant increase in LH pulse amplitude with time for the first 5 pulses measured in samples withdrawn from both control and follicular fluid treated ewes \( (r=0.958, P<0.01 \text{ and } r=0.816, P<0.05 \text{ respectively, see Fig.7.6}) \).

There was no significant difference between treatment groups in the rate of increase in amplitude of these pulses as determined by analysis of the slopes of the individual regression lines. Thereafter in the control group pulse amplitude decreased rapidly and did not significantly change with time throughout the follicular
Overall the mean pulse amplitude in the control group during the follicular phase between zero and +40h after cloprostenol was not significantly different to that observed during Day 6 of the luteal phase (2.0 ± 0.2 versus 2.0 ± 0.3 ng/ml respectively see Fig.7.7). In contrast pulse amplitude in the follicular fluid treated group remained high for up to 16h after cloprostenol injection before declining to a lower baseline (Fig.7.7). Subsequently between +16 and +64h after cloprostenol there was no significant change in the mean pulse amplitude which was similar to that of controls. In addition the pulse amplitude in ewes treated with follicular fluid was significantly higher (P<0.05) on Day 6 of the luteal phase than between +16h and +64h after cloprostenol injection (3.0 ± 0.3 versus 1.9 ± 0.2ng/ml).

Changes in basal and mean LH levels

Basal LH levels did not change significantly throughout the follicular phase in control ewes (2.1 ± 0.1ng/ml). In contrast basal LH levels were initially high (3.4 ± 0.3ng/ml) between zero and +16h in the follicular fluid treated group coincident with the high pulse amplitude observed at this time. Thereafter basal LH levels (2.0 ± 0.1ng/ml) were equivalent to those of controls and did not change significantly with time.

In spite of the slight increase in pulse frequency throughout the follicular phase in control ewes, mean LH levels were relatively constant throughout the follicular phase as demonstrated by analysis of the mean concentrations per 8h block (Fig.7.8). Similarly after the initial 16h of the follicular phase in treated ewes, when LH pulse amplitude was high, the mean LH concentration decreased to a level similar to that of controls and did not fluctuate.

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significantly throughout the remaining sampling period (Fig 7.8b).

Figures 7.9 and 7.10 illustrate the changes in pulsatile LH secretion after cloprostenol-induced luteolysis in a representative control and follicular fluid treated ewe respectively. These specific examples were chosen as they show a complete profile of the changes in pulsatile LH secretion from cloprostenol-induced luteolysis to the onset of the preovulatory LH surge.

Neither LH pulse frequency or amplitude appeared to vary with photoperiod during the follicular phase.

None of the parameters of pulsatile LH secretion measured during the luteal and/or follicular phase of the cycle were correlated with the subsequent ovulation rate either within treatment groups or in the animals as a whole.

**Preovulatory LH surge**

There were no significant differences in basal LH concentrations measured in 2h samples collected 24h before the preovulatory surge or in the magnitude or duration of the preovulatory LH surge between control and follicular fluid treated ewes (Table 7.1). The onset of the preovulatory LH surge was significantly later relative to cloprostenol injection in the treatment than in the control group (P<0.001). Similarly the interval from oestrus to the LH surge was significantly greater in treated ewes (P<0.05) than in control ewes.

**Clearance rate of immunoreactive LH**

The clearance rate of immunoreactive LH in the luteal phase and during the first 4 of the 8h blocks in the follicular phase were compared (Montgomery, Crosbie, Martin & Pelletier, 1984). For each
pulse the slope of decay was calculated by regression analysis of
the log concentration from the peak of the pulse against time
(multiples of 10 minutes). The resulting slopes were pooled across
the pulses within a profile and analysis of variance of repeated
measures used to determine the change in the nature of decay over
time.

The clearance rate of immunoreactive LH was significantly lower
on Day 6 of the luteal phase compared with that measured between
zero and +8h and +8h to +16h after cloprostenol injection in both
control (P<0.025) and follicular fluid treated ewes (P<0.001).
The clearance rate after +16h was similar to that of the luteal
phase in both groups. Similarly there was no significant difference
in the clearance rate on Day 6 of the luteal phase in control
compared with follicular fluid treated ewes. However the clearance
rate was significantly higher in ewes treated with follicular fluid
(P<0.001) compared with controls between 0 to +8h and +8 to +16h
after cloprostenol injection.

Subsequent oestrous cycle

There was no significant effect of follicular fluid treatment
on plasma concentrations of FSH or LH during the subsequent cycle.
Similarly, plasma progesterone levels were characteristic of a
normal cycle in both groups, no differences being found between
basal or mid-luteal levels.

Discussion

Changes in pulsatile LH secretion: control ewes

The results of the present study confirm previous observations
that LH pulse frequency is significantly higher during the
follicular compared with the luteal phase of the oestrous cycle
(Baird et al, 1976; Baird, 1978b; Martensz & Scaramuzzi, 1979; Baird
The initial rapid increase in LH pulse frequency occurring within 6h of cloprostenol-induced luteolysis is most probably the result of falling progesterone levels and is in agreement with the trend observed in a previous study in which samples were withdrawn at 1h intervals after cloprostenol (Baird et al, 1981). Karsch et al (1983) studied ovariectomized steroid-implanted ewes and found that the acute withdrawal of progesterone alone is insufficient to account for the high frequency of LH pulses seen during the follicular phase and have suggested that oestradiol also stimulates the increase in pulse frequency.

It seems unlikely that the initial rapid increase in LH pulse frequency observed in the present study is due to a direct stimulatory effect of oestradiol as it is known that the secretion of oestradiol into the ovarian vein does not begin to increase until between 6 and 12h after cloprostenol injection (Baird et al, 1981). However it is possible that the increase in secretion of oestradiol from the preovulatory follicle(s) is responsible for the more gradual increase in pulse frequency which occurs subsequently.

The transient increase in LH pulse amplitude after cloprostenol injection and before oestradiol secretion has increased suggests that the removal of progesterone has resulted in an increase in pituitary responsiveness to GnRH and hence indicates that progesterone can regulate pulse amplitude. Low levels of progesterone (1ng/ml) have been shown to reduce pituitary responses to GnRH in anoestrous ewes but not in progesterone-treated ovariectomised ewes (Wheaton & Mullet, 1982). This suggests that an ovarian hormone, probably oestradiol, is required to mediate the effects of progesterone. Whether or not the basal oestradiol
secretion at this time was sufficient to mediate this effect in the present study is unknown. Alternatively the initial increase in pulse amplitude may be due to the self-priming effect of GnRH. Crighton & Foster (1976, 1977) using relatively high doses of GnRH (30ug) found that if a second injection of GnRH was administered 1.5h after an initial 'priming' injection there was an increase in the magnitude of the LH response. Thus in the present study the increase in pulse frequency after cloprostenol injection may be facilitating a similar self-priming effect.

There is some disagreement regarding the overall changes in pulse amplitude during the oestrous cycle. Several studies have shown that the amplitude of pulses during the luteal phase were greater than during the follicular phase (Baird, 1978b; Martensz & Scaramuzzi, 1979). A similar reciprocal relationship between frequency and amplitude at different stages of the menstrual cycle was observed in women (Santen & Barden, 1975). It was suggested that this apparent decrease in pulse amplitude during the follicular phase in the ewe was due to a direct effect of the increasing oestradiol levels from the preovulatory follicle(s) (Goodman & Karsch, 1980a). However more recent studies have found that pulse amplitudes were similar during the luteal and follicular phases of the cycle (Baird et al, 1981; Karsch et al, 1983). Similarly in the present study there was no significant change in the pulse amplitude in the luteal compared with the follicular phase of the cycle. Furthermore with the exception of the initial transient increase in pulse amplitude there was no change in amplitude with time throughout the follicular phase in spite of the higher pulse frequency and hence presumably rising oestradiol levels.
Previously, no data comparable to that obtained in the present study during the follicular phase has been available. The classical diagrams of the changes in pulsatile LH secretion throughout the oestrous cycle show a marked rise in basal LH levels and pulse frequency at luteolysis which continue to increase throughout the follicular phase of the cycle until the onset of the preovulatory LH surge (Karsch et al., 1980b; Baird & McNeilly, 1981). It seems likely in view of the present study that the relatively short sampling periods on which these diagrams were based may have resulted in a distorted picture of the changes in pulsatile LH secretion during the follicular phase. The results of the present study show convincingly that neither the mean nor the basal LH levels change throughout the follicular phase after the initial rapid increase in pulse frequency at luteolysis. These observations suggest that while the follicle population is stimulated with a higher frequency of LH pulses than that observed during the luteal phase an increment in pulse frequency and a rise in basal LH level throughout the entire follicular phase is not required. It is not known whether the high frequency pulses themselves convey an important endocrine signal or whether they are merely the most efficient way of ensuring a sustained LH signal to the growing follicles. McNeilly et al., (1984), showed that immunoneutralization of GnRH during the follicular phase resulted in an immediate cessation of pulsatile secretion of LH and oestradiol while basal LH levels were maintained. This suggests that a pulsatile mode of LH release is important to ensure a sustained LH signal to the growing follicles and hence an increase in ovarian oestradiol secretion. Although both pulsatile (at a constant or increasing frequency) and
non-pulsatile administration of LH and GnRH during seasonal anoestrus can result in successful follicle development and ovulation (Karsch et al, 1980a; McNatty et al, 1981; McNeilly et al, 1982; McLeod et al, 1982 a,b,1983), McNatty et al (1981) showed that a pulsatile delivery of exogenous LH to the ovary was more effective than constant administration in that subsequent luteal function was impaired in the latter group. Furthermore it has been shown that constant infusions of GnRH do not induce a constant release of LH in sheep in that levels are initially stimulated and then decline (Sandow, Seeger, Heptner & Konig, 1973; Chakraborty, Adams, Tarnavsky & Reeves, 1974; Hooley et al, 1974). In contrast exogenous pulses of GnRH will maintain high rates of LH secretion for many weeks in the ram (Lincoln, 1979) and for at least 10 days in the female rhesus monkey (Wildt et al, 1981). It seems reasonable to suggest therefore that in the ewe a pulsatile delivery of LH is preferred and that the high LH pulse frequency observed during the follicular phase is the most efficient way in which a sustained LH level, necessary for the final maturation of the preovulatory follicle(s) can be maintained.

Changes in pulsatile LH secretion - follicular fluid treated ewes

From the studies described in Chapters 5 and 6, it was concluded that the high LH pulse frequency observed during the luteal phase in follicular fluid treated ewes occurred because of the suppression of plasma FSH concentrations resulting in a reduction of negative oestradiol feedback. Furthermore, it was suggested that oestradiol was a major negative feedback hormone regulating pulse frequency as these changes occurred in spite of normal (Chapter 5) or high (Chapter 6) progesterone levels. Similarly in the present study the LH pulse frequency in treated
ewes was also significantly higher than in controls during the luteal phase. However a further rapid increase in pulse frequency similar to that observed in control ewes occurred within 6h after cloprostenol injection. This supports the suggestion made previously for control ewes, that the increase in pulse frequency is due to the fall in progesterone levels during luteolysis as the amount of oestradiol being secreted from these follicles in follicular fluid treated ewes was presumably still very low. This further increase in pulse frequency in excess of that observed during the luteal phase suggests that progesterone does operate synergistically with oestradiol to regulate pulse frequency but that its role is probably a minor one.

