THE RELATIONSHIPS BETWEEN OVARIAN ANTRAL FOLLICLE DYNAMICS, LUTEAL FUNCTION AND ENDOCRINE VARIABLES IN EWES

A thesis submitted to the College of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

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Spring 2001

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ABSTRACT

Transrectal ovarian ultrasonography and hormone measurements were used to study ovarian antral follicular dynamics and development of luteal structures during the middle portion of the breeding season in non-prolific cross-bred Western white-faced ewes and prolific Finn sheep. Studies were also done on ovarian activity in Western white-faced ewes during the transition to seasonal anoestrus and at the onset of the breeding season. Lastly, two experiments were carried out to examine ovulatory responses and subsequent luteal function in Western white-faced ewes treated with luteolysin (PGF₂α) and progestogen (medroxyprogesterone acetate-MAP) during the luteal phase of the oestrous cycle and after ovulation induction with gonadotrophin-releasing hormone (GnRH) in mid-anoestrus.

The results of the present experiments showed that the growth of ovine antral follicles reaching ovulatory sizes of ≥ 5 mm in diameter occurred in a wave-like pattern throughout the oestrous cycle in both breeds of sheep under study. There were typically 3 or 4 waves of follicle production throughout the interovulatory period. Ovarian follicular emergence, or beginning of growth from the pool of 3-mm follicles, appeared to be primarily controlled by changes in circulating concentrations of follicle-stimulating hormone (FSH). In cyclic ewes, the largest ovarian follicles acquired the ability to secrete oestradiol from the day of emergence and a peak of oestradiol secretion occurred about the time they reached their maximum diameter. The high ovulation rate in prolific Finn sheep appeared to be achieved mainly by the ovulation of follicles emerging in the last two waves of the interovulatory interval. Interestingly, prolific Finn ewes produced more but smaller corpora lutea (CL) and had lower serum concentrations of progesterone during the luteal phase of the oestrous cycle as compared to non-prolific Western white-faced ewes.

During the transition into seasonal anoestrus in Western white-faced ewes, FSH secretion resembled that during the breeding season but the pattern of emergence of sequential follicular waves was dissociated from FSH and oestradiol secretion. Prior to the first ovulation of the breeding season, there was a distinct elevation in circulating concentrations of progesterone produced by luteinized unovulated follicles and/or interstitial tissue of unknown origin. This increase in serum levels of progesterone, heralding the resumption of ovulatory cycles, did not alter the rhythmic pattern of FSH secretion or follicular wave emergence.

Treatment of non-prolific Western white-faced ewes with PGF₂α and MAP applied late in the oestrous cycle changed follicular dynamics and increased ovulation rate to resemble that in prolific Finn sheep. Effects of MAP on the recruitment and growth of ovulatory follicles in Western white-faced ewes did not have a clear gonadotrophic dependancy, suggesting a possible local regulation of ovarian activity by progestins in ewes.

Following the induction of ovulation with GnRH in anoestrous Western white-faced ewes, an array of ovarian responses were detected with ultrasonography, including failure of ovulation of large antral follicles, normal (full-lifespan) and short-lived CL post-ovulation, and luteinized cystic-like follicles. The normal luteinization of ovulated follicles appeared to be related to the amplitude of episodic elevations in daily serum FSH concentrations before induction of ovulation and characteristics of the preovulatory LH surge.
ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr. Norman C. Rawlings for his supervision and guidance, for patiently teaching me so much, and for his endless support and understanding throughout my studies and research work in the Department of Veterinary Physiological/Biomedical Sciences.

I would also like to thank Susan J. Cook for her excellent technical assistance and help with many other aspects of my studies. Thanks are also extended to the members of my advisory committee, Drs. T.D. Carruthers, P.J. Chedrese, D.L. Hamilton and R.A. Pierson, for their valuable suggestions and guidance in my program; to Dr. A.P. Beard for teaching and spending so much time with me; to Dr. A.C.O. Evans for his suggestions and advise; to Ms. Margot Buckley and employees at the Animals Care Unit, W.C.V.M., for care and management of experimental animals. Many thanks to my fellow graduate students and summer students working in the Department of Veterinary Biomedical Sciences, for sharing my enthusiasm, stimulating discussions and many hours of help during the present experiments, and to Donna Waldbillig and Christine Smetschka for their technical assistance.

Purified hormones were kindly provided by NIDDK and USDA; PC-Pulsar computer program was provided by Drs. V. Ramirez and J. Gitzen; Cycle Detector Program was kindly provided by Dr. D.R. Bergfelt; and RIA-PC program was developed by Dr. D.L. Rieger.

I would also like to acknowledge the financial support of the College of Graduate Studies and Research, University of Saskatchewan, the Alberta Agricultural Research Institute and the Canadian Sheep Federation funding our research program.

I would like to thank my family, for their everlasting encouragement and support in all my activities.

Finally, I would like to thank my wife, Wianeja, to whom I dedicate this thesis, for her love, patience and strength.
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Chapter 1. LITERATURE REVIEW

1.1 Introduction

The experimental work described in this dissertation primarily concerns the patterns of development of ovarian antral follicles and luteal structures, as monitored by transrectal ultrasonography, and their associations with hormone secretion at different times of the year in ewes. Experiments were also carried out to study follicular dynamics, ovulation rate and subsequent luteogenesis in ewes in the breeding season receiving hormonal treatment to manipulate luteal function, and in anoestrous ewes induced to ovulate with gonadotrophin-releasing hormone (GnRH). Literature reviewed in this chapter deals with the above and allied subject matter in the ewe, but where particularly useful information was lacking for sheep, pertinent references to the literature for other domestic species, laboratory animals and primates have been given. In a few instances, examples are also given on new aspects of human reproduction.

During the last two decades, there have been remarkable changes in many aspects of reproductive physiology and endocrinology of farm animals due largely to advances in real-time ultrasonography, and its amalgamation with technologies such as the use of radioimmunoassay and molecular techniques, etc. These developments greatly augmented our understanding of the control of reproductive processes in the female. With the ability to frequently and non-invasively monitor changes in ovarian morphology, in conscious and non-anaesthetised animals, novel aspects of ovarian function and its control by gonadotrophic and steroid hormones have been revealed. Recent ultrasonographic studies have facilitated a further effort to elucidate the mechanisms governing ovarian activity and increase the efficacy of controlled breeding and ovarian manipulation techniques in ewes.
1.2 Reproductive periodicity in the ewe

Patterns of ovarian function in the adult ewe are dominated by two distinct rhythms. The first of these is a 16- to 17-day long oestrous cycle (Marshall, 1904). The other is an annual rhythm of ovarian cyclicity characterized by a season-dependent cessation (anoestrus) and restoration (breeding season) of ovulatory ovarian cycles (Marshall, 1937; Hafez, 1952).

The length of the ovine oestrous cycle is remarkably consistent throughout the breeding season (McKinszie and Terrill, 1937). There are only small differences (≤ 1 day) in the duration of the oestrous cycle between different breeds of sheep (McKinszie and Terrill, 1937) and little effect of age (Asdell, 1946; Hafez, 1952). Cycles of approximately twice the normal length were occasionally seen in cyclic ewes (McKinszie and Terrill, 1937). It was suggested that such cycles might reflect the incidence of two normal oestrous cycles between which behavioural oestrus and/or ovulation failed to occur (Goodman, 1994). More recent studies have shown that abnormally long cycles in ewes may be associated with the prolonged lifespan of corpora lutea (CL; O'Shea et al., 1986). At the other extreme, short ovarian cycles were observed in ewes at the beginning of the breeding season and during the post-partum period. These cycles were associated with insufficient luteinization and short-lived CL occurring in sheep at the time of shifts in the pattern of ovarian function from non-ovulatory to ovulatory (Hunter, 1991). Generally, however, the length of the ovine oestrous cycle remains unchanged from cycle to cycle.

The ewe is a seasonally polyoestrous animal with normal ovulatory cycles occurring, in most breeds, in the autumn and winter (Hafez, 1952). Unlike the length of the oestrous cycle, durations of the breeding season and anoestrus in sheep are highly variable (Goodman, 1994). An annual rhythm of ovarian activity is superimposed on the normal oestrous cycles and interrupts a continuum of ovarian function (Bittman et al., 1983a-b; Goodman, 1994). Thus, seasonal variations in fertility are evident in ewes in that ovulations do not occur throughout the year; however, antral follicular development appears to be maintained during anoestrus (Hutchinson and Robertson, 1966; Smeaton and Robertson, 1971; McNatty et al., 1984a-b).
1.3 The oestrous cycle and its phases

Traditionally, the oestrous cycle is divided into a number of phases (Arthur et al., 1989). Pro-oestrus is the period immediately preceding behavioural oestrus and is characterized by luteal regression and a marked increase in secretory activity of the entire reproductive system. The uterus enlarges, the endometrium becomes oedematous and uterine glands increase in number. The vaginal mucosa becomes hyperaemic, the number of cell layers in the vaginal epithelium increases and superficial layers become cornified. Oestrus is regarded as the period of acceptance of the male. At the peak of the oestrous period, the ewe usually seeks out the ram and “stands” for him to mount and mate her. The uterine, cervical and vaginal glands secrete increased amounts of mucus, the endometrium and vaginal epithelial layer become congested and hyperaemic, and the cervix is relaxed. In ewes ovulation normally occurs just before the end of this phase, which lasts between 24 to 48 h and tends to be longer in prolific breeds of sheep (Land, 1970; Land et al., 1973; Bindon et al., 1979; Quirke et al., 1979; Goodman, 1994). Ovulation in sheep is a spontaneous process (non-induced ovulators) that does not require the act of coitus. During the pro-oestrous and oestrous phases, there is terminal growth and maturation of ovulatory ovarian follicles in the absence of functional CL; the main ovarian products being follicular oestrogens (Arthur et al., 1989). Pro-oestrus and oestrus are frequently referred to collectively as the follicular phase of the oestrous cycle. The metoestrous phase, succeeding oestrus, is the period during which the granulosa and theca cells of ruptured follicles form lutein cells (luteogenesis), which give rise to the CL (Keyes et al., 1983). This phase is sometimes referred to as the phase of the corpus haemorrhagicum. There is a marked reduction in the amount of secretion from the uterine, cervical and vaginal mucosa. The period of the oestrous cycle when there is a fully functional CL secreting large amounts of progesterone is referred to as the luteal phase of the cycle or dioestrus. During this period, the mucosa of the genital organs is pale, the mucosal glands undergo hypotrophy, the secretions of the reproductive tract are scant, and the cervix becomes constricted and rigid.
Changes taking place in the mucosal layers of the reproductive organs, including the vagina, are reflected in changes in their bioelectrical properties. It has been shown that cyclic changes in vaginal electrical resistance (impedance) occur during the oestrous cycle of sheep (Abdel-Rahim and El-Nazier, 1987; Szczepanski et al., 1994), cattle (Canfield and Butler, 1989), pigs (Ko et al., 1989), and many other domestic and non-domestic species (Moller et al., 1984). In sheep, the impedance of the vaginal mucosa decreases dramatically just before the onset of oestrus and it remains low for 24-48 h until ovulation (Szczepanski et al., 1994). Measurements of vaginal impedance provide a useful tool in determining the stage of the oestrous cycle in ewes, particularly the onset of luteolysis and oestrus, and in detecting ovulations (Szczepanski et al., 1994; Bartlewska et al., 1999).

1.3.1 Hormonal profiles during the sheep oestrous cycle

The oestrous cycle is a sequence of interrelated endocrine events regulated by the hypothalamus secreting GnRH, the pituitary gland producing follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin and oxytocin, ovarian antral follicles that secrete oestrogens and inhibin, the CL secreting progesterone and oxytocin, and the uterine endometrium producing prostaglandin F$_{2\alpha}$ (PgF$_{2\alpha}$; Scaramuzzi et al., 1993). Regulation of cyclic activity is mainly under the control of the hypothalamic-pituitary-ovarian axis. Ovarian follicle development and maturation, steroidogenesis, ovulation, and the formation of CL are controlled by the pituitary gonadotrophins. However, the regulation of secretion and bioavailability of gonadotrophic hormones depend on a complex interaction between several internal and external factors. The internal factors in question include amino acids and peptide/protein hormones and neurotransmitters/noromodulators, ovarian steroids, and uterine products. The external factors such as photoperiodic signals, male pheromones, nutrition and stress, are also known to affect the function of the hypothalamic-pituitary-ovarian axis. Regulation can be achieved directly, through effects on GnRH secretion, or indirectly, by altering pituitary responsiveness to GnRH or ovarian sensitivity to gonadotrophins, heterogeneity of LH/FSH (e.g., isoforms), local blood flow, or the counter-current exchange of hormones between lymphatic and blood vessels.
Just before the end of oestrus ("heat") in the ewe, the discharge in circulating concentrations of gonadotrophins produces ovulation. LH is also responsible for the luteinization of follicle remnants after the rupture of ovulating follicles. As the CL forms, circulating concentrations of progesterone begin to rise. Progesterone acting in concert with oestradiol secreted by large ovarian follicles, inhibits LH secretion (Goodman et al., 1980; Karsch et al., 1980). The reduced luteotrophic support and increased production of PgF\(_{2\alpha}\) by the uterine endometrium lead to the onset of luteolysis. A fall in progesterone secretion during luteal regression and a rise in secretion of follicular oestradiol, trigger behavioural oestrus and the preovulatory surge of gonadotrophins. The preovulatory LH surge terminates oestradiol production, by inducing ovulation and luteogenesis, to initiate the next oestrous cycle. A diagram illustrating major hormonal changes during the interovulatory interval in the ewe is given in Fig. 1.1.

1.3.1.1 Gonadotrophic hormone secretion

In the female sheep, there are two distinct modes of gonadotrophin secretion (Dyer, 1985). The preovulatory discharge of LH and FSH, which reaches a peak about 14 h before ovulation (Arthur et al., 1989), is referred to as the phasic mode of gonadotrophin secretion. This gonadotrophin surge is primarily evoked and sustained by decreased progesterone and increased oestradiol secretion during the final stage of the oestrous cycle (Scaramuzzi et al., 1970; Bolt et al., 1971; Baird and Scaramuzzi, 1976; Karsch et al., 1980; Jeffcoate et al., 1984b; Kaynard et al., 1988; Moenter et al., 1990; Joseph et al., 1992; Rawlings et al., 1984). The tonic, episodic or pulsatile mode of LH release, whereby rhythmic LH pulses are generated in response to GnRH (Fig. 1.2), prevails at all reproductive states in ewes, including the period before, during and after the preovulatory surge of gonadotrophins (Rawlings and Cook, 1993), and it is also present in ovariectomized animals (Gay and Sheath, 1972). An increase in tonic LH secretion during the pro-oestrous period results from an increase in LH pulse frequency, from one pulse every 3-4 h during the mid-luteal phase to a maximum of one pulse every 20-30 min just before the LH surge. LH pulse amplitude
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also increases prior to the preovulatory surge (Baird, 1978; Goodman and Karsch, 1981b). In normally cycling ewes, the preovulatory LH discharge consists of high frequency, high amplitude pulses of LH, with the amplitude of LH increases being greater on the downslope than on the upslope of the surge (Rawlings and Cook, 1993). An increment in basal (non-pulsatile) LH release during the surge was also suggested (Rawlings and Cook, 1993).

The frequency and amplitude of LH pulses are modulated by progesterone and oestradiol. During the breeding season, progesterone regulates pulse frequency and oestradiol regulates pulse amplitude (Bjersing et al., 1972; Hauger et al., 1977; Karsch et al., 1979; Goodman and Karsch, 1980; Wheaton et al., 1984). Pulsatile release of LH is inversely related to circulating levels of luteal progesterone (Baird et al., 1976; Hauger et al., 1977; Baird, 1978; Karsch et al., 1979; Wheaton et al., 1984). The frequency of LH pulses is relatively high early in the luteal phase when serum levels of progesterone are low, decreases at mid-cycle during the maximal secretion of progesterone, and increases after the onset of luteolysis when progesterone secretion falls to basal or non-detectable levels. LH pulse amplitude is higher during the mid-luteal phase compared to the follicular phase of the cycle (Hauger et al., 1977; Jackson and Davis, 1979; Wheaton et al., 1979). Basal LH levels (serum concentrations of LH after the removal of pulses) are relatively constant during the middle portion of the luteal phase but tend to be more variable when progesterone levels are low (i.e., at metoestrum and pro-oestrum; Hauger et al., 1977; Wheaton et al., 1979).

The secretion of LH varies with the stage of the breeding season in ewes. Mean serum levels of LH are lower at the beginning than at the middle and end of the breeding season (Rawlings et al., 1977; Robinson and Karsch, 1984; Robinson et al., 1985; Malpaux et al., 1988a-b). Prior to the onset of the ovulatory season, there is an increase in LH pulse frequency from 2-3 pulses per 9 h to 6 pulses per 9 h. The high LH pulse frequency subsequently decreases to 4 pulses per 9 h at the beginning of seasonal anoestrum (McNatty et al., 1984b).

The major characteristics of the pattern of FSH secretion in cyclic ewes is the occurrence of two periovulatory surges. The first of these surges is coincident with the
preovulatory LH surge, and the second occurs between 20-36 h later (Pant et al., 1977; Bister and Paquay, 1983; Wheaton et al., 1984; Findlay et al., 1990). The secondary FSH discharge is lower in amplitude but of a longer duration (20-24 h) compared to the preovulatory surge (11-12 h; Wheaton et al., 1984; Baird et al., 1991). The preovulatory FSH surge is preceded by a relatively low levels of FSH, while serum LH, oestradiol and inhibin concentrations are increasing (Pant et al., 1977; Wheaton et al., 1984; Findlay et al., 1990; Baird et al., 1991). This suggests that the preovulatory FSH surge arises from the action of GnRH that overrides inhibitory effects on FSH release during that time. The second FSH surge occurs after ovulation which effectively terminates the secretion of follicular FSH inhibitors, mainly oestradiol (Findlay et al., 1990; Campbell et al., 1991c). It has been concluded that FSH secretion during the luteal phase of the ewe's oestrous cycle is non-pulsatile (Bister and Paquay, 1983; Wheaton et al., 1984; Wallace and McNeilly, 1986), but others were able to detect FSH pulses in hypophyseal and peripheral circulation during the luteal phase in ewes (Van Cleef et al., 1995).

1.3.1.2 Secretory patterns of oestradiol and progesterone

Oestradiol secretion results from the action of LH binding to its receptor on the follicular theca cells to stimulate androgen synthesis, and of FSH inducing aromatization of this substrate to oestradiol in the granulosa cells (Carson et al., 1979; Armstrong et al., 1981; Fortune and Quirk, 1988). The secretion of both androstenedione and oestradiol begins to increase within 5 min of a pulse of LH, and elevated levels are maintained for about 2 h (Baird, 1978; Martin, 1984). Secretion of androstenedione and oestradiol from large (≥ 5 mm in size) follicles is significantly greater than from smaller antral follicles (Baird and Scaramuzzi, 1976; McNatty et al., 1981; Mann et al, 1992b).

During the interovulatory period in the ewe, there are two major increases in circulating concentrations of oestradiol. The first increase occurs the day after the onset of luteolysis (Hauger et al., 1977; Pant et al., 1977; Baird, 1978; Karsch et al., 1979; Rawlings and Cook, 1993); this increase is concomitant with the increase in LH pulse frequency (Campbell et al., 1990a). The continued increase in oestradiol secretion during the follicular
phase of the oestrous cycle probably reflects increased maturation of preovulatory follicles associated with an increase in LH receptor content in both granulosa and theca cells (Carson et al., 1979; Armstrong et al., 1981; England et al., 1981; Webb and England, 1982). At the time of the preovulatory LH surge, progesterone concentrations in follicular fluid increase while oestradiol levels decline to a minimal value, within 16-24 h of the LH surge (Baird, 1978; England et al., 1981; Campbell et al., 1990). Once serum LH concentrations exceed 5 ng/ml, the largest ovarian follicles are no longer able to respond to LH by producing oestradiol (Baird, 1978). On the day of ovulation, peripheral concentrations of oestradiol fall to non-detectable levels, which coincides with the second peak of FSH (Bister and Paquay, 1983; Wheaton et al., 1984; Findlay et al., 1990; Baird et al., 1991). The second increment in oestradiol secretion occurs early in the luteal phase (Hauger et al., 1977; Pant et al., 1977; Karsch et al., 1979; Campbell et al., 1990). During the remainder of the luteal phase, oestradiol secretion is highly variable (Baird, 1978), although some authors reported another increase in serum levels of oestradiol between days 9 and 11 of the cycle (Hauger et al., 1977).

The pattern of progesterone secretion in the ewe is also episodic (Alecozay et al., 1988). An average of 8 pulses of progesterone per 24 h were observed throughout the luteal phase. The number and height of detectable elevations in progesterone concentrations did not vary throughout dioestrous, and progesterone pulses were not temporarily linked to LH pulses (Alecozay et al., 1988).

1.3.1.3 Secretion of inhibin

Inhibin, an ovarian glycoprotein hormone consisting of two dissimilar subunits (α and β) linked by disulphide bonds, is secreted by the granulosa cells of ovarian follicles (Henderson and Franchimont, 1981; Tsonis et al., 1983), and is present in high concentrations in follicular fluid (de Jong and Sharp, 1976; Tsonis et al., 1983). It has been shown that inhibin suppresses FSH release by the pituitary (McNeilly, 1984; Burger, 1988; Henderson et al., 1988; Martin et al., 1988).
Inhibin is secreted in an episodic pattern throughout the oestrous cycle (McNeilly and Baird, 1989; Murray et al., 1989; Campbell et al., 1990) and during anoestrus (Campbell et al., 1991c) in ewes. Unlike oestradiol, which is primarily produced by large oestrogenic ovarian follicles, inhibin is also produced by large non-oestrogenic and small antral follicles (Campbell et al., 1991b; Mann et al., 1989, 1992a-b).

During the interovulatory interval, there are two prominent increases in inhibin secretion. After luteolysis, the secretion of inhibin gradually increases to reach a peak at the time of the LH surge. A second rise in inhibin production occurs between 16-24 h after the surge, coincident with the second peak of FSH (Campbell et al., 1990). It was, therefore, suggested that the second FSH peak was primarily due to the cessation of oestradiol-negative feedback effects on FSH release after ovulation. During the remainder of the luteal phase, the secretion of inhibin remains relatively constant, with rhythmic pulses occurring approximately every 1 h, and does not appear to be temporarily linked to the secretion of either LH, prolactin (Campbell et al., 1990), oestradiol or FSH (McNeilly and Baird, 1989).

After immunoneutralization of both inhibin and oestradiol, the rise in circulating levels of FSH was significantly greater than with immunization against either hormone alone (Mann et al., 1990; Mann et al., 1993), but immunization against inhibin caused a greater increase in FSH levels than that against oestradiol 17β (Mann et al., 1993). It was concluded that inhibin exerted a general inhibitory effect on FSH secretion while follicular oestradiol was largely responsible for day-to-day fluctuations in serum concentrations of FSH (Baird et al., 1991).

1.4 Regulation of GnRH and gonadotrophin secretion

1.4.1 Localization of GnRH neurons

Gonadotrophin releasing hormone (GnRH), a decapeptide produced by the hypothalamus, is a major stimulus for gonadotrophin secretion (Iwashita and Catt, 1985; Hazum and Conn, 1988). Localization of GnRH-containing neurons in the central nervous system (CNS) of different mammalian species has been previously reviewed by Clarke
(1987), and Thiery and Martin (1991). As in most other species, GnRH has been localized in small cell bodies (10 to 20 μm in diameter) throughout the ovine hypothalamus. The vast majority of GnRH-containing perikarya were found in the rostral portion of the hypothalamus within the medial preoptic area (MPOA; Clarke, 1987). A proportion of scattered cell bodies containing GnRH have also been observed in the medial basal hypothalamus (MBH), mainly in the arcuate and ventro-medial nuclei (Caldani et al., 1988).

The axons arising from the cell bodies in the rostral hypothalamic area form two GnRH fibre tracts terminating in the median eminence (ME; Goodman, 1994). The ME is the major area in which GnRH is stored within neural terminals prior to release into hypophyseal portal blood. Based on experiments involving partial deafferentiation of the MBH, it was shown that most of the GnRH in the ME was produced by the GnRH cell bodies in the rostral hypothalamus (Przekop, 1978; Pau et al., 1982; Goodman, 1994).

1.4.2 Mechanism of action of GnRH on gonadotrophin secretion

GnRH controls both the synthesis and release of pituitary gonadotrophins through binding to specific receptors in the plasma membrane of the gonadotrophs (reviewed by Stojilkovic et al., 1994). Activation of GnRH receptors involves several steps and is followed by a cascade of intracellular responses: 1) following GnRH binding to the extracellular domain of the receptor, G-protein initiates activation of phospholipase C (PLC), leading to hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP₂) and formation of inositol 1,4,5-triphosphate (InsP₃) and diacylglycerol (DAG); 2) InsP₃ increases influx and mobilization of Ca²⁺ cations, and DAG activates protein kinase C (PKC); 3) intracellular Ca²⁺ and PKC serve as interacting signals in the regulation of various processes, including the synthesis, transport and release of gonadotrophins.

Following the administration of exogenous GnRH or GnRH agonists, the number of GnRH receptors in the pituitary initially decreases (down-regulation) and then increases (up-regulation; Nett et al., 1981; Khalid et al., 1987; Hamernik and Nett, 1988). Treatment with a GnRH antagonist completely inhibits GnRH binding and prevents the increase in
GnRH-receptor mRNA levels in ewes (Brooks and McNeilly, 1994). These results imply that GnRH may be required to maintain de novo synthesis and/or recycling of its own receptors.

1.4.3 Pulsatile secretion of GnRH and its relationship with gonadotrophin release

Measurement of GnRH concentrations in the peripheral blood cannot be used to evaluate hypothalamic function because of the approximately 500-fold dilution of GnRH between the hypophyseal portal and jugular veins (Goodman, 1994). Therefore, LH secretion was used as an indirect index of GnRH release; it was assumed that the pulsatile nature of LH release monitored in peripheral circulation reflected the existence of pulsed GnRH secretion. Techniques were developed that permitted direct measurements of GnRH concentrations by either perfusion of the ME or collection of portal blood in ewes (Levine et al., 1982; Clarke and Cummins, 1982; Moenter et al., 1991). The use of these techniques provided evidence for the episodic nature of GnRH release and the close temporal relationship between GnRH and LH pulses. The relationship between pulsatile secretion of GnRH and LH has been shown to be sustained during both the follicular and luteal phase of the oestrous cycle (Baird, 1978; Moenter et al., 1991), and throughout anoestrus in ewes (Scaramuzzi and Baird, 1977; Clarke, 1988; Barrell et al., 1992), albeit during anoestrus the pulse frequency and amplitude of GnRH/LH secretion are significantly lower in comparison to the breeding season. Each LH pulse reaches a peak concentrations within 10 min from the beginning of the rise in LH concentrations (Martin, 1984). The entire LH pulse (nadir-to-nadir) lasts approximately 90 min (Martin, 1984). During luteolysis, GnRH pulses increase in frequency (Clarke et al., 1987; Moenter et al., 1991; Clarke, 1995).

Although each pulse of LH is preceded by a GnRH pulse, there are some “small” elevations in GnRH concentrations that fail to induce LH pulses (Fig. 1.2b; Levine et al., 1982; Clarke and Cummins, 1982). These small GnRH pulses have been suggested to maintain LH synthesis, without pulsatile release, thus leading to accumulation of releasable LH in the pituitary (Clarke and Cummins, 1987). Alternatively, these small GnRH pulses
may increase the population of active GnRH receptors in the pituitary and hence regulate pituitary responsiveness to "large" GnRH pulses.

The pulsatile secretion of GnRH appears to be critical for maintaining optimal gonadotrophin secretion. Perfusion of the pituitary gland with constant levels of GnRH is ineffective in eliciting gonadotrophin secretion in sheep (Chakraborthy et al., 1974). In the study in monkeys, decreasing the frequency of exogenous GnRH pulses from one per hour to one every three hours, caused a decline in circulating LH levels and an increase in serum concentrations of FSH (Wildt et al., 1981). These results suggest that the changes in GnRH pulse frequency in primates may affect the relative amounts of LH and FSH secreted; however, varying the patterns and concentrations of GnRH stimulation failed to alter the ratio of LH and FSH released from perifused sheep pituitary cells (McIntosh and McIntosh, 1986).

During the greater portion of the luteal phase and throughout anoestrus, FSH secretion, unlike that of LH, is non-pulsatile (Wallace and McNeilly, 1986). It was, therefore, suggested that LH and FSH secretion were differentially regulated by GnRH or that FSH secretion did not require GnRH pulses (Clarke et al., 1986). It has been proposed that GnRH is essential to initiate FSH secretion but only minimal exposure to GnRH may be required to maintain secretion (McNeilly et al., 1995). However, more recent studies revealed that during the luteal phase in the ewe, FSH release into the portal vasculature was episodic, with each FSH pulse produced by a GnRH pulse, but the release pattern of FSH monitored in peripheral circulation was uncoupled from the GnRH stimulus (Van Cleeff et al., 1995). Additional pulses of FSH, which did not distinctly coincide with GnRH pulses, were seen in ovariectomized ewes, and the episodes of FSH were detected after administration of a GnRH antagonist to block GnRH secretion during the luteal phase in ovary-intact ewes.

During the sheep oestrous cycle, up to 50% of the pituitary content of FSH is released each day while only 1 to 5% of LH is secreted in the form of pulses (McNeilly, 1995; Taragnat et al., 1998). This fundamental difference in the secretory patterns of gonadotrophin hormones may result from different pathways of gonadotrophin release, but
not differential regulation of LH and FSH synthesis by GnRH in the pituitary. LH is stored in secretory granules which are emptied in response to GnRH, whereas FSH appears to be secreted via constitutive pathway where the rate of secretion closely relates to the rate of synthesis. The mechanisms that control these divergent patterns of gonadotrophin release are not fully understood.

Baird et al. (1991) demonstrated that ovarian inhibin and oestradiol were potent regulators of FSH secretion. In sheep, the changes in peripheral levels of FSH relate primarily to ovarian follicle activity, presumably reflecting output of inhibin and oestradiol (McNeilly, 1995). Both inhibin and oestradiol act directly at the pituitary to decrease FSH subunit gene expression, and hence are thought to be dominant over the GnRH input (McNeilly, 1995). Alternatively, the existence of a GnRH-independent component of pulsed FSH release, and other than ovarian oestradiol and inhibin, can be suggested based on an aforementioned study by Van Cleeff et al. (1995). Lastly, variations in the pattern of gonadotrophin secretion as monitored in peripheral circulation could also be partly due to different half-lives of LH and FSH (Akbar et al., 1974).

1.4.4 Ovarian steroid control of GnRH and gonadotrophin secretion

Oestradiol plays a major role in regulating the secretory activity of the hypothalamus in most mammalian species (reviewed by Fink et al., 1991) including sheep (reviewed by Clarke, 1987). Steroid hormones may exert both positive and negative feedback effects on the secretory activity of the hypothalamus and pituitary gland. The greatest number of oestradiol receptor-immunoreactive neurons are located in the MPOA, the MBH, and in several limbic structures (i.e., amygdala, bed nucleus of the stratum terminalis, and lateral septum; Lehman et al., 1993). Interestingly, GnRH-positive cells do not contain the "classical" oestrogen receptor (Karsch and Lehman, 1988; Herbison et al., 1993; Lehman and Karsch, 1993). This suggests that GnRH secretion is regulated by oestradiol-sensitive neurons that are presynaptic to GnRH neurons (neromodulatory action).
Several types of cells were proposed as the presynaptic cells for this action of oestradiol. Oestradiol receptors have been identified in tyrosine hydroxylase- and β-endorphin-containing cells (Lehman and Karsch, 1993), and in GABAergic neurons in the sheep brain (Robinson et al., 1991). The tyrosine hydroxylase-positive and GABAergic neurons are anatomically linked to GnRH cells in the MPOA, and the β-endorphin immunoreactive cells co-expressed oestrogen receptors mainly within the MBH.

The hypothalamus is the major site of action for positive feedback effects of oestradiol during the follicular phase of the oestrous cycle in ewes (Herman and Adams, 1990; Moenter et al., 1990, 1991, 1992). Another site is the anterior pituitary where oestradiol enhances the response to GnRH (Clarke and Cummins, 1984; Crowder and Nett, 1984; Philips et al., 1990; Herman and Adams, 1990). Full expression of the oestradiol-dependent positive feedback effects on gonadotrophin secretion requires continued hypothalamic input (Clarke et al., 1989). Physical disconnection of the pituitary from hypothalamic GnRH (Clarke et al., 1983; Clarke and Cummins, 1984; Girmus and Wise, 1992) as well as immunosuppression of endogenous GnRH (Adams and Adams, 1986; Herman and Adams, 1990) completely block pituitary response induced by oestradiol. During the luteal phase of the oestrous cycle, oestradiol enhances inhibitory effects of progesterone on pulsatile LH secretion, acting primarily at the level of the hypothalamus (Goodman and Karsch, 1980; Goodman et al., 1981a-b; Martin et al., 1983).

Progesterone receptors were detected in the hypothalamus and pituitary of several species (reviewed by Savouret et al., 1988), but similar evidence is lacking for sheep. However, a number of studies in ewes suggested indirectly that opioid peptides mediated progesterone suppression of tonic LH release (Brooks et al., 1986; Currie and Rawlings, 1987; Yang et al., 1988; Horton et al., 1990). Whisnant et al. (1992) found that the levels of mRNA for pro-opiomelanocortin, the precursor molecule of β-endorphin, was increased in ovariectomized ewes treated with progesterone. β-endorphin reduces both basal and GnRH-stimulated LH release from bovine pituitary cells in vitro (Saleri et al., 1995). These results suggest that the endogenous hypothalamic opioids may play a role in the negative feedback action of progesterone on GnRH and LH secretion in the ewe.
In ovariectomized ewes, treatment with luteal phase levels of progesterone in the absence of oestradiol, decreases LH pulse frequency without altering LH pulse amplitude (Goodman and Karsch, 1980; Rawlings et al., 1984; Tamanini et al. 1986; Thomas et al., 1988). This inhibitory effect of progesterone is all the more pronounced in seasonally anoestrous ewes (Hamernik et al., 1987). The synergistic effects of oestradiol and progesterone on pulsatile LH secretion in ovariectomized ewes (Baird and Scaramuzzi, 1976; Currie et al., 1993) suggest that both steroids control gonadotrophin secretion mainly by regulating hypothalamic GnRH secretion; however, the presence of oestradiol receptors in the gonadotroph cells of rodents and primates (Sprangers et al., 1989; Fox et al., 1990) indicates that steroid hormones may also interact to reduce gonadotrophin production and/or release by the pituitary gland.

The increased number of GnRH receptors in ovariectomized ewes after treatment with oestradiol (Moss et al., 1981) and during in vitro culture of ovine pituitary cells in the media supplemented with oestradiol (Sealfon et al., 1990; Gregg et al., 1990; Laws et al., 1990), indicate that oestradiol 17β is a potent stimulator of the expression of GnRH receptors. Oestradiol alone or in combination with inhibin also increased the amount of GnRH-receptor mRNA in primary cultures of ovine pituitary cells (Wu et al., 1994). During the normal oestrous cycle in ewes, the rate at which the expression of GnRH-receptor mRNA and translation of the GnRH receptor protein change, are correlated with follicular oestradiol production (Brooks et al., 1993); relatively low rate of GnRH-receptor biosynthesis in the luteal phase of the cycle increases abruptly after luteolysis, and then decreases again after the preovulatory LH surge.

In ovariectomized ewes, progesterone blocks the oestradiol-induced LH surge (Kasa-Vubu et al., 1992) by preventing the increase in GnRH pulse frequency and amplitude, but perhaps also by decreasing the sensitivity of pituitary gonadotrophs to oestradiol (Koligian and Stormshak, 1977). An increase in GnRH-receptor mRNA and numbers of GnRH receptors was concomitant with the decline of serum concentrations of progesterone at luteolysis but it occurred before any detectable change in circulating concentrations of oestradiol, in ewes treated with PgF₂α (12 to 24 h after injection of PgF₂α; Turzillo et al., 1994).
1.5 Folliculogenesis

1.5.1 Stages of ovarian follicular development

In most mammalian species, oogonia are transformed, just before or soon after birth, into primary oocytes surrounded by a squamous layer of pre-granulosa cells (primordial follicles; Greenwald and Terranova, 1988). Primordial follicles continuously leave the non-growing pool of follicles by being converted into primary follicles (follicles with a single layer of granulosa cells surrounding the oocyte). Populations of primordial (resting pool) and primary (growing pool) ovarian follicles constitute the reserve pool of follicles that ewes utilize during their entire reproductive life (40,000 to 300,000 of primordial follicles in ewe lambs; Driancourt et al., 1991). The number of primordial follicles in the ovaries of sheep is dramatically reduced from mid-gestation to birth (approximately 20-fold) and further, throughout post-natal life (approximately 35-fold; Driancourt et al., 1991). When ovarian follicles become secondary or preantral follicles, they have two or three layers of granulosa cells (Driancourt et al., 1991). Early antral or tertiary follicles are the next stage of follicular development followed by the formation of the antral (Graafian) follicle (Fig. 1.3). It was estimated that: the period of follicular growth from the primordial to the preovulatory stage in ewes exceeded 6 months (Cahill and Mauleon, 1980); growth from the primordial to the early preantral stage (0.2 mm in diameter) took an average of 130 days (Cahill and Mauleon, 1980; Cahill et al., 1981); 24 to 35 more days were required to reach 0.5 mm in diameter and 5 days to reach 2.2 mm in size (Turbull et al., 1977); and the preovulatory size, a minimum of 4.5 to 5 mm, was attained approximately 4 days later (Turbull et al., 1977; McNeilly, 1984). The daily number of ovarian follicles leaving the reserve pool is 3-4 (Turbull et al., 1977).

A model for folliculogenesis based on physiological properties of ovarian follicles, specifically their responsiveness to and degree of dependency on gonadotrophic hormones, has recently been proposed (Scaramuzzi et al., 1993). According to the authors, ovarian follicles can be divided into the five following classes: primordial, committed, gonadotrophin-responsive, gonadotrophin-dependent and ovulatory. This functional model
**Fig. 1.3.** The growth of primordial ovarian follicles to the periovulatory stage in mammals. The number of primordial follicles (reserve pool) beginning to grow each day (3-4 follicles/day) remains remarkably constant and is controlled by unknown intraovarian factor(s). The growth of antral follicles and terminal stages of follicular development and maturation are primarily gonadotrophin-dependent. Primordial ovarian follicles continue to grow throughout the reproductive life of the female but the majority of follicles, at all stages of development, undergo degeneration (atresia). Reproduced from Hafez (1993), with permission.
is in agreement with the known macroscopic changes occurring throughout the lifespan of ovarian follicles. Ovarian follicles leave the reserve pool and begin the growing phase regulated by gonadotrophins (see: Section 1.6).

1.5.2 The early stage of follicular development and oogenesis

Early follicular development refers to the growth of follicles from the primordial to the preantral stage (Cahill and Mauleon, 1980). In cows and sheep, the phases of early follicle growth can be classified by the changes in the configuration of granulosa cell layers as types 1 (primordial), 1a (transitory), 2 (primary), 3 and 4 (preantral) and 5 (early antral; Cahill and Mauleon, 1980). The control of follicle development from type 1 to type 5 is not fully understood, but it is thought to be independent of gonadotrophic hormones (McNatty et al., 1981). Towards the end of this stage of folliculogenesis, follicles become responsive to gonadotrophins, which is a prerequisite to subsequent antral follicular growth and maturation (Campbell et al., 1995).

Follicular development is associated with progressive structural and functional changes in the oocyte (cytoplasmic and nuclear maturation, and cell division; Monget and Monniaux, 1995). During initial follicular growth, the oocyte remains arrested in the diplotene stage of the first meiotic division (Wasserman and Albertini, 1994). The final oocyte maturation to metaphase II is induced in the mature ovarian follicle by the preovulatory LH surge, during which the oocyte acquires the ability to become fertilized (Hytell et al., 1997).

1.5.3 The wave-like pattern of antral follicle development in ruminants

In evaluating ovarian antral follicular development, a few terms have been introduced and became widely used to describe the patterns of antral follicle turnover (Goodman and Hodgen, 1983). In domestic ruminants, the growing phase is defined as the time taken by the individual follicle to grow to its maximum size. The regressing phase is
the time taken by this follicle to regress to the minimal recordable size (2 or 3 mm in diameter using ovarian ultrasonography), and the time period between the end of the growing phase and the onset of regression is defined as the static phase (Goodman and Hodgen, 1983; Ravindra, 1993; Ravindra et al., 1994). Recruitment refers to the synchronized growth of a group of ovarian antral follicles that eventually gain the ability to fully respond to endocrine (gonadotrophic) stimuli; selection is the process by which only a limited number of these follicles is rescued from atresia and continues to grow to an ovulatory size; dominance is a characteristic of a large selected ovarian antral follicle (dominant follicle) of a wave or cohort of follicles that permits its survival and further development in an endocrine environment suppressive to other co-existing follicles (subordinate follicles); and follicle emergence or follicular wave emergence is the beginning of the growth of a group of antral follicles that subsequently undergo atresia (mid-cycle waves or non-ovulatory follicles) or ovulate.

Ovarian antral follicular dynamics have been most thoroughly studied in cattle. In cows, the gonadotrophin-regulated phases of follicular growth (antral follicles) occur in an orderly succession, in a wave-like pattern (Fig. 1.4; Ginther et al., 1996). The existence of mid-cycle peaks in numbers of antral follicles led to the conclusion that there were two or three waves of follicular development during the interovulatory period in cattle (Rajakoski, 1960; Hackett and Hafs, 1969; Swanson et al., 1972; Ireland et al., 1979). Approximately two decades later, these results were confirmed by more advanced studies using ultrasonography. The incidence of two waves of antral follicular growth, one during metoestrus and the other during the dioestrous period was reported, concurrently with evidence for a three-wave pattern of follicular development characterized by emergence of follicular waves on days 2, 9 and 16 of the cycle (two-wave pattern: Pierson and Ginther, 1988; Knopf et al., 1989; three-wave pattern: Sirios and Fortune, 1988; Savio et al., 1988, 1990; Knopf et al., 1989).

In cattle, the emergence of the first wave of the oestrous cycle is detected by the growth of a cohort of ovarian antral follicles from 3 to 4 mm in diameter just before ovulation (Ginther et al., 1996). During the next few days, one of the follicles becomes dominant, and the others (subordinate) become atretic (Fig. 1.5; Pierson and Ginther, 1987,
Fig. 1.4. The proposed model of bovine ovarian follicle wave dynamics during two-wave (solid lines; ①-②) and three-wave (dashed lines; ①-③) interovulatory intervals and early pregnancy (④-⑤), and their relationships with the pattern of FSH secretion. OV = ovulation. Episodic pulses of LH are schematic and do not represent actual changes in LH pulse frequency and amplitude at oestrus. Hypotheses depicted by arrows in the top panel are: (a) dominant follicle suppresses the growth of its subordinates and (b) the emergence of the next follicular wave; (c) the dominant follicle contributes to its self-growth and (d) self-demise. Courtesy of Dr. G.P. Adams.
Oestrous cycle

Pregnancy

Follicular waves

OV  2-wave pattern

OV  3-wave pattern

FSH

2-wave pattern

3-wave pattern

LH

2-wave pattern

3-wave pattern

Number of days from ovulation

Diameter (mm)

Concentration (ng/ml)
1988; Adams et al., 1993a). This stage of antral follicular development has recently been termed *deviation* (Ginther et al., 1996). It appears that in cattle, all healthy follicles 3 mm in size are recruited on day −1 (day 0 = day of ovulation) and, once selection has occurred, further recruitment is blocked (Ginther et al., 1996). Initially, all emergent follicles of the wave have the ability to become the dominant follicle. Approximately 3 days after wave emergence, the dominant follicle reaches a diameter of 8 mm, and it continues to grow at a higher rate compared to the subordinate antral follicles (Ginther et al., 1996). The selected dominant follicle often possesses the advantage of size, being the first to develop to the largest diameter at the decisive stage of follicle deviation. The duration of the deviation process in cattle is very short (< 8 h; Ginther et al., 1996). A rapid completion of deviation allows for the selection of the dominant follicle before the arrival of the next follicle to the similar stage of development. The ovulatory follicles in cows invariably originate from the last follicle wave of the interovulatory interval (Ginther et al., 1996).

To date, the data describing ovarian antral follicle dynamics in the ewe have largely come from post-mortem (slaughtered animals) studies (Hutchinson and Robertson, 1966; Brand and de Jong, 1973; McNatty et al., 1984a-b) or experiments employing laparoscopy/endoscopy (Smeaton and Robertson, 1971; Noel et al., 1993). Early studies of antral follicle development in cyclic ewes led to the conclusion that follicles begin to grow early in the cycle and then remain in the ovaries to enlarge shortly before ovulation (Hutchinson and Robertson, 1966). In 1971, Smeaton and Robertson demonstrated that there were at least three distinct groups of ovarian antral follicles beginning to grow synchronously during the oestrous cycle in sheep. This early evidence came from experiments employing marking the largest ovarian follicles with non-absorbable dye (carbon or India ink) and following their growth by sequential laparotomy. In 1973, Brand and de Jong suggested a two-phase model of follicular development in cyclic ewes. Their studies were based on the macroscopic examination of ovaries collected from ewes slaughtered at various stages of the cycle. In order to document day-to-day changes in ovarian follicle populations at different times of the year in sheep, eight Suffolk ewes
**Fig. 1.5.** Schematic illustration of the temporal relationships between an elevation in circulating levels of FSH and follicle wave emergence in heifers. The transient rise in mean serum concentrations of FSH permits the growth of the cohort of antral follicles. About 2 days after wave emergence and during a decline in FSH concentrations, the growth profiles of the dominant and subordinate follicles begin to diverge. Modified from Adams et al., 1993b.
underwent daily laparoscopic examination of ovaries for 18 days in August, November, February and May (Noel et al., 1993). A three-wave pattern of follicular turnover (three distinct groups of ovarian antral follicles undergoing growth and atresia) was reported in animals studied during both the breeding season (August, November, February) and anoestrus (May; Noel et al., 1993).

Only recently, transrectal ovarian ultrasonography has been adapted as a method of obtaining morphological and physiological data describing ovarian status in small ruminants (sheep: Ravindra, 1993; Schrick et al., 1993; Ravindra et al., 1994; Ginther et al., 1995; goats: Ginther et al., 1994). Ultrasonographic studies conducted in sheep have revealed a considerable range of variation between breeds and individual animals in terms of the number of emerging waves of antral follicles (2-6 waves or phases of antral follicle development; Ravindra, 1993; Schrick et al., 1993; Ginther et al., 1995), but some workers were unable to demonstrate the rhythmic pattern of antral follicle emergence in ultrasonographically monitored ewes (Lopez-Sebastian et al., 1997). When follicle waves were detected in ewes, they consisted of fewer antral follicles than in cattle (Fig. 1.6) and there appeared to be less evidence for dominance except for the largest follicles growing early in the luteal phase of the cycle, and the ovulatory follicle in its final growth phase before ovulation (Ravindra et al., 1994). The pattern of antral follicle turnover and its relationships with circulating concentrations of gonadotrophins and ovarian steroids in sheep remain to be elucidated.

1.5.4 Follicular atresia

The vast majority of all ovarian follicles in the ovaries of ewes undergo atresia. Atresia is sporadic in ovarian follicles < 1 mm in diameter, but approximately 50 to 80% of follicles > 1 mm are in early, advanced or late stages of atresia (Brand and de Jong, 1973; Turnbull et al., 1977). When developing antral follicles become gonadotrophin-responsive, they are more sensitive to atresia, as hypophysectomy (Dufour et al., 1979; Driancourt et al., 1979; Campbell et al., 1995) or immunization against GnRH (McNeilly et al., 1986) invariably causes regression of all follicles > 2 mm. FSH may prevent the onset of follicular
Fig. 1.6. The growth curves of individual antral follicles ≥ 2 mm in diameter detected with ultrasonography in four individual Western white-faced ewes during the second oestrous cycle of the breeding season. The arrow indicates the diameter of the preovulatory follicle on the day before ovulation. Reproduced from Ravindra (1993), with permission.
regression. The administration of PMSG 24 h after hypophysectomy delayed the atresia of healthy antral follicles by approximately 48 h, but treatment with PMSG at 48 h after hypophysectomy, when most ovarian follicles were already atretic, was ineffective (Driancourt et al., 1987). Alternatively, LH/hCG treatment was associated with an increase in the number of atretic follicles in sheep (Turnbull et al., 1977).

Follicles undergoing atresia have a reduced capacity for aromatization of thecal androgens to oestrogens and are deficient in the granulosa cells (Turnbull et al., 1977). Once the production of follicular oestradiol ends, the follicles are considered to be in an irreversible stage of atresia (Hay et al., 1979). Other histological changes associated with atresia include pyknosis of nuclei of the granulosa cells, hypertrophy of the theca cells and dissociation of the cumulus oophorus complex (Greenwald and Terranova, 1988). The final stages of atresia are characterized by the resumption of meiosis of the oocyte, followed by fibroblast invasion into the antral cavity, disappearance of the interstitial cells and, finally, degeneration of oocytes trapped within the zona pellucida. Recent biochemical studies have demonstrated that the necrosis of granulosa cells during follicular demise occurs by apoptosis (i.e., an active, intrinsic, genetically-controlled process of selective cell deletion; Billig et al., 1994; Tilly et al., 1992; Jolly et al., 1994).

1.6 Hormonal control of folliculogenesis

1.6.1 Gonadotrophic hormones

Gonadotrophins are the most important promoters of ovarian antral follicular emergence and growth (Baird and McNeilly, 1981; Ireland, 1987; Picton et al., 1990). In sheep, LH and FSH receptors can be detected as early as in tertiary follicles and their number increases significantly when follicles continue to grow to 2 mm in diameter (Carson et al., 1979). Initially, LH binding sites are localized in the theca interna cells (Fortune and Armstrong, 1977) and FSH receptors are present in the granulosa cells (Dorrington et al., 1975). However, during the later stages of follicle growth, when follicles in ovaries of sheep are greater than 3 mm in diameter, LH receptors appear also in the granulosa cells (Carson
et al., 1979; Webb and England, 1982). The synthesis of LH receptors by the granulosa cells is stimulated by FSH and oestradiol (Uilenbroek and Richards, 1979; England et al., 1991). During the terminal growth of ovulatory ovarian follicles, serum concentrations of FSH have been found to be minimal while LH concentrations increased prior to the preovulatory LH surge (Roche, 1996). It has been concluded that early antral follicles are predominantly dependent on FSH (FSH dependency), and the terminal phases of folliculogenesis are under the control of LH (LH dependency; Ireland and Roche, 1982, 1983a-b; Ireland, 1987; Ireland and Roche, 1987; Campbell et al., 1995).

