

OVARIAN ANTRAL FOLLICULAR DYNAMICS AND REGULATION IN SHEEP

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ABSTRACT

The main themes of the present thesis was the regulation of ovarian antral follicle growth, the manipulation of follicular dynamics and ovulation rate, as well as the characterization of the ovine corpus luteum (CL). Two treatments with ovine follicle stimulating hormone (oFSH) were used to assess the responsiveness of small antral follicles during different times in a follicular wave. Follicular dynamics were monitored by transrectal ultrasonography and serum FSH concentrations were measured. Two experiments were performed on anestrus Western White Face (WWF) ewes to independently examine whether or not the ovulations during treatment with a medroxyprogesterone acetate (MAP)-containing sponge and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), were due to the direct effects of $PGF_{2\alpha}$ on the ovary or the effects of a rapid decline in progesterone at $PGF_{2\alpha}$ -induced luteolysis. Non prolific Suffolk ewes were used to assess the effectiveness of treatment with medroxyprogesterone acetate (MAP)-containing sponge and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to increase lambing rate. Re-introduction of rams to pre-isolated, mid-anestrus, WWF ewes was used to look at the effect of increased pulsatile secretion of LH on ovarian antral follicular dynamics at different stages of follicular wave development. We also used ovarian transrectal ultrasonography and computer assisted image analysis as non-invasive techniques to investigate whether or not there were correlations between ultrasound image attributes of the ovine CL and changing progesterone concentrations over time, in prolific and non prolific ewes. The results of the present studies showed that, in the ewe, small antral follicles can respond to the injection of FSH to yield a follicular wave more frequently than seen in a normal cycle and in the presence of a large growing antral follicle. Non induced waves can emerge during the growth phase of a wave induced by injection of oFSH. These results bring into question the presence of functional follicular dominance in the ewe. Ovulations occurred after $PGF_{2\alpha}$ injection but during continuous treatment with MAP, but those ewes experiencing a decline in serum progesterone concentrations in the presence of MAP did not ovulate any follicles. We concluded that ovulations occurring after $PGF_{2\alpha}$ injection, in the presence of a MAP sponge could be due to a direct effect of $PGF_{2\alpha}$ at the ovarian level rather than a sudden decline in circulating progesterone concentrations. Treatment of Suffolk ewes with MAP-containing sponges and injection of $PGF_{2\alpha}$ did not increase lambing rate, perhaps due to asynchrony of ovulations. Re-introduction of rams to previously isolated ewes resulted in a subtle increase in LH pulse frequency on the day of ram introduction in ewes in the static phase of a follicular wave. However, there were no consistent changes in follicular dynamics or estradiol secretion in response to this increase in LH pulse frequency. We concluded that changes in LH pulse frequency do not dramatically change ovarian antral follicular dynamics in the anestrus ewe. Both total luteal area and mean spot pixel values for the CL were correlated with the pattern of serum concentrations of progesterone from day 3 to day 15 after ovulation in WWF ewes and from day 3 to day 14 in Finn ewes. There were no significant correlations between progesterone concentrations and spot pixel heterogeneity for either WWF or Finn ewes. We concluded that pixel heterogeneity is a poor indicator of progesterone secretory ability of the CL when compared to mean pixel values. However, luteal area and mean spot pixel values are better but not strong indicators of the functional status of the CL in cyclic ewes.

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Dedicated to ...

Lee

Your loving support helps more than you know

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LIST OF ABBREVIATIONS

CL	Corpus luteum
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
kg	Kilogram
LH	Luteinizing hormone
MAP	Medroxyprogesterone acetate
MHz	Mega hertz
µg	Microgram
mm	Millimeter
oFSH	ovine FSH
PGF _{2α}	Prostaglandin F _{2α}

Chapter 1: REVIEW OF LITERATURE

1.1. Introduction

It is thought that sheep were one of the first mammals to be domesticated by humans because of their potential contribution to providing food and fibre to a growing population. Our knowledge of reproductive physiology and endocrinology in the ewe has advanced greatly over the previous twenty to thirty years due to the combination of immunoassays, real-time ultrasonography and molecular procedures. This greater understanding of ovarian follicular dynamics and regulation of the growth of ovulatory follicles will enable the development of techniques to improve reproductive efficiency in a commercial setting. The ability to manipulate the seasonality and ovulation rate (i.e. potential reproductive rate) will also lead to improved reproductive efficiency of an agriculturally important species.

The experimental work described in this thesis was undertaken to investigate aspects of the responsiveness of small antral follicles to hormonal stimulation. Further studies examined ovulation rate, subsequent luteolysis and luteal function in ewes receiving hormonal treatment, as well as the influence of ram introduction on the secretion of hormones and follicular dynamics. Characteristics of corpora lutea were related to progesterone secretion in different breeds of sheep. The techniques used to aid in these investigations were transrectal ultrasonography, radioimmunoassay, and computer assisted ultrasound image analysis. The literature reviewed in this thesis focuses on the above and associated subject matter in the ewe, but where particularly useful information was

lacking for sheep, pertinent references to the literature for other domestic species, and laboratory animals have been given.

1.2. The ovine estrous cycle

Sheep, originating from temperate climates, are seasonally, polyestrous animals (Gordon, 1997) i.e. they display estrous cycles that occur only during certain seasons of the year. The estrous cycle of the ewe ranges in length from 14 to 18 days, with an average cycle length of 17.5 days (Marshall, 1904), which is highly repeatable (McKinzie and Terrill, 1937; Asdell, 1946; Hafez, 1952). There are some differences in cycle lengths among different breeds of sheep (Merinos and Rambouillet cycles tend to be longer than those recorded for other breeds; Asdell, 1946) and with age (reproductive performance increases up to the age of 3 or 4 years and then gradually declines; McKinzie FF and Terrill, 1937; Hafez, 1952), but these differences are relatively small (≤ 1 day). The ewe is a spontaneous ovulator (Robertson 1977) and repeated estrous cycles provide the female with repeated opportunities to copulate and become pregnant. O'Shea *et al* (1986) reported that abnormally long cycles in ewes may be associated with the prolonged lifespan of corpora lutea. However, short ovarian cycles were observed in ewes during the post-partum period (Bartlewski *et al* 2000). These cycles were associated with insufficient luteinisation and short-lived CL (Hunter 1991).

There is also an annual cycle of ovarian activity that is superimposed on the normal estrous cycle. In most breeds of sheep (Marshall, 1937; McKinzie and Terrill, 1937; Asdell, 1946; Hafez, 1952; Robinson, 1959), normal estrous cycles occur in the fall and

winter (breeding season), but ovarian cycles cease in the spring and summer (anestrus; Bartlewski *et al* 1998). This ensures that lambs are born in the spring, when environmental conditions are favourable for their survival (Gordon, 1997). There is considerable variation in seasonal reproductive patterns between different breeds (Goodman, 1994). The length of the breeding season seems to depend on the location of the breed of ewe (Hafez, 1952; Robinson, 1959). Breeds with marked anestrus periods (e.g. Scottish Blackface) reproduce under much harsher environmental conditions than those with a limited anestrus season (e.g. Merino) (Marshall, 1937; Hafez, 1952; Robinson, 1959). For breeds of sheep in regions of high latitude (temperate regions) the breeding season begins in late summer and continues until late winter (Legan and Karsch 1979; Karsch *et al* 1979). Whereas breeds located closer to the equator (tropical regions) do not show distinct seasonality and some are even able to continue to reproduce throughout the year (Robinson 1959, 1980). Even though some breeds have marked anestrus periods, antral follicular wave development is still maintained throughout the anestrus period (Hutchinson and Robertson 1966; Smeaton and Robertson 1971; Bartlewski *et al* 1998). The annual variation in day length remains unchanged from year to year (Goodman 1994). This explains why photoperiod is one of many environmental variables capable of influencing seasonal breeding in the ewe (Legan and Karsch, 1980). Sheep are short-day breeders because they become fertile (i.e. estrous cycles commence) as day length decreases in the autumn months (Robinson, 1959 and Karsch *et al* 1984).

The estrous cycle can be divided into two distinct phases; the follicular phase and the luteal phase (Senger, 2003). These two phases can then be further sub-divided. The

follicular phase of the estrous cycle includes pro-estrus and estrus (Arthur *et al* 1989). Pro-estrus is characterised by declining serum concentrations of progesterone as a consequence of luteal regression (Arthur *et al* 1989; Senger 2003). There is also an increase in serum estradiol concentrations due to the emergence and growth of the ovulatory follicle (Goodman 1994; Senger 2003). The estrus period immediately follows pro-estrus (Bindon *et al* 1979; Quirke *et al* 1979; Goodman 1994). Estradiol is the dominant hormone during this period and is the cause of major behavioural changes and the period of sexual receptivity and mating, in the ewe (Robertson 1969). Estrus lasts between 24 to 48 hours, depending on the breed (Land 1970; Land 1973). Ovulation in sheep occurs 24 to 30 hours after the onset of estrus behaviour (McKinzie and Terrill 1937; Robertson 1969). The luteal phase of the cycle includes metestrus and diestrus. The first period is metestrus, during which ovulation and the formation of a corpus luteum (CL) occur (Keyes *et al* 1983). A structure called the corpus hemorrhagicum forms prior to the CL and is due to the rupture of blood vessels in the follicle wall (Senger 2003). Once the CL is fully functional and secretes high levels of progesterone, this period is referred to as diestrus and is the longest stage of the estrous cycle (Senger 2003). Cyclic activity in the ewe is mainly regulated by the hypothalamic-pituitary-ovarian axis (Goodman 1994).

1.3. Hormonal profiles during the ovine estrous cycle

Within the brain, the hypothalamus and pituitary are involved in the secretion of gonadotropin releasing hormone (GnRH), whereas the pituitary gland has the role of releasing follicle stimulating hormone (FSH), luteinising hormone (LH), prolactin and

oxytocin. Ovarian follicles secrete estrogens and inhibins and post-ovulation, the corpus luteum (CL) releases progesterone. Finally, the uterine endometrium releases prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

1.3.1. Secretion of gonadotropins

There are two functionally distinct modes of LH secretion in the ewe (Geschwind and Dewey 1968; Dyer 1985; Arthur 1989), and each control different aspects of ovarian function. The preovulatory LH surge (Fig. 1.1 Top panel) which reaches a peak of 39.28 ± 4.21 ng/ml (Rawlings and Cook 1993) 14 hours before ovulation (Arthur *et al* 1989) induces ovulation and formation of the corpus luteum (Goodman 1994). This gonadotropin surge is primarily induced and sustained by decreased progesterone and increased estradiol secretion during the final stage of the estrous cycle (Scaramuzzi *et al* 1970; Kaynard *et al* 1988; Moenter *et al* 1990). Tonic or pulsatile LH secretion (Fig. 1.1 Top panel) occurs throughout the ewes' cycle (Rawlings and Cook 1993) and is important for ovarian steroidogenesis (Goodman 1994). Rhythmic LH pulses are generated in response to GnRH release from the hypothalamus and reach a peak amplitude of 0.33 ng/ml (Bartlewski *et al* 2000). GnRH controls both the synthesis and release of pituitary gonadotropins through binding to specific receptors in the plasma membrane of the gonadotrophs (Stojilkovic *et al* 1994). Intensive blood sampling has revealed low-amplitude pulses of LH occurring 1 to 6 times an hour (Goodman *et al* 1981). Investigations by Baird (1978) demonstrated an increase in tonic LH secretion during the pro-estrus period resulting from an increased LH pulse frequency, from one pulse every 3 to 4 hours during the mid-luteal phase to a maximum of one pulse every 20

to 30 minutes just before the LH surge. During the preovulatory surge release of LH, pulse frequency and amplitude of LH increases (Baird 1978; Goodman *et al* 1981) as does basal serum concentrations of LH (Rawlings and Cook 1993).

The preovulatory surge release of LH is accompanied by an FSH surge with a peak magnitude of 4.36 ± 0.39 ng/ml (Rawlings and Cook 1993) (Fig. 1.1 Top panel; Wheaton *et al* 1984; Baird *et al* 1991). A second FSH surge occurs within 20 to 36 hours after the preovulatory gonadotropin surge and has lower amplitude (3.00 ± 0.53 ng/ml; Bartlewski *et al* 1999) but is longer in duration (20 to 24 hours) as compared to the preovulatory surge (11 to 12 hours) (Fig. 1.1 Top panel; Pant *et al* 1977; Bister and Paquay 1983; Wheaton *et al* 1984; Findlay *et al* 1990). FSH secretion during the ovine estrous cycle is non-pulsatile, when measured from the jugular vein (Bister and Paquay 1983; Wheaton *et al* 1984; Wallace and McNeilly 1986), however there is a day-to-day variation in serum FSH concentrations (Baird *et al* 1981; Cahill *et al* 1981). The combination of ultrasound examination and blood sampling has confirmed that peaks in serum FSH concentrations every 5 days are associated with follicular wave emergence (Ginther *et al* 1995; Bartlewski *et al* 1998; Souza *et al* 1997; Evans *et al* 2000).

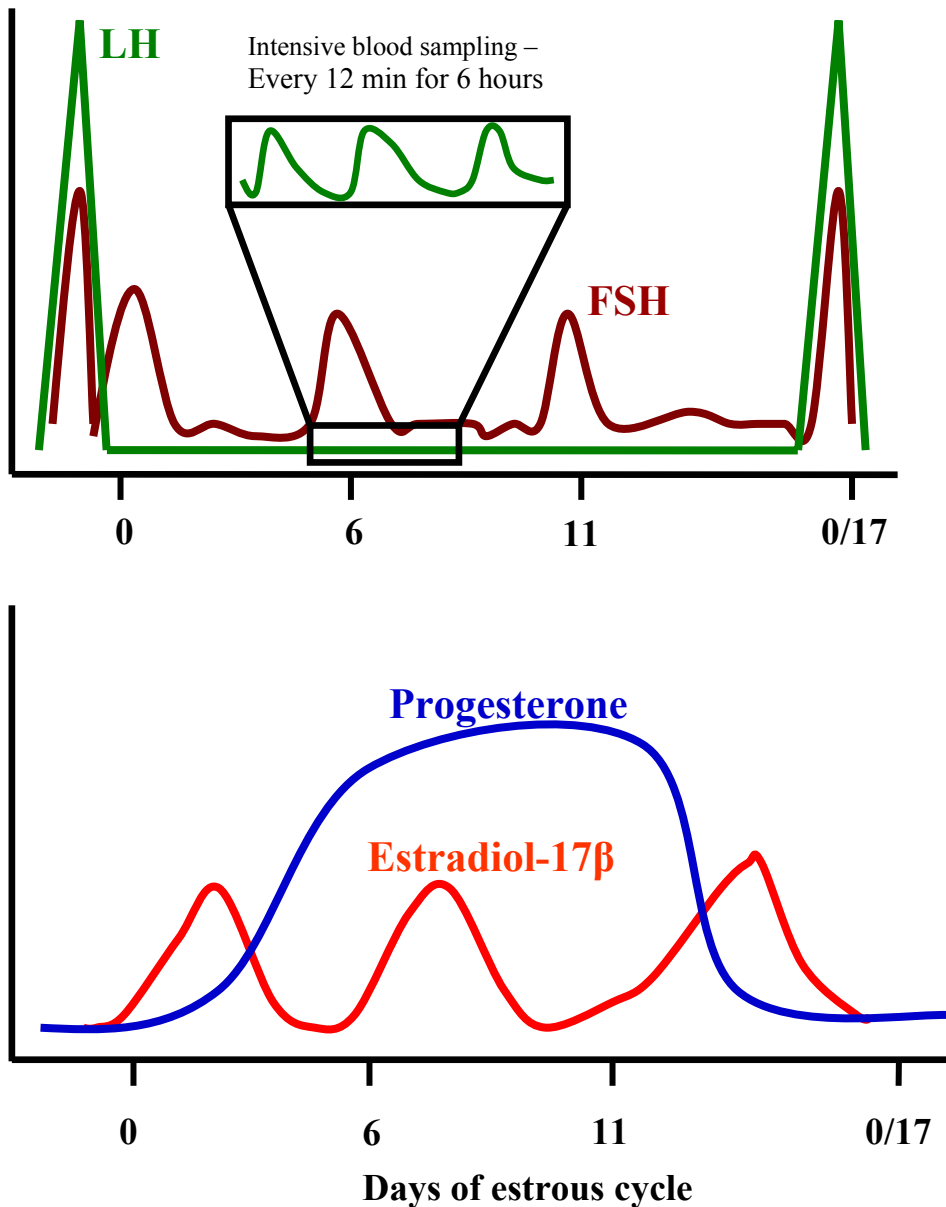


Figure 1.1. Schematic representation of serum profiles of LH and FSH (top panel), and estradiol-17 β and progesterone (bottom panel) throughout an estrous cycle in the ewe (x-axis: d0 = day of ovulation, y-axis: relative concentrations of hormones). Except for high concentrations during preovulatory surge, serum LH concentrations remain basal throughout the luteal phase of the cycle. Pulses of LH secretion are detectable in frequently collected blood samples. FSH secretion remains almost non-pulsatile and periodic peaks in FSH secretion occur once every 4-5 days throughout the estrous cycle. Periodic peaks in estradiol secretion also occur, but they tend to coincide with nadirs in serum FSH concentrations. Serum progesterone concentrations increase from day 0 to day 11 and then reach a nadir by day 15 after ovulation. Based on data from Pant *et al* 1977; Rawlings and Cook 1993; Bartlewski *et al* 1999a; Evans 2003b and reproduced by permission of Duggavathi 2004.

1.4. Regulation of LH secretion

1.4.1. Hypothalamic regulation

GnRH regulates the synthesis and release of pituitary gonadotropins through its specific membrane-bound receptors on the gonadotrophs (Stojilkovic *et al* 1994; Iwashita and Catt 1985; Clarke 1987). LH secretion has been used as an indirect measurement of GnRH release (Goodman 1994); it was assumed that the pulsatile nature of LH release monitored in the peripheral circulation reflected the existence of pulsed GnRH secretion. Techniques developed by Levine *et al* (1982), Clarke and Cummins (1982) and Moenter *et al* (1991) allowed the direct measurement of GnRH concentrations by either perfusion of the median eminence or by sampling portal blood in ewes. These techniques provided evidence for the episodic release of GnRH and the close temporal relationship between GnRH and LH pulses. Although each pulse of GnRH is followed by an LH pulse, there are some small elevations in GnRH concentrations that fail to induce LH pulses (Clarke and Cummins 1982; Levine *et al* 1982). It has been suggested that these small GnRH pulses maintain LH synthesis, leading to accumulation of releasable LH in the pituitary (Clarke and Cummins 1982). The relationship between GnRH and LH is maintained during both the follicular and luteal phase in the breeding season (Baird 1978; Moenter *et al* 1991) as well as throughout the non-breeding season (Scaramuzzi and Baird 1977; Clarke 1988; Barrell *et al* 1992). However, during anestrus the pulse frequency and amplitude of GnRH/LH pulses are significantly lower compared to the breeding season.

1.4.2. Gonadal regulation

LH secretion is regulated either directly or indirectly by the gonadal steroids, estradiol and progesterone. Numerous authors have shown that estradiol regulates LH pulse amplitude while progesterone regulates LH pulse frequency (Bjersind *et al* 1972; Karsch *et al* 1979; Goodman and Karsch 1980; Rawlings *et al* 1984; Wheaton *et al* 1984). It is widely acknowledged that a decrease in progesterone and an increase in estradiol secretion during the preovulatory period of the estrous cycle gives rise to, and maintains the preovulatory LH surge (Scaramuzzi *et al* 1970; Bolt *et al* 1971; Karsch *et al* 1980; Rawlings *et al* 1984; Jeffcoate *et al* 1984b; Kaynard *et al* 1988; Moenter *et al* 1990; Joseph *et al* 1992). During the luteal phase of the ovine estrous cycle, progesterone has an inhibitory effect on pulsatile release of LH (Karsch *et al* 1979; Rawlings *et al* 1984; Wheaton *et al* 1984). Estradiol is also involved in the inhibition of LH secretion, but mainly at the level of the hypothalamus (Goodman and Karsch 1980; Goodman and Karsch 1981; Goodman *et al* 1981; Martin *et al* 1988). During the follicular phase of the estrous cycle in the absence of progesterone, estradiol plays an important role of providing positive feedback, which enhances GnRH secretion in the hypothalamus (Moenter *et al* 1990; Herman and Adams 1990).

1.5. Regulation of FSH secretion

1.5.1. Hypothalamic regulation

During the greater portion of the luteal phase and throughout anestrus, FSH secretion, unlike that of LH, is non-pulsatile (Wallace and McNeilly 1986) suggesting that FSH and LH secretion are differentially regulated by GnRH (Clarke *et al* 1986). Van Cleeff *et al*

(1995) reported that during the luteal phase in the ewe, FSH release was episodic, with each FSH pulse produced by a GnRH pulse, but the release pattern of FSH monitored in peripheral circulation was uncoupled from GnRH stimulus. More recent studies (Padmanabhan and McNeilly 2001; Padmanabhan *et al* 2002; Padmanabhan *et al* 2003) provided evidence of a hypothalamic-independent regulation of FSH secretion.

1.5.2. Gonadal regulation

Estradiol is one of the main regulators of FSH secretion (Baird *et al* 1991) and changes in peripheral concentrations of FSH relate primarily to ovarian follicular activity, reflecting the output of estradiol (McNeilly 1995). Estradiol has the capability to exert both positive and negative feedback effects on FSH secretion (Karsch *et al* 1993). Ovarian inhibin is a strong regulator of FSH secretion. More specifically, there has been found to be an inverse relationship between circulating concentrations of inhibin A and FSH in sheep (Knight *et al* 1998). The role of progesterone in FSH regulation is unclear. Some authors (Dluzen and Ramirez 1987) have shown that, in estrogen-primed rats, an infusion of progesterone had no effect on FSH secretion, whereas others (Tsonis *et al* 1986) have shown that progesterone suppresses the release of FSH from dispersed sheep pituitary cells.

1.6. Secretion and regulation of secretion of estradiol

The main source of estradiol are the largest (≥ 5 mm in diameter), non-atretic follicles (Bjersing *et al* 1972; Evans *et al* 2000). Studies involving the ultrasonographic monitoring of ovarian follicular development and blood sampling have shown 3 to 4

peaks (peak amplitude of 4.6 ± 0.6 pg/ml; Bartlewski *et al* 1999) in serum estradiol concentrations per cycle and those peaks coincide with the attainment of the largest diameter of a follicle in each follicular wave (Fig. 1.1. Bottom panel; Souza *et al* 1998; Bister *et al* 1999; Bartlewski *et al* 1999). Increased estradiol secretion during the follicular phase of the estrous cycle is a reflection of increased maturation of the preovulatory follicles and is 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). The secretion of estradiol results from LH binding to its receptor on the follicular theca cells which stimulates androgen synthesis, and from FSH inducing aromatization of this substrate to estradiol in the granulosa cells (Carson *et al* 1979; Armstrong *et al* 1981; Fortune and Quirke 1988). There is an increase in estradiol secretion within 5 minutes of a pulse of LH, and concentrations remain elevated for around 2 hours (Baird 1978; Martin 1984). In both cyclic (Baird *et al* 1976) and anestrus ewes (Scaramuzzi and Baird 1977), each pulse of LH is followed by a rise in the secretion of estradiol-17 β . Progesterone concentrations in the follicular fluid increase at the time of the preovulatory LH surge, while estradiol concentrations decline to a minimal value, within 16 to 24 hours of the LH surge (Baird 1978; England *et al* 1981; Campbell *et al* 1990). Once serum concentrations of LH exceed 5 ng/ml, the largest ovarian follicles are no longer able to respond to LH by producing estradiol (Baird 1978). In further studies a decline in estradiol concentrations on the day of ovulation was seen coinciding with the secondary peak of FSH secretion (Bister and Paquay 1983; Findlay *et al* 1990; Baird *et al* 1991).

In sheep, estradiol plays an important role in regulating the secretory activity of the hypothalamus (Clarke 1987). Estradiol can exert both a positive and a negative feedback effect on the secretory activity of the hypothalamus and pituitary gland. During the follicular phase of the estrous cycle in ewes, the hypothalamus is the main site for the positive feedback effects of estradiol (Herman and Adams 1990; Moenter *et al* 1990). Estradiol also enhances the response of the anterior pituitary to GnRH (Clarke and Cummins 1984; Crowder and Nett 1984; Phillips *et al* 1990). Physical disconnection of the pituitary from hypothalamic GnRH (Clarke *et al* 1983; Clarke and Cummins 1984; Girmus and Wise 1992) completely blocks pituitary response to estradiol. Therefore, full expression of the estradiol-dependent positive feedback effects on gonadotropin secretion requires continued input from the hypothalamus (Clarke *et al* 1989). However, estradiol enhances the negative feedback effects of progesterone on pulsatile LH secretion, during the luteal phase of the estrous cycle of the ewe, and acts primarily at the level of the hypothalamus (Goodman and Karsch 1980; Goodman *et al* 1981a,b; Martin *et al* 1983). In ovariectomized ewes, Kasa-Vubu *et al* (1992) found that progesterone blocks the estradiol-induced LH surge by preventing the increase in GnRH pulse frequency and amplitude, but Koligian and Stormshak (1977) suggest that perhaps this occurs by decreasing the sensitivity of pituitary gonadotrophs to estradiol. The inhibitory effect of progesterone is all the more pronounced in seasonally anestrous ewes (Karsch *et al* 1987).

1.7. Secretion and regulation of secretion of progesterone

The corpus luteum is a transient endocrine gland, which secretes progesterone, and is formed from follicular cells following ovulation (Juengel and Niswender 1999). Progesterone concentrations follow closely the structural changes of the corpora lutea (Arthur *et al* 1989). Following ovulation, serum progesterone concentrations increase from day 0 to day 11 and then reach a nadir by day 15 after ovulation (Edgar and Ronaldson 1958; Bartlewski *et al* 1999b). The pattern of progesterone secretion in the ewe is episodic, and an average of 8 pulses of progesterone per 24 hours is observed throughout the luteal phase (Alecocay *et al* 1988). It has been demonstrated by several authors (Quirke *et al* 1979; Cahill *et al* 1981) that prolific ewes have higher serum concentrations of progesterone compared to non-prolific breeds, however, contrary to this data, in a more recent study (Bartlewski *et al* 1999b) it was shown that prolific ewes have lower serum progesterone concentrations compared to non-prolific ewes. Further to these observations, other authors have observed that low serum concentrations of progesterone result in the prolonging of the lifespan of large antral follicles in a follicular wave (Johnson *et al* 1996; Flynn *et al* 1999; Vinales *et al* 1999) and a subsequent increase in ovulation rate in non-prolific ewes (Bartlewski *et al* 2003).