After the initial rise in pulse frequency there was an extended period relative to controls during which pulse frequency did not change. This delay before the gradual increase in pulse frequency occurred corresponded to the delay in oestrus onset observed in treated ewes and suggests that the delay in oestradiol production by the follicles was temporarily preventing the subtle increase in pulse frequency.

The LH pulse amplitude was also significantly higher in treated ewes compared with controls during the luteal phase in the present study. However a further initial increase in pulse amplitude was observed after cloprostenol injection which occurred at a similar rate to that of controls. As oestradiol secretion by the follicles is known to be low at this time in follicular fluid treated ewes it seems most probable that this initial stimulation of pulse amplitude is due to a self priming effect of GnRH. As the follicles grow and respond to the high FSH concentrations and elevated LH secretion
the oestradiol production is presumably rising to reach concentrations similar to those of control ewes which in turn results in a gradual fall in pulse amplitude. Thereafter pulse amplitude did not vary throughout the follicular phase and was similar to that of control ewes. It appears therefore that a threshold level of oestradiol secretion is required to maintain normal LH pulse amplitude and that an increase in oestradiol production beyond this threshold, as occurs during the follicular phase, cannot suppress pulse amplitude further.

Changes in FSH secretion

In contrast to LH, the secretion of FSH in control ewes decreased with time during the first 18h after cloprostenol-induced luteolysis and is in agreement with previous observations of a general decline in FSH levels during the follicular phase (Salmonsen et al, 1973; Baird et al, 1981; McNeilly, 1984). The suppression of FSH at this time is probably due to the secretion of oestradiol and inhibin as the secretion of both of these hormones increases in parallel with the development of the preovulatory follicles(s) (Tsonis et al, 1983).

The present study has confirmed previous observations in Chapters 3, 4 and 5 that treatment with bovine follicular fluid resulted in a significant rebound in FSH levels after the cessation of treatment at cloprostenol-induced luteolysis. This rebound may reflect the release of pituitary FSH stores accumulated in the pituitary gonadotrophs during treatment or may equally be a reflection of low endogenous inhibin and steroid production due to insufficient FSH stimulation of the follicle population during follicular fluid treatment. Hence when the follicular fluid
treatment ends there is a hypersecretion of FSH as the pituitary releases its stores and follicle development progresses. As the follicles grow and become oestrogenic in response to LH stimulation they secrete sufficient oestradiol and inhibin into the circulation and the rebound ceases. This is confirmed by the observation that the delay in the onset of oestrus which was most probably due to insufficient oestradiol production by the small follicles was similar to the duration of the hypersecretion of FSH.

Gonadotrophin secretion and ovulation rate

In previous experiments the hypersecretion of FSH after the cessation of follicular fluid treatment was considered to be the most probable cause of the increase in ovulation rate. In the present study follicular fluid treatment did not significantly increase ovulation rate but the ovulation rate of control ewes (3.3) was considerably greater than that normally recorded for Damline ewes (2.25 – 2.5). This highlights one of the problems of working with a prolific breed and suggests that the animals used in the present study were already expressing their maximum ovulation rate. However the number of large follicles (> 5mm diameter) observed at laparoscopy 5 – 6 days after ovulation in the follicular fluid treated group was significantly higher than that of controls and was correlated with the magnitude of the FSH rebound during the initial 24h of the follicular phase. It is tempting to suggest that the hypersecretion of FSH at this time may have resulted in either an increase in the number of antral follicles in the population or a decrease in the incidence of atresia within existing follicles.

In spite of the failure of follicular fluid treatment during the luteal phase to increase ovulation rate in the present
experiment the changes in LH secretion observed were considered to be characteristic of those normally observed after follicular fluid treatment. As discussed in Chapters 3 and 6 it is unlikely that the follicle population was sufficiently developed to benefit from the high pulse amplitude or frequency observed during the luteal phase or indeed from the high pulse amplitude during the early follicular phase. As the subsequent pulsatile LH secretion during the follicular phase is similar in both groups it seems unlikely that quantitative differences at this stage of the cycle could be associated with the change in ovulation rate observed in previous studies.

However it is perhaps not surprising that in this and other studies it has largely proved impossible to quantify differences in gonadotrophin secretion with changes in ovulation rate. The sensitivity of the hypothalamic-pituitary axis to the feedback effects of steroids and inhibin may vary widely between individual ewes at different times of the cycle. Similarly the amount of feedback especially in a prolific breed may vary with ovulation rate but not necessarily in a proportional manner. Thus changes in gonadotrophins may or may not occur. These problems were highlighted by one control ewe in the present study. During both the luteal and follicular phases of the cycle pulsatile LH secretion was largely undetectable by either radioimmunoassay or bioassay. This ewe subsequently ovulated and had four normal corpora lutea. This suggests that the large quantities of oestradiol being produced by the follicles resulted in enhanced negative-feedback and hence low LH secretion.
CHAPTER 8: EXPERIMENT 6

Introduction

The studies detailed in earlier chapters have shown that treatment of prolific Damline ewes with follicular fluid throughout the luteal phase is generally associated with an increase in ovulation rate at the subsequent oestrus. However the baseline ovulation rate in this breed is normally 2.25-2.5 and hence the potential range for an increase in ovulation rate is lower than in less prolific breeds. Furthermore it was emphasised in Chapter 4 that when ovulation rate was increased above the optimum for the breed by follicular fluid treatment there was a reduction in the percentage of ova represented by viable foetuses at the time of slaughter. It is therefore more commercially important to improve fecundity in less prolific breeds.

The present study was therefore undertaken to determine whether the luteal phase follicular fluid treatment regimen used in previous studies would affect ovulation rate in Welsh Mountain ewes (ovulation rate 1.25).

Materials and Methods

Twenty Welsh Mountain ewes were studied during the breeding season in February 1985. The ewes were 2 to 4 years old and weighed $38.7 \pm 1.3$Kg. Oestrus was synchronised as described earlier and the ewes weighed, ranked and allocated to one of two treatment groups. Ten ewes in the treatment group received 8ml of bovine follicular fluid (i.v.) (Batch 6) at 09:00 and 17:00h on Day 1 (oestrus + 24h) to Day 11 of the cycle inclusive. The 10 remaining ewes received bovine serum and acted as controls. Luteal regression was induced at 10:00h on Day 12 of the treatment cycle using cloprostenol. The
subsequent onset of behavioural oestrus was assessed using vasectomised rams at 08:00 and 17:00h daily between 31 and 103h after cloprostenol injection. Ovulation rate was assessed in half the ewes (N=5/group) at laparoscopy between Day 8 and 10 of the subsequent cycle. The remaining ewes were slaughtered on Day 12-13 of the cycle and the number of corpora lutea counted.

**Blood sampling schedule**

Blood samples were taken twice daily throughout the treatment cycle before follicular fluid or serum injection. On Day 9 of the cycle 10 ewes were cannulated (N=5/group) and blood samples collected every 15 minutes between 10:00 and 16:00h. Three blood samples were withdrawn at hourly intervals before cloprostenol-induced luteolysis on Day 12, thereafter samples were taken at 2h intervals for 48h from all 20 ewes. During the subsequent cycle samples were taken daily at 09:00h until Day 13. All samples were assayed for LH and FSH, and the daily samples of the treatment and subsequent samples for progesterone. The LH assay was based on the R29 antiserum.

**Statistics**

Chi-squared was used to test the effects of treatment on ovulation rate. Student's 't' test was used to test the effect of treatment on onset of oestrus and to analyse the characteristics of the pulsatile secretion of LH on Day 9 of the luteal phase. The differences in hormone concentrations between the two groups were analysed by two-way analysis of variance. Linear regression analysis was used to determine the changes in FSH secretion during the follicular phase in control ewes.
Fig. 8.1. Changes in the plasma concentrations of FSH in blood samples withdrawn before injection of bovine follicular fluid (▼) at 09:00h (●) and 17:00h (○) for (a) 0 or (b) 11 days during the luteal phase of the cycle. Values are mean ± s.e.m.
Fig. 8.2. Changes in the plasma concentrations of FSH in blood samples collected at 15-minute intervals from control (○) and follicular fluid treated (●) ewes on Day 9 of the luteal phase. Values are mean ± s.e.m., N=5 per group.
Results

Oestrus and ovulation rate

Twice daily injections of follicular fluid for 11 days of the luteal phase prior to cloprostenol induced-luteolysis resulted in a significant increase in ovulation rate \( (P < 0.001) \) compared to that of controls; \( 1.9 \pm 0.1 \) corpora lutea (s.e.m.), \( N=10 \), compared with \( 1.2 \pm 0.1, N=10 \) in controls (range 1-2 in both groups). Follicular fluid treatment was also associated with a significant delay \( (P < 0.01) \) in the onset of oestrus relative to controls: \( 71.8 \pm 3.7h \) compared with \( 55.8 \pm 2.8h \), in controls \( (N=10/group) \).

Hormone concentrations during treatment

FSH: The changes in plasma levels of FSH measured twice daily throughout the treatment cycle are illustrated in Fig.8.1. In ewes receiving bovine serum FSH levels were initially high and fell gradually during Days 2 and 3. By 17:00h on Day 4 FSH levels began to increase to reach a peak by Day 6 after which they plateaued for the rest of the cycle. In contrast plasma FSH levels in follicular fluid treated ewes were significantly lower \( (P < 0.001) \) than in control ewes throughout the luteal phase. Plasma FSH concentrations were suppressed by approximately 75% by the 17:00h sample taken 8h after the first follicular fluid injection on Day 1. Thereafter FSH concentrations remained suppressed throughout the luteal phase and were at or below the sensitivity of the radioimmunoassay (4ng/ml) in the majority of samples. Similarly, in samples withdrawn at 15 minute intervals on Day 9 of the luteal phase, plasma FSH concentrations in follicular fluid treated ewes were significantly lower \( (P < 0.001) \) than in controls and did not fluctuate with time during the 6h sampling period (Fig.8.2.).

LH: Twice daily LH concentrations were not significantly
Table 8.1. Effect of treatment with bovine follicular fluid on pulsatile LH secretion on Day 9 of the luteal phase in Welsh Mountain ewes.