In cattle, the emergence of each wave of follicle growth is preceded by a transient increase in circulating concentrations of FSH (Adams et al., 1993b). An increment in numbers of small (3 mm in diameter) antral follicles is detected during the rise in serum FSH concentrations (Ginther et al., 1996). The decline in serum FSH levels 48 to 72 h later is a result of the negative feedback exerted by products from emerging follicles; all follicles from 3 to 5 mm in size can contribute to the suppression of FSH release (Gibbons et al., 1999). The suppressive effect of 5-mm follicles is not attributable to an increase in their oestradiol secreting ability, suggesting that oestradiol alone may not be responsible for the cessation of FSH increase during the first 2-3 days after follicle wave emergence (Ginther et al., 1996). The ability of growing follicles to suppress FSH secretion may be due to the secretion of inhibin (Kaneko et al., 1995), or other follicular products (Law et al., 1992), and/or their synergistic actions with oestradiol.

The mechanism of the selection of the dominant follicle of the wave in cattle appears to be a shift by that follicle from FSH- to LH-dependency (Ginther et al., 1996; Roche, 1996). At the time of deviation, or about 3 days after wave emergence, the future dominant follicle develops LH receptors in the granulosa cells. A decline in peripheral levels of FSH begins approximately 16 h before deviation (Ginther et al., 1996). Thus, the growing dominant follicle, utilizing LH for its growth, continues to develop in the face of declining FSH concentrations that are inadequate for development of smaller antral follicles.

The development of sensitive bioassays for FSH led to the discovery that FSH, like many other protein hormones, consists of several isoforms (Chappel et al., 1983). Different isoforms can be isolated on the basis of their molecular weight, electrical charge, clearance
rates and, most importantly, receptor affinity and biological potency (Chappel et al., 1983; Blum and Gupta, 1989; Robertson, 1989; Beitins and Padmanabhan, 1991). Hence, the changes in the proportions of FSH isoforms can alter the net potency of the circulating hormone. The most significant shifts in FSH bioactivity, associated with alterations in the distribution pattern of FSH isoforms, were shown during the experimentally induced onset of puberty in sheep (Padmanabhan et al., 1992), and during the periovulatory phase of the menstrual cycle in women (Padmanabhan et al., 1988), that is during periods characterized by high-frequency GnRH release and elevated oestradiol concentrations. Only recently, a homologous in vitro bioassay for FSH revealed the occurrence of increased biological signal prevailing during the mid- and late luteal phase of the human menstrual cycle (Christinmaître et al., 1996). The postulate is that this transient increase in biological activity of the hormone initiates processes leading to the recruitment of the dominant follicle that matures later during the second portion of the menstrual cycle. These results support the view that FSH heterogeneity may be responsible for stimulation of follicular maturation, follicular selection and deviation.

Recently, a time-wise relationship between ovarian antral follicular emergence (follicles growing from 3 to \( \geq 5 \) mm in diameter) and increases in FSH concentrations in daily blood samples taken during interovulatory periods, has been demonstrated in sheep (Ginther et al., 1995; Fry and Driancourt, 1996). However, Ravindra (1993) failed to find the similar temporal patterns of follicular development and FSH secretion in cycling, transitional and anoestrous ewes.

1.6.2 Intraovarian regulators of folliculogenesis

1.6.2.1 Gonadal steroids

There is evidence from studies in rodents that ovarian steroids can modulate follicular growth, but information is very limited for ruminants. Oestrogens, acting endocrinologically, may enhance the response of ovarian follicles to gonadotrophins in hypophysectomized rats (Richards, 1994). This synergistic effect of oestradiol is mostly due to the induction by FSH and oestradiol of LH receptors in granulosa cells of mature ovarian
follicles (Richards et al., 1995). However, oestradiol implants given to GnRH-deficient mice with hypothalamic lesions, can also stimulate follicle growth (Carlton et al., 1982). Both immunization against follicular steroids and treatment of sheep and cattle with physiological doses of oestradiol can alter follicle growth, although a negative feedback action on the hypothalamic-pituitary axis is probably the major reason for changes in follicle development (Webb et al. 1999).

It has been suggested in ewes, that the CL acts locally to increase the numbers of all follicles visible on the ovarian surface (Dufour et al., 1971, 1972; Fogwell et al., 1977; Dailey et al., 1982). However, in a previous study, more follicles > 4 mm in size were observed in ovaries without CL than within the CL-containing ovaries of ewes that ovulated unilaterally (Ginther, 1971). The effects of the CL/progesterone on the development of ovarian antral follicles in ewes have yet to be determined.

1.6.2.2 Intrafollicular regulators of folliculogenesis

Ovarian inhibins (Cahill et al., 1985b; Findlay, 1993), activin (Findlay, 1993), transforming growth factor α (TGFα; Lobb and Dorrington, 1992), epidermal growth factor (EGF; Skinner et al., 1987), insulin-like growth factors (IGF; Roche, 1996), and their binding proteins, have both autocrine and paracrine effects that can modulate follicular growth. It has been shown that ovarian follicular fluid suppressed follicular development and FSH release from the pituitary (Cummins et al., 1983; Martin et al., 1988; Mann et al., 1990). Follicular fluid contains large amounts of inhibin (de Jong and Sharp, 1976; Tsonis et al., 1983), and it was suggested that inhibin mediated the suppressive effects of follicular fluid on FSH production and follicle growth. However, treatment with inhibin-free ovine follicular fluid resulted in equally strong suppression of follicular development and FSH secretion in experimental animals (Campbell et al., 1991b), suggesting that other compound(s) present in follicular fluid have similar inhibitory potential. Inhibin enhances gonadotrophin-induced steroid secretion by ovine granulosa cells in vitro, and this stimulation in turn stimulates inhibin production by the granulosa cells (Campbell et al.,
1995). These results support the concept of a self-inductive, ultra-short regulatory cascade, involving follicular steroids and inhibin, during the process of granulosa and theca cell differentiation.

Transforming growth factor α (TGFα; Lobb and Dorrington, 1992) and epidermal growth factor (EGF; Skinner et al., 1987) are produced by the theca cells and bind to high-affinity receptors on the granulosa cells in ewes (Wynn et al., 1988). TGFα was shown to acutely suppress oestradiol, inhibin and androstendione production, which caused atresia of large antral follicles (Campbell et al., 1994). EGF inhibits oestradiol 17β production and, consequently, the preovulatory LH surge and manifestations of behavioural oestrus in ewes (Radford et al., 1987). An experiment involving direct infusion of EGF into the ovarian artery during the follicular phase of the oestrous cycle in ewes demonstrated that EGF influenced inhibin and oestrogen production in granulosa cells, but not androgen output by the theca cells (Murray et al., 1993). These results suggest that EGF is an inhibitor of the aromatase complex and directly blocks the aromatization of androgens into oestrogens.

IGF-I is present in high levels in intrafollicular fluid during the growing phase of large (dominant) follicles in cattle (Echternkamp et al., 1990) and sheep (Monger et al., 1993). IGF-I is secreted by the granulosa cells (Oliver et al., 1989) and, in contrast to TGFα and EGF, has been shown to stimulate ovarian follicular development by acting synergistically with FSH in the ovarian dominant follicles in cows (Driancourt, 1991). IGF-I also stimulates granulosa cell proliferation in small (3 mm in diameter) ovine ovarian follicles (Monniaux and Pisselet, 1992), hence synchronizing follicle emergence. In vitro, IGF-I stimulates progesterone secretion by the granulosa cells of large (> 5 mm), but not small, ovine antral follicles (Monniaux and Pisselet, 1992). It has been suggested that IGF-I controls either proliferation or functional transformation of the granulosa cells depending on the stage of individual follicle development. Direct ovarian infusion of an IGF-I analogue significantly enhanced steroid production in ewes (Campbell et al., 1993), indicating that IGF-I is also a potent in vivo stimulator of steroidogenesis.

Changes in follicular content of different growth factors as well as their relative binding proteins were studied in vitro using bovine follicles as a model (Roche, 1996). The
hypothesis was tested that the growth of the dominant follicle and increased oestradiol secretion were associated with relatively low concentrations of inhibins, activin and IGF-binding proteins, whereas the onset of the regressing phase and loss of oestrogenic activity were characterized by relatively high concentrations of inhibins, activin and IGF-binding proteins. The results of recent studies have confirmed that follicular fate is closely correlated with alterations in concentrations of the intrafollicular growth factors mentioned above (Ireland and Roche, 1987; Findley, 1993). These results have led to the speculation that growth factors play a key role in controlling follicular selection, recruitment, maturation and demise.

1.6.3 Control of ovulation rate in the ewe

There are marked variations in ovulation rate among different breeds of sheep (Lahlou-Kassi and Mariana, 1984; Driancourt et al., 1986a-b; Campbell et al., 1995) and among different strains of sheep within breeds (Scaramuzzi and Radford, 1983; Driancourt et al., 1986a-b, 1988). Usually, non-prolific breeds of sheep ovulate 1 to 3 follicles at the end of each interovulatory interval, while prolific sheep have mean ovulation rate ~3.

The mechanisms governing ovulation rate in sheep are unclear. During the follicular phase of the ovine oestrous cycle, there are two distinct populations of ovarian antral follicles, oestrogen-active and oestrogen-inactive, with the number of oestrogen-active follicles being equal to the ovulation rate (England et al., 1981; Webb et al., 1999). There appears to be an intraovarian mechanism that controls the number of oestrogen-active follicles in ewes, as after unilateral ovariectomy the number of such follicles in the remaining ovary doubles (Dufour et al., 1971).

The mean ovulation rate after hCG-induced ovulation during prepubertal and anoestrous periods as well as during the mid-luteal phase was similar to that during the normal oestrous cycles of sexually mature ewes, suggesting that the mechanism controlling ovulation rate operates at stages other than the follicular phase of the oestrous cycle (Driancourt et al., 1988). Although it has been well established that FSH and LH are primary
promoters of antral follicle growth (McNatty et al., 1981; McNeilly et al. 1991), there is no
clear association between serum concentrations of FSH/LH and the number of ovulatory
follicles in ewes differing in prolificacy (McNeilly et al., 1991). Within a breed, ewes
selected for high ovulation rate have higher circulating concentrations of FSH, from
approximately 3 to 5 days before ovulation (McNatty et al., 1994). However, no differences
in serum concentrations of FSH and LH after luteolysis were observed between prolific
Romanov and non-prolific Ile-de-France ewes (Cahill et al., 1981), and the pattern of
FSH/LH secretion prior to ovulation did not vary between Scottish Blackface carriers and
non-carriers of FecB (Booroola) gene (Webb et al., 1999). In other studies, it has been found
that prolific sheep have a higher LH pulse frequency (Thomas et al., 1984) and amplitude
(McNatty et al., 1994) in comparison to less prolific breeds.

Ovarian antral follicles in Romanov, Finnish Landrace and Booroola (Fec++) sheep
(prolific genotypes) produce approximately 70 and 50% less oestradiol and inhibin,
respectively, compared to the follicles of non-prolific breeds (Driancourt et al., 1991).
However, immunization against androgen (Scaramuzzi and Martens, 1975; Campbell et al.,
1990) or inhibin (Henderson et al., 1984), which both result in increased ovulation rate,
were not associated with an increase in serum levels of FSH in ewes. There is, however,
evidence that the ewes carrying high fecundity genes have a higher basal level of FSH and
are more responsive to GnRH (McNatty et al., 1994). The peak value of oestradiol 17β
preceding the preovulatory LH surge was significantly higher in prolific Romanov than in
non-prolific Ile-de-France ewes, and in both breeds it was correlated with the number of
follicles destined to ovulate (Cahill et al., 1981), but there were no differences among the
two breeds in mean oestradiol concentrations at the onset on behavioural oestrus.

The diameter of ovulatory follicles is smaller in prolific than in non-prolific breeds
of sheep. This was suggested to be due mainly to a lower capillary blood supply in animals
with multiple ovulations (Brown and Driancourt, 1989). Reduced numbers of granulosa
cells per follicle were reported for some (Finn and Booroola) but not all (Romanov) prolific
breeds (McNatty et al., 1986; Driancourt and Fry, 1989). Mean numbers of all antral
follicles and the degree of follicle atresia did not differ between Finn and Merino ewes

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selected for high ovulation rate and their respective control lines (Driancourt et al., 1986b, 1988). The high ovulation rate in the more prolific lines above appeared to be related to the terminal development of ovulatory follicles; the more fecund Finn ewes had a lower proportion of atretic follicles > 3 mm in diameter, while the more prolific Merino ewes recruited more follicles with less granulosa cells and considerably smaller diameters at ovulation.

It appears that different genotypes of prolific ewes employ different strategies to increase the number of ovulations (Driancourt et al., 1986a-b; Webb et al., 1999). The prolific Romanov sheep recruit considerably greater numbers of antral follicles but, in the late follicular phase, the intensity of follicle selection and demise through atresia is similar to that of non-prolific breeds. In contrast, the prolific Finn sheep have similar numbers of recruitable follicles to that of non-prolific breeds during the follicular phase of the oestrous cycle, but they achieve a higher ovulation rate through markedly diminished follicle elimination (atresia). Lastly, the high ovulation rate in the carriers of Booroola gene arises mainly from an extended period of follicle recruitment and a low rate of follicular atresia during the follicular phase of the cycle, which permits the “oldest”, or the most mature, ovarian antral follicles to “wait” for ovulation (Driancourt and Jego, 1985). The pattern of ovulatory follicle recruitment and mechanisms governing ovulation rate in sheep remain to be clarified.

1.7 Corpus luteum formation, development and secretion during the ovine oestrous cycle

Previous observations on CL development in ewes have mainly been accomplished at slaughter or using surgical techniques such as laparotomy or endoscopy (Hutchinson and Robertson, 1966; Oldham and Lindsay, 1980; Keisler et al., 1983; McNatty et al., 1984a-b). Approximately 3 days after ovulation, the ovine CL is approximately 6 mm in diameter, and it reaches its maximal size 5-6 days later (Arthur et al., 1989). The diameter of the CL remains unchanged until the next oestrus, during which luteal atrophy occurs abruptly (days 15 to 16 after ovulation; Baird and Scaramuzzi, 1975).
During the ovine oestrous cycle, changes in serum concentrations of progesterone closely reflect physical changes of the CL (Arthur et al., 1989). There are differences between breeds in terms of the maximal serum concentration of progesterone at mid-cycle. It was reported that prolific animals had higher progesterone concentrations than ewes with lower ovulation rates (Quirke et al., 1979). Progesterone secretion is also affected by the stage of the breeding season, with higher serum progesterone concentrations detected in ewes in the middle than at the beginning or end of the season (Wheeler and Land, 1979). The relationship between day-to-day changes in the size of the CL (ultrasonographic estimates of luteal tissue area) and peripheral blood progesterone levels was demonstrated in cyclic and pregnant heifers (Kastelic et al., 1990), but such studies do not exist for sheep.

The CL of the oestrous cycle and pregnancy are formed mainly by the action of LH, involving a cascade of functional and structural changes in the granulosa and theca cells of ovulatory follicles (reviewed by Niswender et al., 1986, and Alila and Dowd, 1991). LH support is also obligatory for the CL development and early luteal function, namely the cellular differentiation and the onset of secretory activity of luteal cells (Niswender et al., 1986; Farin et al., 1987; Wiltbank and Niswender, 1992).

The CL of the ewe is composed of four major cell types: small luteal cells, large luteal cells, fibroblasts and capillary endothelial cells (Farin et al., 1989). Studies of bovine luteal cells in vitro showed that small and large steroidogenic luteal cells derive from the theca and granulosa cells, respectively (Alila and Hansel, 1984; Meidan, 1990; Niswender et al., 1985). However, treatment of cows (Niswender et al., 1985) and ewes (Farin et al., 1988) with LH may promote transformation of small luteal cells into large luteal cells.

Response of the luteal cells to gonadotrophic stimuli depends on their LH receptor (LH-R) content. In ewes, the greatest number of active LH receptors is located on the small luteal cells (Harrison et al., 1987), although the large luteal cells of cyclic ewes and of anoestrous ewes induced to ovulate with GnRH (Harrison et al., 1987; O'Shea et al., 1986) also contain LH binding sites. However, the large luteal cells are virtually unresponsive to LH stimulation as their complement of LH receptors is not coupled with the LH-R/cAMP system responsible for progesterone biosynthesis (Hoyer and Niswender, 1986). Both types
of luteal cells produce progesterone, with the basal production being higher in the large luteal cells (Fitz et al., 1982; Harrison et al., 1987). This implies that large luteal cells do not depend on LH for the production of progesterone (Alila and Dowd, 1991). It has been suggested, in rodents and domestic ruminants, that pituitary prolactin may play an important role in regulating both the basal and LH-stimulated progesterone secretion through increasing an influx of circulating cholesterol essential for steroidogenesis and by controlling the population and affinity of luteal LH receptors (Murphy and Rajkumar, 1985; Kann and Denamur, 1988).

Both small and large luteal cells contribute to the increment in the total luteal mass during the early and mid-luteal phase of the cycle. Large luteal cells increase in size between days 4 and 12 of the cycle but their number remains relatively constant until the onset of luteolysis (O'Shea et al., 1986; Farin et al., 1989). In contrast, the size of small luteal cells does not change but there is an increase in the number of these cells from days 4 to 8 of the oestrous cycle (O'Shea et al., 1986; Farin et al., 1989). The capillary endothelial cells and luteal fibroblasts increase in number between days 4 and 12, and between days 8 and 16 of the cycle, respectively (Farin et al., 1989). The shifts in numbers of the luteal cells above are due to mitotic division (O'Shea et al., 1980). It was also suggested that under certain conditions fibroblasts might differentiate into small luteal cells (Niswender et al., 1985; Schawall et al., 1986), which would explain the existence of luteal cells in ewes that are intermediate in their microscopic morphology between fibroblasts and small luteal cells (O'Shea et al., 1980).

Luteal progesterone acting in concert with follicular oestradiol markedly suppress LH secretion during the luteal phase of the oestrous cycle. Hence, progesterone secretion is highest when the luteotrophic support is minimal (Goodman et al., 1981a; Baird, 1992). This may be due to an increase in the size of large luteal cells that do not rely on LH for progesterone production and/or the onset of full secretory ability of these cells in the face of reduced progesterone secretion by LH-dependent small luteal cells at mid-cycle in ewes (Farin et al., 1987). It was estimated that approximately 80% of luteal progesterone secreted at this stage of the oestrous cycle is produced by the large luteal cells (Niswender et al., 1985).
The large luteal cells in ruminants (cows, sheep and red deer) have been shown to produce oxytocin (Wathes and Swann, 1982; Rodgers et al., 1983; Fields and Fields, 1986; Sawyer et al., 1986). The secretion of oxytocin occurs throughout the luteal phase of the oestrous cycle in the ewe; it increases between days 5 and 8 (Sheldrick and Flint, 1981; Flint and Sheldrick, 1983) and then remains constant until day 15 of the cycle (Ivell et al., 1985).

1.8 Endocrine control of luteolysis

Prostaglandin F\(_{2\alpha}\) (PgF\(_{2\alpha}\)) secreted by uterine endometrial glands is the luteolytic factor in ruminants (reviewed by Knickerbocker et al., 1988). PgF\(_{2\alpha}\) is transported to the ovary through the local passage from the uterine venous and lymphatic vessels to the ovarian artery (reviewed by Krzymowski, 1995). Receptors for PgF\(_{2\alpha}\) in sheep are located mostly in the large luteal cells, whereas small luteal cells are irresponsive to PgF\(_{2\alpha}\) in vitro (Fitz et al., 1982).

Ovarian oestradiol, progesterone and oxytocin are all involved in the control of PgF\(_{2\alpha}\) secretion in ewes. The uterus requires a period of exposure to elevated concentrations of progesterone to prepare the endometrium for PgF\(_{2\alpha}\) production (Silvia et al., 1991). Although concentrations of endometrial receptors for progesterone are highest at oestrus and gradually decline during the luteal phase of the cycle (Zelinski et al., 1982), it has been suggested that the period of exposure to luteal phase levels of progesterone allows for the accumulation of arachidonic acid, prostaglandin endoperoxidase and other substrates needed for PgF\(_{2\alpha}\) synthesis (Knickerbocker et al., 1988; Silvia et al., 1991). Towards the end of the luteal phase, follicular oestradiol promotes the formation of endometrial receptors for oestradiol and oxytocin (Roberts et al., 1975, 1976; Koligian and Stormshak, 1977). Oestradiol increases the secretion of PgF\(_{2\alpha}\) (Ford et al., 1975; Fogwell et al., 1985) and the effect of oestradiol on the recruitment of oxytocin receptors is significantly enhanced by previous exposure to progesterone (McCracken et al., 1984; Homanics and Silvia, 1988; Vallet et al., 1990). The decline in circulating levels of progesterone is associated with the occurrence of an increase in oxytocin receptor levels and pulsatile secretion of PgF\(_{2\alpha}\).
(Sheldrick and Flint, 1985). The increase in endometrial oxytocin receptor can be detected as early as 6 h of the progesterone withdrawal in ewes (Leavitt et al., 1985).

Luteal oxytocin and, possibly, neurohypophysin play an important role in the mechanism that controls PGF$_{2\alpha}$ secretion in sheep (Sharma and Fitzpatrick, 1974; Roberts et al., 1976; Wathes and Swann, 1982; Flint and Sheldrick, 1986). After an increase in endometrial oxytocin receptor levels on ~day 14 of the ewe's oestrus cycle, oxytocin stimulates the pulsatile secretion of PGF$_{2\alpha}$ (Flint and Sheldrick, 1983; Hooper et al., 1986). However, the factor that initiates PGF$_{2\alpha}$ secretion is still unknown. Moore et al. (1986) have shown that the episodic secretion of PGE$_{2\alpha}$ measured in the uterine venous effluent occurs regardless of any detectable change in the pattern of oxytocin secretion. Silvia et al. (1991) suggested that the activation of the utero-ovarian feedback mechanism occurs in the uterine component of the loop.

1.9 Seasonality in ewes

1.9.1 Photoperiodic and neuroendocrine regulation of seasonal reproductive activity in the ewe

In seasonally breeding animals, it is the general rule that animals breed once a year and parturition occurs in the spring, when the time is most favourable to progeny (i.e., during the period of increasing light and warmth, and the time when food for the mother is abundant and adequate lactation may be ensured; Thibault et al., 1993). Animals perceive external signals to regulate their circannual reproductive activity. Of all environmental signals, the annual variation in daylength, remaining unchanged from year to year, constitutes the most dependable cue to synchronize reproductive events with the climatic cycle (Goodman, 1994).

Ewes are referred to as "short-day breeders", which means that exposure to decreasing or short daylengths induces oestrous cycles, whereas increasing or long photoperiods initiate seasonal anoestrus or the non-breeding season (Marshall, 1937; Hammond, Jr., 1944; Yeates, 1949; Hafez, 1952; Legan and Karsch, 1979; Karsch et al.,
1984; Robinson, 1988; Wayne et al., 1990; O'Callaghan et al., 1991; Goodman, 1994). This photoperiodic control of annual cyclicity can be reproduced experimentally by exposing animals to artificial long (inhibitory effect) or short (stimulatory effect) daylengths to either suppress or re-activate ovarian function (Clegg et al., 1965; Walton, 1975; Walton et al., 1977; Legan and Karsch, 1980; Lincoln and Short, 1980; Nicholls et al., 1989). It has also been demonstrated that other environmental and climatic cues, such as fluctuations in temperature, have little or no effect on the seasonal reproductive activity of ewes (Wodzicka-Tomaszewska et al., 1967; Poulton and Robinson, 1980).

In breeds of sheep originating from geographical regions of higher latitudes (temperate regions), the breeding season commences in late summer and continues until late winter (Legan and Karsch, 1979; Karsch et al., 1979). In contrast, breeds developed in areas nearer the equator (tropical regions) do not exhibit as high a degree of seasonality and, in some cases (e.g., Merino sheep of Australia and South Africa), are able to maintain reproductive function throughout the year (Robinson, 1959, 1980); however, a peak of reproductive activity (maximum ovulation rates) in such breeds occurs in the fall, coinciding with the breeding season of breeds with more pronounced seasonality (Dun et al., 1960). More prolific breeds of sheep tend to have a longer breeding season than non-prolific animals under the same climatic conditions (Webster and Haresign, 1983).

Interestingly, shortening and lengthening photoperiods may be only indirectly involved in controlling transitions between fertile and infertile states in seasonal breeders. It was demonstrated that both the beginning and cessation of the breeding season and anoestrus in the ewe were due primarily to photorefractoriness (Hamner, 1968; Robinson and Follet, 1982; Worthy and Haresign, 1983; Robinson and Karsch, 1984; Robinson et al., 1985). Photorefractoriness is a condition of acquired insensitivity to the stimulatory or inhibitory actions of short or long daylengths (Robinson and Follet, 1982). Experimental prolongation of a short daylength beyond the time of the winter solstice fails to prolong the breeding season (Robinson and Karsch, 1984). Similarly, the time of onset of the breeding season is not affected in ewes artificially sustained on the summer solstice photoperiod (Robinson et al., 1985). From these observations, it was concluded that the onset of the next
phase of the annual reproductive cycle in the ewe resulted mainly from the development of insusceptibility to prevailing daylengths and thus cannot be overridden by photoperiodic stimulation (Clegg et al., 1965; Karsch et al., 1980; Goodman and Karsch, 1981a-b; Malpaux et al., 1988b). These data could also be interpreted to suggest a circannual endogenous rhythm that controls reproductive transitions in sheep and is only cued by photoperiodicity.

The pineal gland plays a key role in translating photoperiodic signals into endocrine information (Lincoln, 1992). Pinealectomy prevents all photoperiod-related shifts in annual ovarian activity in the ewe (Kennaway et al., 1982), being equally important for both the transition into the breeding season and anoestrus (Bittman et al., 1983a-b). This function of the pineal gland is due to secretion of an endogenous indoleamine-melatonin (Rollag and Niswender, 1976; Earl et al., 1985). Melatonin release is suppressed by light and increases shortly after the onset of darkness (Rollag and Niswender, 1976; Rollag et al., 1978; Earl et al., 1985). Thus, because the pattern of melatonin secretion closely reflects that of photoperiodic changes (light-dark pattern), it has been suggested that melatonin relays the effects of photoperiod to time seasonal reproductive states in ewes. There is a great deal of evidence supporting this view. Administration of melatonin in sheep kept on a long-day regime advanced the onset of the breeding season (Kennaway et al., 1982, 1983a-b, 1984). Administration of melatonin to pinealectomized animals also mimicked the effects of short days on the production of LH in sheep (Bittman et al., 1983b; Bittman and Karsch, 1984; Yellon et al., 1985, Malpaux et al., 1988a). However, the theory of photorefractoriness is somewhat in conflict with the suggested physiological role of melatonin. Provided that transitions to the breeding season and anoestrus occur independently of the changes in daylength, but are predominantly driven by developing resistance to prevailing photoperiod, the direct role of melatonin in initiating transition periods may be challenged. Although it was suggested that photorefractoriness might result from a transient failure of melatonin secretory patterns to match the annual daylength cycles (Almeida and Lincoln, 1984; Thurm et al., 1984) or was due to an inadequate response of the hypothalamic-pituitary axis to melatonin (Karsch et al., 1986; Malpaux et al., 1987, 1988b; Wayne et al., 1988), the real
nature of the melatonin-dependent mechanism involved in the control of reproductive seasonality in sheep is still ill-understood.

Melatonin receptors were demonstrated in the pars tuberalis, a part of the adenohypophysis, in close vicinity to ME (de Reviers et al., 1989; Bittman and Weaver, 1990; Stankov et al., 1991; Helliwell and Williams, 1992). Surprisingly, much less binding was observed in the ME and pars distalis (de Reviers et al., 1989; Bittman and Weaver, 1990; Stankov et al., 1991; Helliwell and Williams, 1992) which are directly responsible for the control of gonadotrophin secretion (Bittman and Weaver, 1990). There are, however, numerous melatonin receptors present in other parts of the ovine central nervous system, including the rostral hypothalamic region (Bittman and Weaver, 1990; Stankov et al., 1991; Helliwell and Williams, 1992) and the medial-basal hypothalamus, where they co-exist with GnRH neurons (Bittman and Weaver, 1990). In light of these findings, it was hypothesized that melatonin action in sheep was directed towards neural mechanisms controlling the function of the GnRH pulse generator rather than modulating pituitary responsiveness to stimulatory pulses of GnRH (Robinson et al., 1986).

The shifts between different stages of the annual reproductive cycle in ewes are associated with the dramatic changes in the degree of responsiveness of the hypothalamic-pituitary axis to the negative feedback effects of oestradiol on tonic LH secretion (Legan et al., 1977; Karsch et al. 1978; Legan and Karsch, 1979; Goodman et al., 1980, Martin et al., 1983). During the breeding season oestradiol decreases amplitude but does not affect LH pulse frequency (Goodman et al., 1980; Martin et al., 1983), but in anoestru, oestradiol also markedly reduces LH pulse frequency (Goodman et al., 1980, Martin et al., 1983). This is referred to as the "steroid-dependent" action of photoperiod as opposed to the "steroid-independent" effects of photoperiod on LH pulse frequency and amplitude (lower frequency and higher amplitude in anoestrous animals; Karsch et al., 1980; Lincoln and Short, 1980; Goodman et al., 1981; Martin et al., 1983; Robinson, 1988). It has been suggested that melatonin, by controlling activation and/or inactivation of different neurons (both oestradiol-sensitive and oestradiol-independent), synchronizes an annual reproductive rhythm so that GnRH and LH pulse frequency are suppressed under long days, but not in the fall and winter (Goodman, 1994). During anoestrus, dose-dependent oestradiol
suppression of mean serum concentrations of FSH was also found in ewes (Reeves et al., 1974). It was concluded that during seasonal anoestrus in sheep, the pattern of secretion of both gonadotrophic hormones might be affected by the developing negative-feedback effects of oestradiol.

The mode of oestradiol action during anoestrus involves reduction of hypothalamic GnRH pulse frequency (Karsch et al., 1984; Clarke, 1988; Barrell, 1992). Although GnRH neurons in the sheep brain do not express nuclear oestrogen receptors, the effects of oestrogen on the phasic secretion of GnRH arise from its influence on non-GnRH neurons which project to the vicinity of the GnRH network (Herbison, 1995). In anoestrous ewes, catecholaminergic (Meyer and Goodman, 1985, 1986; Goodman, 1989; Havern et al., 1994) and dopaminergic (Meyer and Goodman, 1985, 1986; Havern et al., 1994; Tortonese and Lincoln, 1994; Lincoln and Tortonese, 1995) systems mediate steroid-dependent effects of photoperiod, whereas steroid-independent regulation occurs mainly via the actions of a serotonergic system (Meyer and Goodman, 1985, 1986; Shillo et al., 1985; Wishnant and Goodman, 1990; le Corre and Chemineau, 1993). Most recent studies have also implicated cells generating nitric oxide in oestradiol-dependent neurochemical control of GnRH output (Herbison, 1995).

1.9.2 Endocrine function and ovarian activity during seasonal anoestrus in ewes

Season-related changes in tonic and pulsatile secretion of LH are due solely to alterations in GnRH pulse frequency (Barrel et al., 1992). Since other elements of the hypothalamic-pituitary-ovarian axis remain intact in anoestrous sheep, namely the ability of oestradiol to produce a preovulatory LH surge and the ability of LH to stimulate oestradiol secretion (Legan and Karsch, 1979), low GnRH pulse frequency is a key factor contributing to the maintenance of the anovulatory state in ewes. Although a slight reduction in the degree of ovarian responsiveness to LH has been observed in anoestrous sheep, it was concluded that of all hormonal cues, photoperiod-related suppression of LH secretion is fully adequate to explain the pattern of seasonal breeding in sheep (Karsch et al., 1980).
During transition from the breeding season to anoestrus, the decline in serum progesterone after the last luteal phase is followed by a detectable increase in serum LH concentrations (Karsch et al., 1979) but not by an evident rise in oestradiol secretion (Rawlings et al., 1977). Therefore, as LH pulse frequency fails to rise to a normal follicular phase peak, there is a disruption of sustained ovarian secretion of oestradiol (Karsch et al., 1979; Goodman, 1994). Consequently, the preovulatory gonadotrophin surge does not occur, and LH and progesterone fall to basal levels (Yuthasastrakosol et al., 1977; Jackson and Davis, 1979; Joseph et al., 1992). LH pulse frequency is lower in anoestrous sheep as compared to that recorded during the luteal phase of the oestrous cycle (Scaramuzzi and Martens, 1975; Jackson and Davis, 1979; Scaramuzzi and Baird, 1979; Walton et al., 1980). The frequency of LH pulses does not differ significantly from mid-anoestrus until the end of the anoestrous period (Yuthasastrakosol et al., 1977; Jackson and Davis, 1979; Walton et al., 1980; McNatty et al., 1984a-b).

Ewes remain in anoestrous as long as the photoperiodically mediated inhibition of LH release persists (Goodman, 1994). Towards the end of the non-breeding season, the activity of inhibitory mechanisms holding LH frequency and amplitude in check declines, and restored pulsatile LH secretion stimulates increased follicular oestradiol production, which in turn triggers a first preovulatory LH surge (Karsch et al., 1980). It has been suggested that when this first LH surge is induced, the largest ovarian antral follicles are not always mature enough to respond by ovulating and developing into healthy CL (Legan et al., 1985); short luteal phase patterns of progesterone secretion (1 to 2 days in length) that might result from ovulations of premature ovarian antral follicles (Hunter, 1991) are observed in the majority of ewes at the onset of the breeding season (Legan et al., 1985).

Mean FSH concentrations remain unchanged during transitional periods between the breeding season and anoestrus in ewes (Karsch et al., 1979), and the pattern of FSH secretion in anoestrous is non-pulsatile. There is, however, evidence for periodic fluctuations in FSH release occurring approximately every at 4-5 days (Bister and Paquay, 1983), reflecting daily changes in the mean and basal serum concentrations of FSH.
After the last luteal phase of the breeding season, serum progesterone concentrations rapidly decline to basal or non-detectable levels. There are, however, certain randomly occurring deviations of serum progesterone concentrations above the basal level throughout anoestrus (Thorburn et al., 1969; Yuthasastrakosol et al., 1975; Walton et al., 1977; l’Anson, 1983; Ravindra, 1993). In addition, a small increase in plasma progesterone concentrations was seen approximately four days prior to the first ovulation of the breeding season (Walton et al., 1977; Berardinelli et al., 1979; Foster and Ryan, 1979). The source of progesterone detected during anoestrus and at the transition to the breeding activity in ewes has yet to be determined.

Mean serum concentrations of oestradiol recorded during mid-anoestrus, increased within the period from the first to the second oestrous cycle of the ovulatory season (Yuthasastrakosol et al., 1975), and the first well defined peak of oestradiol was reported to occur during the second cycle of the breeding season (Yuthasastrakosol et al., 1975). Pulsatile tonic secretion of oestradiol is maintained and remains coupled with episodic LH secretion throughout anoestrus in ewes (Scaramuzzi and Baird, 1977).

Earlier post-mortem studies in ewes revealed that ovaries of anoestrous ewes were not inactive (Hutchinson and Robertson, 1966; Smeaton and Roberston, 1971; McNatty et al., 1984b). Maximum follicle diameter and mean number of ovarian antral follicles did not differ between animals sacrificed during anoestrus and those slaughtered from day 5 of the oestrous cycle onwards (Brand and de Jong, 1973). Some studies reported that there was a tendency for the ovaries of anoestrous ewes to bear significantly more small ovarian antral follicles relative to the ovaries of cyclic ewes (Hutchinson and Robertson, 1966; Ravindra, 1993). Ultrasound examination of ovaries was done during two 5-day periods in anoestrus, approximately 55 and 30 days prior to first ovulation of the breeding season (Ravindra, 1993). No antral follicles ≥ 6 mm in diameter were recorded during either scanning period, and the total numbers of all antral follicles ≥ 2 mm in size did not differ between the two scanning periods in anoestrus. The numbers of ovarian follicles in different size-classes varied from day to day, but these changes were not associated with concentrations of FSH and oestradiol measured in daily blood samples. In another study using repeated laparoscopy
(Noel et al., 1993), the growth of the largest ovarian follicles in anoestrous ewes exhibited a distinct wave-like pattern. Thus, although ovarian follicle populations have been studied at slaughter and, more recently, with the use of surgical techniques and ultrasonography, and hormone secretion has been studied in ewes at different reproductive states, the information on the relationships between ovarian activity and concurrent endocrine changes in ewes is still very limited. There is, particularly, a paucity of studies on the pattern of ovarian follicular development and luteal function in ewes varying in prolificacy, and no definitive attempt has been made to gain detailed information regarding ovarian function in ewes during transitional periods between the breeding activity and anoestrus.
Chapter 2. GENERAL OBJECTIVES

The main objective of the present studies was to use transrectal ovarian ultrasonography and hormone measurements to describe ovarian antral follicle dynamics and the development of CL/luteal structures in relation to circulating concentrations of gonadotrophic hormones and ovarian steroids in ewes.

A better understanding of the follicular and luteal function and their endocrine dependency may help us devise improved management systems and practical techniques to increase fertility in commercial flocks of sheep. In this context, the aim of the first study in the breeding season was to document and compare the patterns of antral follicle kinetics and luteal function throughout the normal (non-synchronized) oestrous cycle, as related to prolificacy, in different breeds of sheep (non-prolific Western white faced ewes and prolific Finn sheep). Of particular interest were any differences in the patterns of development of antral follicles and concurrent hormone secretion that might explain differences in ovulation rate in the ewes under study.

Gonadotrophic hormones (LH and FSH) are essential for antral follicle growth and maturation; however, alterations in the pulsatile characteristics of LH/FSH secretion around the time of follicle emergence and during later stages of follicle lifespan, have not been studied to date, and it is not known whether changes in the pattern of LH/FSH release occur during formation of the corpus luteum (CL) in ewes. The objective was to see if the patterns of episodic secretion of FSH and LH were correlated with various stages of large antral follicular development, from the day of emergence until regression, and luteinization of ovulated follicles in ewes.

A study of ovarian follicular dynamics during both transitional periods (into anoestrus and from anoestrus to the breeding activity) could be intriguing and help us to understand the processes of the circannual “turning on and off” of oestrous cyclicity, typical
of seasonal breeders like sheep. The control of ovarian follicular activity and luteal function during the transitional reproductive states in sheep are still unclear. The goal was to describe ovarian follicular dynamics and CL development during the periods encompassing the first and the final luteal phase of the breeding season, and to see if photoperiod-cued changes in LH secretion affected the numbers and growth characteristics of ovarian antral follicles and the formation of luteal structures.

Manipulating ovarian function to increase fertility in less prolific genotypes of sheep is one of the priorities in contemporary sheep breeding. Earlier studies in cattle and sheep led us to hypothesize that the increased ovulation rate in prolific breeds of sheep might be related to circulating concentrations of luteal progesterone at the mid-luteal phase of the oestrous cycle; it has been shown that serum levels of progesterone in these species are inversely related to the lifespan and viability of the largest, ovulatory-sized antral follicles. An experiment was designed to see if the induction of luteolysis followed by the treatment with progestogen-impregnated sponges (creation of “low progesterone” environment) would stimulate prolonged follicle growth and increase ovulation rate in non-prolific Western white-faced ewes.

In some ewes, the CL that develops from the first ovulation following the period of “reproductive quiescence” (e.g., seasonal or lactational anoestrus, puberty) is short-lived. In the absence of concomitant healthy CL, short-lifespan CL lead to a lack of synchrony of cyclic events, pregnancy loss, management problems, and ultimately reduced fertility. Elucidation of the mechanism underlying untimely luteolysis and subsequent work to minimize these effects could prevent the consequences of luteal insufficiency. This would permit us to overcome some of the reproductive restraints placed on ewes bred out of season, and during post-partum and peripubertal periods. GnRH induced ovulations in seasonally anoestrous ewes are a good model for the study of abnormal luteal function. We, therefore, examined the relationships between antral follicle development, secretion of LH, FSH, oestradiol and progesterone, ovulations and formation of luteal structures in response to GnRH in anoestrous ewes.
Chapter 3. HYPOTHESES

1. Growth and regression of ovarian antral follicles in cyclic ewes occur in waves, and this pattern of antral follicle development is not affected by differences in prolificacy.

2. Emergence of ovarian follicles is associated with detectable fluctuations in daily serum concentrations of FSH, and is reflected in peaks of mean serum concentrations of follicular oestradiol throughout the ewe's oestrous cycle.

3. The high ovulation rate in prolific Finnish Landrace sheep depends on the increased numbers of ovulatory-sized follicles recruited during the final stage of the oestrous cycle or a longer period of recruitment of ovulatory follicles (follicles emerging from the mid- to late luteal phase). In contrast, non-prolific Western white-faced ewes have fewer emergent follicles that maintain ovulatory diameters during the follicular phase of the cycle, recruited over a shorter period of time.

4. Differences in the number and size of CL in ewes differing in ovulation rates are positively correlated with and reflected in changes in mean serum levels of progesterone.

5. The beginning of the growth of the largest antral follicles during metoestrus and dioestrus in ewes is preceded by a distinct increase in the secretion of FSH, and later stages of the follicle lifespan are associated with progressive changes in oestrogenicity and the pulsatile patterns of LH and FSH secretion.
6. Formation and initial development of CL alter the pulsatile secretion of LH and FSH in the ewe.

7. During the transition from the breeding season to anoestrus in ewes, the onset of photoperiod-cued suppression of tonic LH secretion results in the disruption of antral follicular growth and depresses the development and/or secretory potential of CL/luteal structures.

8. Restoration of ovarian cyclicity at the transition from anoestrus to the breeding season in ewes is associated with the occurrence of short luteal phases resulting from incomplete luteinization of ovulated follicles and/or the shortened lifespan of CL/luteal structures.

9. Treatment of cyclic Western white-faced ewes (non-prolific breed) with PgF₂α/progestogen from late in the oestrous cycle will prolong the growth of the largest antral follicles and increase the ovulation rate to that of more prolific genotypes of sheep (e.g., Finnish Landrace).

10. Ovulation induction with GnRH during deep anoestrus in ewes results in both normal and aberrant patterns of luteal function post-ovulation, and different ovarian responses are related to the stage of follicular lifespan, or the degree of follicle maturation, and hormone secretion around the time of treatment.
Chapter 4. GENERAL METHODOLOGICAL CONSIDERATIONS

4.1 Animals and management

All experiments were carried out in Saskatoon, Saskatchewan, Canada, at 52° N, between years 1995 and 1999, using sexually mature Western white-faced (aged from 3 to 9 years) and Finnish Landrace ewes (aged 3 to 4 years). The Western White Face is largely of Rambouillet x Columbia breeding, and the average number of lambs born per ewe is 1.5 ± 0.2 (Jeffcoate et al., 1984a). The Western white-faced ewes were nulliparous and Finn sheep had lambed twice; the average number of offspring born per ewe was 2.4 ± 0.3. Animals were housed outdoors in sheltered dry lots and received daily maintenance rations of alfalfa pellets while water, hay and cobalt iodized salt licks were available ad libitum.

4.2 Oestrous detection

Oestrous was detected with vasectomized crayon-harnessed rams and an electronic oestrous detector (Firma Draminski, Olsztyn, Poland). The instrument measures changes in vaginal mucous impedance near the cervix uteri and was validated for the present application in sheep (Szczechanski et al., 1994). A decline in vaginal electrical resistance below 40 ohms occurs at the beginning of behavioural oestrus and it persists for approximately 24-48 h until ovulation (Szczechanski et al., 1994; Bartlewske et al., 1999).

4.3 Ultrasound technique

Ovarian ultrasonographic imaging was performed using a B-mode, real-time echo camera (Aloka SSD-500; Overseas Monitor Corp. Ltd., Richmond, BC, Canada) equipped
with a 7.5-MHz human prostate transducer (shaft length 35 cm; shaft diameter 1.6 cm) or a stiffened 7.5-MHz veterinary use transrectal probe, modified and validated for the application in sheep (Schrick et al., 1993; Ravindra et al., 1994). Ewes were examined in a standing position, restrained by a head gate in a sheep cart. For greater consistency, all examinations were done by a single operator. Images were displayed at 1.5x magnification. Number, diameter and relative position of all detected ovarian follicles and luteal structures were sketched on ovarian charts. All ovarian images were recorded on high-grade video tapes (Fuji S-VHS, ST-120N), using a compatible cassette recorder (Panasonic, Super VHS, AG 1970), for retrospective analysis of ovarian data.

4.4 Blood sampling and hormone analyses

Daily blood samples were collected by jugular venipunctures, using vacutainers (Becton Dickson, Rutherford, NJ, USA). For the intensive blood sampling, indwelling jugular catheters (vinyl tubing; 1.00 mm i.d. x 1.50 mm o.d.; SV70, Critchley Electrical Products Pty Ltd., Auburn, NSW, Australia) were inserted 24 h before the bleed and were filled with heparinized saline (1000 U.S.P. units of heparin sodium per 1 l of saline; Hepalean, Organon Teknika Inc., Toronto, ON, Canada). All blood samples were allowed to clot for 18-24 h at room temperature, and serum was harvested and stored at −20°C until assayed. Serum samples were analysed by radioimmunoassay for concentrations of FSH (Currie and Rawlings, 1989), LH (Rawlings et al., 1984), oestradiol (Joseph et al., 1992) and progesterone (Rawlings et al., 1984). Concentrations of LH and FSH are given in terms of NIAMDD-oLH-24 and NIAMDD-oFSH-1, respectively. The sensitivities of assays were: 0.1 ng/ml (FSH and LH), 1.0 pg/ml (oestradiol) and 0.03 ng/ml (progesterone). The range of standard curves was from 0.06 to 8.0 ng/ml, 0.12 to 16.0 ng/ml, 1.0 to 50 pg/ml and 0.1 to 10 ng/ml in the LH, FSH, oestradiol and progesterone assays, respectively. Serum samples with concentrations of hormones below the assay sensitivity were assigned a concentration equal to the sensitivity of the assay.
The Cycle Detector Program (Clifton and Steiner, 1983) was used to identify peaks in daily serum concentrations of FSH and oestradiol in individual ewes. The program determines threshold concentrations of the hormone based on the values of intraassay coefficients of variation or the variation between sample replicates. Concentrations that are greater than the threshold values are the peak concentrations of a fluctuation. A cycle or fluctuation is defined as a progressive increase and decrease in hormone concentrations that encapsulate a peak concentration (nadir-to-peak-to-nadir).

The PC-PULSAR program (Gitzen and Ramirez, 1988) was used to estimate LH/FSH pulse frequency, amplitude and duration as well as mean and basal serum concentrations of LH and FSH obtained by frequent blood sampling. The basal serum level ("smoothed series") was generated after the removal of short-term variations in hormone concentrations, including possible pulses. Standard deviation criteria (G and Baxter parameters) were used for pulse detection (Merriam and Wachter, 1982).
Chapter 5. OVARIAN ANTRAL FOLLICULAR DYNAMICS AND THEIR RELATIONSHIPS WITH ENDOCRINE VARIABLES THROUGHOUT THE OESTROUS CYCLE IN BREEDS OF SHEEP DIFFERING IN PROLIFICACY

5.1 Abstract

Daily transrectal ultrasonography of ovaries was performed in non-prolific Western white-faced (n = 12) and prolific Finn ewes (n = 7), during one oestrous cycle in the middle portion of the breeding season (October-December), to record the number and size of all follicles ≥ 3 mm in diameter. Blood samples collected once a day were analysed by radioimmunoassay for concentrations of LH, FSH and oestradiol. The cycle-detection computer program was used to identify transient increases in concentrations of FSH and oestradiol in individual ewes. Follicular and hormonal data were then analysed for associations between different stages of the lifespan of the largest follicles of follicular waves and detected fluctuations in serum concentrations of FSH and oestradiol. A follicular wave was defined as a follicle or a group of follicles that began to grow from 3 to ≥ 5 mm in diameter within a 48-h period. An average of 4 follicular waves per ewe were seen to emerge during the interovulatory interval in both breeds of sheep studied. The last follicular wave of the oestrous cycle contained ovulatory follicles in all ewes, and the penultimate wave in 10% of white-faced ewes but in 57% of Finn sheep. Transient increases in serum concentrations of FSH were detected in all animals and peak concentrations occurred on days approximating follicle wave emergence. Follicular wave emergence was associated with the onset of transient increases in serum concentrations of oestradiol, and the end of the growth phase of the largest follicles (≥ 5 mm in diameter) was associated with peak serum concentrations of oestradiol. Serum FSH concentrations were higher in Finn than in Western white-faced ewes during the follicular phase of the cycle (P < 0.05). There were no
significant differences in serum concentrations of LH between Western white-faced and Finn ewes (P > 0.05). Mean serum concentrations of oestradiol were higher in Finn compared to Western white-faced ewes (P < 0.01). It was concluded that follicular waves (follicles growing from 3 to ≥ 5 mm in diameter) occurred in both prolific and non-prolific genotypes of ewes and were closely associated with increased secretion of FSH and oestradiol. The increased ovulation rate in prolific Finn ewes appeared to be due primarily to an extended period of ovulatory follicle recruitment.

5.2 Introduction

In sheep, ovulation rate is genetically pre-determined and it can vary between breeds from ~1 in mainly monovular Merino del Pais (Lopez-Sebastian et al., 1997) and Australian Merino ewes (Campbell et al., 1995), to ~2 in breeds with a high incidence of twinning, like the Javanese thin-tail sheep (Sutama et al., 1988), and to greater than 3 in prolific breeds such as the Finnish Landrace and Romanov (Campbell et al., 1995). There are also specific genes, for example the Fee or Booroola genotype, that result in ovulation rates greater than 5 (Cummins et al., 1983). Higher ovulation rates are often accompanied by smaller ovulatory follicles (Driancourt, 1991; Driancourt et al., 1993) and fewer granulosa cells per follicle with less oestradiol production (Avdi et al., 1997). It has been suggested that increased ovulation rates could be due to a wider window of time for follicle recruitment or an increase in the numbers of follicles recruited (Scaramuzzi et al., 1993). The physiological regulation of increased ovulation rate is unclear and although the growth of antral follicles is largely regulated by FSH (Scaramuzzi et al., 1993), the evidence that increased FSH secretion is responsible for increased ovulation rates is contradictory (Scaramuzzi et al., 1993; Campbell et al., 1995; Fry and Driancourt, 1996; Avdi et al., 1997; Souza et al., 1997). In one recent study using ultrasonography, ovarian follicular dynamics did not differ between sheep with or without the Booroola gene (Souza et al., 1997). However, ovulatory follicles and CL were smaller in ewes carrying the fecundity gene but serum gonadotrophin and ovarian hormone concentrations did not differ between the prolific and non-prolific ewes.
Early observations on the development of antral follicles in ewes led to the conclusion that follicles begin to grow early in the cycle and then remain in the ovaries to enlarge shortly before ovulation (Hutchinson and Robertson, 1966). In 1971, Smeaton and Robertson reported that there were at least three distinct groups of ovarian follicles beginning to grow synchronously throughout the normal oestrous cycle in ewes. In 1973, Brand and de Jong suggested a two-phase model of follicular development in the cyclic ewe, with two distinct cohorts of antral follicles beginning to grow at about days 2 and 11 of the cycle. Studies using ink marking of follicles suggested waves of follicle production during the oestrous cycle (Driancourt, 1991). Recent studies using daily endoscopy or ovarian ultrasonography have revealed that the growth of follicles to ≥ 5 mm in diameter occurs in an orderly fashion, at approximately 5-day intervals, throughout the oestrous cycle in the ewe (Noel et al., 1993; Ginther et al., 1995). However, studies summarized by Lopez-Sebastian et al. (1997), including the data of Schrick et al. (1993), did not find waves of follicle production.