The mechanisms involved in the synthesis and secretion of progesterone are complex in nature. Niswender and Nett (1988) reviewed, in detail, the steroidogenic pathways involved in progesterone synthesis and secretion. In brief, cholesterol bound to low density lipoprotein (LDL) produced by the liver is the primary substrate for progesterone synthesis. The steroidogenic luteal cells contain LDL receptors that are involved in the

transport of lipoprotein from outside to inside the cell, where cholesterol is liberated. For the biosynthesis of progesterone, cholesterol is transported to the mitochondria. LH is the single most important endocrine factor involved in the regulation of synthesis and secretion of progesterone in the corpus luteum (Schomberg *et al* 1967; Niswender *et al* 1976). Several authors have shown that, LH administration consistently increases progesterone secretion (Cook *et al* 1969; McCracken *et al* 1971; Baird and Collett 1973) and maintains luteal function in hypophysectomised ewes (Kaltenbach *et al* 1968). Elevations in LH also prolong the luteal life span in normal ewes (Karsch *et al* 1970), whereas injections of LH antisera cause premature luteal regression (Fuller and Hansel 1970). However, other reviewers (Goodman 1994) have suggested that this may well be a pharmacological effect. Endogenous LH pulses have no obvious effect on progesterone secretion in late luteal phase ewes (Baird 1978; Campbell *et al* 1990). These *in vivo* observations are consistent with *in vitro* data demonstrating that most progesterone secretion derives from large luteal cells that are unresponsive to LH (Goodman 1994). However, it has been suggested that these luteal cells normally function at maximal capacity so that they cannot respond further to an LH stimulus (Niswender *et al* 1985; Wiltbank *et al* 1991).

1.8. Follicular growth and development

1.8.1. Folliculogenesis

Populations of primordial (resting pool; primary oocytes surrounded by a squamous layer of pre-granulosa cells; Greenwald and Terranova 1988) and primary (growing pool; single layer of granulosa cells surrounding the oocyte) ovarian follicles constitute the

reserve pool of follicles formed just before or soon after birth (40,000 to 3000,000 primordial follicles in ewe lambs; Driancourt *et al* 1991). There is a continual migration of (3 to 4 ovarian follicles per day) primordial follicles from the non-growing pool of follicles into the growing pool of primary follicles (van Wezel and Rodgers 1996; Turnbull *et al* 1977). When follicles leave the resting pool, they become secondary or preantral follicles with two or three layers of granulosa cells (Driancourt *et al* 1991). At this stage the granulosa cells become cuboidal and begin to express markers of cell proliferation (Wandji *et al* 1997; Fortune 2003). The next stage is early antral or tertiary follicular development followed by the formation of a complete antrum (i.e. the Graafian follicle; Driancourt *et al* 1991). The period of follicular growth from the primordial to the preovulatory stage in ewes exceeds 6 months (Cahill and Mauleon 1980). Growth from the primordial to the early preantral stage (0.2 mm in diameter) takes an average of 130 days (Cahill and Mauleon 1980; Cahill *et al* 1981). It takes an additional 24 to 35 days to reach 0.5 mm in diameter, 5 days to reach 2.2 mm in size (Turnbull *et al* 1977) and about 4 days to reach a preovulatory size of 4.5 to 5 mm in diameter (Turnbull *et al* 1977; McNeilly 1984).

1.8.2. The early stage of follicular development

The growth of follicles from the primordial to the preantral stage is termed early follicular development (Cahill and Mauleon 1980). The control of early follicular development is not fully understood, but is thought to be independent of gonadotropic hormones (McNatty *et al* 1981). Tisdall *et al* (1995) provided evidence, in sheep, suggesting FSH receptors are present on granulosa cells as early as the primary follicular

stage, although several other authors (reviewed by Fortune 2003) have demonstrated a varying ability of FSH to stimulate preantral follicle development. Wu *et al* (2000), from *in vitro* experiments in mice, indicated that LH is needed for development of smaller preantral follicles to the antral follicle stage; however, in general, there is a poor understanding of the potential effects of LH on the growth of preantral follicles (Fortune 2003). Nevertheless, follicles become responsive to gonadotropins towards the end of this early stage of folliculogenesis, and this is a prerequisite to subsequent antral follicular growth and maturation (Campbell *et al* 1995).

1.8.3. Antral follicular waves in sheep

There are two stages of ovarian antral follicular development in both sheep and cattle (Mihm and Bleach 2003). The first is a 'slow growth phase' which, as discussed earlier, is believed to be independent of gonadotropins (Cahill 1981; Lussier *et al* 1987). The second is a 'fast growth phase' that requires gonadotropin support, and is usually described as a follicular wave (Sunderland *et al* 1994). In sheep, a follicular wave is defined as a follicle or group of follicles that grows from 2 or 3 mm in diameter to an ostensibly ovulatory size of ≥ 5 mm in diameter, with emergence restricted to a 24 hour period (Duggavathi *et al* 2003). Of the mammalian species studied, ovarian follicular dynamics has been most closely studied in cattle (Adams and Ginther 1995), therefore follicular dynamics in sheep will be compared to that of cattle in the following section. It is the antral follicular wave stage that will be focused on next; however, in order to aid in the interpretation of this information a few terms must first be defined.

In domestic ruminants, the *growth phase* is defined as the time taken by the individual antral follicle to grow from emergence (e.g. 2 or 3 mm in diameter in sheep as recorded by transrectal ultrasonography), to its maximum size. The time taken by this follicle to regress to the minimal recordable size is termed the *regression phase*, 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; Schrick *et al* 1993; Ravindra *et al* 1994). Follicle *recruitment* refers to the synchronised growth of a group of ovarian antral follicles that eventually gain the ability to fully respond to endocrine (gonadotropic) stimuli. *Selection* is the process by which only limited numbers of these follicles are rescued from atresia and continue 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). *Follicle emergence or follicular wave emergence* is the beginning of the growth of a group of follicles from the minimum recordable size, which subsequently ovulate or undergo atresia (Ginther *et al* 1996).

The development and application of transrectal ultrasonography brought about a large increase in the understanding of follicular waves in both cyclic and anestrous ewes (Schrick *et al* 1993; Ravindra *et al* 1994; Ginther *et al* 1995; Souza *et al* 1998; Leyva *et al* 1998b; Bartlewski *et al* 1999a; Gibbons *et al* 1999; Vinales *et al* 1999; Evans *et al* 2000). The wave-like pattern of antral follicular emergence and growth occurs more frequently in sheep (every 4 to 5 days; Ginther *et al* 1995; Bartlewski *et al* 1998; Evans *et al* 2000) than in cattle (every 7 to 10 days; Savio *et al* 1988; Sirois and Fortune 1988;

Pierson and Ginther 1988; Knopf *et al* 1989), and each wave is preceded by a transient increase in serum FSH concentrations (Adams *et al* 1992; Ginther *et al* 1995; Bartlewski *et al* 1998; Souza *et al* 1998; Bister *et al* 1999; Bartlewski *et al* 1999a; Bartlewski *et al* 1999c; Evans *et al* 2000; Evans *et al* 2001; Duggavathi *et al* 2003a; Duggavathi *et al* 2004). In the ewe, a follicular wave consists of 1 to 4 follicles growing from 2 to 3 mm in diameter to a maximum size of 4 to 12 mm in diameter before regression or ovulation (Noel *et al* 1993; Ravindra *et al* 1994; Ginther *et al* 1995; Bartlewski *et al* 1999a; Evans *et al* 2000; Vinales *et al* 2001). The number of follicular waves per cycle can vary between breeds of sheep, but range from 2 to 4 waves per cycle (Noel *et al* 1993; Ravindra *et al* 1994; Ginther *et al* 1995). In cattle, there is a significant increase in the number of small antral follicles at the time of follicular wave emergence (6 to 9 follicles in the 4 to 6 mm diameter range; Gong *et al* 1993; Ginther *et al* 1996), and then a gradual reduction in the number of small follicles during the growth of the dominant follicle (Ginther *et al* 1996). In a recent study in sheep, Duggavathi *et al* (2003) surmised that, unlike in cattle, there is no increase in the numbers of small antral follicles (2-3 mm in diameter) at follicular wave emergence. In cattle, one of the follicles of the wave becomes dominant, and the others become atretic (subordinate; Ginther *et al* 1989b); this stage has been given the term deviation (reviewed by Ginther *et al* 1996). In sheep, ovulatory follicles originate from the final follicular wave of the cycle, like in cattle (Ginther *et al* 1995; Bartlewski *et al* 1999a). However, in some prolific breeds of sheep (Finnish Landrace and Rambouille x Booroola ewes) about 50% of all ovulatory sized follicles from the penultimate wave ovulate along with the ovulatory follicles from the final wave of the cycle (Bartlewski *et al* 1999a; Gibbons *et al* 1999). The ovulatory

follicles (8 to 20 mm in diameter) in cattle originate only from the last follicular wave of the interovulatory interval (Ginther *et al* 1996). Therefore, selection, deviation and dominance are not obvious in sheep.

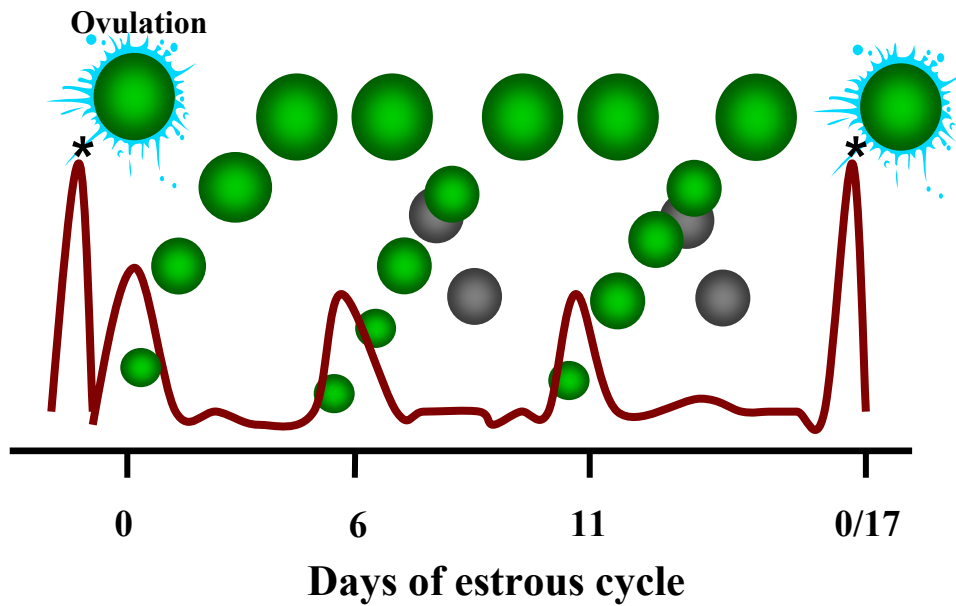


Figure 1.2. A schematic representation of ovarian antral follicular waves in sheep during an estrous cycle. Three follicular waves (defined as 1 or more 2-3 mm follicles emerging and growing together to reach an ovulatory diameter of ≥ 5 mm, before regression (dark colored spheres) or ovulation) are shown here. * indicates preovulatory FSH surge that is coincidental with preovulatory LH surge (not shown). Also shown are the periodic peaks in serum FSH concentrations that precede each follicular wave emergence. Based on data from Ginther *et al* 1995; Bartlewski *et al* 1999a; and reproduced by permission of Duggavathi 2004.

1.9. Regulation of antral follicular growth and development

1.9.1. Gonadotropic hormones

Gonadotropins have the greatest influence on ovarian antral follicular emergence and growth (Baird and McNeilly 1981; Ireland 1987; Picton *et al* 1990). In sheep, LH receptors are initially found localised in the theca cells of large preantral follicles (Fortune and Armstrong 1977; Logan *et al* 2002). However, when follicles reach around 4 mm in diameter, LH receptors can also be found in the granulosa cells (Carson *et al* 1979; Webb and England 1982; Logan *et al* 2002). Both FSH and estradiol have been found to stimulate the synthesis of LH receptors by the granulosa cells (Uilenbroek and Richards 1979; England *et al* 1981). However, FSH receptors are found to be present, on the granulosa cells, as early as the primary follicle stage (Tisdall *et al* 1995). As follicles continue growth to 2 mm in diameter, FSH receptor numbers increase in sheep (Carson *et al* 1979). These observations suggest that early antral follicles are predominantly dependent on FSH whereas the terminal phase of folliculogenesis is under the control of LH (Baird and McNeilly 1981; Campbell *et al* 1995). Further to these investigations, it was discovered that, FSH alone, but not LH alone, could stimulate the growth of follicles to a preovulatory size in long-term GnRH agonist treated ewes (Picton *et al* 1990). There is unequivocal evidence that a transient peak in serum FSH concentrations precedes emergence of each follicular wave in both cyclic (Ginther *et al* 1995; Souza *et al* 1998; Bartlewski *et al* 1999a; Bartlewski *et al* 2000a; Evans *et al* 2001; Duggavathi *et al* 2004) and anestrus ewes (Bartlewski *et al* 1998; Evans *et al* 2001), as well as in cattle (Adams *et al* 1992; Adams *et al* 1993).

1.9.2. Gonadal steroids as regulators of follicular growth

Richards (1994) suggested that estrogens, acting endocrinologically, may enhance the response of ovarian follicles to gonadotropins in hypophysectomised rats. From the results of other studies in rodents (Findlay *et al* 2000; Richards 2001; Britt and Findlay 2003) it was concluded that estradiol is required for early folliculogenesis. It is also thought that the combination of FSH and estradiol enhances the formation of LH receptors in granulosa cells of mature ovarian follicles (Richards *et al* 2002). There is contradictory evidence for the role of the CL in follicle growth. Dailey *et al* (1982) deduced that the CL acts locally to increase the numbers of all follicles visible on the ovarian surface. However, more recently, Bartlewski *et al* (2001) suggested that the presence of the CL locally inhibits the numbers of antral follicles not growing beyond 3 mm in diameter in ewes. Bartlewski *et al* (2001) also concluded that there was no inhibitory effect of the CL on the numbers of follicles growing beyond 3 mm in diameter.

1.10. Ovulation in the ewe

There are notable 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). The mean ovulation rate of non-prolific breeds of sheep is 1 to 3 follicles whereas the mean ovulation rate of prolific sheep is roughly 3 (Bartlewski *et al* 1999).

Ovulation, in the ewe, is a distinct biological phenomenon that requires the rupture of healthy tissue at the surface of the ovary (for structural anatomy of the follicular wall see fig. 1.3.; Espey and Lipner 1994). This is achieved through finely orchestrated biochemical changes regulated by multiple pathways and modulated by an even larger number of factors and processes (Tsafriri and Chun 1996).

In 1932, Hartman gave the first major review of the literature on ovulation, but it wasn't until 30 years later that Asdell (1962) summarised the principle theories. The general assumption at that time was that mammalian follicles rupture as a consequence of increasing follicular pressure (Heape 1905). It was also thought that contraction of smooth muscle tissue in the ovarian stroma promoted the increase in pressure (Thomson 1919). As it gradually became apparent that neither the smooth-muscle theory nor the pressure theory adequately explained the mechanical events leading to ovulation, more attention was given to the possibility that the morphologic changes that occur at the apex of an ovulatory follicle might be the result of enzymic degradation of the thecal connective tissue (Schochet 1916; McKenzie and Terrill 1937). However, Espey (1994) hypothesised that mammalian ovulation is comparable to an inflammatory reaction. This hypothesis is supported by evidence from Cajander (1976) who demonstrated that any potent nonsteroidal anti-inflammatory agent (such as indomethacin) will inhibit ovulation if the drug is administered during the first 80% of the ovulatory process.

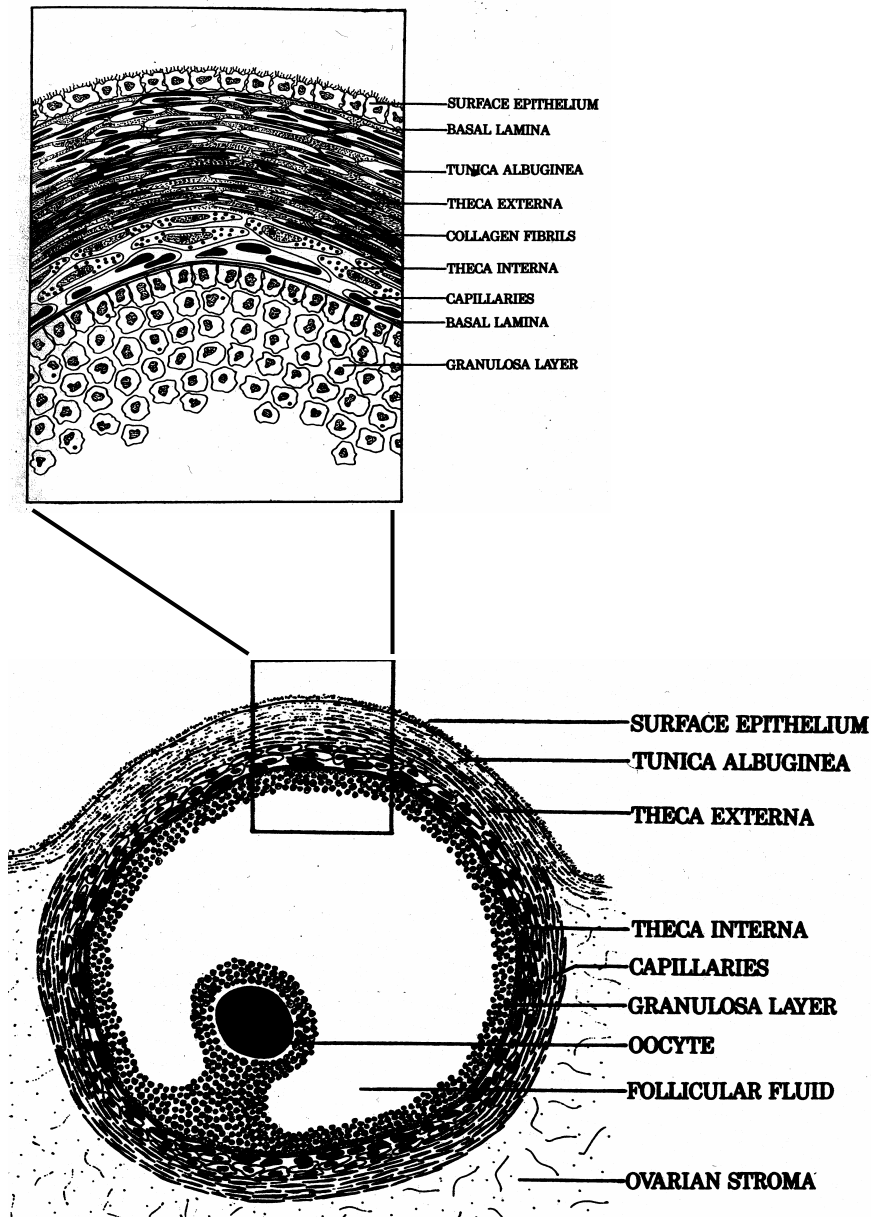


Figure 1.3. The structural organization of a mature ovarian follicle and above a close-up view of the components of the follicle wall. At the apex of a mature follicle, where a stigma forms and the follicle ruptures, there are five different layers of cells. The outermost layer is the surface epithelium, a single-cell layer of cuboidal epithelial cells. The second layer is the tunica albuginea, consisting of fibroblasts and collagen, that forms a tenacious sheath around the entire ovary. The third layer is the theca externa, the follicle's own capsule of collagenous connective tissue, which delineates its boundary. The fourth layer consists of the secretory cells of the theca interna, just inside the theca externa. The fifth and innermost layer is the stratum granulosum, from which extend the cumulus mass and its oocyte. Reproduced from Espey and Lipner (1994).

As a follicle grows and develops within the ovary it produces increasing amounts of estradiol which promotes the expression of LH and/or FSH receptors on the plasma membranes of follicular cells (Espey 1999). A review of the literature suggests that ovulation can only occur in mature ovarian follicles that have acquired adequate concentrations of LH and/or FSH receptors (Richards and Hedin 1976; McFarland *et al* 1989; Leung and Steele 1992). At this stage of the estrous cycle, increasing concentrations of circulating estradiol induce a sudden increase in GnRH secretion, in turn causing a surge in LH and FSH secretion from the pituitary gland (Espey 1999). The preovulatory LH surge is important because it sets in motion a cascade of biochemical events that lead to ovulation and functional and structural changes in the granulosa and theca cells of the ovulatory follicle (reviewed by Niswender *et al* 1986; Alila and Dowd 1991; Espey 1999). The most important structural changes leading to ovulation are those of the connective tissue of the tunica albuginea and theca externa (Tsafiriri and Chun 1996). As the time of rupture nears, the apex of a mature follicle protrudes above the surface of the ovary and eventually forms a stigma (Espey 1999). As ovulation approaches, there is degradation of the collagenous connective tissue in the follicle wall (Espey 1967) and an intrafollicular pressure of about 20 mm Hg (Espey 1999). These changes in the connective tissue are accompanied by increased permeability of the blood vessels, resulting in leakage of blood cells and edema of follicular tissue (Parr 1975; Abisogun *et al* 1988). Once the egg-bearing cumulus mass is expelled from the ovary, ovulation is complete (Espey 1999).

1.11. Corpus luteum formation and development

The corpus luteum is a transient endocrine organ formed from cells of the follicle following ovulation (Juengel *et al* 1999). In cattle it has been demonstrated that the granulosa and theca cells, of the follicular wall, give rise to large and small luteal cells, respectively (Alila and Hansel 1984; Niswender *et al* 1985; Meidan *et al* 1990). However, research has shown that small luteal cells may differentiate into large luteal cells when LH is administered to ewes (Farin *et al* 1988) and cows (Niswender *et al* 1985). The transition of follicular tissue into luteal tissue is a dynamic process that includes differentiation, migration, and proliferation of cells (reviewed by Juengel *et al* 1999).

In ewes, the greatest number of active LH receptors is located on the small luteal cells (Harrison *et al* 1987). Luteal cells require LH receptors in order to respond to gonadotropic stimuli. However, the large luteal cells are unresponsive to LH stimulation (Hoyer and Niswender 1986), suggesting that large luteal cells are not dependent on LH for the production of progesterone (Alila and Dowd 1991). The increase in total luteal mass during the early and mid-luteal phase of the cycle is due to both small and large luteal cells. Between days 4 and 12 there is an increase in size of large luteal cells; however, the number of cells remains constant until the onset of luteolysis (O'Shea *et al* 1986; Farin *et al* 1989). On the other hand, there is an increase in the number of small luteal cells from days 4 to 8 but no change in the actual size of the small luteal cells (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 (Farin

et al 1989). Many of the proliferating cells contribute to the extensive capillary network of the corpus luteum (Juengel *et al* 1999). Once established, the capillary network supports blood flow to the corpus luteum at a rate that exceeds that in other tissues (Juengel *et al* 1999).

1.12. Endocrine regulation of luteolysis

The luteolytic factor in ruminants is prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and is released from the endometrial glands of the uterus (reviewed by Knickerbocker *et al* 1988). $PGF_{2\alpha}$ travels to the ovary by way of the uterine venous and lymphatic vessels and ovarian artery (Koziorowski *et al* 1989). In the ewe, small luteal cells are insensitive to $PGF_{2\alpha}$, while large luteal cells contain $PGF_{2\alpha}$ receptors (Fitz *et al* 1982). Functional luteolysis (a decline in the capacity to release progesterone) can be induced by $PGF_{2\alpha}$ through interference with the transfer of cholesterol through membranes of the mitochondria and by way of large luteal cell receptors (Spencer 1998). However, the CL of the ewe is only responsive to $PGF_{2\alpha}$ between days 4 and 14 of the estrous cycle (Day 0 = oestrous; Chamley *et al* 1972).

Ovarian estradiol, progesterone, and oxytocin are regulators of $PGF_{2\alpha}$ secretion, in the ewe. Exposing the uterus to high levels of progesterone for a specific period of time prepares the endometrium for $PGF_{2\alpha}$ synthesis (Silvia *et al* 1991). Zelinski *et al* (1982) reported high concentrations of endometrial receptors for progesterone at estrus but then a gradual decline during the luteal phase of the cycle. The exposure to luteal phase progesterone allows the build up of prostaglandin endoperoxidase and arachidonic acid,

which are required for $\text{PGF}_{2\alpha}$ production (Knickerbocker *et al* 1988; Silvia *et al* 1991). Towards the end of the luteal phase, the formation of endometrial receptors for oxytocin and estradiol increases and is stimulated by follicular estradiol (Roberts *et al* 1975, 1976; Koligan and Stormshak 1977; Spencer 1988; Juengel and Niswender 1999). Early exposure to progesterone greatly amplifies the effect of estradiol on the recruitment of oxytocin receptors and estradiol amplifies the secretion of $\text{PGF}_{2\alpha}$ (Ford *et al* 1975; McCracken *et al* 1984; Fogwell *et al* 1985; Homanics and Silvia 1988; Vallet *et al* 1990). It is interesting to note that an increase in pulsatile $\text{PGF}_{2\alpha}$ secretion and an elevation in the number of oxytocin receptors are related to the decrease in circulating progesterone concentrations (Sheldrick and Flint 1985). Leavitt *et al* (1985) found that the increase in endometrial oxytocin receptors can be detected as early as 6 hours after the withdrawal of progesterone in the ewe.

1.13. Transrectal ultrasonography

Real-time B-mode ultrasonographic imaging of the reproductive tract was developed over several decades. However, significant advancements since the 1970's have seen the application of this technique in humans (Kambe *et al*, 1977), and the 1980's in farm animal species (Ginther 1983; Pierson and Ginther 1986; Adams *et al*. 1989). Transrectal ultrasonography of the ovine ovary was first reported comprehensively by Schrick *et al* in 1993, and is a non-invasive technique for the collection of real-time data whilst at the same time allowing repeated observations from the same individual; making it possible to study the dynamic interactions within the ovarian follicular population over time (Pierson and Ginther 1988). Schrick *et al* (1993) argued against the existence of a “wave” like

pattern of follicular development in sheep. However, numerous authors (Bartlewski *et al* 1999a ; Evans *et al* 2000) have put forward convincing evidence that supports the theory of “wave” like follicular development in sheep, similar to that seen in cattle (Pierson and Ginther 1984; Ginther *et al* 1996). With the advent of high-resolution ultrasound equipment it is now possible to identify and quantify all antral follicles ≥ 1 mm in diameter in the ovine ovary. This technology has enabled authors (Duggavathi *et al* 2003a) to surmise that there is no increase in the numbers of small follicles at follicle wave emergence in cyclic ewes, in contrast to cattle (Gong *et al* 1993; Fortune 1994; Ginther *et al* 1996). Early detection of the CL after ovulation allows assessment of the developing structure and the correlation of its ultrasound (size) characteristics to functional attributes such as circulating progesterone concentrations (Kastelic *et al* 1990, heifer; Bartlewski *et al* 1999b, ewe; Checura *et al* 2002, mare).

