DYNAMICS AND REGULATION OF OVARIAN ANTRAL FOLLICULAR WAVES IN SHEEP

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ABSTRACT

The focus of the present thesis was on ultrasonographic, endocrine and molecular characterization of ovarian antral follicular waves in sheep. Transrectal ultrasonography and computer assisted image analysis were used to determine the feasibility of detecting ovulation and the forming corpus luteum (CL) and to non-invasively monitor CL differentiation and growth. High resolution transrectal ultrasonography and hormone measurements were used to assess changes in numbers of small ovarian antral follicles and their relationships to the emergence of follicular waves in cyclic ewes and to correlate pulsatile secretion of gonadotropins with follicular growth in a wave, during the mid to late-luteal phase of the ovine estrous cycle. A series of experiments were conducted, using treatment with injections of ovine follicle stimulating hormone (oFSH) and measurement of serum concentrations of FSH, in cyclic and anestrous sheep, to investigate the existence of follicular dominance. We also evaluated the characteristics of secretory patterns of FSH that are critical for follicular wave emergence, in anestrous ewes. The possible existence of an endogenous rhythm of FSH secretion, independent of ovarian antral follicular dynamics, was studied in ovariectomized ewes. Finally, ovarian antral follicles at defined stages of growth in a follicular wave (based on transrectal ultrasonographic observations) were collected from separate groups of sheep by ovariectomy, to profile the expression patterns of steroidogenic enzymes (3β-HSD, 17α-OH and aromatase) using immunohistochemistry and gray-scale densitometric analysis.

The results of the present studies showed that it is possible to detect ovulation and visualize developing CL as early as 12-24 h after ovulation in the ewe. Changes in echotexture of the CL were closely associated with its morphological and functional characteristics, and we concluded that computer assisted image analysis holds promise for the noninvasive monitoring of CL differentiation and growth. Follicles reaching ovulatory diameter (≥ 5 mm) emerged and grew in a wave-like pattern in sheep, but without variation in the number of small follicles (1-3 mm in diameter), as seen in cattle. We concluded that all follicles that are recruited to grow beyond 2-3-mm in diameter, to 4-mm diameter in a wave, succeed in reaching an ovulatory diameter of ≥ 5 mm in the ewe. The emergence and growth of ovarian antral follicles in follicular waves, in sheep, do not require changes in LH secretion and may perhaps involve changes in the follicular sensitivity to LH. The largest follicle of a wave, in sheep, appears to have limited effects on other small follicles and on the time of emergence of the next follicular wave. Thus, functional dominance, as is present in cattle, may be absent in sheep. An endogenous rhythm for periodic peaks in serum FSH concentrations that is independent of ovarian follicular dynamics may exist in sheep. The expression patterns of steroidogenic enzymes, in the theca and granulosa compartments of antral follicles growing in each follicular wave in the ewe, paralleled serum estradiol concentrations, with the exception of the concentrations of 3β-HSD in granulosa cells, which increased continuously from follicles 3 mm in diameter to the preovulatory follicle after the LH surge. The largest follicle of any follicular wave, irrespective of the stage of the cycle, would appear to be mature enough to ovulate if a gonadotropin surge is provided.

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Dedicated to...

My Parents…
for showing me this very world

My Brother…
for his support throughout my academic ventures

My Teachers…
for all their efforts towards my learning

My Friends…
for reinforcing my strength and morale

My Family…
for understanding my emotions

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LIST OF ABBREVIATIONS

3β-HSD	3 β hydroxysteroid dehydrogenase
17α-OH	Cytochrome P450 17 α hydroxylase
Aromatase	Cytochrome P450 aromatase
BMP	Bone morphogenetic protein
CL	Corpus luteum
EGF	Epidermal growth factor
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
IGF-I	Insulin-like growth factor I
Kg	Kilogram
LH	Luteinizing Hormone
LH-R	LH receptor
MHz	Mega hertz
μg	Microgram
mm	Millimeter
mRNA	messenger Ribonucleic Acid
oFSH	ovine FSH
P450scc	Cytochrome P450 side chain cleavage
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
StAR	Steroidogenic acute regulatory enzyme
TGFα	Transforming growth factor α