In sheep, it was concluded that the growth of antral follicles is primarily dependent on FSH and the terminal phase of follicular development, culminating in ovulation, is under the control of LH (Baird et al., 1976; Scaramuzzi et al., 1993; Campbell et al., 1995). During the ovine oestrous cycle, serum concentrations of FSH increase and decrease at relatively regular intervals (Campbell et al., 1991b; Ginther et al., 1995), and there is a temporal relationship between the emergence of follicular waves (follicles growing from 3 to ≥ 5 mm in diameter) and these transient elevations in daily serum FSH concentrations (Ginther et al., 1995; Fry and Driancourt, 1996).

The aim of the present study was to use daily ultrasound imaging of ovaries to describe and compare the patterns of ovarian antral follicle turnover throughout the oestrous cycle in two breeds of sheep with different ovulation rates (non-prolific Western white-faced and prolific Finn sheep). We were particularly interested in differences that could explain the mechanism utilized to increase ovulation rate in prolific sheep. In this respect, we also attempted to correlate alterations in circulating concentrations of FSH and oestradiol, and the development of the largest ovarian follicles.
5.3 Materials and methods

5.3.1 Animals and experimental procedures

Twelve Western white-faced (aged approximately 5 years, average body weight 90 ± 7 kg) and seven Finn ewes (3-4 years of age, average body weight 57 ± 4 kg) were used in this study during the middle portion of the breeding season (October-December). Each animal underwent daily transrectal ovarian ultrasonography for one ovulatory cycle starting on the day on which the ewe was first marked by the rams or a decline in vaginal impedance readings below 40 ohms was recorded. Days of ovulation were regarded as the days on which large ovarian antral follicles that had been identified by ultrasonography were no longer detected.

5.3.2 Follicular data summary and analysis

Follicular data (follicles ≥ 3 mm in diameter) were combined for both ovaries. Data from two Western white-faced ewes were excluded from analyses as they had abnormally long cycles (both 23 days). A follicular wave was defined as one or more antral follicles that grew from 3 to ≥ 5 mm in diameter; the day the follicles were first detected at 3 mm was the day of wave emergence. Groups of follicles emerging within 48 h were regarded as a single follicular wave. For follicular waves in which follicles emerged within 24 h, the day of wave emergence was the day on which the first follicle of the group was detected at 3 mm and for follicular waves in which follicles emerged within 48 h, the central day was considered the day of wave emergence (see: Fig. 5.1 and 5.2). The following characteristics of follicular waves were determined for each ewe: 1) number of emerging follicular waves; 2) days of wave emergence; 3) number of follicles per wave; 4) maximum diameter attained by the largest follicle of the wave; 5) durations of the follicle growing, static and regressing phases as well as a total lifespan of the largest follicle, and 6) number of days between emergence of sequential follicular waves (interwave intervals). The growing phase was defined as the time taken by a single follicle to grow from 3 mm to its maximum size; the
regressing phase was the time taken by that follicle to regress from its maximum size to 3 mm, and the static phase was the time between the end of the growing phase and beginning of the regressing phase or ovulation. If more than one follicle reached the same maximum size, the follicle that first attained the maximum diameter and/or remained at its maximum size for the longest period of time was regarded as the largest follicle of the wave. Preliminary inspection of follicular data revealed that 13 out of 17 ewes studied had four waves of large follicle growth per oestrous cycle. Therefore, for each of the end points above, a 4 (number of waves) by 2 (breeds) factorial analysis of variance was used (General Linear Model procedures in the Statistical Analysis System SAS/STAT®, version 6; 1990, Cary, NC, USA). Comparisons between sequential waves, within the oestrous cycle, and amongst the two breeds, for each follicular wave, were only done for the ewes that had four waves per cycle, but an additional analysis was performed in which the data for animals with three or four waves per cycle were combined. The characteristics of ovulatory and non-ovulatory antral follicles (follicles growing to ≥ 5 mm in diameter) emerging in the last follicular wave in Western white-faced ewes and in the penultimate wave in Finn ewes were compared by ANOVA. This was not done for the final follicular wave in Finn ewes as all follicles ≥ 5 mm in the wave ovulated, nor for the penultimate wave in Western white-faced ewes, because out of ten white-faced ewes only one had an ovulatory follicle that emerged in the penultimate wave of the cycle.

Daily numbers of emerging 3-mm follicles that did not grow any larger before regression and of 3-mm follicles that subsequently reached 4 or ≥ 5 mm in diameter, were normalized to the day of the first ovulation of the interovulatory interval (day 0), for the period from days −1 to 15 (the entire growing phase could not be determined for non-ovulatory follicles emerging on days 16 and 17 after ovulation) for ewes in each breed that had four waves of follicle growth per cycle, and analysed for day and breed effects and day-by-breed interactions using multivariate analysis of variance (SAS/STAT®, 1990). If the main effect of day was significant (P < 0.05), an analysis of variance, followed by Fisher's protected least significant difference (LSD), were used to compare individual (daily) means within each breed. If there was a significant main effect of breed or if the day-by-breed
interaction was significant, means within each day were compared between breeds by a t-test using Statistix® Analytical Software (version 4.1, 1997; Tallahassee, FL, USA).

5.3.3 Hormone analysis

For reference sera with mean LH concentration of 0.14 or 1.13 ng/ml, the intra- and interassay coefficients of variation CVs were 11.3 and 5.0% or 10.1 and 6.9%, respectively. For FSH reference sera with mean concentration of 2.42 or 3.72 ng/ml, the intra- and interassay CVs were 5.6 and 3.9% or 8.1 and 10.1%, respectively. For oestradiol reference sera with mean concentration of 9.3 or 18.9 pg/ml, the intra- and interassay CVs were 15.0 and 10.1% or 16.3 and 10.9%, respectively. Daily serum concentrations of LH, FSH and oestradiol for the period from day -1 to 17, were normalized to the day of first ovulation of the interovulatory interval (day 0), for ewes of both breeds that had four waves of follicular emergence per oestrous cycle. Data from the two Western white-faced ewes with abnormally long cycles were excluded from analysis. Main effects of breed, day and the breed-by-day interaction were determined by multivariate analysis of variance.

The following characteristics of detected peaks/fluctuations in daily serum concentrations of FSH and oestradiol were determined for each breed: 1) the mean number of peaks per ewe per oestrous cycle, 2) the mean duration of sequential fluctuations, 3) the mean peak concentration and 4) the mean length of intervals between peaks of adjacent fluctuations. Analyses of variance were used to compare means among breeds and within the oestrous cycle for ewes with four follicular waves per cycle.

Follicular and hormonal data were analysed for associations between various stages of follicular wave development (follicles growing from 3 to ≥ 5 mm) and peaks in serum concentrations of FSH and oestradiol. For this purpose, the data for ewes with three or four follicular waves per cycle were combined. The mean number of identified follicular waves and the mean number of FSH or oestradiol peaks per ewe, per oestrous cycle, were compared using a paired t-test. Spearman correlations were done between the lengths of interpeak intervals for detected FSH and oestradiol fluctuations, and intervals between
adjacent days of follicular wave emergence (interwave intervals) as well as different phases of the growth of the largest ovarian follicles of waves, namely the day of emergence, the beginning of the static phase, the end of the static phase, and the end of the regressing phase (regression to 3 mm in diameter).

Oestradiol fluctuations appeared to begin in the proximity of the days of follicle wave emergence. Therefore, we also correlated the lengths of intervals between sequential days of onset of oestradiol fluctuations with the lengths of interwave intervals. The distribution of peaks of FSH fluctuations and onset of oestradiol fluctuations, over the 2 days prior to or after the day of wave emergence and on the day of emergence, were determined. Similarly, the distribution of oestradiol peaks within 2 days before and after the day that the static phase of the largest follicle of a wave began was calculated. The distribution of FSH peaks and onset of oestradiol fluctuations in relation to days of follicular wave emergence was analysed by $\chi^2$-test. A similar analysis was done for peaks of daily oestradiol concentrations and days on which the largest follicles of waves reached their maximum diameters.

5.4 Results
5.4.1 Oestrus detection and cycle length

At the first observed ovulation of the interovulatory interval studied, all the ewes but one were well marked by rams, one animal was poorly marked. At the second ovulation of the interovulatory interval, all ewes were well marked. Vaginal impedance decreased below 40 ohms in all ewes (mean $33.2 \pm 2.7$ and $28.7 \pm 2.4$ ohms for white-faced and Finn ewes, respectively; $P > 0.05$) on the day they were first marked by rams, at the beginning and end of the oestrous cycle studied. Two Western white-faced ewes had prolonged interovulatory intervals (both 23 days), associated with prolonged lifespan of corpora lutea (recorded for $\sim 16$ consecutive days). Excluding these two ewes, the mean interovulatory interval was $17.0 \pm 0.3$ and $16.6 \pm 0.2$ days, for Western white-faced and Finn ewes, respectively ($P > 0.05$). In Western white-faced ewes, the length of the interovulatory
interval in two ewes with three follicular waves per cycle was 17 and 19 days, respectively, and in ewes with four follicular waves (n = 8), the range was 16 to 18 days. Finn ewes with three follicular waves per cycle (n = 2) each had 16 day interovulatory intervals and of the Finn ewes with four emerging follicular waves (n = 5), one animal had a 16-day and four ewes had 17-day interovulatory intervals.

5.4.2 Follicular dynamics

Individual follicle profiles (follicles growing from 3 to ≥ 5 mm in diameter) are shown for 4 different animals, for each breed (including a white-faced ewe with a prolonged cycle), to illustrate variations in the number of follicular waves per ewe, follicle size and the number and time of emergence of ovulatory follicles (Fig. 5.1 and 5.2). The mean ovulation rate was 1.8 ± 0.2 and 2.7 ± 0.2, for Western white-faced and Finn sheep, respectively (P < 0.05).

For ewes with four waves of follicular growth per cycle, follicular data were aligned to the day of first ovulation of the interovulatory interval studied (day 0). When normalized on this basis, a significant main effect of breed (P < 0.001), but not of day (P > 0.05) or day-by-breed interaction (P > 0.05), was seen for mean daily numbers of emerging 3-mm follicles not growing beyond 3 mm in diameter (Fig. 5.3a). The Western white-faced ewes exceeded (P < 0.05) Finn sheep in daily numbers of follicles reaching a maximum size of 3 mm from day −1 to 0, 4 to 6, 9 to 11 and 13 to 15 of the oestrous cycle studied. There was no significant main effect of day (P > 0.05), breed (P > 0.05) or day-by-breed interaction (P > 0.05) for mean daily numbers of 3-mm follicles growing to a maximum diameter of 4 mm before regression.

Daily numbers of emerging 3-mm follicles which subsequently grew to ≥ 5 mm in diameter differed by day (P < 0.05), but there was no significant difference between the two breeds of sheep studied (P > 0.05) nor a significant day-by-breed interaction (P > 0.05). Follicles growing to ≥ 5 mm in diameter emerged from the pool of 3-mm follicles on each day of the cycle except for days 2 and 15 in Western white-faced ewes, and days −1, 7 and
Fig. 5.1. The diameter profiles of individual antral follicles growing to a size of ≥ 5 mm and recorded during the oestrous cycle in four Western-white faced ewes (a-d), with accompanying daily serum concentrations of FSH (middle panels; ■) and oestradiol (bottom panels; □). The arrows along the X-axis (top panels; †) denote the days of follicle wave emergence, and the arrows within the upper chart area (−) indicate the ovulatory follicles on the day before ovulation. A capital letter L marks a large antral follicle that became luteinized and gradually transformed into a solid luteal structure. The diameters of ovulatory follicles observed at the first ovulation of the interovulatory interval studied are not shown. Asterisks denote the peak values of fluctuations (nadir-to-peak-to-nadir) in circulating concentrations of FSH and oestradiol (the two dashed lines encompass a fluctuation) as determined by the cycle-detection computer program (Clifton and Steiner, 1983).
Fig. 5.2. The diameter profiles of individual antral follicles growing to a size of $\geq 5$ mm and recorded during the oestrous cycle in four Finn ewes (a-d), with accompanying daily serum concentrations of FSH (middle panels; □) and oestradiol (bottom panels; □). The arrows along the X-axis (top panels; †) denote the days of follicle wave emergence, and the arrows within the upper chart area (・) indicate the ovulatory follicles on the day before ovulation. The diameters of ovulatory follicles observed at the first ovulation of the interovulatory interval studied were not shown. Asterisks denote the peak values of fluctuations (nadir-to-peak-to-nadir) in circulating concentrations of FSH and oestradiol (the two dashed lines encompass a fluctuation) as determined by the cycle-detection computer program (Clifton and Steiner, 1983).
Fig. 5.3. Mean (± SEM) daily numbers of 3-mm follicles reaching a maximum diameter of 3 mm before regression (a) and of 3-mm follicles that subsequently grew up to ≥ 5 mm in diameter (b), in cyclic Western white-faced (n = 8; ■) and Finn ewes (n = 5; □) that underwent daily transrectal ovarian ultrasonography for one ovulatory cycle during the mid-breeding season (October-December) and had four waves of follicular growth during the cycle. Follicular data were normalized to the day of first ovulation of the oestrous cycle studied (day 0) and analysed for the period from day −1 to day 15. See text for statistical details.
15 in Finn ewes. There were peaks in the numbers of 3-mm follicles that subsequently reached or exceeded 5 mm in diameter on days 1, 5, 8, 10 and 12 of the cycle in Western white-faced ewes, and similarly on days 1, 6, 8, 10, 12 and 14 in Finn sheep (Fig. 5.3b).

5.4.3 The characteristics of follicular waves

A total of 64 follicular waves (follicles growing from 3 to ≥ 5 mm in diameter) was obtained in the present study; 38 waves were identified in cyclic Western white-faced ewes and 26 waves in Finn sheep. The characteristics of follicular waves are given, for both breeds, in Table 5.1. The data for two Western white-faced ewes with prolonged (23 days) interovulatory intervals were excluded. Often Western white-faced sheep, two animals had 3 and eight ewes had 4 follicular waves per oestrous cycle. Two Finn sheep had 3 and five had 4 waves per cycle. The last follicular wave of the interovulatory period contained ovulatory follicles in all ewes. Of all follicles growing from 3 to ≥ 5 mm in diameter in this wave, 77.3 and 100% ovulated, in Western white-faced and Finn ewes, respectively. The penultimate wave of the oestrous cycle bore ovulatory follicles in one white-faced ewe with 4 waves per cycle and in four Finn sheep (three ewes with 4 and one with 3 waves of large follicle growth). In the penultimate follicular wave of the cycle, 8% (1/12) and 50% (6/12) of follicles growing to ≥ 5 mm in diameter were ovulatory, in white-faced and Finn sheep, respectively.

The following results are for ewes with four waves of follicular growth per cycle. There was no significant difference in the mean days on which follicular waves emerged between non-prolific white-faced and prolific Finn sheep studied; however, the difference for the last follicular wave (Wave 4) approached significance (P = 0.05). The mean number of follicles per wave (1.6 ± 0.1) did not vary (P > 0.05) among white-faced and Finn sheep nor among the four follicular waves for each breed. The maximum diameter attained by the largest ovarian follicle of the wave emerging late in the cycle (Wave 4) was greater (P < 0.05) in Western white-faced than in Finn sheep, and the largest follicle emerging during the early and mid-luteal phase (Waves 2 and 3) tended to be larger in non-prolific white-
Table 5.1. Characteristics of ovarian follicular waves (follicles growing from 3 to ≥ 5 mm in diameter; mean ± SEM) identified in Western white-faced (n = 8, regular font) and Finn sheep (n = 5, italics) that underwent daily ovarian ultrasonography during one ovulatory cycle in the mid-breeding season (October-December), and had four waves per cycle. For comparison, data for two ewes of each breed that only had three waves of follicle growth per cycle are given in parentheses. Day 0 = day of ovulation.

<table>
<thead>
<tr>
<th>End point</th>
<th>Wave 1</th>
<th>Wave 2</th>
<th>Wave 3</th>
<th>Wave 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of waves containing ovulatory follicles</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (2)</td>
<td>8 (-)</td>
</tr>
<tr>
<td></td>
<td>0 (0)</td>
<td>0 (1)</td>
<td>3 (2)</td>
<td>5 (-)</td>
</tr>
<tr>
<td>Mean day of wave emergence</td>
<td>0.2 ± 0.3 (0.1)</td>
<td>4.7 ± 0.4 (7.7)</td>
<td>8.2 ± 0.4 (11.12)</td>
<td>11.8 ± 0.4 (-,-)</td>
</tr>
<tr>
<td></td>
<td>0.8 ± 0.4 (2.2)</td>
<td>5.6 ± 0.2 (7.8)</td>
<td>9.0 ± 0.4 (11.11)</td>
<td>13.0 ± 0.4 (-,-)</td>
</tr>
<tr>
<td>No. of follicles/wave</td>
<td>1.6 ± 0.3 (1.3)</td>
<td>1.6 ± 0.4 (1.1)</td>
<td>1.2 ± 0.2 (1.2)</td>
<td>2.4 ± 0.5 (-,-)</td>
</tr>
<tr>
<td></td>
<td>2.0 ± 0.3 (2.3)</td>
<td>1.2 ± 0.2 (1.2)</td>
<td>1.4 ± 0.2 (2.4)</td>
<td>1.4 ± 0.3 (-,-)</td>
</tr>
<tr>
<td>Largest follicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>6.2 ± 0.3 (5.7)</td>
<td>6.0 ± 0.3 (5.6)</td>
<td>6.0 ± 0.3 (6.7)</td>
<td>6.7 ± 0.2(-,-)</td>
</tr>
<tr>
<td></td>
<td>5.4 ± 0.2 (6.6)</td>
<td>5.2 ± 0.2 (5.5)</td>
<td>5.4 ± 0.2 (5.6)</td>
<td>5.6 ± 0.2(-,-)</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>1.2 ± 0.08 (0.8, 1)</td>
<td>1.1 ± 0.1 (1, 1)</td>
<td>1.1 ± 0.09 (0.75, 0.8)</td>
<td>1.0 ± 0.08 (-,-)</td>
</tr>
<tr>
<td></td>
<td>1.0 ± 0.2 (1.1)</td>
<td>1.1 ± 0.2 (0.5, 1)</td>
<td>1.1 ± 0.2 (0.7, 1)</td>
<td>1.2 ± 0.2 (-,-)</td>
</tr>
<tr>
<td>Growing phase (days)</td>
<td>3.3 ± 0.4 (2.5)</td>
<td>3.0 ± 0.4 (2.3)</td>
<td>3.0 ± 0.5 (4, 5)</td>
<td>3.9 ± 0.1(-,-)</td>
</tr>
<tr>
<td></td>
<td>2.6 ± 0.4 (3, 3)</td>
<td>2.2 ± 0.4 (2.4)</td>
<td>3.0 ± 0.5 (3.3)</td>
<td>2.4 ± 0.2(-,-)</td>
</tr>
<tr>
<td>Static phase (days)</td>
<td>2.9 ± 0.7 (2, 5)</td>
<td>1.4 ± 0.3 (1, 2)</td>
<td>1.6 ± 0.3 (2, 2)</td>
<td>1.4 ± 0.2(-,-)</td>
</tr>
<tr>
<td></td>
<td>3.8 ± 0.4 (1, 1)</td>
<td>1.4 ± 0.4 (2, 3)</td>
<td>1.8 ± 0.6 (2, 2)</td>
<td>1.4 ± 0.2(-,-)</td>
</tr>
<tr>
<td>Regressing phase (days)</td>
<td>4.9 ± 0.8b (3, 4)</td>
<td>4.1 ± 0.8b (2, 3)</td>
<td>2.7 ± 0.6 (-,-)</td>
<td>1.7 ± 0.3 (-,-)</td>
</tr>
<tr>
<td></td>
<td>2.2 ± 0.4b (3, 4)</td>
<td>2.4 ± 0.6 (-,-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lifespan (days)</td>
<td>11.0 ± 0.7b (7, 13)</td>
<td>8.2 ± 1.0b (4, 7)</td>
<td>7.0 ± 0.6b (5, 6)</td>
<td>5.3 ± 0.2b (-,-)</td>
</tr>
<tr>
<td></td>
<td>8.6 ± 0.4bc (6, 7)</td>
<td>6.0 ± 0.6b (6, 6)</td>
<td>5.8 ± 0.5c (4, 4)</td>
<td>3.8 ± 0.4bc (-,-)</td>
</tr>
<tr>
<td>Interwave interval(^1) (days)</td>
<td>4.5 ± 0.2a (7, 7)</td>
<td>3.5 ± 0.3b (4, 5)</td>
<td>3.5 ± 0.3b (-,-)</td>
<td>4.0 ± 0.3 (-,-)</td>
</tr>
<tr>
<td></td>
<td>4.2 ± 0.2 (5, 6)</td>
<td>3.8 ± 0.4 (4, 4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means with different superscripts are different (P < 0.05); \(^{abc}\) within rows, \(^{AB}\) within cells.

\(^1\) Interwave interval refers to the number of days between emergence of the wave in that column and emergence of the next sequential wave.
faced than in Finn ewes (Wave 2: \( P = 0.06 \) and Wave 3: \( P = 0.09 \)). The mean growth rate of the largest follicles of sequential waves (1.1 \( \pm \) 0.03 mm/day) did not differ (\( P > 0.05 \)) throughout the oestrous cycle within each breed nor between the two breeds studied. The growth phase of large follicles late in the cycle (Wave 4) was significantly longer in Western white-faced than in Finn ewes (\( P < 0.001 \)), and it was longer for Wave 4 than for preceding follicular waves (\( P < 0.05 \)) in white-faced ewes. The static phase of the follicular lifespan did not vary (\( P > 0.05 \)) between the 2 breeds studied, but the duration of the static phase of wave 1 was significantly longer relative to subsequent waves (Waves 2-4), in both white-faced and Finn ewes. The regressing phase of the first follicular wave was longer (\( P < 0.05 \)) in white-faced than in Finn sheep, and this phase was also longer for Wave 1 than Wave 3 in white-faced ewes (\( P < 0.001 \)). Follicular lifespan decreased significantly from Wave 1 to 2 and from Wave 3 to 4, in both breeds under study, and it was longer for Waves 1 (\( P < 0.05 \)) and 4 (\( P < 0.01 \)) in Western white-faced compared to Finn ewes. The difference between breeds in mean lifespan of the largest follicles emerging in mid-cycle (Waves 2 and 3) approached significance (Wave 2: \( P = 0.09 \) and Wave 3: \( P = 0.07 \)). The duration of interwave intervals did not differ between the 2 breeds studied but the interval between the first and the second follicular wave of the cycle in Western white-faced ewes was longer (\( P < 0.01 \)) compared to interwave intervals separating the following waves of large follicle growth.

Adding the data for ewes with 3 follicular waves to those of ewes with 4 waves per cycle did not alter any of the statistical trends determined for the groups of white-faced and Finn ewes with four waves per oestrous cycle. In ewes with three waves per cycle, Wave 3 appeared to emerge up to 2-4 days later than in ewes with four follicular waves per cycle. Therefore, the mean day of emergence of Wave 3 averaged day 9 (8.9 \( \pm \) 0.5) and 10 (9.7 \( \pm \) 0.6) when ewes with three or four waves per cycle were combined, as opposed to day 8 (8.2 \( \pm \) 0.4) and 9 (9.0 \( \pm \) 0.4) after ovulation when only ewes with four waves per cycle were considered, for white-faced and Finn ewes, respectively.
5.4.4 The growth characteristics of ovulatory and non-ovulatory antral follicles

The comparisons between ovulatory and non-ovulatory antral follicles originating from the last follicular wave of the cycle in white-faced ewes and from the penultimate wave in Finn sheep are shown in Table 5.2. In white-faced ewes, there were no differences (P > 0.05) in terms of the mean day of emergence or the duration of follicle static phases between ovulatory and non-ovulatory follicles that emerged in the final wave of the interovulatory interval. However, ovulatory follicles were seen to grow over a longer (P < 0.05) period of time and reached considerably greater diameters (P < 0.05) compared to non-ovulatory follicles in this wave. The mean growth rate was higher (P < 0.05) for non-ovulatory than for ovulatory ovarian follicles in the last wave in white-faced ewes. Non-ovulatory follicles that began to grow in the penultimate wave in Finn sheep, emerged earlier in the cycle (P < 0.05), grew for a shorter duration (P < 0.05) and to smaller maximum diameters (P < 0.05), and had significantly shorter static phases in comparison to follicles that emerged in the same wave and subsequently ovulated.

5.4.5 Serum concentrations of LH

Including all ewes with 3 or 4 waves of follicular growth per cycle, there was a significant main effect of day (P < 0.001), but not of breed (P > 0.05) or day-by-breed interaction (P > 0.05) for mean daily serum concentrations of LH normalized to the day of first ovulation. In both breeds of sheep, serum concentrations of LH increased prior to ovulation (P < 0.05). Peaks in daily serum concentrations of LH, during the luteal phase of the oestrous cycle, were only detected in 5 ewes (3 Western white-faced and 2 Finn ewes), and so no further analysis was done.

5.4.6 Serum concentrations of FSH

When daily serum FSH concentrations were normalized to the day of first ovulation (day 0) for ewes with 4 follicle waves per cycle, there was a significant main effect of day
Table 5.2. Comparisons of characteristics of ovulatory and non-ovulatory antral follicles (≥ 5 mm in diameter; means ± SEM), originating from the last follicular wave of the oestrous cycle in ten Western white-faced ewes (regular font) and from the penultimate wave in seven Finn ewes (italics); the data were combined for ewes with three or four follicular waves. Day 0 = day of first ovulation of the interovulatory interval.

<table>
<thead>
<tr>
<th>End point</th>
<th>Western white-faced ewes last follicular wave</th>
<th>Finn ewes penultimate follicular wave</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ovulatory follicles (n = 17)</td>
<td>Non-ovulatory follicles (n = 5)</td>
</tr>
<tr>
<td>Mean day of emergence</td>
<td>11.8 ± 0.3</td>
<td>11.6 ± 0.5</td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>6.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>0.9 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Growing phase (days)</td>
<td>3.6 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Static phase (days)</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>ab</sup> Means with different letter superscripts, within each breed, are different (P < 0.05).
(P < 0.01), breed (P < 0.001), and breed-by-day interaction (P < 0.01; Fig. 5.4a). Mean daily concentrations of FSH were higher (P < 0.05) in Finn than in white-faced ewes around the day of ovulation, at the beginning and end of the oestrous cycle (days −1, 0 and 16), and on day 7 after ovulation. Distinct increases in mean circulating concentrations of FSH could be seen on days 0, 5 and 16 in Finn and on days 0, 5 and 8 of the oestrous cycle in Western white-faced ewes studied.

The characteristics of peaks in serum FSH concentrations, in blood samples taken daily during the oestrous cycle in Western white-faced and Finn ewes, as determined by the cycle-detection computer program (Clifton and Steiner, 1983), are given in Table 5.3. Peaks of transient increases in daily serum concentrations of FSH were detected in all ewes studied. Mean numbers of identified FSH peaks per ewe were 3.7 ± 0.2 and 3.6 ± 0.2, for Western white-faced and Finn ewes, respectively (P > 0.05). Further statistical analyses were limited to ewes with 4 waves of follicular growth per cycle. The amplitude of FSH peaks occurring early in the cycle was higher in Finn compared to Western white-faced ewes (P < 0.05). The duration of fluctuations for daily serum FSH concentrations did not differ significantly throughout the cycle nor between the two breeds of sheep studied (P > 0.05). Mean durations of interpeak intervals for daily serum FSH concentrations did not vary significantly between breeds, but the mean interpeak interval between first two FSH peaks of the cycle was longer (P < 0.05) compared to subsequent interpeak intervals in Western white-faced ewes.

5.4.7 Serum concentrations of oestradiol

There were significant main effects of day and breed (P < 0.001) for daily serum concentrations of oestradiol normalized to the day of first ovulation, but the interaction was not significant (P > 0.05). In the Western white-faced ewes studied, mean concentrations of oestradiol increased significantly from day 0 to day 2 (Fig. 5.4b). During the luteal phase, oestradiol concentrations were variable and there was a sharp decline (P < 0.05) from day 16 to day 17, at the time of the second ovulation of the interovulatory period. In the group
Fig. 5.4. Mean (± SEM) serum concentrations of FSH (a) and oestradiol (b) from blood samples collected daily during the oestrous cycle of Western white-faced (n = 8; ■, ▲) and Finn (n = 5; □, △) ewes with four follicular waves per oestrous cycle. Concentrations of the hormones were normalized to the day of first ovulation of the interovulatory interval studied (day 0). See text for statistical descriptions.
Table 5.3. Characteristics of sequential (1-4) fluctuations in serum oestradiol (E$_2$) and follicle-stimulating hormone (FSH) concentrations in blood samples collected daily throughout an oestrous cycle in Western white-faced (n = 10, regular font) and Finn sheep (n = 7, italics) that underwent transrectal ovarian ultrasonography during the middle portion of the breeding season (October-December) and had four waves of follicle growth per cycle. Data for two ewes in each breed that had three follicular waves per cycle are given in parentheses. All mean values are mean ± SEM.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Endpoint</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak concentration</td>
<td>2.32 ± 0.37$^a$</td>
<td>3.13 ± 0.27</td>
<td>2.83 ± 0.33</td>
<td>3.00 ± 0.53</td>
<td>4.9 ± 0.8$^a$</td>
<td>4.1 ± 0.7$^a$</td>
<td>4.6 ± 0.6</td>
<td>4.2 ± 1.0$^a$</td>
</tr>
<tr>
<td></td>
<td>FSH (ng/ml)</td>
<td>(3.69, 4.39)</td>
<td>(1.71, 3.11)</td>
<td>(3.59, 5.86)</td>
<td>(4.60, 7.47)</td>
<td>(3.7, 4.4)</td>
<td>(1.7, 3.1)</td>
<td>(3.6, 5.9)</td>
<td>(, -)</td>
</tr>
<tr>
<td></td>
<td>E$_2$ (pg/ml)</td>
<td>4.43 ± 0.11$^b$</td>
<td>3.81 ± 0.36</td>
<td>3.00 ± 0.28</td>
<td>2.91 ± 0.30</td>
<td>7.2 ± 0.6$^a$</td>
<td>7.2 ± 0.6$^a$</td>
<td>6.2 ± 1.0$^a$</td>
<td>8.0 ± 0.9$^b$</td>
</tr>
<tr>
<td></td>
<td>E$_2$ (pg/ml)</td>
<td>(3.38, 3.93)</td>
<td>(2.74, 3.36)</td>
<td>(2.70, 2.80)</td>
<td>(-, -)</td>
<td>(8.4, 8.7)</td>
<td>(6.3, 7.2)</td>
<td>(7.6, 14.8)</td>
<td>(-, -)</td>
</tr>
<tr>
<td></td>
<td>Duration of fluctuation</td>
<td>ND</td>
<td>3.7 ± 0.4</td>
<td>3.2 ± 0.4</td>
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<td>4.0 ± 0.3</td>
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<td>(days)</td>
<td>(5, 6)</td>
<td>(5, 7)</td>
<td>(ND, ND)</td>
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<td>(3, 3)</td>
<td>(5, 6)</td>
<td>(-, -)</td>
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<tr>
<td></td>
<td>ND</td>
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<td>(6, 7)</td>
<td>(5, 5)</td>
<td>(-, -)</td>
<td>(4, 6)</td>
<td>(4, 4)</td>
<td>(2, 5)</td>
<td>(-, -)</td>
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<td></td>
<td>Interpeak interval$^1$</td>
<td>4.7 ± 0.2$^a$</td>
<td>3.6 ± 0.3$^b$</td>
<td>3.7 ± 0.4$^b$</td>
<td>4.1 ± 0.3</td>
<td>3.9 ± 0.6</td>
<td>4.5 ± 0.3</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>(days)</td>
<td>(6, 8)</td>
<td>(3, 4)</td>
<td>(6, 6)</td>
<td>(3, 4)</td>
<td>(5, 6)</td>
<td>(-, -)</td>
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<td>4.6 ± 0.4</td>
<td>3.8 ± 0.4</td>
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<td>(6, 6)</td>
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Means with different letter superscripts, for each hormone, are different (P < 0.05); $^{ab}$ within cells, $^{ab}$ within rows.

$^1$ Interpeak interval refers to the number of days between the peak in the indicated column and the next sequential peak.

ND - the duration of fluctuations was not determined as complete fluctuations (nadir-to-nadir) were not seen at the beginning and end of the scanning period.
of Finn ewes, mean concentrations of oestradiol increased from day 0 to day 3 after ovulation \((P < 0.05)\), fell from day 3 to day 8 \((P < 0.05)\), increased again from day 11 to day 15 \((P < 0.05)\) and finally declined to the lowest values on days 16-17 of the oestrous cycle \((P < 0.05)\), around the time of the second observed ovulation of the interovulatory interval studied. On days 1-5, 14-15 and 17 of the cycle, mean serum concentrations of oestradiol were significantly higher in Finn compared to white-faced ewes \((P < 0.05)\).

Using the cycle-detection program, peaks in serum oestradiol concentrations were identified in all ewes studied. The mean number of peaks per ewe, per oestrous cycle was \(3.5 \pm 0.2\) and \(3.4 \pm 0.2\) for white-faced and Finn ewes, respectively \((P > 0.05; \text{Table 5.3})\). For ewes of both genotypes that had four follicular waves per cycle, mean peak concentrations of transient increases in serum oestradiol concentrations did not differ \((P > 0.05)\) throughout the cycle in both breeds studied, but were higher in Finn than in white-faced ewes \((P < 0.05)\) for Peaks 1, 2 and 4 of the cycle. The average duration of fluctuations and interpeak intervals for daily serum concentrations of oestradiol did not vary \((P > 0.05)\) among breeds nor throughout the oestrous cycle within each breed of sheep studied.

5.4.8 The relationship between follicle wave development and serum concentrations of FSH

In this analysis, ewes with 3 or 4 waves of follicular emergence per cycle were included. The number of emerging follicular waves and the number of identified FSH peaks per ewe, did not differ in both breeds of sheep under study \((3.7 \pm 0.2 \text{ vs. } 3.8 \pm 0.1 \text{ and } 3.6 \pm 0.2 \text{ vs. } 3.7 \pm 0.2; \text{ FSH peaks vs. follicular waves, for Western white-faced and Finn ewes, respectively; } P > 0.05)\). The lengths of intervals between adjacent days of wave emergence (interwave intervals) were positively correlated with the lengths of interpeak intervals for FSH fluctuations \((r = 0.76, P < 0.001 \text{ and } r = 0.84, P < 0.001, \text{ for white-faced and Finn sheep, respectively})\). The lengths of interpeak intervals for FSH concentrations were also correlated with the intervals between emergence \((r = 0.72, P < 0.01)\), beginning \((r = 0.64, P = 0.01)\) and end \((r = 0.63, P = 0.01)\) of the static phase, but not the end of regression to a
3-mm size ($r = 0.45$, $P > 0.05$), for the largest follicles of waves in Finn ewes. In white-faced ewes, however, the lengths of interpeak intervals for FSH were only correlated with intervals between emergence of the largest follicles of waves ($r = 0.73$, $P < 0.001$), but not intervals between beginning ($r = 0.24$, $P > 0.05$) and end ($r = -0.10$, $P > 0.05$) of the follicle static phase, or the end of regression ($r = -0.10$, $P > 0.05$). All identified FSH peaks occurred from day $-1$ to day $2$ relative to the nearest day of wave emergence (day $0$) in Western white-faced ewes, and from day $-2$ to day $1$ in Finn ewes (Fig. 5.5a). The highest proportion of FSH peaks occurred on day $0$ in white-faced ($64.9\%$; $P < 0.05$) and on day $-1$ in Finn sheep ($43.5\%$). In Finn sheep, the combined percentage of peaks that occurred on days $-1$ and $0$ ($73.9\%$) was significantly higher than for the three remaining days, before and after the day of wave emergence.

5.4.9 The relationship between follicle wave development and oestradiol secretion

In this analysis, the data for ewes with 3 or 4 waves of follicular emergence per cycle were used. The number of identified peaks of oestradiol fluctuations as well as the number of days on which oestradiol fluctuations began did not differ from the number of emerging follicular waves in both breeds of sheep studied ($3.5 \pm 0.2$ vs. $3.8 \pm 0.1$ and $3.4 \pm 0.2$ vs. $3.7 \pm 0.2$; oestradiol fluctuations vs. follicular waves, for Western white-faced and Finn ewes, respectively; $P > 0.05$). The lengths of intervals between days on which oestradiol fluctuations began were positively and significantly correlated with interwave intervals, in both Western white-faced and Finn ewes ($r = 0.42$, $P < 0.05$ and $r = 0.53$, $P < 0.05$, for white-faced and Finn ewes, respectively). When the correlations above were done for days of emergence of the largest follicles of sequential waves, the correlation coefficients were $r = 0.42$, $P < 0.05$ and $r = 0.73$, $P < 0.001$, for white-faced and Finn ewes, respectively. In both breeds studied, intervals between onset of oestradiol fluctuations were also significantly correlated with intervals between ends of follicle growing phases ($r = 0.38$, $P < 0.05$ and $r = 0.83$, $P < 0.001$, for white-faced and Finn ewes, respectively), but not with intervals between ends of follicle static phases and days of regression to 3 mm in diameter.
Fig. 5.5. (a) Relative frequency of the occurrence of identified FSH peaks \( (n = 37 \text{ and } n = 23, \text{ for Western white-faced and Finn ewes, respectively}) \) and (b) distribution of days of the beginning of oestradiol fluctuations \( (n = 36 \text{ and } n = 24, \text{ for white-faced and Finn ewes, respectively}) \) within two days before and after the day of follicular wave emergence; (c) The percentage of peaks in daily serum oestradiol concentrations \( (n = 34 \text{ and } n = 24, \text{ for white-faced and Finn ewes, respectively}) \), expressed for the two days before and after the end of the growing phase of the largest follicle of a wave; (d) A graph illustrating changes in the diameter of the largest follicles of waves on the day of oestradiol peak ± 2 days. The graphs were compiled from follicular and endocrine data obtained in Western white-faced (◼; \( n = 10 \)) and Finn ewes (□; \( n = 7 \)) which underwent daily ovarian ultrasonography during one oestrous cycle in the mid-breeding season (October-December), and had three or four waves of follicular development per cycle.
(r = -0.02, P > 0.05 and r = 0.26, P > 0.05 or r = 0.21, P > 0.05 and r = 0.09, P > 0.05, for ends of static phases and days of regression, for white-faced or Finn ewes, respectively). The proportion of oestradiol fluctuations that began on the day of the emergence of sequential waves was 52.8% (P < 0.05) in Western white-faced and 45.8% (P < 0.05) in Finn ewes studied (Fig. 5.5b).

In both Western white-faced and Finn ewes, the intervals between adjacent peaks of oestradiol increases were strongly and positively correlated with interwave intervals (r = 0.89, P < 0.001 and r = 0.67, P < 0.01, respectively), intervals between days of emergence of the largest follicles (r = 0.93, P < 0.001 and r = 0.79, P < 0.001, respectively), and intervals between ends of follicle growing phases (r = 0.49, P < 0.01 and r = 0.83, P < 0.001, respectively). In addition, there was a significant correlation between intervals for the ends of static phases and interpeak intervals for daily oestradiol concentration in Finn (r = 0.66, P < 0.01), but not in white-faced ewes (r = -0.05, P > 0.05), and there was no correlation between the interpeak intervals and days of the largest follicle regression to 3 mm (r = -0.03, P > 0.05 and r = -0.12, P > 0.05, for Western white-faced and Finn ewes, respectively). An analysis of the frequency and distribution of the oestradiol peaks within two days before and after the end of follicle growing phases revealed that most peaks (85.2%, P < 0.05 and 87.5%, P < 0.05, for Western white-faced and Finn sheep, respectively) occurred one day before and on the day the largest follicles of waves reached their maximum sizes (Fig. 5.5c-d).

5.5 Discussion

Previous studies on ovarian follicle dynamics done at slaughter led to contrasting conclusions that follicular growth was either continuous (Hutchinson and Robertson, 1966) or that there were two (Brand and de Jong, 1973) or three (Smeaton and Robertson, 1971) phases of large antral follicle development during the oestrous cycle in the ewe. Waves of follicles were suggested based on studies using ink marking of follicles (Driancourt, 1991). Results of studies employing daily ultrasound scanning revealed that the growth of ovarian
follicles from a pool of 2-mm follicles could be seen on most days of the interovulatory interval in ewes, with peaks occurring on days 2 and 11 of the cycle (Ravindra et al., 1993). In the study of Noel et al. (1993) using laparoscopy, three waves of follicles appeared to emerge during the cycle but other studies failed to show a wave-like pattern (Schrick et al., 1993; Landau et al., 1996). In a recent study using ultrasound imaging of ovaries in cyclic Polypay ewes, follicles only reaching 3 or 4 mm in diameter did not exhibit an organized pattern of growth and demise (Ginther et al., 1995), but 3-mm follicles began to grow to ≥ 5 mm approximately every 5 days during the interovulatory interval (Ginther et al., 1995). The present data obtained in two breeds of sheep with different ovulation rates, strongly support the conclusion that the development of ovarian follicles reaching an ovulatory size occurs in an orderly succession during the oestrous cycle in the ewe; a phenomenon apparently not influenced by differences in prolificacy.

Ginther et al. (1995) reported that the percentage of interovulatory intervals with 3 waves was 8%, 4 waves - 58%, and 5 or 6 waves - 34%. Three waves of follicular development were recorded mainly during short interovulatory intervals (9-14 days) that occurred towards the end of the breeding season, and 5 or 6 waves occurred during prolonged interovulatory intervals (22-24 days). As the length of the ovine oestrous cycle in the breeding season is remarkably consistent (17 ± 1 day; Goodman, 1994) and there is little effect of breed and age (Hafez, 1952), we concluded that the prolonged interovulatory intervals (23 days each) seen in two Western white-faced ewes in the present study (Fig. 5.1d), reflected aberrant hormonal control of the oestrous cycle. In the remaining ewes of both breeds, the vast majority of interovulatory intervals (76.5%; 13/17) were associated with the emergence of 4 follicular waves. Based on this and on previous observations in Polypay ewes (Ginther et al., 1995), we conclude that during the normal oestrous cycle in the ewe, if follicular waves are identified, there are typically 4 waves of large antral follicle growth. However, based on the results of Ravindra et al. (1993) and Noel et al. (1993), three waves may be the consistent pattern in some ewes.

In cyclic Polypay ewes, sequential follicular waves emerged, on average, on days 0, 5, 9 and 14 of the oestrous cycle (day 0 = day of ovulation; Ginther et al., 1995). In the
present study, the mean days of wave emergence (follicles growing from 3 to \( \geq 5 \) mm in
diameter) were 0, 5, 9 and 12 or 1, 6, 10 and 13, for all Western white-faced or Finn sheep
studied, respectively. This breed difference is difficult to explain, but as follicles in Finn
ewes achieve smaller maximum sizes, all phases of follicular development, including
follicle recruitment, may occur at smaller sizes than in the white-faced ewes. If recruitment
into the waves occurred on set days of the cycle, then recruited follicles would be at a
smaller follicle diameter in Finn ewes on each day of recruitment. This would mean that
follicles in Finn ewes would reach our arbitrary follicle size of 3 mm at which we define
emergence later than in Western white-faced ewes. Previous studies of follicular dynamics
in prolific and non-prolific genotypes of ewes indicated that follicles attained maturity at a
smaller diameter in prolific animals (Scaramuzzi and Radford, 1983; Webb and Gauld,
1985; Driancourt et al., 1986a-b; Souza et al., 1997). In addition, ovulatory follicles in ewes
can be recruited from a wide range of sizes (Tsoni et al., 1984). It is likely that there is a
difference between breeds of sheep with different ovulation rates in terms of the size of
follicles that acquire the ability to respond to hormonal stimuli and grow to the
periovulatory size during the oestrous cycle.

In a previous study in cyclic white-faced ewes (Ravindra et al., 1994), the ovulatory
follicle that began to grow from 2 mm in diameter at about day 11 of the cycle, grew to a
significantly larger size and over a longer duration than did any other follicle during the
oestrous cycle. In addition, the rate at which the ovulatory follicle grew was significantly
higher than that for large antral follicles emerging during the early period of the cycle (~ day
2). In the present study, ovulatory follicles (Wave 4) only reached greater maximum
diameters when compared to the largest non-ovulatory follicles within the same wave, but
not when compared to the largest non-ovulatory follicles of other waves.

In the present Western white-faced ewes, the length of follicular lifespan was higher
for follicles that emerged during the early period of the oestrous cycle than for subsequent
follicular waves, including waves containing non-ovulatory follicles that emerged during
the luteal phase. A similar trend was observed in the prolific Finn sheep. The longer lifespan
of the first follicular wave of the cycle was primarily due to the longer static phase. The
longer follicular lifespan in Western white-faced compared to Finn sheep could be partly due to the larger maximum diameter attained by the ovarian follicles in the white-faced ewes studied. Growth rates did not differ between breeds but the length of the growing and regression phases tended to be longer in Western white-faced ewes. However, the reason for the drastic decline in the length of the static phase between the first and the next two follicular waves of the oestrous cycle in ewes is not clear. It is attractive to speculate that the extended static phase of follicles growing early in the cycle may reflect the presence of developing or non-fully functional luteal structures; follicles growing in the mid-luteal phase may have a shorter static phase because of decreased gonadotrophic support under progesterone dominance. The effects of progesterone dominance on gonadotrophic support were also reflected in the shorter interwave intervals seen during the luteal phase in both breeds of ewes. In a previous study (Johnson et al., 1996), creation of lower than normal serum concentrations of progesterone (≤ 1 ng/ml) in ewes resulted in larger follicles than normal. Prolific Finn ewes, however, had smaller, shorter-lived follicles compared to Western white-faced ewes even though their serum progesterone concentrations were lower.

The proportion of ovulatory antral follicles that began growing in the penultimate wave of follicular emergence during the oestrous cycle in Finn sheep was exactly 50%. All these follicles were maintained and ovulated along with follicles that emerged after the onset of luteolysis (~day 13 of the cycle). Clearly, large antral follicles are maintained in prolific Finn ewes towards the end of the cycle and added to the ovulatory follicles emerging just prior to ovulation, to give a higher ovulation rate compared to white-faced ewes. The potential effect of follicular rescue from regression by PMSG has been demonstrated in sheep and cattle in vitro (sheep: Hay and Moor, 1977; cattle: Monniaux et al., 1984). In Finn sheep, the ovulatory follicles originating from the penultimate wave appeared to emerge approximately 48 h later compared to non-ovulatory follicles in the same wave. It is, therefore, likely that regression of these follicles in Finn ewes was prevented by the next increase in serum concentrations of FSH. Ovulation of ovarian follicles that began to grow to ≥ 5 mm in size before day 10 of the cycle in Western white-faced ewes was sporadic (1 out of 10 animals) and a proportion (22.7%) of all antral follicles that emerged in the last
follicular wave in white-faced ewes became atretic and did not ovulate, compared to 100\% ovulation of follicles in this wave in Finn ewes. The latter could possibly be attributed to lower circulating concentrations of FSH just before ovulation in Western white-faced than in Finn ewes. However, there does not seem to be agreement as to whether increased ovulation rate in sheep is caused by increased secretion of FSH (Scaramuzzi et al., 1993; Campbell et al., 1995; Fry and Driancourt, 1996; Avdi et al., 1997; Souza et al., 1997). Differences in LH pulsatility during the follicular phase could also be important (Scaramuzzi et al., 1993); we did not assess this. In cattle (Pierson and Ginther, 1988; Ginther et al., 1989), goats (Ginther and Kot, 1994) and sheep (Ravindra et al., 1994; Ginther et al., 1995), the follicles that ovulate at the end of a normal cycle originate from the last wave of the cycle but, in Finn ewes, ovulatory follicles can originate not only from the last follicular wave of the oestrous cycle but also from a previous wave. Landau et al. (1996) also concluded that with increased ovulation rate, the time width for recruitment of ovulatory follicles increases. This mechanism for increasing ovulation rate was suggested by Scaramuzzi et al. (1993).

Analysis of mean daily concentrations of FSH and oestradiol for groups of ewes did not permit the detection of distinct fluctuations in serum concentrations of hormones as the timing, duration and amplitude of these increases varied between ewes, particularly during the luteal phase (Fig. 5.4). The occurrence of peaks of FSH (Ginther et al., 1995) and, presently, oestradiol concentrations was only revealed using a statistical technique based on the assessment of daily concentrations of the hormones in individual animals (Clifton and Steiner, 1983).

The present data confirm the existence of a close temporal relationship between transient increases in circulating concentrations of FSH and follicular growth to periovulatory sizes (follicles growing from 3 to \( \geq \) 5 mm in diameter) throughout the oestrous cycle in Western white-faced and Finn ewes, and indicate that differences in prolificacy do not influence this pattern of follicle emergence. In Western white-faced ewes, the periodicity of fluctuations in daily FSH concentrations was correlated with emergence of the largest follicle, but later stages of the lifespan of individual follicles were not
associated with the occurrence of FSH peaks. In Finn ewes, a significant correlation was seen between increases in daily FSH concentration and days of emergence, days on which the static phases commenced as well as days on which the static phases of the largest follicle ended. In both breeds studied, there was a tight clustering of days on which FSH peaks were detected around the days of follicle wave emergence. Based on these results, we suggest that rhythmically generated increases in serum FSH concentrations may induce the growth of large follicles and/or maintain follicles during the static phase, but follicular demise is independent of the periodicity of FSH secretion. Moreover, as LH secretion is considerably suppressed during the luteal phase of the oestrous cycle (Karsch et al., 1980), our data are consistent with FSH being the primary gonadotrophin that stimulates ovarian antral follicle development up to periovulatory size in cyclic ewes (McNeilly et al., 1991).