1.13.1. Image analysis

Computer-assisted image analysis is a useful extension to ultrasonography. Ultrasonography is based on the abilities of different tissues to reflect high frequency sound waves (Pierson and Adams 1995). A piezoelectric transducer is used to emit acoustic pressure waves and transmit them into the adjacent tissues (Ginther 1995). A proportion of the wave is reflected by the tissue interfaces, received by the transducer and displayed as a grey-scale image (Powis and Powis 1984; Zagzebski 1996). Grey-scale images are composed of thousands of picture elements, known as pixels (Ginther 1995). A single tissue reflector is represented by a single pixel and is designated one of 256 shades of grey (ranging from black to white) in an 8-bit grey-scale image (Singh *et al*

2003). Subjective scoring (Townson and Ginther 1989) has been used to quantify changes in image attributes; however, the accuracy and repeatability of this procedure is under question (Tom *et al* 1998a) because the human eye can only distinguish between 18 and 20 shades of grey (Baxes 1994). Therefore, computer algorithms have been designed specifically for a more objective analysis of ultrasound images, and providing a quantitative approach to echotextural analysis (SYNERGYNE Version 2.8[©], Sasaktoon, Saskatchewan, Canada). The application of this technology has enabled investigators to obtain vast quantities of information on the topic of the echotexture dynamics of antral follicles (Tom *et al* 1998b) and CL (Duggavathi *et al* 2003b) and has allowed correlation with endocrine variables (Kastelic *et al* 1990; Bartlewski *et al* 1999b).

1.14. The ‘Ram Effect’

The seasonality of breeding activity in sheep represents an important constraint in the breeding program of commercial flocks (Rosa and Bryant 2002). From the farmer’s point of view, the economic return from his or her sheep will depend primarily on their reproductive efficiency (Gordon 1997), and low lamb output per ewe is a major factor limiting the energetic efficiency of sheep meat production (Blaxter, 1964). Increasing the frequency of lambing under some sheep farming conditions may be a means of achieving greater reproductive efficiency, levelling out the flow of lambs to the market and utilising buildings, capital and labour more effectively (Hulet, 1977). There are several techniques available to manipulate reproduction during the anoestrous season (Martin 1995; Gordon 1997); however, these can be expensive, require sufficient labour to implement, and may increase waiting time for sending animals to slaughter (Rosa and Bryant 2002).

If seasonally anestrus ewes are preconditioned by a period of isolation from rams (at least one month), they respond to the re-introduction of the male by displaying a reasonably well synchronised estrus about one estrous cycle after ram exposure (Underwood *et al.*, 1944; Edgar and Bilkey, 1963; Fulkerson *et al.*, 1981; Knight *et al.*, 1981; Gordon, 1997). This occurrence is known as the “ram effect,” and can be used in commercial sheep flocks (Gordon, 1997). The “ram effect” has been observed in a wide variety of breeds (Martin and Scaramuzzi 1983), but there is considerable variation in the percentage of ewes responding (Martin 1984). Some of this variation appears to reflect the “depth” of anestrus (Martin 1984). Breeds with limited anestrus periods respond more readily to rams (Schinckel 1954; Martin *et al* 1983; Nugent *et al* 1988) than do breeds with more prolonged anestrus (Chesworth and Tait 1974; Nugent *et al* 1988). Ewes are also generally more responsive in late or early anestrus than they are in the middle of the anovulatory period (Edgar and Bilkey 1963; Cushwa *et al* 1992).

The “ram effect” was first documented by Underwood *et al* (1944); however, it was Watson and Radford (1960) who first investigated pheromones as the stimuli for inducing the “ram effect.” Behavioural stimuli are also thought to be involved (Signoret *et al* 1982; Pearce and Oldham 1988); however, tactile and auditory cues are not required (Watson and Radford 1960). The pheromones mediating this effect are produced by the sudoriferous glands in the skin (Knight and Lynch 1980) and their production is controlled by androgens (Goodman 1994). This effect of androgens may account for the observations that rams with higher reproductive activity are more effective in inducing ovulation in anestrus ewes (Signoret *et al* 1982; Iglesias *et al* 1991).

There are reported to be two different responses that are caused by the ram effect: an increased ovulation rate (Cognie *et al.*, 1980; Oldham, 1980) and advancing the breeding season (Coop and Clark, 1968; Gordon, 1997). Introducing ewes to the rams towards the end of the non-breeding season will often induce a proportion of anestrus ewes to ovulate within 2-3 days (Knight *et al.*, 1978; Oldham *et al.*, 1979); however, behavioural signs of estrus are not shown until about three weeks later. Chesworth and Tait (1974) documented increases in LH concentrations within one hour of exposing Greyface ewes to the ram just prior to the breeding season. Other studies revealed that LH pulse frequency increases markedly within minutes of ram introduction (Martin *et al.*, 1980). In addition to this, ewes stimulated by the ram effect experience a preovulatory LH surge, similar to that of spontaneously ovulating sheep and have been observed to ovulate about 40h after exposure to the male (Oldham *et al.*, 1979). The increased pulse frequency of LH is the critical step in the ram effect; it leads up to the preovulatory LH surge and subsequent ovulation (Rosa and Bryant 2002). Rosa and Bryant (2002) proposed that LH secretion increases because (1) the presence of rams attenuates the negative feedback effect of the steroids, reversing the effect of photoperiod, or (2) that a direct mechanism independent of the negative feedback of steroids exists. In contrast to LH, the secretion of FSH has been reported either to remain unchanged (Martin *et al.* 1980) or to decrease and remain low in ewes with ram exposure (Atkinson and Williamson 1985).

1.15. General objectives

With this background information, the following were the general objectives of the research work described in this thesis.

- 1) The number of small antral follicles in the sheep ovary (≥ 1 mm but ≤ 3 mm in diameter) remains constant throughout the estrous cycle except for the periovulatory period, even though there are 3 or 4 periods of wave emergence. Each wave is preceded by a peak in FSH secretion. In cattle, as each follicular wave emerges there is an increase in number of small follicles in the ovary, the growth of the dominant follicle of the wave is associated with a decrease in the number of small follicles and an inhibition of follicular wave emergence. The objective of the first study of this thesis was to investigate whether small follicles, in the ewes' ovaries, were able to respond to a physiological peak in FSH secretion at different times in a follicular wave, and in the presence of a large (≥ 4 mm in diameter) follicle, with the induction of a follicular wave. In other words, are small follicles receptive to FSH stimulation at any phase of the estrous cycle. In addition, it was of interest to see if the next expected endogenously derived follicular wave could emerge in the presence of an induced wave. In other words does follicular dominance occur in the ewe?

- 2) Prolific breeds of sheep have lower circulating concentrations of progesterone during the luteal phase than non prolific breeds of sheep. Experimental studies have shown that creating low serum concentrations of progesterone in the ewe

resulted in the prolonging of the lifespan of large antral follicles in a follicular wave (Johnson *et al* 1996; Vinoles *et al*, 1999). Prolonging follicle lifespan was also achieved by treating ewes with medroxyprogesterone acetate (MAP) releasing intravaginal sponges. Bartlewski *et al* (2003) investigated the effects on follicular development and ovulation rate of treating non-prolific Western White Face (WWF) ewes, for 6 days with a medroxyprogesterone acetate (MAP) releasing intravaginal sponge, starting on day 8 after ovulation and coupled with a single administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) given on day 8. One to 6 days after $PGF_{2\alpha}$ administration, during the period sponges were in place, several large antral follicles ovulated; these ovulations were not preceded by a preovulatory LH/FSH surge, and none of these ovulations were followed by the formation of corpora lutea (CL), only transient corpora hemorrhagica (CH). Prostaglandin treated animals experienced the expected drop in serum progesterone concentrations after treatment and progesterone concentrations remained low throughout the MAP treatment period. Thus, the objectives of the second study of this thesis were to determine whether the ovulations that occurred after $PGF_{2\alpha}$ treatment in the presence of an intravaginal sponge releasing a progestagen, in cyclic ewes, could be due to a direct effect of $PGF_{2\alpha}$ on the ovary or due to the sharp decline in serum progesterone concentrations caused by the luteolytic dose of $PGF_{2\alpha}$.

- 3) The findings of the previously mentioned study by Bartlewski *et al* (2003), included an increase in ovulation rate of approximately 50% at the end of the treatment, when compared to control ewes or the pre-treatment cycle of treated ewes. This was attributed to the ovulation of follicles from the penultimate follicular wave before ovulation, and their addition to ovulatory follicles from the final wave before ovulation. The objective of the third study of this thesis was to see if this treatment would have commercial viability, through an increase in lambing rate, without any negative effects on lamb weights or sex ratios.

- 4) If seasonally anestrus ewes are preconditioned by a period of isolation from rams (at least one month), they respond to the re-introduction of the male with increased LH secretion and a well synchronized oestrus, about 17 days after ram exposure. The response is variable and depends on breed and stage of anestrus, being more limited during mid-anestrus in ewes with a distinct seasonality. There has been limited investigation on the effect of ram introduction on ovarian follicular wave dynamics using ovarian ultrasonography combined with extensive hormone measurements. It was hoped that if ram introduction was done in mid anestrus in distinctly seasonal ewes that only subtle increases in LH secretory pulse frequency would be seen, allowing us to examine the role of such a change on ovarian follicular dynamics. It has been previously reported by Bartlewski *et al* (2000) that LH secretory patterns change throughout the estrous cycle, and that increases in LH pulse amplitude are associated with the end of the growth phase of the largest follicle of a wave, in cyclic ewes. Thus, the objective of the fourth

study of this thesis was to investigate the effect of ram introduction on ovarian follicular dynamics and LH secretion in anestrus ewes at different stages of follicle wave development.

- 5) In an article by Bartlewski *et al* (1999b) it was demonstrated that CL volume (taken from ultrasonographic images) was correlated with progesterone concentrations during the formation and demise of the CL in Western White Face ewes (non-prolific breed). However, during the period after formation and before demise of the CL, CL volumes did not correlate with circulating progesterone concentrations in Western White Face ewes. Conversely, circulating progesterone concentrations and CL volume were correlated throughout the luteal phase in prolific Finn sheep. Therefore, the objectives of the final study of this thesis were to investigate whether or not there were any correlations between mean pixel value and heterogeneity of ultrasonographic images of CL and changing serum progesterone concentrations over time, in prolific (Finn Sheep) and non-prolific (Western White Face Sheep) ewes.

Chapter 2: HYPOTHESES

1. Small antral follicles (≥ 1 mm but ≤ 3 mm in diameter) in the ewe are able to respond to a physiological peak in serum concentrations of FSH, leading to follicular wave emergence, in the presence of a large (≥ 4 mm in diameter) follicle and at different phases of the first wave of the cycle, without disrupting the emergence of the second follicular wave of the cycle.
2. Ovulations, in non prolific ewes, after $\text{PGF}_{2\alpha}$ treatment and in the presence of a progestagen releasing intravaginal sponge are due to a direct effect of $\text{PGF}_{2\alpha}$ on the ovary.
3. Treatment of non prolific ewes for 6 days with a progestagen releasing intravaginal sponge, starting on day 8 after ovulation and coupled with a single injection of $\text{PGF}_{2\alpha}$ given on day 8, will increase lambing rate without any negative effects on lamb weight or sex ratios.
4. Re-introduction of rams to anestrus ewes, after a period of isolation, will induce an increase in LH pulse frequency in the ewe, which will not affect ovarian follicular dynamics, regardless of the stage of the follicular wave.
5. Ultrasound image attributes of CL, in the ewe, are correlated to serum progesterone concentrations during the luteal phase of prolific and non prolific breeds of sheep.

Chapter 3: AN INVESTIGATION INTO THE RESPONSIVENESS OF SMALL ANTRAL FOLLICLES (≥ 1 MM BUT ≤ 3 MM IN DIAMETER) TO FSH STIMULATION DURING A FOLLICULAR WAVE IN THE EWE

Davies KL, Duggavathi R and Rawlings NC.

3.1. Abstract

In the ewe 1 to 3 ovarian antral follicles (≥ 1 mm but ≤ 3 mm in diameter) emerge or continue growth in a wave-like pattern every 4 to 5 days. Waves are preceded by a peak in FSH secretion. To see if small follicles were able to respond to FSH at different times in a follicular wave, cyclic ewes (n=7) were given ovine FSH (oFSH; two s.c. injections of 0.5 $\mu\text{g}/\text{kg}$, 8 h apart) at 36 h and 72 h after ovulation, in the period between the endogenous peaks preceding waves 1 and 2 of the cycle. Five control ewes received vehicle only. Blood samples were collected every 12 hours, prior to each ovarian ultrasonographic scanning session. Peaks in exogenous serum FSH concentration were seen on days 2.1 ± 0.1 and 3.6 ± 0.1 after ovulation and induced follicular waves on days 2.0 and 3.5 after ovulation. There were no differences in maximum follicle diameter and peak estradiol concentration amongst follicular waves ($P > 0.05$). The induced follicular waves had significantly shorter growth phases, and tended ($P = 0.08$) to have faster growth rates, than follicles in waves 1 and 2. Induced waves did not delay the emergence of the next expected follicular wave of the cycle (Wave 2). We concluded that, in the ewe, small antral follicles can respond to FSH administration to yield a follicular wave more frequently than seen in a normal cycle and in the presence of a large growing antral follicle. Non induced waves can emerge during the growth phase of an induced wave. The results from the present study therefore bring into question the presence of functional dominance in the ewe.

3.2. Introduction

In cattle, there are 2 or 3 waves of ovarian antral follicular growth during an estrous cycle (Pierson and Ginther 1988; Sirois and Fortune 1988). Wave emergence occurs at 7 to 10 day intervals (Savio *et al* 1988; Knopf *et al* 1989) and the emergence of each wave is preceded by a peak in serum FSH concentrations (Adams *et al* 1992). The emergence of a follicular wave, in cattle, is characterized by an increase in the number of small antral follicles (between 6 and 9 follicles, 4 to 6 mm in diameter; Ginther *et al* 1996). Within the next few days one of these follicles attains dominance, and the others become atretic (subordinate; Ginther *et al* 1989). It was suggested that during this period it is the dominant follicle that suppresses the growth of the subordinate follicles and prevents the emergence of a new follicular wave (Armstrong and Webb 1997). Mechanisms for this dominance included the early acquisition of LH receptors by the dominant follicle allowing it to become LH dependent, in parallel with the suppression of FSH secretion by secretory products of the dominant follicle, removing the FSH support of subordinate follicles (indirect dominance; Armstrong and Webb 1997; Austin *et al* 2002; Ginther *et al* 2003). In addition, treatment of cattle with physiological or supraphysiological concentrations of FSH, in the presence of a growing dominant follicle, failed to show the emergence of a new follicular wave, supporting the concept of direct follicle to follicle dominance (Guilbault *et al* 1991; Adams *et al* 1993).

In sheep, antral follicular growth occurs in a wavelike pattern, with waves emerging every 4 to 5 days (Ginther *et al* 1995; Bartlewski *et al* 1999a; Evans *et al* 2000) There are 3 to 4 waves of antral follicle growth per cycle (Noel *et al* 1993; Ginther *et al* 1995;

Bartlewski *et al* 1999a) and each wave is preceded by a transient peak in serum FSH concentrations (Ginther *et al* 1995; Souza *et al* 1998). In sheep, a follicular wave is regarded as a follicle or group of follicles that emerges or grows from 2 or 3 mm in diameter to an ovulatory size of ≥ 5 mm in diameter, with emergence restricted to a 24 h period (Duggavathi *et al* 2003a). In contrast to cattle, the use of high-resolution transrectal ultrasonography in sheep revealed that the number of small antral follicles (1-3 mm in diameter) did not increase at wave emergence except for the periovulatory period (Duggavathi *et al* 2003a). Again, in contrast to cattle, in a previous study in our laboratory with sheep (Duggavathi *et al* 2004), follicular wave emergence was induced by treatment with physiological concentrations of ovine FSH (oFSH), in the presence of a large growing follicle, bringing the existence of direct follicle to follicle dominance into question in the ewe. The objective of the present study was to investigate whether small antral follicles, in the ewe, are able to respond to a physiological peak in FSH secretion at different times in a follicular wave, with the induction of follicular wave emergence. In addition, we were interested to see if the next expected endogenously stimulated follicular wave would emerge in the presence of an induced wave, bringing the concept of indirect follicular dominance into question in the ewe.

3.3. Materials and Methods

3.3.1. Animals

Twelve adult, clinically healthy, cyclic Western White Face ewes were used for this experiment (mean body weight of 76.3 ± 3.9 kg). Ewes were housed outside in sheltered pens and were fed maintenance rations of hay with water and cobalt iodized salt available

ad libitum. Experimental procedures were performed according to the standards of the Canadian Council on Animal Care.

3.3.2. *Experimental Procedures*

Seven ewes were injected with ovine FSH (oFSH; 0.5 µg/kg) at 36 h after ovulation. The oFSH used was NIDDK-oFSH-18. Each 1 mg of oFSH had a biological potency of FSH equivalent to 65.6 x NIH-oFSH-S1 or 1640 IU and a biological potency of LH equivalent to 0.1 x NIH-oLH-S1 or 106 IU. The oFSH was prepared in saline with 0.05% BSA (w/v; Sigma, St. Louis, MO) and 50% polyvinylpyrrolidone (w/v; Sigma). A second injection of oFSH, at the same dose, was given 8 h after the first injection. Five control ewes received two injections of vehicle only. This treatment regimen, of two injections 8 h apart, was repeated at 72 h after ovulation. This treatment regimen was designed to create two physiological peaks in serum FSH concentrations equally spaced between the endogenous peaks that preceded the first and second follicular waves of the cycle. The induced peaks were designed to occur early and late in the growth phase of the first follicular wave of the cycle.

3.3.3. *Ultrasonography*

Transrectal ultrasonography of ovaries was performed using a high-resolution, real-time B-mode echo camera (Aloka SSD-900; Aloka Co. Ltd., Tokyo, Japan) connected to a 7.5 MHz transducer. The number, diameter and relative position of all follicles ≥ 1 mm in diameter and corpora lutea (CL), were sketched onto ovarian charts, and all ovarian images were recorded on high-grade video tapes (Fuji S-VHS, ST-120 N; Fujifilm,

Tokyo, Japan), using a compatible VCR (Panasonic, Omnivision Super VHS 2002, Model number PV-VS4821-K), for retrospective analysis of ovarian data. Ewes were scanned twice daily (0800 and 2000 h), starting from the time of estrus (assigned by the day ewes were marked by a crayon harnessed ram). Scanning continued until the identification of the emergence of the second wave of the cycle (non induced). Subsequently, ewes were scanned once a day until the emergence of the third non induced wave of the cycle.

3.3.4. Blood Sampling

Blood samples (10 ml) were collected prior to each scanning session, by jugular venipuncture, using vacutainers (Becton Dickinson, Rutherford, NJ, USA). Blood samples were allowed to clot for 18-24 h at room temperature, and serum was harvested and stored at -20°C until assayed.

3.3.5. Hormone Assays and Data Analyses

All serum samples were analyzed for circulating concentrations of FSH and estradiol by validated radioimmunoassays (Currie and Rawlings, 1987; Joseph *et al*, 1992). The range of the standard curves was from 0.1-16.0 ng/ml and 1.0-50.0 pg/ml for FSH and estradiol, respectively. The sensitivities of assays (defined as the lowest concentration of hormone capable of significantly displacing labeled hormone from the antibody; unpaired t-test, $P < 0.05$) were as follows: FSH, 0.1ng/ml and estradiol, 1pg/ml. All FSH samples were analyzed in one assay. The intra-assay coefficient of variation (CV) was 8.4%, for a reference serum with mean FSH concentration of 1.01 ng/ml. The intra- and inter-assay

CV's were 11% or 10%, and 12% or 11%, for reference sera with mean estradiol concentrations of 3.3 pg/ml or 43.1 pg/ml, respectively. Peaks in serum concentrations of FSH, in samples taken twice daily, were determined using the cycle-detection computer program (Clifton and Steiner, 1983). Serum concentrations of FSH and estradiol were normalized to the day of ovulation, and analyzed for the period from 0.5 days before to 8 days after ovulation.

3.3.6. Follicular Data Analyses

A follicular wave was regarded as a follicle or group of follicles that emerged or grew from 2 or 3 mm in diameter to an ovulatory size of ≥ 5 mm in diameter (Bartlewski *et al*, 1999a), with emergence restricted to a 24 h period (Duggavathi *et al*, 2003a). Treatments were given during the growth phase of the first wave of the cycle (wave 1). The first follicular wave induced by treatment was designated as wave A and the second follicular wave induced by treatment was designated as wave B. The follicular wave emerging after the second induced wave (oFSH treated ewes) or 4-5 days after the emergence of wave 1 (control ewes), was wave 2 of the cycle; the third wave of the cycle was also observed. The lengths of the growing, static, and regression phases of the largest follicle of the wave are presented. The day of follicular wave emergence was determined in relation to the day of ovulation. The maximum diameter of the follicle in a wave, the growth rate of the largest follicle in the wave and the number of follicles in a wave are also presented. The inter-wave intervals presented were defined as the interval between the time of wave emergence (i.e., time at which the largest follicle[s] of a wave was 2 or 3 mm in diameter) for two consecutive follicular waves (Duggavathi *et al*, 2004). Preliminary

analyses for numbers of 1-2 mm, 2 mm, and 3 mm follicles showed similar patterns. These follicles were therefore grouped as a single size-class (≥ 1 mm, but ≤ 3 mm in diameter) for subsequent analyses and presentation. Daily patterns are presented from day 0 to day 8 after ovulation, normalized to the day of ovulation.

3.3.7. *Statistical Analyses*

Due to missing follicular data, one ewe from the oFSH-treated group was removed from analysis. Statistical differences were assessed by two-way repeated-measures Analysis of Variance (ANOVA), one-way repeated-measures ANOVA (SigmaStat[®] Statistical Software, for Windows Version 2.03, 1997, SPSS Inc., Chicago, IL, USA). The mean length of the regression phase for waves B and 2 were not determined because not all ewes had a regressing follicle on the last day of the experiment. Multiple comparisons were made by the method of Fisher's least significant difference (LSD). All values are presented as means \pm S.E.M.

3.4. **Results**

3.4.1. *Administration of exogenous oFSH*

Analysis of serum FSH concentrations, by the cycle-detection program, identified two peaks in serum concentrations of FSH in both oFSH-treated and control ewes and these peaks preceded the emergence of waves 1 and 2 of the cycle (Table 3.1). Two additional peaks (preceding waves A and B) were identified at 2.10 ± 0.10 and 3.58 ± 0.08 days after ovulation respectively, in oFSH-treated ewes only (Table 3.1). ANOVA showed significant group and time effects, and a group x time interaction ($P < 0.05$) for mean

serum FSH concentrations (Figure 3.1). Fishers LSD confirmed the serum FSH peaks identified by the cycle detection program for FSH peaks A and B (Figure 1).

Table 3.1. Mean day of peak in serum FSH concentrations (ng/ml; detected using the cycle detection program) and mean day on which wave emergence was detected, in ewes given oFSH (n=6) or vehicle (n=5) 1.5 days and 3 days after ovulation.

Parameter	Group	Peak 1	Peak A	Peak B	Peak 2
Day of FSH-peak	oFSH-treated	0.50 ± 0.13	2.10 ± 0.10	3.58 ± 0.08	5.42 ± 0.20
	Control	0.10 ± 0.25	No Peak A	No Peak B	4.80 ± 0.47
Day of wave emergence		Wave 1	Wave A	Wave B	Wave 2
	oFSH-treated	0.33 ± 0.11	2.00 ± 0.00	3.50 ± 0.00	5.33 ± 0.11
	Control	0.30 ± 0.26	No Wave A	No Wave B	5.10 ± 0.19

Day 0 = Day of ovulation. Data presented as mean ± SEM.

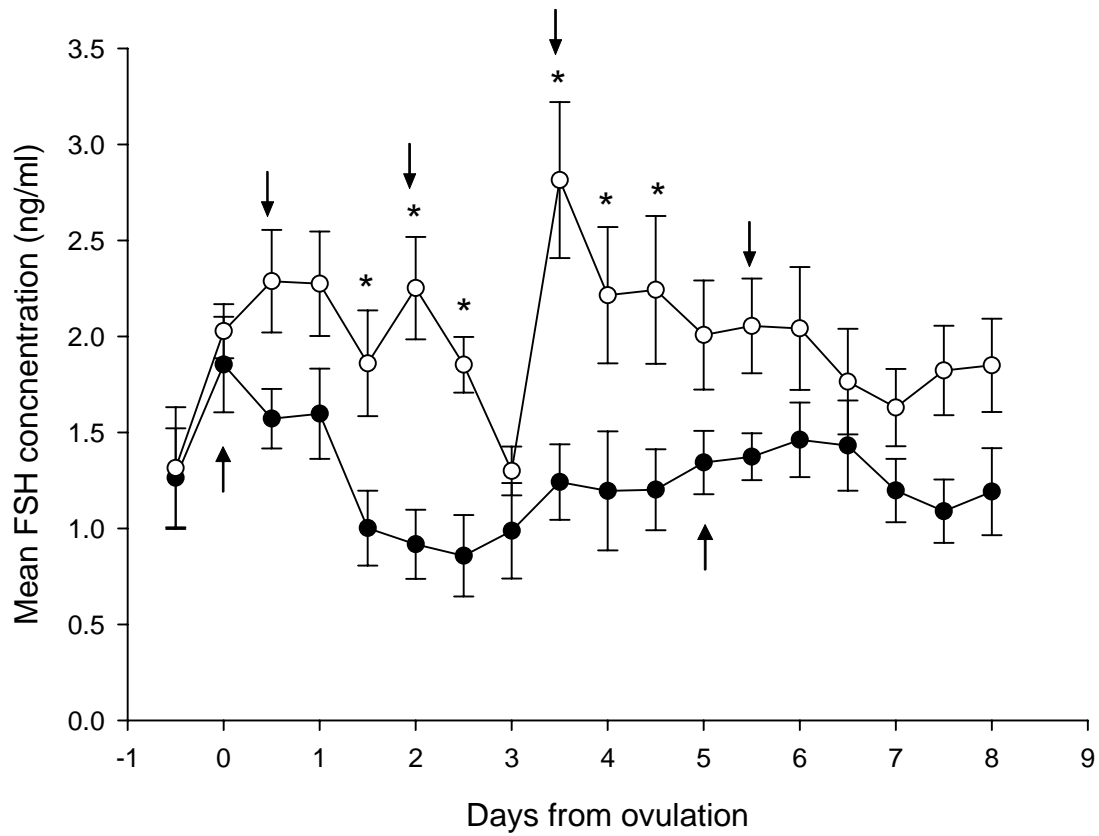


Figure 3.1. Mean circulating serum concentrations of FSH (ng/ml) from -0.5 days to 8 days after ovulation, in ewes given oFSH (○; n=6) or vehicle (●; n=5) 1.5 days and 3 days after ovulation. An arrow denotes a peak in FSH concentration identified by the cycle detection program for oFSH-treated (↓) or vehicle (↑). Day 0 = Day of ovulation. Data presented as mean ± SEM. * denotes a significant induced peak in serum concentrations of FSH (P<0.05).