Chapter 1: REVIEW OF LITERATURE

1.1. Introduction

The female reproductive system in mammals plays a major role in the process of reproduction as important steps such as gametogenesis (oogenesis), fertilization, pregnancy, parturition and lactation occur in the female. The ovary is the major functional unit of the female reproductive system. It is a very dynamic organ system, with complex processes such as follicle formation, oocyte maturation, cellular proliferation, differentiation and apoptosis taking place in a cyclic manner (estrous/menstrual cycle and seasonality). Adding to the complexity, these phenomena are regulated at multiple levels, such as locally at the ovary itself and also at the level of the pituitary and hypothalamus. The physiological mechanisms behind such amazing phenomena such as the growth of a follicle by about 150-200 times in diameter (40 μ to 5-7 mm), concomitant cytoplasmic and nuclear maturation of the oocyte, selection of ovulatory follicle(s), growth of the corpus luteum (about 25 fold in 12 days), timed luteal regression in the absence of pregnancy, have all intrigued researchers for more than 300 years. Even though, the first reference to the reproductive system was made by Aristotle (384-322 BCE), significant interest in ovarian physiology began in the mid 17th century (Short 1977).

In the recent past, there have been remarkable advancements in our understanding of ovarian physiology due largely to advances in real-time ultrasonography. This, along with developments in cell and molecular biological techniques, has greatly augmented

our understanding of the control of ovarian antral follicular dynamics and the regulation of the growth of ovulatory follicles in several species such as human, cattle and sheep.

This thesis describes the experimental work done to investigate certain aspects of the dynamics and regulation of ovarian antral follicular waves in sheep using the techniques of transrectal ultrasonography, radioimmunoassay, immunohistochemistry and computer assisted image analysis. Literature reviewed in this chapter focuses on our current understanding of follicular waves and allied aspects of ovarian follicular dynamics in the ewe, but where particularly useful information was lacking for sheep, pertinent references to the literature for other domestic species, laboratory animals and primates have been given.

1.2. Estrous cyclicity in the ewe

The ewe is a seasonally polyestrous animal with normal ovulatory cycles occurring, in most breeds, in the autumn and winter (Hafez 1952). The annual rhythm of ovarian cyclicity is characterized by a season-dependent cessation (anestrus) and restoration (breeding season) of ovulatory cycles (Marshall 1937; Hafez 1952). The estrous cycle is 16 to 17 days in length in sheep (Marshall 1904). The length of the ovine estrous cycle is remarkably consistent among different breeds (McKinszie and Terrill 1937), ages (Hafez 1952) and stages of the breeding season (McKinszie and Terrill 1937). Unlike the length of the estrous cycle, the duration of the breeding and non-breeding seasons in sheep of different breeds is highly variable (Goodman 1994).

Traditionally, the estrous cycle is divided into a number of phases (Goodman 1994). Pro-estrus is the period immediately preceding behavioral estrus and is characterized by

luteal regression, and the emergence and growth of the ovulatory follicle. Estrus is regarded as the period of sexual receptivity and mating. In ewes the estrous phase lasts between 24 to 48 h, depending on the breed (Land 1970a; Land *et al.* 1973; Quirke *et al.* 1979; Bindon *et al.* 1979; Goodman 1994). Ovulation in sheep is a spontaneous process that does not require the act of coitus and it occurs 24 to 30 h after the onset of estrous behavior (McKinszie and Terrill 1937; Robertson 1969). Pro-estrus and estrus are frequently referred to collectively as the follicular phase of the estrous cycle. Metestrus is the period during which the remnants of the ovulated follicle transform into an endocrine gland, the corpus luteum (CL) (Keyes *et al.* 1983). The luteal structure in this phase is also called the corpus haemorrhagicum. The period of the estrous cycle when there is a fully functional CL, secreting large amounts of progesterone, is referred to as diestrus. Metestrus and diestrus are frequently referred to collectively as the luteal phase of the estrous cycle. Regulation of cyclic activity is mainly under the control of the hypothalamic-pituitary-ovarian axis.