At any stage of the ovine oestrous cycle, the largest non-atretic ovarian follicles are the main source of oestradiol (Bjersing et al., 1972). A peak of oestradiol has been shown to occur on the day preceding ovulation (Scaramuzzi et al., 1970; Cox et al., 1971), but also during mid-cycle, on days 3-4 (Cox et al., 1971) and days 6-9 (Scaramuzzi et al., 1970; Mattner and Braden, 1972), suggesting the existence of periods of increased oestradiol secretion throughout the oestrous cycle in the ewe. Considerable variability among ewes in the production of oestradiol during the luteal phase has been noted by Baird (1978). Using the cycle-detection program, peaks in daily serum concentration of oestradiol were identified that appeared to coincide with the culmination of the growth of the largest ovarian follicles, in all ewes studied. This confirms the observations of Souza et al. (1997). Moreover, there was a temporal relationship between the beginning of consecutive oestradiol fluctuations and the days of follicular wave emergence (follicles growing from 3 to ≥ 5 mm in diameter). These results imply that the largest antral follicles (growing from 3 to ≥ 5 mm in diameter) are capable of producing significant amounts of oestradiol at different stages of the sheep oestrous cycle. Hence, the periovulatory pattern of gonadotrophin release is not critical for the establishment of oestradiol production by the large antral follicles in cyclic ewes. Additionally, the net production of oestradiol appeared to be significantly higher for most of the cycle in prolific Finn as compared to non-prolific
white-faced ewes, despite a lack of significant differences in serum concentrations of gonadotrophic hormones, except for FSH during the periovulatory stage of the cycle. However, more frequent sampling may reveal differences. Based on results of previous studies (Driancourt, 1991), it was concluded that increased ovulation rate resulted in decreased oestradiol production from follicles due to decreased granulosa cell numbers. However, in a recent study (Driancourt et al., 1996) it was found that oestradiol production from granulosa cells was higher in Finn ewes selected for ovulation rate compared to Finn ewes with lower ovulation rates. Serum concentrations of oestradiol in some Western white-faced ewes, but not in Finn sheep, were on many days lower than the assay sensitivity (1 pg/ml; Fig. 5.1b), and in some of these ewes, luteinization of large follicles was detected ultrasonographically. Based on the latter observation, we suggest that ovarian follicles in ewes of different genotypes differ in physiological properties and responsiveness to FSH and LH. The nature of these divergent patterns of follicular secretion and morphology remains to be elucidated.

5.6 Conclusions

In summary, the growth of ovine antral follicles reaching ovulatory sizes exhibits a distinct wave-like pattern throughout the oestrous cycle in both prolific and non-prolific genotypes of sheep. Ovarian follicular emergence from the pool of 3-mm follicles and the initial stages of the growth of the largest follicles appear to be primarily controlled by increases in FSH secretion. The largest ovarian follicles acquire the ability to produce large amounts of oestradiol from the day of emergence (beginning of growth from 3 to ≥ 5 mm in diameter) and a peak of oestradiol release occurs about the time they reach their maximum diameter. The higher ovulation rate in the prolific Finn ewes appears to be due to the maintenance of follicles emerging in the penultimate follicular wave and their addition to ovulatory follicles emerging in the last wave of the interovulatory interval. The dissimilar patterns of ovulatory follicular recruitment in prolific Finn and non-prolific Western white-faced sheep may be caused by differences in circulating concentrations of FSH but may also reflect different intrinsic properties of ovarian antral follicles.
Chapter 6. AN ULTRASONOGRAPHIC STUDY OF LUTEAL FUNCTION IN BREEDS OF SHEEP WITH DIFFERENT OVULATION RATES

6.1 Abstract

Development and demise of luteal structures were monitored using daily transrectal ultrasonography in two breeds of sheep differing in ovulation rates (non-prolific Western white-faced cross-bred, n = 12 and prolific pure-bred Finn sheep, n = 7), during one oestrous cycle in the mid-breeding season. Jugular blood samples were collected once a day for radioimmunoassay of progesterone. The mean diameter of ovulatory follicles was greater in Western white-faced than in Finn ewes (6.4 ± 0.2 and 5.3 ± 0.2 mm, respectively; P < 0.001). The mean volume of luteal structures was greater (P < 0.05) in Western white-faced compared with Finn sheep from days 5 to 15 of the cycle (day 0 = day of ovulation). This accounted for the higher (P < 0.05) total luteal volumes recorded in Western white-faced ewes on day 7 and from days 11 to 15, despite the greater ovulation rate in Finn ewes (2.7 ± 0.3 and 1.7 ± 0.2, respectively; P < 0.05). Mean serum progesterone concentrations were higher (P < 0.05) in Western white-faced than in Finn ewes from days 4 to 14. Daily total luteal volumes were positively correlated with daily serum progesterone concentrations throughout the cycle in Finn sheep (r ≥ 0.40, P < 0.02), and during luteal growth and regression (r > 0.60, P ≤ 0.00001) but not during mid-cycle, in white-faced ewes (r = 0.16, P = 0.22). During the growth of the CL, luteal tissue volume increased faster (P < 0.05) than serum progesterone concentrations in both breeds of sheep. During luteolysis, the decrease in luteal volumes paralleled that in serum progesterone concentrations in Finn (P = 0.11) but not in Western white-faced ewes, where luteal volumes decreased more slowly (P = 0.02) in relation to progesterone secretion. Increased ovulation rate in prolific Finn ewes resulted in more but smaller CL, and lower serum progesterone levels compared with non-
prolific Western white-faced ewes. We conclude that breed-specific mechanisms exist to control the formation of luteal tissue and progesterone secretion in cyclic ewes differing in prolificacy. The mechanisms may involve ovulation of Graafian follicles at different sizes and inhibitory paracrine effects of CL on co-existing CL.

6.2 Introduction

In the ewe, serum progesterone concentrations are low during the first 3 days of the oestrous cycle, rise gradually from days 3 to 8 (day 0 = onset of oestrus), remain relatively constant from days 8 to 14 and then rapidly decline (over the next 1 to 2 days) to basal or undetectable levels (Stabenfeldt et al., 1969; Thorburn et al., 1969; Yuthasastrakosol et al., 1975; Edgar and Ronaldson, 1985). This qualitative pattern of progesterone secretion is markedly consistent between breeds (Goodman, 1994). There are, however, differences between breeds in terms of the maximal serum concentration of progesterone at mid-cycle. It was reported that prolific animals had higher progesterone concentrations than ewes with lower ovulation rates (Quirke et al., 1979). However, an increase in the number of CL does not result in a proportional increase in progesterone production, as evidenced by comparative studies conducted in breeds of sheep differing in ovulation rates (Wheeler and Land, 1977; Quirke et al., 1979; Cahill et al., 1981) or in single and double ovulators within the same breed (Quirke et al., 1979). In addition, progesterone secretion is affected by the stage of the breeding season, with higher serum progesterone concentrations detected in ewes in the middle than at the beginning or end of the season (Wheeler and Land, 1979).

Changes in the size of the CL throughout the oestrous cycle parallel those in progesterone secretion (Arthur et al., 1989). By the fifth day of dioestrus, the ovine CL is approximately 6 mm in diameter, and it reaches its maximal size by the middle of the dioestrous phase. The diameter of the CL remains unchanged until the next oestrus, during which luteal atrophy occurs abruptly. Luteal regression in ewes involves two stages, namely functional luteal regression, in which a gradual fall in progesterone secretion begins on day 13, and structural luteolysis occurring on days 15 to 16 of the oestrous cycle (Baird and Scaramuzzi, 1975).
Previous observations on ovarian function in small ruminants have mainly been accomplished at slaughter or using surgical techniques such as laparotomy or endoscopy (Hutchinson and Robertson, 1966; Oldham and Lindsay, 1980; Keisler et al., 1983; McNatty et al., 1984). Transrectal ovarian ultrasonography has only recently been adapted for use in small ruminants (sheep: Schrick et al., 1993; Ravindra et al., 1994; Ginther et al., 1995; goats: Ginther and Kot, 1994). The relationship between ultrasonographic estimates of luteal tissue area and peripheral blood progesterone levels was demonstrated in cyclic and pregnant heifers (Kastelic et al., 1990), but such studies do not exist for sheep.

The objective of the present study was to use ultrasonography and serum progesterone measurements to describe and compare luteal morphology and function throughout the oestrous cycle in and between two breeds of sheep with different ovulation rates (non-prolific Western white-faced and prolific Finn sheep). With the added ability to non-invasively determine CL size on a daily basis we hoped to relate changes in the volumes of the luteal tissue to circulating concentrations of progesterone (CL function) during the ovine oestrous cycle.

6.3 Materials and methods
6.3.2 Hormone analysis

Intra- and inter-assay coefficients of variation (CVs) for ovine reference sera with mean progesterone concentrations of 0.49 or 1.56 ng/ml were 11.9 and 4.2% or 14.8 and 11.2%, respectively. Progesterone concentrations were normalized to the day of first ovulation of the interovulatory interval studied (day 0) and daily mean concentrations were determined for both breeds, for the period from days −1 to 17 after ovulation. All values are given as mean ± SEM.

6.3.3 Statistical analyses

Data from two Western white-faced ewes with prolonged interovulatory intervals (each 23 days) were withdrawn from analyses. Ovulation rates, mean numbers of luteal
structures per ewe and the mean lengths of the lifespan of CL/luteinized follicles for the two breeds were compared using Student's t-test. The volume of all luteal structures in the ovaries of ewes was determined using a formula for the volume of a sphere ($\pi \times d^3/6; d =$ diameter); for cavitated CL and luteinized follicles, the volume of the central cavity was subtracted from the volume of the outer sphere. The mean luteal volume was then defined as the total volume of luteal tissue divided by the number of identified luteal structures per ewe. Daily total and mean luteal volumes were determined for each ewe for the period during which luteal structures were observed, and the resulting values were normalized to the day of first ovulation of the interovulatory period (day 0).

Daily total and mean luteal volumes and mean progesterone concentrations were analysed by a two-way repeated measures analysis of variance (RM-ANOVA). Main effects of day and breed, and the day-by-breed interaction were determined for each of the three end points. If the main effect of day was significant, Fisher's protected least significant difference (LSD) was used as the post-ANOVA test to compare individual (daily) means. If there was a significant effect of breed or day-by-breed interaction, differences between breeds within each day were determined by ANOVA.

In evaluating the relationship between daily luteal volumes and serum progesterone concentrations during various stages of CL/luteal structure lifespan, the interovulatory interval was partitioned into four periods, based on the ultrasonographic detection of CL and assessment of daily mean concentrations of progesterone. Period 1, or the phase of luteal tissue formation, was defined as the time after ovulation during which CL/luteal structures were not identifiable with ultrasonography (days 0 to 2). Period 3, or the phase of mid-cycle luteal function, was regarded as the period encompassing the days on which daily mean serum concentrations of progesterone did not differ ($P < 0.05$) from the maximal mean concentration of the hormone seen, in both breeds, on day 11. This period lasted from days 7 to 12 in both breeds studied. The time between the end of luteal formation and the beginning of the period of mid-cycle luteal function was Period 2, or the phase of luteal growth/development (days 3 to 6). Period 4, or luteal regression, was the average time (in days) from the end of the mid-cycle phase of luteal function until the complete regression
of luteal structures as confirmed by ultrasonography (days 13 to 15 in Finn sheep and days 13 to 16 in Western white-faced ewes). The relationship between daily total luteal volumes and circulating concentrations of progesterone was studied by simple linear regression; the independent variable was total luteal volume, while the dependent variable was progesterone concentration. When a significant linear relationship was seen, slopes of regression lines for each breed were compared using one-way ANOVA. Where there was no significant correlation between daily serum progesterone concentrations and total luteal volumes, sequential polynomial regression analyses (degrees from 2 to 5) were applied to assess the mathematical relation between the two sets of data points within that period.

In addition to normal CL, a luteinized unovulated follicle was detected in the ovaries of 3 white-faced ewes studied. Therefore, the regression analyses above were initially done for all 10 Western white-faced ewes studied and then, separately, for the 7 Western white-faced ewes that only had normal CL and the 3 ewes with luteinized ovarian follicles.

The rates of increase for daily total luteal volumes and serum concentrations of progesterone, using the data from day 3 (mean day of CL detection) to day 7 (first day of the plateau phase of progesterone secretion) of the cycle, were determined by a two-variable regression for each breed separately. Similarly, the rates at which total luteal volumes and serum progesterone concentrations decreased were calculated based on the period from day 12 (the day prior to the onset of functional luteolysis in both breeds) to day 15 (Finn sheep) or day 16 (Western white-faced ewes; i.e., until complete regression of CL/luteinized follicles). For the purpose of these analyses, data for each animal were converted into a percentage of the maximal value determined for each animal during the 2 periods above (Kastelic et al., 1990). The input variable was day and the outcome variable was percentage of the maximum value. Therefore, the slopes of the regression lines were the rates at which luteal tissue volume/serum progesterone concentrations increased/decreased (expressed as a percentage of the maximum/day). The slopes of the two regression lines were compared within each of the two periods and the slopes for serum progesterone concentrations and luteal volumes were compared between the two breeds by ANOVA.
6.4 Results

6.4.1 Characteristics of luteal structures and progesterone secretion

The mean ovulation rate, as determined for all ewes at the beginning of the oestrous cycle studied, was 1.7 ± 0.2 and 2.7 ± 0.3, for Western white-faced and Finn sheep, respectively (P < 0.05). The mean diameter of ovulatory follicles recorded prior to the first ovulation of the interovulatory interval was significantly higher in Western white-faced than in Finn ewes (6.4 ± 0.2 and 5.3 ± 0.2 mm, for Western white-faced and Finn ewes, respectively; P < 0.001).

In all ewes, CL could be detected by day 5 after ovulation (an average of 3.4 ± 0.3 and 3.0 ± 0.2 days after ovulation, for Western white-faced and Finn sheep, respectively). In addition to a CL, a luteinized uniovulated follicle was seen to form in the ovaries of 3 of the 12 white-faced ewes. These structures were identified in each of the 3 ewes on Days 3, 4 and 7, respectively. An 8-mm short-lived CL, recorded only once on day 3 post-ovulation, was found in 1 animal; this ewe also had a luteinized follicle and a normal CL present in the ovary contralateral to that containing the short-lived CL. Due to the incidence of follicular luteinization and premature regression of the CL in the Western white-faced ewes, the mean number of all luteal structures per ewe (2.0 ± 0.2) was different from the ovulation rate above. This value, however, was still lower than the mean number of CL present in ovaries of Finn sheep (2.7 ± 0.3; P < 0.05).

The mean lifespan of CL and luteinized follicles was 12.4 ± 0.5 and 10.9 ± 0.3 days (P < 0.05), for white-faced and Finn sheep, respectively. In the group of Western white-faced ewes, two animals had prolonged interovulatory intervals (both 23 days) associated with prolonged lifespan of the CL (followed ultrasonographically for ~16 days). When the durations of lifespan for the CL recorded in these ewes were excluded from analysis, the mean luteal lifespan observed in cyclic Western white-faced sheep was 11.7 ± 0.3 days, but it still differed from the mean lifespan of CL of Finn sheep (10.9 ± 0.3 days; P < 0.05). Data from the 2 white-faced ewes were withdrawn from subsequent statistical analyses. The mean interovulatory interval was 17.0 ± 0.3 and 16.6 ± 0.2 days, for white-faced (n = 10) and Finn ewes (n = 7), respectively (P > 0.05).
Central cavities of 2 to 3 mm in diameter were seen in growing CL for an average of 3 days (range: from day 2 to day 8) in 6 of the 7 Finn and in all Western white-faced ewes. Cavities were also recorded for an average of 3 days during luteolysis (days 13 to 16) in all ewes. From day 9 to day 12, inner cavities had filled in and CL appeared as solid and uniform structures in all animals. The maximum diameter attained by CL during mid-cycle ranged from 12 to 14 mm and from 9 to 12 mm in Western white-faced ewes and Finn sheep, respectively. The two Western white-faced ewes with atypically long interovulatory intervals had CL containing central cavities from days 5 to 7 and from days 14 to 16; however, the CL appeared solid again on day 17 and small internal cavities (2 mm) could be seen over the 3 days before complete luteal atrophy (i.e., from days 21 to 23). In 2 out of the 3 Western white-faced sheep with ovaries bearing luteinized follicles, central cavities within these structures persisted until days 13 and 14, respectively. The follicles that luteinized in these ewes were first detected at 5 mm in diameter on day –1 before ovulation; prior to luteinization these follicles attained maximum diameters of 7 and 14 mm on days 2 and 3, respectively, and were first identified as luteinized structures on days 3 and 7, respectively. After the onset of luteinization of their walls, these follicles reached maximum sizes of 14 and 13 mm in diameter, with central cavities of 7 and 8 mm in diameter, respectively. In the other white-faced ewe with a luteinized follicle, this structure transformed into a solid luteal structure on day 8. This follicle was first detected at 3 mm on day 1 after ovulation and was seen to grow to reach 7 mm in diameter on day 4, when a 3-mm thick band of echogenic tissue surrounding the follicle antrum was first detected by ultrasonography. The maximum diameter attained by this luteinized follicle was 10 mm (days 9 to 12). All luteinized follicles described above underwent luteolysis on day 16 of the cycle. The different types of luteal structures detected in the present study in cyclic ewes are illustrated in Fig. 6.1 and Fig. 6.2. Graphs showing daily diameters of CL/luteinized follicles, serum progesterone concentrations, and total and mean luteal volumes in 4 individual ewes are given in Fig. 6.3.

In the group of Western white-faced ewes, mean daily serum progesterone concentration rose from day 0 to reach a peak of 3.58 ± 0.18 ng/ml on day 11 of the cycle.
Fig. 6.1. Photographic reproductions of ultrasonograms of ovaries in Western white-faced and Finn sheep examined during the middle portion of the ovulatory season. (a) Corpus luteum containing a small central cavity in the ovary of a Finn ewe on day 5 of the estrous cycle (day 0 = day of ovulation); (b) A short-lifespan CL identified ultrasonographically only once on day 3 after ovulation in a Western white-faced ewe; (c) Corpus luteum present in the ovary of a Finn sheep during mid-cycle (day 11); (d) Corpus luteum recorded on day 11 in the ovary of a white-faced ewe; (e) Corpora lutea seen in the ovary of a Finn sheep on day 15 of the cycle; (f) A band of echogenic luteal tissue enveloping a persistent unovulated ovarian follicle in a Western white-faced ewe (day 8). Each scale bar represents 10 mm.
Fig. 6.2. Ultrasound images of the ovary in a Western white-faced ewe, recorded on three consecutive days (a-c; days 6 to 8 after ovulation), and showing an individual anovulatory follicle that underwent luteinization of its walls. Boundaries of the follicular antrum (a) and of a band of luteal tissue enveloping the follicle (b-c) are delineated by arrowheads. Scale bars represent 10 mm.
Fig. 6.3. Daily diameter profiles of CL and luteinized follicles (top), serum concentrations of progesterone (middle), and total (△) and mean (▲) luteal volumes (bottom) determined in a Finn ewe (a) and 3 white-faced ewes (b-d), scanned from day −1 (day 0 = day of ovulation) until the following ovulation during the middle portion of the breeding season. An arrow and a capital letter L indicate the day on which luteinization of a large unovulated antral follicle (■) was detected (c-d).
(P < 0.05; Fig. 6.4a). Serum progesterone concentrations declined to 1.97 ± 0.17 ng/ml by day 13 (P < 0.05) and reached a low level of 0.11 ± 0.05 ng/ml on day 15 of the cycle. In the Finn ewes, mean serum progesterone concentration increased to a maximum of 2.54 ± 0.20 ng/ml on day 11, declined to 1.11 ± 0.27 ng/ml (P < 0.05) on day 13 and further to 0.23 ± 0.11 ng/ml (P < 0.05) on day 14; there was no significant difference from days 7 to 12 of the cycle (P > 0.05). Mean daily serum progesterone concentrations were higher (P < 0.05) in Western white-faced compared to Finn sheep from day 4 to day 14.

6.4.2 Luteal volumes and serum progesterone concentrations

Daily total and mean luteal volumes for the period when luteal structures were observed for both breeds are shown in Fig. 6.4b-c. Daily total luteal volumes differed by day (P < 0.05) and between the two breeds of sheep studied (P < 0.05). In white-faced ewes, daily total luteal volumes increased gradually from 221 ± 47 mm³ on day 3 to a maximum of 1580 ± 105 mm³ on day 9 (P < 0.05); there were no significant changes from days 7 to 12 of the cycle. The total luteal volume reached a nadir on day 16 (238 ± 32 mm³). In Finn sheep, daily total luteal volumes rose from a minimum of 334 ± 67 mm³ on day 3 to reach a peak of 1377 ± 191 mm³ on day 9 (P < 0.05). From days 5 to 13, daily total volumes of luteal tissue did not differ (P > 0.05). Daily total luteal volumes declined subsequently to a low value (251 ± 106 mm³) on day 15 of the oestrous cycle in Finn sheep (P < 0.05). The Western white-faced ewes exceeded (P < 0.05) Finn sheep in daily total volumes of luteal tissue on day 7 and days 11 to 15 of the oestrous cycle.

A significant main effect of day (P < 0.001), breed (P < 0.05) and interactions of day by breed (P < 0.05) were seen for daily mean luteal volumes during the period when CL/luteal structures were observed. The daily mean luteal volume was highest in both breeds on day 9 of the cycle (842 ± 66 and 477 ± 46 mm³ for white-faced and Finn sheep, respectively). From days 5 to 14 in Western white-faced ewes and from days 5 to 13 in Finn sheep, daily mean volumes of luteal structures did not differ (P > 0.05). The luteal volumes declined gradually to reach a minimum of 164 ± 34 mm³ on day 16 and 111 ± 30 mm³ on
Fig. 6.4. Mean daily serum progesterone concentrations (a) in 10 Western white-faced cross-bred and 7 pure-bred Finn sheep that underwent daily transrectal ultrasonography of ovaries during the mid-breeding season, and daily total (b) and mean (c) luteal volumes calculated for the period during which CL/luteinized follicles were observed. Data were normalized to the day of first ovulation of the interovulatory interval (day 0). All values are mean ± SEM. Asterisks denote days on which difference in means between the white-faced (■) and Finn sheep (□) was statistically significant (P < 0.05).
day 15 in Western white-faced and Finn ewes, respectively. Daily mean luteal volumes were significantly higher in Western white-faced than in Finn sheep from days 5 to 15 of the oestrous cycle.

There was a significant positive correlation between daily serum progesterone concentrations and daily total luteal volumes in both breeds of sheep, during the period of luteal growth and regression (Periods 2 and 4; Table 6.1). In Western white-faced and Finn sheep, the correlation coefficients were 0.63 and 0.71, and 0.54 and 0.52 for Periods 2 and 4, respectively. The difference in the slopes between the 2 breeds for the period of luteal growth approached significance ($P = 0.07$). During luteolysis, these slopes did not differ ($P >0.10$) between the 2 breeds. During the phase of mid-cycle luteal function (Period 3; Table 6.1), there was a significant correlation between daily serum progesterone concentrations and total luteal volumes in Finn ($r = 0.40$, $P < 0.007$) but not in white-faced ewes ($r = 0.16$, $P = 0.22$). In Western white-faced ewes, a significant coefficient of correlation ($r = 0.45$, $P < 0.05$) was obtained with a fourth degree polynomial regression (Table 6.1).

In the 7 Western white-faced ewes that only had normal CL during the oestrous cycle studied, daily serum progesterone concentrations and total luteal volumes were correlated during the phase of luteal growth and regression (Periods 2 and 4 of analysis: $r = 0.61$, $P = 0.003$ and $r = 0.67$, $P = 0.0002$, respectively), but not during mid-cycle (Period 3: $r = 0.17$, $P = 0.29$). Corresponding correlation coefficients determined for the 3 white-faced ewes that had luteinized follicles were $r = 0.77$, $P = 0.005$; $r = 0.29$, $P = 0.25$; and $r = 0.82$, $P = 0.0009$ for Periods 2, 3 and 4, respectively.

The slopes of regression lines for daily increases in total luteal volumes were greater than the slopes for increases in progesterone concentrations in both breeds studied (19.56 or 17.75 % of maximum/day; $P = 0.03$, and 13.15 or 6.83 % of maximum/day; $P = 0.0002$, for daily total luteal volumes or progesterone concentrations, in Western white-faced and Finn ewes, respectively; Fig. 6.4). The slopes of regression equations for luteal volumes did not differ ($P = 0.54$) between the 2 breeds during the period above. The slope for daily increments in serum progesterone concentrations was greater ($P = 0.03$) for white-faced than for Finn sheep. During functional luteolysis, there was no difference between the
Fig. 6.5. Regression lines for total luteal tissue volumes (Δ, □) and serum progesterone concentrations (▲, ■) during luteal development and regression in cyclic Western white-faced (Δ, ▲) and Finn (□, ■) sheep. During luteal development, the slopes of lines for total luteal volumes were greater than for progesterone concentrations in Western white-faced (P = 0.03) and Finn sheep (P = 0.0002), and the slope for serum progesterone concentrations was greater in white-faced than in Finn ewes (P = 0.03). During luteolysis, the slopes for progesterone concentrations were greater than for total luteal volumes in white-faced ewes (P = 0.02) and the difference in slopes of total luteal volumes for the two breeds approached significance (P = 0.05). Symbols differentiate parameters and breeds only, and are not data points.
Percentage (%) of maximum

Days from first ovulation of the interovulatory interval
Table 6.1. Regression equations, coefficients of correlation and significance (P-values) of determined coefficients of correlation for daily serum progesterone concentrations (dependent variable) against daily total volumes of luteal tissue (independent variable), during three different stages of the luteal phase (Period 2 or luteal growth, Period 3 or mid-cycle luteal function and Period 4 or luteal regression), in cyclic Western white-faced and Finn sheep.

<table>
<thead>
<tr>
<th></th>
<th>Luteal growth</th>
<th>Mid-cycle luteal function¹</th>
<th>Luteal regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western white-faced sheep</td>
<td>( y = 0.0013 , x + 1.3534 )</td>
<td>( y = -0.1335 , x + 0.0001 , x^2 + 0.0000 , x^3 ) ( + 0.0000 , x^4 + 52.3576 ) ( r = 0.45, , P &lt; 0.05 )</td>
<td>( y = 0.0016 , x - 0.3464 ) ( r = 0.71, , P &lt; 0.000001 )</td>
</tr>
<tr>
<td>Finn sheep</td>
<td>( y = 0.00063 , x + 1.1670 )</td>
<td>( y = 0.00052 , x + 1.5568 ) ( r = 0.40, , P &lt; 0.02 )</td>
<td>( y = 0.00097 , x + 0.0160 ) ( r = 0.52, , P &lt; 0.02 )</td>
</tr>
</tbody>
</table>

\( y \) - daily serum progesterone concentrations (ng/ml)
\( x \) - daily total luteal volumes (mm³)

¹Values of coefficients for variables \( x^3 \) and \( x^4 \) were less than 0.00001.
rate of decline in total luteal volume and daily serum progesterone concentrations \((P = 0.11)\) in Finn ewes. However, the slope of the regression line for total luteal volumes differed from that for daily serum concentrations of progesterone during luteolysis in white-faced ewes \((-19.73\) and \(-26.40\%\) of maximum/day, for daily total luteal volumes and serum progesterone concentrations, respectively; \(P = 0.02\)). The difference in slopes of regression lines for total luteal volumes for Western white-faced and Finn sheep approached significance \((-19.73\) and \(-26.49\%\) of maximum/day; \(P = 0.05\)), but the slopes for declining progesterone concentrations did not differ between the two breeds \((P = 0.10)\).

### 6.5 Discussion

Data from the present study contrast with earlier findings that ewes with higher ovulation rates have higher serum progesterone concentrations during mid-cycle (Quirk et al., 1979). For 11 consecutive days, commencing on day 4 after ovulation, non-prolific Western white-faced ewes had higher circulating concentrations of progesterone compared to prolific Finn sheep. Higher serum progesterone concentrations in the Western white-faced ewes could be partly explained by the greater total luteal volumes first seen at day 7 of the oestrous cycle. However, mean serum progesterone concentration was higher in Western white-faced ewes by day 4 after ovulation; this and the regression analyses of daily progesterone concentrations against total luteal volumes, suggested that progesterone synthetic ability of the white-faced ewes could be greater than for the Finn ewes in the present study. This divergence in progesterone synthetic capability is all the more marked when the differences in body weight and therefore blood volume of the two breeds are considered. Serum concentrations of any hormone depend on the rates at which hormones are biosynthesized, distributed and metabolized (Little et al., 1975). The average metabolic clearance rate (MCR) of progesterone \((3.28 \pm 0.24 \text{ l blood/min})\) did not differ between cyclic and pregnant sheep within the same breed (Paterson et al., 1976). In sheep, progesterone clearance rates were also shown to be inversely related to feed level (Parr et al., 1993). The ewes of both breeds employed in the present study were kept on the same
nutrition regimen, minimizing possible differences in progesterone production and metabolism due to feed intake. However, we did not determine the metabolic clearance rate of progesterone in this study. Inter-breed differences in the clearance rate of progesterone have apparently not been investigated in sheep.

In the present study, the Finn ewes were 3-4 years old and had lambed twice while the Western white-faced ewes were 5 years old and nulliparous. In both prolific and non-prolific genotypes of sheep, ovulation rate increases with age; it reaches a maximum between 3 to 6 years, then declines gradually (Hafez, 1993). However, this difference is mainly due to the low ovulation rate in very young ewes (i.e., yearlings; Gordon, 1996). In a previous study using Targhee range sheep, there was no difference in the mean ovulation rate or percentage of ewes lambing between nulliparous (aged 18 to 19 months) and primiparous ewes (aged 30 to 31 months; Hulet et al., 1969). In the sexually mature ewe, ovulation rate at successive oestrous cycles is highly repeatable (Dermody et al., 1966), except that at the beginning and end of the ovulatory season the number of ovulations is lower than at mid-season (Gordon, 1996). We therefore concluded that differences in patterns of CL formation and progesterone levels in the present ewes were unlikely to be influenced by the age and reproductive history of Western white-faced and Finn sheep under study.

Corpora lutea induced in anoestrous ewes produce less progesterone than spontaneously generated CL in normally cycling ewes, largely because the induced CL have fewer luteal cells, and individual volumes of both small and large luteal cells are lower than for non-induced CL (O'Shea et al., 1984). In vitro, luteal cells from induced CL also produce less progesterone than the cells from CL of normal cyclic ewes, even though their LH binding is similar (O'Shea et al., 1984). It is attractive to speculate that the smaller CL of Finn ewes might contain fewer and smaller luteal cells, with a diminished progesterone synthetic capability, compared with CL from Western white-faced ewes.

The maximum diameter reached by preovulatory follicles is higher in non-prolific breeds of sheep (Driancourt et al., 1986a-b; Castonguay et al., 1990; Webb and Gauld, 1995). A smaller ovulatory follicle size has consistently been reported for prolific breeds.
such as Booroola (Scaramuzzi and Radford, 1983), Finn (Wheeler and Land, 1977), Romanov (Driancourt et al., 1986b), Booroola x Suffolk and Booroola x Finnish Landrace (Castonguay et al., 1990). There is increasing evidence that the size of ovarian antral follicles destined to ovulate determines the size of subsequent CL. The mean weight of individual corpora lutea in sheep decreases as the ovulation rate increases (Henderson et al., 1988). In Romney ewes with a single ovulation, most corpora lutea weighed > 600 mg, but with an increase in the ovulation rate, after FSH pretreatment, the proportion of corpora lutea weighing < 400 mg rose steadily (Henderson et al., 1988). Although these results may reflect the influence of the size of ovulatory follicles, the CL themselves may also exert local inhibitory effects on the growth of co-existing CL. The present data for non-prolific Western white-faced and prolific Finn sheep seem to support the occurrence of an inverse relationship between the number and volume of individual CL as well as an influence of ovulatory follicle size on CL volume.

A variety of luteal structures were detected during the oestrous cycle in Western white-faced ewes. Luteinization of unovulated ovarian antral follicles has been seen in cyclic ewes (Schrick et al., 1993) and in sheep during the transition into the breeding season. Oestrous cycles of nearly twice the normal duration (Goodman, 1994) and associated with prolonged lifespan of CL (Oldham and Lindsay, 1980) have also been recorded in ewes. In a previous study (Carriere et al., 1995), ovarian function in cattle was monitored by ultrasonography following a single injection of a PgF$_{2a}$ analogue (Cloprostenol) and oestradiol valerate on either days 17, 18 or 19 of the oestrous cycle. It was suggested that differences in the stage of folliculogenesis at the time of treatment led to the array of ovarian responses seen, including a premature ovulation followed by a CL which persisted for over 30 days, ovulation of hypertrophic ovarian follicles, development of several waves of follicular cysts or luteinization of an unovulated cystic-like follicle. Such variation in the degree of follicle maturation in oestrous ewes may explain the formation of atypical luteal structures in Western white-faced ewes in this study. Seemingly, luteolytic mechanisms in the present ewes effectively abolished all of the types of luteal structures present to reinitiate oestrus and ovulations.
In cyclic Finn sheep, the relationship between daily serum progesterone concentrations and total luteal volume was maintained throughout the entire period when CL were observed. In contrast, the relation between the luteal volume and circulating concentrations of progesterone during mid-cycle in Western white-faced sheep was non-linear, regardless of the presence or absence of luteinized anovulatory follicles. These observations imply that during the mid-cycle period, production of progesterone in non-prolific white-faced ewes may be controlled by factors other than changes in the size of the CL/luteinized follicles.

In cattle, ultrasonographically determined luteal tissue area and plasma progesterone concentrations increased at a similar rate during luteal growth, but during luteal regression, luteal tissue area was seen to decline more slowly than plasma progesterone concentrations (Kastelic et al., 1990). The patterns of functional and structural development of the CL in cattle are different from those observed in the present ewes where luteal volume increased at a higher rate than progesterone secretion. Changes in progesterone secretion and luteal volume during luteolysis in cyclic non-prolific white-faced ewes resembled those seen in heifers, but in Finn sheep luteal volume and progesterone concentrations declined at a similar rate. Changes in the luteal tissue volume and progesterone secretion during the growth and regression of the CL are remarkable. It was also intriguing that luteal cavities re-appeared during luteolysis, probably reflecting rapid structural degradation and/or fluid accumulation in the regressing CL.

6.6 Conclusions

It is intriguing that prolific Finn ewes produce more but smaller CL and have lower serum concentrations of progesterone to support the greater number of fetuses they carry compared with non-prolific Western white-faced ewes. There appear to exist breed-specific mechanisms controlling luteal tissue formation and the adequacy of progesterone secretion in cyclic sheep of varying ovulation rates. The present study suggests that these mechanisms could involve ovulation of mature ovarian follicles at different sizes and possibly inhibitory effects of CL on co-existing CL.
Chapter 7. AN ULTRASOUND-AIDED STUDY OF TEMPORAL RELATIONSHIPS BETWEEN THE PATTERNS OF LH/FSH SECRETION, DEVELOPMENT OF OVULATORY-SIZED ANTRAL FOLLICLES AND FORMATION OF CORPORA LUTEA IN EWES

7.1 Abstract

To characterize the pulsatile secretion of LH and FSH and their relationships with various stages of follicular wave development (follicles growing from 3 to ≥ 5 mm) and formation of CL, 6 Western white-faced ewes underwent ovarian ultrasonography and intensive blood sampling (every 12 min for 6 h) each day, for 10 and 8 consecutive days, commencing 1 and 2 days after oestrus, respectively. Basal serum concentrations of LH and LH pulse frequency declined, whereas LH pulse duration and FSH pulse frequency increased by day 7 after ovulation (P < 0.05). LH pulse amplitude increased (P < 0.05) at the end of the growth phase of the largest ovarian follicles in the first follicular wave of the cycle. The amplitude and duration of LH pulses rose (P < 0.05) 1 day after CL detection. Mean and basal serum FSH concentrations increased (P < 0.05) on the day of emergence of the second follicular wave, and also at the beginning of the static phase of the largest ovarian follicles in the first follicular wave of the cycle. FSH pulse frequency increased (P < 0.05) during the growth phase of emergent follicles in the second follicle wave. The detection of CL was associated with a transient decrease in mean and basal serum concentrations of FSH (P < 0.05), and it was followed by a transient decline in FSH pulse frequency (P < 0.05). These results indicate that LH secretion during the luteal phase of the sheep oestrous cycle reflects primarily the stage of development of the CL, and only a rise in LH pulse amplitude may be linked to the end of the growth phase of the largest follicles of waves. Increases in mean and basal serum concentrations of FSH are tightly coupled with
the days of follicular wave emergence, and they also coincide with the end of the growth phase of the largest follicles in a previous wave, but FSH pulse frequency increases during the follicle growth phase, especially at mid-cycle.

7.2 Introduction

Although gonadotrophins do not seem to be essential for antrum formation and early stages of antral follicle growth, they promote follicular development to ovulatory diameters in the ewe (Driancourt et al., 1979; McNatty et al., 1990; McNeilly et al., 1991; Picton and McNeilly, 1991). FSH appears to be the primary stimulator of this follicle development but changes in LH secretion may also influence the growth of large antral follicles in ewes (Picton and McNeilly, 1991; McNeilly et al., 1991).

The growth of ovarian follicles reaching ostensibly ovulatory sizes throughout the sheep oestrous cycle exhibits a distinct wave-like pattern, and there is a temporal relationship between elevations in mean daily serum concentrations of FSH and emergence of successive follicular waves (defined as follicles growing from 3 to ≥ 5 mm in diameter; Ginther et al., 1995). Alterations in the pulsatile characteristics of secretion of LH/FSH around the time of follicular wave emergence and during later stages of follicle wave development have not been studied to date, and it is not known whether changes in the pattern of LH/FSH release occur during formation of the CL in ewes.

The recent application of transrectal ovarian ultrasonography in the ewe allows us to monitor day-to-day changes in follicle size and to detect CL. The objective of this study was to see if the patterns of episodic secretion of FSH and LH were correlated with various stages of follicular wave development and luteinization of ovulated follicles in ewes. Of particular interest were any changes in the pattern of gonadotrophin release that were associated with transitions between different phases of large antral follicle lifespan in the cyclic ewe, from the day of emergence until the onset of regression.
7.3 Materials and methods

The 12 sexually mature Western white-faced cross-bred ewes were employed in the present study in November. Oestrous cycles were synchronized with 2 injections of PGF\textsubscript{2\alpha}, as dinoprost tromethamine (Lutalyse\textsuperscript{®}, 15 mg i.m.; Upjohn, Orangeville, ON, Canada), given 9 days apart. The first 6 ewes that exhibited oestrus at the end of the synchronized oestrous cycle (i.e., 17 days after synchronized oestrus) were selected for intensive blood sampling and ultrasonographic examination of ovaries. Blood samples (4 ml) were collected every 12 min for 6 h, for 8 consecutive days (between 9 a.m. and 3 p.m.), commencing 2 days after the first signs of oestrus were seen. During sampling, ewes were kept in pens in a barn with large windows to ensure ambient light exposure. Hematocrits were checked each day, and there was no more than a 9.5% decrease in packed cell volume (PCV) from the beginning to the end of the 8-day period of blood sampling. Daily ultrasonographic scanning was performed between 11 a.m. and 12 p.m., from 1 day after oestrus was first detected until 1 day after the end of the period of intensive blood sampling (10 consecutive days).

For FSH reference sera with mean concentrations of 2.27 or 3.78 ng/ml, the intra- and interassay coefficients of variation (CVs) were 9.6 and 8.3% or 5.9 and 8.8%, respectively. For reference sera with mean LH concentrations of 0.13 or 0.99 ng/ml, the intra- and interassay CVs were 11.3 and 9.9% or 12.1 and 13.0%, respectively.

To analyse the hormonal and ovarian data, the parameters determined using the PULSAR program were converted into the percentage of the maximal value recorded during the period from days 0 to 7 after ovulation in each ewe, and then aligned as follows: 1) day 0 (day of ovulation) + 7 days; 2) day of emergence of the second follicular wave of the cycle (EM2) ± 2 days; 3) the end of the growth phase (maximal follicle size or onset of the static phase) of the largest non-luteinized follicle of the first wave (ST1) ± 2 days; 4) the end of the static phase of the largest non-luteinized follicle of the first wave (ST2) ± 2 days; and 5) mean day of CL detection ± 2 days. Means normalized on this basis were analysed by one-way ANOVA (MINITAB\textsuperscript{®} Statistical Software, Minitab Inc., Enhanced Version, Release 9.1 for VMS/VAX 6.2; 1992, State College, PA, USA). When an overall analysis
was significant (P < 0.05), the LSD test was done to determine differences between individual (daily) means for each of the 5 alignments above. These analyses were not done for the amplitude and duration of FSH pulses, because such pulses were recorded on only some days in the present ewes. All results are expressed as mean ± SEM.

Ovarian data were combined for the 2 ovaries of each ewe. A follicular wave was defined as one or more antral follicles growing from 3 to ≥ 5 mm in diameter before regression; the day the follicles were first detected at 3 mm was the day of wave emergence. For follicular waves in which follicles started to grow from 3 mm onward within a 24-h period, the day of wave emergence was the day on which the first follicle of the group was detected at 3 mm. The growing phase of the largest non-luteinized follicle of the wave was the time taken by that follicle to grow from 3 mm to its maximum diameter, and the static phase was regarded as the time during which that follicle was seen to maintain its maximum diameter. If more than one follicle attained the same maximum size, the follicle that reached the maximum diameter first and/or remained at its maximum size for the longest period of time was regarded as the largest follicle of the wave.

7.4 Results

Individual follicle profiles (follicles growing from 3 to ≥ 5 mm in diameter) are shown for 2 ewes, with accompanying results of the PULSAR analyses, in Fig. 7.1. Diagrams depicting all ovarian follicles ≥ 3 mm and luteal structures identified by ultrasonography in 1 other ewe, with corresponding serum concentrations of LH and FSH determined during 6-h periods of intensive blood collection, are given in Fig. 7.2.

All of the 6 ewes studied were marked by rams within a 24-h period at the end of the synchronized cycle; 3 ewes were well marked, 2 ewes moderately marked, and 1 ewe poorly marked. Vaginal impedance decreased to < 40 ohms (mean 30.0 ± 1.6 ohms) in all ewes on the day of estrus, and then increased (P < 0.05) to 51.5 ± 2.4 ohms 48 h later. This and ultrasonographic observations confirmed that all ewes ovulated between 24 and 48 h after the onset of oestrus. In 1 ewe, a second ovulation occurred, in the contralateral ovary, 2 days
Fig. 7.1. The panels depict (from top to bottom): diameter profiles of individual ovarian antral follicles growing from 3 mm to a minimum size of 5 mm; mean (solid bars) and basal (hollow bars) serum levels of LH; LH pulse frequency (hollow bars), amplitude (solid bars) and duration (angle hatched bars); mean (solid bars) and basal (hollow bars) serum FSH concentrations; and FSH pulse frequency (hollow bars), amplitude (solid bars) and duration (angle hatched bars) in 2 Western white-faced ewes (a and b) that underwent daily intensive blood sampling (samples taken at 12-min intervals for 6 h) and ultrasonographic examination of ovaries from ovulation to the mid-luteal phase of the estrous cycle. Data for a ewe with 2 ovulations detected 2 days apart are shown (Fig. 7.1a). Arrows in the top panels (↓ OV or ← OV) indicate the number and diameters of preovulatory antral follicles on the day before ovulation; EM = day of emergence of the first follicular wave of the oestrous cycle studied; EM2 = day of emergence of the second wave of the cycle; ST1 = beginning of the static phase of the largest non-luteinized follicle of the first follicular wave; and ST2 = end of the static phase of the largest antral follicle of the wave. L denotes a large uniovulated follicle that transformed into a luteal structure. Day 0 = day of ovulation.
Fig. 7.2. A schematic representation of ovarian antral follicles (solid) and corpora lutea (hatched) detected by ultrasonography, with accompanying serum concentrations of LH (□) and FSH (■), measured in serial blood samples collected daily, every 12 min for 6 h, throughout the early and into the mid-luteal phase of the oestrous cycle in a representative Western white-faced ewe. Day 0 = day of ovulation.
after the first detected ovulation, and it was preceded by a second decline in vaginal impedance (23 ohms) and signs of behavioural oestrus.

Each ewe studied had 2 emerging follicular waves over the scanning period. The major characteristics of identified waves are shown in Table 7.1. A summary of ultrasonographic observations including days of ovulation, mean days of CL detection, days of follicle wave emergence, and days on which the growth and static phases of the largest follicles of waves ended, is given in Table 7.2. In all ewes, CL were detected by day 5 after ovulation (average of 3.1 ± 0.3 days). In addition to a CL, a luteinized unovulated follicle was seen to form in the ovaries of 2 ewes, on days 4 and 8 (Fig. 7.1b), respectively. One of the ewes with a luteinized follicle also had a short-lived CL detected for only 2 days (days 3 to 4 after ovulation). In the ewe with 2 ovulations that occurred 2 days apart (days 0 and 2; Fig. 7.1a), two follicles ovulated on day 0, giving rise to 1 normal CL detected on day 2 and a short-lived CL only seen on day 2. Three follicles ovulated on day 2, one from the previous follicular wave and 2 from the first wave of the cycle, but resulted in only 1 CL detected on day 4. One follicle from the first follicle wave (emerging 24 h after the first ovulation) grew and regressed with a normal growth and regression profile, and therefore was the follicle characterized for Wave 1 in this ewe. In the ewe that luteinized a follicle on day 4 after ovulation, the other non-luteinized follicle of Wave 1 was characterized, and in the ewe that luteinized a follicle on day 8 (Fig. 7.1b), the luteinization occurred 4 days into the follicle regression phase, and hence this follicle was characterized as a Wave 1 follicle. The growth and regression curves of the follicles ≥ 5 mm in diameter in both ewes above appeared normal.

The PULSAR characteristics for serum concentrations of LH and FSH were converted into the percentage of the maximal value for each ewe and analysed for the period from days 0 to 7, for the 6 Western white-faced ewes studied. When normalized on this basis, there was a significant effect of day for basal LH concentrations (P = 0.01), LH pulse frequency (P = 0.02) and duration (P = 0.0001), and FSH pulse frequency (P = 0.01), but not for LH pulse amplitude (P = 0.29). The day effect for mean serum concentrations of LH (P = 0.06) and mean (P = 0.08) and basal (P = 0.09) serum concentrations of FSH
Table 7.1. Characteristics of ovarian follicular waves (follicles growing from 3 to ≥ 5 mm in diameter; mean ± SEM) identified in six Western white-faced ewes that underwent daily ovarian ultrasonography and intensive blood sampling from ovulation to the mid-luteal phase. Day 0 = day of ovulation.

<table>
<thead>
<tr>
<th>End point</th>
<th>Wave 1</th>
<th>Wave 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean day of wave emergence</td>
<td>0.3 ± 0.2</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>Range</td>
<td>0-1</td>
<td>4-7</td>
</tr>
<tr>
<td>No. of follicles/wave</td>
<td>2.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td><strong>Largest non-luteinized follicle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>6.7 ± 0.3</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>1.2 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>
Table 7.2. Summary of ultrasonographic observations conducted from ovulation to the mid-luteal phase of the oestrous cycle (November) in 6 Western white-faced ewes. OV = ovulation; EM1-emergence of the first follicular wave (follicles growing from 3 to ≥ 5 mm in diameter) of the cycle; EM2-emergence of the second follicular wave of the cycle; ST1-beginning of the static phase of the largest follicle in the first follicular wave; ST2-end of the static phase of the largest follicle in the first follicular wave; and CL-mean day of CL detection. Day 0 = day of ovulation.

<table>
<thead>
<tr>
<th>Day of cycle</th>
<th>Ewe #1</th>
<th>Ewe #2</th>
<th>Ewe #3</th>
<th>Ewe #4</th>
<th>Ewe #5</th>
<th>Ewe #6(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>OV1 EM1</td>
<td>OV EM1</td>
<td>OV EM1</td>
<td>OV</td>
<td>OV EM1</td>
<td>OV</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EM1</td>
<td>EM1</td>
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<tr>
<td>2</td>
<td></td>
<td>OV2</td>
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<td>CL</td>
<td></td>
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<tr>
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<td></td>
<td>CL ST1</td>
<td>CL ST1</td>
<td>ST1</td>
<td>CL</td>
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<tr>
<td>4</td>
<td></td>
<td>EM2</td>
<td></td>
<td>ST2</td>
<td></td>
<td>ST1</td>
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<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>EM2</td>
<td>EM2 ST2</td>
<td>EM 2 ST2</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>ST2</td>
<td></td>
<td></td>
<td></td>
<td>ST1</td>
</tr>
<tr>
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<td>EM2</td>
<td></td>
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</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ST2</td>
</tr>
</tbody>
</table>

\(^1\) End of the static phase was not determined (see: Fig. 7.1d).
approached significance (Fig. 7.3). Basal serum concentrations of LH were highest on day 2 and then decreased steadily (P < 0.05) from days 2 to 7 after ovulation. Mean LH pulse frequency was highest on day 1 after ovulation and then declined (P < 0.05) by day 7 of the oestrous cycle. The duration of detected LH pulses increased significantly from days 3 to 5 after ovulation. The frequency of FSH pulses was minimal on day 4 after ovulation, but it subsequently increased (P < 0.05) between days 5 and 6 of the oestrous cycle.

There were no significant trends (P ≥ 0.06) for any of the characteristics of episodic LH secretion determined for the period from 2 days before to 2 days after the day of wave emergence (the second wave of the estrous cycle studied; EM2). The trend for LH pulse amplitude during that period approached significance (P = 0.06). However, mean and basal serum levels of FSH (P = 0.0001) and FSH pulse frequency (P = 0.004; Fig. 7.4) differed by day; both mean and basal FSH concentrations increased 1 day before and on the day of wave emergence (P < 0.05), and FSH pulse frequency increased (P < 0.05) from 1 day before to 2 days after wave emergence.

Mean and basal LH concentrations did not vary (P ≥ 0.40) during the period encompassing the end of the growth phase (ST1) of the largest ovarian follicles in the first follicular wave of the estrous cycle studied. There was a significant increase in LH pulse amplitude on the day the largest follicles of the wave reached their maximum diameters (ST1; Fig. 7.5b). LH pulse frequency declined (P < 0.05) from 2 days before to 1 day after the end the follicular growth phase. The duration of LH pulses increased (P < 0.05) from 1 day before to 2 days after the onset of the static phase. Mean and basal FSH concentrations increased (P < 0.05) on the day of and 2 days after the end of the growth phase of the largest follicles of the wave (Fig. 7.5c). None of the analysed parameters appeared to be affected by the end of the static phase of the largest follicle growth curves (ST2; P ≥ 0.38).