<table>
<thead>
<tr>
<th></th>
<th>Control ewes</th>
<th>Treated ewes</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 5</td>
<td>N = 5</td>
<td></td>
</tr>
<tr>
<td><strong>Day 9 Luteal Phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal LH (ng/ml)</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>N.S</td>
</tr>
<tr>
<td>LH pulses /6h</td>
<td>1.6 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>N.S</td>
</tr>
<tr>
<td>LH pulse amplitude/6h (ng/ml)</td>
<td>3.2 ± 0.6</td>
<td>4.1 ± 1.0</td>
<td>N.S</td>
</tr>
<tr>
<td>Mean LH level/6h (ng/ml)</td>
<td>1.8 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>N.S</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
Changes in the plasma concentrations of (a) FSH and (b) LH after the injection of cloprostenol (100ug i.m.) on Day 12 of the cycle in control ewes (O) and ewes injected twice daily with bovine follicular fluid (●) throughout the luteal phase. The blood samples were collected at 2h intervals and values are mean ± s.e.m.
different between treatment groups throughout the luteal phase. Similarly, there was no significant difference in the pulsatile LH secretion measured in 5 control and 5 treatment ewes on Day 9 of the cycle (Table.8.1.).

**Progesterone:** There was no significant difference in plasma progesterone concentrations between groups. Initially plasma progesterone concentrations were all $< 0.2$ng/ml, characteristic of oestrus. Concentrations began to rise on Day 3 of the cycle and reached peak values of $4.9 \pm 0.4$ and $5.8 \pm 0.6$ng/ml for the control and treated ewes respectively on Day 12.

**Hormone concentrations after luteal regression**

**FSH:** The changes in plasma FSH concentrations after cloprostenol-induced luteal regression are illustrated in Fig.8.3.a. Plasma FSH levels in follicular fluid treated ewes were initially low on Day 13 and increased after luteolysis to reach a maximum of $82.2 \pm 11.3$ ng/ml (range 49-169 ng/ml) between 8 and 36h after cloprostenol injection ($18.0 \pm 2.9$h). In contrast linear regression analysis of the individual slopes of control group ewes showed that there was a significant negative regression with time between 0 and $+24$h after cloprostenol injection in 9/10 ewes (range $r = -0.463$ to $-0.978$, $P<0.001$). This represented a fall of approximately 30% compared with initial levels at time zero. There was no change in FSH concentration with time in the remaining ewe. Thereafter FSH concentrations in control ewes remained low until the onset of the preovulatory FSH surge which began in 2 control ewes $+42$h after cloprostenol. Consequently the data were analysed until $+40$h. Plasma FSH concentrations in follicular fluid treated ewes were significantly greater ($P<0.005$) than in controls between $+2$ and
+40h after cloprostenol injection. This represents an approximately 2-fold increase in FSH concentrations in follicular fluid treated ewes as reflected by the mean FSH concentrations during this period, (22.7 ± 1.4ng/ml in control ewes and 40.3 ± 4.6ng/ml in treated ewes).

Since only 2 control ewes had a double and 1 treated ewe a single ovulation it was not possible to correlate FSH levels during the follicular phase with ovulation rate within treatment groups.

LH: The changes in plasma LH concentrations after luteolysis are shown in Fig.8.3.b. Within 8h of cloprostenol injection in control ewes there was a significant rise (P<0.01) in the concentration of LH reaching a maximum at 9.9 ± 0.9h after injection. Thereafter plasma LH concentrations declined slightly but remained higher than that observed prior to luteolysis throughout the follicular phase sampling period. Similarly in follicular fluid treated ewes plasma LH levels increased significantly (P<0.01) within 4h of luteolysis induction, reaching maximum concentrations by 10.1 ± 0.8h after cloprostenol. The follicular fluid treated ewes had significantly higher (P<0.005) plasma LH levels than controls in the period +2 to 24h after cloprostenol injection. Thereafter LH levels did not vary significantly between groups.

Subsequent oestrous cycle

There was no significant effect of treatment on plasma concentrations of FSH or LH during the subsequent oestrous cycle. Plasma progesterone levels were characteristic of a normal cycle in ewes of both groups with no differences being found in basal or mid-luteal values in spite of the observed differences in ovulation
rate between groups. Similarly, when the individual progesterone profiles were grouped and analysed according to ovulation rate irrespective of previous treatment no significant differences were found in either the basal or mid-luteal levels.

**Discussion**

Administration of bovine follicular fluid to Welsh Mountain ewes throughout the luteal phase of the cycle resulted in a highly significant increase in ovulation rate. This confirms previous observations using prolific Damline ewes and suggests that inhibin containing preparations could also be successfully used to increase fecundity in a less prolific breed. The percentage increase above baseline ovulation rate in Welsh Mountain ewes was greater than that observed in the prolific breed. Similarly previous studies using immunization against androstenedione to increase ovulation rate have been more successful in less prolific breeds (Scaramuzzi, Geldard, Beels, Hoskinson & Cox, 1983).

In contrast to previous observations in Damline ewes (Chapters 3-7) the twice daily injection schedule in the present study was associated with the complete suppression of plasma FSH concentrations throughout the luteal phase, with no 'day to day' rebound occurring. This may reflect differences in sensitivity to inhibin activity between the two breeds. Conversely previous observations have shown that ovariectomised Booroola Merino ewes were more sensitive to injections of follicular fluid than the less prolific control Merino strain, with FSH suppression occurring 24h earlier in the former group (Cummins et al, 1983). However it is not known whether this difference in sensitivity occurs in intact
Merino ewes. Alternatively as the weight of the Welsh Mountain ewes used in the present study was approximately 66% of that of Damline ewes the greater suppression of FSH levels may simply be a dose effect. Similarly as the plasma FSH concentrations in control Welsh Mountain ewes were generally between 10 and 15 ng/ml lower than that of Damline ewes throughout the luteal phase, the minimum effective dose of follicular fluid required to suppress FSH levels was probably less in Welsh Mountain ewes.

In the present study there was no detectable effect of follicular fluid treatment on any of the parameters of pulsatile LH secretion. In view of the fact that FSH levels were suppressed throughout follicular fluid treatment, oestradiol secretion by the follicle population is assumed to have been very low. It seems reasonable to expect that the loss of negative feedback by oestradiol would result in an increase in pulsatile LH secretion as observed previously in Damline ewes. However the amount of steroid feedback normally observed in the two breeds may differ substantially. The number of mature follicles in Welsh Mountain ewes (from the same original flock) secreting > 500pg oestradiol/h in vitro was shown to be approximately 1.3 per ewe during both the luteal and follicular phase of the cycle (Webb & Gauld, 1985b) and is similar to the ovulation rate of 1.2. In Chapter 6 the number of mature follicles dissected from Damline ewes on Day 10 of the luteal phase was 2.3 per ewe. As these mature follicles secrete a high proportion of the oestradiol leaving the ovary (Webb & Gauld, 1985b) it is reasonable to suggest that there may be considerably less oestradiol available to feedback on the hypothalamic-pituitary axis in Welsh Mountain ewes. Presumably
follicular fluid treatment in the present study prevented the growth and development of these mature follicles but the reduction in the negative feedback signal may have been less than that observed in Damline ewes and consequently LH secretion was not significantly effected. Alternatively Welsh Mountain ewes may simply be less sensitive to the negative feedback effects of oestradiol and hence can tolerate low oestradiol levels without a marked change in LH secretion. However these suggestions are made tentatively as pulsatile LH secretion was only measured in 10 ewes (5 per group) during one period of 6h and hence subtle differences in LH secretion may well have been missed.

Although pulsatile LH secretion was not measured during the follicular phase in the present study the rise in the mean LH concentrations after cloprostenol injection in both groups was similar to that observed in Damline ewes (Chapter 7) and probably reflects a marked increase in LH pulse frequency as progesterone levels decline. Furthermore the high LH concentrations in follicular fluid treated ewes compared with controls during the first 24h of the follicular phase is probably a reflection of high amplitude pulses as observed previously in Damline ewes. As the onset of behavioural oestrus was also delayed in this study this suggests that the follicle population were secreting insufficient concentrations of oestradiol to (a) initiate the onset of behavioural oestrus and (b) maintain negative feedback on LH pulse amplitude.

As no significant differences in LH pulse amplitude were observed during the luteal phase, the high LH secretion in follicular fluid treated ewes after the decline in progesterone
levels suggests that the normal progesterone levels observed during the luteal phase are playing a major role in regulating pulse amplitude. The observations are in direct contrast with those discussed previously (Chapter 5-7) and suggest that the feedback relationship between the ovary of this normally monovular breed and the hypothalamic-pituitary axis may differ considerably from that of the prolific Damline breed.

It seems unlikely that the follicles from follicular fluid treated ewes were sufficiently developed at luteolysis to respond to the initially high LH secretion observed during the early follicular phase. In vitro evidence (McNatty, 1982) suggests that in the sheep FSH is required for the increase in granulosa cell aromatase activity which normally occurs during preovulatory follicle development and results in the increase in preovulatory oestradiol secretion by increasing the conversion of thecal androgens (Baird & McNeilly, 1981). Thus it seems probable that the hypersecretion of FSH in the present study resulted in more follicles being recruited to ovulate before oestradiol and inhibin secretion by the preovulatory follicles suppressed the secretion of FSH and prevented further growth of those follicles at a slightly less advanced stage of development (see Baird, 1983). In addition these results indicate that even in a characteristically monovular breed there are many follicles which can develop into normal preovulatory follicles when exposed to a stimulatory gonadotrophic environment. Similarly the results of a recent study showed that the infusion of exogenous FSH to increase the level of FSH by approximately 100% around the time of luteolysis in Welsh Mountain ewes resulted in 6-fold increase in ovulation rate (Baird et al, 1984).
These results suggest that the manipulation of endogenous FSH secretion in the ewes by administration of follicular fluid during the luteal phase can result in a significant increase in ovulation rate. Furthermore this controlled increase in ovulation rate from 1 to 2 in a traditionally low fecundity breed may be more commercially useful than treatments such as PMSG where excessive ovulation rates (>3) often result.
CHAPTER 9: EXPERIMENT 7

Introduction

All studies described previously suggest that the most probable reason for the observed increase in ovulation rate is the hypersecretion of FSH during the follicular phase after the cessation of follicular fluid treatment at luteolysis.

However the 10-day treatment regime used in most of these studies is obviously impracticable for the sheep industry. As it is desirable to minimise intensive animal handling it seems reasonable to suggest that follicular fluid treatment could be most successfully used if combined with existing oestrus synchronisation techniques. A small preliminary trial was therefore undertaken to determine if the endogenous rebound in FSH concentrations could be manipulated with only 1 or 2 injections of follicular fluid around the time of cloprostenol-induced luteolysis.

Materials and Methods

Experimental design

Twenty-one Damline ewes were studied during the breeding season in January, 1985. The ewes were 3 to 6 years old and weighed 61.6 ± 1.5Kg. Oestrus was synchronised as described previously and the ewes weighed, ranked and allocated to one of three treatment groups so that weights were equivalent between treatments at the start of the study.