1.3. Hormonal profiles during the ovine estrous cycle

The hormones that are involved in the orchestrated communication among the individual components associated with the regulation of the estrous cycle are largely: gonadotropin releasing hormone from the hypothalamus; follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin and oxytocin from the pituitary gland; estrogens and inhibins from the ovarian antral follicles; progesterone and oxytocin from the CL; and prostaglandin F2α (PgF2α) from the uterine endometrium.

1.3.1. Secretion of gonadotropins

In the ewe, there are two distinct modes of gonadotropin secretion (Arthur *et al.* 1989). The preovulatory discharge of LH and FSH (Arthur *et al.* 1989), is referred to as the phasic mode of gonadotropin secretion (Fig. 1.1 Top panel). This surge of gonadotropins lasts 8-12 h and is primarily evoked and sustained by decreased progesterone and increased estradiol secretion during the final stage of the estrous cycle (Scaramuzzi *et al.* 1970; Bolt *et al.* 1971; Baird and Scaramuzzi 1976; Karsch *et al.* 1980; Rawlings *et al.* 1984; Jeffcoate *et al.* 1984a; Kaynard *et al.* 1988; Moenter *et al.* 1990; Joseph *et al.* 1992). The pulsatile or tonic mode of LH release is generated in response to pulsatile GnRH release from the hypothalamus (Levine *et al.* 1982). Pulsatile LH release (Fig. 1.1 Top panel) prevails at all reproductive states in ewes, including the period before, during and after the preovulatory surge of gonadotropins (Rawlings and Cook 1993), and it is also present in ovariectomized ewes (Gay and Sheth 1972). In cyclic ewes, low-amplitude pulses occur from 1 to 6 times in a 6 hr period (Goodman *et al.* 1981). An increase in tonic LH secretion during the pro-estrous period results from an increase in pulse frequency (Baird 1978). An increase in pulse frequency and amplitude of LH occurs during the preovulatory surge (Goodman *et al.* 1981). An increment in basal (non-pulsatile) LH release during the surge was also suggested (Rawlings and Cook 1993).

An FSH surge (Fig. 1.1 Top panel) coincides with the preovulatory LH surge (Wheaton *et al.* 1984; Baird *et al.* 1991). The secondary FSH surge, which is lower in amplitude but longer in duration, occurs between 20-36 h after the preovulatory gonadotropin surge (Pant *et al.* 1977; Bister and Paquay 1983; Wheaton *et al.* 1984; Findlay *et al.*

1990). Unlike LH, tonic FSH secretion during the ovine estrous cycle is non-pulsatile (Bister and Paquay 1983; Wheaton *et al.* 1984; Wallace and McNeilly 1986), but some researchers were able to detect FSH pulses in hypophyseal and peripheral circulation during the luteal phase in ewes (Van Cleeff *et al.* 1995). Apart from this tonic release of FSH, many researchers have observed considerable day-to-day (periodic) variation in serum FSH concentrations (Cahill *et al.* 1981; Baird *et al.* 1981). These observations were confirmed by documentation of a 5-d rhythm of peaks in FSH secretion in cyclic, anestrous and pregnant ewes (Bister and Paquay 1983). These rhythmic fluctuations in serum FSH concentrations were previously suggested to be coincidental with waves of follicular growth (Smeaton and Robertson 1971; Brand and de Jong 1973). This concept of temporal association between FSH peaks (Fig. 1.1 Top panel) and follicular wave emergence was confirmed unequivocally in the experiments in which antral follicular development was monitored by ultrasonography (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).

During the ovine estrous cycle, up to 50% of the pituitary content of FSH is released every day while only 1 to 5% of LH is secreted in the form of pulses on a daily basis (Taragnat *et al.* 1998). LH is stored in secretory granules which are emptied in response to GnRH. FSH appears to be secreted constitutively, where the rate of secretion closely relates to the rate of synthesis (McNeilly *et al.* 2003). The mechanisms that control these divergent patterns of gonadotropin release are not fully understood (Padmanabhan *et al.* 2003).