The amplitude and duration of identified LH pulses increased (P < 0.05) 1 day after the detection of CL in ewes. Mean and basal FSH levels fell (P < 0.05) on the day of CL detection and subsequently increased (P < 0.05), 1 and 2 days later. FSH pulse frequency declined (P < 0.05) 1 day after CL detection and remained low 24 h later (Fig. 7.6).
**Fig. 7.3.** Summary of the PULSAR characteristics of LH and FSH secretion determined for the day of ovulation + 7 days in six Western white-faced ewes studied from ovulation to the mid-luteal phase of the oestrous cycle. All values were converted to the percentage of maximal value recorded for each ewe from day 0 to day 7 after ovulation. Results are expressed as mean ± SEM. Within each parameter, means denoted by different letters are significantly different (LSD test, P < 0.05). See text for statistical details of overall analyses of variance. The graphs represent: (a) mean (solid bars) and basal (hollow bars) serum levels of LH; (b) LH pulse frequency (hollow bars), amplitude (solid bars) and duration (angle hatched bars); (c) mean (solid bars) and basal (hollow bars) serum FSH concentrations; and (d) FSH pulse frequency (hollow bars).
Fig. 7.4. Summary of the PULSAR characteristics of LH and FSH secretion for the day of emergence of the second wave of the cycle (EM2) ± 2 days in six Western white-faced ewes studied from ovulation to the mid-luteal phase of the oestrous cycle. All values were converted to the percentage of maximal value recorded for each ewe from day 0 to day 7 after ovulation. Results are expressed as mean ± SEM. Within each parameter, means denoted by different letters are significantly different (LSD test, P < 0.05). See text for statistical details of overall analyses of variance. The graphs represent: (a) mean (solid bars) and basal (hollow bars) serum levels of LH; (b) LH pulse frequency (hollow bars), amplitude (solid bars) and duration (angle hatched bars); (c) mean (solid bars) and basal (hollow bars) serum FSH concentrations; and (d) FSH pulse frequency (hollow bars).
Fig. 7.5. Summary of the PULSAR characteristics of LH and FSH secretion for the day of and 2 days prior to and after the end of the growth phase of the largest follicles in the first follicular wave of the cycle (ST1), in six Western white-faced ewes studied from ovulation to the mid-luteal phase of the oestrous cycle. All values were converted to the percentage of maximal value recorded for each ewe from day 0 to day 7 after ovulation. Results are expressed as mean ± SEM. Within each parameter, means denoted by different letters are significantly different (LSD test, P < 0.05). See text for statistical details of overall analyses of variance. The graphs represent: (a) mean (solid bars) and basal (hollow bars) serum levels of LH; (b) LH pulse frequency (hollow bars), amplitude (solid bars) and duration (angle hatched bars); (c) mean (solid bars) and basal (hollow bars) serum FSH concentrations; and (d) FSH pulse frequency (hollow bars).
Days from end of the growth of the largest follicles
Fig. 7.6. Summary of the PULSAR characteristics of LH and FSH secretion aligned to the mean day of CL detection ± 2 days, for six Western white-faced ewes studied from ovulation to the mid-luteal phase of the oestrous cycle. All values were converted to the percentage of maximal value recorded for each ewe from day 0 to day 7 after ovulation. Results are expressed as mean ± SEM. Within each parameter, means denoted by different letters are significantly different (LSD test, P < 0.05). See text for statistical details of overall analyses of variance. The graphs represent: (a) mean (solid bars) and basal (hollow bars) serum levels of LH; (b) LH pulse frequency (hollow bars), amplitude (solid bars) and duration (angle hatched bars); (c) mean (solid bars) and basal (hollow bars) serum FSH concentrations; and (d) FSH pulse frequency (hollow bars).
7.5 Discussion

In previous studies using ultrasonography and blood sampling once daily, it was shown that large antral follicles (attaining ≥ 5 mm in size) grew in waves across the ewe's oestrous cycle, and that around the time of wave emergence (growth from the 3-mm pool of follicles) there was a transient elevation (2 to 3 days) in serum concentrations of FSH (Ginther et al., 1995). In the present study, using daily periods of blood sampling every 12 min for 6 h, with daily ultrasonography, we confirmed this pattern of FSH secretion. Interestingly, when FSH concentrations were normalized to follicle wave emergence (EM2; Fig. 7.4c), the peak in mean serum FSH concentrations appeared to be due to a similar pattern of basal FSH secretion (the FSH concentrations left after pulse removal). This would suggest that the periodic increase in FSH secretion occurring around follicle emergence reflected increased FSH synthesis and constitutive secretion, not changes in pulsed secretion of FSH.

With data normalized to follicle wave emergence (Fig. 7.4), there was a clear increasing trend in FSH pulse frequency from follicle emergence onwards (Wave 2). This is intriguing and suggests that as basal FSH secretion peaks and then begins to decline after follicle emergence, pulsed FSH secretion increases or becomes more obvious. The increase in pulsatile secretion of FSH paralleled the growth phase of the emergent follicles, and it also appeared to be more pronounced after CL detection (Wave 2) compared with immediately after ovulation (Wave 1; Fig. 7.4d).

In the present study, observations were made from ovulation into the mid-luteal phase of the oestrous cycle (days 0 to 7). Emergence of the second follicle wave occurred at a time when progesterone secretion from the developing CL was increasing. In this study, looking at patterns of serum FSH concentrations in the alignments to various stages of cyclic ovarian function strongly suggested a link between FSH secretion and follicle growth patterns, but much less to CL function. However, over the period of the cycle when progesterone levels would have been expected to be increasing, all of the parameters of serum FSH concentrations we examined tended to increase. Corpora lutea were detected on
average 3 days after ovulation. In previous work, we have shown that the day of ultrasonographic CL detection is the day on which serum progesterone concentrations exhibit a first increase above the basal or non-detectable level. It has been established that ovarian oestradiol and inhibin are the major negative feedback regulators of FSH secretion (Clarke et al., 1986; Campbell et al., 1991b; Mann et al., 1992a), but progesterone may also affect FSH secretion in the ewe (Rawlings et al., 1984). The duration of the interval between adjacent peaks in mean serum FSH concentrations is longer early in the luteal phase than at the mid- and late luteal phase of the oestrous cycle in Western white-faced ewes.

Mean and basal serum concentrations of FSH started to increase from around the time of the end of the growth phase/onset of the static phase of the first follicle wave, suggesting that secretion of a follicular inhibitor of FSH release declined at that time (Fig. 7.5). This inhibitor was probably not oestradiol, because oestradiol secretion is maximal on the day of peak follicle size, but inhibin secretion could have changed. New follicle waves emerged within 1 to 2 days of the onset of the static phase of the previous wave (Table 7.2), suggesting that changed secretory activity of the follicle in the static phase permits the increase in FSH secretion that heralds the next follicle wave. This would mirror the concepts proposed for cattle (Ginther et al., 1996), except that follicular dominance is less apparent in the ewe, because several follicles can emerge and grow to a similar final stage in a single wave.

Apart from wanting to determine the characteristics of FSH secretion that gave rise to the pre-emergent peaks of mean serum concentrations of FSH, we were also interested in possible changes in pulsatile LH secretion from the early to mid-luteal phase. In considering the data for the various alignments of serum LH parameters with follicular and luteal function, it appeared that LH was most influenced by the development of the CL/progesterone secretion. When LH data were normalized to the day of ovulation, LH pulse frequency declined and pulse duration increased by day 7 of the cycle. Progesterone regulates LH pulse frequency and oestradiol pulse amplitude (Baird et al., 1976; Karsch et al., 1979; Goodman and Karsch, 1980; Wheaton et al., 1984; Goodman, 1994). In addition, it has been suggested that LH pulse frequency and amplitude vary in a reciprocal manner
(Goodman and Karsch, 1980), probably reflecting diminished GnRH/LH secretion into each pulse as frequency increases and vice versa. In Fig. 7.6, it was clear that LH pulse amplitude and duration increased after CL detection, even though the concomitant decline in LH pulse frequency was not significant in that data alignment. There was little evidence for any association between LH secretory parameters and any stage of antral follicle growth and regression in the present study. However, although LH pulse amplitude appeared to increase over the period of CL development as expected, the change in amplitude and pulse duration were abrupt, occurring the day after CL detection or on the day of the onset of the static phase of the largest follicles in the first follicle wave of the oestrous cycle (Fig. 7.4). The onset of the static phase occurred on the day of or 1 day after CL detection in most ewes (Table 7.2). Therefore, it is difficult to know whether the effect on LH pulse amplitude is caused by the first increase in progesterone secretion, which occurs on the day of CL detection, or reflects changes in the secretion of feedback factors by the antral follicle at its zenith. This could not involve oestradiol, because oestradiol decreases LH pulse amplitude (Goodman and Karsch, 1980) and the follicle produces the greatest amount of oestradiol on the day it reaches its maximum size (onset of the static phase), which is when LH pulse amplitude increased. In addition, as neither LH pulse frequency nor mean LH levels changed considerably at terminal growth of large ovine antral follicles, the increased oestrogenic secretory ability of the follicles appears to arise from the increase in follicular LH responsiveness, probably due to synthesis of LH receptors enhanced by FSH and oestradiol (England et al., 1981).

It has been suggested in ewes that FSH is important for the initiation of antral follicle growth to ovulatory diameters and it may also maintain large follicles during the static phase, but later stages of follicular lifespan are independent of FSH periodicity (Bartlewska et al., 1998). In the present study, there were no changes in either FSH or LH secretory characteristics around the time when follicular regression started, indicating that the onset of follicular demise is likely to be associated with changes in the ability of antral follicles to utilize gonadotrophins rather than with the availability of gonadotrophic hormones.
With the use of daily transrectal ovarian ultrasonography, we have found that normal cyclic Western white-faced ewes may produce short-lived CL and luteinized follicles in otherwise normal cycles (i.e., with a normal CL). These extra luteal structures do not appear to disrupt the cycle, successive waves of follicle growth, or alter the expected serum profiles of progesterone. In the present study, ewes with such structures were analysed with the whole group of ewes. One ewe ovulated at oestrus, and then ovulated again 2 days later, evidently from the 2 waves of follicles (Fig. 7.1a). We have previously seen ovulations from follicular waves with an extended lifespan in Western white-faced and especially prolific Finn ewes. The ewe with the 2 periods of ovulation in the present study was included in the analysis, because she produced 2 waves of follicle growth during the early luteal phase like all other ewes.

7.6 Conclusions

We conclude that mean and basal (pulses removed) FSH secretion peaks at or around antral follicle emergence (follicles growing from 3 mm in diameter to ≥ 5 mm) and postulate that these increases in secretion might be caused by changes in function of the follicles in the previous follicular wave entering their static phase. FSH pulse frequency increased during follicle growth as basal secretion declined. FSH secretion appeared to be linked to and/or regulated mainly by follicle growth and function, whereas pulsed secretion of LH was more closely related to development of the CL/progesterone secretion. Apart from an increase in LH pulse amplitude on the day of the onset of the antral follicle static phase (first wave of the cycle), none of the parameters of pulsatile LH secretion appeared to be temporally associated with any stage of follicle growth and regression.
Chapter 8. OVARIAN FUNCTION IN EWES DURING THE TRANSITION FROM THE BREEDING SEASON TO ANOESTRUS

8.1 Abstract

Transrectal ovarian ultrasonography was conducted in 6 Western white-faced ewes for 35 days from the last oestrus of the breeding season, to record the number and size of all ovarian follicles ≥ 3 mm in diameter and luteal structures. Blood samples were collected once a day for estimation of serum concentrations of FSH, oestradiol and progesterone. Each ewe had 5 follicular waves (follicles growing from 3 to ≥ 5 mm in diameter) over the scanning period. The duration of the growth phase of the largest ovarian follicles did not differ (P > 0.05) between waves, but follicular static and regressing phases decreased significantly (P < 0.05) after the decline in serum progesterone concentrations at the end of the last luteal phase of the breeding season. The intervals between the 5 follicular waves were: 9.2 ± 0.4, 5.2 ± 0.7, 8.3 ± 0.8 and 5.8 ± 0.7 days; the 2 shorter intervals differed (P < 0.05) from the 2 longer intervals. Using the cycle-detection program, rhythmic increases in serum FSH concentrations were detected in all ewes; the amplitude, duration and periodicity of FSH fluctuations did not vary (P > 0.05) throughout the period of study. The number of identified FSH peaks (7.8 ± 0.5 peaks per ewe, per scanning period) was greater (P < 0.05) than the number of emerging follicular waves. Serum concentrations of oestradiol remained low (≤ 1 pg/ml) on most days, in 5 out of the 6 ewes studied, and sporadic elevations in oestradiol secretion above the non-detectable level were not associated with the emergence of follicular waves. The ovulation rate was lower than that seen during the middle portion of the breeding season (November-December) in Western white-faced ewes but the transitional ewes had larger CL. Maximal serum concentrations of progesterone appeared to be lower and the plateau phase of progesterone secretion appeared to be shorter during
the last luteal phase of the ovulatory season in comparison to the mid-breeding season of Western white-faced ewes.

During the transition into anoestrus in ewes, the endogenous rhythm of FSH release is remarkably robust but the pattern of emergence of sequential follicular waves is dissociated from FSH and oestradiol secretion. Luteal progesterone secretion is suppressed because of fewer ovulations and diminished total luteal volume, but it may also result from diminished gonadotrophic support. These season-related alterations in the normal pattern of ovine ovarian cycles appear to be due to reduction in ovarian responsiveness to gonadotrophins and/or attenuation in secretion of LH occurring at the onset of the anovulatory season in ewes.

8.2 Introduction

The ewe is a seasonally polyoestrous animal with normal ovulatory cycles occurring, in most breeds, in the autumn and winter (Hafez, 1952). After the last luteal phase of the breeding season, the decline in serum concentrations of progesterone is not accompanied by a prominent rise in oestradiol secretion (Rawlings et al., 1977; Legan and Karsch, 1979). The lack of a normal preovulatory peak of oestradiol may be, in part, due to reduction in ovarian responsiveness to gonadotrophic hormones (Legan and Karsch, 1979), but it is primarily caused by an enhanced negative feedback suppression of LH secretion by oestradiol (Legan et al., 1977; Goodman et al., 1981; Karsch et al., 1993). LH pulse frequency fails to increase to a normal follicular phase level, removing the stimulus for production of oestradiol and hence the positive feedback effect on LH secretion. Consequently, oestrus and ovulation do not occur, and progesterone and LH fall to basal or non-detectable levels (Rawlings et al., 1977). Mean serum FSH concentrations, however, appear to remain unchanged during the period of transition into anoestrus in ewes (Karsch et al., 1984).

The growth of ovulatory-sized ovarian follicles occurs at regular intervals, approximately every 4-5 days, both in cyclic (Ginther et al., 1995) and anoestrous sheep.
(Bartleowski et al., 1998). In the ewe, the emergence of follicular waves (defined as follicles growing from 3 to ≥ 5 mm in diameter) is closely associated with transient elevations in serum FSH concentrations (Ginther et al., 1995; Bartleowski et al., 1998). There is also a temporal relationship between the onset of increased oestradiol secretion and the beginning of the growing phase of the follicles as well as between peaks of serum oestradiol concentrations and the end of the growth of the largest ovarian follicles of waves, throughout the sheep oestrous cycle, but not during anoestrus (Souza et al., 1996; Bartleowski et al., 1998). Ovarian follicular development and its relationship with the secretory profiles of FSH and oestradiol during the transition from the breeding season to anoestrus in sheep have not been studied.

During the normal oestrous cycle in the ewe, serum concentrations of progesterone are related to the total volume of luteal tissue and vary between prolific and non-prolific breeds of sheep. It has also been shown that progesterone secretion is affected by the stage of the ovulatory season in ewes, with higher serum progesterone concentrations detected in the middle than at the beginning or end of the season (Wheeler and Land, 1977).

It is remarkable that despite decreased LH secretion in anoestrous ewes, waves of growth of large antral follicles continue in a similar pattern to the breeding season; each wave preceded by an increase in serum concentrations of FSH. The purpose of the present study was to use daily ovarian ultrasonography and blood sampling to examine ovarian follicular dynamics and serum concentrations of FSH, oestradiol and progesterone in the transitional period between the breeding season and seasonal anoestrus in ewes. We wanted to see if the abrupt change in LH secretion at that time was reflected in any change in oestradiol and progesterone secretion, and follicular dynamics/luteal function. We hypothesized that the abrupt cessation of the breeding season would be reflected in alterations in the orderly secretion of FSH and production of large, ovulatory-sized follicles, and that this "natural perturbation" of endocrine and follicular patterns could reveal important clues as to the regulation of the orderly wave production of ovarian antral follicles in the ewe.

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8.3 Materials and methods

8.3.1 Animals and experimental procedures

Twelve Western white-faced (aged approximately 4 years, weighing between 75 and 90 kg), nulliparous and clinically healthy ewes, were used in the present study during the transition to anoestrus (January-March). From 15 January, ewes were checked daily for oestrus with 3 vasectomized crayon-harnessed rams and a vaginal impedometer (Electronic Oestrous Detector; Firma Draminski, Olsztyn, Poland). Daily transrectal ultrasonography of ovaries commenced for each ewe when it was marked by the rams or a decline in vaginal impedance to ≤ 40 ohms was recorded; ultrasonography continued until 35 consecutive days of scanning, from the last oestrus of the breeding season, were collected for 6 ewes.

8.3.2 Hormone analysis

All samples for FSH were analysed in a single assay; intraassay coefficients of variation (CVs) for ovine reference sera with mean hormone concentrations of 2.33 and 3.67 ng/ml were 12.9 and 8.7%, respectively. For oestradiol reference sera with mean concentrations of 3.5 or 12.0 pg/ml, the intra- and interassay CVs were 16.5 and 7.8% or 14.5 and 8.9%, respectively. For progesterone reference sera with mean concentrations of 0.42 or 2.36 ng/ml, the intra- and interassay CVs were 10.3 and 4.8% or 13.7 and 6.0%, respectively.

The following characteristics of detected peaks/fluctuations in serum concentrations of FSH were determined: 1) the mean number of peaks per ewe, per scanning period; 2) the mean duration of sequential fluctuations; 3) the mean peak concentration; and 4) the mean length of intervals between peaks of adjacent fluctuations (interpeak intervals). This was not done for oestradiol as serum concentrations of oestradiol were significantly suppressed over the period of study and the range of detected peaks of oestradiol concentration (1-8 peaks/ewe) did not allow further analyses of serial peaks in all animals. Repeated measures analyses of variance (RM-ANOVA; General Linear Model procedures in MINITAB®
Statistical Software, Minitab Inc., Enhanced Version, Release 9.1 for VMS/VAX 6.2; 1992, State College, PA, USA) were used to compare the amplitude, duration and periodicity (interpeak intervals) of FSH peaks/fluctuations over time.

8.3.3 Follicle populations and dynamics

Follicular data (follicles ≥ 3 mm in diameter) were combined for both ovaries of each ewe. Daily numbers of 3-mm follicles that did not grow any larger, and of 3-mm follicles that subsequently attained 4 or ≥ 5 mm, were normalized to the day of the last observed ovulation of the breeding season (day 0), for the period from days -1 to 31 (the entire growth phase was not seen for the 3-mm follicles emerging during the last 2 days of the scanning period), and analysed by RM-ANOVA (MINITAB®, 1992).

The following characteristics of follicular waves (follicles growing from 3 to ≥ 5 mm in size) were determined for each ewe: 1) number of emerging follicular waves per scanning period; 2) days of wave emergence, relative to day of ovulation (day 0); 3) number of follicles per wave; 4) maximum diameter attained by the largest follicle of the wave; 5) durations of the follicle growing, static and regressing phases, and the total lifespan of the largest follicle; and 6) number of days between emergence of sequential follicular waves (interwave intervals). Analyses of variance (MINITAB®, 1992) were done to compare the characteristics above between sequential follicular waves detected in ewes during the period of study.

8.3.4 Luteal structures and progesterone secretion

The number of ovulations as well as the number, day of detection and total lifespan of luteal structures were determined for each ewe. Daily total luteal volumes were normalized to the day of the last ovulation of the breeding season (day 0), for the period during which luteal structures were observed with ultrasonography (days 3 to 16 after ovulation), and were analysed by RM-ANOVA (MINITAB®, 1992). Daily serum
progesterone concentrations were normalized to the day of ovulation (day 0) and analysed for the period from days −1 to 33. When an overall analysis of variance was significant (P < 0.05), Fisher’s protected least significant difference (LSD) was used as the post-ANOVA test to compare individual (daily) means for total luteal volumes and progesterone concentrations. In all of the analyses above, the statistical models contained the following terms: ewe, time (days or sequential events), and error. All results are given as mean ± SEM.

In order to study the relationship between daily luteal volumes and serum progesterone concentrations, the luteal phase was divided into 4 periods, based on the ultrasonographic detection of CL/luteal structures and assessment of daily mean concentrations of progesterone. Period 1, or the phase of luteal tissue formation, was defined as the time after ovulation during which CL/luteal structures were not detectable with ultrasonography (days 0 to 2). Period 3, or the mid-luteal phase, was regarded as the period encompassing all the days on which daily mean serum concentrations of progesterone did not differ (P > 0.05) from the maximal concentration of the hormone seen on day 9 after ovulation; this period lasted from day 9 to 11 in the ewes under study. The time between the end of luteal formation and the beginning of the mid-luteal phase was Period 2, or the phase of luteal growth (days 3 to 8). Period 4, or luteal regression, was the time from the end of the mid-luteal phase until complete demise of luteal structures, determined by ultrasonography (days 12 to 16). The relationship between daily total luteal volumes and circulating concentrations of progesterone for all ewes, for each of the 4 periods above, was studied by simple linear regression (MINITAB®, 1992). The independent variable was total luteal volume and the dependent variable was progesterone concentration.

8.4 Results

8.4.1 Oestrus detection

The Western white-faced ewes in the present study became anoestrous during the period from late January to mid-February. At the last ovulation of the breeding season, all

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the ewes but 1 were well marked by rams. Vaginal impedance decreased to ≤ 40 ohms (mean: 37.5 ± 1.9 ohms) in all ewes on day -1, and then increased to 45.2 ± 4.5 ohms on the day of ovulation (day 0; P < 0.05). In 1 ewe, a second ovulation occurred, in the contralateral ovary, 4 days after the first detected ovulation and it was preceded by a second decline in vaginal impedance (38 ohms; day 2) and manifestations of behavioural oestrus (day 3). One other ewe was marked by rams on day 7 after ovulation; this was not preceded by a decline in vaginal impedance to ≤ 40 ohms, but it was accompanied by a peak in serum concentration of oestradiol (Fig. 8.1a). After the final luteolysis of the breeding season, vaginal mucous impedance fell to ≤ 40 ohms in 4 out of the 6 ewes studied, for an average of 2 days (range: 1 to 5 days), between days 14 and 20 after ovulation; no ewe was marked by the rams at this time.

8.4.2 Follicle populations and dynamics

Individual follicle profiles of follicles growing from 3 to ≥ 5 mm in diameter before regression are shown for 4 individual ewes, with corresponding serum FSH and oestradiol concentrations, in Fig. 8.1. Five ewes ovulated only 1 follicle and one ewe ovulated 2 follicles (2 ovulations detected 4 days apart; Fig. 8.1d). Daily numbers of 3-mm follicles not growing beyond 3 mm in diameter and daily numbers of 3-mm follicles growing to a maximum size of 4 or ≥ 5 mm in diameter before regression did not change significantly with time (P > 0.05; Fig. 8.2). However, follicles that attained ≥ 5 mm in diameter emerged from the pool of 3-mm follicles only on days 0 to 3, 9 to 16, 18, 22 to 27 and 29 to 31 after ovulation (Fig. 8.2c).

A total of 30 follicular waves (follicles growing from 3 to ≥ 5 mm in diameter) was obtained in the present study; each ewe had 5 waves per 35 day scanning period. The characteristics of follicular waves are given in Table 8.1. The mean number of follicles per wave (1.3 ± 0.2), the maximum diameter (6.1 ± 0.2 mm), the mean growth rate (1.1 ± 0.1 mm/day), and the duration of the growth phase of the largest ovarian follicles (3.2 ± 0.4 days) did not differ (P > 0.05) among successive follicular waves. The static phase of the
Fig. 8.1. The diameter profiles of individual antral follicles growing to a size of ≥ 5 mm recorded from the last oestrus of the breeding season, through the last luteal phase and into early anoestrus in 4 Western-white faced ewes (a-d), with accompanying daily serum concentrations of FSH (middle panels; ■) and oestradiol (bottom panels; □). The arrows along the X-axis (top panels; †) denote the days of follicle wave emergence. The arrows in the upper chart area (†OV) indicate the number and diameter of ovulatory follicles on the day before ovulation. L denotes a large antral follicle that became luteinized and gradually transformed into a solid luteal structure. Asterisks denote the peak values of fluctuations (nadir-to-peak-to-nadir) in circulating concentrations of FSH and oestradiol (the 2 dashed lines encompass a fluctuation) as determined by the cycle-detection computer program (Clifton and Steiner, 1983).
Fig. 8.2. Mean (± SEM) daily numbers of 3-mm follicles not growing beyond 3 mm before regression (a), and of 3-mm follicles that subsequently grew up to 4 (b) or ≥ 5 mm in diameter (c), in 6 Western white-faced ewes that underwent daily transrectal ovarian ultrasonography for 35 days from the last oestrus of the breeding season. Follicular data were aligned to the day of ovulation (day 0) and analysed for the period from day −1 to day 31. See text for statistical details.
Days from last ovulation of the breeding season
Table 8.1. Characteristics of ovarian follicular waves (follicles growing from 3 to ≥ 5 mm in diameter; mean ± SEM) identified in 6 Western white-faced ewes that underwent daily ovarian ultrasonography from the last oestrus of the breeding season, through the final luteal phase and into early anoestrus. Day 0 = day of last ovulation of the breeding season.

<table>
<thead>
<tr>
<th>End point</th>
<th>Wave 1</th>
<th>Wave 2</th>
<th>Wave 3</th>
<th>Wave 4</th>
<th>Wave 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean day of wave emergence</td>
<td>1.5 ± 0.3</td>
<td>10.7 ± 0.3</td>
<td>15.8 ± 0.9</td>
<td>24.2 ± 0.5</td>
<td>30.0 ± 0.7</td>
</tr>
<tr>
<td>Range</td>
<td>0-2</td>
<td>9-11</td>
<td>13-18</td>
<td>23-26</td>
<td>27-31</td>
</tr>
<tr>
<td>No. of follicles/wave</td>
<td>1.2 ± 0.2</td>
<td>1.7 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td><strong>Largest follicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>6.0 ± 0.5</td>
<td>6.5 ± 0.3</td>
<td>5.8 ± 0.3</td>
<td>6.0 ± 0.3</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>1.2 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.08</td>
</tr>
<tr>
<td>Growing phase (days)</td>
<td>3.0 ± 0.8</td>
<td>3.5 ± 0.5</td>
<td>3.0 ± 0.7</td>
<td>3.3 ± 0.6</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Static phase (days)</td>
<td>(2.6 ± 0.8^a)</td>
<td>(2.7 ± 0.5^a)</td>
<td>(1.3 ± 0.2^b)</td>
<td>(1.7 ± 0.3^{ab})</td>
<td>ND</td>
</tr>
<tr>
<td>Regressing phase (days)</td>
<td>(3.0 ± 0.7^{ab})</td>
<td>(4.5 ± 0.6^a)</td>
<td>(2.7 ± 0.7^b)</td>
<td>(3.3 ± 0.3^b)</td>
<td>ND</td>
</tr>
<tr>
<td>Lifespan (days)</td>
<td>(9.0 ± 1.6^{ab})</td>
<td>(10.5 ± 0.9^a)</td>
<td>(7.0 ± 1.0^b)</td>
<td>(8.3 ± 0.8^b)</td>
<td>ND</td>
</tr>
<tr>
<td>Interwave interval(^1) (days)</td>
<td>(9.2 ± 0.4^a)</td>
<td>(5.2 ± 0.7^b)</td>
<td>(8.3 ± 0.8^a)</td>
<td>(5.8 ± 0.7^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^{ab}\) Within rows, means with different letter superscripts are different (P < 0.05).

\(^1\) Interwave interval refers to the number of days between emergence of the wave in that column and emergence of the next sequential wave.

ND-the entire follicular lifespan was not seen for follicular waves emerging just prior to the end of the scanning period.
lifespan of the largest follicles of waves was longer (P < 0.05) for the first two waves than for the third wave detected during the observation period. The regression phase and total lifespan of the second follicular wave was longer (P < 0.05) in comparison to subsequent waves of follicular emergence (Waves 3 and 4). The intervals between Waves 1 and 2, and Waves 3 and 4, were longer than the intervals between Waves 2 and 3, and Waves 4 and 5 (P < 0.05).

3.4.3 FSH concentrations

Using the cycle-detection program (Clifton and Steiner, 1983), peaks of transient increases in daily serum concentrations of FSH were detected in all ewes studied (Fig. 8.1). The mean number of identified FSH peaks per ewe, per scanning period was 7.8 ± 0.5 (range: 6 to 9). The mean amplitude (3.03 ± 0.10 ng/ml) and duration (3.6 ± 0.2 days) of sequential fluctuations as well as mean interpeak interval for serum FSH concentrations (4.2 ± 0.3 days) did not vary (P > 0.05) throughout the period of study.

8.4.4 Oestradiol concentrations

In 5 out of the 6 ewes, daily serum concentrations of oestradiol were, on most days, below assay sensitivity (1 pg/ml; Fig. 8.1). Using the cycle-detection program, peaks of daily oestradiol concentrations were identified in all ewes studied. There were three animals with a single peak, one ewe with 2 peaks, one animal had 3, and one had 8 peaks of oestradiol over the 35-day period of blood sampling during the transition into anoestrus. In the three ewes with a single peak of oestradiol, a peak was detected on days 7, 11 and 26 after ovulation, respectively. In the ewe with 2 peaks, the peaks occurred on days 19 and 31, in the animal with 3 peaks, on days 19, 26 and 29 after ovulation, and in the ewe with 8 sequential oestradiol peaks, on days 3, 6, 10, 13, 16, 19, 22 and 28 after ovulation.
8.4.5 Luteal structures and progesterone concentrations

In all ewes, CL could be identified by day 6 after ovulation (detected on average 2.8 ± 0.7 days after ovulation). In the ewe with 2 ovulations detected 4 days apart, the second ovulation was not followed by formation of a CL. In addition to a CL, a luteinized follicle was seen to form in one ewe (Fig. 8.1b). This structure was first detected on day 5 after ovulation when its outer diameter was 9 mm and the diameter of the central cavity was 5 mm. The cavity was seen to fill in by day 10, when the outer diameter of the luteinized follicle was 12 mm. The maximum diameter attained by CL during mid-cycle ranged from 12 to 14 mm. The mean lifespan of all identified luteal structures was 13.5 ± 0.5 days. All of the luteal structures described above regressed between days 15 and 19 after ovulation (mean: day 16.3 ± 0.6).

Mean daily total luteal volumes increased (P < 0.05) from 335 ± 92 mm³ on day 3 to 754 ± 71 mm³ on day 5, and then to a maximum of 1273 ± 184 mm³ on day 11 (P < 0.05); there were no significant differences from days 6 to 13 after ovulation (Fig. 8.3). The total luteal volume reached a nadir on day 16 (426 ± 243 mm³). Mean daily serum progesterone concentration rose from day 0 to a peak value of 2.92 ± 0.30 ng/ml on day 9 after ovulation (P < 0.05; Fig. 8.3). Serum progesterone concentrations declined to 1.10 ± 0.60 ng/ml by day 14 (P < 0.05) and reached a low level of 0.08 ± 0.03 ng/ml on day 16 after ovulation; there was no significant change from days 9 to 11 (P > 0.05). Serum concentrations of progesterone remained basal (P > 0.05) for the remainder of the scanning period in all ewes.

There was a significant positive correlation between serum progesterone concentrations and the total luteal volume during the period of luteal growth and regression (r = 0.47, P = 0.005 and r = 0.56, P = 0.0017; Periods 2 and 4 of analysis, respectively), but not during the mid-luteal phase (Period 3: r = 0.23, P = 0.35). Regression equations, and lower and upper 95% confidence interval limits for serum progesterone concentrations ([P₄]; ng/ml) and daily total luteal volumes ([TLV]; mm³), for Periods 2 and 4, were as follows: [P₄] = 0.0007 x [TLV] + 0.80; 0.0002 and 0.0012; and [P₄] = 0.0014 x [TLV] - 0.0033; 0.0006 and 0.0023. When the data for the ewe that had a luteinized follicle were withdrawn
Fig. 8.3. Mean daily serum progesterone concentrations (−) and total volumes of luteal tissue (△) in 6 Western white-faced ewes studied for 35 days from the last oestrus of the breeding season (August-October). Data were normalized to the day of last ovulation of the breeding season (day 0). See text for statistical details.
Days from last ovulation of the breeding season
from analysis, significant correlations between daily serum progesterone concentrations and total luteal volumes was seen for Periods 2 and 4, but not Period 3 \( (r = 0.48, P = 0.01; r = 0.03, P = 0.91; \text{ and } r = 0.65, P = 0.0005, \text{ for Periods 2, 3 and 4, respectively}) \).

8.5 Discussion

Based on the measurement of oestradiol concentrations in blood samples collected once a day, the last luteal phase of the breeding season in ewes was not followed by an increment in serum concentrations of oestradiol. Oestradiol secretion appeared to be markedly attenuated during the entire period of transition into seasonal anoestrus. During the mid-breeding season in Western white-faced ewes, 3 to 4 peaks of oestradiol per oestrous cycle, occurring at approximately 4-day intervals, could be detected in all animals. As ewes move further into anoestrus, more frequent fluctuations in circulating concentrations of oestradiol reappear in most animals (Bartlewski et al., 1998). In ewes, the frequency of LH pulses that drives oestradiol production, depends on seasonal variation in the negative feedback effect of oestradiol; the response to oestradiol is low throughout the breeding season, rises during the transition to anoestrus and remains strong until the beginning of the next mating season (Legan and Karsch, 1979). In addition, a transitory decline in ovarian follicular responsiveness to gonadotrophins, particularly LH, has previously been reported in ewes at the end of the breeding season (Legan and Karsch, 1979). It appears that depressed ovarian sensitivity to gonadotrophic hormones with enhanced negative feedback suppression of LH secretion, resulting in very low serum levels of oestradiol, may be important for the rapid cessation of oestrus and ovulations after the final luteal phase of the breeding season in ewes. As anoestrus progresses, a partial recovery of ovarian responsiveness to LH/FSH and restoration of oestradiol production may occur.

Recently, it was demonstrated in sheep that there is a functional relationship between transient elevations in FSH release and the emergence of preovulatory size follicles throughout the breeding season (Ginther et al., 1995) and anoestrus (Bartlewski et al., 1998). In the present study, rhythmically generated fluctuations of FSH were detected in all ewes.
The mean amplitude of identified FSH peaks during the mid-breeding season, the transition into anoestrum (present report), and the anoestrous period (Bartleewski et al., 1998) in Western white-faced ewes, were as follows: 2.82 ± 0.17, 3.03 ± 0.10 and 2.75 ± 0.18 ng/ml, respectively. In the present study, despite the occurrence of a repeated FSH signal for follicular growth, there were fewer follicular waves (follicles growing from 3 to ≥ 5 mm in diameter) than FSH peaks (5.0 ± 0.0 and 7.8 ± 0.5 per ewe, per scanning period, respectively; P < 0.05). The mechanism blocking the emergence of follicular waves during the transition into anoestrum is unclear. Development of multiple antral follicles of periovulatory size can be induced in sheep by treatment with FSH alone (Picton et al., 1990). However, suppression of basal LH release during administration of FSH completely blocks this follicular growth (McNeilly et al., 1991). In the present ewes, the emergence of large antral follicles was interrupted in the mid-luteal phase and after luteolysis, as judged by the duration of intervals between adjacent follicular waves. This may be due to an attenuated follicular sensitivity to gonadotrophic hormones or increased negative feedback effects of oestradiol on LH secretion; we did not determine the pulsatile pattern of LH secretion in the present ewes. The question arises, why is the relationship between periodic increases in serum FSH concentrations and the emergence of ovarian follicular waves restored later in anoestrum, when LH secretion remains strongly depressed? The ability of growing ovine follicles to utilize FSH and/or LH may be temporarily altered during the transition into anoestrum. Alternatively, the change in the mean duration of FSH fluctuations (nadir-to-peak-to-nadir; 3.6 ± 0.2 vs. 4.5 ± 0.2 days, transition to anoestrus vs. anoestrum, respectively) may play a role in resumption of the rhythmic wave-like pattern of follicle emergence in anoestrum. During anoestrum, a dose-dependent suppression of FSH secretion by oestradiol was found in ewes (Joseph et al., 1992). It has also been shown that inhibin, a potent inhibitor of FSH production and secreted by large ovarian follicles in response to FSH (Findlay et al., 1986; Mann et al., 1989), is significantly less responsive to FSH in anoestrous animals (Campbell et al., 1991). Relatively low concentrations of oestradiol (Yuthasastrakosol et al., 1975; Scaramuzzi and Baird, 1977; Bartleewski et al., 1998) combined with reduced production of inhibin during anoestrum in the ewe may result in
prolonged increases in serum concentrations of FSH, which in turn may compensate for the lower LH support and maintain an orderly emergence of follicular waves.

Earlier post-mortem studies, using animals sacrificed during deep anoestrus, revealed that the number of small and medium-sized follicles was greater in anoestrous ewes than in ewes during the luteal phase of the oestrous cycle (Brand and de Jong, 1973). The mean number of large ovarian follicles (growing to ≥ 5 mm in diameter) per wave appeared to decrease from the mid-breeding season (1.6 ± 0.1) to the transition to anoestrus (1.3 ± 0.2, present study) and to later stages of anoestrus (1.2 ± 0.1; Bartlewski et al., 1998). Daily numbers of antral ovarian follicles in the 3- to 4-mm size-class increased from early (March-April) to mid-anoestrus (May) in Western white-faced ewes (Bartlewski et al., 1998). It appears that daily numbers of follicles growing to a maximum diameter of 3, 4 or ≥ 5 mm before regression are not immediately influenced by the onset of seasonal anoestrus. The rise in numbers of small and medium antral follicles, as anoestrus advances, may reflect a change in ovarian follicular responsiveness to gonadotrophins (Bartlewski et al., 1998).

In the present Western white-faced ewes, the duration of follicle lifespan was seen to decline after luteolysis (Table 8.1). The shorter lifespan of follicular waves that emerged after luteal regression was due to the shorter regressing and initially a shorter static phase. Previous studies in cyclic Western white-faced ewes showed that the static phase of large follicles early in the cycle was longer relative to subsequent waves, and the regressing phase of the first follicular wave was longer in comparison to the last non-ovulatory wave (emerging during the mid-luteal phase of the cycle). Johnson et al. (1996) demonstrated that lower than normal luteal-phase levels of progesterone in ewes stimulated prolonged follicular lifespan, particularly the static phase ("persistent" ovarian follicles). These observations suggest that large antral follicles developing under strong progesterone dominance (mid-luteal phase) have a shorter static/regressing phase compared to follicles growing at submaximal progesterone concentrations (e.g., during CL formation and regression), probably due to greater LH pulse frequency when progesterone levels are low (Campbell et al., 1995). As ewes become anoestrous, however, progesterone secretion is
lower than that observed during the mid-breeding season, but LH pulse frequency is 
suppressed because of an enhanced negative feedback effect of oestradiol on LH secretion, 
removing LH support of follicle growth. This and decreased follicular sensitivity to 
gonadotrophins may explain the shorter follicle lifespan seen in the early anoestrous ewes 
in the present study, and in ewes across anoestrus (7.7 ± 0.9 days; Bartlewski et al., 1998).

In cyclic ewes, peripheral concentrations of oestradiol are highest around the time 
when the largest follicles of waves attain their maximum size, and then oestradiol levels 
begin to decline, whereas the follicles often remain at a maximum diameter for up to 2 more 
days. These observations taken with the reduced oestrogenicity of follicles during the 
transition into anoestrus led us to speculate that oestrogens are not critical for the 
maintenance of large antral follicles during the static phase and/or that follicles in their static 
phase may start a transition into regression even though their size does not change. Recent 
observations in heifers (Bo, 1995) showed that oestradiol injected into the ovarian stroma 
in the close proximity of a dominant follicle did not have any direct effects on its 
development.

The time during which CL were not identifiable with ultrasonography did not appear 
to vary with the stage of the breeding season in ewes (3.4 ± 0.3 vs. 2.8 ± 0.7 days after 
ovulation, mid-breeding season vs. transition into anoestrus; present study). The mean day 
on which identified CL and luteinized follicles regressed did not differ between the mid-
breeding season and the transition into anoestrus (16.1 ± 0.5 and 16.3 ± 0.6 days after 
ovulation, respectively), indicating the maintenance of a normal luteolytic mechanism. 
However, the mean lifespan of all luteal structures in Western white-faced ewes differed 
between the middle and terminal portion (present study) of the breeding season (11.7 ± 0.3 
and 13.1 ± 0.5 days, respectively), probably reflecting the difference in the proportion of 
animals (3/10 vs. 1/6 ewes, mid-breeding season vs. transition into anoestrus, respectively) 
with luteinized uniovulated follicles, which had a shorter lifespan (≤ 10 days) than normal 
CL.

The mean maximum total luteal volume (1580 ± 105 mm³ on day 9 of the oestrous 
cycle; day 0 = day of ovulation), recorded during the mid-breeding season (November-
December) in Western white-faced ewes, was higher than that seen in the present ewes (1273 ± 184 mm\(^3\) on day 11 after ovulation), and the mean ovulation rate was lower in the transitional ewes (average ovulation rate in the mid-breeding season was 1.8 ± 0.2). The maximum size of individual CL/luteal structures was lower during the mid-breeding season than during the transition into anoestrus in white-faced ewes (842 ± 66 mm\(^3\) and 1101± 98 mm\(^3\), respectively). The size of CL in sheep decreases as the ovulation rate increases. In Romney ewes with a single ovulation, most CL weighed > 600 mg, but with an increase in the ovulation rate, after FSH pre-treatment, the proportion of corpora lutea weighing < 400 mg rose steadily (Henderson et al., 1988). It is possible that CL exert local inhibitory effects on the growth of co-existing CL as the lower ovulation rate towards the end of the breeding season resulted in ostensibly larger sizes of CL. As the largest diameter attained by the ovulatory follicles in white-faced ewes apparently did not vary between the mid-breeding season and the transition into anoestrus (6.4 ± 0.4 and 6.3 ± 0.3 mm, respectively), this rules out the possibility that the difference in CL size, during the two periods in question, was due to differences in the size of follicles destined to ovulate. Such a mechanism was suggested for prolific and non-prolific breeds of ewes; a smaller ovulatory follicle size has consistently been reported for prolific sheep (Webb and Gauld, 1985; Driancourt et al., 1986a; Castonguay et al., 1990), and the mean volume of luteal structures was found to be significantly higher in non-prolific (Western white-faced) than in prolific (Finn) ewes.

8.6 Conclusions

During the transition from the breeding season into anoestrus in ewes, the endogenous rhythm of FSH release fails to maintain an orderly emergence of follicular waves (follicles growing from 3 to ≥ 5 mm). The cessation of ovulatory cycles in Western white-faced ewes is associated with significantly reduced secretion of oestradiol by ovarian follicles and lower serum concentrations of progesterone, compared to ewes in the mid-breeding season. Decreased concentrations of progesterone may reflect the lower ovulation rate and smaller total volumes of luteal tissue, but very low concentrations of oestradiol do
not result from the absence of large antral follicles. All of the above alterations in the normal cyclic pattern of ovarian function are likely due to diminished ovarian responsiveness to gonadotrophic stimuli and/or the onset of photoperiod-cued suppression of LH release.
Chapter 9. OVARIAN FUNCTION IN EWES AT THE ONSET OF THE BREEDING SEASON

9.1 Abstract

Transrectal ultrasonography of ovaries was performed each day, during the expected transition from anoestrus to the breeding season (mid-August to early October), in 6 Western white-faced cross-bred ewes, to record ovarian antral follicles ≥ 3 mm in size and luteal structures. Jugular blood samples were collected daily for radioimmunoassay of FSH, oestradiol and progesterone. The first ovulation of the breeding season was followed by the full-length oestrous cycle in all ewes studied. Prior to the ovulation, all ewes exhibited a distinct increase in circulating concentrations of progesterone, yet no CL were detected and luteinized uniovulated follicles were seen in only 3 ewes. FSH secretion was not affected by the cessation of anoestrus and peaks of episodic FSH fluctuations were associated with the emergence of ovarian follicular waves (follicles growing from 3 to ≥ 5 mm). During the 17 days prior to the first ovulation of the breeding season, there were no apparent changes in the pattern of emergence of follicular waves. Mean daily numbers of small antral follicles (not growing beyond 3 mm in diameter) declined (P < 0.05) after the first ovulation. The ovulation rate, maximal total and mean luteal volumes and maximal serum progesterone concentrations, but not mean diameters of ovulatory follicles, were ostensibly lower during the first oestrous cycle of the breeding season compared with the mid-breeding season of Western white-faced ewes. Oestradiol secretion by ovarian follicles appeared to be fully restored, compared to anoestrous ewes, but it was not synchronized with the growth of the largest antral follicles of waves until after the beginning of the first oestrous cycle. An increase in progesterone secretion preceding the first ovulation of the breeding season does not result, as previously suggested, from the ovulation of immature ovarian follicles and
short-lived CL, but progesterone may be produced by luteinized unovulated follicles and/or interstitial tissue of unknown origin. This increase in serum concentrations of progesterone does not alter the pattern of follicular wave development, hence it seems to be important mainly for inducing oestrous behaviour, synchronizing it with the preovulatory surge of LH, and preventing premature luteolysis during the ensuing luteal phase. Progesterone may also enhance ovarian follicular responsiveness to circulating gonadotrophins through a local mechanism.

9.2 Introduction

Towards the end of seasonal anoestrus in ewes, the activity of inhibitory mechanisms holding LH pulse frequency and amplitude in check declines, and restored LH secretion stimulates oestradiol production by ovarian follicles, which in turn triggers a first preovulatory LH surge (Karsch et al., 1980). It has been suggested that when the first LH surge is induced, the largest follicles are not always mature enough to respond to the LH discharge by developing into healthy CL (Legan et al., 1985; Hunter, 1991). During the transition to the breeding season in ewes, short luteal phase patterns of progesterone secretion have been observed that may result from ovulation of premature ovarian antral follicles (Legan et al., 1985). An increase in plasma progesterone concentrations was also seen approximately 4 days prior to the first ovulation of the breeding season (Walton et al., 1977), suggesting the possibility of luteinization of anovulatory follicles. However, with ultrasonography no luteal structures were detected in ewes during the 15 days prior to the first ovulation of the breeding season (Ravindra and Rawlings, 1997). Brief increases in progesterone concentrations during the transition to the breeding season in ewes may play a role in synchronizing the anoestrous pattern of antral follicle turnover with an ensuing LH surge (Legan et al., 1985). After administration of luteal phase levels of progesterone for 2 days, followed by induction of an LH surge, full-length luteal phases were observed in 100% of experimental anoestrous ewes (Legan et al., 1985).
Secretion of FSH is less affected by annual changes in daylength than LH release (Karsch et al., 1979). There is evidence of periodic increases in circulating concentrations of FSH occurring at 4- to 5-day intervals throughout the year in the ewe (Bister and Paquay, 1983). These fluctuations in serum FSH concentrations are temporarily associated with emergence of ovulatory-sized antral follicles, both during (Ginther et al., 1995) and outside of the breeding season (Bartlewski et al., 1998).

As the regular emergence of follicular waves (follicles growing from 3 to ≥ 5 mm in diameter) continues throughout anoestrus in ewes (Bartlewski et al., 1998), it is unlikely that when the first LH surge of the ovulatory season occurs, there are mainly small and immature (Legan et al., 1985; Ravindra and Rawlings, 1997) ovarian antral follicles. However, large antral follicles at various stages of their development (i.e., growing, static or regressing phase) may respond differently to an LH stimulus (Rubianes et al., 1997), and during the transition from anoestrus to the breeding season there may be an array of luteal structures including normal CL, luteinized unovulated follicles and short-lifespan CL. The aim of the present study was to use transrectal ovarian ultrasonography and hormone measurements to identify the luteal structures and the source of progesterone associated with the transition to the breeding season in ewes, and to see if transition had any effect on the wave pattern of antral follicle growth and the associations of such waves with circulating levels of FSH and oestradiol.

9.3 Materials and methods

Experimental procedures and data analyses employed in this study are similar to those described for the Western white-faced ewes in the transition from the breeding season into anoestrus. In the following paragraphs, experimental procedures and statistical analyses unique to the present experiment will be detailed.

The 12 Western white-faced, nulliparous and clinically healthy ewes used in the present experiment were approximately 5 years old and weighed between 77 and 95 kg. Daily transrectal ultrasonography of ovaries commenced in 12 ewes on 15 August and it
continued until the end of the first oestrous cycle of the breeding season was confirmed in
the 6 ewes. Days of ovulation were determined by the collapse of large antral follicles that
had been identified and followed for several days with ultrasonography.

9.3.1 Hormone assays

All samples for FSH were analysed in a single assay; intraassay coefficients of
variation (CVs) for ovine reference sera with mean hormone concentrations of 2.46 and 3.99
ng/ml were 14.8 and 6.9%, respectively. For oestradiol reference sera with mean
concentrations of 6.5 or 13.7 pg/ml, the intra- and interassay CVs were 6.8 and 12.6% or 2.1
and 6.9%, respectively. For progesterone reference sera with mean concentrations of 0.50
or 1.60 ng/ml, the intra- and interassay CVs were 9.4 and 15.4% or 7.4 and 9.4%,
respectively. FSH and oestradiol data for individual ewes were normalized to the peak
nearest to the day of the first ovulation of the breeding season in order to obtain
characteristics of successive fluctuations in hormone secretion for the entire period of study.

9.3.2 Data analysis

Daily numbers of 3-mm follicles that did not grow any larger, and of 3-mm follicles
that subsequently attained 4 or ≥ 5 mm in diameter, were aligned to the day of the first
detected ovulation of the breeding season (day 0), for the period from days −16 to 15 (the
entire growth phase was not seen for the 3-mm follicles emerging during the last 2 days of
the scanning period). Follicular and hormonal data were analysed for associations between
various stages of follicular wave development (follicles growing from 3 to ≥ 5 mm) and
peaks in serum concentrations of FSH and oestradiol. The mean number of identified
follicular waves and the mean number of FSH or oestradiol peaks per ewe, per scanning
period, were compared using a paired t-test. Spearman correlations were done between the
lengths of interpeak intervals for detected FSH and oestradiol fluctuations, and intervals
between adjacent days of follicular wave emergence (interwave intervals) as well as
different phases of the lifespan of the largest ovarian follicles of waves, namely the day of
emergence, and the beginning and end of the static phase. As the regressing phase was not
recorded for the waves containing ovulatory follicles, the correlation between interpeak
intervals above and intervals between ends of follicle regression phases was not analysed.