Group FFA (N=7) were injected (i.v.) with 10ml of bovine follicular fluid (Batch 6) at 19:00h on Day 10 (13h before cloprostenol injection) and again at 08:00h on Day 11 of the luteal phase (Day 1 = oestrus + 24h). Group FFB (N=7) received 10ml follicular fluid at 08:00h on Day 11 only and a further group (N=7)
Table 9.1. Effect of treatment of ewes with bovine follicular fluid on time to onset of oestrus from cloprostenol-induced luteal regression, ovulation rate and the number of large follicles at laparoscopy.

<table>
<thead>
<tr>
<th>Group</th>
<th>Onset of oestrus (h)</th>
<th>Ovulation rate (range)</th>
<th>No. of large follicles (&gt;5mm diameter) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43.4 ± 2.9</td>
<td>2.7 ± 0.2 (2 - 3)</td>
<td>1.4 ± 0.4c (0 - 3)</td>
</tr>
<tr>
<td>N=7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group FFA</td>
<td>57.1 ± 5.9</td>
<td>3.3 ± 0.3 (2 - 4)</td>
<td>2.6 ± 0.4a (1 - 4)</td>
</tr>
<tr>
<td>N=7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group FFB</td>
<td>50.3 ± 1.5</td>
<td>3.1 ± 0.4 (2 - 5)</td>
<td>3.6 ± 0.2b (3 - 4)</td>
</tr>
<tr>
<td>N=7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ac,ab,P<0.05; bc,P<0.01

Values are mean ± s.e.m.
received bovine serum at both injection times and acted as controls. Luteolysis was induced in all ewes using cloprostenol within 10 minutes of the 08:00h injection on Day 11. The subsequent onset of oestrus was assessed using a vasectomised ram at 08:00 and 16:00h between 24 and 88h after cloprostenol injection. Ovulation rate was determined by laparoscopy between Days 6 and 8 of the subsequent cycle.

Blood sampling was kept to a minimum with samples being taken from all ewes at -15, -13, -2 and 0h before cloprostenol-induced luteolysis. Thereafter samples were taken at 4h intervals for 12h and than at +24 and +32h after cloprostenol injection, just before oestrus detection. All samples were assayed for FSH.

Statistics

One-way analysis of variance was used to test the effects of treatment on onset of oestrus, ovulation rate and the number of large follicles at laparoscopy. Duncan's New Multiple Range test was then used where appropriate.

Results

Oestrus and ovulation rate

The injection of bovine follicular fluid 13h before and coincident with cloprostenol-induced luteolysis (Group FFA) resulted in a marginal increase in ovulation rate and delay in the onset of oestrus compared with control animals. Similar results were observed in ewes which received a single injection of follicular fluid at the time of cloprostenol-induced luteolysis only. (Table 9.1). There were significantly more large follicles (>5mm diameter) as assessed at laparoscopy in both follicular fluid treated groups than in controls (P<0.05 for Group FFA and P<0.01 for Group FFB).
Fig. 9.1. Changes in the concentration of FSH around cloprostenol-induced luteolysis in (a) control ewes, (b) ewes injected with 10ml bovine follicular fluid coincident with cloprostenol and (c) ewes injected with 10ml bovine follicular fluid 13h before and coincident with cloprostenol. Values are mean ± s.e.m.
In addition 2 ewes in Group FFA had a large pre-ovulatory follicle at laparoscopy (i.e. Day 6 of the subsequent cycle). No correlation existed between the delay in onset of oestrus and the subsequent ovulation rate or the number of large follicles.

**Plasma FSH concentrations**

The changes in plasma FSH concentrations before and after cloprostenol-induced luteolysis are illustrated in Fig.9.1. The initial plasma FSH levels at -15 and -13h before cloprostenol injection were similar in all 3 groups. Linear regression analysis showed that in 5/7 ewes receiving bovine serum FSH levels declined significantly with time by approximately 30%, between zero and +32h after cloprostenol (P<0.05). In the 2 remaining ewes FSH concentrations did not change during the early follicular phase.

The injection of follicular fluid coincident with cloprostenol injection in Group FFB resulted in a decrease in FSH levels to approximately 27% of pretreatment levels by 12h after cloprostenol. By +24h after injection FSH levels had returned to pretreatment values and were similar to those of control ewes. In contrast the injection of follicular fluid at -13h before cloprostenol injection resulted in a significant suppression of plasma FSH concentrations as measured 11h later (P<0.001). At the time of cloprostenol injection FSH levels in Group FFA were approximately 34% of pretreatment levels and significantly lower than in controls (P<0.01). The second injection of follicular fluid coincident with cloprostenol maintained this FSH suppression until at least +12h after injection. By between +24h and +32h after cloprostenol FSH levels had rebounded to reach concentrations approximately 2 fold
greater than in controls in 6/7 ewes. FSH concentrations continued to be suppressed in the remaining ewe and it was interesting to note that this ewe had the lowest ovulation rate in the group (2 ovulations).

**Discussion**

The results of the present study show that injection of bovine follicular fluid 13h before and/or coincident with cloprostenol-induced luteolysis was associated with a marginal increase in ovulation rate at the following oestrus.

Follicular fluid administration resulted in a significant decrease in plasma FSH concentrations in both groups, although only the double injection regime (Group FFA) was subsequently associated with a detectable rebound in FSH secretion. The lack of a detectable rebound in FSH levels in Group FFB suggests that the suppression of FSH concentrations by a single injection of follicular fluid was of insufficient duration to grossly effect oestradiol production by the follicles. Furthermore previous observations have shown that the magnitude of the rebound in FSH concentrations was correlated with the length of follicular fluid treatment (Miller et al, 1982; Chapter 4). The marginal delay in the onset of oestrus also appeared to be related to the length of follicular fluid treatment and hence the duration of FSH suppression and suggested that normal follicle growth had been interrupted. However the effect of the abrupt suppression of FSH before and/or coincident with luteolysis on the follicle population in the present study is not known. Normally the decline in FSH concentrations after cloprostenol is associated with a major decline in the incidence of healthy follicles (McNatty et al, 1982a). It seems
probable therefore that the withdrawal of FSH around luteolysis in the present study would result in either a delay in preovulatory follicle growth or an increase in atresia in the large follicle population. The subsequent increase in FSH concentrations between 12 and 24h after cloprostenol injection would presumably allow the smaller antral follicles to be recruited. The occurrence of a significantly higher number of large follicles at laparoscopy in follicular fluid treated ewes was similar to that described earlier (Chapter 7). Although the mechanism involved in this response is not known the results of the present study suggest that even a relatively short alteration in plasma FSH levels can markedly affect follicle recruitment and/or selection processes.

The use of short-term follicular fluid treatments to manipulate endogenous FSH concentrations and hence ovulation rate is promising. However further investigation is needed to determine a more effective injection schedule. In addition short-term treatments require to be tested in less prolific breeds as this is where any potential commercial benefits exist.
CHAPTER 10: EXPERIMENT 8

Introduction

Seasonal anoestrus can place an economically significant limitation on the reproductive potential of ewes, and hence many attempts have been made to develop methods for the induction of ovulation in anoestrous ewes.

Previous studies have shown that pulsatile or continuous infusion of GnRH will induce preovulatory follicle development and ovulation in seasonally anoestrous ewes (Crighton et al, 1975; McNatty et al, 1982b; McLeod et al, 1982a,b,1983; McLeod & Haresign, 1984). Similarly pulsatile injection of LH alone, at a constant or increasing frequency over a 72h period during anoestrus, will induce ovulation in Finn-Merino (McNeilly et al, 1982) and progesterone primed Romney ewes (McNatty et al, 1981). These results have led to the suggestion that seasonal anoestrus in the ewe is due to an inadequate episodic secretion of LH (McNeilly et al, 1982) and that an alteration in the secretion of FSH is unimportant to the aetiology of seasonal anoestrus. However it has been shown that long term GnRH therapy, administered once every 2h for 40 to 80 days will induce and maintain cyclic activity in anoestrous Romney ewes (McNatty et al, 1982b) while a similar study using LH alone failed to do so beyond two consecutive cycles (McNatty et al, 1984b). As a decrease in plasma FSH levels during LH administration was observed these authors suggested that FSH supplementation as well as LH may be required for the maintenance of cyclic ovarian function in seasonally anoestrous ewes (McNatty et al, 1984b). It seems that in ewes treated with LH alone there is continual negative feedback on FSH release at the pituitary by
oestradiol and inhibin from the follicles induced to develop by LH treatment. In contrast exogenous GnRH treatments maintain FSH secretion at the pituitary so FSH supplementation is not required. Many of the treatments discussed above were associated to some degree with inadequate luteal function. However pretreatment of ewes with PMSG results in ovulation and normal luteal function (Haresign & Lamming, 1978) which also suggests that FSH as well as LH may be required to ensure adequate gonadotrophin priming of the follicles(s) before the preovulatory LH surge.

The basic objective of the present study was to determine the relative contributions of LH and FSH to the induction of ovulation during seasonal anoestrus by administering LH and FSH alone or in combination. Limited data (Haresign, 1975; Land et al, 1976) has suggested that a breed difference may exist in the ability to respond to exogenous treatment during anoestrus. In addition it has recently been suggested that the mechanism controlling the number of mature follicles (and hence ovulation rate) is functional during seasonal anoestrus as well as during the breeding season (Webb & Gauld, 1985a,b). With this in mind, the response to exogenous hormone treatment in a prolific (Damline) and non-prolific (Welsh Mountain) breed were examined. The hypothesis under test was that ovulation and normal luteal function would be easier to induce in the prolific breed with a 'shallow' anoestrus.

Materials and Methods

Experimental animals

Twenty Welsh Mountain and nineteen Damline ewes were studied during seasonal anoestrus in June, 1983. All ewes were known to have been reproductively active as assessed by laparoscopy and heat
Fig. 10.1 Injection schedule for LH and/or FSH in anoestrous Welsh Mountain and Damline ewes.
detection records during the previous breeding season. A
vasectomised ram was introduced into the flock to check that the
ewes were anoestrous.

Plasma LH and FSH before treatment

To determine if there were any significant differences in
plasma gonadotrophin concentrations between the two breeds in
anoestrous before the start of the main experiment, 4 Welsh Mountain
and 4 Damline ewes were randomly selected from the experimental
flock, cannulated and placed in metabolism crates three days before
the start of the main experiment. The following day blood samples
were withdrawn at 15 minute intervals for 12h between 07:00 and
19:00h.

Experimental design

To facilitate intensive handling of the large number of ewes
involved, the study was carried out using two groups of twenty ewes
spaced one week apart. All results were initially analysed
separately. However as no differences in the response to treatment
were found the data were combined for analysis. The ewes were
weighed, ranked and allocated to one of 4 groups so that weights
were equivalent between treatments prior to the start of the
experiment. The experimental design is summarized in Fig.10.1.