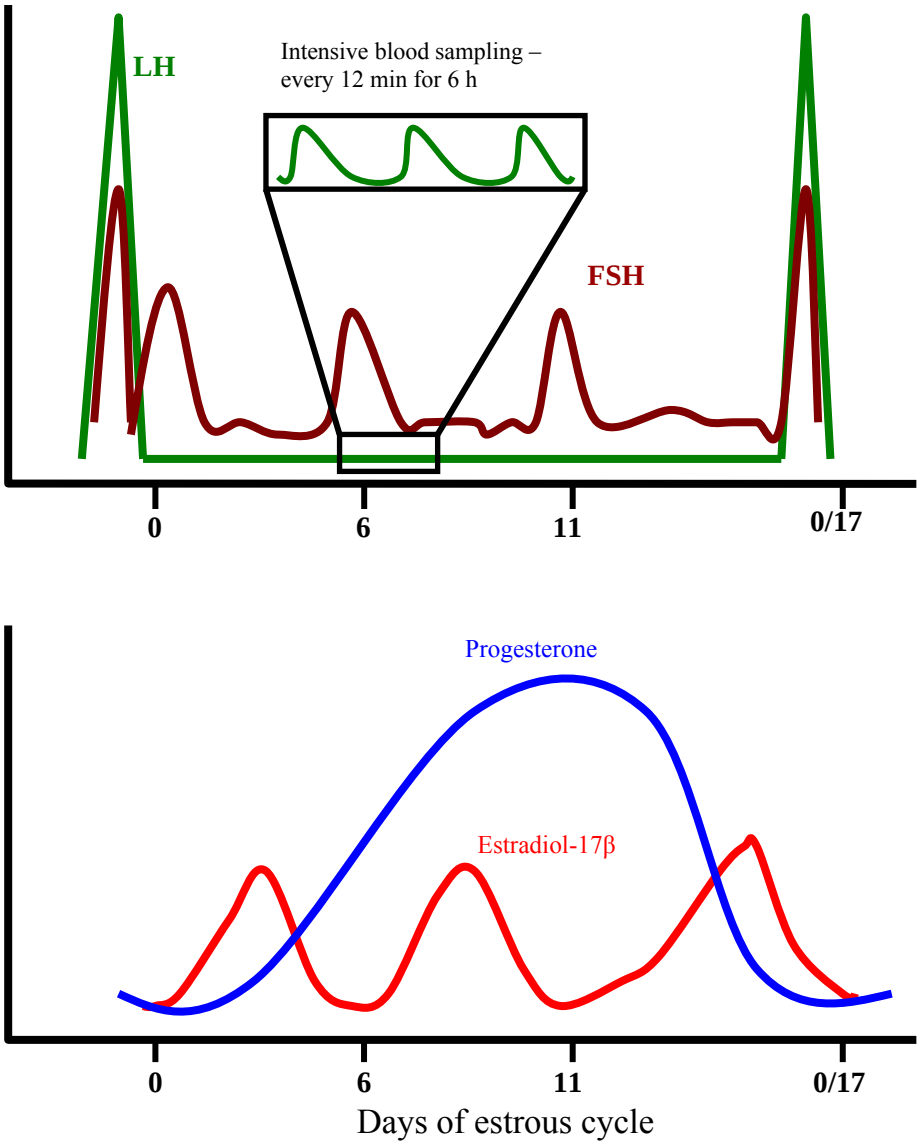

Figure 1.1. Schematic representation of serum profiles of LH and FSH (top panel), and estradiol-17β and progesterone (bottom panel) throughout an estrous cycle in the ewe (x-axis: d0 = day of ovulation, y-axis: relative concentrations of hormones). Except for high concentrations during preovulatory surge, 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 d 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)

1.3.2. Secretion of estradiol

The pattern of estradiol secretion in the ewe is pulsatile (Baird 1978). Each pulse of LH is followed by a rise in the secretion of estradiol-17β in cyclic (Baird *et al.* 1976b) and anestrous (Scaramuzzi and Baird 1977) ewes with utero-ovarian autotransplants. The largest non-atretic follicles are the main source of estradiol (Bjersing *et al.* 1972; Evans *et al.* 2000). During the ovine estrous cycle, there are periods of increased circulating concentrations of estradiol (Scaramuzzi *et al.* 1970; Cox *et al.* 1971; Campbell *et al.* 1995). In previous studies where antral follicular growth was monitored using ultrasonography, 3 to 4 peaks (Fig. 1.1 Bottom panel) in serum estradiol concentrations were shown to occur in a cycle, each coinciding with the end of the growth phase of the largest follicle of a follicular wave (Bister *et al.* 1999; Bartlewski *et al.* 1999a; Souza *et al.* 1998). On the day of ovulation, peripheral concentrations of estradiol fall to non-detectable levels, which coincides with the secondary peak of FSH secretion (Bister and Paquay 1983; Wheaton *et al.* 1984; Findlay *et al.* 1990; Baird *et al.* 1991).