3.4.2. Follicular wave emergence

The mean days of wave emergence for waves 1 and 2 of the cycle did not differ between oFSH-treated and control ewes (Table 3.1). Two additional waves (waves A and B) emerged after oFSH treatment in oFSH-treated ewes (Table 3.1). The interval (days) between the emergence of waves 1 and 2 did not differ ($P>0.05$) between oFSH-treated and control ewes (4.42 ± 0.33 and 4.90 ± 0.36 , respectively). The interval (days) between the emergence of waves 2 and 3 did not differ ($P>0.05$) between oFSH-treated and control ewes (3.75 ± 0.33 and 4.00 ± 0.36 , respectively).

3.4.3. Characteristics of the largest follicle of the follicular wave

Within the oFSH-treated ewes, the length of the growth phase did not differ amongst waves 1 and 2 (Table 3.2). However, the largest follicle of wave 1 had a significantly longer growth phase than both waves A and B; the largest follicle of wave 2 had a growth phase significantly longer than the growth phase of wave A (Table 3.2). There was no significant effect ($P>0.05$) of wave, or group, or a wave x group interaction for the lengths of the static phases (Table 3.2).

Table 3.2. The length of the growing, static, and regression phases (days), maximum follicle diameter (mm) and growth rate of the largest of a wave. Serum estradiol concentrations (pg/ml) at maximum follicle diameter within each wave, and the number of follicles in a wave. All parameters were measured for waves 1 and 2 in control ewes (n=5) given vehicle, and waves 1, A, B, and 2 in ewes given oFSH (n=6) 1.5 days and 3 days after ovulation.

Parameter	Group	n	Wave 1	Wave A	Wave B	Wave 2
Growth phase (days)	oFSH-treated	6	3.1 ± 0.3 ^a	1.4 ± 0.2 ^b	1.9 ± 0.3 ^{bc}	2.7 ± 0.3 ^{ac}
	Control	5	2.7 ± 0.4	No Wave	No Wave	2.6 ± 0.2
Static phase (days)	oFSH-treated	6	3.4 ± 0.4	4.4 ± 1.2	3.0 ± 0.7	2.0 ± 0.5
	Control	5	3.6 ± 0.6	No Wave	No Wave	2.5 ± 0.4
Regression phase (days)	oFSH-treated	6	1.9 ± 0.4	3.1 ± 0.4	Not determined	Not determined
	Control	5	2.7 ± 0.2	No Wave	No Wave	Not determined
Maximum diameter (mm)	oFSH-treated	6	6.3 ± 0.3	5.7 ± 0.5	5.8 ± 0.4	5.8 ± 0.2 ^y
	Control	5	7.0 ± 0.3	No Wave	No Wave	6.6 ± 0.2 ^x
Estradiol concentration (pg/ml)	oFSH-treated	6	3.9 ± 0.5 ^y	4.3 ± 0.6	3.1 ± 1.2	4.2 ± 1.5
	Control	5	6.4 ± 0.6 ^x	No Wave	No Wave	5.1 ± 1.1
Growth rate (mm/day)	oFSH-treated	6	1.4 ± 0.1 ^{ey}	2.5 ± 0.3 ^d	2.2 ± 0.6 ^d	1.4 ± 0.1 ^e
	Control	5	2.0 ± 0.2 ^x	No Wave	No Wave	1.9 ± 0.3
Number of follicles in a wave	oFSH-treated	6	1.0 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.2 ^y
	Control	5	1.6 ± 0.4	No Wave	No Wave	1.8 ± 0.2 ^x

Data presented as mean ± S.E.M.

^{a,b,c} denotes a significant difference (P<0.05) between waves within a group.

^{d,e} denotes a tendency for significant differences (P=0.08) between waves within a group.

^{x,y} denotes a significant difference (P<0.05) between groups within a wave.

3.4.4. Maximum follicle diameter and estradiol concentrations

There were no significant differences ($P>0.05$) amongst waves in maximum follicle diameter and peak estradiol concentrations at maximum follicle diameter within oFSH-treated ewes (waves 1, 2, A, and B of the cycle) and control ewes (waves 1 and 2 of the cycle; Table 3.2). Within wave 2, control ewes had a greater maximum follicle diameter than the oFSH-treated ewes ($P<0.05$; Table 3.2). Within wave 1, the control ewes had a higher serum estradiol concentration at maximum follicle diameter than the oFSH-treated ewes ($P<0.05$; Table 3.2).

3.4.5. Follicle growth rate and number of follicles in a wave

Follicle growth rate did not differ ($P>0.05$) between waves within the control group (Table 3.2). However, the growth rate of follicles in wave 1 was greater ($P<0.05$) for the control ewes than the oFSH-treated ewes (Table 3.2). Follicle growth rate within the oFSH-treated ewes tended ($P=0.08$) to be greater for follicles in waves A and B as compared to those follicles in waves 1 and 2 (Table 3.2). Within wave 2, oFSH-treated ewes had a significantly lower number of follicles in the wave as compared to ewes in the control group.

3.4.6. Numbers of small follicles

There was no significant effect of group or a group x time interaction for mean numbers of small follicles (≥ 1 mm but ≤ 3 mm in diameter) analyzed for the period from the day of ovulation (Day 0) to eight days after ovulation. However, there were an increased number of small follicles around the time of ovulation. Numbers of small follicles declined

($P < 0.05$) by day 2 in oFSH-treated ewes and by day 7 in the control ewes (Figure 3.2).

The numbers of small follicles from 0 to 8 days after ovulation ranged from 9 to 16 in oFSH-treated ewes and from 9 to 13 in control ewes.

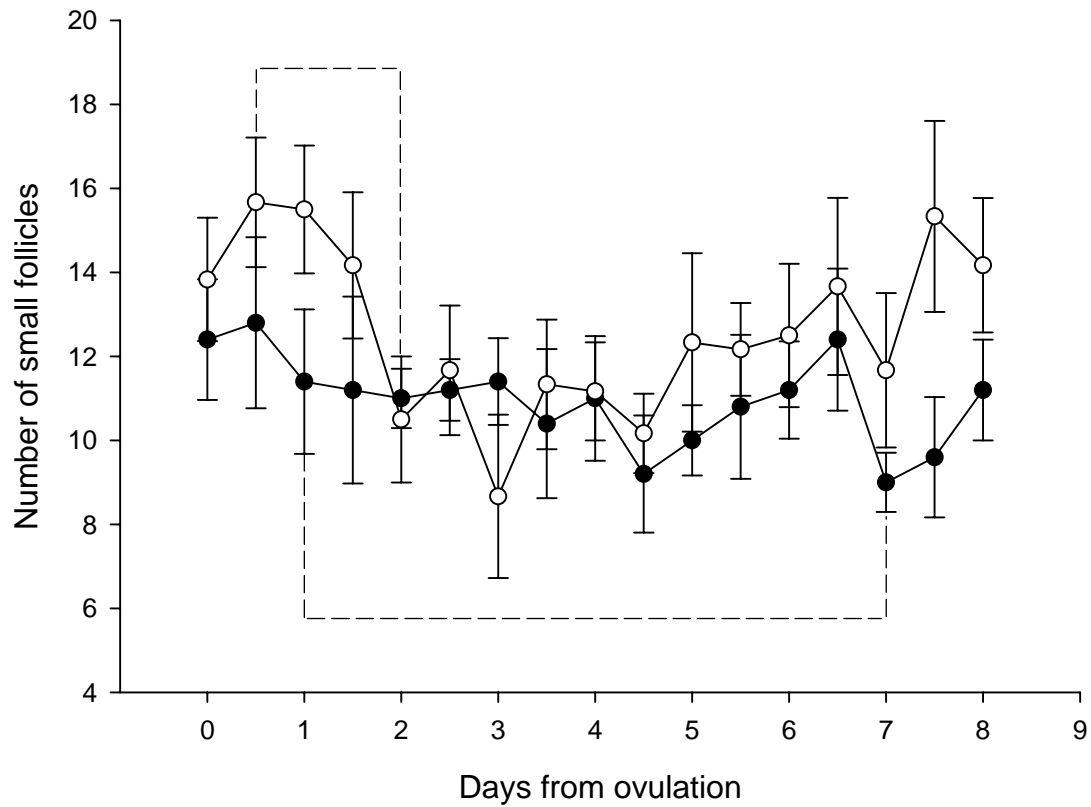


Figure 3.2. Mean daily number of small follicles (≥ 1 mm to ≤ 3 mm in diameter) in ewes given oFSH (\circ ; $n=6$) or vehicle (\bullet ; $n=5$) on day 1.5 and day 3 after ovulation. Dashed lines represent significant differences between points in the number of small follicles. Day 0 = Day of ovulation. Data presented as \pm SEM.

3.5. Discussion

In the present study, injections of oFSH resulted in peaks in serum concentrations of FSH on days 2.1 ± 0.1 and 3.6 ± 0.1 after ovulation, creating a series of 4 peaks fairly equally spaced (2 endogenous peaks surrounding 2 induced peaks). Injections of oFSH in turn induced follicular wave emergence with similar time relationships. The induced follicular waves did not delay the emergence of the next expected endogenous follicular wave of the cycle (wave 2). The induced waves emerged in the presence of a large (≥ 4 mm in diameter) growing follicle and the second non induced wave of the cycle (Wave 2) emerged during the growth phase of the second induced follicle wave (Wave B). Therefore, it appeared that follicular waves could be induced in the presence of Wave 1 of the cycle and that induced waves did not have any inhibitory effect on the growth of Wave 1 or the emergence of Wave 2 of the cycle, even though Wave 2 emerged while induced-Wave B was growing. These findings bring direct and indirect dominance in sheep into question.

The findings of the present study contradict those of similar studies performed in cattle. Adams *et al* (1993) reported an inability of injection of recombinant bovine FSH (rbFSH) to stimulate wave emergence in the cow during the growth phase (dominance) of the first wave of the cycle suggesting a direct follicle to follicle dominance. The same authors speculated that follicular dominance in cattle does not involve suppression of FSH (indirect dominance) secretion as postulated by other authors (Ginther *et al* 2003). Several recent studies involving sheep also bring into question the presence of follicular dominance in the ewe. Some of the relevant findings include those of Driancourt *et al*

(1991) who found that eCG-induced growth of follicles in the ewe was not suppressed by the presence of a large follicle. In addition to this, Bartlewski *et al* (1999a) found that Finnish Landrace ewes frequently ovulate follicles from the penultimate wave of the estrous cycle along with follicles from the final wave of the cycle. There are also numerous reports of follicular wave emergence occurring in the presence of a growing ovulatory-size follicle when ewes were treated with exogenous progesterone (Johnson *et al* 1996; Leyva *et al* 1998; Flynn *et al* 2000). Considering all of this information together, it appears that there may be distinct differences between cattle and sheep in terms of their mechanisms of regulation of ovarian follicular wave patterns.

In cattle, follicle wave emergence is accompanied by an increase in the number of small antral follicles (Ginther *et al* 1996). However, in the present study in sheep, there was only an increase in the number of small follicles around the time of ovulation. This is in agreement with a recent study involving sheep, where it was found that the number of small antral follicles remained constant throughout the estrous cycle, except for the periovulatory period (Duggavathi *et al* 2003a).

Maximum follicle diameter and serum estradiol concentrations at maximum follicle diameter did not differ ($P>0.05$) between oFSH-induced and non induced follicular waves, suggesting that induced follicles were functionally normal. However, the induced follicular waves in oFSH-treated ewes had significantly shorter growth phases, and tended ($P=0.08$) to have faster growth rates, than follicles in waves 1 and 2. It could be argued that the differences in growth profiles between the oFSH-induced and non-

induced follicular waves may be related to the characteristics of the FSH peak created with exogenous oFSH. In the present study, distinct FSH peaks were created in ewes given FSH but because 4 FSH peaks occurred within about 6 days the complete profiles of each peak could not be described. However, in a previous study (Duggavathi *et al* 2004), when a single FSH peak was induced between endogenous FSH peaks, using the same regimen of injections of oFSH employed in the present study, it was found that the amplitude of the induced FSH peak was similar to the amplitude of endogenous FSH peaks but the induced peak was of shorter duration than non induced endogenous peaks. However, as in the present study, induced follicles appeared functionally normal and growth rate and the length of the growth phase did not differ from non induced follicular waves. The higher frequency of follicular wave induction in the present study, early in the cycle, may have increased pulsatile LH secretion (not measured) by giving more peaks of serum concentrations of estradiol. Such an effect could have been subsequently suppressed by rising serum concentrations of progesterone by the time of emergence of Wave 2 of the cycle. These intriguing findings await further study.

In conclusion, injection of oFSH increased serum FSH concentrations and induced follicular wave emergence from the pool of small follicles, in the presence of a large (≥ 4 mm in diameter) follicle (wave 1 of the cycle), without disrupting the emergence of the second follicular wave of the cycle. In the ewe, small follicles can respond to FSH peaks to yield a follicular wave more frequently than seen in a normal cycle and in the presence of a growing follicle; and non induced waves can emerge during the growth phase of an

induced wave. The results from the present study therefore bring into question the presence of functional dominance in the ewe.

Chapter 4: DOES INJECTION OF PROSTAGLANDIN F_{2α} (PGF_{2α}) CAUSE OVULATION IN ANESTROUS WESTERN WHITE FACE EWES?

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4.1. Abstract

In a previous study in our laboratory, treatment of non-prolific Western White Face (WWF) ewes with PGF_{2α} and medroxyprogesterone acetate (MAP)-containing intravaginal sponges on ~Day 8 of a cycle (Day 0=first ovulation of the interovulatory interval), resulted in ovulations during the subsequent 6 days when MAP sponges were in place. Two experiments were performed on WWF ewes during anestrus to allow us to independently examine if such ovulations were due to the direct effects of PGF_{2α} on the ovary or the effects of rapid decline in progesterone at PGF_{2α}-induced luteolysis. Experiment 1: Ewes fitted with MAP sponges for 6 days (n=12), were injected with PGF_{2α} (n=6; 15 mg i.m.), or saline (n=6) on the day of sponge insertion. Experiment 2: Ewes received progesterone-releasing subcutaneous implants (n=6) or empty implants (n=5) for 5 days. Six hours prior to implant removal, all ewes received a MAP sponge, which remained in place for 6 days. Ewes from both experiments underwent ovarian ultrasonography and blood sampling once daily for 6 days before and twice daily for 6 days after sponge insertion. Additional blood samples were collected every 4 hours during sponge treatment. Experiment 1: Four of six (67%) PGF_{2α}-treated ewes ovulated ~1.5 d after PGF_{2α} injection; these ovulations were not preceded by estrus or a preovulatory surge release of LH, and did not result in corpora lutea (CL). The growth phase was longer (P<0.05) and the growth rate slower (P<0.05) in ovulating as compared to non ovulating follicles in PGF_{2α}-treated ewes. Experiment 2: In ewes given

progesterone implants, serum progesterone concentrations reached a peak (1.72 ng/ml; $P < 0.001$) on the day of implant removal and fell to basal concentrations (< 0.17 ng/ml; $P < 0.001$) within 24 hours of implant removal. No ovulations occurred in either the treated or the control ewes. We concluded that ovulations occurring after $\text{PGF}_{2\alpha}$ injection, in the presence of a MAP sponge could be due to a direct effect of $\text{PGF}_{2\alpha}$ at the ovarian level rather than a sudden decline in circulating progesterone concentrations.

4.2. Introduction

During the estrous cycle, ewes have three to four waves of antral follicle growth (Noel *et al*, 1993; Schrick *et al* 1993; Ginther *et al* 1995; Bartlewski *et al* 1999a; Gibbons *et al* 1999; Evans *et al* 2000; Duggavathi *et al* 2003a). Each wave consists of 1 to 3 antral follicles that grow from a pool of follicles 1-3 mm in diameter and reach a maximum diameter of about 5 to 6 mm before regression or ovulation (Ginther *et al*, 1995; Souza *et al* 1997; Bartlewski *et al* 1999a; Evans *et al* 2000; Vinales *et al* 2001). Each wave is preceded by a transient increase in serum FSH concentrations (Ginther *et al*, 1995; Bartlewski *et al* 1999a, 2000; Duggavathi *et al* 2005). In non-prolific breeds of sheep such as the Western White Face (WWF), an antral follicle(s) from the final wave of the cycle will ovulate rather than regress (Bartlewski *et al*, 1999a). However, in prolific Finnish Landrace ewes, follicles also ovulate from the penultimate wave of the cycle. These follicles from the penultimate wave have a prolonged lifespan, allowing them to ovulate with follicles from the final wave (Bartlewski *et al*, 1999a). In prolific breeds of sheep, such as the Finnish Landrace, mean serum progesterone concentrations during the

luteal phase of the cycle were lower than in the non prolific WWF sheep (Bartlewski *et al*, 1999b).

In other experimental studies, creation of lower than normal luteal phase concentrations of progesterone in cyclic ewes resulted in the prolonged lifespan of large antral follicles in a follicular wave (Johnson *et al* 1996; Vinales *et al*, 1999). Similar effects were observed when luteolysis was induced with PGF_{2α} on Day 6 and intravaginal sponges containing MAP inserted, from Days 5 to 19 after ovulation (Flynn *et al* 1999), or when MAP-impregnated sponges were inserted on Day 12 after ovulation and ovaries were exposed to MAP in the absence of functional CL (Leyva *et al* 1998). It was suggested that MAP treatment applied in the absence of endogenous progesterone effectively mimicked a low progesterone regimen in sheep (Bartlewski *et al* 2003). The potential for short-term treatment with MAP intravaginal sponges to increase ovulation rate in non-prolific breeds of sheep by mimicking the low progesterone environment seen in prolific Finn sheep, was further studied by Bartlewski *et al* (2003).

Bartlewski *et al* (2003) investigated the effects on follicular wave development and ovulation rate of treating non-prolific WWF ewes, for 6 days with a MAP-releasing intravaginal sponge, starting on day 8 after ovulation and coupled with a single administration of PGF_{2α} on the day of sponge insertion. This treatment resulted in the extension of the lifespan of large antral follicles and an increased ovulation rate after the treatment. However, surprisingly, one to 6 days after PGF_{2α} administration, during the period sponges were in place, several large antral follicles ovulated; these ovulations

were not preceded by a preovulatory LH/FSH surge, and none of these ovulations were followed by the formation of CL, only transient corpora hemorrhagica (CH; Bartlewski *et al* 2003). Prostaglandin treated animals experienced the expected drop in serum progesterone concentrations after treatment and progesterone concentrations remained low throughout the MAP treatment period.

The objectives of the present study were to determine whether the ovulations that occurred after PGF_{2α} treatment in the presence of an intravaginal sponge releasing a progestagen, in cyclic ewes, could be due to a direct effect of PGF_{2α} on the ovary or due to the sharp decline in serum progesterone concentrations caused by the luteolytic dose of PGF_{2α}. To study this we used seasonally anovular anestrous ewes. This allowed us to look at the effects of PGF_{2α} in the absence of a decline in serum progesterone concentrations and to use progesterone releasing implants to create an increase and precipitate drop in serum concentrations of progesterone independent of luteolysis induced by PGF_{2α}.

4.3. Materials and Methods

4.3.1. Animals

Experiment 1: Twelve adult, clinically healthy anestrous (May-June) Western White Face ewes were used in this experiment, with a mean body weight of 72 ± 4 kg. Experiment 2: Eleven (May-June) Western White Face ewes were used for this experiment, with a mean body weight of 76 ± 3 kg. All ewes were housed outdoors in sheltered pens and were fed maintenance rations of alfalfa hay daily. Ewes were kept with vasectomized crayon-

harnessed rams. Experimental procedures were performed according to the standards of the Canadian Council on Animal Care.

4.3.2. *Experimental Procedure*

Transrectal ultrasonography of ovaries was performed using real-time, high-resolution, B-mode ultrasound scanning equipment (Aloka SSD-900, Aloka Co. Ltd., Tokyo, Japan) and a stiffened 7.5 MHz transducer. Experiment 1: All ewes underwent daily transrectal ultrasonography for 6 days prior to the start of the treatment. On day 7, all ewes were treated with progestagen-releasing intravaginal sponges (Medroxyprogesterone acetate (MAP), 60mg; Veramix[®], Upjohn, ON, Canada), sponges were left in place for 6 days. Six ewes were treated with a single injection of Prostaglandin F_{2α} (PGF_{2α}), (15mg i.m.; Lutalase, Upjohn, Orangeville, ON, Canada) on the day of MAP sponge insertion; ewes in the control group (n=6) were treated with 3ml of sterile saline. From the day of sponge insertion onwards all ewes were scanned twice daily until the end of treatment with MAP sponges. Experiment 2: All ewes underwent daily transrectal ultrasonography for 6 days prior to the start of the treatment. On the seventh day after the start of scanning, six ewes (treatment group) received subcutaneous progesterone-releasing silastic rubber implants (22cm x 0.48cm; Rawlings *et al*, 1984; Elastomer from Factor II, Lakeside, Arizona, USA; Progesterone from Sigma-Aldrich, Oakville, Ontario, Canada). Implants were placed in the axillary region under local anaesthetic (Rawlings *et al* 1984). The five ewes in the control group (n=5) received empty silastic rubber implants (containing no progesterone). Implants remained in place for 5 d and all ewes underwent once daily scanning during the period when implants were in place. All ewes had a MAP

intravaginal sponge inserted 6 hours prior to the removal of the silastic implants. MAP sponges remained in place for 6 days, during which time ewes underwent twice daily transrectal ultrasonography. The treatments of experiment 2 were aimed to mimic intravaginal sponge treatment at Day 8 after ovulation in cyclic ewes and the precipitous drop in serum concentrations of progesterone that PGF_{2α} treatment would induce at that stage of the cycle (Bartlewski *et al* 2003).

4.3.3. *Blood Sampling*

Experiment 1: Blood samples were collected (10ml) by jugular venipuncture using vacutainers (Becton Dickinson, Rutherford, NJ, USA) prior to each scanning session for the first 6 days of the experiment; in addition, ewes were bled every 4 hours (4 ml) during MAP sponge treatment, via indwelling jugular catheters (vinyl tubing, 1.00 mm inside diameter × 1.50 mm outside diameter; SV70, Critchley Electrical Products Pty Ltd., Auburn, NSW, Australia). Blood samples were allowed to clot for 18 to 24 hours at room temperature, and serum was harvested and stored at -20°C until assayed. Experiment 2: All ewes were bled before each scanning session, in addition, ewes were also bled every 4 hours during MAP sponge treatment.

4.3.4. *Hormone Analyses*

Circulating concentrations of LH (Rawlings *et al*, 1998) and FSH (Joseph *et al*, 1992) were determined by previously validated radioimmunoassay. All serum samples were analyzed for concentrations of LH and FSH in a single assay. The range of standards was from 0.1 to 8 ng/ml for LH (NIAMMD-oLH-24) and 0.1 to 16 ng/ml for FSH

(NIAMMD-oFSH-RP-1). The sensitivities of the assays (lowest concentration of hormone capable of significantly displacing labelled hormone from the antibody) were both 0.1 ng/ml. The intra-assay coefficient of variation (CV) was 12.6% for a reference serum with mean LH concentration of 0.16 ng/ml. For reference sera with mean FSH concentrations of 0.57 or 1.35 ng/ml, intra-assay CVs were 9.4 and 13.7%, respectively.

Circulating concentrations of progesterone (Ravindra *et al*, 1994) and estradiol (Joseph *et al*, 1992) were determined by previously validated radioimmunoassays. Two samples per day were analyzed for concentrations of estradiol and one sample per day for progesterone concentrations. The range of standards was from 1.0 to 50 pg/ml for estradiol with a sensitivity of 1 pg/ml, and 0.1 to 10.0 ng/ml for progesterone with a sensitivity of 0.03 ng/ml. Experiment 1: Intra- and inter-assay CV's for the progesterone assay were 11.2 and 15.2% or 4.2 and 6.8% for reference sera with mean concentrations of 0.45 or 0.92 ng/ml respectively. Intra- and inter-assay CV's for the estradiol assay were 16.6 and 17.80% or 11.2 and 16.30% for reference sera with mean concentrations of 5.0 or 15.9 pg/ml, respectively. Experiment 2: Intra- and inter-assay CV's for the progesterone assay were 9.2 and 12.4% for a reference serum with mean concentration of 0.62 ng/ml, respectively. Intra- and inter-assay CV's for the estradiol assay were 10.9 and 8.0% or 12.4 and 11.4% for reference sera with mean concentrations of 8.8 or 23.5 pg/ml, respectively.

4.3.5. Follicular Data Analyses

Follicular data (follicles ≥ 2 mm in diameter) were combined for both ovaries of each ewe. A follicular wave was defined as 1 or more antral follicles that grew from 2 mm to ≥ 5 mm in diameter; the day the follicles were first detected at 2 mm was the day of wave emergence (Duggavathi *et al*, 2003a). Experiment 1: The following characteristics of follicles and follicular waves were determined for each ewe in which ovulation was detected during the period of MAP treatment (1) the number of follicles ovulating; (2) the time from PGF_{2 α} injection to ovulation; (3) the interval from emergence to ovulation; (4) the number of large follicles (follicles ≥ 5 mm in diameter) from 24 hours before to 36 hours after ovulation (n=4); (5) the mean diameter of the ovulating follicles at 12 h before ovulation; (6) the time from attainment of maximum follicular diameter to ovulation; (7) the duration of corpus hemorrhagicum (CH) detection. The following follicular characteristics were determined for all ewes (1) the number of follicular waves emerging during MAP sponge treatment; (2) the maximum follicle diameter of follicles that reached their maximum diameter during MAP sponge treatment; (3) the length of the growth phase of follicles that reached their maximum diameter during MAP sponge treatment; (4) the growth rate of follicles that reached their maximum diameter during MAP sponge treatment. The day of ovulation of a follicle, as detected by ultrasonography, was the disappearance of a follicle ≥ 5 mm in diameter from one scanning session to the next, and the demonstration of a corpus hemorrhagicum (CH: Duggavathi *et al* 2003b). Experiment 2: The following characteristics of follicles and follicular waves were determined for each ewe; (1) the number of follicular waves emerging during MAP sponge treatment; (2) the numbers of large follicles (≥ 5 mm in

diameter) present in both ovaries each day were determined per ewe from 2 days before until 10 days after implant insertion.

4.3.6 Statistical Analyses

One-way repeated measures analysis of variance (ANOVA; SigmaStat[®] for Windows Version 2.03, 1997, SPSS Inc., Chicago, IL, USA) was employed for comparisons of emerging follicular waves and individual antral follicles attaining ≥ 5 mm in diameter in experiment 1. Two-way repeated measures ANOVA was employed to analyse: (1) the changes in number of large follicles (follicles ≥ 5 mm in diameter) from 2 days before implant insertion to 6 days after MAP sponge insertion in treated and control ewes (Experiment 2) (2) mean serum hormone concentrations (LH, FSH, progesterone and estradiol) from 2 days before prostaglandin injection and insertion of MAP sponge to 6 days afterwards (Experiment 1) and from 2 days before implant insertion to 6 days after MAP sponge insertion (Experiment 2). The numbers of follicular waves emerging during MAP sponge treatment (Experiment 2) were compared between treated and control ewes using a t-test. Statistical significance was defined as $P < 0.05$. Data are presented as mean \pm S.E.M.