**LH alone:** 5 Welsh and 5 Damline ewes were injected with pulses of
LH for 72h with increasing frequency, initially once every 3h for
24h, then once every 2h for 24h and once every h for the final 24h.

**FSH alone:** 5 Welsh and 5 Damline ewes were pulsed with FSH for 36h
followed by saline for 36h. Initially FSH was injected once every 3h
for 24h and then once every 2h for 12h.

**LH & FSH:** 5 Welsh and 4 Damline ewes were injected with LH for 72h

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and simultaneously with FSH (36h) and then saline (36h) as described above.

**Saline:** 5 Welsh and 5 Damline ewes were injected with saline for 72h with increasing frequency and acted as controls.

**Hormones and blood sampling**

Stock solutions of ovine LH S-23 and ovine FSH S-14 [NIH:500ug/ml sterile saline, (9gNaCl/L)] were prepared and diluted appropriately in saline (9gNaCl/L) containing 10% sheep plasma previously obtained from each ewe. Plasma was added to the hormone preparation as this prevents non-specific adsorption of the hormone to the plastic storage tube (A.S. McNeilly, unpublished observations).

The concentration of LH injected (10ug in 1ml) had previously been shown to give a pulse similar in magnitude and duration to that seen in the normal preovulatory period (McNeilly et al, 1982). FSH was initially injected at a concentration of 30ug in 3mls and then 20ug in 2mls. At the injection frequencies used this resulted in a relatively constant level of exogenous FSH, while LH levels remained basal. To minimise stress and prevent contamination of blood samples both jugular veins were cannulated on the day before hormone treatment began. Blood samples were withdrawn at 3h intervals throughout the 72h treatment period which commenced at 09:00h on Day 1. In addition, to check that the exogenous hormone was entering the blood stream a further sample was taken at 09:30h on Days 1, 2 and 3 of treatment 30 minutes after hormone injection. With the exception of these latter samples all blood samples were taken before hormone injection and were withdrawn via the second jugular cannula. After the final hormone injection blood samples were
withdrawn for a further 12h (at 4h intervals) and then at 2 to 3 day intervals for the duration of an expected oestrous cycle.

All blood samples were assayed for LH and FSH. Samples withdrawn at 09:00 and 21:00h on Days 1 to 4 inclusive and all subsequent samples were assayed for progesterone. The LH assay was based on the R3 1/1 antiserum.

Ovulation rate was determined by laparoscopy 6 or 7 days after the final hormone injection.

Definitions

A preovulatory LH surge was defined as occurring when there was a sustained elevation in LH concentrations above 15ng/ml for > 6h. The time of onset of the preovulatory LH surge was taken as the time when LH levels first exceeded 15ng/ml. If more than one rise in plasma LH concentrations was evident the first elevation in LH levels above 15ng/ml coincident with an elevation in FSH was assumed to be the true LH surge. One Welsh Mountain ewe (9M344) did not meet these criteria in that no coincident rise in FSH was detected. However she had a transitory elevation in progesterone concentration following a rise in LH which was subsequently designated as the preovulatory LH surge.

Normal luteal function was defined as an elevation in progesterone concentrations for at least 8 days, starting within 4 days of the preovulatory LH surge and exceeding 1.5ng/ml on at least one occasion.

Statistics

Where appropriate results were analyzed using either Student's 't' test, or a combination of one-way analysis of variance followed by Duncan's New Multiple Range Test where treatment effects were significant.

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Table 10.1  Number of corpora lutea (C.L.) and/or corpora albicantia, characteristics of the preovulatory LH and coincident FSH surges and adequacy of C.L. function in terms of progesterone secretion in anoestrous Welsh and Damline ewes injected with LH or LH + FSH.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Ewe No</th>
<th>Treatment</th>
<th>No. of Corpora lutea</th>
<th>No. of Corpora albicantia</th>
<th>Time from 1st injection to onset of LH surge (h)</th>
<th>Maximum Peak Height of LH Surge (ng/ml)</th>
<th>Maximum Peak Height of Coincident FSH Surge (ng/ml)</th>
<th>Corpus luteum Function</th>
<th>Days of progesterone above baseline</th>
<th>Plasma progesterone above 1.5 ng/ml</th>
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<tr>
<td>Damline</td>
<td>OJ249</td>
<td>LH</td>
<td>0</td>
<td>2</td>
<td>24</td>
<td>43.0</td>
<td>124</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>IJ464</td>
<td>LH</td>
<td>2</td>
<td>0</td>
<td>39</td>
<td>27.2</td>
<td>99</td>
<td></td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>OJ208</td>
<td>LH</td>
<td>0</td>
<td>1</td>
<td>18</td>
<td>50.0</td>
<td>54</td>
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<td>2</td>
<td>-</td>
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<tr>
<td></td>
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<td>2</td>
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<td>131</td>
<td></td>
<td>4</td>
<td>-</td>
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<tr>
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<td>IJ292</td>
<td>LH</td>
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<td>1</td>
<td>24</td>
<td>32.0</td>
<td>102</td>
<td></td>
<td>1</td>
<td>-</td>
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<td>Mean ± s.e.m.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>29.4 ± 4.7</td>
<td>39.6 ± 13</td>
</tr>
<tr>
<td>Welsh</td>
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<td>LH</td>
<td>0</td>
<td>2</td>
<td>69</td>
<td>117.0</td>
<td>206</td>
<td></td>
<td>12</td>
<td>+</td>
</tr>
<tr>
<td>Mountain</td>
<td>8M344</td>
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<td>0</td>
<td>1</td>
<td>45</td>
<td>16.9</td>
<td>9**</td>
<td></td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8M349</td>
<td>LH</td>
<td>0</td>
<td>3</td>
<td>*60</td>
<td>27.8</td>
<td>91</td>
<td></td>
<td>11</td>
<td>+</td>
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<td></td>
<td>IM451</td>
<td>LH</td>
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<td>0</td>
<td>30</td>
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<td>153</td>
<td></td>
<td>11</td>
<td>-</td>
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<td></td>
<td>8N420</td>
<td>LH</td>
<td>0</td>
<td>1</td>
<td>66</td>
<td>59.5</td>
<td>114</td>
<td></td>
<td>7</td>
<td>-</td>
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<tr>
<td>Mean ± s.e.m.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>54.0 ± 7.3</td>
<td>54.6 ± 17.4</td>
</tr>
<tr>
<td>Damline</td>
<td>OJ220</td>
<td>LH + FSH</td>
<td>3</td>
<td>3</td>
<td>30</td>
<td>45.3</td>
<td>231</td>
<td></td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IJ463</td>
<td>LH + FSH</td>
<td>2</td>
<td>1</td>
<td>*27</td>
<td>21.0</td>
<td>169</td>
<td></td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>IJ030</td>
<td>LH + FSH</td>
<td>1</td>
<td>0</td>
<td>*24</td>
<td>54.5</td>
<td>208</td>
<td></td>
<td>9</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>IJ285</td>
<td>LH + FSH</td>
<td>4</td>
<td>0</td>
<td>*24</td>
<td>26.3</td>
<td>90</td>
<td></td>
<td>12</td>
<td>+</td>
</tr>
<tr>
<td>Mean ± s.e.m.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26.2 ± 1.4</td>
<td>36.8 ± 7.8</td>
</tr>
<tr>
<td>Welsh</td>
<td>8M468</td>
<td>LH + FSH</td>
<td>4</td>
<td>0</td>
<td>*27</td>
<td>66.5</td>
<td>231</td>
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<td>8</td>
<td>+</td>
</tr>
<tr>
<td>Mountain</td>
<td>IM351</td>
<td>LH + FSH</td>
<td>1</td>
<td>0</td>
<td>*30</td>
<td>103.5</td>
<td>245</td>
<td></td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8M370</td>
<td>LH + FSH</td>
<td>1</td>
<td>0</td>
<td>21</td>
<td>43.0</td>
<td>154</td>
<td></td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IM401</td>
<td>LH + FSH</td>
<td>0</td>
<td>0***</td>
<td>*30</td>
<td>90.0</td>
<td>258</td>
<td></td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8M312</td>
<td>LH + FSH</td>
<td>0</td>
<td>1</td>
<td>*18</td>
<td>59.0</td>
<td>262</td>
<td></td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Mean ± s.e.m.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25.2 ± 2.4</td>
<td>72.4 ± 10.8</td>
</tr>
</tbody>
</table>

* 2 or > LH peaks.  ** No coincident FSH surge with LH surge in this ewe.  *** This animal had a preovulatory LH surge but no visible corpus luteum or corpus albicans. + Ewe had one corpus luteum but no detectable rise in progesterone.
Fig. 10.2. Concentrations of FSH in blood samples collected at 15 minute intervals during a 12h pretreatment profile in Welsh Mountain (O), and Damline (●) ewes. N=4 per group. Values are mean ± s.e.m.
Table 10.2  Pulsatile secretion of LH in anoestrous Welsh Mountain and Damline ewes during a 12h pretreatment profile. Samples were withdrawn at 15 minute intervals.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Basal</th>
<th>LH pulse Frequency (per 12h)</th>
<th>LH pulse Amplitude (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Welsh Mountain</td>
<td>1.1 ± 0.1</td>
<td>2.3 ± 0.5</td>
<td>10.1 ± 2.3</td>
</tr>
<tr>
<td>(N=4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Damline</td>
<td>1.1 ± 0.3</td>
<td>2.5 ± 0.5</td>
<td>8.1 ± 1.5</td>
</tr>
<tr>
<td>(N=4)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
Results

Ovarian response

At laparoscopy 6 or 7 days after the final hormone injection there was evidence of recent ovulation in all ewes in the LH group (10/10) and in 8/9 receiving LH in combination with FSH. Since no corpora lutea or corpora albicans were seen in any of the ewes treated with saline or FSH alone (N=20), ovulation rate has been assessed by combining both corpora lutea and corpora albicans as shown in Table 10.1, since corpora albicans could only have arisen as a result of the treatment with LH or LH+FSH. The corpora lutea were macroscopically normal and indistinguishable from those observed during the breeding season.

Pretreatment gonadotrophin profile

The plasma levels of FSH, during a 12h profile carried out prior to the start of treatment, were higher in Welsh Mountain than in Damline ewes, but this difference was not significant (Fig.10.2). Analysis of the pulsatile LH secretion during the same period showed no significant differences in LH pulse frequency or amplitude between the breeds at this stage of anoestrus (Table 10.2) with both breeds having approximately 1 pulse per 5h.

LH concentrations during treatment

(a) response to hormone injections

There was no significant change in the concentration of LH in those ewes receiving FSH alone or saline throughout the experiment as determined in samples withdrawn (i) daily at 09:30h, 30 minutes after injection and (ii) at 3h intervals throughout the study.