1.3.3. Secretion of progesterone

Even though the pattern of progesterone secretion in the ewe is pulsatile during the luteal phase, progesterone pulses are not temporally associated with LH pulses (Baird *et al.* 1976b). Following ovulation, serum progesterone concentrations (Fig. 1.1 Bottom panel) 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). Serum concentrations of progesterone seem to vary between breeds. Interestingly, some authors (Quirke *et al.*

1979; Cahill *et al.* 1981) have shown that prolific ewes have higher serum concentrations of progesterone compared to non-prolific breeds, while others (Bartlewski *et al.* 1999b) have shown that prolific ewes have lower serum progesterone concentrations compared to non-prolific ewes. However, the latter observation has been supported by a recent study (Bartlewski *et al.* 2003), where treatment with PGF2α and an intra-vaginal progestogen sponge, during the mid-luteal phase of the cycle, to create a sub-luteal or low progesterone environment, increased ovulation rate in non-prolific ewes.

1.3.4. Secretion of inhibins, activins and follistatin

Inhibins and activins are disulphide-linked dimeric glycoproteins belonging to the transforming growth factor β (TGF-β) superfamily (de Kretser *et al.* 2000; Knight and Glister 2003). Inhibins are dimers of a unique α subunit linked to either a βA or βB subunit to give inhibin A or B, respectively (Knight and Glister 2001). Dimerization of β subunits alone gives rise to activins, activin A, activin B and activin AB (Knight and Glister 2001). Follistatin, a cysteine-rich monomeric glycoprotein, is not related to the TGF-β superfamily but is a binding protein for activins (Knight and Glister 2001). Follistatin was originally identified and isolated from both bovine and porcine follicular fluids (Xia *et al.* 2003). In sheep, expression of the inhibin A and B genes is confined to the granulosa layer of ovarian follicles (Tisdall *et al.* 1994) and is positively related to both the size and estrogen secretory capability of antral follicles (Campbell and Baird 2001). Thus it appears that large follicles are the major source of inhibin. However, gene expression for inhibins have also been detected in the placenta, pituitary, adrenal, bone marrow, kidney, spinal cord and brain (Meunier *et al.* 1988) in rats. Follistatin

gene expression is detected in the granulosa layer of preantral, antral and early atretic follicles, at all stages of the ovine estrous cycle (Tisdall *et al.* 1994). In a previous study (Souza *et al.* 1998), the relationship between plasma inhibin A concentrations and follicular growth (as monitored by ultrasonography) was investigated using ewes with an ovarian autotransplant. In that study, the mean plasma concentrations of inhibin A increased during the first day of growth of the first wave after ovulation, but did not change significantly during the remainder of the experiment, which encompassed the emergence of the next follicular wave. The increase in inhibin A concentration was associated with the development of the largest follicle of the first wave from 2.5 mm to 4 mm in diameter. In another study (Evans *et al.* 2001), follicular waves were not associated with fluctuations in inhibin A concentration. There are specific assays for ovine inhibin A, but not for ovine inhibin B (Souza *et al.* 1997b). However, from studies in humans (Groome *et al.* 1996) and rats (Woodruff *et al.* 1996), it appears as if inhibin B, unlike inhibin A, is primarily produced by small antral follicles. Moreover, inhibin B is 10 times less potent than inhibin A in suppressing FSH release by cultured sheep pituitary cells (Robertson *et al.* 1996).