4.4. Results

4.4.1. Experiment 1

Occurrence of ovulation

There were no ovulations in the control ewes, during the period MAP sponges were in place. However, one control ewe ovulated two follicles before the day of saline injection

and MAP sponge insertion and formed two corpora lutea. At a mean of 1.2 ± 0.4 days after treatment with $\text{PGF}_{2\alpha}$ and insertion of progestogen sponges (Day 0), 4 of 6 (67%) treated ewes ovulated, resulting in a total of 5 ovulations. There were three ewes which ovulated once (On day 0, pm; day 1, am; day 2, am), and one ewe which ovulated twice (On day 1, am; day 2, pm). All of these ovulations occurred during the period of MAP sponge treatment; no mature corpora lutea resulted.

Ovulating Follicles

For those follicles ovulating after $\text{PGF}_{2\alpha}$ injection and during MAP treatment, the interval from emergence of the follicular wave to ovulation was 6.6 ± 0.7 days. The mean follicle diameter before ovulation was 5.4 ± 0.2 mm. The time from attainment of maximum follicle diameter to ovulation was 2.4 ± 0.9 days. The duration of corpus hemorrhagicum detection was 3.2 ± 0.6 days. In the ewes that ovulated, after treatment, there was a significant decline in the number of large follicles from 12 hours before to the time of ovulation (Figure 4.1; $P < 0.001$).

Follicle Comparisons

The number of follicle waves emerging during MAP sponge treatment did not differ between treatment and control groups (1.5 ± 0.2 waves and 1.8 ± 0.2 waves, respectively; $P > 0.05$). The maximum follicle diameter (mm) of follicles that reached their maximum diameter during MAP sponge treatment did not differ between the treated and control ewes for non ovulating follicles (5.3 ± 0.2 mm and 5.2 ± 0.1 mm, respectively; $P > 0.05$) or between ovulating and non ovulating follicles within the treatment group (5.6 ± 0.2

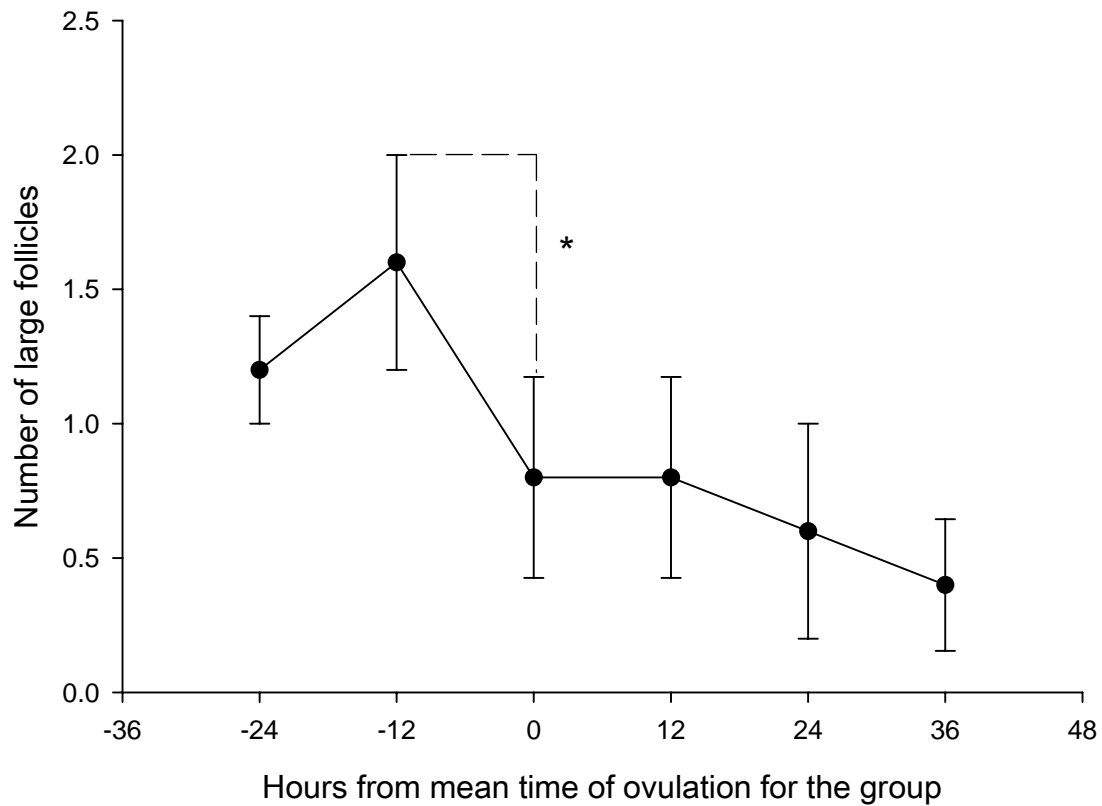


Figure 4.1. Mean (\pm SEM) number of large follicles (follicles ≥ 5 mm in diameter) from 24 hours before to 36 hours after ovulations in ewes ($n=4$) given $\text{PGF}_{2\alpha}$ injection on the first day of a 6 day treatment with intra vaginal sponges releasing medroxyprogesterone acetate (Hours from time of ovulation). (* $P<0.001$). Ovulations occurred 1.2 ± 0.4 days after $\text{PGF}_{2\alpha}$ treatment.

mm and 5.3 ± 0.2 mm, respectively; $P > 0.05$). The length of the growth phase (days) of follicles reaching maximum diameter during MAP sponge treatment did not differ between treated and control ewes for non ovulating follicles (2.3 ± 0.5 days and 2.6 ± 0.4 days, respectively; $P > 0.05$) but was longer for ovulating than non ovulating follicles within the treatment group (4.8 ± 0.6 days and 2.3 ± 0.5 days, respectively; $P < 0.05$). The growth rate (mm/day) of follicles reaching maximum diameter during MAP sponge treatment did not differ between treatment and control groups for non ovulating follicles (2.8 ± 0.4 mm/day and 2.6 ± 0.5 mm/day, respectively; $P > 0.05$) but was lower for ovulating than non ovulating follicles within the treatment group (1.3 ± 0.2 mm/day and 2.8 ± 0.4 mm/day, respectively; $P < 0.05$).

Mean Serum LH and FSH Concentrations

There was a significant day effect for both mean serum concentrations of LH and FSH. The day effect for mean serum FSH concentrations was due to the natural fluctuations in serum FSH concentrations associated with follicle wave emergence. The day effect for mean serum LH concentrations was due to an immediate decline on day 0 (Day 0 = day of $\text{PGF}_{2\alpha}$ injection). There was no evidence of a surge in secretion of LH prior to any of the ovulations occurring after $\text{PGF}_{2\alpha}$ injection and during the period of MAP sponge treatment (see Figure 4.2).

Mean Serum Progesterone and Estradiol Concentrations

The overall mean circulating progesterone concentration for this experiment was 0.1 ± 0.0 ng/ml and there was no significant difference in progesterone concentrations between

treatment and control groups and no significant day effect. Mean circulating estradiol concentrations were 2.3 ± 0.1 pg/ml and did not differ between the two groups; there was no significant day effect.

4.4.2. Experiment 2

Occurrence of ovulation

There were no ovulations observed in either the treatment or control ewes.

Mean Serum Progesterone Concentrations

Serum Progesterone concentrations in control ewes remained basal and did not change throughout the experiment. After implant insertion (day 0), serum progesterone concentration significantly increased reaching a peak of 1.72 ng/ml, on the day of implant removal (day 5). Progesterone concentrations then declined ($P < 0.05$) to basal concentrations within 24 hours of implant removal (see Figure 4.3).

Follicle Characteristics

The daily number of large follicles present in each ewe did not differ significantly between the treatment and control groups from 2 days before to 10 days after implant insertion. There was also no statistically significant difference between treatment and control ewes for the number of follicular waves emerging during MAP sponge insertion (1.3 ± 0.2 and 1.6 ± 0.2 ; $P > 0.05$, respectively).

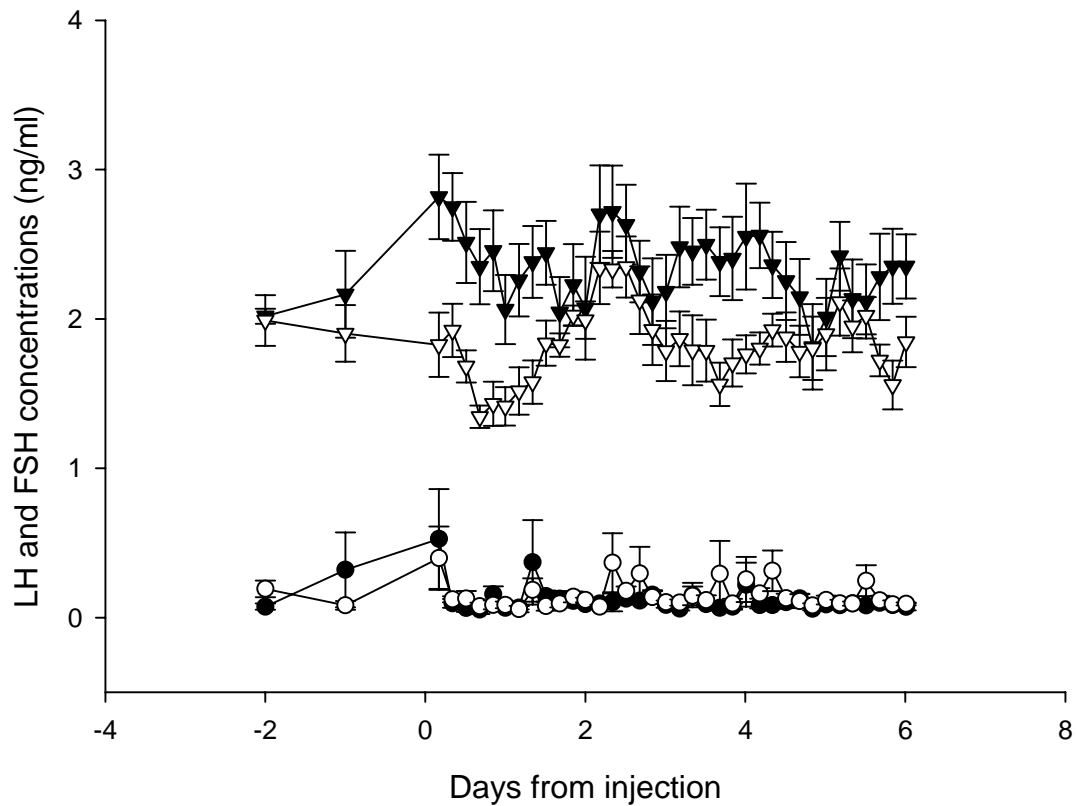


Figure 4.2. Mean (\pm SEM) serum concentrations of LH (circles) and FSH (triangles) in ewes for the period from 2 days before to 6 days after the day of injection of $\text{PGF}_{2\alpha}$ (filled symbols; $n=6$) or saline (open symbols; $n=6$) and start of a 6 day treatment with intra vaginal sponges releasing medroxyprogesterone acetate. There was a significant day effect for both LH ($P<0.05$) and FSH ($P<0.001$).

Mean Serum Estradiol, LH and FSH Concentrations

There was no significant difference between treatment and control ewes for serum concentrations of estradiol (mean serum concentration of estradiol were 5.4 ± 0.4 pg/ml), LH or FSH (Figure 4.4) from 2 days before until 11 days after implant insertion.

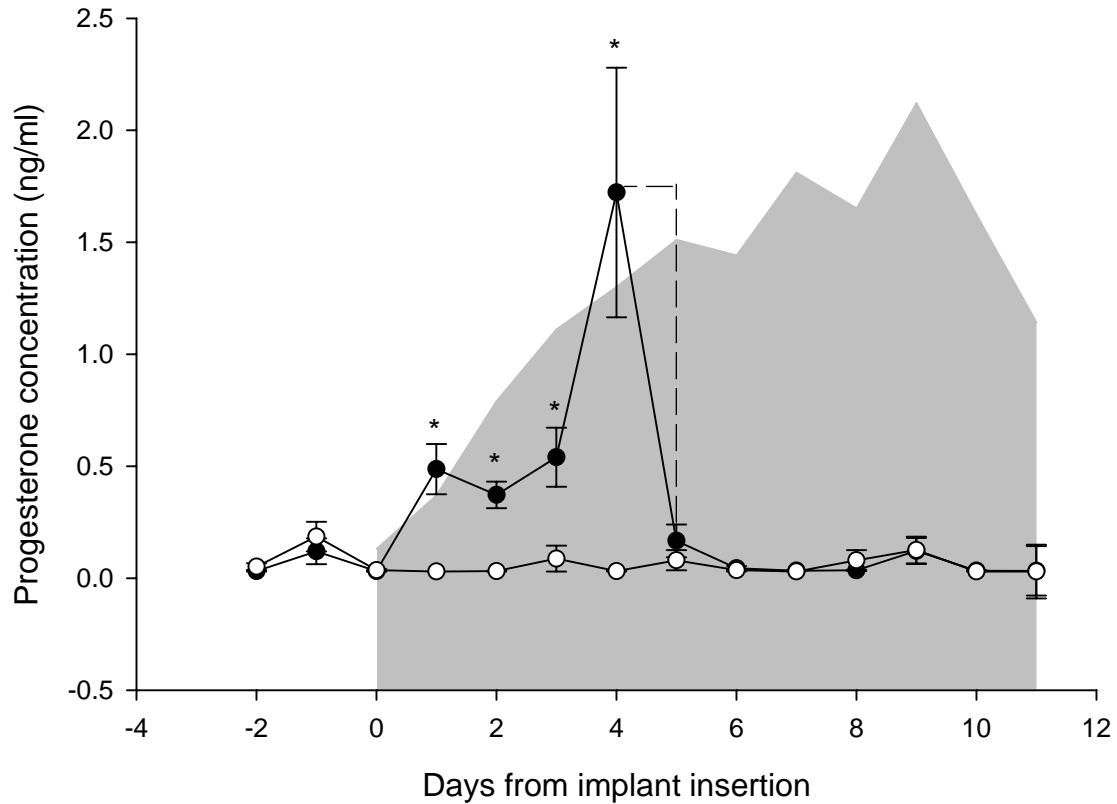


Figure 4.3. Mean serum concentrations of progesterone (mean \pm SEM) in 6 ewes given silastic rubber implant releasing progesterone (filled symbols) and 5 ewes given blank implants (open symbols), on day 0; implants were removed on day 5. Intra vaginal sponges releasing medroxyprogesterone acetate were inserted 6 hours before implant removal and left in place for 6 days. The shaded area is the profile of serum progesterone concentrations in cyclic ewes. Differences between groups ($P < 0.05$). The dashed line on the graph denotes a significant decline in serum progesterone concentrations in the treatment ewes from Day 4 to Day 5 ($P < 0.001$).

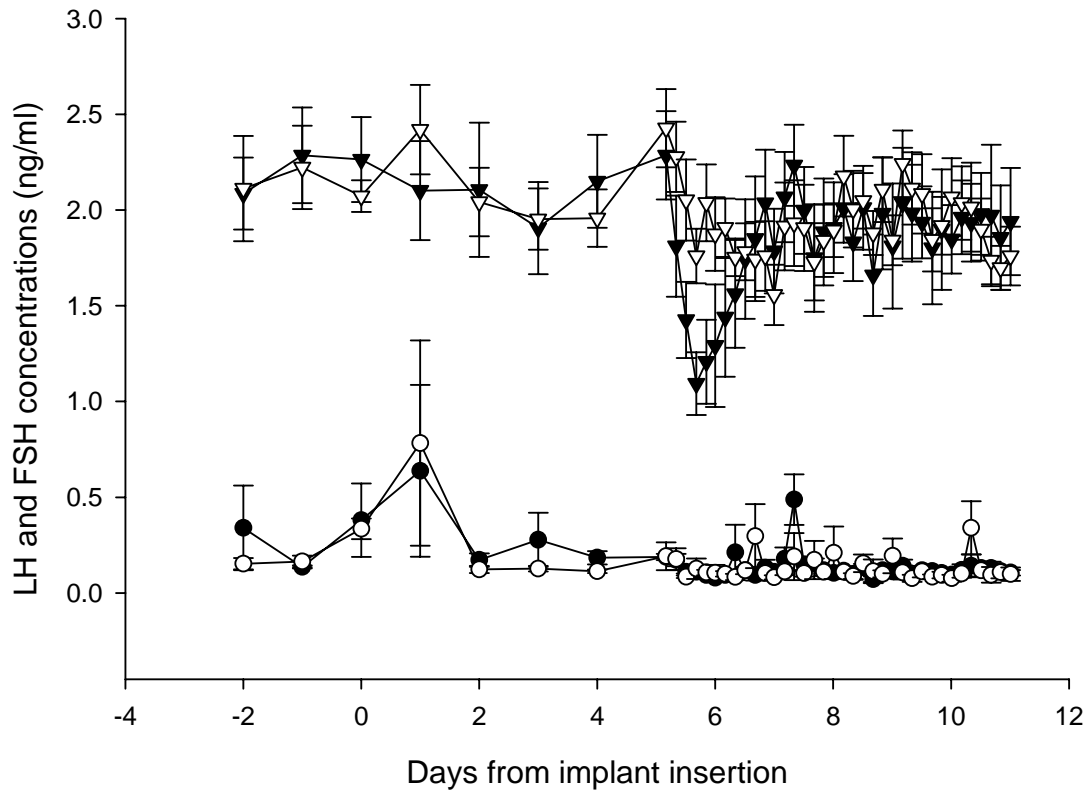


Figure 4.4. Mean (\pm SEM) serum concentrations of LH (circles) and FSH (triangles) in ewes for the period from 2 days before insertion of silastic rubber implants releasing progesterone (open symbols; n=6) or empty silastic rubber implants (filled symbols; n=5), to 6 days after intra vaginal sponges releasing medroxyprogesterone acetate were inserted (day 4).

4.5. Discussion

In the present study, six anestrous Western White Face ewes were given an injection of PGF_{2α} and had an intravaginal MAP sponge inserted. Within approximately 1.5 d of injection of PGF_{2α}, four of the six treated ewes ovulated, with one of the ewes ovulating twice. Signs of estrus or a preovulatory LH/FSH surge did not precede these ovulations, and none of these ovulations were followed by the formation of CL, only transient CH. However, when six anestrous Western White Face ewes were fitted with progesterone-releasing implants which were subsequently removed five days later, creating a rapid decline in circulating serum concentrations of progesterone, no ovulations were seen. These findings are in agreement with an earlier study by Bartlewski *et al* (2003) in which treatment of cyclic ewes for 6 days with a MAP releasing intravaginal sponge, starting on ~Day 8 after ovulation and coupled with a single administration of PGF_{2α} given on the day of sponge insertion, resulted in the ovulation of several large follicles; most ovulations occurred between 1 to 3 days after PGF_{2α}. As in our present study, a preovulatory surge of gonadotropins did not precede these ovulations, and none of these ovulations were followed by the formation of CL. There was an immediate decline in serum LH concentrations following injection of PGF_{2α} and intravaginal MAP sponge insertion (experiment 1). It is uncertain why this occurred; however, mean serum LH concentrations remain within normal basal levels. By separating any direct effects of treatment with PGF_{2α} from the sudden decline in circulating serum progesterone concentrations at luteolysis, it appeared that ovulations that occurred during the period of MAP sponge insertion in PGF_{2α}-treated anestrous ewes were due to a direct effect of

PGF_{2α} at the ovarian level rather than a sudden decline in circulating progesterone concentrations.

Ovarian PGF_{2α} is involved in the ovulatory process. Increases in follicular tissue concentrations of PGF_{2α} occur within 4 hours of the preovulatory surge in luteinising hormone (Murdoch *et al* 1993; Silvia 1999). PGF_{2α} is implicated in the reduction of follicular blood flow, via vasoconstriction, particularly at the rupture point, during the later portion of the ovulatory process (O'Grady *et al* 1972; Ford *et al* 1977; Silvia 1999). It has also been suggested that prostaglandins activate proteolytic enzymes that contribute to the breakdown of the follicular wall, particularly collagenase (LeMaire and Marsh 1975; Espey 1980; Silvia 1999). In the present study, injection of PGF_{2α} may have begun the ovulatory process through the mechanisms previously described. Several authors have likened the effects of PGF_{2α} on ovulation as being similar to an acute inflammatory reaction (Armstrong and Grinwich 1972; O'Grady *et al* 1972; Tsafiri *et al* 1972; Espey *et al* 1982). Administration of indomethacin (a potent nonsteroidal anti-inflammatory agent; Espey *et al* 1982; Espey *et al* 1988) either before or after the initiation of the ovulatory process, consistently inhibits prostaglandin synthesis and follicular rupture (O'Grady *et al* 1972; Armstrong and Grinwich 1972; Espey *et al* 1982). Therefore, in the present study, administration of PGF_{2α} may have led to direct effects on the ovary, including the disruption of the cellular matrix of the follicular wall through several different mechanisms. It was interesting that follicles induced to ovulate in the present study were older follicles with a slower growth rate compared to non ovulating follicles. Such follicles could have been exposed to PGF_{2α} for a longer period and hence more

susceptible to disruption by $\text{PGF}_{2\alpha}$. It is intriguing to speculate that ovulation at the end of a normal estrous cycle could be initiated by the luteolytic release of endometrial $\text{PGF}_{2\alpha}$ and then further induced or synchronised by the preovulatory LH surge through mechanisms involving local ovarian follicular secretions of $\text{PGF}_{2\alpha}$ (Murdoch *et al* 1993; Silvia 1999). Presumably the normal rapid metabolism of $\text{PGF}_{2\alpha}$ and the strictly controlled timing of release of $\text{PGF}_{2\alpha}$ from the uterus (Knickerbocker *et al* 1988) would prevent the untimely ovulation of follicles during the luteal phase of the ovine estrous cycle.

Administration of $\text{PGF}_{2\alpha}$ to cyclic ewes in which 4 to 14 day old CL are present, results in regression of the CL and a decline in serum progesterone concentrations (Chamley *et al* 1972; Sheldrick and Flint 1985). A decline in serum progesterone concentrations leads to a preovulatory surge in LH and the cascade of events that result in ovulation of a follicle (Niswender *et al* 1986; Alila and Dowd 1991). In the second experiment of the present study, removal of progesterone-releasing implants created a decline in circulating serum progesterone concentrations, without administration of exogenous $\text{PGF}_{2\alpha}$. This decline in circulating progesterone concentrations appeared to have no effect on circulating serum concentrations of LH or FSH and no ovulations occurred in these animals. These results further support a notion that a direct action of $\text{PGF}_{2\alpha}$ within the follicle, rather than changes in gonadotropin concentrations, were responsible for the ovulations seen in the present study.

The failure of normal luteogenesis of ovulated follicles, in the present study, may be attributed to several different factors. Gordon (1997c) reported that the average time from PGF_{2α} administration to ovulation, in cyclic ewes, is 70 hours. However, in the present study, the average time from PGF_{2α} administration to ovulation averaged 29 hours. Therefore, the failure of normal luteal formation could be attributed to the inadequate developmental competence of the preovulatory follicle as reported by other others (Legan *et al* 1977; Bartlewski *et al* 2003). These follicles failed to stimulate the preovulatory mode of LH secretion, which is essential for normal luteogenesis of ovulated follicles (Niswender *et al* 1986).

In summary, a PGF_{2α}/MAP treatment of anestrus ewes resulted in ovulation of follicles during the period of MAP sponge insertion, but these ovulations were not preceded by a preovulatory LH or FSH surge (Experiment 1). However, creating a rapid decline in circulating progesterone concentrations, in MAP treated ewes, resulted in no ovulations (Experiment 2). Therefore, ovulations occurring after PGF_{2α} injection in anestrus ewes, and in the presence of a MAP sponge were likely due to a direct effect of PGF_{2α} at the ovarian level rather than a sudden decline in circulating progesterone concentrations.

**Chapter 5: THE EFFECT OF A 6 DAY TREATMENT WITH
MEDROXYPROGESTERONE ACETATE (MAP), AFTER PROSTAGLANDIN
F_{2α} (PGF_{2α})-INDUCED LUTEOLYSIS AT MID-CYCLE ON LAMBING RATE,
BIRTH WEIGHT AND SEX RATIOS IN SUFFOLK EWES**

Davies KL, Duggavathi R, Barrett DMW and Rawlings NC.

5.1. Abstract

Increasing the ovulation rate of non-prolific breeds of sheep, may enable producers to overcome one of the major limiting factors to efficient sheep meat and wool production, that is, low lamb output per ewe. In a previous study in our laboratory, treatment of Western White Face ewes with PGF_{2α} on day 8 of a cycle and insertion of medroxyprogesterone acetate (MAP) sponges for 6 days from day 8 of the cycle, resulted in an approximately 50% increase in ovulation rate at the end of the treatment. We hypothesized that the MAP sponge and PGF_{2α} treatment would give an increased lambing rate and that the treatment would have no negative effects on lamb weights or sex ratios, and therefore have commercial viability. Twenty eight adult and thirty eight primiparous Suffolk ewes were synchronized with a 14 day progestagen treatment (MAP sponge) and then randomized assigned for age to either the treatment group (n=33) or control group (n=33). Eleven days after sponge withdrawal, ewes in the treatment group were fitted with MAP sponges for 6 days and injected with PGF_{2α} on the day of sponge insertion. The control ewes received an injection of saline at the time of PGF_{2α} injection. After sponge removal the treated and control ewes were mixed and assigned to one of 4 groups and rams were introduced to the ewes. There were no overall differences in lambing rate, lamb birth weights or sex ratios between the treatment and control groups (P>0.05). Within the treatment group there was a significantly higher number of lambs born to multiparous ewes than to primiparous ewes (P<0.05). We concluded, that the inability to

increase lambing rate in Suffolk ewes was not due to the inability to increase ovulation rate or the ovulation of aged follicles but was rather more a lack of insemination of ewes at the appropriate time with respect to ovulation.

5.2. Introduction

During the estrous cycle, ewes have three to four waves of antral follicular growth (Noel *et al*, 1993), each preceded by a transient increase in serum FSH concentrations (Ginther *et al*, 1995). In non-prolific breeds of sheep such as the Western White Face and Suffolk, a follicle(s) will only ovulate from the final wave of the cycle (Bartlewski *et al*, 1999a). On the other hand, in prolific breeds of sheep such as the Finn, follicles can ovulate from the penultimate as well as the final wave of the cycle (Bartlewski *et al*, 1999a). By increasing the ovulation rate of non-prolific breeds of sheep, producers may be able to overcome one of the major limiting factors to efficient sheep meat and wool production, that is, low lamb output per ewe (Blaxter, 1964; Gordon 1997b). In a previous study in our laboratory (Bartlewski *et al*, 2003) Western White Face ewes were treated for 6 days with medroxyprogesterone acetate (MAP) sponges, starting on day 8 after ovulation. The sponge treatment was coupled with a single injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on the day of MAP sponge insertion. The findings of this study included an approximately 50% increase in ovulation rate at the end of the treatment, when compared to control ewes or the pre-treatment cycle of treated ewes. This was attributed to the ovulation of follicles from the penultimate follicular wave before ovulation, and their addition to ovulatory follicles from the final follicular wave before ovulation. In cattle, there is evidence for (Austin *et al* 1999) and against (Ahmad *et al* 1997), compromised oocyte quality in

follicles with an extended lifespan, leading to low fertility. However, this does not appear to be the case in sheep (Evans *et al* 2001b). We hypothesized that the 6 day MAP sponge treatment, coupled with PGF_{2α} treatment, would give an increased lambing rate and that the treatment would have no negative effects on lamb weights or sex ratios. The purpose of the present study was to see if this treatment would lead to an increased lambing rate and therefore have commercial viability.