Preliminary inspection of the data revealed that the number of fluctuations in serum
concentrations of oestradiol appeared to differ from the number of emerging follicular
waves during the period preceding the first ovulation, but not during the first interovulatory
interval of the breeding season. Therefore, additional analyses were done for oestradiol
peaks and follicular waves, in which the data from only the first interovulatory interval were
used. During the first interovulatory interval, the serial oestradiol fluctuations appeared to
begin in the proximity of the days of follicle wave emergence. Therefore, the lengths of
intervals between sequential days of onset of oestradiol fluctuations were correlated with
the lengths of intervals between different points on the largest follicle growth curves, as
described for the oestradiol peaks above. The distribution of peaks of FSH fluctuations and
the onset of oestradiol fluctuations in relation to the day of wave emergence ± 2 days were
analysed by $\chi^2$-test. A similar analysis was done for oestradiol peaks and days on which the
largest follicles of waves reached their maximum sizes.

Daily total luteal volumes were normalized to the day of the first ovulation of the
breeding season (day 0), for the period during which luteal structures were observed with
ultrasonography in all ewes (days 4 to 16), and were analysed by RM-ANOVA (MINITAB®,
1992). Daily serum progesterone concentrations were aligned to the day of ovulation and
analysed for the period from days −16 to 17. To study the relationship between daily luteal
volumes and serum progesterone concentrations during the first interovulatory interval of
the breeding season, the luteal phase was divided into 4 periods. Period 1, or the phase of
luteal tissue formation, was defined as the time after ovulation during which CL/luteal
structures were not detectable with ultrasonography (day 0 to 3). Period 3, or the mid-luteal
phase, was regarded as the period encompassing all the days on which daily mean serum
concentrations of progesterone did not differ ($P > 0.05$) from the maximal concentration of
the hormone seen on day 11 after ovulation (days 7 to 13). The time between the end of
luteal formation and the beginning of the mid-luteal phase was period 2, or the phase of luteal growth (days 4 to 6). Period 4, or luteal regression, was the time from the end of the mid-luteal phase until complete demise of luteal structures, determined by ultrasonography (days 14 to 16). The relationship between daily total luteal volumes and circulating concentrations of progesterone was studied by simple linear regression. The independent variable was total luteal volume and the dependent variable was progesterone concentration.

Prior to the first oestrous cycle of the breeding season, luteinized unovulated follicles were identified in 3 out of the 6 ewes studied. Additional analyses were done to compare various endocrine and ovarian variables between ewes with or without luteinized follicles detected before the first ovulation. Multivariate analysis of variance (RM-ANOVA; MINITAB®, 1992) was used to determine the main effect of group (the presence or absence of luteinized follicles), day and the day-by-group interaction for daily numbers of follicles and hormone concentrations aligned to the day of ovulation (day 0). The statistical model included the following terms: ewes, ewes within group, time (day), group x day interaction, and residual error. The characteristics of sequential follicular waves recorded during the period of study were compared between the 2 groups of ewes by a Student t-test (MINITAB®, 1992). Proportions are expressed as percentage values and all other results are presented as mean ± SEM.

9.4 Results
9.4.1 Detection of oestrus and ovulations

Two out of the 6 ewes studied were moderately marked by rams at days −18 and −17 before the first ovulation of the breeding season, respectively. None of the ewes showed a decline in vaginal mucous impedance below 40 ohms at that time. In 1 of these 2 ewes, a luteinized follicle was detected 3 days after the ewe was marked by rams. The first ovulation of the breeding season occurred between 10 and 25 September (i.e., 26 to 41 days after ultrasonographic examination began), and it was followed by the full-length oestrous cycle in all ewes studied. All ewes were well marked by rams and exhibited a decline in
vaginal impedance to < 40 ohms on the day of or 1 day before ovulation (means for days
-1, 0 and 1: 33.2 ± 2.9, 46.7 ± 4.5 and 48.2 ± 2.5 ohms, respectively). At the second
observed ovulation of the breeding season, all ewes were well marked by rams. Vaginal
impedance fell below 40 ohms in all ewes on the day they were marked by rams (mean: 30.8
± 1.8 ohms) and it increased to 47.8 ± 3.5 ohms on the day of ovulation. The mean duration
of the first interovulatory interval of the breeding season was 17.5 ± 0.4 days (range: 16 to
19 days).

9.4.2 Ovarian follicle populations

Individual follicle growth curves are shown for 4 individual ewes, with corresponding serum FSH and oestradiol concentrations, in Fig. 9.1. Analysis of variance was significant (P = 0.002) for mean daily numbers of 3-mm follicles not growing beyond
3 mm in diameter during the period from days -16 to 15 (day 0 = day of first ovulation of
the breeding season); the number of these follicles was lower (P < 0.05) on days 1, 6 to 9
and 12 to 13 than on days -16, -12 to -9 and -6 to -1 (Fig. 9.2). Daily numbers of 3-mm follicles growing to a maximum size of 4 or ≥ 5 mm in diameter did not vary with time (P
> 0.05). However, follicles that reached ≥ 5 mm in diameter emerged from the pool of 3-
mm follicles only on days -16 to -14, -12 to -9, -5 to -3, -1 to 3, 5 to 7, 9 to 12 and 14
to 15 (Fig. 9.2). Daily numbers of 3-mm follicles in the 3 categories above did not differ (P
> 0.05) between ewes with or without luteinized follicles detected before the first ovulation
of the breeding season.

9.4.3 Characteristics of follicular waves

There were three ewes with 7 and three ewes with 6 emerging follicular waves
(follicles growing from 3 to ≥ 5 mm in diameter) over the period of study (mean: 6.5 ± 0.2).
During the 17-day period before the first ovulation of the breeding season, one ewe had 2,
four ewes had 3, and one ewe had 4 waves (mean: 3.0 ± 0.2). During the first oestrous cycle,
**Fig. 9.1.** The diameter profiles of individual antral follicles growing to \( \geq 5 \) mm in size, recorded during the 16 days prior to the first ovulation and throughout the first interovulatory interval of the breeding season, in 4 Western-white faced ewes (a-d), with accompanying daily serum concentrations of FSH (middle panels; ■) and oestradiol (bottom panels; □). The arrows along the X-axis (top panels; †) denote the days of follicle wave emergence. The arrows in the upper chart area indicate the number and diameter of ovulatory follicles on the day before ovulation. L denotes a large antral follicles that became luteinized and gradually transformed into a solid luteal structure. Asterisks denote the peak values of fluctuations (nadir-to-peak-to-nadir) in circulating concentrations of FSH and oestradiol (the 2 dashed lines encompass a fluctuation), determined by the cycle-detection computer program (Clifton and Steiner, 1983).
Days from first ovulation of the breeding season
Fig. 9.2. Mean (± SEM) daily numbers of 3-mm follicles not growing beyond 3 mm before regression (dashed line), and of 3-mm follicles that subsequently grew up to ≥ 5 mm in diameter (solid line), in six Western white-faced ewes that underwent daily transrectal ultrasonography of ovaries during the transition from anoestrus to the breeding season (August-October). Follicular data were normalized to the day of first ovulation of the season (day 0) and analysed for the period from day –16 to day 15. See text for statistical details.
Days from first ovulation of the breeding season

Numbers of follicles
three ewes had 3 and three ewes had 4 follicular waves (mean: 3.5 ± 0.2). The characteristics of successive waves are summarized in Table 9.1. In these analyses, data for ewes with 6 or 7 waves per scanning period were combined. The mean number of follicles per wave (1.6 ± 0.1), the maximum diameter (6.2 ± 0.2 mm), and the duration of the static (1.7 ± 0.2 days) and regressing (3.4 ± 0.2 days) phases of the largest ovarian follicles as well as the mean interwave interval (4.9 ± 0.3 days) did not differ (P > 0.05) among sequential follicular waves. The follicular growing phase was longer (P < 0.05) for the last 2 waves (Waves 6 and 7) than for the first 2 waves (Waves 1 and 2) detected during the observation period and for the second wave of the interovulatory interval (Wave 5). The growth rate of the largest follicles of waves was higher (P < 0.05) for the first 2 waves (Waves 1 and 2) than the last 2 waves (Waves 6 and 7) recorded during the study period. The lifespan of the largest follicles of waves that contained mainly ovulatory follicles (Waves 3 and 7) was shorter (P < 0.05) relative to the remaining waves, and the lifespan was longest (P < 0.05) for the first wave emerging after ovulation (Wave 4). None of the characteristics of follicular waves varied among ewes with or without unovulated luteinized follicles recorded before the first ovulation of the breeding season. At the first ovulation, all ovulatory follicles emerged in the final follicular wave preceding ovulation, and at the second observed ovulation, one ewe had an ovulatory follicle that originated from the penultimate wave of the oestrous cycle (first detected at 3 mm at 9 days before ovulation) and ovulated along with a follicle that started to grow in the last wave of the cycle (6 days before ovulation; Fig. 9.1b). Ovulatory follicles emerged, or started to grow from 3 mm onwards, an average of 4.4 ± 0.4 days before the first ovulation, and 5.9 ± 0.5 days before the second observed ovulation (P < 0.05). The percentage of antral follicles attaining ≥ 5 mm in diameter that ovulated in the final follicular wave was 90 and 89%, for the first and the second ovulation of the breeding season, respectively (P > 0.05).

9.4.4 FSH concentrations

Using the cycle-detection computer program, peaks of serum FSH concentrations were identified in all ewes under study. The mean number of identified FSH peaks per ewe,
<table>
<thead>
<tr>
<th>End point</th>
<th>Wave 1</th>
<th>Wave 2</th>
<th>Wave 3</th>
<th>Wave 4</th>
<th>Wave 5</th>
<th>Wave 6</th>
<th>Wave 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean day of wave emergence</td>
<td>-15.0 ± 0.4</td>
<td>-10.3 ± 0.5</td>
<td>-4.7 ± 0.4</td>
<td>0.5 ± 0.3</td>
<td>6.0 ± 0.4</td>
<td>9.8 ± 0.3</td>
<td>13.7 ± 0.9</td>
</tr>
<tr>
<td>Range</td>
<td>-16 to -14</td>
<td>-11 to -8</td>
<td>-6 to -3</td>
<td>0 to 2</td>
<td>5 to 7</td>
<td>9 to 11</td>
<td>12 to 15</td>
</tr>
<tr>
<td>No. of follicles/wave</td>
<td>1.3 ± 0.3</td>
<td>1.6 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>2.3 ± 0.4</td>
<td>1.3 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td><strong>Largest follicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>5.7 ± 0.5</td>
<td>5.8 ± 0.4</td>
<td>6.5 ± 0.2</td>
<td>6.7 ± 0.2</td>
<td>5.7 ± 0.3</td>
<td>6.0 ± 0.3</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>1.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.1 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.3 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.8 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Growing phase (days)</td>
<td>2.2 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.7 ± 0.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.7 ± 0.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.5 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.2 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Static phase (days)</td>
<td>1.5 ± 0.5</td>
<td>1.8 ± 0.5</td>
<td>1.2 ± 0.2</td>
<td>2.5 ± 0.8</td>
<td>2.3 ± 0.7</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Regressing phase (days)</td>
<td>2.7 ± 0.7</td>
<td>3.5 ± 0.6</td>
<td>ND</td>
<td>4.0 ± 0.4</td>
<td>3.5 ± 0.6</td>
<td>3.5 ± 1.5</td>
<td>ND</td>
</tr>
<tr>
<td>Lifespan (days)</td>
<td>6.3 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.2 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.8 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.6 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.8 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Interwave interval&lt;sup&gt;1&lt;/sup&gt; (days)</td>
<td>5.7 ± 0.6</td>
<td>5.0 ± 0.8</td>
<td>5.1 ± 0.5</td>
<td>5.4 ± 0.3</td>
<td>4.0 ± 0.4</td>
<td>4.0 ± 0.6</td>
<td>4.0 ± 0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Within rows, means with different letter superscripts are different (P < 0.05).

<sup>1</sup> Interwave interval refers to the number of days between emergence of the wave in that column and emergence of the next sequential wave.

ND-not determined.
per scanning period was $7.0 \pm 0.5$ (range: 7 to 9). The mean amplitude ($3.11 \pm 0.06$ ng/ml) and duration ($4.0 \pm 0.1$ days) of sequential fluctuations as well as mean interpeak interval for serum FSH concentrations ($4.6 \pm 0.3$ days) did not vary ($P > 0.05$) throughout the period of study (Table 9.2) nor between ewes with or without luteinized follicles detected before the first oestrous cycle of the breeding season. The main effect of group ($P = 0.23$), day ($P = 0.20$) and the interaction ($P = 0.68$) were not significant for daily serum concentrations of FSH normalized to the day of first ovulation of the breeding season, for the 2 groups of ewes in question.

### 9.4.5 Oestradiol concentrations

Peaks of serum concentrations of oestradiol were identified in all ewes studied (range: 5 to 9 peaks/ewe). The mean peak values of oestradiol fluctuations immediately preceding the first ovulation of the breeding season was higher ($P < 0.05$) than for earlier peaks of serum oestradiol concentrations. The mean peak concentration of oestradiol just before the first ovulation ($7.6 \pm 0.4$ pg/ml) was higher ($P < 0.05$) than before the second observed ovulation ($5.6 \pm 0.5$ pg/ml). The duration of oestradiol fluctuations decreased transiently before the first preovulatory elevation in serum oestradiol concentrations, and subsequently during the mid- and late luteal phase of the oestrous cycle ($P < 0.05$; Table 9.2). Preovulatory peaks of oestradiol concentrations occurred an average of $2.3 \pm 0.8$ and $0.7 \pm 0.2$ days before the first and second ovulations, respectively ($P < 0.05$). The amplitude, duration and periodicity (interpeak intervals) of serum oestradiol fluctuations did not differ ($P > 0.05$) among ewes with or without luteinized follicles seen before the first ovulation of the breeding season, but ewes with luteinized follicles tended to have more oestradiol peaks during that period ($4.0 \pm 0.6$ vs. $2.3 \pm 0.3$, luteinized follicles vs. no luteinized follicles, $P = 0.07$). The main effect of group ($P = 0.23$), day ($P = 0.65$) and the interaction ($P = 0.95$) were not significant for daily serum concentrations of oestradiol aligned to the day of first ovulation, for the 2 groups of ewes above.
Table 9.2. Characteristics of sequential fluctuations in serum follicle-stimulating hormone (FSH) and oestradiol (E₂) concentrations in blood samples collected daily during the 16 days prior to the first ovulation and throughout the first interovulatory interval of the breeding season in 6 Western white-faced ewes. Data were centralized to the peak nearest to the first ovulation of the breeding season confirmed with ultrasonography. All values are mean ± SEM.

<table>
<thead>
<tr>
<th>End point</th>
<th>Successive FSH/E₂ fluctuations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peak concentration</strong></td>
<td></td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>3.16 ± 0.44 2.99 ± 0.29 3.01 ± 0.39 3.43 ± 0.80 3.05 ± 0.41 3.16 ± 0.33 2.97 ± 0.98</td>
</tr>
<tr>
<td>E₂ (pg/ml)</td>
<td>- 4.9 ± 0.5a 4.8 ± 0.4a 7.6 ± 0.5b 6.3 ± 0.7ab 6.2 ± 0.7ab 5.8 ± 0.5ab</td>
</tr>
<tr>
<td><strong>Duration of fluctuation</strong></td>
<td></td>
</tr>
<tr>
<td>(days)</td>
<td>4.3 ± 0.8 4.3 ± 0.6 3.8 ± 0.5 4.0 ± 0.6 3.7 ± 0.3 3.6 ± 0.7 4.3 ± 0.3</td>
</tr>
<tr>
<td>-</td>
<td>- 5.2 ± 1.0a 2.5 ± 0.2b 4.5 ± 0.7a 4.2 ± 0.7ab 3.2 ± 0.4b 3.0 ± 0.3b</td>
</tr>
<tr>
<td><strong>Interpeak interval (days)</strong></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>4.8 ± 0.5 3.8 ± 0.6 5.5 ± 0.8 3.7 ± 0.4 4.7 ± 0.6 5.4 ± 0.5 -</td>
</tr>
<tr>
<td>-</td>
<td>- 4.2 ± 0.6 5.2 ± 1.5 6.8 ± 0.5 3.5 ± 0.4 5.2 ± 0.9 -</td>
</tr>
</tbody>
</table>

*ab* Within rows, means with different letter superscripts are different (P < 0.05).
9.4.6 Relationship between follicle wave development and serum concentrations of FSH and oestradiol

The number of emerging follicular waves and the number of identified FSH peaks per ewe, per scanning period, did not differ (6.8 ± 0.4 vs. 6.5 ± 0.2, FSH peaks vs. days of wave emergence; P > 0.05). The lengths of interpeak intervals for serum FSH concentrations were positively correlated with the lengths of interwave intervals (r = 0.50, P = 0.004), intervals between emergence of the largest follicles of waves (r = 0.49, P = 0.006), and intervals between ends of follicle static phases (r = 0.45, P = 0.03), but not with intervals between the beginning (r = 0.29, P = 0.13) of follicular static phases. When the analysis was done for the first interovulatory interval of the breeding season, the interpeak intervals were only correlated with interwave intervals (r = 0.54, P = 0.01) and the intervals between emergence of the largest follicles of waves (r = 0.47, P = 0.01), but not with intervals between the beginning (r = 0.25, P = 0.36) and end (r = −0.05, P = 0.80) of the follicle static phase. Most peaks of serum FSH concentrations occurred from days −1 to +1 relative to the day of wave emergence (83.3%; P < 0.05; Fig. 9.3a).

The number of oestradiol fluctuations was greater than the number of emerging follicular waves (7.2 ± 0.3 vs. 6.5 ± 0.2, oestradiol fluctuations vs. days of wave emergence, P < 0.05). There was no significant correlation between interpeak intervals for serum concentrations of oestradiol and different stages of follicle wave lifespan for the duration of the study. During the first oestrous cycle of the breeding season, the intervals between onset of sequential oestradiol fluctuations were positively correlated with interwave intervals (r = 0.52, P = 0.03), but not with intervals between days of emergence (r = 0.42, P = 0.16) or beginning (r = 0.29, P = 0.31) and end (r = 0.19, P = 0.57) of the static phase of the largest follicles of waves. The percentage of oestradiol fluctuations that began on the day of and each of 2 days after the wave emergence was 88.9% (P < 0.05; Fig. 9.3b).

The interpeak intervals for serum oestradiol fluctuations were not correlated with different phases of follicle wave development over the study period. However, during the first oestrous cycle of the breeding season, there was a positive correlation between
interpeak intervals and intervals between beginning \( r = 0.51, P = 0.04 \) and end \( r = 0.60, P = 0.01 \) of follicle static phases, but not interwave intervals \( r = -0.05, P = 0.85 \) or intervals between days of emergence of the largest follicles of waves \( r = -0.11, P = 0.85 \). An analysis of the distribution of the oestradiol peaks in relation to the beginning or end of follicle static phases revealed that all peaks clustered around the beginning of the static phase (day 0); 60.0\% of all peaks were detected from days -2 to -1 and 40.0\% from days 0 to +1 (Fig. 9.3c).

### 9.4.7 Ovulation rates and characteristics of luteal structures

Graphs showing daily total luteal volumes and serum progesterone concentrations in 4 individual ewes are given in Fig. 9.4. Before the first ovulation of the breeding season, luteinized unovulated follicles were seen in 3 ewes, but in the 3 other ewes no luteal structure was detected. The luteinized follicles were observed from days -15 to -9 and from days -6 to 0 (day 0 = day of ovulation) in a ewe with a luteinized follicle detected in each ovary, from days -16 to -14 and from days -10 to -2 in the other ewe with 2 luteinized follicles detected in the same ovary, and from days -11 to -3 in a single ewe that had 1 luteinized follicle. The mean lifespan of luteinized follicles identified before the first ovulation was 7.0 ± 1.1 days.

The mean ovulation rate at the first ovulation of the season was 1.3 ± 0.2. The mean diameter of ovulatory follicles on the day before ovulation was 6.2 ± 0.2 mm. In all ewes, CL were detected by day 6 after ovulation (average of 4.2 ± 0.5 days). A luteinized follicle was detected in 2 ewes during the first interovulatory interval; in both ewes, a luteinized follicle was positioned in the ovary contralateral to the ovary containing a CL. The mean number of all luteal structures per ewe was 1.5 ± 0.2. The mean lifespan of CL and luteinized follicles was 11.6 ± 1.1 days. One ewe had a short-lived CL observed for only 4 consecutive days (days 5 to 8 after ovulation), which attained a maximum size of 10 mm in diameter 1 day prior to regression. This ewe also had a luteinized follicle (observed from days 6 to 17 of the cycle).
Fig. 9.3. (a) Relative frequency of the occurrence of identified FSH peaks and (b) distribution of days of the beginning of oestradiol fluctuations within 2 days before and after the day of follicular wave emergence; (c) the percentage of peaks in daily serum oestradiol concentrations, expressed for the 2 days before and after the end of the growing phase of the largest follicle of a wave. The graph (a) was compiled from follicular and FSH data obtained in 6 Western white-faced ewes for the period of the 16 days prior to the first ovulation and throughout the first interovulatory interval of the breeding season, and the graphs (b) and (c) from the data obtained only during the first interovulatory interval of the season.
Fig. 9.4. Daily total luteal volumes (▲, △) and serum concentrations of progesterone (—), determined in 4 Western white-faced ewes (a-d) that underwent daily ovarian ultrasonography during the transition from anoestrus to the breeding season (August-October). The luteal volumes denoted by (▲) show the volumes of unovulated luteinized follicles detected in 3 ewes before the first ovulation of the breeding season, and (△) represent the volume of corpora lutea and/or luteinized antral follicles identified during the first oestrous cycle of the season. The data presented in these graphs are for the same 4 ewes as the follicular profiles, and serum concentrations of FSH and oestradiol given in Fig. 9.1.
Central cavities of 2 to 5 mm in diameter were seen in developing CL, in all Western white-faced ewes studied, for an average of 4 days (between days 3 and 10) after the first ovulation. During luteolysis, the central luteal cavity re-appeared in only 1 ewe, 1 day before complete luteal regression (day 16). The maximum diameter attained by CL during the mid-luteal phase ranged from 10 to 12 mm. In 2 ewes with ovaries containing luteinized follicles during the first luteal phase of the breeding season, central cavities within these structures persisted until days 11 and 6 after ovulation, respectively. The follicles that luteinized in these ewes began to grow from 3 mm in diameter on days 1 and 2 of the cycle, attained maximum diameters of 7 and 6 mm on days 5 and 4, and were first identified as luteinized structures 1 day later. After the onset of luteinization of their walls, both these follicles reached a maximum size of 11 mm in diameter, and their central cavities filled in on days 12 and 7, respectively. At the second observed ovulation of the breeding season, the average ovulation rate was 1.5 ± 0.2 and the mean diameter of pre-ovulatory follicles was 6.2 ± 0.3 mm. The mean ovulation rate and size of follicles destined to ovulate did not differ between the first and the second observed ovulation (P > 0.05).

Daily total luteal volumes rose from 358 ± 61 mm³ on day 4 to reach a maximum value of 903 ± 113 mm³ on day 10 after ovulation (P < 0.05; Fig. 9.5). From days 6 to 13, daily total volumes of luteal tissue did not differ (P > 0.05). Total luteal volumes subsequently declined to 571 ± 73 mm³ (P < 0.05) on day 14, but they did not vary (P > 0.05) for the remainder of the luteal phase in Western white-faced ewes studied (days 14 to 16 after ovulation).

9.4.8 Serum concentrations of progesterone

A distinct increase in serum concentrations of progesterone (maximum concentrations were from 1.47 to 2.88 ng/ml) occurred in all ewes before the first ovulation of the breeding season (day 0; Fig. 9.5). Mean serum progesterone concentrations rose (P < 0.05) from day −16 to reach a peak of 1.74 ± 0.28 ng/ml on day −7 before ovulation. Serum progesterone concentrations subsequently decreased (P < 0.05) to 0.28 ± 0.19 ng/ml
Fig. 9.5. Mean daily serum progesterone concentrations (−) and total volumes of luteal tissue (△) in 6 Western white-faced ewes studied during the transition into the breeding season (August-October). Data were normalized to the day of first ovulation of the breeding season (day 0). See text for statistical descriptions.
Days from first ovulation of the breeding season
on day 0; there was no difference (P > 0.05) from day -11 to -1 before ovulation. During the first interovulatory interval of the breeding season, mean serum progesterone concentrations did not vary from day 0 to day 4 after ovulation (P > 0.05), increased from day 4 to 5 (P > 0.05) and then to a maximum of 2.82 ± 0.25 ng/ml on day 11, declined to 1.92 ± 0.28 ng/ml (P < 0.05) on day 14, then to 0.62 ± 0.28 ng/ml (P < 0.05) on day 15, and finally to 0.10 ± 0.04 ng/ml on day 16. There was no significant difference between mean serum progesterone concentrations determined on days 7 to 8 and from days 10 to 13 after ovulation (P > 0.05).

There was a significant main effect of group (P < 0.001) and day (P < 0.001), but the interaction was not significant (P = 0.28), for daily serum concentrations of progesterone in ewes with or without luteinized follicles detected prior to the first ovulation (day 0). Ewes that had luteinized follicles exceeded ewes without identifiable luteal structures in mean serum progesterone concentrations on days -6, -4 to 0 and 2 to 5 (P < 0.05; Fig. 9.6)

9.4.9 Relationship between luteal volumes and progesterone secretion

There was a significant positive correlation between daily serum progesterone concentrations and total luteal volumes during the period of luteal growth (Period 2 of analysis; r = 0.61, P = 0.006), but not during the phase of mid-cycle luteal function (Period 3; r = -0.01, P = 0.95) in the 6 Western white-faced ewes studied. During luteolysis (Period 4), the correlation between serum concentrations of progesterone and total luteal volumes approached significance (r = 0.41, P = 0.09). The regression equation, and lower and upper 95% confidence interval limits for serum progesterone concentrations ([P₄]; ng/ml) and daily total luteal volumes ([TLV]; mm³) for Period 2 were as follows: [P₄] = 0.002 x [TLV] + 0.04; 0.0008 and 0.004. When the data for the two ewes which had a luteinized follicle during the first oestrus cycle of the breeding season were withdrawn from analysis, coefficients of correlation and corresponding P-values for the three periods above were as follows: r = 0.90, P = 0.00003, r = -0.16, P = 0.42, and r = 0.32, P = 0.33, for Periods 2, 3 and 4, respectively.
Fig. 9.6. Mean (± SEM) daily serum concentrations of progesterone in Western white-faced cross-bred ewes with (n = 3, □) or without (n = 3, □) luteinized unovulated follicles detected prior to the first ovulation of the breeding season. The 6 ewes underwent daily transrectal ultrasonography of ovaries during the period of transition from anoestrus to the breeding season (August-October). The endocrine data were normalized to the day of first ovulation of the breeding season (day 0) and analysed for the period from day -16 to day 17. See text for statistical details.
Progesterone concentration (ng/ml)

Days from first ovulation of the breeding season
9.5 Discussion

There was a distinct period of increased progesterone secretion in all ewes before the first ovulation and the beginning of the first full-length oestrous cycle of the breeding season. In the present study, ultrasonographic examination of ovaries performed from 26 to 41 days prior to the first ovulation of the breeding season revealed the presence of luteinized follicles in 3 out of the 6 Western white-faced ewes studied, during the 17-day period preceding the ovulation, but no luteal structure was detected in 3 other ewes. Ovarian ultrasonography performed in all ewes just before and during the early increase in serum progesterone levels did not reveal ovulations and subsequent formation of CL. An increase in serum progesterone concentrations has previously been seen before the first ovulation of the ovulatory season in ewes (Yuthasatrakosol et al., 1975; Walton et al., 1977; l'Anson, 1983; Ravindra and Rawlings, 1997). Such an increase has also been reported in peripubertal ewe lambs (Berardinelli et al., 1980; Foster and Ryan, 1979; Keisler et al., 1983). No fully formed luteal structures were observed in the studies above, but luteal tissue embedded within the ovarian stroma, and not palpable or observable macroscopically on the ovarian surface, was described (Berardinelli et al., 1980). Our observations suggest that luteinized follicles might be the site of progesterone production by the ovaries of ewes during the transition to the breeding season. It has been demonstrated that an LH surge precedes the transient increase in serum progesterone concentrations during the transition to the breeding season in ewes (Walton et al., 1977; Ravindra and Rawlings, 1997). Even though this LH surge does not result in ovulation, it might stimulate luteinization of follicles in some ewes. It is also possible that partial luteinization of large antral follicles was not identified with ultrasonography, and these may be the source of progesterone secretion in anoestrous ewes. However, luteinization of unovulated follicles in cyclic ewes is invariably associated with a marked reduction in serum oestradiol concentrations; no such decline was observed in the present study. Whether luteinized ovarian antral follicles in transitional ewes are capable of simultaneously producing large amounts of both oestrogens and gestagens, or progesterone is secreted by ovarian structures other than CL and luteinized follicles, remains to be elucidated.
The pattern of follicular wave emergence appeared confluent in the transition between anoestrus and the breeding season due mainly to the continuum of episodic FSH secretion whose magnitude and periodicity was not affected by the end of anoestrus. During the first oestrous cycle of the breeding season, follicular lifespan was significantly longer for the largest follicles in the first compared to the following follicular waves (follicles growing from 3 to \( \geq 5 \) mm in size); a similar pattern of large antral follicle development was seen during the later portion of the breeding season in Western white-faced ewes. The fact that the number of small antral follicles declined between anoestrus and the beginning of the breeding season, confirmed previous findings in ewes (Hutchinson and Robertson, 1966; McNatty et al., 1984; Ravindra and Rawlings, 1997). A decrease in numbers of small (not growing beyond 3 mm in diameter) follicles occurred abruptly after the first ovulation of the breeding season. This decrease appeared to be due to reduced growth of antral follicles to a 3-mm size range, but not the more rapid turnover of follicles growing to larger diameters. It has been suggested in cattle (Adams et al., 1992) and sheep (Johnson et al., 1997) that high circulating concentrations of progesterone suppress antral follicle growth. It is possible that follicles developing under strong progesterone dominance at mid-cycle have a shorter lifespan in comparison to follicles growing during the formation of CL, and that luteal phase levels of progesterone suppress the growth of small antral follicles. However, daily numbers of small antral follicles (3 mm in diameter) do not change during the transition from the breeding season to anoestrus in ewes, despite the rapid cessation of progesterone secretion after the final luteal phase of the season, but they increase more gradually, from early to mid-anoestrus (Bartlewski et al., 1998). It is, therefore, possible that alterations in serum concentrations of progesterone are only partly responsible for the change in small antral follicle populations and the lifespan of large (\( \geq 5 \) mm in size) antral follicles between anoestrus and the breeding season.

It has been suggested that short periods of increased progesterone secretion are important in restoring and re-organizing oestrous cycles at the onset of the breeding season in ewes as anoestrous ewes primed with progesterone for 2 days and receiving pulses of GnRH produced a normal LH surge followed by the full-length luteal phase (Legan et al., 1989).
1985). The effect of the sudden introduction of rams into a flock of anoestrous sheep, referred to as the "ram effect", has also been shown to increase the frequency of LH pulses, resulting in ovulation (Pearce et al., 1985; Martin et al., 1986). However, the ovulations induced using the ram effect are rarely accompanied by signs of oestrous and they are typically followed by short-lived CL (Martin et al., 1986). The premature regression of CL is prevented and a synchronized oestrus is assured when the introduction of rams is preceded by a 12- to 14-day period of progestogen priming (Martin et al., 1986). This progestogen pre-treatment can be substituted by a single intramuscular injection of 20 mg of progesterone per ewe (Hunter et al., 1986), after which fully functional CL are observed, but oestrous behaviour is inconsistent. In a previous study in Western white-faced ewes, an early increase in progesterone secretion preceding the first ovulation of the breeding season did not completely normalize the ensuing interovulatory interval; the duration of the oestrous cycle was less variable and consistency of follicular emergence (follicles growing from 2 mm in diameter onwards) appeared to be higher in the second than in the first cycle of the breeding season (Ravindra and Rawlings, 1997). We initially hypothesized that the effects of progesterone in ewes approaching the breeding season might manifest in alterations in the lifespan and/or periodicity of follicular waves. In the present study, there was no apparent change in follicular wave development (follicles growing from 3 to ≥ 5 mm in diameter) prior to the first ovulation of the breeding season, except for an increase in the growth rate of the largest follicles of waves emerging during the initial and middle portion of the early increase in progesterone secretion. The growth rate of large antral follicles has previously been seen to increase towards the end of the anoestrous period in Western white-faced ewes (Bartlekowski et al., 1998), probably reflecting an increase in ovarian follicle responsiveness to gonadotrophins as anoestrous wanes (Legan et al., 1985). Our conclusion is that elevated circulating concentrations of progesterone prior to the first ovulation of the breeding season do not alter the pattern of the growth of ovulatory-sized follicles in ewes, and hence they are important primarily for inducing oestrous behaviour, synchronizing it with the first preovulatory LH surge (Goodman, 1994), and preventing premature luteal regression during the following luteal phase (Beard and Hunter, 1996).
It is attractive to speculate that progesterone may also enhance the sensitivity of Graafian follicles to gonadotrophins, and facilitate ovulation and subsequent luteinization of ovulated follicles. It has been suggested that the CL exerts stimulatory effects on follicular development and ovulation through a local (intraovarian) mechanism (Hafez, 1987). In cyclic ewes, the ovary that contained CL had greater follicle development then the ovary without CL, in animals that ovulated unilaterally (Dufour et al., 1972). The presence of the previously formed CL or injection of progesterone into the ovary of anoestrous ewes increased the efficiency of pregnant mare serum gonadotrophin (PMSG) in inducing ovulation (Rexroad and Casida, 1977). Systemic progesterone is crucial for the normal luteal function in ewes; treatment with progesterone prevents luteolysis by reducing the uterine response to oxytocin early in the luteal phase (Beard and Hunter, 1996). The summation of the local and systemic effects of progesterone may play a role in the establishment of oestrous cycles in ewes during the transition to the cyclic activity.

In cyclic ewes, serum concentrations of oestradiol are highest around the time when the largest follicles of waves reach their maximum diameters, but such a relationship has not been seen in anoestrous ewes (Souza et al., 1996; Bartlewska et al., 1998). The close temporal relationship between the emergence of antral follicular waves and serum concentrations of oestradiol re-established after the first ovulation of the breeding season in ewes. The onset of increased oestradiol secretion occurred around the time of follicle wave emergence and peaks of serum oestradiol concentrations towards the end of the growth phase of the largest ovarian follicles of waves. These observations suggest that the pulsatile pattern of gonadotropic hormone secretion during the luteal phase of the sheep oestrous cycle is more organized and/or ovarian antral follicles are more responsive to circulating gonadotropins compared to at the end of the breeding season and across anoestrus.

The time after ovulation during which CL were not detectable with ultrasonography appeared to be slightly longer at the first cycle of the breeding season (present study) compared with the mid-breeding season and the transition into anoestrus in the same group of Western white-faced ewes (4.2 ± 0.5, 3.4 ± 0.3 and 2.8 ± 0.7 days after ovulation,
respectively). The mean day on which identified CL and luteinized follicles regressed also appeared to be somewhat delayed at the first interovulatory interval of the breeding season than in the mid-breeding season or the transition into anoestrus (17.0 ± 0.4, 16.1 ± 0.5 and 16.3 ± 0.6 days after ovulation, respectively). The maximum total luteal volume recorded during the mid-breeding season (November-December, 1580 ± 105 mm³ on day 9 of the oestrous cycle, day 0 = day of ovulation) and during the final luteal phase of the breeding season in Western white-faced ewes (1273 ± 184 mm³ on day 11 after ovulation), were higher than that seen in the present study (903 ± 113 mm³ on day 10 after ovulation). The maximum size of individual CL/luteal structures was lower during the mid-breeding season and in the present study than during the transition into anoestrum in white-faced ewes (842 ± 66, 701 ± 48 and 1101 ± 98 mm³, respectively). The mean ovulation rate was higher in the mid-breeding season ewes (1.8 ± 0.3) than at the final (1.2 ± 0.2) or first (1.3 ± 0.2) ovulation of the breeding season. However, the maximum diameter attained by the ovulatory follicles did not vary between the mid-breeding season, the transition into anoestrum, and the present study (6.4 ± 0.4, 6.3 ± 0.3 and 6.2 ± 0.2 mm, respectively).

The difference in maximal serum concentrations of progesterone observed after the last ovulation of the breeding season (2.92 ± 0.30 ng/ml on day 9 after ovulation), during the mid-breeding season (3.58 ± 0.18 ng/ml on day 11 after ovulation, and during the first oestrous cycle of the breeding season of Western white-faced ewes (2.82 ± 0.25 ng/ml on day 11 after ovulation), probably reflects differences in the total luteal tissue content due to the number of ovulations and average sizes of luteal structures. In addition, the diminished luteotrophic support and/or ovarian responsiveness to LH (Niswender and Nett, 1988) might influence progesterone production, especially towards the end of the ovulatory season in ewes, which may account for similar maximal serum progesterone concentrations seen during both transitional periods (into and out of anoestrum) despite the higher total luteal volume in ewes during the transition to anoestrum.

Daily total luteal volumes were positively correlated with daily serum concentrations of progesterone during luteal growth and regression, but not during the mid-luteal phase, in white-faced sheep studied in November-December as well as during the final oestrous cycle.
of the breeding season. In the present study, there was a correlation between total luteal volumes and circulating concentrations of progesterone during the growth of CL, but not during the mid-luteal phase, and during luteolysis the correlation only approached significance. The lack of a linear relationship between the total luteal volume and progesterone secretion during the mid- and late luteal phase in the present ewes may be, in part, due to increased progesterone synthesis by ovarian antral follicles and/or progesterone-secreting cells in the ovarian stroma.

9.6 Summary and conclusions

The first ovulation of the breeding season in ewes was preceded by a transient increase in circulating progesterone secreted by either luteinized unovulated follicles or interstitial tissue of unknown origin (Berardinelli et al., 1980), but not by short-lived CL resulting, as previously suggested, from the ovulations of immature ovarian follicles (Legan et al., 1985). Prior to the first ovulation, production of follicular oestradiol, suppressed during anoestrus, appeared to be fully restored, but it was not synchronized with the growth of the largest antral follicles of waves until after the onset of the first luteal phase of the breeding season. The endogenous rhythm of FSH secretion was not affected by the cessation of seasonal anoestrus and it remained coupled with the emergence of follicular waves (follicles growing from 3 to ≥ 5 mm). There was no apparent change in the pattern of emergence or growth characteristics of successive follicular waves before the beginning of the first oestrous cycle of the breeding season, except for the higher growth rate of the largest antral follicles emerging, on the average, 15 and 10 days before the first ovulation. The first follicular wave after ovulation had a longer lifespan compared with all other waves, including all the waves that contained non-ovulatory follicles, indicating the resumption of the normal cyclic pattern of follicle wave development. During the first interovulatory interval of the breeding season, there was a rapid decline in the number of small antral follicles (not growing beyond 3 mm in diameter). The ovulation rate, total and mean luteal volumes and maximal serum progesterone concentrations, but not mean
diameters of ovulatory follicles, appeared to be reduced at the onset of the breeding season compared with the mid-breeding season in Western white-faced ewes. The increase in progesterone secretion did not alter the pattern of follicle wave development prior to the first ovulation of the breeding season, hence it seems to be important mainly for inducing oestrous behaviour, synchronizing it with the preovulatory LH surge, and preventing untimely luteolysis during the following oestrous cycle. Progesterone may also enhance ovarian follicular responsiveness to circulating gonadotrophins through a local mechanism.
Chapter 10. EFFECTS OF MEDROXYPROGESTERONE ACETATE (MAP) IN THE ABSENCE OF LUTEAL PROGESTERONE ON ANTRAL FOLLICLE DEVELOPMENT AND OVULATION RATE IN CYCLIC WESTERN WHITE-FACED EWES

10.1 Abstract

Luteolysis was induced with PgF$_{2\alpha}$ and intravaginal sponges containing medroxyprogesterone acetate (MAP) were inserted for 6 days, on ~day 8 after ovulation, in 7 non-prolific Western white-faced ewes; seven untreated ewes served as controls. This was done to mimic the low circulating progesterone levels seen in prolific Finn ewes. Low progesterone concentrations in cyclic Finn ewes may be a determining factor in their high ovulation rate. Ovarian follicle dynamics and development of CL were monitored daily using transrectal ultrasonography. Blood samples were collected each day and also every 12 min for 6 h, mid-way through the period of treatment with progestogen sponges. Six out of 7 ewes ovulated 2 to 5 days after PgF$_{2\alpha}$ (1.9 ± 0.6 ovulations/ewe), but this did not affect the emergence of ensuing follicular waves (follicles growing from 3 to ≥ 5 mm in diameter). These ovulations did not result in CL. Following the removal of sponges, the mean ovulation rate was 3.1 ± 0.4 in treated and 2.0 ± 0.3 in control ewes (P < 0.05). Of all follicles ≥ 5 mm in size in the last follicular wave before the final ovulation of the study period, 85 and 74% ovulated in treated and control ewes, respectively. Ovulations from the penultimate wave were seen in 5 treated but only in 2 control ewes (70 and 21% of follicles in this wave were ovulatory, in treated and control ewes, respectively), and in 3 treated ewes 4 ovulations were seen from follicular waves before the penultimate wave. There were no significant differences between the 2 groups in daily serum concentrations of FSH and oestradiol, and no differences in the parameters of LH/FSH secretion, based on an intensive
bleed. In summary, treatment with \( \text{PgF}_2 \)/MAP changed follicular dynamics and increased the ovulation rate of non-prolific Western white-faced ewes to resemble that of prolific Finn sheep. These effects of low serum progesterone concentrations or progestogen treatment did not appear to have a clear gonadotrophic dependancy.

10.2 Introduction

Marked genetic variation in prolificacy among breeds of sheep (Driancourt et al., 1986a-b, 1988; Fry et al., 1988; Goodman, 1994; Fry and Driancourt, 1996; Webb et al., 1999) provides a useful tool for the study of the mechanisms governing ovulation rate. Earlier studies failed to produce convincing evidence that differences in circulating concentrations of gonadotrophic hormones were solely responsible for differences in ovulation rate (Webb et al., 1999). It has been suggested that the high ovulation rate in prolific sheep is due to intraovarian rather than pituitary factors (McNatty et al., 1993; Campbell et al., 1996; Souza et al., 1997; Webb et al., 1999).

The largest antral follicles (reaching \( \geq 5 \) mm in diameter before regression or ovulation) grow in waves during the ewe’s oestrous cycle (Noel et al., 1993; Ginther et al., 1995; Bister et al., 1999). There are typically 4 or 3 waves of follicle emergence throughout the interovulatory interval. In non-prolific breeds such as the Western White Face, Suffolk, Texel and Ile-de-France (Bister et al., 1999), the ovulatory follicles originate mainly from the last follicular wave, but in prolific Finn ewes, ovulatory follicles are recruited also from the penultimate wave of the cycle that emerges during the mid-luteal phase.

During the mid-breeding season (October to December), mean serum concentrations of progesterone are higher in non-prolific Western white-faced ewes compared to prolific Finn sheep. It has been shown that lower than normal luteal phase levels of progesterone result in prolonged follicle lifespan in ewes (Johnson et al., 1996; Vinoles et al., 1999). Similarly, the lifespan of large antral follicles was seen to be prolonged in ewes injected with \( \text{PgF}_2 \) on day 6 and treated with vaginal sponges soaked with medroxyprogesterone acetate (MAP), from days 5 to 19 after ovulation (Flynn et al., 1999). Progestogen treatment
in the latter study also resulted in increased LH pulse frequency on day 18 after ovulation. We proposed that the difference in the pattern of follicle development between breeds of sheep differing in prolificacy might be due to a difference in circulating concentrations of progesterone. The present experiment was undertaken to see if induction of luteolysis and treatment with progestogen-impregnated (MAP) sponges late in the sheep oestrous cycle would alter the pattern of antral follicle growth and/or secretion of gonadotrophins and oestradiol, and increase ovulation rate in non-prolific Western white-faced ewes.

10.3 Materials and methods

10.3.1 Animals and experimental procedures

Fourteen sexually mature, clinically healthy Western white-faced ewes were used in this study from October to November. Oestrus was synchronized by a 14-day treatment with progestogen-releasing intravaginal sponges (MAP; Veramix®, Upjohn, ON, Canada; 60 mg). Ewes were checked for oestrus with 2 vasectomized crayon-harnessed rams and an electronic oestrous detector measuring changes in vaginal mucous impedance (Szczechanski et al., 1994). Ovulations were confirmed by ultrasonography. All of the 14 ewes employed in this study were in oestrus within a 24-h period at the end of the cycle after the synchronized oestrus, and ovulated between 24 to 48 h after the onset of oestrus. Daily transrectal ultrasonography of ovaries started at ~day 5 after the ovulation. The size and position of CL and ovarian antral follicles ≥ 3 mm in size were recorded for 19 days. Nine days after oestrus, or on ~day 8 after ovulation (7.7 ± 0.2 days), seven ewes received a single injection of PgF$_{2\alpha}$ (Lutalyse®, Upjohn, Orangeville, ON, Canada; 15 mg) and a progestogen sponge which was put in place for 6 days. Treatment with progestogen-releasing sponges was designed to encapsulate the emergence of the last 2 follicle waves of the cycle. The remaining 7 ewes served as untreated controls. Blood samples were collected daily prior to each ultrasonographic examination and also every 12 min for 6 h, three days after injection of PgF$_{2\alpha}$ and insertion of progestogen sponges.
10.3.2 Ovarian data summary and analyses

As in previous studies, the number of emerging follicular waves during the period of observation and days of wave emergence were determined for each ewe. Preliminary inspection of follicular data revealed that the ewes of the present study had an average of 4 waves of large follicle growth during the 19-day scanning period (range: 3 to 5). For the purpose of statistical analyses, the follicular waves were grouped according to the mean day of emergence in the 19 days of observation and relative to the day of \( \text{PgF}_2 \) administration (Wave A: days -3 to -1; Wave B: days 0 to 3; Wave C: days 4 to 7; Wave D: days 9 to 11; and Wave E: days 12 to 14). Letters were assigned to the waves as in some ewes with 3 or 4 waves over the period of study, these waves did not necessarily emerge at a time that placed them in consecutive groupings as described above (i.e., in ewes with 3 waves, the waves could have been Waves A, B and D or Waves B, C and D, etc.). The distribution of emerging follicle waves in individual ewes is shown in Fig. 10.4. The characteristics of ovulatory antral follicles emerging in the last and penultimate follicular waves before the final ovulation of the observation period were compared within and between the 2 groups by ANOVA (SigmaStat\textsuperscript{®} 2.0, 1997). This was not done for the ovulatory follicles emerging in the preceding waves and contributing to the final ovulation of the study, because none of the control ewes and only 3 out of 7 treated animals had ovulatory follicles that emerged prior to the penultimate wave.

The number of ovulations and time at which they occurred as well as mean numbers of luteal structures per ewe were compared between the 2 groups of ewes as well as between the first and last ovulation of the study period within each group, by ANOVA (SigmaStat\textsuperscript{®} 2.0, 1997). Daily total luteal volumes were determined for each ewe, for the period during which luteal structures were observed, and the resulting values were normalized to the day of treatment with \( \text{PgF}_2 \) (day 0). Daily total luteal volumes were then analysed by a two-way RM-ANOVA (SigmaStat\textsuperscript{®} 2.0, 1997).
10.3.3 Hormone analyses

Earlier studies in our laboratory (unpublished) showed that the antiserum to progesterone did not cross-react with medroxyprogesterone acetate (MAP) which permitted the measurement of endogenous progesterone in progestogen-treated ewes. All LH analyses were done in a single assay; the intraassay coefficients of variation (CVs) for mean LH concentration of 0.19 or 0.97 ng/ml were 12.3 or 8.9%, respectively. For FSH reference sera with mean concentration of 1.28 or 2.41 ng/ml, the intra- and interassay CVs were 6.4 and 2.9% or 7.1 and 5.9%, respectively. For oestradiol reference sera with mean concentration of 5.1 or 13.9 pg/ml, the intra- and interassay CVs were 14.0 and 12.2% or 10.3 and 7.2%, respectively. Intra- and inter-assay CVs for reference sera with mean progesterone concentrations of 0.29 or 0.69 ng/ml were 13.2 and 9.4% or 18.5 and 7.3%, respectively. Daily serum concentrations of FSH, oestradiol and progesterone for the entire study period, were normalized to the day of treatment with PgF₂₆ (day 0), for ewes of both groups. Main effects of group, day and the group-by-day interaction were determined by RM-ANOVA (SigmaStat® 2.0, 1997). The Cycle Detection Program (Clifton and Steiner, 1983) was used to identify peaks and fluctuations in daily serum concentrations of FSH and oestradiol in individual ewes. Characteristics of detected peaks/fluctuations in daily serum concentrations of FSH and oestradiol were evaluated as described previously. Analyses of variance (SigmaStat® 2.0, 1997) were used to compare means for FSH peaks/fluctuations among groups and within the study period for each group. This was not done for oestradiol concentrations, as in treated ewes, elevations in serum levels of oestradiol were irregular and dissociated from the pattern of follicle wave emergence, precluding our being able to compare successive peaks in all ewes.

10.3.4 The relationship between follicle wave development and serum concentrations of FSH/oestradiol

Follicular and hormonal data were analysed for associations between various stages of follicular wave development (follicles growing from 3 to ≥ 5 mm) and peaks in serum
concentrations of FSH and oestradiol. A statistical method for evaluating temporal relationships between elevations in serum concentrations of FSH/oestradiol and follicle wave development has been previously described.

10.3.5 Additional analyses

Daily serum concentrations of FSH, oestradiol and progesterone in the 6 treated ewes that ovulated after \( \text{PgF}_2 \) and before the removal of progestogen sponges were aligned to the day of ovulation in each ewe (day 0), and analysed for the period from days −2 to 2 by one-way RM-ANOVA (\text{SigmaStat}® 2.0, 1997).

Preliminary inspection of the present data and initial statistical analyses of mean hormone concentrations aligned to the day of treatment with \( \text{PgF}_2 \), revealed differences between the treated and control ewes in progesterone levels after the final ovulation of the study period. Therefore, ovarian and endocrine data were aligned to the day of the last ovulation (day 0) and analysed, by RM-ANOVA (\text{SigmaStat}® 2.0, 1997), for the period from days −1 to 5 (hormone concentrations) or from days 3 to 5 (total luteal volumes). Lastly, ultrasonographic examinations of the ovaries showed that after the final ovulation, some ovulated follicles in 3 treated and 3 control sheep failed to form CL (inadequate CL). In order to assess the differences between the ewes with normal CL only and the ewes that had both normal and inadequate CL, ovarian and hormonal data were compared between the 2 groups of ewes in question, for the whole study period and after alignment to the day of final ovulation, as described above for the treated and control ewes.