An increment in plasma LH levels ($3.7 \pm 0.6$ng/ml) was detected
Fig. 10.3. Plasma concentrations of LH (●) and FSH (○) in 4 individual ewes receiving LH injections (i.v.) at an increasing frequency for 72h plus FSH for 36h. Ewe 8M370 (a) had a single LH peak and Ewe IM351(c) had a second LH peak. Both ewes had one corpus luteum at laparoscopy which were subsequently determined to be inadequate. In contrast both Ewes 8M468(b) and IJ285(d) had 2 or more LH peaks and had 4 corpora lutea secreting normal quantities of progesterone.
at 09:30h on Day 1 of treatment in all ewes (N=19) receiving LH alone or in combination with FSH. There were no significant breed differences in the magnitude of this response nor were there any differences between ewes injected with LH alone or LH+FSH. Furthermore there were no significant breed or treatment differences in the basal LH concentrations during the first 24h treatment period. By 09:30h on Days 2 and 3 of treatment, preovulatory LH peaks were evident in a high proportion of ewes, (Table 10.1) and hence, it was not possible to determine the magnitude of response to the LH injections at 09:00h.

(b) preovulatory LH peak(s)

All ewes receiving LH or LH+FSH showed one (N=10) or more (N=9) preovulatory type LH discharge(s) (Fig. 10.3). Two or more LH surges occurred in both breeds and predominantly in the LH+FSH treatment group as indicated in Table 10.1. The interval between the surges varied from 12 to 42h (26 ± 2.9h) and the second peak generally occurred at the end of the 1 per 2h injection period or during the 1 per h period. The presence of 2 or more LH peaks was not related to the subsequent luteal function. These peaks were of similar magnitude and duration but only the first peak was associated with a concurrent rise in FSH levels and thus was considered to be the true preovulatory surge.

The mean interval from the first hormone injection to the onset of the preovulatory LH surge was significantly longer for the Welsh ewes receiving LH alone (P<0.01) compared with the combination treatment and the corresponding Damline groups. The surge in these latter 3 groups began as early as 18h after the first hormone injection. This interval was considerably shorter than the
comparable interval between cloprosentol-induced luteolysis and the onset of the LH surge in the breeding season for Welsh Mountain (N=20; 50.2 ± 7.7h; D.T. Baird, A.S. McNeilly and R. Webb, unpublished observations) and Damline ewes (N=7; 55.1 ± 2.3h; Chapter 7) respectively.

Within a breed there was no significant difference in the mean peak height between treatment groups. However the mean peak height for Welsh Mountain ewes receiving LH alone or in combination with FSH was significantly higher (P < 0.05) than that of the corresponding Damline groups. During the breeding season the LH peak height estimated as 137 ± 20ng/ml for Welsh Mountain ewes (N=20; D.T. Baird, A.S. McNeilly and R. Webb, unpublished observations) and 140.1 ± 24.7 ng/ml for Damline ewes (N=7; Chapter 7), was significantly greater (P<0.01 and P<0.001) for Welsh and Damline ewes respectively than those induced during seasonal anoestrus.

There was no relationship between the occurrence of either corpora albicantia at laparoscopy and the onset and/or magnitude of the preovulatory LH surge.

**FSH concentrations during treatment;**

**(a) response to hormone injections**

There was no significant change in the concentration of FSH in ewes receiving LH alone or saline in samples withdrawn daily at 09:30h, 30 minutes after injection or at 3h intervals throughout the experiment. However there was a significant increase in FSH in both Welsh Mountain (P < 0.01) and Damline (P < 0.05) ewes following the first injection of FSH.

There was no significant difference within breeds between the
response to FSH alone compared with that observed when FSH+LH was administered. Plasma FSH concentrations increased from 51.2 ± 7.4ng/ml to 94.1 ±8.3 ng/ml in Welsh Mountain ewes compared with 41.7 ± 6.5ng/ml to 63.4 ± 5.7ng/ml in Damline ewes. Although the FSH concentration 30 minutes after injection was significantly higher (P < 0.01) in Welsh Mountain than in Damline ewes there was no significant difference when expressed as % change from the pretreatment value (95.7 ± 13.2% versus 69.7 ± 17.5% respectively).

(b) Preovulatory FSH surge

The maximum peak height of the FSH surge coincident with the preovulatory LH surge for individual ewes is shown in Table 10.1. The mean FSH peak height was significantly higher (P<0.05) in Welsh Mountain ewes receiving LH+FSH than in the LH only group. A similar but non-significant trend was observed in the Damline ewes on the same treatment.

Irrespective of treatment the mean FSH peak was higher in Welsh Mountain (N=9, 190.4 ± 21.4ng/ml) compared with Damline ewes (N=9; 134.2 ± 19.3ng/ml). This breed difference is also apparent during the breeding season where the mean FSH peak height, coincident with the preovulatory LH surge was estimated as 147.0 ± 21.5ng/ml for Welsh Mountain (N=20; D.T. Baird, A.S. McNeilly and R. Webb, unpublished observations) and 65.4 ± 16.3ng/ml for Damline ewes (N=7, Chapter 7). Furthermore the mean FSH peak was higher during anoestrus compared with the breeding season in both breeds reaching significance in the Damline ewes (P<0.02).

Progesterone concentrations

The distribution of ewes with corpora lutea at laparoscopy showing normal and abnormal luteal phases are shown in Table 10.1.
Fig. 10.4. Plasma concentrations of LH (●) and FSH (○) in 2 Damline ewes receiving LH+FSH (a) and (c) and in 2 Welsh Mountain ewes receiving LH alone (b) and (d). Despite the similarity between gonadotrophin profiles within groups, (a) and (b) subsequently had a normal progesterone profile while those of (c) and (d) were abnormal.
No trend in distribution between breed or treatment groups was evident. In addition the presence or absence of small transient increases in progesterone above baseline (range 0.3-0.7 ng/ml, Table 10.1) were detected in 4/6 ewes with corpora albicantia visible at laparoscopy. Of the ewes with corpora lutea at laparoscopy only one Welsh Mountain receiving LH+FSH had no detectable rise in progesterone. Irrespective of treatment the mean interval between the first hormone injection and the onset of the preovulatory LH surge was not significantly different in those ewes designated as having normal compared with abnormal luteal function. This is illustrated in Fig.10.4, which emphasises the similarity in gonadotrophin profiles in 2 ewes of each breed which subsequently show abnormal and normal progesterone profiles respectively.

Discussion

These results show that injection of LH, alone or in combination with FSH during seasonal anoestrus will induce a preovulatory LH surge and ovulation in both Welsh Mountain and Damline ewes. This confirms and extends previous observations using LH in Finn-Merino (McNeilly et al., 1982) and Romney ewes (McNatty et al., 1981) and both LH and LH plus FSH in Ile de France ewes (Oussaid, 1982).

The occurrence of corpora albicantia 7-8 days after the preovulatory LH surge was assumed to represent a recent ovulation in response to treatment, the resulting corpus luteum having regressed by the time of laparoscopy. This was supported by the observation that no corpora albicantia were evident in either breed receiving saline or FSH alone. Furthermore some of the corpora lutea which failed to secrete normal levels of progesterone appeared
macroscopically normal at laparoscopy suggesting that the occurrence of a corpus luteum (which subsequently regresses) or a corpus albicans is largely dependent on the time at which laparoscopy is carried out with respect to the time of ovulation. There was no difference in the number of ewes of either breed ovulating in response to LH versus LH+FSH. Welsh Mountain ewes have previously been considered to have a 'deep' and long anoestrous period. However, it appears that during early anoestrus at least they can be induced to ovulate as readily as Damline ewes which are considered to have a 'shallow', short anoestrus largely due to the Finnish Landrace and Dorset Horn components of their genetic make-up. Furthermore although numbers were small, normal luteal function was observed in 2/5 Welsh Mountain ewes injected with LH and 1/5 injected with LH+FSH suggesting that the addition of FSH did not improve the response to LH alone. Similarly, in Damline ewes normal luteal function was observed in 1/5 ewes injected with LH and 3/4 injected with LH+FSH. These results suggest that FSH secretion during seasonal anoestrus was sufficient in both breeds to allow mature follicle development and was not significantly limiting the induction of ovulation and normal luteal function. The results of the present study confirm previous observations that an increase in the number of LH pulses alone is sufficient to induce follicular maturation, resulting in ovulation during seasonal anoestrus (McNeilly et al, 1982; McNatty et al, 1981). All ewes in the present study injected with LH at an increasing frequency had a preovulatory-type LH surge. Although oestradiol was not measured it is assumed that the injection of additional LH induced and sustained oestradiol secretion from the follicles resulting in the
induction of an endogenous preovulatory LH surge and ovulation as shown previously (McNeilly et al., 1982).

However, the rapid onset of the LH surge in Damline ewes injected with LH and both Damline and Welsh ewes injected with LH+FSH suggests that in the present study the majority of the 2-hourly and all the once hourly injections in these groups were not required to induce the preovulatory LH surge. Thus the frequency of LH pulses which induced ovulation (1 per 3h) in the majority of ewes was well below that found during the normal breeding cycle (see Chapter 7). Similarly, other studies have shown that while an increase in plasma LH levels prior to the preovulatory LH surge is critical for follicular maturation and subsequent luteal function, an increase in LH pulse frequency is not a prerequisite for the induction of ovulation (McNatty et al., 1981; McLeod et al., 1983; see discussion Chapter 7).

It has previously been suggested that the rapid onset of the LH surge in post-partum ewes during GnRH treatment allowed insufficient time for follicular development resulting in inadequate luteal function (Wright, Geytenbeek, Clarke & Findlay, 1983). In the present study the time between the first hormone injection and the onset of the LH surge in Damline ewes injected with LH and in both breeds injected with LH+FSH, was considerably shorter than the comparable interval between prostaglandin-induced luteolysis and the onset of the LH surge in the breeding season. This contrasts with a previous observation following LH injection in Finn-Merino ewes (McNeilly et al., 1982) where the two intervals were similar and associated with normal luteal function. However, although the incidence of normal luteal function was low in the present study,
there was no correlation between the interval to the LH surge and subsequent luteal function, indicating that follicular maturation was sufficiently advanced in certain individual ewes prior to treatment to respond quickly and successfully to gonadotrophin stimulation whilst in others the follicles recruited to ovulate were probably immature and resulted in inadequate luteal function. In support of this possibility was the delayed response in Welsh Mountain ewes where fewer follicles were presumably available to respond to LH alone (Webb & Gauld, 1985b) leading to a longer time period for oestradiol to increase sufficiently to elicit a preovulatory surge. Indeed with LH alone the interval to the onset of the LH surge was similar to that observed during the breeding season. It seems probable that when FSH was added follicular development and oestradiol secretion was enhanced and so the preovulatory LH surge occurred some 29h earlier, at a time comparable to that of the LH treated prolific Damline ewes which probably have more mature follicles before exogenous treatment.