5.3. Materials and Methods

5.3.1. Animals

Twenty eight adult and thirty eight primiparous (bred at 7 months of age and lambed at 1 year of age), clinically healthy cyclic Suffolk ewes, from the Department of Animal and Poultry Science sheep flock at the University of Saskatchewan, were used for this experiment. Ewes were housed outside in sheltered pens and were fed rations of hay, *ad libitum*, and 11lb of whole grain barley daily. Experimental procedures were performed according to the standards of the Canadian Council on Animal Care.

5.3.2. Experimental Procedures

All ewes (n=66 Suffolk ewes) were synchronized by treating with a progestagen-releasing intravaginal sponge (Medroxyprogesterone acetate (MAP), 60mg; Veramix[®], Upjohn, ON, Canada) for 14 days. Ewes were then assigned to either the treatment group (n=33) or the control group (n=33). Eleven days after sponge withdrawal (approximately day 8 of the cycle), the ewes in the treatment group, had an intravaginal MAP sponge inserted and left in place for 6 days. These ewes were also injected with PGF_{2α} on the day

of MAP sponge insertion. The control ewes were not resynchronized and only received an injection of saline at the time of PGF_{2α} injection to the treatment ewes. After the removal of the second MAP sponge from the treatment ewes, the treated and control ewes were mixed and assigned to one of 4 groups and rams were introduced to the ewes at an average ewe to ram ratio of 6.5:1. At lambing, numbers of lambs born, the weight, and the gender of each lamb and the date of birth were recorded. There were 7 primiparous ewes in the treatment group and 3 primiparous ewes in the control group which did not lamb. All analysis (except for pregnancy rate) was performed after the removal of the data from these animals.

5.3.3. Statistical Analyses

Two way ANOVA (SigmaStat[®] for Windows Version 2.03, 1997, SPSS Inc., Chicago, IL, USA) was employed to compare data within and between groups (both treatment versus control as well as multiparous versus primiparous) for pregnancy rate, lambing rate (i.e. the number of live lambs born per ewe), birth weight and sex ratios.

5.4. Results

5.4.1. Pregnancy Rate

There was no overall difference in pregnancy rate between the treatment (1.3 ± 0.1 lambs/ewe) and control (1.5 ± 0.1 lambs/ewe) groups ($P > 0.05$).

5.4.2. Lambing Rate

Within the treatment group there was a significantly higher number of lambs born to multiparous ewes than to primiparous ewes ($P < 0.05$; Table 5.1.). There were no differences between multiparous and primiparous ewes within the control group ($P > 0.05$; Table 5.1.). There was no overall difference in lambing rate between the treatment and control groups ($P > 0.05$).

5.4.3. Lamb Birth Weights and Sex Ratios

There were no significant differences ($P > 0.05$) found between or within groups for lamb birth weights or for the percentage of male lambs born per ewe (Table 5.1.).

Table 5.1. Mean (\pm S.E.M) lambing rate, lamb birth weight (kg) and sex ratios (% of males born per ewe) for treated ewes that lambed (primiparous, n=13 and multiparous, n=13) and control ewes (primiparous, n=15 and multiparous, n=15).

Factor	Treatment Group		Control Group	
	Primiparous (n=13)	Multiparous (n=13)	Primiparous (n=15)	Multiparous (n=15)
Lambing Rate	1.15 \pm 0.16 ^a	1.92 \pm 0.16 ^b	1.40 \pm 0.15	1.80 \pm 0.15
Birth Weight (kg)	4.67 \pm 0.24	4.64 \pm 0.19	4.68 \pm 0.20	4.97 \pm 0.18
Sex Ratio (% of males born per ewe)	53.85 \pm 11.80	50.00 \pm 11.80	43.33 \pm 10.00	54.40 \pm 10.00

^{a,b} denotes significant difference ($P < 0.05$) within treatment group.

Between groups there were no significant differences ($P > 0.05$) for any of the factors studied.

5.5. Discussion

The results from this preliminary trial suggest that short-term treatment of Suffolk ewes with MAP sponges, coupled with an injection of PGF_{2α} does not increase lambing rate. The treatment neither affected birth weights nor the sex ratio of lambs born, as compared to control animals. The findings of a previous by Bartlewski *et al* (2003) included an approximately 50% increase in ovulation at the end of the treatment and therefore we expected to see this increase in ovulation rate reflected in an increase in lambing rate when this protocol was applied to ewes that were bred after treatment.

Transrectal ovarian ultrasonography was not performed on these experimental animals and therefore it is difficult to ascertain with certainty that the treatment actually increased ovulation rate in the ewes. However, Barltewski *et al* (2003) reported an increase in ovulation rate, as detected by transrectal ovarian ultrasonography, due to the ovulation of follicles from the penultimate follicular wave before ovulation as well as follicles from earlier waves. Reports in cattle have shown varying oocyte quality from follicles with an extended lifespan and this resulted in low fertility (Austin *et al* 1999; Ahmad *et al* 1997). This does not appear to be the case in sheep (Evans *et al* 2001b). Evans *et al* (2001b) reported that the ovulation of aged follicles produced oocytes that were equally capable of being fertilized and develop into good quality embryos and full-term lambs as compared to follicles of shorter duration. Therefore, other factors may have lead to the lack of increase in lambing rate in the treated group of ewes when compared to the control ewes.

Bartlewski *et al* (2003) demonstrated that the treatment protocol with MAP sponges and PGF_{2α} lead to asynchronous ovulation and estrus behavior. In the ewe, ovulation normally occurs just before the end of estrus, which lasts on average 36 hours (Quirke *et al* 1979; Goodman 1994). However, in the study by Bartlewski *et al* (2003) treated ewes ovulated follicles over a range of time periods from 48 hours before to 48 hours after the onset of estrus behavior. After ovulation an oocyte will remain viable for 10 to 25 hours; however, abnormal development and lowered viability increases with the age of the oocyte (Jainudeen *et al* 2000; Pineda 2003). In the present study rams were introduced to ewes after the 6 day treatment with a MAP sponge. Therefore follicles ovulating out of synchrony with the period of estrus would have provided oocytes of varying viability at mating; with some oocytes ovulated too late.

A closer look at the ewes within the treatment group showed that lambing rate was lower in primiparous ewes compared to multiparous ewes. This suggests that the treatment may have in fact reduced the lambing rate of primiparous ewes, compared to multiparous ewes. However, a lower lambing rate in primiparous compared to multiparous ewes was expected as there is a gradual increase in the performance of sheep, as judged by lambing rate and ovulation rate, up to the age of 3 or 4 years (Marshall and Potts 1924; McKenzie and Terrill 1937). In conclusion, the inability of a short term MAP sponge and PGF_{2α} treatment to increase lambing rate in Suffolk ewes was probably not due to the inability to increase ovulation rate or the ovulation of aged follicles but was rather more a lack of insemination of ewes at the appropriate time with respect to ovulation.

Chapter 6: EFFECT OF RAM INTRODUCTION ON OVARIAN ANTRAL FOLLICULAR DYNAMICS AND LH SECRETION IN ANESTROUS EWES AT DIFFERENT STAGES OF FOLLICULAR WAVE DEVELOPMENT

Davies KL, Patullo K, Duggavathi R, Barrett DMW, Ewen K, Bagu ET, Cook SJ and Rawlings NC.

6.1. Abstract

Ram introduction at mid anestrus and to ewes with a distinct anestrus, would be expected to cause an increase in pulsatile LH secretion. The objective of the present study was to use ram introduction to look at the effect of increased pulsatile secretion of LH on ovarian antral follicular dynamics at different stages of follicular wave development in mid anestrus in Western White Face ewes. Twenty Western White Face ewes were isolated from the flock five weeks prior to the start of the experiment. Ten ewes were selected and re-introduced to rams; five ewes had follicle(s) in the growth phase of a follicular wave and the other five had follicle(s) in the static phase of a follicular wave at ram introduction. A further five ewes remained separated from the flock (control group). For 3 days after ram introduction, all ewes underwent twice daily transrectal ovarian ultrasonography; subsequently, scanning was then done daily for a further 10 days. Blood samples were collected prior to each ultrasound examination, every 4 hours for 72 hours from ram introduction and every 12 minutes for 6 hours from ram introduction and daily for two days from the day of ram introduction. Following ram introduction no ewes were marked by rams or ovulated. However, there was a significant increase in LH pulse frequency on the day of ram introduction in ewes in the static phase of a follicular wave as compared to the ewes in the control group. There were no consistent changes in follicular dynamics or estradiol secretion. We concluded that changes in LH pulse

frequency do not dramatically change ovarian antral follicular dynamics in the anestrous ewe.

6.2. Introduction

If seasonally anestrous ewes are preconditioned by a period of isolation from rams (at least one month), they respond to the re-introduction of the male by displaying a reasonably well synchronized estrus at a length of time equivalent to one estrous cycle after ram exposure (Underwood *et al* 1944; Edgar and Bilkey, 1963; Fulkerson *et al* 1981; Knight *et al* 1981; Gordon, 1997b). Introducing ewes to rams towards the end of the non-breeding season will also often induce ovulation in a proportion of ewes within 2-3 days (Coop and Clarke 1968; Knight *et al* 1978; Oldham *et al* 1979); however, these ovulations are not associated with estrus. Chesworth and Tait (1974) documented increases in serum LH concentrations within one hour of exposing Greyface ewes to the ram just prior to the breeding season. Other studies revealed that LH pulse frequency increased markedly within minutes of ram introduction (Martin *et al* 1980). In addition to this, ewes stimulated by the ram experience a preovulatory LH surge similar to that of spontaneously ovulating sheep (Oldham *et al* 1979).

There is considerable variation between breeds in the percentage of ewes responding to ram introduction (Martin 1984). Breeds with short periods of anestrous respond more readily to rams (Schinckel 1954; Martin *et al* 1983; Nugent *et al* 1988) than do breeds with more prolonged anestrous (Nugent *et al* 1988; Chesworth and Tait 1974). Ewes are

also generally more responsive in late or early anestrous than they are in the middle of the anovulatory period (Cushwa *et al* 1992; Edgar and Bilkey 1963).

Using ultrasonography it was shown that during both the breeding season and anestrous, ovarian antral follicles grow in a wavelike pattern in the ewe (Bartlewski *et al* 1998; Bartlewski *et al* 1999a). Ovarian antral follicles grow or emerge from a pool of follicles 1-3 mm in diameter and reach a diameter of 5-7 mm; follicular waves emerge every 4 to 5 days (Souza *et al* 1996; Bartlewski *et al* 1998; Bartlewski *et al* 1999a; Duggavathi *et al* 2003a). The emergence of each wave of follicle growth is preceded by a transient peak in serum FSH concentrations (Bartlewski *et al* 1998; Bartlewski *et al* 1999a; Evans *et al* 2001a; Duggavathi *et al* 2003a). Prior to the availability of ultrasonography to study antral follicular dynamics it was concluded that the development of ovulatory sized follicles was largely dependent on FSH with perhaps a role for LH in final growth and maturation (Picton *et al* 1990; Campbell *et al* 1995). Although the pulsatile secretory pattern of LH changes during the estrous cycle in the ewe has been characterized, its relationship to various phases of a follicular wave and its requirement for the genesis, function and regression of waves are unclear (Karsch *et al* 1979; Bartlewski *et al* 2000a; Duggavathi *et al* 2005a). In heifers, several authors have speculated a role for LH in antral follicle deviation (Ginther *et al* 2001a; Ginther *et al* 2001b; Sartori *et al* 2001).

We hypothesized that if ram introduction was done in mid anestrous, in distinctly seasonal ewes, that only a subtle increase in LH secretory pulse frequency would be seen, allowing us to examine the role of such a change on ovarian follicular waves. Thus, the

objective of the present study was to investigate the effect of ram induced changes in LH secretion, in anestrus ewes, on ovarian follicular dynamics at different stages of the follicular wave. Using the anestrus ewe allowed us to remove the confounding affect of cyclicity and the development and regression of the corpus luteum, on LH secretion and follicular dynamics.

6.3. Materials and Methods

6.3.1. Animals

Twenty clinically healthy, nulliparous, anestrus (May – June) Western White Face (WWF) ewes and three mature, vasectomised rams (2 Suffolk, 1 Katahdan; age 5-8 yrs old) were used in this experiment; mean body weights were 87 ± 3 kg and 94 ± 7 kg, respectively. All ewes and rams were housed outside in sheltered paddocks and were fed daily maintenance rations of hay, with water and cobalt iodized salt bar available *ad libitum*. Experimental procedures were performed according to the standards of the Canadian Council on Animal Care.

6.3.2. Experimental Procedures and Ultrasonography

Ewes were isolated from any form of contact (sight, smell and sound) with rams and the rest of the flock for 5 weeks prior to the start of the experiment. All ewes underwent daily transrectal ultrasonography of the ovaries for 7 days using a real-time, high-resolution, B-mode echo camera (Aloka SSD-900, Aloka Co. Ltd., Tokyo, Japan) and a stiffened 7.5 MHz transducer, in order to establish the status of follicular wave development. Subsequently, ultrasonography was done twice daily until 3 days after ram introduction

and then daily for a further 10 days. The numbers of follicles 1-2 mm in diameter were counted and the number, diameter and relative position of all follicles ≥ 2 mm in diameter were sketched onto ovarian charts. All ovarian images were recorded on high-grade video tapes (Fuji S-VHS, ST-120 N; Fujifilm, Tokyo, Japan), using a compatible VCR (Panasonic, Omnivision Super VHS 2002, model number PV-VS4821-K, Matsushia Kotobuki Electronics, Cibitung Bekasi, Indonesia), for retrospective analysis of ovarian data. Ten ewes were introduced to 3 crayon-harnessed, mature rams. These ewes were split into two groups. Five ewes had follicle(s) in the growing phase of a follicular wave; this was based on the status of the previous wave and the emergence of follicles 3-4 mm in diameter (growing group). The other 5 ewes had follicle(s) in the static phase; this was based on the existence of follicles ≥ 5 mm in diameter but no longer growing (static group). A further 5 ewes remained separated from the rest of the flock and rams (control group). The control group of ewes consisted of 4 ewes in the static phase of a follicular wave and 1 ewe in the growth phase of a follicular wave.

6.3.3. Blood Sampling

Blood samples were collected (10 ml) by jugular venipuncture using vacutainers (Becton Dickinson, Rutherford, NJ, USA) prior to each ultrasound examination, except during the period below. All ewes underwent intensive blood sampling (every 12 minutes for 6 hours, starting at 8 am; 4 ml per sample), via indwelling jugular catheters (vinyl tubing, 1.00 mm inside diameter x 1.50 mm outside diameter; SV70, Critchley Electrical Products Pty Ltd., Auburn, NSW, Australia), from the time of ram introduction and daily for two days after the day of ram introduction, in order to characterize circulating

concentrations of LH and FSH. In addition, ewes were bled every 4 hours for a period of 72 h after ram introduction (4 ml per sample). Blood samples were allowed to clot for 18 to 24 h at room temperature, and serum was harvested and stored at -20 °C until assayed.

6.3.4. *Hormone Analyses*

Circulating concentrations of LH (Rawlings *et al* 1998), FSH (Joseph *et al* 1992), and estradiol (Joseph *et al* 1992) were determined by validated radioimmunoassays. Circulating concentrations of FSH and estradiol were analyzed in a blood sample collected at the same time each day. Samples from intensive bleeds and those collected every 4 hours, were analyzed for circulating concentrations of FSH and LH. Samples collected every 4 hours for 32 hours after ram introduction were also analyzed for circulating concentrations of estradiol. The range of standards was from 0.10 to 9.0 ng/ml, 0.12 to 12.0 ng/ml, and 1.0 to 50 pg/ml, for the LH, FSH, and estradiol assays, respectively. The sensitivities of assays (defined as the lowest concentration of hormone capable of significantly displacing labeled hormone from the antibody; unpaired t-test, $P < 0.05$) were as follows: LH, 0.1 ng/ml, FSH, 0.1 ng/ml, and estradiol 1 pg/ml. The intra- and inter-assay coefficients of variation (CV's) were 2.37% and 10.26% or 6.92% and 7.07% respectively for reference sera with mean LH concentrations of 0.08 ng/ml or 3.42 ng/ml respectively. The intra- and inter-assay CV's were 4.33% and 8.76% or 6.92% and 10.43% respectively for reference sera with mean FSH concentrations of 0.27 ng/ml or 1.22 ng/ml respectively. The intra- and inter-assay CV's were 14.8% and 16.5% or 9.5% and 12.1% respectively for reference sera with mean estradiol concentrations of 3.6 pg/ml or 63.6 pg/ml respectively.

LH and FSH data from intensive bleeds were analyzed using the PULSAR program (Gitzen and Ramirez, 1998) to identify basal and mean hormone concentrations as well as pulse frequency and amplitude. The basal serum concentrations (“smoothed series”) were generated after the removal of short-term variations in hormone concentrations, including possible pulses. In blood samples collected daily, peaks in FSH concentration were detected using the cycle detector computer program (Clifton and Steiner, 1983).

6.3.5. Follicular Data Analyses

The temporal pattern of the numbers of follicles from different size classes (1-2 mm, 2 mm, 3 mm, 4 mm, and ≥ 5 mm in diameter) were analyzed from the day of ram introduction to 2 days after ram introduction. The maximum follicle diameter from the first and second waves after ram introduction were analyzed, as was the length of the first inter-wave interval after ram introduction.

6.3.6. Statistical Analyses

Data were assessed by one-way Analysis of Variance (ANOVA) or two-way repeated-measures ANOVA (SigmaStat[®] Statistical Software, for Windows Version 2.03, 1997, SPSS Inc., Chicago, IL, USA). Multiple comparisons were made by the method of Fisher’s least significant difference (LSD). All results were normalized to the day of ram introduction (Day 0 = Day of ram introduction). All data are presented as mean \pm S.E.M.

6.4. Results

6.4.1. General Results

None of the ewes were marked by rams following ram introduction and no ewes ovulated, as determined by ultrasound examinations.

6.4.2. Serum LH concentrations

Based on the intensive bleeds, on the day of ram introduction (Day 0) ewes in the growth phase of a follicular wave had significantly ($P < 0.05$) higher mean serum LH concentrations as compared to the control ewes (Table 6.1). Ewes in the static phase of a follicular wave had higher basal LH concentrations ($P < 0.05$) on day one after ram introduction than on day 2 after ram introduction. There were no significant ($P > 0.05$) changes in LH pulse amplitude within the groups of ewes over the two days after ram introduction. For ewes in the static phase of a follicular wave LH pulse frequency was highest on the day of ram introduction and had significantly ($P < 0.05$) declined the second day after ram introduction. On day 0, LH pulse frequency was greater for ewes in the static phase of a follicular wave, compared to the control ewes; pulse frequency was intermediate for ewes in the growing phase of a follicular wave.

6.4.3. Serum FSH concentrations

Based on the intensive bleeds, mean and basal concentrations of FSH after ram introduction did not differ ($P > 0.05$) between or within groups (Table 6.1). Based on blood samples collected daily, FSH inter-peak interval and the FSH peak amplitude after ram introduction did not differ ($P > 0.05$) between or within groups (Table 6.2).

Table 6.1. Serum LH, FSH, and estradiol characteristics on the day of ram introduction (0), and the first (1), and second day after ram introduction (2) for ewes in the static phase of a follicular wave (n=5), ewes in the growth phase of a follicular wave (n=5) and control ewes. Blood samples were collected every 12 minutes for 6 hours from ram introduction and each day for 2 days after ram introduction. For circulating serum estradiol concentration, day 0 is the first 8 samples from samples taken every 4-hours.

Parameter	Group			
	<i>Static</i>	<i>Growing</i>	<i>Control</i>	
Mean LH (ng/ml)	0	0.34 ± 0.09 ^{ab}	0.50 ± 0.09 ^a	0.22 ± 0.09 ^b
	1	0.24 ± 0.09	0.42 ± 0.09	0.23 ± 0.10
	2	0.25 ± 0.09	0.31 ± 0.09	0.15 ± 0.10
Basal LH (ng/ml)	0	0.12 ± 0.01 ^{xy}	0.12 ± 0.01	0.11 ± 0.01
	1	0.14 ± 0.01 ^x	0.12 ± 0.01	0.12 ± 0.01
	2	0.10 ± 0.01 ^y	0.14 ± 0.01	0.10 ± 0.01
LH pulse amplitude	0	0.98 ± 0.84	2.99 ± 0.84	1.01 ± 0.94
	1	0.81 ± 0.84	2.44 ± 0.84	0.56 ± 0.94
	2	1.46 ± 0.84	2.52 ± 0.84	0.49 ± 0.94
Number of LH pulses per 6 hours	0	2.00 ± 0.21 ^{ax}	1.40 ± 0.21 ^{ab}	1.00 ± 0.21 ^b
	1	1.40 ± 0.21 ^{xy}	1.20 ± 0.21	1.00 ± 0.21
	2	0.80 ± 0.21 ^y	1.00 ± 0.21	0.60 ± 0.21
Mean FSH (ng/ml)	0	2.06 ± 0.24	1.31 ± 0.24	1.76 ± 0.27
	1	1.99 ± 0.24	1.07 ± 0.24	1.42 ± 0.27
	2	1.64 ± 0.24	1.14 ± 0.24	1.20 ± 0.27
Basal FSH (ng/ml)	0	1.89 ± 0.24	1.28 ± 0.24	1.79 ± 0.27
	1	2.01 ± 0.24	1.04 ± 0.24	1.45 ± 0.27
	2	1.63 ± 0.24	1.11 ± 0.24	1.20 ± 0.27
Mean estradiol (pg/ml)	0	2.5 ± 0.6	1.9 ± 0.6	2.1 ± 0.6
	1	2.7 ± 0.8	2.7 ± 0.8	1.5 ± 0.8
	2	3.5 ± 0.8	2.0 ± 0.8	2.4 ± 0.8

^{a,b} denote values that are significantly different (P<0.05) amongst groups within days.

^{x,y,z} denote values that are significantly different (P<0.05) within groups amongst days.

Data are presented as mean ± SEM.

Table 6.2. FSH peak amplitude (the peak at the time of ram introduction or the first peak after ram introduction), FSH inter-peak interval (the first inter-peak interval after ram introduction), and length of the inter-wave interval after ram introduction for ewes in the static phase of a follicular wave (n=5), ewes in the growth phase of a follicular wave (n=5) and control ewes.

Parameter	Group		
	Static	Growing	Control
FSH peak amplitude	1.25 ± 0.44	0.81 ± 0.31	1.45 ± 0.35
FSH inter-peak interval (days)	4.1 ± 1.1	3.6 ± 0.7	3.7 ± 0.7
Inter-wave interval (days)	4.8 ± 1.3	5.0 ± 0.7	4.7 ± 1.0

Data are presented as mean ± SEM.

6.4.4. Number of follicles in different size classes

There were no significant differences between groups (ewes in the static or growth phase of a follicular wave and control ewes) or with time, in the number of follicles 1-2 mm (Figure 6.1; panel A) and 3 mm in diameter (Figure 6.1; panel C). On the day of ram introduction (Day 0), ewes in the growth phase of a follicular wave had significantly ($P<0.05$) greater numbers of follicles 2 mm in diameter than ewes in the static phase of a follicular wave (Figure 6.1; panel B). Both ewes in the static phase of a follicular wave and control ewes showed an increase in the number of follicles 2 mm in diameter from day 0 to 1 day after ram introduction after which the number of follicles 2 mm in diameter in the control group declined (Figure 6.1; panel B). On day 1 after ram introduction, control ewes had significantly greater numbers of follicles 2 mm in diameter than ewes in the static phase of a follicular wave (Figure 6.1; panel B). Ewes in the growth phase of a follicular wave had significantly ($P<0.05$) greater numbers of follicles 2 mm in diameter than both ewes in the static phase of a follicular wave and control ewes on day 2 after ram introduction (Figure 6.1; panel B). However, ewes in the static phase of a follicular wave had significantly greater ($P<0.05$) numbers of follicles 4 mm in diameter than ewes in the growth phase of a follicular wave on day 2 after ram introduction (Figure 6.1; panel D). Ewes in the growth phase of a follicular wave had significantly greater numbers of follicles ≥ 5 mm in diameter than ewes in the static phase of a follicular wave on day 2 after ram introduction (Figure 6.1; panel E). The number of follicles ≥ 5 mm in diameter increased on day 1 and then declined on day 2 after ram introduction in ewes in the static phase of a follicular wave, compared to the control ewes (Figure 6.1; panel E).