10.4 Results

10.4.1 Ovulation rates and characteristics of ovulatory follicles

The mean ovulation rate for the ovulation preceding treatment was \( 1.9 \pm 0.3 \) (range: 1 to 3) and \( 1.4 \pm 0.2 \) ovulations/ewe (range: 1 to 2), for treated and control ewes, respectively (\( P > 0.05 \)). Within 2 to 5 days of the injection of \( \text{PgF}_2 \) and insertion of
progestogen sponges (day 0), ovulations were detected in 6 of 7 treated ewes, with an average ovulation rate of 1.9 ± 0.6 (range: 0 (Fig. 10.1a) to 5 (Fig. 10.1b)). One ewe ovulated on day 2, three ewes on day 3, one ewe ovulated on days 2, 3 and 4, and one ewe on days 4 and 5 after 
PgF\textsubscript{2a}. Inspection of growth profiles for individual antral follicles around the time of the treatment revealed that 50% (8/16) of all follicles ≥ 5 mm in diameter detected on the day of injection ovulated. In addition, one treated ewe had 2 follicles that emerged on day 2 and ovulated on day 5 post-treatment, and in one other ewe, a follicle that emerged on day 1 ovulated on day 4 after 
PgF\textsubscript{2a}. No ewes were marked by rams and none of the follicles that ovulated after 
PgF\textsubscript{2a} and before the withdrawal of sponges, formed an observable CL. In the 6 ewes that ovulated 2 to 5 days after 
PgF\textsubscript{2a} treatment, there were no significant changes (P > 0.05) in serum concentrations of FSH and progesterone in daily blood samples from 2 days before to 2 days after ovulation, but mean serum concentrations of oestradiol declined from 4.1 ± 0.2 pg/ml on day −1 before ovulation to 1.5 ± 0.5 pg/ml on the day of ovulation (P < 0.001), and subsequently increased (P < 0.05) to 3.4 ± 0.7 pg/ml on day 2 after ovulation.

Following the removal of sponges, the mean ovulation rate was 3.1 ± 0.4 (range: 2 to 5) in treated ewes vs. 2.0 ± 0.3 (range: 1 to 3) in control ewes (P < 0.05). The mean ovulation rate after treatment (3.1 ± 0.4) was greater (P < 0.05) than the pre-treatment ovulation rate (1.9 ± 0.3) for ewes given 
PgF\textsubscript{2a} and progestogen sponges. All but one ewe were well marked by rams at the final ovulation of the study period; one treated ewe was poorly marked. Ovulations occurred 9.5 ± 0.1 days (range: 8 to 10 days) and 9.4 ± 0.3 days (range: 8 to 12 days) after 
PgF\textsubscript{2a}, in treated and control ewes, respectively (P > 0.05). The interval between the first and the last ovulation of the study period, was 16.9 ± 0.5 days (range: 15 to 18 days) and 16.9 ± 0.3 days (range: 16 to 18 days), for treated and control ewes, respectively (P > 0.05). The last follicular wave before ovulation contained ovulatory follicles (follicles that ovulated after the removal of sponges in the treated ewes) in all ewes studied. Of all follicles reaching ≥ 5 mm in diameter in this wave, 85% (11/13) and 74% (11/15) ovulated, in the treatment and control groups, respectively. The penultimate wave before the final ovulation contained ovulatory follicles in 5 of 7 treated ewes but only in 2
control sheep (Fig. 10.2d). In the penultimate follicular wave, 70% (7/10) and 21% (3/14) of follicles attaining ≥ 5 mm in diameter were ovulatory, in the treated and control ewes, respectively. In addition, one treated ewe had a follicle that emerged in the follicular wave before the penultimate wave (day -2 before PgF₂α) and it ovulated 11 days after emergence (Fig. 10.1c). One treated ewe ovulated a follicle from a wave that emerged > 3 days before PgF₂α and the time elapsed from emergence to ovulation was > 12 days. Finally, one treated ewe had an ovulatory follicle that emerged in each of the 2 waves before the penultimate wave.

None of the parameters of follicle growth for ovulatory follicles in the final wave before ovulation differed between the treated and control ewes (Table 10.1). In both groups of ewes, ovulatory follicles in the penultimate follicle wave emerged approximately 3 days earlier than ovulatory follicles in the final wave before ovulation (P < 0.05). Ovulatory follicles in the penultimate wave in the treated ewes had a significantly longer growing phase (5.1 ± 0.5 vs. 3.0 ± 0.0 days) but a shorter static phase (2.7 ± 0.6 vs. 5.0 ± 1.0 days) compared to the control ewes (P < 0.05). The comparisons between the final and penultimate follicular waves within each group of ewes revealed that in the treated ewes, ovulatory follicles in the penultimate wave before ovulation grew to larger maximum sizes (7.0 ± 0.3 and 6.2 ± 0.2 mm, for the penultimate and final waves, respectively; P<0.05) and over a longer duration (5.1 ± 0.5 and 3.4 ± 0.6 days, for the penultimate and final waves, respectively; P < 0.05) in comparison to ovulatory follicles in the final follicle wave. In contrast, the ovulatory follicles from the penultimate wave in the control ewes had significantly longer static phases in comparison to the follicles in the final wave before ovulation (5.0 ± 1.0 and 2.0 ± 0.3 days, for the penultimate and final waves, respectively; P < 0.05).

10.4.2 CL/luteal structures and progesterone secretion

In the ewes of the present study, all CL were detected by day 4 after the last observed ovulation of the study period (both groups: 3.3 ± 0.2 days after ovulation; P > 0.05). A short-lived CL (observed once only on day 3 after ovulation) was seen in one control ewe,
Fig. 10.1. The diameter profiles of individual antral follicles growing from 3 to ≥ 5 mm (top panels) with accompanying daily serum concentrations of FSH (middle panels) and oestradiol (bottom panels) in four (a-d) Western white-faced ewes that received Pgf2α on ~day 8 and a progestogen sponge from ~days 8 to 14 after ovulation. Day 0 = day of treatment with Pgf2α. The arrows along the X-axis (top panels; †) denote the days of follicle wave emergence, and the arrows within the upper chart area (−OV) indicate the ovulatory follicles on the day before ovulation. Asterisks denote the peak values of fluctuations (nadir-to-peak-to-nadir) in circulating concentrations of FSH and oestradiol (the two dashed lines encompass a fluctuation) as determined by the cycle-detection computer program (Clifton and Steiner, 1983).
**Fig. 10.2.** The diameter profiles of individual antral follicles growing from 3 to ≥ 5 mm (top panels) with accompanying daily serum concentrations of FSH (middle panels) and oestradiol (bottom panels) in four (a-d) control Western white-faced ewes. Day 0 = day of treatment with PgF₂α of the treated ewes. The arrows along the X-axis (top panels; ↑) denote the days of follicle wave emergence, and the arrows within the upper chart area (−OV) indicate the ovulatory follicles on the day before ovulation. LF (Fig. 10.2d) denotes a large uniovulated antral follicle that luteinized. Asterisks denote the peak values of fluctuations (nadir-to-peak-to-nadir) in circulating concentrations of FSH and oestradiol (the two dashed lines encompass a fluctuation) as determined by the cycle-detection computer program (Clifton and Steiner, 1983).
Table 10.1. Characteristics of ovulatory antral follicles (growing to ≥ 5 mm in diameter; means ± SEM), originating from the last (regular font) and penultimate (italicized) follicular wave before ovulation, and as monitored by daily transrectal ultrasonography in seven Western white-faced ewes that received Pgf₂ₐ injection on ∼day 8 and a progestogen sponge from ∼day 8 to 14 after ovulation, and in seven untreated white-faced ewes. Day 0 = day of treatment with Pgf₂ₐ.

<table>
<thead>
<tr>
<th>End point</th>
<th>Final follicular wave</th>
<th>Penultimate follicular wave</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Treated ewes (n = 11)</td>
<td>Control ewes (n = 11)</td>
</tr>
<tr>
<td>Mean day of emergence</td>
<td>4.5 ± 0.6</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>Time from emergence to ovulation (days)</td>
<td>5.3 ± 0.6ᵃ</td>
<td>5.5 ± 0.4ᶜ</td>
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<tr>
<td>Growing phase (days)</td>
<td>3.4 ± 0.6ᵃ</td>
<td>3.5 ± 0.4</td>
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<tr>
<td>Static phase (days)</td>
<td>1.8 ± 0.2</td>
<td>2.0 ± 0.3ᶜ</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>6.2 ± 0.2ᵃ</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>Diameter on the day before ovulation (mm)</td>
<td>6.0 ± 0.2</td>
<td>6.4 ± 0.3</td>
</tr>
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</table>

|                                                | Treated ewes (n = 7) | Control ewes (n = 3)     |
|                                                | 2.1 ± 0.4             | 2.0 ± 0.0                  |
| 7.9 ± 0.3ᵇ                                    | 8.0 ± 1.0ᵇ           |
| 5.1 ± 0.5ᵃᵇ                                   | 3.0 ± 0.0ᵇ           |
| 2.7 ± 0.6ᶜ                                    | 5.0 ± 1.0ᵈᵇ          |
| 0.9 ± 0.1                                     | 1.2 ± 0.1             |
| 7.0 ± 0.3ᵇ                                    | 6.7 ± 0.3             |
| 6.3 ± 0.4                                     | 6.0 ± 0.6             |

Means with different letter superscripts are significantly different (P < 0.05): ᵃᵇ between the final and penultimate waves for treated ewes; ᶜᵈ between the final and penultimate waves for control ewes; and ˢᵇ between treated and control ewes for the penultimate follicle wave.
and in this same ewe, a luteinized unovulated follicle was detected (Fig. 10.2d). After the final ovulation, three treated ewes and 3 control animals had fewer CL than ovulated follicles. The mean number of all luteal structures per ewe (2.4 ± 0.3 and 1.4 ± 0.2 luteal structures/ewe, for treated and control ewes, respectively; P < 0.05) was different from the mean ovulation rates (3.1 ± 0.4 and 2.0 ± 0.3 ovulations/ewe, for treated and control ewes, respectively; P < 0.05).

Daily total luteal volumes and mean serum concentrations of progesterone for the ewes given \( \text{PgF}_{2\alpha} \) and control ewes, are shown in Fig. 10.3. All treated ewes responded to \( \text{PgF}_{2\alpha} \) as evidenced by an abrupt decline in circulating concentrations of progesterone and the ovarian content of luteal tissue. In the treated ewes, daily total luteal volumes decreased 24 h after \( \text{PgF}_{2\alpha} \) and again 48 h post-treatment (P < 0.05; Fig. 10.3a). Circulating levels of luteal progesterone declined (P < 0.05) to a basal level 24 h after injection of \( \text{PgF}_{2\alpha} \) and remained low (P > 0.05) during the entire period of treatment with progestogen sponges (Fig. 10.3b). Mean serum concentrations of progesterone and daily total luteal volumes were higher in the control than in treated ewes for 6 and 8 consecutive days, respectively, from days 1 to 6 and from days 1 to 8 after \( \text{PgF}_{2\alpha} \), but progesterone concentrations and luteal volumes did not differ (P > 0.05) between the 2 groups prior to treatment (days –3 to 0) and after luteolysis in the control ewes (days 9 to 12 after \( \text{PgF}_{2\alpha} \)).

10.4.3 Follicle wave emergence over the period of study

Individual follicle profiles (follicles growing from 3 to ≥5 mm in diameter) are shown, with accompanying serum concentrations of FSH and oestradiol, for 4 different animals for each group, to illustrate the pattern of follicular wave emergence in relation to hormone secretion, follicle size and the number and time of emergence of ovulatory follicles during the study period (Fig. 10.1 and 10.2). A total of 56 follicular waves (follicles growing from 3 to ≥5 mm in diameter) were obtained in the present study; 29 waves were identified in treated and 27 waves in control ewes. The distribution of detected follicular waves is shown, for both groups of ewes, in Fig. 10.4. Out of the 7 treated ewes, two ewes had 3 waves (Fig. 10.1b, d), two ewes had 4 (Fig. 10.1c), and three ewes had 5 emerging follicle
Fig. 10.3. Daily total volumes of luteal tissue (a) and serum concentrations of progesterone (b) (mean ± SEM) in seven Western white-faced ewes that received Pgf$_{2\alpha}$ injection on ∼day 8 and a progestogen sponge from ∼days 8 to 14 after ovulation (treated ewes, filled symbols), and seven untreated white-faced ewes (control ewes, hollow symbols). Day 0 = day of treatment with Pgf$_{2\alpha}$ of the treated ewes. Data were normalized to the day of treatment with Pgf$_{2\alpha}$ and analysed for the period from days −3 to 15. See text for statistical descriptions.
Fig. 10.4. Distribution of days of follicle wave emergence determined with ultrasonography in seven Western white-faced ewes that received PgF$_{2a}$ injection on ~day 8 and a progestogen sponge from ~days 8 to 14 after ovulation (treated ewes, filled symbols), and seven untreated white-faced ewes (control ewes, hollow symbols). Day 0 = day of treatment with PgF$_{2a}$ of the treated ewes. Letters (A-E) are used to designate groupings of days of follicle wave emergence in individual ewes across the 19-day observation period, as for statistical analysis the days of emergence were grouped according to days of the study and not by consecutive numbering of waves in each ewe. Asterisks denote the days of wave emergence in a single treated ewe that did not ovulate after PgF$_{2a}$ and before sponge withdrawal.
waves over the scanning period (Fig. 10.1a). Two control ewes had 3 waves (Fig. 10.2c), four ewes had 4 (Fig. 10.2a, d), and one ewe had 5 emerging follicle waves over the study period (Fig. 10.2b). The mean number of emerging follicle waves per ewe per study period did not vary between the 2 groups (4.1 ± 0.3 and 3.9 ± 0.3, for treated and control ewes, respectively; P > 0.05). Mean days of follicle wave emergence, relative to the day of treatment with \( \text{PgF}_2 \) (day 0), were as follows: Wave A: −2.2 ± 0.2 and −1.8 ± 0.4; Wave B: 1.4 ± 0.4 and 1.8 ± 0.3; Wave C: 5.2 ± 0.4 and 5.8 ± 0.6; Wave D: 9.7 ± 0.3 and 9.5 ± 0.2; and Wave E: 13.7 ± 0.3 and 13.0 ± 0.4, in the treated and control ewes, respectively (P > 0.05).

10.4.4 Serum concentrations of gonadotrophins

There was a significant main effect of day (P < 0.05), but the main effect of group (P = 0.42) and interaction (P = 0.93) were not significant for daily serum concentrations of FSH, for the duration of the study (Fig. 10.5a). FSH secretion is linked to the pattern of follicle wave emergence in each individual ewe and so group trends for mean daily FSH concentrations were not analysed further. The mean serum concentrations of FSH, based on the intensive bleed, did not differ between groups (1.32 ± 0.16 and 1.53 ± 0.26 ng/ml, for treated and control ewes, respectively; P > 0.05).

Using the cycle-detection computer program (Clifton and Steiner, 1983), peaks of transient increases in daily serum concentrations of FSH were detected in all ewes studied. Mean numbers of identified FSH peaks per ewe per study period (3.8 ± 0.2 peaks/ewe), mean peak concentrations (2.10 ± 0.09 ng/ml), the duration of sequential FSH increases (nadir-to-nadir: 4.2 ± 0.1 days) and of the interpeak intervals for serum concentrations of FSH (4.2 ± 0.2 days) did not differ (P > 0.05) over the study period nor between treated and control ewes.

The secretory parameters of LH in the present ewes, based on the intensive blood sampling, were not affected by the treatment with \( \text{PgF}_2 \) and progestogen. Mean and basal level of LH, LH pulse frequency, amplitude and duration were: 0.20 ± 0.02 and 0.20 ± 0.03
ng/ml, 0.13 ± 0.02 and 0.15 ± 0.01 ng/ml, 0.29 ± 0.09 and 0.27 ± 0.07 pulse/h (range: 0 to 3 pulses/6h in both treated and control ewes); 0.47 ± 0.10 and 0.47 ± 0.12 ng/ml; and 54 ± 15 and 48 ± 11 min; for treated and control ewes, respectively (P > 0.05).

10.4.5 Serum concentrations of oestradiol

Circulating concentrations of oestradiol in the ewes of the present study differed by day (P < 0.001) but there was no significant effect of treatment (P = 0.77) and interaction of the main effects was not significant (P = 0.12; Fig. 10.5b). As with FSH, group means for serum levels of oestradiol are not further described.

10.4.6. The relationships between follicle wave development and daily serum concentrations of FSH and oestradiol

The number of emerging follicular waves and the number of identified FSH peaks per ewe per scanning period, did not differ (3.9 ± 0.3 vs. 4.1 ± 0.3 and 3.6 ± 0.2 vs. 3.9 ± 0.3; FSH peaks vs. follicular waves, for treated and control ewes, respectively; P > 0.05), and the lengths of intervals between adjacent days of wave emergence (interwave intervals) were positively correlated with the lengths of interpeak intervals for FSH fluctuations (r = 0.77, P = 0.00007 and r = 0.82, P = 0.00003, for treated and control ewes, respectively). In both groups of ewes, all identified FSH peaks occurred from days −2 to 1 relative to the nearest day of wave emergence (day 0), and the highest proportion of FSH peaks occurred on day 0 (63 and 56%, for treated and control ewes, respectively; $\chi^2$ test: P < 0.05).

The number of identified peaks of oestradiol fluctuations did not differ from the number of emerging follicular waves in both groups of ewes studied (4.3 ± 0.3 vs. 4.1 ± 0.3 and 3.9 ± 0.3 vs. 3.9 ± 0.3; oestradiol peaks vs. follicular waves, for treated and control ewes, respectively; P > 0.05). The lengths of intervals between days on which oestradiol fluctuations began were strongly and positively correlated with interwave intervals in control ewes (r = 0.84, P = 0.001), but not in treated ewes (r = 0.32, P = 0.18). The intervals
Fig. 10.5. Mean (± SEM) daily serum concentrations of FSH (a) and oestradiol (b) in seven Western white-faced ewes that received PGF$_2\alpha$ injection on ~day 8 and a progestogen sponge from ~days 8 to 14 after ovulation (treated ewes, filled symbols), and seven untreated white-faced ewes (control ewes, hollow symbols). Day 0 = day of treatment with PGF$_2\alpha$ of the treated ewes. Data were normalized to the day of treatment with PGF$_2\alpha$ and analysed for the period from days −3 to 15.
Figure (a) shows the FSH concentration (ng/ml) over days before or after PGF$_{2a}$ treatment. The graph compares treated ewes (n = 7) with control ewes (n = 7). The PGF$_{2a}$ treatment was administered on day 8 after ovulation.

Figure (b) depicts the Oestradiol concentration (pg/ml) over days before or after PGF$_{2a}$ treatment. The treatment again compares treated ewes (n = 7) with control ewes (n = 7), with PGF$_{2a}$ administered on day 8 after ovulation.
between adjacent peaks of oestradiol increases were positively and significantly correlated with intervals between ends of follicle growing phases in control \( r = 0.54, P = 0.03 \) but not in treated ewes \( r = 0.41, P = 0.10 \).

### 10.4.7 Hormone secretion and luteal volumes after the final ovulation of the study period

There was a significant difference between the treated and control ewes in terms of progesterone secretion during the period from 1 day before to 5 days after the last ovulation of the study period (day 0), but daily total volumes of luteal tissue, from days 3 to 5 after ovulation, did not differ \( P > 0.05 \). The treated ewes exceeded control ewes in mean serum levels of progesterone on days 4 to 5 after ovulation. Mean daily concentrations of FSH and oestradiol did not vary between the 2 groups during that period \( P > 0.05 \).

### 10.4.8 Comparisons between ewes with normal and inadequate CL after the final ovulation of the study period

After the final ovulation of the study period, eight ewes formed as many CL as ovulated follicles (ewes with normal CL), but 6 ewes had fewer CL than ovulations (3 treated and 3 control ewes; ewes with inadequate CL). Mean ovulation rate was \( 2.1 \pm 0.3 \) and \( 3.2 \pm 0.4 \) ovulations/ewe, for ewes with normal and inadequate CL, respectively \( P < 0.05 \). Mean number of detected luteal structures per ewe was \( 2.1 \pm 0.3 \) and \( 1.7 \pm 0.2 \) \( P > 0.05 \), and the time from ovulation to detection of CL averaged \( 3.1 \pm 0.2 \) and \( 3.1 \pm 0.3 \) days, for ewes with normal and inadequate CL, respectively \( P > 0.05 \). With the data analysed for the whole study period, the ewes with normal CL exceeded \( P < 0.05 \) ewes that had inadequate CL in mean serum concentrations of oestradiol on days -3 to -2, 3, 9 to 10 and 15 relative to \( \text{PgF}_2 \) injection. When data were normalized to the day of last ovulation (Day 0), mean serum levels of oestradiol were significantly higher in ewes with normal CL on days 1 to 2, and daily serum concentrations of progesterone were higher \( P < 0.05 \) on days 3 and 4 after ovulation.
10.5 Discussion

In the present study, treatment of non-prolific Western white-faced ewes with medroxyprogesterone acetate (MAP), in the absence of luteal progesterone, resulted in a significant increase in ovulation rate, by approximately 50%, in comparison to control ewes (3.1 ± 0.4 and 2.0 ± 0.3, respectively) and the control (pre-treatment) cycle of treated ewes (3.1 ± 0.4 and 1.9 ± 0.3, respectively). This increase was due mainly to the ovulation of follicles from the penultimate follicular wave before ovulation, and some follicles from earlier waves. There was also a greater percentage of follicles ovulating from the last wave before ovulation as compared to untreated controls (85 % vs. 74%), but the total number of ovulatory follicles in this wave did not vary among the treated and control ewes. The high ovulation rate in prolific Finn sheep is achieved by ovulation of ~50% of follicles that begin growing from 3 to ≥ 5 mm in diameter in the penultimate follicular wave and ovulate along with the follicles from the final wave of the oestrous cycle. The occurrence of 2 ovulatory waves has also recently been shown in 3/4 Rambouillet x 1/4 Booroola ewes (Gibbons et al., 1999). This contrasts with non-prolific Western white-faced ewes where very few ovulatory-sized follicles ovulate from the penultimate wave of the cycle. In addition, in Finn ewes 100% of ovulatory-sized follicles in the last wave ovulated, whereas only 77% of follicles ≥ 5 mm in size in the last follicular wave of the cycle in Western white-faced ewes were ovulatory. There is, therefore, a striking similarity in the pattern of ovulatory follicle recruitment between the present non-prolific Western white-faced ewes treated with PgF₂α/progestogen and normally cycling prolific Finn sheep.

It has been shown that creating subluteal levels of progesterone in cyclic ewes prolongs the lifespan of large antral follicles (Johnson et al., 1996; Vinoles et al., 1999). A similar effect is seen when luteolysis is induced with PgF₂α and progesterone is replaced by MAP released from intravaginal sponges (Flynn et al., 1999) or when MAP-impregnated sponges are inserted on day 12 after ovulation and ovaries are exposed to MAP in the absence of functional CL (Leyva et al., 1998). Therefore, the MAP treatment mimics a low, subluteal progesterone regimen. During the luteal phase, non-prolific Western white-faced
ewes exceed prolific Finn ewes in serum concentrations of progesterone. We suggest that treatment of non-prolific Western white-faced ewes with MAP after PgF$_{2\alpha}$-induced luteolysis at mid-cycle, created the equivalent of the "low progesterone environment" of cyclic Finn ewes, and hence promoted an increase in ovulation rate to that seen in Finn sheep during the breeding season (2.7 ± 0.2 ovulations/ewe).

In this experiment, the ovulatory follicles in the penultimate follicular wave of the treated ewes had significantly longer growing phases in comparison to ovulatory follicles in the last follicle wave, and before ovulation they grew to larger maximum diameters, as illustrated in Table 10.1. This is in agreement with previous studies in cattle (Adams et al., 1992) and sheep (Johnson et al., 1996; Flynn et al., 1999; Vinoles et al., 1999) describing the effects of submaximal levels of progesterone or a progestogen on the turnover of large antral follicles.

In earlier studies, prolongation of follicle lifespan was seen in ewes given PgF$_{2\alpha}$ early in the cycle with a subsequent treatment to create low serum concentrations of progesterone (Johnson et al., 1996; Vinoles et al., 1999) or with MAP (Flynn et al., 1999). The mechanism underlying extended follicle growth when the treatments started early in the cycle appeared to be an increase in LH pulse frequency (Flynn et al., 1999), similar to the explanation given for cattle (Stock and Fortune, 1993; Taft et al., 1996); however, LH pulse frequency was only measured once after 13 days of MAP treatment in sheep (Flynn et al., 1999). In the present study, a shorter treatment with MAP was applied from late in the cycle (~days 8 to 14 after ovulation) to encompass emergence of the last 2 follicular waves of the interovulatory interval, and no increase in LH pulse frequency, as determined 3 days into the 6-day treatment with MAP, was noted. In a previous study in which intravaginal MAP sponges were put in place for 12 days on day 12 after ovulation (i.e., just prior to natural luteolysis), prolonged follicle lifespan has been seen but LH pulse frequency did not change over the first 6 days of treatment and a numerical, but not significant, increase was seen after 12 days (Leyva et al., 1998). Therefore, the increased ovulation rate seen after PgF$_{2\alpha}$/MAP treatment commenced late in the cycle and applied for a short period of time, may not be dependent on changes in LH pulse frequency.
It is possible that lower than normal luteal phase levels of progesterone or progestogen treatment could have direct effects on the ovary. Certainly, the CL can exert local effects on developing follicles in sheep (Ginther, 1971; Dufour et al., 1971, 1972; Rexroad and Casida, 1977; Dailey et al., 1982) in spite of morphological barriers between the luteal and follicular compartments, and the high levels of progesterone in ovine follicular fluid (10- to 40-fold serum concentrations; Evans et al., 2000). Progesterone may also modify follicular responsiveness to gonadotrophins, in both luteal and non-luteal ovaries (Rexroad and Casida, 1977; McLeod and Haresign, 1984; Hunter and Southre, 1987; Scaramuzzi and Downing, 1999). It is also feasible that circulating concentrations of progesterone/progestogen could affect ovarian follicles indirectly, by extraovarian mechanisms not involving changes in gonadotrophin secretion. Luteal progesterone may alter the counter-transfer of hormones in the subovarian vascular plexus, probably via changes in oestrogen : progesterone ratios and their effects on the constriction of blood vessels (Koziorowski et al., 1989). In the present study, no differences were seen between treated and control ewes in serum concentrations of FSH, including the various characteristics of episodic peaks in daily serum concentrations of FSH.

In the ewes of the present study, injection of a luteolytic dose of PgF<sub>2α</sub> on ~day 8 after ovulation resulted in ovulation of some large follicles. Most ovulations occurred by day 3, but in 2 ewes ovulations were detected on days 4 and 5 after PgF<sub>2α</sub>. This was not prevented by treatment with MAP sponges inserted on the day of PgF<sub>2α</sub> injections. Breakthrough ovulations do occur in MAP- and CIDR-treated ewes (E.K. Inskeep - personal communication). In the present study, ovulations were detected ultrasonographically by the collapse of ovariatory-sized (≥ 5 mm in diameter) antral follicles that had been followed for several days; their disappearance was obvious, leaving only 2- and 3-mm follicles. A rapid decline in daily serum concentrations of oestradiol in the 6 ovulating ewes after PgF<sub>2α</sub>/progestogen treatment coincided with the day of ovulation (see also Fig. 10.1 and Fig. 10.5b). Interestingly, there were no differences in serum concentrations of FSH in samples taken daily during that period. Frequent blood sampling conducted on day 3 after PgF<sub>2α</sub> did not indicate the occurrence of phasic secretion of LH in the treated ewes nor differences in
any parameter of LH/FSH secretion between the treated and control animals. Prostaglandin 
F₂₆-induced luteolysis in the presence of MAP may have resulted in aberrant or truncated
periovulatory changes in LH secretion. Clearly, in ovulating ewes there is tremendous
variation in the magnitude and duration of the preovulatory LH discharge (Rawlings and
Cook, 1993). None of the ovulations above were followed by the formation of CL, as
evidenced by both ultrasonographic examination and radioimmunoassay of serum
progesterone. The failure of normal luteogenesis has previously been reported in ewes
induced to ovulate during the luteal phase (Rahmanian and Murdoch, 1987). Although MAP
effectively blocked behavioural oestrus and CL formation, its effect on the suppression of
ovulations appeared to be significantly less than that of luteal progesterone in the present
Western white-faced ewes.

Differences in circulating levels of progesterone or treatment with a progestogen in
the absence of luteal progesterone, did not alter the pattern of FSH secretion and follicular
wave emergence. However, peaks in daily serum concentration of oestradiol were only
associated with the development of follicular waves in the present control but not in treated
ewes. Therefore, the presence of endogenous progesterone appears to organize the rhythmic
pattern of oestradiol secretion in cyclic ewes; this role of progesterone was also evident in
a study in ewes during the transition from anoestrus to the breeding activity where the
correlation between follicle wave development and oestradiol secretion re-established
during the first full-length luteal phase of the ovulatory season. Alterations in the length of
follicle lifespan and the pattern of oestradiol production, within physiological levels, did not
appear to affect the endogenous rhythm of FSH release and the orderly emergence of
follicular waves in the present Western white-faced ewes. The latter is in agreement with
previous suggestions that follicular oestradiol in ewes may be relatively less important for
the regulation of FSH secretion than other follicular products (e.g., inhibin; Mann et al.,
1993; Souza et al., 1996).

Some earlier studies suggested that follicular dominance, as seen in cattle, is absent
or weaker in ewes (Castonguay et al., 1990; Driancourt et al., 1991; Driancourt, 1994), but
other reports indicated that functional dominance might be exerted by follicles in the first
wave of the cycle, which develop during increasing production of progesterone (Vinoles et al., 1999), or by the preovulatory follicles during the follicular phase (Ravindra et al., 1994; Lopez-Sebastian et al., 1999). Alternatively, a few researchers concluded that dominance in sheep is evident at all stages of the oestrous cycle (Rubianes et al., 1997b; Evans et al., 2000a) as well as during anoestrus (Rubianes et al., 1995). In this study, the presence of ovulatory-sized follicles with a prolonged lifespan in progestogen-treated ewes did not block or delay the emergence of subsequent follicular waves (Fig. 10.4), similar to earlier observations by Johnson et al. (1996) and Leyva et al. (1998). However, others reported that follicle wave emergence was suppressed after administration of PgF$_{2\alpha}$ and progestogen/low progesterone treatment early in the luteal phase of ewes (Flynn et al., 1999; Vinoles et al., 1999). Based on our present and earlier results, variations in follicle lifespan and interwave intervals during the sheep oestrous cycle appear to be due largely to changes in the circulating levels of progesterone, but follicle dominance in sheep is seemingly not exerted beyond the growth phase of the largest follicles of waves, because the emergence of successive follicular waves is associated with, or heralded by, the largest follicles in preceding waves entering the static phase of their lifespan or ovulating. The fact that 2 or more follicles emerging from the pool of 3-mm follicles in a wave (i.e., within $\leq$ 48 h) can attain ostensibly ovulatory diameters, also indicates that even during the growth phase of emergent follicles the suppression of follicle development by co-existing follicles is very weak or non-existent.

Impaired luteogenesis of some ovulated follicles was seen in both treated and control ewes after the final ovulation in the present study. Luteal inadequacy in ewes may be due to a lack of luteinization of some ovulated follicles (Rahmanian and Murdoch, 1987) or premature regression of CL (Beard and Hunter, 1994). A report by Mann and Lamming (1995) showed that in cattle, low serum concentrations of oestradiol around ovulation were associated with an earlier increase in an oxytocin receptor level and augmented PgF$_{2\alpha}$ response to oxytocin stimulation (i.e., stronger luteolytic signal). In the present study, mean serum levels of oestradiol were significantly higher in ewes producing as many CL as oovulations compared to animals with inadequate CL, on days 1 and 2 after ovulation, but
normal CL were produced in both groups of ewes in question. This suggests that luteal insufficiency may be a product of inadequate developmental competence of preovulatory follicles and/or the premature onset of luteolysis; this would explain the co-existence of apparently normal and inadequate CL in a ewe, or even in an ovary of a ewe.

Induction of multiple births has been identified as one of the primary goals in controlled sheep breeding (Gordon, 1996). The results of the present study may provide a basis for devising practical follicle manipulation techniques to increase ovulation rate in commercial flocks of sheep. However, in cattle there is evidence (Austin et al., 1999; Mihm et al., 1999), but not unequivocal evidence (Ahmad et al., 1997), that oocyte quality is compromised in follicles with an extended lifespan/dominance, resulting in low fertility. Recent studies suggest that this is not the case in sheep (Evans et al., 2000b), which means that the addition of prolonged lifespan follicles to the ovulatory cohort could effectively increase fertility. In the study by Johnson et al. (1996) fertility was depressed by a \( \text{Pgf} \text{2a} \)/low progesterone treatment in ewes, but in that study treatment was applied early in the cycle and for a longer duration (days 5 to 15 after ovulation), and did not result in ovulations of the largest ("oldest") follicles during the period of progesterone supplementation.

10.6 Conclusions

In conclusion, a \( \text{Pgf} \text{2a} \)/progestogenic regimen applied late in the cycle of non-prolific Western white-faced ewes increased the ovulation rate to that of prolific Finnish Landrace sheep mainly through maintenance of ovulatory-sized follicles in the penultimate and earlier waves, and their addition to ovulatory follicles from the final follicular wave before ovulation. The pattern of ovulatory follicle recruitment in the present non-prolific Western white-faced ewes treated with \( \text{Pgf} \text{2a} \)/progestogen closely resembled that in prolific Finn ewes. As serum progesterone concentrations are higher during the luteal phase in Western white-faced than in Finn sheep and as treatment with progestogen (MAP) appears to mimic the effects of low levels of progesterone, we concluded that the low serum progesterone concentrations in Finn ewes and \( \text{Pgf} \text{2a} \)/MAP treatment of Western white-faced ewes were
critical in determining the ovulation rate in both situations. We were unable to show a
dependency of this effect on LH pulse frequency, suggesting that the increased ovulation
rate was likely to be related to other direct or indirect effects, independent of changes in
gonadotrophin secretion.
Chapter 11. OVARIAN RESPONSES TO GnRH IN ANOESTROUS EWES: FOLLICULAR AND ENDOCRINE CORRELATES WITH LUTEAL OUTCOME

11.1 Abstract

The relationships between the development of antral follicles (growing to ≥ 5 mm in diameter), hormone secretion (LH, FSH, oestradiol and progesterone), ovulations and formation of luteal structures in response to GnRH were examined in 24 anoestrous Western white-faced ewes (May-July). The ewes were monitored by transrectal ovarian ultrasonography for 34 days, commencing 15 days before the administration of GnRH. Following the treatment with GnRH, 83% (20/24) ewes ovulated; 25% of all ewes (6/24) subsequently had normal (full-lifespan) CL, 37% (9/24) had inadequate CL, 17% (4/24) had both normal and inadequate CL, 17% of ewes (including 3 of 4 anovular ewes and 1 ewe with inadequate CL) formed luteinized follicles, and only 4% of ewes (1/24) did not ovulate or produce any luteal structure. None of the parameters of follicle growth (follicles reaching ≥5 mm in diameter) differed between the follicles that ovulated or failed to ovulate, and there was no evident correlation between the age or stage of development of ovulatory-sized antral follicles and the type of luteal structure formed, except for luteinized unovulated follicles; these follicles all emerged within 3 days of GnRH injections. Mean serum concentrations of FSH and oestradiol before treatment did not differ (P > 0.05) between ewes with different ovarian responses, but peaks of fluctuations in daily serum FSH concentrations were higher in ewes that produced normal CL only compared to ewes with inadequate CL only. After the GnRH treatment, oestradiol secretion was higher in ewes that formed luteinized unovulated follicles than in all ewes with inadequate CL (P < 0.05). The peak concentration of the GnRH-induced LH surge was higher and the interval from GnRH to the peak of LH discharge was shorter in ewes with inadequate CL only compared to ewes...
that had normal CL only after ovulation (P < 0.05). In conclusion, ovulatory-sized antral follicles at a similar stage of their lifespan can give rise to either normal or inadequate CL, and a proportion of these follicles do not ovulate in response to GnRH in seasonally anoestrous ewes. The normal luteinization of ovulated follicles does not appear to be influenced by the secretion of follicular oestradiol around the time of ovulation and during the later stages of the luteal phase, but high concentrations of oestradiol are associated with the formation of unovulated luteinized follicles in GnRH-treated anoestrous ewes. The amplitude of episodic elevations in daily serum FSH concentrations and characteristics of the preovulatory LH surge may both be critical for luteogenesis following ovulation.

11.2 Introduction

In some ewes, the CL that develops from the first ovulation after a period of "reproductive quiescence" (e.g., seasonal or lactational anoestrus, puberty) is short-lived (Hunter et al., 1988; Southey et al., 1988; Baird, 1992; Garverick et al., 1992). Similarly, following induction of ovulation with GnRH in anoestrous ewes, a proportion of ewes produce inadequate CL which secrete low levels of progesterone and/or undergo premature regression.

Not infrequently, the number of CL/luteal structures differs from that of developing embryos in pregnant ewes (Hulet et al., 1969; Bartleewski et al., 2000). A higher number of luteal structures compared with that of embryos may be explained, at least in some instances, by unfertilized ova and/or early embryonic death, but it may also result from luteinization of unovulated follicles, whereas a lower number of luteal structures may be due to a lack of luteinization of some ovulated follicles or premature regression of some CL. Recent studies using ovarian ultrasonography in ewes have revealed short-lifespan CL and luteinized follicles in otherwise normal oestrous cycles (i.e., with normal CL), and ovulations that do not result in CL. Premature luteal regression could be due to the premature induction of the luteolytic signal (oxytocin-induced uterine PGF₂α secretion; Beard and Hunter, 1994b). The diminished secretion of oestradiol by large antral follicles
before ovulation (Mann and Lamming, 1995) and/or increased oestradiol production during CL formation (Beard and Hunter, 1994b; Beard and Lamming, 1994) may both cause an earlier and stronger luteolytic signal. The apparent lack of luteinization may be due to inadequate development of preovulatory follicles. It has been suggested that subtle differences in the degree of follicle maturation at oestrus might account for the occurrence of atypical luteal structures and luteal inadequacy, regardless of the endocrine milieu to which preovulatory follicles were exposed (cattle: Carriere et al., 1995). Abnormal luteal function in the breeding season ewes may be a product of reduced developmental competence of preovulatory follicles (Haresign and Lamming, 1978; McNatty et al., 1981; McLeod et al., 1982b; Murdoch and Dunn, 1983; Legan et al., 1985b) rather than the premature onset of luteolysis; this would explain the co-existence of apparently normal and short-lived CL in a ewe or in an ovary of a ewe. Alternatively, the luteolytic signal resulting in premature luteal regression may be responsible for lysis of all or only some CL in ewes. The aetiology of luteal inadequacy in domestic ruminants remains ill-understood.

GnRH-induced ovulations in seasonally anovular ewes are a good model for the study of abnormal luteal function (McLeod et al., 1982a-b; Southey et al., 1988). The objective of the present experiment was to use transrectal ovarian ultrasonography and hormone measurements to see if the development of luteal structures was related to the stage of development of ovarian antral follicles induced to ovulate with GnRH and/or to the circulating concentrations of LH, FSH and ovarian steroids.

11.3 Materials and methods

11.3.1 Animals and experimental procedures

This study was performed during mid-anoestrus (May-July) on 24 Western White Face cross-bred ewes (aged between 3 and 6 years and weighing between 65 and 102 kg). Daily transrectal ultrasonography of ovaries was initially done for 15 days to record all follicles \( \geq 3 \) mm in diameter. On the 16th day of ultrasonography, all ewes received 250 ng of GnRH i.v. at 2-h intervals for 24 h followed by a bolus injection of 125 \( \mu \text{g} \) of GnRH i.v.
to induce a synchronous LH surge. Ewes were then examined by ultrasonography every 12 h (06:00 a.m. and 06:00 p.m.) for 6 days, to detect ovulations and monitor the formation of luteal structures and waves of follicle growth (follicles growing from 3 to ≥ 5 mm in size). Daily ovarian ultrasonography continued for 11 more days, or until the end of a 17-day period after the bolus injection of GnRH. Blood samples (10 ml) were collected prior to each ultrasound examination. In addition, all ewes were bled at 15 min intervals for 8 h after the bolus injection of GnRH, via indwelling jugular cannulae (vinyl tubing; 1.0 mm i.d., 1.5 mm o.d.; Critchley Electrical Products Pty Ltd., Auburn, NSW) inserted 24 h before frequent blood sampling.

11.3.2 Ovarian data summary

Initially, follicular data were combined for both ovaries of each ewe. Data from a ewe that ovulated before the bolus injection of GnRH were excluded from analyses. A follicular wave, the day of wave emergence, the growing, static and regressing phases of follicle growth curves, and the largest follicle of the wave have been previously defined. The static phase of luteinized anovulatory follicles was defined as the time from the end of the growth to ultrasonographically detectable luteinization of follicle walls. As in earlier studies, various characteristics of emergent follicles were determined for each ewe, for the duration of the observation period. Days of follicle emergence relative to the time of the bolus injection of GnRH and/or ovulation were also noted for each ewe.

Ovulation rates, mean numbers of luteal structures per ewe and the mean lengths of the lifespan of CL/luteinized follicles were determined for each ewe. Characteristics of detected luteal structures in the ovaries of ewes were analyzed as described previously.

11.3.3 Hormone analyses

The intra- and interassay coefficients of variation (CVs) for ovine reference sera analyzed for FSH (means: 2.61 or 4.19 ng/ml) were 7.5 and 8.7% or 4.3 and 5.5%, respectively; for LH (means: 0.26 or 1.19 ng/ml) were 9.8 and 11.3% or 6.4 and 6.9%,
respectively; for progesterone (means: 0.25 or 1.56 ng/ml) were 11.0 and 9.6% or 14.0 and 10.0%, respectively; and for oestradiol (means: 6.9 and 15.4 pg/ml) were 13.4 and 16.3% or 5.8 and 10.1%, respectively. The Area Under Curve (AUC) for serum concentrations of LH in samples taken after the bolus injection of GnRH was computed (NCSS Statistical Software; Kaysville, UT; http://www.ncss.com).

11.3.4 Statistical analyses

Preliminary inspection of the data revealed differences in ovulatory responses and the formation of luteal structures after the GnRH treatment. Ewes were therefore assigned to 1 of 6 response categories, namely (a), where each ewe ovulated and had normal (full-lifespan) CL (n = 5; NCL); (b), where each ewe ovulated and produced both normal and inadequate CL (n = 4; NCL + ICL); (c), where each ewe ovulated and only had inadequate CL (n = 9; ICL); (d), a ewe that had an inadequate CL after ovulation but also formed 2 luteinized unovulated follicles; (e), ewes that did not ovulate but each ewe subsequently formed a luteinized follicle (n = 3; LF); and (f), a ewe that did not ovulate and did not have any luteal structures detectable with ultrasonography. Inadequate CL or luteal inadequacy in this study were regarded as: 1) absence of ultrasonographically recordable CL after confirmed ovulation; or 2) CL detected and followed with ultrasonography for ≤ 7 days. Normal CL were defined as CL that formed from the remnants of ovulated follicles, were detected for ≥ 11 days, and regressed before or around the end of the 17 day scanning period after the GnRH treatment, as confirmed by ultrasonography and/or radioimmunoassay of progesterone. Out of 9 Western white-faced ewes with inadequate CL after GnRH, seven ewes had short-lifespan CL, detected for 1 to 7 days, one ewe had no detectable luteal structures after ovulation, and 1 ewe that ovulated 2 follicles only had 1 short-lived CL identifiable with ultrasonography.

For analysis, the study was divided into: 1) the 15 days prior to the GnRH treatment; 2) the period from 1 day before to 6 days after the beginning of the GnRH treatment; 3) the 11 day period of daily ultrasonography and blood sampling from 7 to 17 days after the
beginning of the GnRH treatment; and 4) the 17 day period of blood sampling after the end of transrectal ovarian ultrasonography in ewes. Statistical analyses were done, on a per ewe basis, to compare various hormonal and ovarian parameters between ewes that ovulated (n = 19) and ewes that failed to ovulate after GnRH (n = 4). Comparisons were also made based on luteal outcome; comparisons were made between ewes that ovulated and formed normal CL (n = 5; NCL), ewes that ovulated and had both normal and inadequate CL (n = 4; NCL + ICL), ewes that ovulated but only had inadequate CL (n = 9; ICL), and ewes that did not ovulate but formed luteinized follicles (n = 3; LF). RM-ANOVA (SigmaStat® Statistical Software, version 2.0 for Windows® 95, NT & 3.1, 1997; Chicago, IL) was used to determine main effects of group, time and the interaction of these terms.

Additional analyses were done in which the data for individual ovarian follicles ≥ 5 mm in diameter at the time of the GnRH bolus injection were used (Fig. 11.1). The characteristics of ovulatory and non-ovulatory antral follicles, and of follicles that formed normal or inadequate CL or luteinized unovulated follicles were compared by ANOVA (SigmaStat® 2.0, 1997). The following parameters were assessed: 1) the interval from emergence to the bolus injection of GnRH; 2) the interval from the bolus injection of GnRH to ovulation (ovulatory follicles) or luteinization of follicle walls (luteinized unovulated follicles); 3) durations of the growing and static phases of follicle growth curves; 4) maximum follicle diameter; and 4) the mean growth rate of the follicles.

The parameters of the GnRH-induced LH discharge determined by frequent blood sampling (samples taken every 15 min for 8 h after the bolus injection of GnRH), namely the maximum LH concentration, the time from the GnRH bolus injection to peak LH concentration, and the Area Under Curve (AUC) for serial LH concentrations, were compared among the 4 groups of ewes with different luteal structures by ANOVA (SigmaStat® 2.0, 1997).
Fig. 11.1. The grouping of individual ovarian antral follicles ≥ 5 mm in diameter at the time of the GnRH bolus injection that ovulated or failed to ovulate in response to the GnRH treatment, and of follicles that formed different types of luteal structures, as determined with ultrasonography in 23 anoestrous Western white-faced ewes.
Follicles ≥5 mm in size on the day of GnRH bolus injection that **ovulated** and **formed normal CL**
(13 follicles)

| from ewes with NCL only (9 follicles) | from ewes with NCL and ICL (4 follicles) |

Follicles ≥5 mm in size on the day of GnRH bolus injection that **ovulated** and **formed inadequate CL**
(19 follicles)

| from ewes with ICL only (13 follicles) | from ewes with ICL and NCL (5 follicles) | from a ewe with ICL and LF (1 follicle) |

Follicles ≥5 mm in size on the day of GnRH bolus injection that did **not ovulate**
(14 follicles)

| from ewes with NCL only (3 follicles) | from ewes with NCL and ICL (3 follicles) | from ewes with ICL only (5 follicles) | from a ewe with ICL and LF (1 follicle) | from a ewe with LF only (1 follicle) | from a ewe that did not ovulate (1 follicle) |

**Unovulated luteinized follicles**
(5 follicles)

| from ewes with LF only (3 follicles) | from a ewe with LF and ICL (2 follicles) |

NCL-normal (full-lifespan) CL
ICL-inadequate CL
LF-luteinized follicles
11.4 Results

11.4.1 Patterns of ovarian responses to GnRH

Nineteen ewes ovulated within 72 h of the bolus injection of GnRH, with a mean ovulation rate of $1.8 \pm 0.2$ ovulations/ewe (range: 1 to 3), and 4 ewes did not ovulate. Out of 19 ewes that ovulated, five ewes had normal CL only, four ewes had both normal and inadequate CL, nine ewes formed inadequate CL only, and 1 ewe that produced an inadequate CL after ovulation formed 2 luteinized unovulated follicles. Out of the 4 anovulatory ewes, three ewes formed luteinized follicles and 1 ewe did not have any luteal structures.

11.4.2 Ovulations and characteristics of luteal structures

After the GnRH treatment, the mean ovulation rate was higher ($P < 0.05$) in ewes that formed both normal and inadequate CL than in ewes with inadequate CL only. Mean ovulation rates were as follows - NCL: $1.8 \pm 0.4$; NCL + ICL: $2.2 \pm 0.2$; ICL: $1.4 \pm 0.2$ ovulations/ewe. In all groups of ewes above, ovulatory follicles originated in both the final and penultimate waves emerging before the GnRH treatment. The percentage of all ovulated follicles that emerged in the final or penultimate follicular waves before GnRH was 89, 62 and 77% or 11, 38 and 23%, for ewes with normal CL only, normal and inadequate CL, or inadequate CL only, respectively. In the 3 subsets of ewes above, a proportion of follicles $\geq 5$ mm in diameter on the day of the bolus injection of GnRH did not ovulate (31, 18 and 32% of follicles; NCL, NCL + ICL and ICL, respectively).

In all but two ovulating ewes, CL were detected by day 5 after the bolus injection of GnRH (range: 3 to 5 days) or by day 4 after ovulation (range: 2 to 4 days). The mean interval from the bolus injection to the detection of CL ($3.6 \pm 0.2$ days) and from ovulation to CL detection ($2.7 \pm 0.2$ days) did not differ ($P > 0.05$) between normal and inadequate CL. The mean lifespan of normal CL averaged $12.6 \pm 0.4$ days (range: 11 to 14 days), and of inadequate CL detected with ultrasonography - $2.0 \pm 0.4$ days (range: 1 to 7 days). The time from the bolus injection of GnRH to the ultrasonographic detection of the luteinization
of follicle walls (unovulated luteinized follicles) was $6.1 \pm 0.4$ days (range: 5 to 7 days), and the time from emergence to the luteinization of these follicles was $4.8 \pm 0.5$ days (range: 3.5 to 6 days). Mean lifespan of luteinized follicles was $10.8 \pm 0.7$ days (range: 9 to 13 days).

Mean daily total luteal volumes during the 17 days after the bolus injection of GnRH are shown, for the 4 groups of ewes with different luteal structures, in Fig. 11.2. The total luteal volumes were highest ($P < 0.05$) in ewes with normal CL only, were higher ($P < 0.05$) in ewes with both normal and inadequate CL than in ewes with inadequate CL only and ewes that formed luteinized follicles, and were higher ($P < 0.05$) in ewes with luteinized unovulated follicles than in ewes with inadequate CL only.

11.4.3 Follicle wave development in the 15 days before the GnRH treatment

The characteristics of follicular waves (follicles growing from 3 to $\geq 5$ mm in diameter before regression or ovulation) emerging before the GnRH treatment are shown, for different subsets of ewes, in Table 11.1. The mean number of follicular waves per ewe ($3.0 \pm 0.4$), the mean number of follicles per wave ($1.6 \pm 0.1$), and the duration of interwave intervals ($4.2 \pm 0.3$ days) did not differ ($P > 0.05$) between ovulating and non-ovulating ewes, but the largest follicles of waves grew to a greater maximum diameter, and the growing, static and regressing phases of the follicle lifespan were significantly longer in ovulating than in non-ovulating ewes ($P < 0.05$). The mean growth rate of the largest follicles of waves tended to be higher ($P = 0.07$) in non-ovulating than in ovulating ewes.