A lack of LH at the induced preovulatory LH surge has been suggested as a possible cause of inadequate luteal function (Foster & Crighton, 1974). In the present study, although the maximum peak height of the LH surge was highly variable and lower than that recorded during the breeding season, there was no correlation between the magnitude of the LH surge and subsequent luteal function. This is in agreement with previous studies carried out during the breeding season which demonstrated that there was no relationship between ovulation rates or luteal function and the magnitude and duration of the preovulatory LH surge (Land et al., 1973; Bindon et al., 1978; Quirke et al., 1979; Haresign, 1981).
The occurrence of more than one LH peak in 9/19 animals that ovulated is difficult to interpret. These surges were of similar magnitude and duration but only the first elevation above 15ng/ml was associated with a coincident rise in FSH levels. Furthermore there was no relationship between the occurrence of these multiple LH surges and subsequent luteal function. A previous study (McLeod et al, 1982b) recorded instances of 2 preovulatory LH surges 4-5 days apart during an 8-day GnRH treatment period. However, in the present study the interval between surges was much shorter (26h) suggesting that luteinisation of a further follicle(s) could probably not have occurred. Alternatively it has been observed in normal cycling ewes that small LH surges often occur in association with the large follicle(s) occurring on Day 2 or 3 of the luteal phase prior to the inhibitory rise in progesterone from the corpus luteum (Baird & Scaramuzzi, 1976). As these second LH peaks occurred during the 72h treatment period it is possible that a combination of the continued stimulation of the ovary with LH pulses and the absence of progesterone from the induced corpus luteum result in a second LH surge being produced.

There was no detectable differences in the pattern of FSH secretion immediately prior to the LH surge between ewes injected with LH alone compared with those injected with LH+FSH. Individual ewes varied widely irrespective of breed and treatment with FSH concentrations apparently increasing, decreasing or remaining unchanged before the coincident preovulatory LH/FSH surge. In contrast, during the normal oestrus cycle FSH levels fell by between 30 and 60% during the preovulatory period before the onset of the preovulatory surge (Chapter 7 and 8). This fall in FSH concentrations has been attributed to the rise in oestradiol and
Inhibin production from the developing follicles (Tsonis et al., 1983; McNeilly, 1984). However, the patterns of FSH secretion in the present study are confounded by the early onset of the LH surge and the relatively infrequent blood sampling regimen, as well as the injection of exogenous FSH. Nevertheless, a peak of FSH occurred coincidently with the LH surge in 18/19 ewes which ovulated and was higher than that observed for ewes from the same flocks during the breeding season. A breed difference in the peak height of the FSH surge was measured irrespective of treatment confirming the earlier observation in the pretreatment gonadotrophin profile that FSH levels are generally higher in Welsh Mountain compared with Damline ewes. However, Oussaid (1982) reported considerably higher basal FSH levels in Ile de France ewes studied in May (early anoestrous) compared with June (mid-anoestrous) and suggested that FSH levels may vary with the stage of anoestrus. Similarly, the frequency of LH pulses for Welsh ewes (1 per 5h) measured during the pretreatment sampling period in the present study was greater than that previously recorded (1 per 12h) for this breed in this laboratory (Scaramuzzi & Martensz, 1975; Martensz et al., 1979), indicating that stage of anoestrus may be an important variable to consider when comparing breeds.

Of the 18 ewes with recent evidence of ovulation in the present study only 6 had a normal progesterone profile during the subsequent luteal phase. Similarly, inadequate or short cycles have been observed following induction of ovulation during anoestrus with single (Crighton et al., 1973; Haresign et al., 1975; McNeilly & Land, 1979) or multiple (McLeod et al., 1982a) injections of GnRH and with long term treatment with LH alone (McNatty et al., 1984b).
Conversely, other studies have observed normal luteal function following induction of ovulation with multiple injections of LH (McNeilly et al, 1982) and GnRH (McLeod et al, 1982b). Collectively these studies suggest that the defects in corpus luteum function arise because of defects in the hormonal priming of the follicles from which they arise. Alternatively, it has recently been suggested that suboptimal luteotrophic support in the post ovulatory period may partially explain deficiencies in GnRH-induced corpora lutea as reflected by suboptimal luteal cell number, size and progesterone production (O'Shea, Rogers & Wright, 1984). Certainly evidence obtained from Damline ewes on Days 1-2 of the luteal phase of the normal oestrous cycle indicates that LH pulse frequency is high during the post-ovulatory period (Chapter 5). However, although numbers are small, the continuation of LH injections for up to 48h after the pre-ovulatory surge had been induced in many of the ewes in the present study did not appear to have promoted luteal growth as reflected by plasma progesterone concentrations. Nevertheless it seems probable that the pulsatile LH pattern which was used both before and after induction of the preovulatory LH surge may have been inappropriate in the present study.

In conclusion the present study has confirmed that (a) for two breeds the relative infrequency of LH pulses limits the development of pre-ovulatory follicles during seasonal anoestrus. (b) induced ovulation during seasonal anoestrus is frequently followed by inadequate luteal function and probably reflects either defects in hormonal priming of the pre-ovulatory follicle and/or inappropriate luteotrophic support after ovulation (c) that the requirement for FSH remains unknown, although it may be related to stage of anoestrus.
CHAPTER 11

GENERAL DISCUSSION AND CONCLUSIONS

Our knowledge of the endocrine factors controlling ovulation rate is rudimentary. The growth, development and subsequent ovulation of the preovulatory follicle(s) depends on gonadotrophin stimulation by both FSH and LH. However, while the role of LH in the final stages of follicular maturation and ovulation is relatively well documented, little is known about the role of FSH. Classically FSH stimulates the growth and development of ovarian follicles. During the luteal phase in the ewe FSH secretion is normally sufficient to ensure the development of large antral follicles at all times and has been considered to have a permissive role (Baird, 1983). The administration of exogenous FSH to a variety of species increases the ovulation rate (Greenwald, 1962; Elsdon, Nelson & Seidel, 1978; Wright, Bondioli et al, 1981; Armstrong & Evans, 1983) and suggests that short term regulation of FSH may be important in the control of ovulation.

The initial study described in Chapter 3 was carried out to investigate the role of FSH in follicular development throughout the luteal phase of the oestrous cycle by suppressing plasma levels of FSH with follicular fluid and determining whether ovulation rate was affected at the following oestrus. In this and all subsequent experiments administration of follicular fluid was associated with a marked suppression of plasma FSH concentrations in agreement with previous studies in intact ewes (Miller et al, 1982; McNeilly, 1984, 1985a). It is believed that inhibin is the active component of follicular fluid suppressing FSH release from the pituitary gland in these studies. However it is recognised that other proteins may
exist in follicular fluid and that these may act directly on the ovary. As progress in the isolation and characterisation of inhibin from follicular fluid has been slow (de Jong & Robertson, 1985) pure inhibin preparations are not yet available to confirm or refute whether inhibin is the only component of follicular fluid actively suppressing FSH concentrations in the present studies.

The suppression of plasma FSH levels throughout the luteal phase was associated with a failure of follicle growth beyond 2.7mm diameter (Chapter 6). This provided conclusive evidence that antral follicle growth is FSH dependent as high LH concentrations were maintained throughout the treatment period. However it is recognised that both FSH and LH may be required in that GnRH immunoneutralization results in a failure of follicle growth beyond 2.5mm diameter (McNeilly et al., 1985). The basal in vitro oestradiol production by follicles incubated from ewes treated with follicular fluid was significantly lower than that of controls indicating that the suppression of FSH in follicular fluid treated ewes had resulted in a reduction in the aromatization of androgen to oestrogen. As a consequence of this low oestradiol production the negative feedback signal on pulsatile LH secretion was reduced.

Treatment of Damline ewes with follicular fluid for the entire luteal phase was consistently associated with high LH pulse frequency characteristic of Day 1 of the cycle and a rise in pulse amplitude throughout the luteal phase treatment (Chapters 3-7). The high LH pulse frequency was maintained throughout the cycle despite the attainment of normal or high progesterone levels. This indicated that LH pulse frequency at least in prolific Damline ewes was largely independent of progesterone feedback influences and that
oestradiol is a major negative feedback hormone controlling both LH pulse frequency and amplitude changes during the luteal phase.

After the cessation of follicular fluid treatment at the induced luteolysis a rebound increase in FSH concentrations occurred. The magnitude of this rebound was correlated with the length of follicular fluid treatment and is thought to reflect the release of an accumulation of FSH stores or substances involved in FSH synthesis in the pituitary once the effects of follicular fluid have ceased. In addition the hypersecretion of FSH may partially be the result of low endogenous inhibin and steroid(s) production due to the insufficient FSH stimulation of follicular growth during treatment. When the treatment ends the pituitary releases its stores of FSH and follicular development progresses. As the small follicles grow they presumably secrete sufficient inhibin and steroids into the circulation to feedback on the hypothalamic-pituitary axis and so the FSH rebound ceases. After cloprostenol-induced luteolysis there was also a transient rise in mean LH levels (Chapters 3 and 8). Examination of the pulsatile LH secretion during this period showed that this was due to a rise in pulse amplitude while LH pulse frequency was similar to that of controls.

In the majority of studies described the suppression of follicular growth by follicular fluid treatment resulted in a delay in the onset of oestrus (Chapters 3, 5, 7, 8). As behavioural oestrus is an oestrogen-dependent event (Robinson, 1959) this delay is almost certainly due to insufficient oestradiol production by the developing follicle population. This is supported by the low basal oestradiol production by follicles dissected from treated ewes on Day 10 of the cycle (i.e. on the day before luteolysis is normally
induced). Similarly the length of delay in the onset of behavioural oestrus was similar to the duration of the FSH rebound. Treatment with follicular fluid did not alter the magnitude and duration of the preovulatory LH or coincident FSH surge nor their timing relative to behavioural oestrus (Chapters 3 and 7). This suggests that the periovulatory events were delayed due to lack of follicular development rather than being altered in any way.