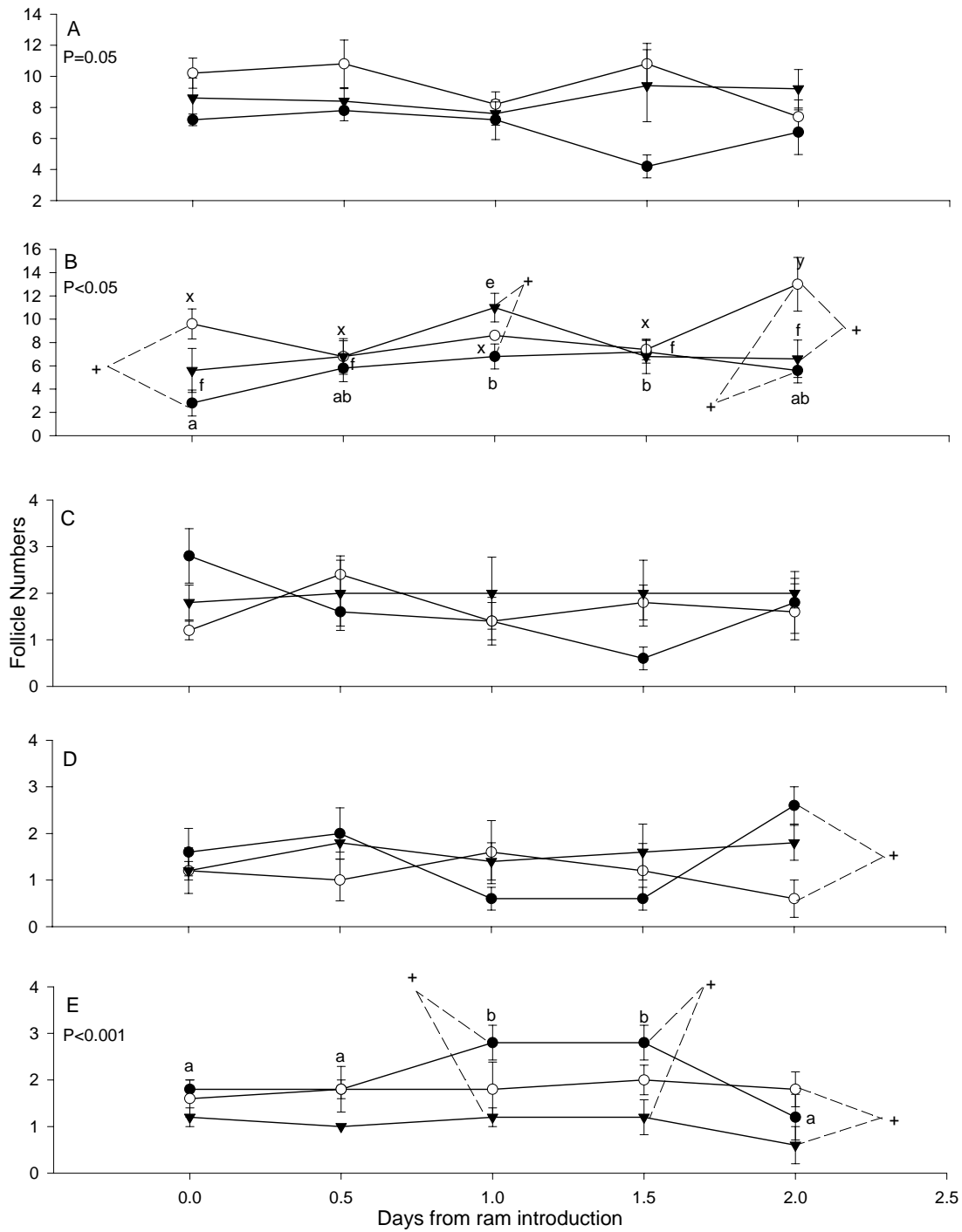


Figure 6.1. Number of ovarian follicles in different size classes (A) 1-2 mm, (B) 2 mm, (C) 3 mm, (D) 4 mm, and (E) ≥ 5 mm in diameter per ewe from the day of ram introduction (Day 0) to 2 days after ram introduction for ewes in the static phase (●; n=5), or growth phase of a follicular wave (○; n=5) and control ewes (▼; n=5). Data are presented as mean \pm SEM. + with dashed line represents significant (P<0.05) difference between groups. Letters denote significant (P<0.05) day effects within a group.

6.4.5. Maximum follicle diameter and estradiol concentrations

There were no differences ($P>0.05$) between or within groups (ewes in the static phase or growth phase of a follicular wave and control ewes) for maximum follicle diameter (mm) for wave 1 (5.6 ± 0.2 , 5.2 ± 0.2 , and 5.4 ± 0.2 ; respectively) or wave 2 (5.6 ± 0.2 , 5.6 ± 0.2 , and 5.0 ± 0.2 ; respectively) after ram introduction. Mean serum estradiol concentrations (Table 6.1 and Figure 6.2) after ram introduction did not differ ($P>0.05$) between or within groups.

6.4.6. Inter-wave interval after ram introduction

There were no differences ($P<0.05$) between groups for the length of the first inter-wave interval after ram introduction (i.e. between the first and second wave after ram introduction; Table 6.2).

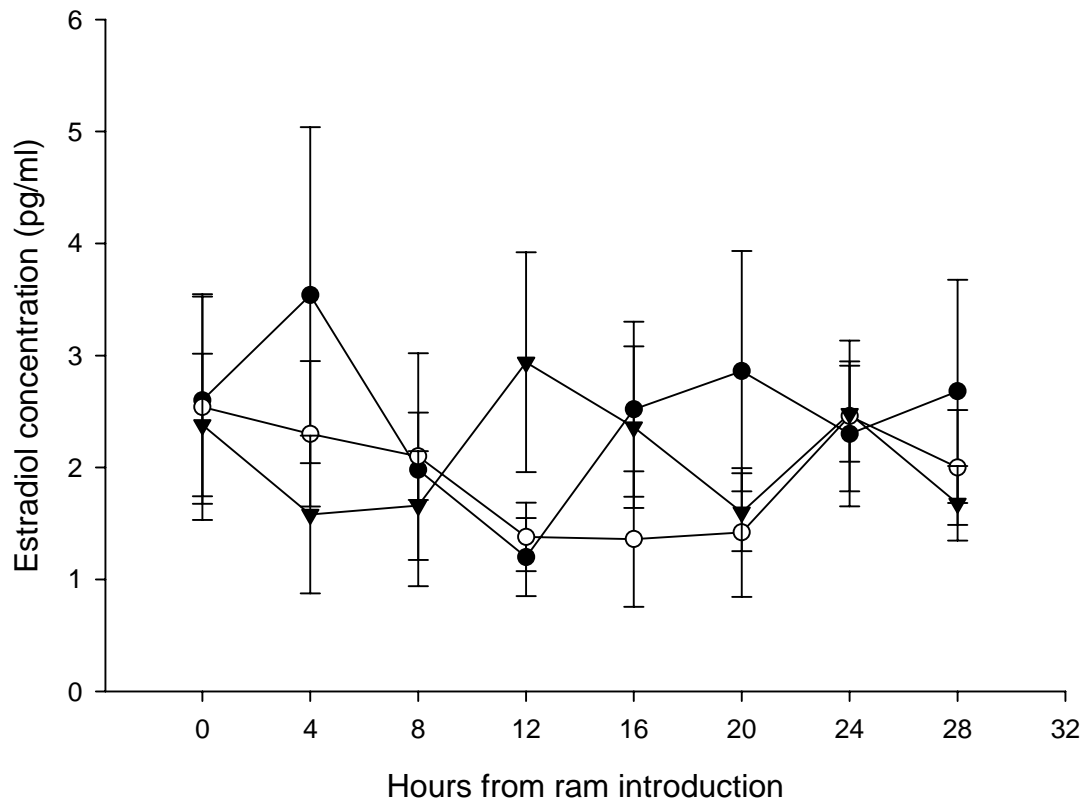


Figure 6.2. Mean serum estradiol concentrations (pg/ml) in ewes in the static phase of a follicular wave (●; n=5), ewes in the growth phase of a follicular wave (○; n=5) and control ewes (▼; n=5) with samples taken every 4 hours from the time of ram introduction (Hour 0) to 28 hours after ram introduction. Blood samples were analyzed daily. Data are presented as mean \pm SEM.

6.5. Discussion

Introduction of rams to previously isolated anestrus, non prolific Western White Face ewes resulted in subtle changes in LH secretion, based on frequent blood sampling. Most notably was the increase in LH pulse frequency on the day of ram introduction in the ewes in the static phase of a follicular wave as compared to the control ewes and the intermediate values in ewes in the growth phase of a follicular wave. This increase in LH pulse frequency was analogous to the change seen from anestrus to the luteal phase of a cycle (Karsch *et al* 1979). Ram introduction had no significant effect on FSH secretory characteristics. Following ram introduction no ewes were marked by rams and no evidence of ovulations was seen during ultrasonographic examinations. The rather subtle effect of ram introduction in this study was probably due to the fact the ewes employed in the present study were in deep anestrus (Martin 1984), and of a breed with a significant period of anestrus (Martin *et al* 1986).

Previous studies have shown that re-introduction of ram's can lead to the induction of a preovulatory LH surge and ovulation of follicle(s) (Chesworth and Tait 1974; Knight *et al* 1978; Oldham *et al* 1979; Martin *et al* 1980), but this did not occur in the present study. In the present study we did not see consistent trends in the temporal pattern of follicle numbers in any size class (figure 6.1) with the exception of an increase in the number of follicles 2 mm in diameter from day 0 to day 1 after ram introduction in control ewes and ewes in the static phase of a follicular and an increase in follicles ≥ 5 mm in diameter in ewes in the static phase from day 0.5 to 1.0. Due to the fact that the

control group of ewes consisted of 4 ewes in the static phase of a follicular wave and only 1 ewe in the growth phase of a follicular wave we speculated that the increase in the number of follicles 2 mm in diameter was not a treatment effect but rather a reflection of the ewes' status with regard to the stage of the follicular wave. The increase in the number of follicles 2 mm in diameter, after ram introduction, represented follicles entering the growth phase of the next follicular wave. The transient rise in the number of follicles ≥ 5 mm in diameter in ewes in the static phase of a follicular wave was probably due to the fact that three out of the five ewes experienced emergence of follicles of the next follicular wave that grew to be ≥ 5 mm in diameter on days 1 and 1.5 after ram introduction.

In cattle, there is selection of a dominant follicle which occurs at the point of follicle deviation (Ginther *et al* 1996). At the point of deviation there is a distinct change in the growth rates of follicles in a wave (Ginther *et al* 2003). At deviation the largest follicle of the wave continues to grow while the subordinate follicles of the wave show a decline in growth rate (Ginther *et al* 1997). It has been suggested that LH is involved in the deviation mechanism (Ginther *et al* 1996). Reports have shown that suppression of pulsatile LH secretion leads to the restriction of growth of follicles beyond 7-9 mm (Gong *et al* 1995) and conversely the lifespan of a dominant follicle could be extended by increasing LH pulse frequency (Fortune *et al* 1991; Savio *et al* 1993). These findings are supportive of a role for LH secretion in the maintenance and growth of dominant follicles, in cattle. Prior to the availability of ultrasonography, to study antral follicle dynamics, it was concluded that the development of ovulatory sized follicles in the wave

was largely dependent on FSH with perhaps a role for LH in final growth and maturation (Picton *et al* 1990; Campbell *et al* 1995). However, there have been conflicting reports for the role of LH secretion in follicular development in the ewe (McNeilly *et al* 1991). Karsch *et al* (1979) reported that LH secretory pulse frequency is higher during the follicular phase as compared with the luteal phase of the ovine estrous cycle. In a study using ultrasonography in the luteal phase of a cycle, there was no change in LH pulsatility associated with the emergence or growth of the largest follicle of a wave, but there was a significant increase in LH concentrations associated with the decline in serum progesterone concentrations at the end of the luteal phase (Duggavathi *et al* 2005a). It was shown that increases in LH pulse amplitude were associated with the end of the growth phase of the largest follicle of the first wave of the cycle (Bartlewski *et al* 2000a); however, serum progesterone concentrations increased over this period of time and this in itself causes a decrease in LH pulse frequency and increase in amplitude. In the ovine estrous cycle LH secretory patterns may be influenced by and coupled more to the formation and regression of the corpus luteum than follicular wave dynamics. In the present study although ram introduction caused changes in LH pulse frequency of the order of change seen from anestrus to the luteal phase of a cycle in sheep this appeared to have no significant impact on follicular dynamics or estradiol secretion. This and the observation discussed above led us to suggest that the role of LH in antral follicular growth and maturation in the ewe is permissive or secondary to FSH.

In summary, ram introduction to anestrus ewes produced subtle but significant changes in pulsatile LH secretion within the physiological range seen in cyclic ewes (Rawlings

and Cook 1993; Bartlewski *et al* 2000a). However, this did not have marked effects on antral follicular wave dynamics in the anestrous ewe. We concluded that changes in LH pulse frequency within a physiological range did not influence antral follicular dynamics in the growth or static phase of a follicular wave in anestrous ewes. We suggest that changes in LH pulse frequency are not critical for the normal pattern of antral follicular waves in sheep.

Chapter 7: COMPUTER ASSISTED IMAGE ANALYSIS OF CORPORA LUTEA IN RELATION TO PERIPHERAL CONCENTRATIONS OF PROGESTERONE: A COMPARISON BETWEEN BREEDS OF SHEEP WITH DIFFERENT OVULATION RATES

Davies KL, Bartlewski PM, Pierson RA, and Rawlings NC.

7.1. Abstract

Transrectal ovarian ultrasonography is a non-invasive technique that permits the real-time serial visualization of ovarian structures. Previous studies have reported correlations between serum progesterone concentrations and physical characteristics of the corpus luteum (CL) in different species. The aim of the present study was to investigate whether or not there were correlations between ultrasound image attributes of the CL and changing progesterone concentrations over time, in prolific and non prolific ewes. Ultrasonographic images of CL were taken, once daily, from 12 Western White Face ewes and 7 Finn ewes for the duration of one luteal phase. Blood samples were collected daily prior to each scanning session and assayed to determine mean serum concentrations of progesterone. Analysis of ultrasound images was performed using a series of custom-developed computer algorithms optimized for ultrasonography on a computer graphics workstation. Both total luteal area and mean pixel values were correlated with the pattern of serum concentrations of progesterone from day 3 to day 15 after ovulation in Western White Face ewes and from day 3 to day 14 in Finn ewes. There was no significant correlation between progesterone concentrations and spot pixel heterogeneity for either Western White Face ewes' or Finn ewes.' We concluded that pixel heterogeneity is a poor indicator of progesterone secretory ability of the CL when compared to mean pixel

values. However, luteal area and mean spot pixel values are better but not strong indicators of the functional status of the CL in cyclic ewes.

6.2. Introduction

Real-time transrectal ultrasonography of the reproductive tract, for use in different farm animal species, has been developed over several decades (Pierson and Ginther, 1986; Adams *et al* 1989; Kot and Ginther, 1999). Ultrasonographic imaging is based on the ability of different tissues to reflect high frequency sound waves differentially, and reflection varies with tissue density (Pierson and Adams, 1995; Singh *et al* 1997). A portion of the waves are reflected by tissue interfaces, while other parts of the waves are propagated (Zagzebski, 1996). Images are displayed as 2 dimensional maps of grey-scale based upon location and strength of the echoes returning from the tissue interfaces (Zagzebski, 1996). Grey-scale images are composed of thousands of picture elements, known as pixels (Zagzebski, 1996). A single tissue reflector is represented by a single pixel and is designated one of 256 shades of grey (ranging from black (0) to white (255)) (Zagzebski, 1996).

Transrectal ovarian ultrasonography in the ewe allows for repeated, non-invasive collection of real-time data on the status of reproductive organs from the same individual. This technology allows the study of dynamic changes within the ovarian follicular population and luteal structures during the estrous cycle (Pierson and Ginther, 1988). Subjective scoring has been used to quantify changes in image attributes such as the pixel values in a specific tissue over time (Pierson and Ginther, 1985); however, the accuracy

and repeatability of this procedure is questionable (Tom *et al* 1998a) because the human eye can only distinguish between 18 and 20 shades of grey (Baxes, 1994). Therefore, computer algorithms have been designed specifically for a more objective analysis of ultrasound image characteristics. Due to the repeatability of the application of this technique, computerized ultrasound measurements from a particular tissue can be evaluated and compared with time.

Progesterone secretion during the ovine estrous cycle has been well described (Edgar and Ronaldson 1958; Stabenfeldt *et al* 1969; Bartlewski *et al* 1999b). Several authors (Quirke *et al* 1979; Cahill *et al* 1981) have demonstrated that there are differences in the overall mean circulating concentrations of progesterone amongst prolific and non prolific breeds of sheep. A recent study by Bartlewski *et al* (1999b) showed that prolific Finnish Landrace ewes have lower circulating concentrations of progesterone as compared to non prolific Western White Face (Columbia x Rambouillet) ewes. Changing progesterone concentrations measured in the circulation reflect physical changes in the CL (Arthur *et al* 1989). Singh *et al* (1997) reported correlations between ultrasound image attributes of the CL and circulating concentrations of progesterone in heifers. And Baerwald *et al* (2005) concluded that numerical pixel values reflect the morphological and endocrinological changes in the CL during an inter-ovulatory interval in women. However, such detailed studies have not been performed in ewes.

In 1999b, Bartlewski *et al* reported correlations between daily total luteal volumes (assessed by ultrasonography) and daily circulating concentrations of progesterone in

prolific Finnish Landrace sheep. In the same study, these correlations were only seen during luteal growth and regression in non prolific Western White Face ewes. Therefore, the aim of this study was to investigate whether or not there are correlations between ultrasound image attributes of the CL and changing progesterone concentrations over time, in prolific and non prolific ewes. The progesterone data and the ultrasonographic images of CL collected by Bartlewski *et al* (1999b) were used in the present study. It was hoped that computer assisted image analysis of the CL could be used as a non invasive and immediate indicator of the progesterone secretory ability of the ovine CL, as has been suggested for other species (Singh *et al* 1997).

7.3. Materials and Methods

7.3.1. Animals

Twelve cross-bred Western White Face (approximately 5 yr of age and an average body weight of 90 ± 7 kg) and 7 pure-bred Finn (3 to 4 yr of age and an average body weight of 57 ± 4 kg), ewes were used during the mid-breeding season (October to December) (Bartlewski *et al* 1999b). The average number of lambs born per ewe for the Western White Face is 1.5 ± 0.2 (Rawlings *et al* 1987). The Finn sheep in this study had lambed twice, with an average number of offspring per ewe of 2.4 ± 0.4 . Ewes were housed in sheltered dry lots. Maintenance rations of alfalfa pellets were fed once a day and water, hay and cobalt iodized salt licks were available ad libitum. Estrus was detected with 3 crayon-harnessed rams and an electronic estrus 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 (Szczepanski *et al* 1994;

Bartlewski *et al* 1999b). A decline in electrical resistance of the vaginal tissue below 40 ohms typically occurs at the onset of behavioral estrus and persists for 24 to 48 h (Szczepanski *et al* 1994; Bartlewski *et al* 1999b).

7.3.2. *Ultrasonography*

Transrectal ovarian ultrasonography was performed daily using a B-mode, real time echo camera (Aloka SSD 500, Overseas Monitor Corp. Ltd., Richmond, BC, Canada) equipped with a 7.5MHz linear-array transducer and images were recorded on high grade videotape (Fuji S-VHS, ST-120N; Fujifilm, Tokyo, Japan), using a compatible VCR (Panasonic, Super VHS, AG 1970; Matsushita Electric of Canada Ltd, Mississauga, Ontario) (Bartlewski *et al* 1999b). Ultrasound examinations began either on the day on which each ewe was marked by rams or when a decline in vaginal impedance readings below 40 ohms was first recorded. Daily examinations were continued until ovulation at the next estrus. The day of ovulation was regarded as the day on which a large ovarian antral follicle(s) that had been detected ultrasonographically and followed for several days, was no longer seen (Ravindra *et al* 1994). From both ovaries, the number, diameter and relative position of all ovarian antral follicles ≥ 3 mm in diameter were recorded. Similarly, the size (outer diameter and diameters of inner cavities) and position of CL were also recorded.

7.3.3. *Blood Sampling*

Blood samples (10ml) were collected every day prior to scanning by jugular venipuncture, using vacutainers (Becton Dickson, Rutherford, NJ, USA) (Bartlewski *et al*

1999b). Blood samples were allowed to coagulate for 18 to 24 hours at room temperature. After removal of blood clots and centrifugation, serum was harvested and stored at -20°C.

7.3.4. *Hormone Analysis*

Serum samples were analyzed by radioimmunoassay for concentrations of progesterone (Rawlings *et al* 1987; Bartlewski *et al* 1999b). The sensitivity of the assay defined as the lowest concentration of unlabelled progesterone that significantly displaced labeled progesterone from the antibody (unpaired t-test, $P < 0.05$), was 30 pg/ml. The range of standards was from 10 pg/ml to 10 ng/ml. Intra- and inter-assay coefficients of variation (CV's) 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.

7.3.5. *Image acquisition and processing*

Images of the ovary from the day of first visualization until luteal structures could no longer be detected were digitized using a real-time B-mode echo camera at a resolution of 640 x 480 pixels. Analysis of ultrasound images was performed using a series of custom-developed computer algorithms optimized for ultrasonography on a computer graphics workstation (SYNERGYNE Version 2.8[®], Saskatoon, Saskatchewan). Numerical pixel value and pixel heterogeneity within the CL were calculated for the whole cross-sectional area of each CL (area pixel values and area pixel heterogeneity). The area encompassing the CL was outlined in order to calculate the total luteal area, mean area pixel value and the mean area pixel heterogeneity within the CL. Fluid-filled cavities, when detected within a CL, were outlined and the data subtracted from the CL so that only luteal tissue

was evaluated. Four computer generated spots were then randomly placed over the area of the image representing the CL but avoiding fluid-filled areas of the CL. Numerical pixel value (spot pixel value) and pixel heterogeneity (spot pixel heterogeneity) were calculated within each spot and then averaged over the 4 spots. Spot size was selected so that approximately 70% of the area of the CL was included in the analysis. There were no differences in the data analyzed among the four spots; therefore, data were combined. The mean pixel value was the mean of the grey-scale values of all the pixels within the outlined area of the CL (area pixel value) or within the area of the four measuring spots (spot pixel value). Pixel heterogeneity was the standard deviation of grey-scale values of all the pixels falling within the outlined area of the CL (area pixel heterogeneity) or within the area of the four measuring spots (spot pixel heterogeneity) (Figure 7.1). Data were combined for each animal; values analyzed were on a per ewe basis.

7.3.6. Statistical analysis

Data from 4 of the Western White Face ewes were discarded; two ewes had abnormally long interovulatory intervals (each 23 d) and luteinized unovulated follicles were detected in the other two animals. Total luteal area, serum progesterone concentrations, mean spot pixel values and spot pixel heterogeneity were analyzed by one way repeated measures Analysis of Variance (SigmaStat[®] Statistical Software, for Windows Version 2.03, 1997, SPSS Inc., Chicago, IL, USA) from 3 to 15 days after ovulation and 3 to 14 days after ovulation in Western White Face and Finn sheep, respectively. Days 3 to 15 and 3 to 14 were chosen because these were the days on which complete image data for each CL was available for each animal (i.e. CL images could be seen within the limits of the resolution

imposed by the Aloka SSD 500). Correlations between mean spot pixel values and mean area pixel values, and between mean spot pixel heterogeneity and mean area pixel heterogeneity for both Western White Face and Finn sheep were analyzed using Pearson's Correlation (SigmaStat[®] Statistical Software, for Windows Version 2.03, 1997, SPSS Inc., Chicago, IL, USA). Correlations were also examined between serum progesterone concentrations and total luteal area, mean spot pixel value or mean spot pixel heterogeneity, for both Western White Face ewes' and Finn ewes'. All values are presented as means \pm S.E.M.

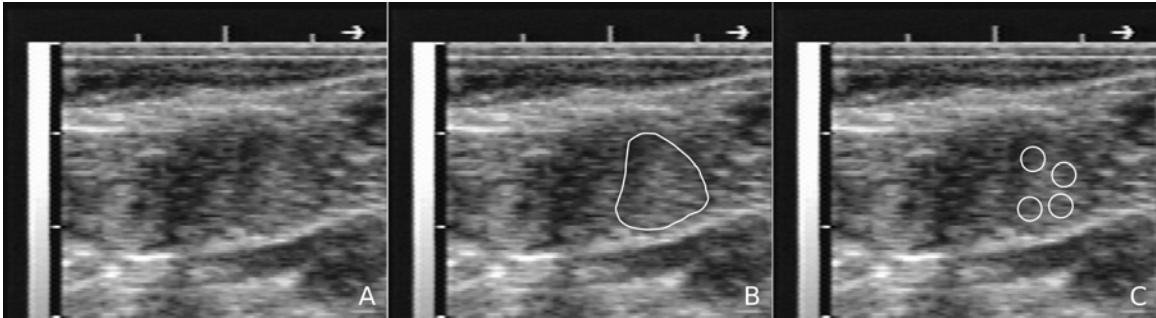


Figure 7.1. (A) Sample region for computer-assisted image analysis of an ultrasound image of a day 7 (Day 0 =day of ovulation) ovine ovarian corpus luteum. (B) Area analysis of the CL measures the pixel value and pixel heterogeneity of the total area of the CL excluding the fluid-filled cavities. (C) Spot analysis of the CL measures the pixel value and pixel heterogeneity of the CL by placing a measuring circle at four different locations over the CL to cover approximately 70% of the area of the CL.

7.4. Results

7.4.1. Spot versus area analysis

Mean spot pixel values and mean area pixel values were correlated for both Western White Face ewes and Finn ewes ($r^2=0.92$ and $r^2=0.89$, respectively; $P<0.001$). However, mean spot heterogeneity and mean area heterogeneity, were not as highly correlated for either Western White Face ewes or Finn ewes ($r^2=0.28$ and $r^2=0.36$, respectively; $P<0.001$). We opted to use spot analysis to represent the data as this technique is faster and easier to implement than area analysis and would be a more suitable technique to use in a practical setting.

7.4.2. Progesterone concentrations and total luteal area

The serum progesterone concentrations determined by Bartlewski *et al* (1999b) were used to correlate with the ultrasound image attributes of CL in the present study. Mean daily progesterone concentrations in 8 Western White Face ewes showed a significant increase from day 3 to day 5 after ovulation, did not change significantly from day 5 to day 13 after ovulation and then decreased ($P<0.05$) from day 13 to day 15 (Figure 7.2; top panel). In Western White Face ewes, total luteal area showed a significant increase from day 3 to day 7 after ovulation, did not change significantly from day 7 to day 13 after ovulation and then declined ($P<0.05$) from day 13 to day 14 after ovulation (Figure 7.2; top panel). There was a correlation between progesterone concentrations and total luteal area ($r^2=0.590$, $P<0.001$) from day 3 to day 15 after ovulation.