Ewes that formed luteinized unovulated follicles had fewer follicle waves and longer interwave intervals compared to ewes that had both normal and inadequate CL after the GnRH treatment ($P < 0.05$). The maximum size attained by the largest follicles of waves was lower ($P < 0.05$) in ewes that formed luteinized unovulated follicles than in ewes with both normal and inadequate CL post-treatment. The growing phase of the largest follicles of waves was longest for ewes with both normal and inadequate CL, and the static phase of follicle development was shortest in ewes with luteinized unovulated follicles after GnRH ($P < 0.05$).
Fig. 11.2. Mean (± SEM) total luteal volumes determined with ultrasonography in 23 GnRH-treated anoestrous Western white-faced ewes over the 17 days after the bolus injection on GnRH, and summarized for the 4 groups of ewes forming different luteal structures post-treatment - NCL: where each ewe ovulated and had normal (full-lifespan) CL; NCL + ICL: where each ewe ovulated and produced both normal and inadequate CL; ICL: where each ewe ovulated and only had inadequate CL; and LF: ewes that did not ovulate but each ewe subsequently formed a luteinized follicle.
Table 11.1. Characteristics of follicular waves (defined as follicles growing from 3 to ≥ 5 mm in diameter before regression or ovulation) detected with ultrasonography during the 15 day period prior to the GnRH treatment in 23 anoestrous Western white-faced ewes. The grouping of ewes with different luteal structures after the GnRH treatment is as follows - NCL: where each ewe ovulated and had normal (full-lifespan) CL; NCL + ICL: where each ewe ovulated and produced both normal and inadequate CL; ICL: where each ewe ovulated and only had inadequate CL; and LF: ewes that did not ovulate but each ewe subsequently formed a luteinized follicle. All values are mean ± SEM.

<table>
<thead>
<tr>
<th>End point</th>
<th>Ovulating ewes (n = 19)</th>
<th>Non-ovulating ewes (n = 4)</th>
<th>NCL (n = 5)</th>
<th>NCL + ICL (n = 4)</th>
<th>ICL (n = 9)</th>
<th>LF (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of follicular waves/ewe</td>
<td>3.5 ± 0.2</td>
<td>2.5 ± 0.9</td>
<td>3.4 ± 0.2&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.0 ± 0.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.3 ± 0.4&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>2.0 ± 1.0&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Number of follicles/wave</td>
<td>1.7 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td><strong>Largest follicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>6.0 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.8 ± 0.2&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>6.5 ± 0.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.9 ± 0.2&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>5.3 ± 0.2&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Growing phase (days)</td>
<td>3.5 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9 ± 0.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>4.3 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9 ± 0.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.9 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Static phase (days)</td>
<td>2.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3 ± 0.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.4 ± 0.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.1 ± 0.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Regressing phase (days)</td>
<td>3.1 ± 0.2</td>
<td>2.4 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9 ± 0.4</td>
<td>2.9 ± 0.7</td>
<td>3.2 ± 0.2</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>1.1 ± 0.06</td>
<td>1.5 ± 0.2</td>
<td>1.0 ± 0.09</td>
<td>0.9 ± 0.1</td>
<td>1.2 ± 0.09</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Interwave interval (days)</td>
<td>4.1 ± 0.2</td>
<td>4.4 ± 0.6</td>
<td>3.9 ± 0.4&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>3.8 ± 0.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>4.1 ± 0.3&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>5.0 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means denoted by different letter superscripts are different (P < 0.05):<sup>ab</sup> between ovulating and non-ovulating ewes;<sup>AB</sup> between the 4 groups of ewes with different luteal structures.
11.4.4 Characteristics of ovulatory and non-ovulatory antral follicles and of follicles that gave rise to different luteal structures

The time from emergence to the bolus injection of GnRH did not differ (P > 0.05) between anovulatory follicles (3.4 ± 0.6 days) and the follicles that ovulated and subsequently formed normal (4.0 ± 0.4 days) or inadequate CL (2.9 ± 0.5 days), but the follicles that luteinized emerged later compared to all ovulatory-sized follicles that ovulated or regressed after GnRH (0.2 ± 0.9 days after the bolus injection of GnRH; P < 0.05). The time from emergence to ovulation (5.0 ± 0.5 days) and the time from the GnRH bolus injection to ovulation (28 ± 3 h) did not vary (P > 0.05) between ovulatory follicles that formed normal or inadequate CL. The duration of follicle growing phases (3.1 ± 0.5 days) did not differ among the 4 groups of follicles (P > 0.05), but the static phase of follicle lifespan was longer (P < 0.05) for the follicles that formed inadequate CL (3.3 ± 0.5 days) compared to anovulatory and luteinized unovulated follicles (1.7 ± 0.5 and 1.4 ± 0.4 days, respectively). The mean growth rate and maximum follicle size were greatest (P < 0.05) for the luteinized unovulated follicles (growth rate: 1.2 ± 0.2, 1.3 ± 0.1, 1.0 ± 0.09 and 2.6 ± 0.7 mm/day; maximum follicle diameter: 6.1 ± 0.4, 6.3 ± 0.3, 5.9 ± 0.3 and 11.0 ± 1.0 mm; for individual follicles forming normal CL, inadequate CL, anovulatory follicles and luteinized unovulated follicles, respectively).

11.4.5 Serum concentrations of FSH, oestradiol and progesterone over the 15 days before the GnRH treatment

During the 15 day period prior to the GnRH treatment, mean daily concentrations of FSH (1.85 ± 0.22 ng/ml) did not vary (P > 0.05) between the ewes that ovulated and those that did not ovulate in response to GnRH, nor among the 4 groups of ewes that subsequently formed different luteal structures. Peaks of transient increases in daily serum concentrations of FSH were detected in all ewes studied (range: 3 to 5). None of the parameters of detected FSH peaks/fluctuations varied between ovulating and non-ovulating
ewes (P > 0.05) but mean peak concentrations of FSH fluctuations were higher (P < 0.05) in ewes with normal CL only than for ewes that only had inadequate CL (2.69 ± 0.17 and 2.15 ± 0.10 ng/ml, respectively).

Mean daily serum concentrations of oestradiol (3.1 ± 0.6 pg/ml) did not differ (P > 0.05) between ovulating and non-ovulating ewes nor between ewes allocated to the 4 groups of ewes differing in luteal outcomes. Peaks in daily serum oestradiol concentrations were identified in 22 of 23 ewes studied (no peaks were detected in a single ewe with inadequate CL). None of the parameters of oestradiol peaks/fluctuations differed between ovulating and non-ovulating ewes nor between ewes allocated to the 4 groups of ewes above (P > 0.05).

No CL/luteal structures were detected with ultrasonography and serum concentrations of progesterone were basal in all ewes prior to the GnRH treatment. Mean daily concentrations of progesterone (0.05 ± 0.008 ng/ml) did not differ (P > 0.05) between ovulating and non-ovulating ewes nor between the ewes that subsequently formed different luteal structures.

11.4.6 Characteristics of the GnRH-induced LH surge

None of the parameters of the GnRH-induced LH surge (Table 11.2) differed among ovulating and non-ovulating ewes (P > 0.05). The maximum LH concentration was higher for ewes with inadequate CL only than for ewes that only had normal CL after ovulation (P < 0.05). The interval from the GnRH bolus injection to the peak of LH surge was shorter for ewes with inadequate CL only than for ewes with normal CL only (P < 0.05). Mean Area Under Curve (AUC) for serum LH concentrations during the 8 h period after the bolus injection of GnRH was significantly lower for ewes with both normal and inadequate CL after ovulation compared to ewes with inadequate CL only and anovular ewes that formed luteinized uniovulated follicles.
Table 11.2. Characteristics of the GnRH-induced LH surge determined in serum samples collected every 15 min for 8 h after the bolus injection of GnRH in 23 anoestrous Western white-faced ewes. The grouping of ewes with different luteal structures after the GnRH treatment is as follows - NCL: where each ewe ovulated and had normal (full-lifespan) CL; NCL + ICL: where each ewe ovulated and produced both normal and inadequate CL; ICL: where each ewe ovulated and only had inadequate CL; and LF: ewes that did not ovulate but each ewe subsequently formed a luteinized follicle. All values are mean ± SEM.

<table>
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<tr>
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<th>NCL + ICL (n = 4)</th>
<th>ICL (n = 9)</th>
<th>LF (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum LH concentration (ng/ml)</td>
<td>28.1 ± 5.1</td>
<td>23.0 ± 6.5</td>
<td>19.0 ± 2.8a</td>
<td>31.7 ± 16.7b</td>
<td>33.2 ± 7.9b</td>
<td>26.9 ± 7.5ab</td>
</tr>
<tr>
<td>Time from GnRH bolus injection to the peak of LH surge (pg/ml)</td>
<td>103 ± 19</td>
<td>105 ± 20</td>
<td>159 ± 69a</td>
<td>105 ± 18b</td>
<td>75 ± 5b</td>
<td>110 ± 27ab</td>
</tr>
<tr>
<td>Area Under Curve (AUC) for serum LH concentrations during the 8-h period after the bolus injection of GnRH (square units)</td>
<td>3554 ± 489</td>
<td>3837 ± 1433</td>
<td>3112 ± 663ab</td>
<td>2377 ± 306a</td>
<td>4571 ± 842b</td>
<td>4607 ± 1109ab</td>
</tr>
</tbody>
</table>

Means denoted by different letter superscripts are different (P < 0.05): ab between the 4 groups of ewes with different luteal structures.
11.4.7 Serum concentrations of oestradiol and progesterone from 1 day before to 6 days after the beginning of the GnRH treatment

Circulating levels of oestradiol were significantly higher in non-ovulating than in ovulating ewes from 3 to 4 days after the bolus injection of GnRH (group by time interaction: \( P < 0.05 \)). Three days after the bolus injection of GnRH, ewes that formed luteinized follicles exceeded \( (P < 0.05) \) all ewes with inadequate CL in mean serum concentrations of oestradiol (Fig. 11.3a).

Mean serum concentrations of progesterone did not differ \( (P > 0.05) \) between ovulating and non-ovulating ewes, as 3 of 4 ewes that failed to ovulate formed luteinized follicles and only 1 ewe did not have any luteal structures, but mean progesterone concentrations differed between the 4 groups of ewes studied (group effect: \( P < 0.05 \); group by time interaction: \( P < 0.001 \); NCL: 0.22 ± 0.03; NCL + ICL: 0.18 ± 0.04; ICL: 0.08 ± 0.02; and LF: 0.16 ± 0.04 ng/ml; Fig. 11.3b). The mean value was higher \( (P < 0.05) \) for ewes that had only normal CL compared to ewes with inadequate CL only. The difference between the 2 groups above was significant from 4 to 5.5 days after the bolus injection of GnRH. In addition, 5.5 days after the bolus injection of GnRH, ewes with luteinized unovulated follicles had higher \( (P < 0.05) \) circulating concentrations of progesterone than ewes with inadequate CL only.

11.4.8 Serum concentrations of oestradiol and progesterone from 7 to 18 days after the beginning of the GnRH treatment

The difference in mean serum concentrations of oestradiol between ovulating and non-ovulating ewes approached significance \( (2.5 ± 0.3 \) and \( 4.0 ± 0.7 \) pg/ml, respectively; group effect: \( P = 0.07 \)). When mean daily concentrations of oestradiol for the groups of ewes that produced different luteal structures were considered, the difference between ewes with luteinized unovulated follicles and all other groups approached significance \( (P ≤ 0.08; \) NCL: 2.9 ± 0.6; NCL + ICL: 2.4 ± 0.7; ICL: 2.5 ± 0.5; and LF: 4.5 ± 0.8 pg/ml).
Table 11.3. Characteristics of peaks/fluctuations in serum oestradiol (E$_2$) concentrations in blood samples collected daily during the 11 days, from 7 to 17 days after the bolus injection of GnRH, in 23 anoestrous Western white-faced ewes. The grouping of ewes with different luteal structures after the GnRH treatment is as follows - NCL: where each ewe ovulated and had normal (full-lifespan) CL; NCL + ICL: where each ewe ovulated and produced both normal and inadequate CL; ICL: where each ewe ovulated and only had inadequate CL; and LF: ewes that did not ovulate but each ewe subsequently formed a luteinized follicle. All values are mean ± SEM.

<table>
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<tr>
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<th>Non-ovulating ewes (n = 4)</th>
<th>NCL (n = 5)</th>
<th>NCL + ICL (n = 4)</th>
<th>ICL (n = 9)</th>
<th>LF (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of peaks/ewe</td>
<td>1.9 ± 0.3</td>
<td>2.2 ± 0.6</td>
<td>1.8 ± 0.5</td>
<td>2.0 ± 0.4</td>
<td>1.9 ± 0.5</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>Peak concentration (pg/ml)</td>
<td>3.8 ± 0.3$^a$</td>
<td>5.1 ± 0.5$^b$</td>
<td>4.2 ± 0.8$^{AB}$</td>
<td>3.3 ± 0.3$^A$</td>
<td>4.1 ± 0.4$^{AB}$</td>
<td>5.0 ± 0.5$^B$</td>
</tr>
<tr>
<td>Duration of fluctuations (days)</td>
<td>3.2 ± 0.2$^a$</td>
<td>4.0 ± 0.4$^b$</td>
<td>3.2 ± 0.5$^{AB}$</td>
<td>2.9 ± 0.3$^A$</td>
<td>3.3 ± 0.3$^{AB}$</td>
<td>3.7 ± 0.4$^B$</td>
</tr>
<tr>
<td>Interpeak intervals (days)</td>
<td>4.5 ± 0.5</td>
<td>3.8 ± 0.7</td>
<td>4.5 ± 0.9</td>
<td>4.2 ± 0.8</td>
<td>4.0 ± 0.8</td>
<td>3.8 ± 0.7</td>
</tr>
</tbody>
</table>

Means denoted by different letter superscripts are different (P < 0.05): $^{ab}$ between ovulating and non-ovulating ewes; $^{AB}$ between the 4 groups of ewes with different luteal structures.
Fig. 11.3. Mean (± SEM) serum concentrations of oestradiol (a) and progesterone (b) in blood samples collected from 1 day before to 6 days after the beginning of the GnRH treatment in the 4 groups of anoestrous Western white-faced ewes that formed different luteal structures - NCL: where each ewe ovulated and had normal (full-lifespan) CL; NCL + ICL: where each ewe ovulated and produced both normal and inadequate CL; ICL: where each ewe ovulated and only had inadequate CL; and LF: ewes that did not ovulate but each ewe subsequently formed a luteinized follicle. The line in the upper chart area denotes the 24-h period during which GnRH injections were given every 2 h (250 ng/ewe i.v.) prior to the bolus injection of GnRH (125 μg/ewe i.v.). See text for statistical details.
Fig. 11.4. Mean (± SEM) serum concentrations of progesterone in blood samples collected from 7 to 17 days after the beginning of the GnRH treatment in the 4 groups of anoestrous Western white-faced ewes that formed different luteal structures - NCL: where each ewe ovulated and had normal (full-lifespan) CL; NCL + ICL: where each ewe ovulated and produced both normal and inadequate CL; ICL: where each ewe ovulated and only had inadequate CL; and LF: ewes that did not ovulate but each ewe subsequently formed a luteinized follicle. See text for statistical descriptions.
Peaks of fluctuations in daily serum concentrations of oestradiol were identified in 21 of 23 ewes (range: 1 to 4; Table 11.3). One ewe with normal CL and 1 ewe with inadequate CL after ovulation did not have any detectable peaks of oestradiol secretion. The number of peaks per ewe (2.0 ± 0.4) and the interval between sequential oestradiol peaks (4.1 ± 0.6 days) did not differ (P > 0.05) among ovulating and non-ovulating ewes, but mean peak concentrations and the duration of oestradiol fluctuations were lower (P < 0.05) in ovulating than in non-ovulating ewes. There were no differences in mean numbers of oestradiol peaks per ewe (2.1 ± 0.5) nor the length of interpeak intervals (4.1 ± 0.8 days) between the 4 groups of ewes with different luteal structures, but mean peak concentrations and the duration of oestradiol fluctuations were lower (P < 0.05) in ewes with both normal and inadequate CL than in ewes that had luteinized unovulated follicles.

Circulating concentrations of progesterone did not vary (P > 0.05) between ovulating and non-ovulating ewes, but progesterone concentrations were significantly lower in ewes that only had inadequate CL compared to all other groups of ewes (Fig. 11.4). The ewes with normal CL and the ewes that had both normal and inadequate CL exceeded the ewes that only had inadequate CL in mean daily serum concentrations of progesterone from days 6 to 13 (P < 0.05). Ewes that had luteinized unovulated follicles had higher (P < 0.05) concentrations of progesterone compared to the ewes with inadequate CL only, from days 6 to 12 after the bolus injection of GnRH. Mean daily concentrations of progesterone did not differ significantly between ewes with normal CL only, both normal and inadequate CL, and inadequate CL only, but mean progesterone concentrations tended to be higher (P = 0.05) in ewes with normal CL only than in ewes with luteinized follicles, on days 6, 12 and 13 after the bolus injection of GnRH.

11.5 Discussion

The present results are in agreement with earlier ultrasonographic observations of Western white-faced ewes in the breeding season, in that: 1) a proportion of antral follicles attaining an ostensibly ovulatory size of ≥ 5 mm in diameter at the time of ovulation of
other follicles fail to ovulate; 2) some ewes may produce both normal and inadequate CL after ovulation; and 3) the luteinization of unovulated antral follicles may occur.

The reasons for the failure of ovulation, impaired luteogenesis of ovulated follicles and shortened lifespan of the inadequate CL in ewes have yet to be determined. In earlier studies, the occurrence of inadequate CL after GnRH-induced ovulations in anoestrous ewes was attributed mainly to the premature onset of luteolysis (Beard and Hunter, 1994b), insufficient luteotrophic support post-ovulation (O’Shea et al., 1984; Legan et al., 1985), or inability of developing CL to utilize gonadotrophins because of diminished levels of luteal LH receptors (Kesler et al., 1981). It has also been suggested that luteal insufficiency in ruminants may result from inadequate follicle development before the induction of ovulation with GnRH (Haresign and Lamming, 1978; McNatty et al., 1981; McLeod et al., 1982b; Legan et al., 1985). The co-existence of inadequate and full-lifespan CL in 17% of the present anoestrous ewes following the treatment with GnRH, indicates that luteal inadequacy is most likely due to the differences in the ability of individual ovarian follicles to respond to endocrine signals by ovulating and developing into healthy CL rather than the premature luteal regression. The failure of ovulation of some follicles that reached an ostensibly ovulatory diameter at the time of GnRH treatment is also supportive of the hypothesis that variable ovarian responses in the present anoestrous ewes were caused by the differences in the degree of follicular maturation. The fact that even in face of suppressed LH secretion during anoestrus in ewes a proportion of all ovulated follicles formed full-lifespan CL and some anovulatory follicles underwent luteinization, appears to rule out diminished luteotrophic support as an exclusive reason for luteal inadequacy. It is possible that the premature luteolytic signal may have resulted in regression of all or only some of the forming CL; however, an apparent lack of luteinization of some ovulated follicles and the short lifespan (≤ 2 days) of most inadequate CL detected with ultrasonography, suggest that incomplete follicular maturation was the most plausible explanation of the abnormal luteal function after ovulation induction in the present anoestrous ewes.
In the present study, none of the parameters of follicle growth (follicles growing from 3 to ≥ 5 mm in diameter) differed between individual follicles that ovulated or failed to ovulate after GnRH, and there was no apparent relationship between the age of large antral follicles and the type of luteal structure formed, except for luteinized unovulated follicles. These follicles all emerged within 3 days of the GnRH treatment and most of them started growing from 3 mm onwards after the bolus injection of GnRH.

The largest follicles of waves recorded during the 15 days before the GnRH treatment of the present anoestrous ewes grew to a greater maximum diameter and had a longer lifespan in ovolating than in non-ovulating ewes. Although these differences appeared to be due largely to the differences between ewes with both normal and inadequate CL and ewes that had luteinized follicles after GnRH, the duration of the follicle static phase in 3 anovular ewes that formed luteinized follicles was significantly shorter in comparison to all ewes that ovulated after GnRH (Table 11.2). In a recent study in Spanish Merino ewes treated with a PgF$_{2\alpha}$ analogue (Cloprostrenol) and FSH-P on day 13 of the oestrous cycle (Lopez-Sebastian et al., 1999), the ovulatory response was related to the growth characteristics of the largest antral follicles detected on the day of treatment. If the largest follicle did not change its diameter by ≥ 1 mm during a 24 h period after the treatment then 78% of ewes ovulated > 3 follicles, but if the largest follicle increased or decreased in size immediately after the treatment, only 33% of ewes had > 3 ovulations. These results may be interpreted to suggest that the optimal time for ovulation is when the largest ovine follicles enter the static phase of their lifespan, whereas the presence of follicles in their growing or regressing phases is associated with lower ovulation rates. The stage of follicle development may, therefore, determine the ability of all large antral follicles to ovulate, particularly after ovulation induction in ewes. Such a possibility was also suggested by Houghton et al. (1994) and Rubianes et al. (1997a-b) in ewes treated with PgF$_{2\alpha}$ and/or GnRH at different stages of the luteal phase. As the largest emergent follicles in non-ovulating ewes were characterized by shorter static phases compared with ovulating ewes prior the GnRH treatment in this study (1.3 ± 0.1 vs. 2.7 ± 0.2 days, respectively), there is a probability that all ovarian follicles ≥ 5 mm in diameter at the time of the bolus GnRH
injection in non-ovulating ewes may have been in the transition into their regressing phase; this could partly explain the failure of ovulation.

In an earlier study by Carriere et al. (1995), ovarian function in cattle was monitored by ultrasonography following a single injection of Cloprostenol and oestradiol valerate on either day 17, 18 or 19 of the oestrous cycle. It was suggested that differences in the degree of follicle maturation at the time of treatment led to the array of ovarian responses seen, including a premature ovulation resulting in a CL which persisted for \( > 30 \) days, ovulation of hypertrophic ovarian follicles, development of several waves of follicle cysts or luteinization of an unovulated cystic-like follicle. As in the present anoestrous ewes the age and stage of development did not vary significantly between ovulated follicles forming different luteal structures, we concluded that the differences in the ability to utilize gonadotrophic hormones (e.g., FSH/LH receptor levels) and/or the synthesis of intraovarian regulators of folliculogenesis (Roche, 1996; Khalid et al., 1997; Monniaux et al., 1997; Webb et al., 1999) might be responsible for the fate of large antral follicles after the GnRH treatment. Seemingly, ovine antral follicles of the same size and phase of development differ in their responsiveness to gonadotrophins.

LH plays a key role in the control of ovulation and CL function (Niswaender and Nett, 1988; Baird, 1992). The synthesis of follicular LH receptors is stimulated by both FSH and oestradiol (Uilenbroek and Richards, 1979; England et al., 1981). In the ewes of the present study, mean peak concentrations of daily serum FSH concentrations before the GnRH treatment were higher in ewes with normal CL than in ewes that had only inadequate CL after GnRH. Peaks of FSH secretion in ewes occur just before the emergence of follicle waves and they also coincide with the end of the growth phase of the largest follicles of waves. The difference in the height of FSH peaks could account for the formation of either normal or inadequate CL in the 2 subgroups of ewes above; this might be due to effects of FSH on the synthesis of LH receptors in preovulatory follicles. However, the occurrence of both normal and inadequate CL in some ewes and the fact that a proportion of follicles reaching \( \geq 5 \) mm in diameter in all ewes studied did not ovulate after GnRH, suggest that differences in serum FSH concentrations are not solely responsible for ovulation rate and
subsequent development of luteal structures. Again, variations in the degree of follicle responsiveness to FSH/LH could have resulted in different responses to GnRH in the ewes of the present study.

The present observations indicate that discrete patterns of the GnRH-induced LH surge may be important for the luteinization of ovulated follicles. In the ewes that had only inadequate CL after ovulation, the peak concentrations of the LH discharge were significantly greater but the time from the GnRH bolus injection to LH peak was shorter compared with the ovulating ewes that only produced normal CL. Mean values for these 2 parameters were intermediate for the ewes with both normal and inadequate CL post-treatment. The total amount of LH released over the 8 h period after the GnRH bolus injection was probably less important for the ensuing luteogenesis, as the estimates of Area Under Curve (AUC) for serial LH concentrations during that period did not vary between ewes with normal or inadequate CL. However, the ewes that had both normal and inadequate CL appeared to secrete less LH into the LH surge than ewes that had only inadequate CL or non-ovulating ewes with luteinized follicles.

Earlier research implicated follicular oestrogens in the aetiology of luteal inadequacy in ruminants. It has been proposed that low levels of circulating oestradiol around ovulation (Mann and Lamming, 1995) or a high concentration of oestradiol early in the luteal phase (Beard and Lamming, 1994) are associated with premature luteolysis. The present results do not support this notion. The pattern of oestradiol secretion prior to ovulation and during the formation of luteal structures did not differ between anoestrous Western white-faced ewes with normal and inadequate CL after the GnRH treatment. The only significant differences in mean serum concentrations of oestradiol in this study were those between ovulating and non-ovulating ewes at 3-4 days after the bolus injection of GnRH, or between ewes that formed luteinized unovulated follicles (3 anovular ewes) and animals with both normal and inadequate CL or inadequate CL only, at 3 days post-treatment. These differences probably reflect the presence of hypertrophic antral follicles emerging around the time of GnRH treatment which subsequently luteinized. It is intriguing that the presence of highly oestrogenic follicles did not preclude the formation of luteal tissue. On the
contrary, it appears that increased production of oestradiol, in the absence of fully-functional CL, may have stimulated the luteinization of large unovulated follicles in anoestrous ewes.

It has been suggested that progesterone may affect subsequent luteal function by modulating the development of preovulatory follicles, since as little as 2 days of exposure to elevated levels of progesterone (McLeod and Haresign, 1984; Legan et al., 1985b; Hunter et al., 1986; Hunter and Southoe, 1987) or as much as 12 days of progesterone priming of anoestrous ewes (McLeod and Haresign, 1984), followed by ovulation induction with GnRH, result in full-length luteal phases. Progesterone increases the growth rate but hastens the regression of large antral follicles in anoestrous ewes, through a local/intraovarian mechanism (i.e., following intraovarian injections of progesterone; Rexroad and Cassida, 1977). An increase in LH receptor content of large oestrogenic follicles was observed in GnRH-treated anoestrous ewes after progesterone priming (Khalid et al., 1997). However, in a previous study in Western white-faced ewes during the transition from anoestrus to the breeding activity, it was shown that a transient increase in progesterone secretion preceding the first ovulation of the breeding season did not affect the growth characteristics of preovulatory follicles. It was suggested that endogenous progesterone was important mainly for inducing oestrous behaviour and synchronizing it with the preovulatory LH surge (Pearce et al., 1985), and for preventing premature luteolysis during the ensuing luteal phase by reducing the uterine response to oxytocin (Beard and Hunter, 1996). In this study, only 42% of all anoestrous ewes receiving GnRH without prior progesterone priming produced full-lifespan CL. Based on this and previous results, it appears that increased serum levels of progesterone before ovulation may enhance the number of full-lifespan CL after ovulation induction in anoestrous ewes, possibly through its direct actions on large antral follicles (e.g., increased LH binding; Khalid et al., 1997), but without apparent alterations in circulating concentrations of oestradiol or growth patterns of preovulatory follicles. The combined effects of progesterone on the preovulatory antral follicles and the uterine luteolytic apparatus may be the primary mechanism during the transition from anoestrus to the breeding season in ewes.
11.6. Conclusions

In summary, large antral follicles of a similar age and stage of development can ovulate and give rise to either normal or inadequate CL, and a proportion of these follicles fail to ovulate after the GnRH treatment in anoestrous ewes. This suggests that ovine antral follicles of the same size and phase of the lifespan vary in their responsiveness to gonadotrophic hormones. The present results indicate that the amplitude of periodic increases in daily serum concentrations of FSH and the pattern of the GnRH-induced LH surge may both be critical for normal luteinization of ovulated follicles. Therefore, both the physiological status of the follicle and gonadotrophic stimuli before and at the time of treatment may be important for the fate of ovulatory-sized antral follicles after the administration of GnRH in anoestrous ewes. Serum levels of oestradiol appear to play a relatively less important role in the regulation of ovulatory response and the aetiology of luteal insufficiency compared to other hormonal factors (i.e., gonadotrophic hormones), but elevated concentrations of oestradiol are associated with the formation of unovulated luteinized follicles in GnRH-treated anoestrous ewes.
Chapter 12. SUMMARY DISCUSSION, CONCLUSIONS AND FUTURE STUDIES

12.1 Summary discussion

The results of experiments described in this thesis answered several questions regarding the pattern of ovarian antral follicle development, luteal function and their hormonal regulation in the ewe. At all stages of the breeding season, the growth of the largest ovarian follicles (attaining $\geq 5$ mm in diameter before regression or ovulation) exhibited a distinct wave-like pattern. This is in accordance with earlier observations using repeated laparoscopy in Suffolk ewes (Noel et al., 1993) and ovarian ultrasonography in cycling Polypay ewes (Ginther et al., 1995). During the mid-breeding season in normally cycling Western white-faced (non-prolific) and Finn sheep (prolific breed), there were either 3 or 4 waves of follicle emergence per interovulatory interval. In both breeds, this pattern of follicular development was found to be closely associated with periodic elevations in daily serum concentrations of FSH. Peaks of transient increases in serum FSH concentrations were detected in all animals, and peak concentrations occurred on days approximating follicle wave emergence. In addition, follicular wave emergence was associated with the onset of transient increases in serum concentrations of oestradiol, and the end of the growth phase of the largest follicles of successive waves coincided with peak serum concentrations of oestradiol. From the studies described in Chapters 5 and 7 in this thesis, it appears that the initial stage of antral follicle growth (follicle emergence) is primarily controlled by FSH, but subsequent follicular development and demise are independent of FSH fluctuations in ewes.

The wave-like pattern of antral follicle growth in sheep differs from the orderly emergence of follicular waves in cattle (Ginther et al., 1996) in that emergence of sequential
waves in ewes occurs more frequently and there is little evidence for follicular dominance. Follicular waves in ewes consist of 1-3 follicles attaining a similar final stage of development. It has been suggested that the largest (“dominant”) follicles beginning to grow early in the luteal phase (Vinoles et al., 1999) and preovulatory follicles during the follicular phase of the cycle (Ravindra et al., 1994) may suppress the growth of their “subordinates”. However, the fact that more than one follicle acquires the ability to reach an ostensibly ovulatory size in a single wave suggests that deviation does not occur and dominance is weak or absent in the ewe. In cyclic Western white-faced ewes, there was a divergence in the growth pattern of the largest follicles of waves between the first and subsequent waves of follicle growth; the largest follicles in the first wave of the cycle had a longer lifespan than all non-ovulatory follicles emerging during dioestrus (Chapter 5). A similar tendency was noted in cyclic Finn sheep. This difference appears to be due primarily to changes in serum concentrations of progesterone across the ewe’s oestrous cycle; the prolonged follicle growth early in the luteal phase may be associated with the presence of developing or non-fully functional CL secreting low levels of progesterone. Whether these effects of subluteal concentrations of progesterone on antral follicle lifespan are local or systemic (i.e., mediated by changes in pulsed secretion of gonadotrophic hormones) remains to be determined.

The last follicular wave of the oestrous cycle contained ovulatory follicles in all ewes and the penultimate in only 10% of white-faced ewes but in 57% of Finn sheep (Chapter 5). There was also a greater percentage of ovulatory follicles in the final wave of the cycle in Finn (100%) as compared to Western white-faced ewes (77%). The increased ovulation rate in prolific Finn ewes appeared to be due primarily to an extended period of ovulatory follicle recruitment; 50% of all emergent follicles in the penultimate wave of the cycle in Finn ewes were maintained and added to the cohort of ovulatory follicles originating in the final wave. The existence of two ovulatory waves has also recently been shown in Rambouillet x Booroola ewes (Gibbons et al., 1999). The mechanism underlying the dissimilar patterns of ovulatory follicle recruitment in non-prolific Western white-faced and prolific Finn sheep is not known. Serum FSH concentrations were higher in Finn than in Western white-faced ewes during the follicular phase of the cycle and mean serum
concentrations of oestradiol were higher in Finn ewes both during the luteal phase and prior to ovulation. However, there is no consensus as to the role of FSH and oestradiol in determining the ovulation rate in the ewe (Webb et al., 1999). A number of earlier studies failed to provide evidence for pituitary gonadotrophins and follicular oestradiol being responsible for the difference in ovulation rate in sheep (Webb et al., 1999).

There is now increasing evidence that luteal progesterone can affect the growth of antral follicles in the ovary of the ewe, both through intra- and extraovarian mechanisms (Rexroad and Casida, 1987; Johnson et al., 1996; Flynn et al., 1999; Vinoles et al., 1999). In this context, and because of generally accepted knowledge that prolific animals have higher progesterone concentrations than ewes with lower ovulation rates (Quirke et al., 1979), our finding that non-prolific Western white-faced ewes exceed prolific Finn sheep in progesterone secretion (Chapter 6) was both unexpected and intriguing. Mean serum progesterone concentrations were significantly higher in Western white-faced than in Finn ewes from days 4 to 14 after ovulation. This can be partly explained by the difference in mean and total luteal volumes, but the difference in serum levels of progesterone was first detected early in the luteal phase, at which time the total content of luteal tissue did not vary between the two breeds, suggesting that progesterone synthetic ability of Western white-faced ewes could be greater than for the Finn ewes. We concluded that breed-specific mechanisms exist that control the formation of luteal tissue and progesterone secretion in cyclic ewes varying in prolificacy, and that these mechanisms might involve ovulation of Graafian follicles at different sizes and inhibitory effects of CL on co-existing CL. However, the variation in the degree of luteotrophic support and/or ovarian responsiveness to gonadotrophic stimuli may also play a role in determining the adequacy of luteal function in breeds of sheep differing in ovulation rates. Similar mechanisms appear to be in effect during different stages of the ovulatory season in ewes (Chapters 6, 9 and 10). It is also feasible that CL/luteal structures exert local effects on co-existing CL as they do on ovarian follicles present in the ovine ovary.

Based on intensive bleeds conducted for 8 days from early in the luteal phase of Western white-faced ewes, mean and basal serum FSH concentrations increased on the day
of follicular wave emergence, which coincided with the beginning of the static phase of the largest ovarian follicles in the preceding wave (Chapter 7). Interestingly, FSH pulse frequency increased during the growth phase of emergent follicles, as mean and basal serum FSH concentrations started to decline. LH pulse amplitude increased at the end of the growth phase of the largest ovarian follicles in the first follicular wave of the cycle. Basal serum concentrations of LH and LH pulse frequency decreased, whereas LH pulse duration increased by day 7 after ovulation. The amplitude and duration of LH pulses rose one day after CL detection. The detection of CL was also accompanied by a transient decrease in mean and basal serum concentrations of FSH, and it was followed by a transient decline in FSH pulse frequency. These results indicate that FSH secretion appears to be linked to and/or regulated mainly by follicle growth and function, whereas pulsed secretion of LH is more closely related to development of CL/progesterone secretion. Apart from an increase in LH pulse amplitude on the day of the onset of the antral follicle static phase (first wave of the cycle), none of the parameters of LH secretion appeared to be linked to any stage of follicle growth and regression. Changes in FSH secretion associated with the formation of CL in ewes appeared to be only temporary. No major changes in any parameter of FSH/LH secretion appeared to be associated with the onset of the regressing phase of the largest follicles of waves, suggesting the importance of intraovarian regulation of follicle demise.

During the transition from the breeding season to anoestrus in ewes (Chapter 8), follicle wave emergence was dissociated from FSH secretion. In all ewes, some FSH peaks failed to initiate the emergence of a follicle wave. The transition into seasonal anoestrus was also associated with markedly depressed oestradiol production and lower concentrations of progesterone as compared to the mid-breeding season of Western white-faced ewes. Low progesterone production in transitional ewes appeared to arise from a reduced number of CL and diminished total luteal volume, but very low serum concentrations of oestradiol did not result from the absence of large antral follicles. The onset of anoestrus in ewes is believed to be cued mainly by the photoperiod-dependent suppression of GnRH release and concomitant enhancement of negative-feedback effects of oestradiol on tonic LH secretion (Karsch et al., 1977). During the final luteal phase of the breeding season circulating
concentrations of oestradiol were found to be minimal (≤ 1 pg/ml) and it appears that the inhibitory action of oestradiol on LH secretion may play a relatively less important role in the onset of anoestrum in ewes. LH support of follicle growth could be significantly reduced during that period because of oestradiol-independent suppression of LH release (Goodman, 1994); however, LH secretion was apparently sufficient to maintain follicle growth to ovulatory diameters. Immunoneutralization of LH in ewes completely blocks follicular development beyond 2-2.5 mm in diameter (McNeilly et al., 1991). We suggest that diminished ovarian responsiveness to gonadotrophins could also contribute to the abrupt cessation of ovulatory cycles at the end of the breeding season in ewes. In summary, alterations in ovarian follicular dynamics at the transition to anoestrum (asynchrony of FSH peaks and follicle wave emergence, and diminished oestradiol production) were not due to changes in FSH secretion, and are probably a product of decreased ovarian sensitivity to gonadotrophic hormones and/or attenuated LH secretion.

Prior to the first ovulation of the breeding season, all Western white-faced ewes studied exhibited a distinct increase in circulating concentrations of progesterone, yet no CL were detected and luteinized unovulated follicles were seen in only three of six animals (Chapter 9). An increase in progesterone secretion prior to the first ovulation of the breeding season did not result, as previously suggested, from the ovulation of immature ovarian follicles and short-lived CL (Legan et al., 1985), but progesterone may be produced by luteinized unovulated follicles and/or interstitial tissue of unknown origin (Berardinelli et al., 1977; Schrick et al., 1993). This increase in serum concentrations of progesterone did not alter the pattern of follicular wave development, hence it seems to be important mainly for inducing oestrous behaviour, synchronizing it with the preovulatory surge of LH (Goodman, 1994), and preventing premature luteolysis during the ensuing luteal phase (Beard and Hunter, 1994). Progesterone may also enhance ovarian follicular responsiveness to circulating gonadotrophins through a local mechanism (Rexroad and Casida, 1977). FSH secretion was not affected by the cessation of anoestrum and FSH peaks were closely associated with the emergence of follicular waves (follicles growing from 3 to ≥ 5 mm). Oestradiol secretion appeared to be fully restored, compared to anoestrous ewes, but it was
not synchronized with the growth of the largest antral follicles of waves until after the beginning of the first luteal phase. The latter finding may be interpreted to suggest that luteal progesterone organizes the pattern of oestradiol secretion in cyclic ewes; this role of progesterone was also manifest in a study in ewes treated with \( \text{PgF}_{2\alpha}/\text{MAP} \) (Chapter 10) where the correlation between follicle wave development and oestradiol secretion from the mid- to late luteal phase was absent in treated but not in control animals.

A 6-day treatment of non-prolific Western white-faced ewes with progestogen (medroxyprogesterone acetate -MAP) in the absence of endogenous progesterone, from late in the oestrous cycle (~day 8 after ovulation), changed follicular dynamics and increased ovulation rate to resemble that in prolific Finn sheep (Chapter 10). The increased ovulation rate in Western white-faced ewes after \( \text{PgF}_{2\alpha}/\text{MAP} \) treatment was due to more ovulations of antral follicles from the final and earlier waves before ovulation. This is intriguing and suggests that the low serum concentrations of progesterone in Finn ewes and treatment of Western white-faced ewes with \( \text{PgF}_{2\alpha}/\text{MAP} \) both could be critical in determining a high ovulation rate. It is, therefore, attractive to speculate that differences in the pattern of ovulatory follicle recruitment and ovulation rate between non-prolific Western white-faced ewes and some prolific strains of sheep (e.g., Finnish Landrace) are due to differing serum concentrations of progesterone. In the present study, the effect of MAP on ovulatory follicle kinetics in Western white-faced ewes did not have a clear gonadotrophic dependancy; none of the analysed parameters of LH/FSH secretion appeared to be altered by a \( \text{PgF}_{2\alpha}/\text{MAP} \) regimen applied at mid-cycle of Western white-faced ewes. More studies are required to provide direct evidence for the existence of gonadotrophin-independent control of ovulation rate by progesterone in the ewe. It is, however, likely that the present results will help to develop practical follicle manipulation techniques to increase fertility in commercial flocks of sheep.

Using daily transrectal ovarian ultrasonography in cyclic Western white-faced ewes we found out that: 1) a proportion of antral follicles attaining an ostensibly ovulatory size of \( \geq 5 \text{ mm} \) in diameter at the time of ovulation of other follicles fail to ovulate (Chapter 5); 2) some ewes may produce both normal and inadequate CL after ovulation (Chapters 6, 7,
8 and 9); and 3) the luteinization of unovulated antral follicles may occur (Chapters 6, 7, 8, and 9). The reasons for the failure of ovulation, impaired luteogenesis of ovulated follicles and shortened lifespan of inadequate CL in ewes are still unknown. The results of the present experiment in anoestrous Western white-faced ewes induced to ovulate with GnRH (Chapter 11) indicated that ovulatory-sized ovarian follicles at a similar stage of their lifespan could give rise to either normal or inadequate CL, and a proportion of these follicles did not ovulate in response to GnRH. This suggests that ovarian antral follicles of a similar age may differ in their responsiveness to gonadotrophic stimuli. The normal luteinization of ovulated follicles did not appear to be influenced by the secretion of follicular oestradiol, as previously suggested (Beard and Hunter, 1994b; Beard and Lamming, 1994; Mann and Lamming, 1995), but high concentrations of oestradiol were associated with the formation of unovulated luteinized follicles in some GnRH-treated anoestrous ewes. Interestingly, peaks of fluctuations in daily serum FSH concentrations were higher in ewes that produced normal CL only compared to ewes with inadequate CL only, and the peak concentration of the GnRH-induced LH surge was higher but the interval from GnRH to the peak of LH discharge was shorter in ewes with inadequate CL only compared to ewes that formed normal CL only after ovulation. Therefore, the amplitude of periodic increases in daily serum FSH concentrations before ovulation and parameters of the preovulatory LH surge both could be critical for luteogenesis post-ovulation. This might be due to the regulation of the synthesis of FSH/LH receptors in preovulatory follicles, thus affecting the ensuing luteal function (Haresign and Lamming, 1978, McNatty et al., 1981; McLeod et al., 1982b; Murdoch and Dunn, 1983).
12.3 Conclusions

1. The growth of ovine antral follicles reaching ovulatory sizes of $\geq 5$ mm in diameter exhibits a distinct wave-like pattern throughout the oestrous cycle in both prolific (Finnish Landrace) and non-prolific (Western White Face) breeds of sheep.

2. Ovarian follicular emergence from the pool of 3-mm follicles and the initial stages of the growth of the largest follicles of waves appear to be primarily controlled by increases in FSH secretion.

3. The largest ovarian follicles in cyclic ewes acquire the ability to secrete oestradiol from the day of emergence (beginning of growth from 3 to $\geq 5$ m in diameter) and a peak of oestradiol release occurs about the time they reach their maximum size.

4. The high ovulation rate in prolific Finn sheep is achieved by the maintenance of follicles emerging in the penultimate follicular wave and their addition to ovulatory follicles emerging in the last wave of the oestrous cycle.

5. Prolific Finn ewes produce more but smaller CL and have lower serum concentrations of progesterone during the luteal phase of the oestrous cycle as compared with non-prolific Western white-faced ewes. There appear to exist breed-specific mechanisms controlling luteal tissue formation and the adequacy of progesterone secretion in cyclic sheep differing in ovulation rates.

6. In cyclic ewes, mean and basal FSH secretion increases just prior to antral follicle emergence and FSH pulse frequency increases during follicle growth as basal FSH secretion declines. This temporal pattern of FSH release appears to be regulated mainly by changes in the function of the largest follicles of successive waves.
7. Pulsed secretion of LH during metoestrus and dioestrus in ewes is more closely related to development of the CL/progesterone secretion rather than to the stage of antral follicle growth and regression.

8. During the transition into anoestrus in ewes, the endogenous rhythm of FSH release is remarkably robust but the pattern of emergence of sequential follicular waves is dissociated from FSH and oestradiol secretion. Luteal progesterone secretion is suppressed because of fewer ovulations, diminished total luteal volume and/or depressed gonadotrophic support. This season-related disruption of the normal pattern of ovarian cycles appears to be due to reduction in ovarian sensitivity to gonadotrophins and/or attenuation in secretion of LH occurring at the onset of the anovulatory season in ewes.

9. Prior to the first ovulation of the breeding season, there was a distinct elevation in circulating concentrations of progesterone produced by luteinized unovulated follicles and/or interstitial tissue of unknown origin. This increase in circulating concentrations of progesterone did not alter the rhythmic pattern of FSH secretion or follicular wave emergence.

10. Treatment of non-prolific Western white-faced ewes with progestogen (medroxyprogesterone acetate-MAP) in the absence of endogenous progesterone (after \( \text{PgF}_2 \) given late in the cycle), changed follicular dynamics and increased ovulation rate to resemble that in prolific Finn sheep (i.e., induction of ovulations of follicles originating from the last and earlier follicle waves before ovulation). Effects of MAP on ovulatory follicle kinetics in Western white-faced ewes did not have a clear gonadotrophic dependency.

11. Ovulatory-sized antral follicles at a similar stage of their lifespan can give rise to either normal or inadequate CL, and a proportion of these follicles do not ovulate
in response to GnRH in seasonally anoestrous ewes. The normal luteinization of ovulated follicles appeared to be linked to the amplitude of episodic elevations in daily serum FSH concentrations before ovulation induction and characteristics of the preovulatory LH surge.
12.2 Future studies

1. The bulk of the data generated in experiments described above are descriptive in nature and document major trends in antral follicle populations and luteal function at various physiological states in ewes. Having determined the patterns of large antral follicular development at different times of the year in the ewe, many experimental studies can now be designed to examine the roles of FSH, LH and ovarian steroids, or the interactions of these hormones, in the control of folliculogenesis and ovulation rate in this species. The requirement of both gonadotrophins for antral follicle emergence and growth can be tested by manipulating the patterns of LH/FSH secretion with GnRH antagonists or inhibin and/or using exogenous gonadotrophins. Exogenous steroids or their synthetic analogues can be used to assess the effects of progesterone and oestradiol. An experiment described in Chapter 7 was a first attempt to relate the changes in pulsed secretion of FSH/LH with different stages of follicle wave development and CL formation in the ewe. We plan to repeat this study during the later stages of the luteal phase and in anoestrus, using both prolific and non-prolific breeds of sheep.

2. There is increasing evidence that gonadal steroids, especially progesterone, can affect ovarian antral follicle development both through paracrine and endocrine mechanisms in domestic ruminants (Ginther, 1971; Dufour et al., 1971, 1972; Rexroad and Casida, 1977; Dailey et al., 1982; Adams et al., 1992; Johnson et al., 1996; Leyva et al., 1998; Flynn et al., 1999; Vinoles et al., 1999). In addition, the presence of multiple CL appeared to have inhibitory effects on the development of co-existing CL (see: Chapter 6). Unilaterally ovulating ewes may provide a useful model to further investigate whether the effects of progesterone are local or systemic. Ultrasonographic imaging of ovaries combined with analysis of the changes in circulating concentrations of gonadotrophins and ovarian steroids in ewes that ovulated unilaterally, could augment our knowledge of the intra- and extraovarian regulation of folliculogenesis and luteal function in farm animals.
3. The results of the present studies (Chapters 5, 6, 8, 9 and 11) suggest that ovulation rates, normal luteogenesis of ovulated follicles and subsequent progesterone secretion in the ewe may, at least in part, depend on hormonal milieu affecting follicular maturation during the preovulatory period. It is, therefore, necessary to examine the differences in the pattern of ovulatory follicle recruitment and its temporal relationships with hormonal profiles in ewes of varying ovulation rates, within and between breeds, from the pro-oestrous to dioestrous period. Based on the present observations in non-prolific Western white-faced and prolific Finnish Landrace sheep, a number of experiments can be done in which hormonal supplementation or manipulations of circulating hormone levels could be applied to alter the pattern of follicle wave dynamics and the viability of large emergent follicles in ewes (Chapter 10). The possible studies may include varying serum concentrations of progesterone and oestradiol in the late luteal phase, and manipulating the patterns of the periovulatory secretion of LH and FSH in the ewes of both genotypes.

4. Some previous studies (Rexroad and Casida, 1977; McLeod and Haresign, 1984; Hunter and Southee, 1987; Scaramuzzi and Downing, 1999) and experimental work described in Chapters 8 and 10 in this thesis, indicated that the degree of ovarian responsiveness to gonadotrophic stimulation may be an important component of the control of ovarian activity in the ewe. Further ultrasound-aided studies aimed to elucidate structural and functional changes in ovarian antral follicles at different stages of their lifespan and after hormonal treatments are needed. Of particular interest would be investigations into the synthesis of follicular and luteal receptors for gonadotrophic hormones, and of intrafollicular regulators of folliculogenesis.

5. Fertility after different oestrous synchronization techniques in ewes is variable (Gordon, 1996). Ovarian activity during oestrus and ovulation induction regimens in cyclic and anoestrous ewes still remains inadequately understood. Similarly, the
mechanism of the “ram effect” (i.e., stimulation of follicular growth and ovulation by introduction of the novel ram; Pearce and Oldham, 1988) is not known. The assessment of ovarian antral follicular development and hormonal changes associated with the treatments above could help us identify and minimize the causes of suboptimal or inconsistent fertility in controlled sheep breeding.

6. More studies are also required on follicular kinetics and the resumption of luteal function in ewes during the post-partum period. A better understanding of the onset of cyclic ovarian activity after parturition in ewes could help us remove some restraints imposed on frequently bred ewes.

7. Very little is known about follicular dynamics during the period preceding puberty in ewes. Transrectal ovarian ultrasonography may serve as a useful method of obtaining physiological and morphological data describing follicular development in prepubertal ewe lambs.

8. In our studies, we aim to correlate changes in ovarian function, as recorded with the use of ultrasonography, with endocrine profiles in ewes. It is a concern that ultrasonographic examinations themselves may stress the ewe. Evaluation of the level of stress associated with transrectal ultrasonography should be done using the estimation of adrenal cortisol secretion during the scanning periods in ewes.

9. Ultrasound images are composed of elementary square units (pixels) differing in brightness. Echotextural properties of tissues (i.e., the degree to which ultrasound beams are dispersed and/or deflected by examined organs) are related to their histophysiological properties (cellular composition, vascularity, fluid content, etc.). It has been demonstrated that microscopic changes associated with alterations in the rates of hormone production and storage by endocrine structures such as CL and ovarian antral follicles are correlated with their ultrasonographic attributes and can
be assessed with computer-assisted pixel intensity analysis of ultrasonograms (Singh et al., 1997, 1998; Pierson and Adams, 1999). We propose to perform pixel intensity analyses on selected ovarian images to see if ultrasonographic characteristics of ovarian structures can be used to estimate circulating concentrations of gonadal steroids, and the health and fate of individual antral follicles and luteal structures in ewes.
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