The suppression of plasma levels of FSH during the luteal phase was associated with an increase in ovulation rate at the subsequent oestrus. The results reported in Chapter 6 show that had luteolysis been induced the follicles selected to ovulate would have been of a similar steroidogenic capacity and from a less variable follicle size range ($\leq 2.7$mm diameter) than controls. The hypersecretion of FSH following the cessation of follicular fluid treatment presumably allows more of these follicles to be recruited and become oestrogenic before oestradiol secretion from the selected, developing follicles suppressed the secretion of FSH and prevented further growth of these follicles at a slightly less advanced stage of development. The possible contribution of high pulsatile LH secretion to the observed increase in ovulation rate in these studies cannot be ignored. However it seems unlikely that the follicle population was sufficiently developed either before or immediately after luteolysis to benefit from the high pulsatile LH secretion. In Damline ewes the increase in ovulation rate was greatest in ewes treated with follicular fluid for 10 days prior to luteolysis compared with 2 and 6 day groups. This increase in ovulation rate was reflected by a greater number of embryos surviving at Day 30-34 of gestation in the 6 and 10 day treatment
groups (Chapter 4). This emphasises the flexibility of the follicle population in this breed and indicates that a large number of follicles containing viable eggs are available for selection at any one time. Conception rate was not altered by treatment nor was there any evidence of visible embryo abnormalities. However a higher % embryonic loss was observed in the 10 day treatment group. As the optimum ovulation rate for maximum embryonic survival in this study was found to be 3 ova this reflected the increase in ovulation rate (5 ova) above the optimum. Indeed it is clear that stimulation of too great an ovarian response may in fact be detrimental to the productivity of the ewe flock. It is apparent therefore that the use of follicular fluid treatment to increase ovulation rate in less prolific breeds may be more commercially important. With this in mind the 10-day luteal phase treatment was administered to Welsh Mountain ewes (Chapter 8). The resulting controlled increase in ovulation rate from 1 to 2 suggests that inhibin containing preparations could be successfully used to increase productivity in traditionally low fecundity breeds. This controlled increase in ovulation rate, produced by manipulating endogenous FSH secretion within the ewes, is more desirable than established methods of increasing ovulation rate such as the use of PMSG which is associated with a wide variability in response to a standard dose (Bindon & Piper, 1977). In its present form a 10-day follicular fluid treatment regimen is obviously impractical for the sheep industry. However, as soon as inhibin has been purified and sequenced it may be feasible to develop synthetic slow-release preparations to be administered in an implant, minipump or pessary form. Alternatively it may be possible to manipulate the endogenous
Fig. 11.1. Hormone changes throughout the oestrous cycle of the ewe, based on the results presented in Chapters 3-7. Luteolysis was induced on Day 11 of the cycle using cloprostenol. The insets show the change in pulsatile LH secretion during the luteal and follicular phase of the cycle. The concentration of oestradiol in jugular venous plasma is based on that published by Hauger et al. (1977).
FSH secretion in the ewe using only 1 or 2 injections of follicular fluid in combination with existing oestrus synchronisation techniques. With this in mind a small preliminary trial was undertaken using Damline ewes (Chapter 9). Although the results were promising in that a marginal increase in ovulation rate and a higher number of large follicles were observed when follicular fluid was injected prior to and/or coincident with cloprostenol a more effective injection schedule is needed. Furthermore short term treatments require to be tested in less prolific breeds as this is where any possible benefits to the sheep industry exist.

As all the endocrine changes in ewes treated with follicular fluid were assessed relative to control ewes a wealth of control data was obtained which suggests that the published literature on the gonadotrophin changes throughout the oestrous cycle is inadequate.

The sequence of events during the periovulatory period is considered to be similar whether luteal regression occurs spontaneously or whether it is induced by surgical enucleation or cloprostenol injection (Baird & Scaramuzzi, 1976; Legan & Karsch, 1979; Karsch et al, 1979). For convenience the endocrine changes described in this thesis were studied following cloprostenol-induced luteal regression. While it is recognised that the gonadotrophin changes described particularly in the follicular phase may be subtly different from those occurring naturally no comparable data of changes in pulsatile LH secretion in naturally cycling ewes is available. With the exception of the oestradiol profile, Fig. 11.1 summarizes the endocrine changes described in this thesis during the oestrous cycle in Damline ewes.
Within 6h of cloprostenol-induced luteolysis LH pulse frequency increases rapidly from 1 pulse / 2-3h to 1 pulse / h in response to the falling progesterone levels (Chapter 7). These pulses of LH presumably stimulate the secretion of oestradiol and androgens from the follicle as it is known that oestradiol secretion into the ovarian vein begins to rise 6 to 12h after cloprostenol injection (Baird et al, 1981). This increase in oestradiol secretion from the preovulatory follicles is probably responsible for the less dramatic increase in pulse frequency which occurs subsequently. Thereafter pulse frequency reaches a plateau at approximately 1 pulse / 50 minutes and does not increase further prior to the LH surge. The transient increase in pulse amplitude after cloprostenol-induced luteolysis has not been reported previously and may reflect the self-priming effect of GnRH or the removal of progesterone feedback. Thereafter pulse amplitude was similar to that found during the luteal phase. The observation of high LH pulse amplitude during periods of low oestradiol secretion in follicular fluid treated ewes suggests that a minimum threshold oestradiol level is required to maintain normal LH pulse amplitude but that an increase in oestradiol secretion above this threshold as occurs during the follicular phase in control and follicular fluid treated ewes cannot suppress pulse amplitude further.

The combination of the high LH pulse frequency and the increasing amount of oestradiol secreted in response to each LH pulse results in a net secretion of oestradiol which exceeds the rate of clearance and allows the concentration to increase (Baird, 1978a). At some critical threshold concentration or after a period of prolonged high oestradiol levels the preovulatory surge of
LH begins.

The results described in Chapter 5 provided comprehensive data on the changes in pulsatile LH secretion during the luteal phase. On Day 1 of the cycle LH pulse frequency was high and progesterone levels undetectable. As progesterone concentrations increased during the luteal phase LH pulse frequency fell to reach a minimum on Day 10 when progesterone concentrations are at a maximum. It was because of this inverse relationship between progesterone and LH that progesterone was originally proposed as the major negative feedback hormone regulating pulse frequency during the oestrous cycle (Scaramuzzi & Baird, 1976, Hauger et al, 1977). However it is now generally accepted that both luteal progesterone and follicular oestradiol are required to inhibit pulse frequency during the breeding season (see Martin, 1984). Indeed as LH pulse frequency remained high in follicular fluid treated ewes despite a normal rise in progesterone concentrations and low oestradiol levels it is proposed that progesterone can only exert a potent inhibitory effect on LH pulsatility when there is sufficient oestrogen present to sensitise the hypothalamic pulse generator to progesterone. Thus during the luteal phase progesterone appears to determine the overall pattern of LH release while oestrogen determines the upper and lower limits within which LH pulse frequency can fluctuate. When the normal oestradiol negative feedback signal is reduced by follicular fluid treatment or immunization against oestradiol (Scaramuzzi et al, 1980a) normal progesterone levels alone are unable to inhibit LH pulse frequency. LH pulse amplitude did not change throughout the control oestrous cycles examined in the present studies while baseline levels increased in the follicular
compared with the luteal phase (Chapters 3, 5 and 7). Using ovariectomized ewes after hypothalamo-pituitary disconnection, Clarke et al (1984) showed that decreasing GnRH pulse frequency from once per hour to once per 2 hours caused a decrease in plasma LH baseline and an increase in pulse amplitude. Similarly increases in GnRH pulse frequency caused a rise in LH baseline and a reduction in pulse amplitude. However these studies were carried out in the absence of ovarian steroids. As changes in ovarian steroids are almost certainly the signals for changing GnRH release this may account for the apparent discrepancy between these studies and the pulse amplitude data presented in this thesis.

The profile of FSH secretion during the periovulatory period observed in these studies was similar to that described previously in both natural and synchronised cycles (Bindon et al, 1979; Baird et al. 1981; Cahill et al 1981). FSH concentrations declined gradually after luteolysis reflecting the increased secretion of inhibin and oestradiol from the preovulatory follicle(s). At the time of the preovulatory LH surge there was a parallel increase in FSH concentrations followed approximately 24h later by a second more prolonged FSH peak (Chapters 3 and 7). Many studies have reported wide variability within and between ewes in the timing of FSH fluctuations during the luteal phase (L'Hermite et al; Salmonsen et al, 1973; McNeilly et al, 1976; Pant et al, 1977). While it is largely agreed that waves of FSH secretion do exist within individual ewes it has generally been concluded that these fluctuations do not occur at fixed time intervals during the luteal phase (Miller et al, 1981a; Bister & Paquay, 1983; Wheaton et al, 1984). However in the cloprostenol-synchronised cycles studied in
this thesis a definite pattern of FSH secretion emerged (Chapters 3-8). After the secondary FSH peak on Day 1 of the cycle FSH levels fell to reach a nadir on Days 2-3. A third peak occurred on Days 5-6 of the luteal phase followed by a shallow trough on Days 7-9. On Day 10 a fourth peak showing the lowest amplitude was observed in the majority of ewes (Fig. 11.1). Similarly, Lahlou Kassi et al, (1984) found that when the individual secretory patterns based on 3-6h samples during the natural cycle were analysed peaks of FSH occurred on Day 0, 1, 6 and 10 with a fifth peak 87-66h before the next preovulatory LH surge. These peaks were demonstrated in both the prolific D'man and less prolific Timahdite ewes but were not evident using the average secretory pattern. Ink-labelling studies suggest that 2-3 waves of follicle growth occur during the oestrous cycle (Smeaton & Robertson, 1971; Brand & de Jong, 1973; Bherer et al, 1977). Similarly peaks of oestradiol-17B have been observed on Days 3-4 and 6-9 of the luteal phase (Cox et al, 1971; Mattner & Braden, 1972; Baird & Scaramuzzi, 1976) and are presumably an indication of periodic antral follicle activity. If we accept that FSH secretion is controlled by both inhibin and oestradiol feedback systems, then, as the follicles grow in response to FSH, more inhibin and oestradiol will be secreted resulting in a greater negative feedback signal and hence a fall in FSH secretion (Days 2-3). As these follicles become atretic the negative feedback signal decreased and FSH levels rise (Day 5-6). In addition as LH pulse frequency declines during the luteal phase presumably less oestrogen is secreted from the follicle population and hence the control of FSH release by oestradiol at least is diminished. Thus the shallow trough (Day 7-9) and subsequent rise (Day 10) in FSH

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secretion may be largely attributed to the inhibin component of the feedback system. The results of the studies described in this thesis suggest that inhibin is the major negative feedback hormone controlling FSH secretion in the ewe as administration of follicular fluid completely suppressed FSH secretion to undetectable levels in many cases. However it is recognised that pharmacological doses of inhibin were being used and that these may have overridden any possible effects of oestradiol.

The recent development of a highly sensitive bioassay for inhibin based on the inhibition of ovine pituitary FSH secretion in vitro (Tsonis, McNeilly & Baird, 1985) suggests that it will soon be possible to measure inhibin activity in the ovarian vein and peripheral circulation throughout the oestrous cycle. Thus our knowledge of the negative feedback control of inhibin and steroids on FSH secretion will, hopefully, be clarified.

While the physiology of inhibin has yet to be elucidated the results presented in this thesis have shown conclusively that inhibin, in the form of follicular fluid, is a potent regulator of FSH secretion. Furthermore the manipulation of FSH levels during the oestrous cycle has considerable commercial potential in terms of increasing ovulation rate. The data on the changes in FSH and pulsatile LH secretion during the oestrous cycle has expanded our knowledge of the endocrine changes throughout the normal cycle and their consequences for follicle growth.
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