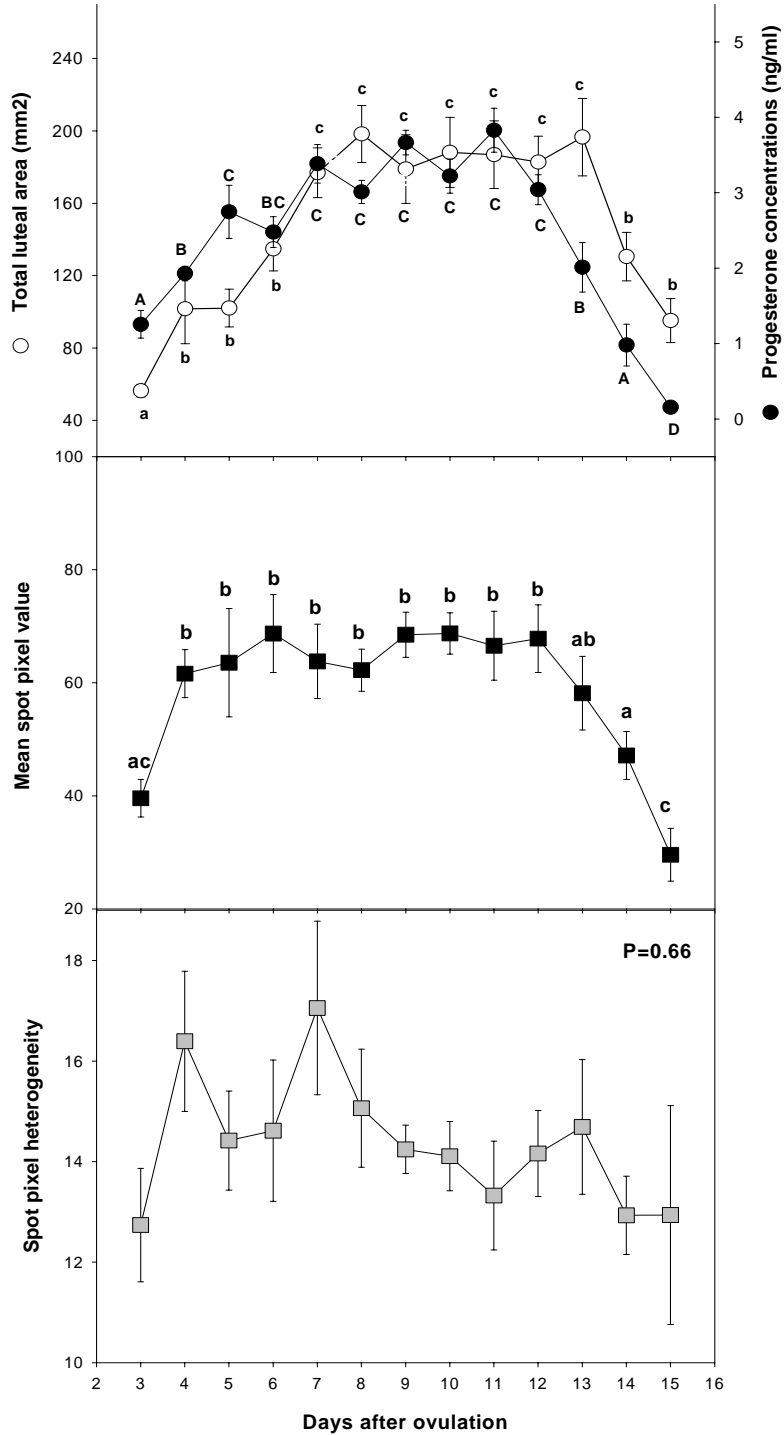


Figure 7.2. Total luteal area (○; mm²), mean spot pixel values (■) and spot pixel heterogeneity (◻) of the CL and serum progesterone concentrations (●; ng/ml) from day 3 of the cycle (Day 0 = Day of ovulation) to day 15 in Western White Face ewes (n=8). Significantly different values (P<0.05) are represented by different letters.

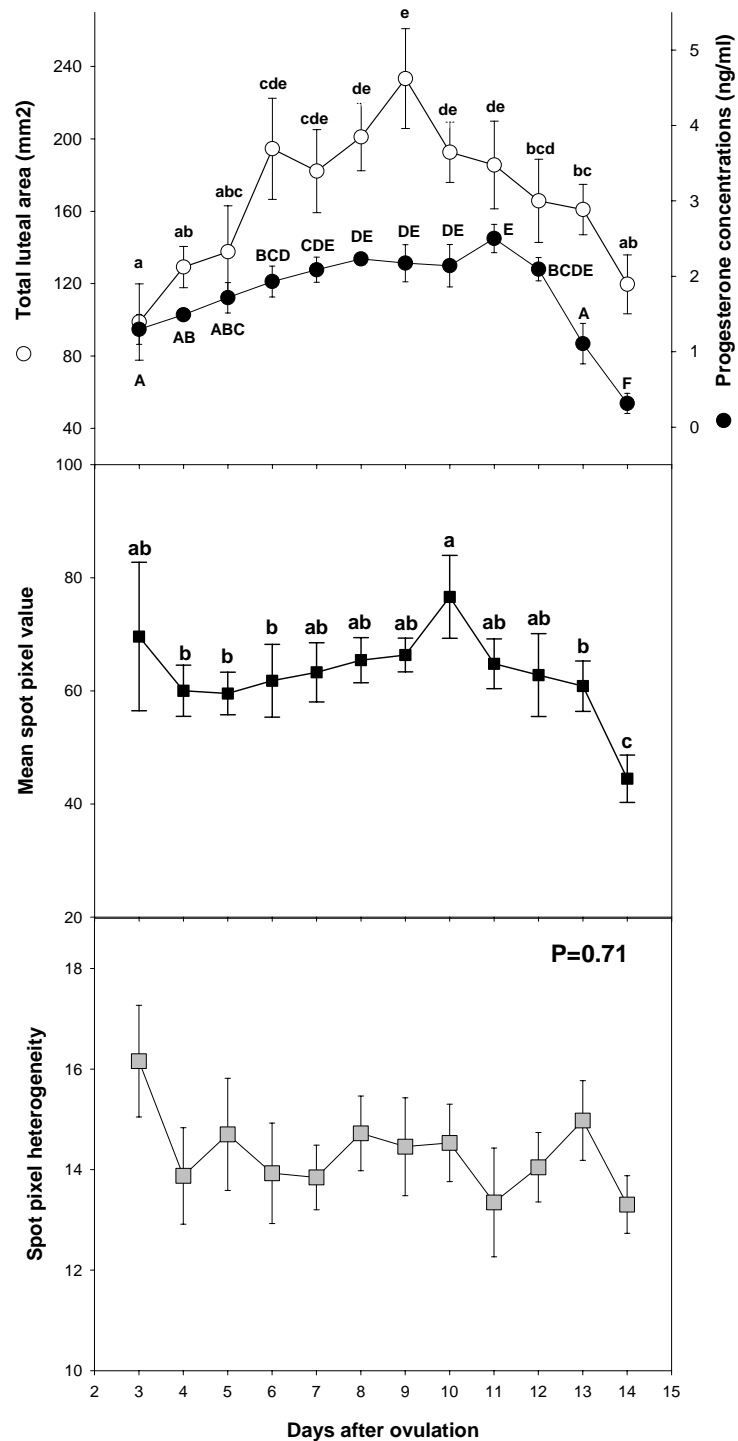


Figure 7.3. Total luteal area (○; mm²), mean spot pixel values (■) and spot pixel heterogeneity (■) of the CL and serum progesterone concentrations (●; ng/ml) from day 3 of the cycle (Day 0 = Day of ovulation) to day 14 in Finn sheep (n=7). Significantly different values (P<0.05) are represented by different letters.

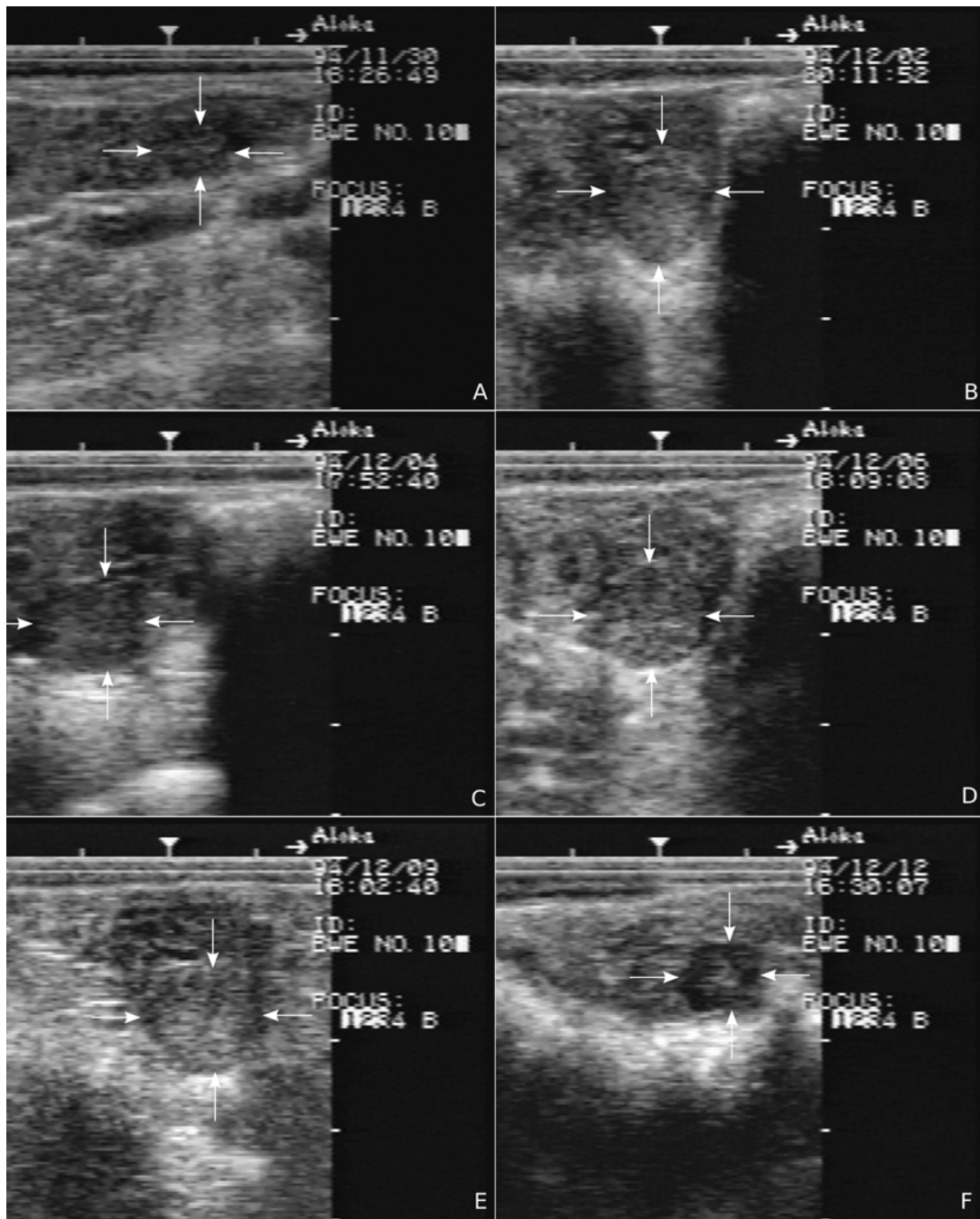


Figure 7.4. Selected ultrasound images of Western White Face ewe ovaries showing corpora lutea in various stages of development (A-F). The borders of luteal structures are indicated by arrows. (A) Day 3, (B) Day 5, (C) Day 7, (D) Day 9 (E) Day 12 and (F) Day 15 of the ovine estrus cycle.

Progesterone concentrations in Finn ewes increased significantly from day 3 to day 11 after ovulation. From day 11 to day 14 after ovulation there was a significant decline in progesterone concentrations (Figure 7.3; top panel). Total luteal area increased from day 3 to day 9 after ovulation ($P < 0.05$), after which there was a significant decline from day 9 until day 14. There was a significant correlation between progesterone concentrations and total luteal area ($r^2 = 0.365$; $P < 0.05$) from day 3 to day 14 after ovulation.

7.4.3. Mean spot pixel values

Mean spot pixel values of CL for Western White Face ewes' showed a significant increase from day 3 to day 4 after ovulation and then did not change significantly from day 4 until day 13, after which there was a significant decline (Figure 7.2; centre panel and figure 7.4). There was a correlation between progesterone concentrations and spot pixel values ($r^2 = 0.483$, $P < 0.001$) from day 3 to day 15 after ovulation.

Mean spot pixel values of CL for Finn ewes' showed an increase ($P < 0.05$) from day 4 to day 10 after ovulation and then a decline ($P < 0.05$) from day 10 to day 14 ($P < 0.05$; Figure 7.3; centre panel and figure 7.4). There was a correlation between progesterone concentrations and spot pixel values ($r^2 = 0.267$, $P < 0.05$) from day 3 to day 14 after ovulation.

7.4.4. Mean spot pixel heterogeneity

Mean spot pixel heterogeneity of CL did not differ significantly throughout the luteal phase for both Western White Face and Finn ewes (Figures 7.2 and 7.3; bottom panel and

figure 7.4). There was no significant correlation between circulating progesterone concentrations and spot pixel heterogeneity ($r^2=0.133$; $P>0.05$) for Western White Face ewes' from day 3 to day 15 after ovulation. There was no significant correlation between progesterone concentrations and spot pixel heterogeneity ($r^2=-0.037$; $P>0.05$) for Finn ewes' from day 3 to day 14 after ovulation.

7.5. Discussion

As noted previously (Bartlewski *et al* 1999b), peak serum progesterone concentrations were greater in Western White Face ewes than in Finn ewes. Interestingly, despite the differences in serum progesterone concentrations, the pattern of luteal area did not differ between breeds and resembled that reported previously for luteal tissue volumes (Bartlewski *et al* 1999b). In the present study both total luteal area and mean spot pixel values were correlated with the pattern of serum progesterone concentrations in both Western White Face and Finn ewes for the entire period when CL were detected with ultrasonography (Figure 7.4). The relationship between daily serum progesterone concentrations and total luteal volume in Western White Face and Finn ewes was reported previously (Bartlewski *et al* 1999b). In the study by Bartlewski *et al* (1999b) a mathematical formula was used to calculate total luteal volume of the CL based on measurements of the diameter of the CL from ultrasonographic images. Bartlewski *et al* (1999b) found that in Finn sheep, the relationship between daily serum progesterone concentrations and total luteal volume was maintained throughout the entire period that the CL could be observed. However, in the same study there was no correlation between the total luteal volume and circulating concentrations of progesterone during mid-cycle in

Western White Face ewes; correlations were noted only during luteal growth and regression. In the present study, the correlations between luteal area or spot pixel values and serum progesterone concentrations were greater for the Western White Face ewes than the Finn ewes ($r^2=0.590$ or $r^2=0.365$ and $r^2=0.483$ or $r^2=0.267$, respectively). This would appear to partly reflect a greater variability in pixel values at day 3 of the cycle in Finn compared to Western White Face ewes. Interestingly, although the profiles and peak values of serum progesterone concentrations varied between breeds the temporal patterns of luteal area and pixel values were similar. This would bring into question the ability of the latter parameter to predict functionality of the CL in the ewe. Clearly pixel analysis for the CL gave a clear pattern during the luteal phase reflecting tissue organization, growth and regression but obviously this analysis did not reflect the subtle differences in functionality between breeds. In cattle, Singh *et al* (1997) reported that pixel values of ultrasound images of corpora lutea were correlated with plasma progesterone concentrations; however, these images were collected in a water bath using CL dissected from ovariectomized heifers at specific time points, not on a daily basis.

In sheep, cattle and women there is a rise in mean pixel values from 2 to 3 days after ovulation. The rise in pixel values may represent the gradual reorganization of tissue cells and multiplication of luteal cells; thereby forming a structure with increased density and therefore a higher echogenicity. Following the rise in pixel values there is a plateau, where pixel values remain constant and therefore, presumably there is little tissue restructuring occurring. During the period of CL regression there is a decline in pixel values which is attributed to tissue reorganization from increased vascularization of luteal

tissue and the resulting decrease in tissue density (Baerwald *et al* 2005), which in turn leads to a decrease in echogenicity. In cattle (Tom *et al* 1998a) and women (Baerwald *et al* 2005), during the first 2 days after ovulation there is a sharp decrease in pixel values which represents the sudden increase in vascularity as blood vessels permeate the corpus hemorrhagicum. The last few days of the cycle in cattle (Tom *et al* 1998a) and women (Baerwald *et al* 2005) are characterized by a sharp increase in pixel values and coincides with the regression of the CL. The increase in pixel values represents the restriction of blood vessels and restructurization of the tissue forming the corpus albicans. In the present study it was not possible to record images of CL from the first 2 to 3 nor the last 2 to 3 days after ovulation due to the difficulty of scanning small ruminants and limitations of resolution. Putting the very early and late points mentioned above aside our present data parallels the observations for the luteal phase in women and cattle.

In contrast to sheep, cattle and women the mare has a very different pattern of changing pixel values for its CL during its estrous cycle. In the mare, on the day of ovulation, pixel values are brightest due to the collapse of the follicular wall and the presence of relatively low vascular perfusion (Pierson and Ginther 1985). After ovulation mean pixel value decreases and is maintained at lower values during the period of maximum progesterone production which corresponds to increased blood flow (Pierson and Ginther 1985). Finally, during luteal regression pixel values begin to increase which is representative of decreased blood flow, increased tissue density and fibrin infiltration (Pierson and Ginther 1985). At this time it is unclear as to why the pattern of pixel values in sheep, cattle and women differ from those of the mare.

In both breeds of sheep in the present study there was a greater correlation between luteal area and serum progesterone concentrations than between pixel values and serum progesterone concentrations. However, none of the correlations between progesterone and luteal area or pixel values were great enough to consider them to be of predictive value. Mean spot pixel heterogeneity did not differ significantly throughout the time period analyzed for both Western White Face and Finn ewes. There was a lack of correlation between serum progesterone concentrations and pixel heterogeneity in both breeds of sheep studied in the present experiment. It has been suggested that pixel heterogeneity is a product of the presence of echoic luteal cells of various types, blood clots, and extruded non-echoic serum in the clot, creating hyper- and hypo-echoic areas in the luteal tissue (Duggavathi *et al* 2003b). Therefore, the pixel heterogeneity of CL may be more of a reflection of structural rather than functional characteristics of the CL, which may explain the lack of correlation between pixel heterogeneity and circulating serum progesterone concentrations, in the present study.

In summary, circulating progesterone concentrations during the luteal phase of the ewe were correlated to luteal area and mean pixel values but not to pixel heterogeneity of ultrasound images of the CL. None of the correlations between progesterone and luteal area or pixel values were great enough to consider them to be of predictive value. The differences between breeds in serum progesterone concentrations but not for luteal area and pixel values led us to conclude that these parameters were not good predictors of functionality.

Chapter 8: GENERAL DISCUSSION AND FUTURE DIRECTIONS

8.1. General Discussion

The results of the experiments described in this thesis have answered questions and expanded our knowledge on follicular dynamics and CL function in the ewe. The application of some of the findings described in this thesis in a commercial setting have the potential to increase productivity and efficiency and therefore be of benefit to the sheep industry as whole. In the ewe, one to three antral follicles emerge and grow from a pool of small follicles (≥ 1 mm but ≤ 3 mm in diameter) every 4 to 5 days (Ginther *et al* 1995; Bartlewski *et al* 1999a), each wave is preceded by a peak in serum concentrations of FSH (Ginther *et al* 1995; Souza *et al* 1998) and follicles in a wave grow to ≥ 5 mm in diameter (Duggavathi *et al* 2003a). In cattle, there is a dominant follicle of a wave that suppresses the growth of other follicles (subordinate follicles) and prevents the emergence of a new follicular wave (Armstrong and Webb 1997). The emergence of a follicular wave in cattle is characterized by an increase in the number of small antral follicles (4 to 6 mm in diameter; Ginther *et al* 1989). However, in the ewe, the number of small antral follicles (≥ 1 mm but ≤ 3 mm in diameter) remains constant throughout the ovine estrous cycle, except for the periovulatory period (Duggavathi *et al* 2003a). In the present study (chapter 3) injections of oFSH at 36 hours and 72 hours after ovulation, in the ewe, resulted in peaks in serum concentrations of FSH which in turn induced follicular wave emergence in the presence of a large (≥ 4 mm in diameter) growing follicle. The fact that we were able to create follicle wave emergence in the presence of a large growing follicle and that the induced waves did not disrupt the emergence of the second endogenous wave of the cycle, brings into question the presence of both direct

and indirect dominance in the ewe. In addition to the findings of the present study, other authors have found that prolific Finnish Landrace ewes ovulate follicles from the penultimate along with follicles from the final wave of the cycle (Bartlewski *et al* 1999a). Further to these findings it has been demonstrated in the present study (chapter 6) that there may be other differences between cattle and sheep with regard to the control of follicular wave dynamics, in particular, LH pulse frequency.

In order to assess the affect of LH pulse frequency on follicular dynamics in the ewe the “ram effect” was employed. Isolation of seasonally anestrous ewes from rams for at least one month and then re-introduction of rams leads to synchronized estrus (Underwood *et al* 1944; Edgar and Bilkey 1963) and will often induced ovulation in a proportion of ewes within 2 to 3 days (Coop and Clarke 1968; Knight *et al* 1978). It is known that LH pulse frequency increases markedly within minutes of ram introduction (Martin *et al* 1980) and ewes stimulated by the ram experience a preovulatory LH surge similar to that of spontaneously ovulating sheep (Oldham *et al* 1979). In heifers, there is speculated to be a role for LH in antral follicle deviation (Ginther *et al* 2001a; Sartori *et al* 2001), however, the relationship between LH and follicular development in the ewe is unclear (Karsch *et al* 1979; Bartlewski *et al* 2000; Duggavathi *et al* 2005). In the present study (chapter 6), introduction of rams to previously isolated anestrous, non-prolific Western White Face ewes resulted in a subtle increase in LH pulse frequency. However, these changes in LH secretion were not reflected by consistent trends in the temporal pattern of follicular dynamics. Considering the results of the present study (chapter 6) and findings from other authors (Picton *et al* 1990; McNeilly *et al* 1991) we suggest that changes in LH pulse

frequency are not critical for the normal pattern of antral follicular waves in sheep. In cattle, selection of a dominant follicle occurs at the point of follicle deviation (Ginther *et al* 1996). It has been suggested that LH, in particular LH pulse frequency, has a role to play in the deviation mechanism of that species (Fortune *et al* 1991; Gong *et al* 1995; Ginther *et al* 1996). This further emphasizes the subtle differences between species with regard to the mechanisms controlling follicular wave dynamics.

In a previous study in our laboratory (Bartlewski *et al* 2003), treatment of non prolific Western White Face ewes with PGF_{2α} and MAP-containing intravaginal sponges on ~Day 8 of a cycle (Day 0 = first ovulation of the interovulatory interval), resulted in ovulations during the subsequent 6 days when MAP sponges were in place. Ovulations occurring during the MAP sponge period were not preceded by a preovulatory LH/FSH surge, and none of these ovulations were followed by the formation of CL, only transient corpora hemorrhagica (CH: Bartlewski *et al* 2003). Performing two experiments in anoestrous ewes enabled us to investigate whether these ovulations were caused by a sharp decline in serum progesterone concentrations or due to a direct affect of PGF_{2α} on the ovary without the confounding presence of a CL (chapter 4). The combination of PGF_{2α}/MAP treatment resulted in ovulations of follicles during the period of MAP sponge insertion, but these ovulations were not preceded by a preovulatory LH or FSH surge (Experiment 1). However, creating a rapid decline in circulating progesterone concentrations, in MAP treated ewes, resulted in no ovulations (Experiment 2). Therefore, ovulations occurring after PGF_{2α} injection in anoestrous ewes, and in the presence of a MAP sponge were likely due to a direct effect of PGF_{2α} at the ovarian level

rather than a sudden decline in circulating progesterone concentrations. In non prolific breeds of sheep such as the Western White Face and Suffolk, a follicle(s) will only ovulate from the final wave of the cycle (Bartlewski *et al* 1999a). Whereas prolific breeds, such as the Finn, can ovulate follicles from the penultimate as well as the final wave of the cycle (Bartlewski *et al* 1999a). By increasing the ovulation rate of non prolific breeds of sheep, producers may be able to overcome one of the major limiting factors to efficient sheep meat and wool production, that is, low lamb output per ewe (Blaxter 1964; Gordon 1997b). When Bartlewski *et al* (2003) treated non prolific Western White Face ewes for 6 days with a MAP-containing intravaginal sponge, coupled with an injection of PGF_{2α}, on day 8 after ovulation, it resulted in an approximately 50% increase in ovulation rate at the end of the treatment, as compared to control ewes. Therefore applying the treatment given in the study by Bartlewski *et al* (2003) to non prolific Suffolk ewes prior to mating may influence the outcome of lambing rate. However, in the present study (chapter 5) there was no affect of the treatment protocol on lambing rate, birth weights nor sex ratios. We concluded that the short-term MAP sponge and PGF_{2α} treatment lead to the untimely ovulation of follicles with respect to breeding. In order to further investigate the differences between prolific and non prolific breeds of sheep an assessment of the ultrasound image attributes of CL was performed (chapter 7) in two different breeds of sheep.

With transrectal ovarian ultrasonography researchers have the ability to collect real-time serial images of ovarian structures, such as the CL (Pierson and Ginther 1986). In the ewe, changing progesterone concentrations measured in the circulation reflect physical

changes in the CL (Arthur *et al* 1989). Previous studies have reported correlations between serum progesterone concentrations and ultrasound image attributes of the CL in different species (Tom *et al* 1998; Baerwald *et al* 2005). However, such detailed studies have not been performed in ewes. In the present study (chapter 7) both total luteal area and mean spot pixel values were correlated with the pattern of serum progesterone concentrations in both non prolific Western White Face and prolific Finn ewes for the entire period when CL were detected with ultrasonography. However, none of the correlations between progesterone and luteal area or pixel values were great enough to consider them to be of predictive value. Interestingly, despite the differences in serum progesterone concentrations between the Western White Face and Finn ewes, the pattern of luteal area did not differ between breeds. The differences between breeds in progesterone concentration but not for luteal area and pixel values led us to conclude that these parameters were not good predictors of functionality.

In conclusion, it appears from the present studies (chapters 3 to 7) that there are differences between species as well as within breeds of sheep with regard to follicular dynamics and regulation of follicular growth. Furthering our understanding of the regulation of follicular dynamics and ovulation rate in the ewe, through the use of techniques such as radioimmunoassays, ultrasound image analysis or the manipulation of treatment protocols would be of benefit to producers and the sheep industry as a whole.

8.2. Future directions

1. Injections of oFSH during an inter-wave interval in the ewe, increase the number of follicular waves emerging during the cycle as compared to a “normal” cycle (chapter 3). Other authors have demonstrated that prolific Finn ewes ovulate follicles from the penultimate wave of the cycle along with follicles from the final wave of the cycle (Bartewski *et al* (1999a). In the present study (chapter 3) this treatment was applied to the first wave of the cycle. It would be worth investigating the effects of administering the same treatment in the penultimate or final wave of the cycle as an effective method to increase ovulation rate in non prolific breeds of sheep.
2. Administration of the PGF_{2α}/MAP treatment to cyclic ewes in this thesis did not result in the expected increased lambing rate. In order to check that ovulations actually occurred in these animals at the expected time, ultrasonography of the ovaries could be performed. In addition, the aspiration of ovulated oocytes during treatment and then subsequent *in vitro* fertilization and maturation would enable us to examine if the treatment results in oocytes capable of producing a viable embryo.
3. In order to assess the effect of LH pulse frequency on follicular dynamics in the ewe, it may be prudent to repeat the present study (chapter 6) with ewes that have a less distinct breeding season and rams that are novel to the ewes. Also the isolation of ewes and re-introduction of rams closer to the transition from one

season to the other may result in more dramatic effects on LH pulse frequency and a more rigorous test of its effects on follicle dynamics.

4. Ultrasound image attributes of the ovine CL in the present study (chapter 7) were collected from 3 days to 14 or 15 days after ovulation. We were unable to collect observations of CL throughout the entire ovine estrous cycle due to the difficulty of scanning small ruminants and the limitations of resolution. Future studies may benefit from the use of higher resolution ultrasonographic equipment (Aloka 900-SSD, Aloka Co. Ltd., Tokyo, Japan) with which it is possible to detect follicle rupture and CL differentiation as early as 12 to 24 hours after ovulation in sheep (Duggavathi *et al* 2003b). With the collection of images of CL throughout the entire ovine estrous cycle it would be possible to compare the pixel characteristics of “normal” CL to those of the abnormal structures seen in chapter 4, of this thesis, when a PGF_{2α}/MAP treatment was administered.

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