There is scant information on the secretory pattern of activin in sheep. However, it appears that all of the activin present in the circulation is bound to follistatin (Woodruff 1998). Follistatin is known to neutralize the biological activities of activins (Mather *et al.* 1993) and several bone morphogenetic proteins (Iemura *et al.* 1998). Follistatin concentrations remain unchanged throughout the estrous cycle and during pregnancy in sheep (Xia *et al.* 2003). However, similar amounts of follistatin mRNA have been detected in ovine granulosa cells of preantral, antral, and early atretic follicles at all

stages of the estrous cycle and in the CL, except for the preovulatory follicles that have low amounts of the transcript in the granulosa cells (Tisdall *et al.* 1994).

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). Measurements of GnRH concentrations in portal blood or median eminence perfusates in sheep (Levine *et al.* 1982; Clarke and Cummins 1982), have provided evidence for 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 (Levine *et al.* 1982;Clarke and Cummins 1982). These small GnRH pulses have been suggested to 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 breeding (Baird 1978) and non-breeding seasons (Scaramuzzi and Baird 1977). During anestrus the frequency and amplitude of GnRH/LH pulses are significantly lower compared to the breeding season (Scaramuzzi and Baird 1977).

1.4.2. Pituitary regulation

The potential paracrine regulators in the pituitary include inhibins, activins and follistatin (Padmanabhan *et al.* 2002). However, neither inhibin (Mercer *et al.* 1987) nor activin (Gharib *et al.* 1990) affect the amounts of mRNA encoding LHβ subunit in pituitary gonadotrophs.

1.4.3. Gonadal regulation

Gonadal steroids, estradiol and progesterone regulate LH secretion either directly or indirectly. Progesterone regulates tonic secretory pulse frequency while estradiol regulates pulse amplitude (Bjersing *et al.* 1972; Karsch *et al.* 1979; Goodman and Karsch 1980; Rawlings *et al.* 1984; Wheaton *et al.* 1984). The preovulatory LH surge is evoked and sustained by decreased progesterone and increased oestradiol secretion during the preovulatory period of the estrous cycle (Scaramuzzi *et al.* 1970; Bolt *et al.* 1971; Baird and Scaramuzzi 1976; Karsch *et al.* 1980; Rawlings *et al.* 1984; Jeffcoate *et al.* 1984b; Kaynard *et al.* 1988; Moenter *et al.* 1990; Joseph *et al.* 1992). In sheep, estradiol provides positive feedback during the follicular phase of the estrous cycle by enhancing GnRH secretion at the hypothalamic level (Moenter *et al.* 1990; Herman and Adams 1990) and by increasing the responsiveness to GnRH of the gonadotrophs at the pituitary level (Herman and Adams 1990). During the luteal phase of the ovine estrous cycle, pulsatile release of LH is inversely related to circulating levels of luteal progesterone (Karsch *et al.* 1979; Rawlings *et al.* 1984; Wheaton *et al.* 1984). Estradiol enhances the inhibitory effects of progesterone on LH secretion by acting primarily at the level of the hypothalamus (Goodman and Karsch 1980; Goodman and Karsch 1981; Goodman *et al.* 1981; Martin *et al.* 1988). The inhibitory effect of progesterone is all the more pronounced in seasonally anestrous ewes (Karsch *et al.* 1987). In ovariectomized ewes, progesterone blocks the estradiol-induced LH surge (Legan and Karsch 1979) by preventing the increase in pulsatile GnRH release and perhaps also by decreasing the sensitivity of pituitary gonadotrophs to estradiol (Koligian and Stormshak 1977).

1.5. Regulation of FSH secretion

1.5.1. Hypothalamic regulation

Like LH, each GnRH pulse is followed by a pulse of FSH secretion in the hypothalamic-pituitary portal circulation (Padmanabhan *et al.* 1997). However, there is evidence for GnRH-independent FSH pulses in the portal circulation in ovariectomized ewes (Padmanabhan *et al.* 2003). An episodic pattern of FSH secretion in peripheral blood persists after the blockade of GnRH action in ovariectomized rabbits (Pau *et al.* 1991). These pieces of evidence suggest the existence of a hypothalamic-independent regulation of FSH secretion (Padmanabhan *et al.* 2002). Moreover, there appears to be some evidence for the existence of a separate hypothalamic releasing factor for FSH, even though its identity still remains unknown (Padmanabhan and McNeilly 2001).

1.5.2. Pituitary regulation

Inhibins, activins and follistatin are known to be produced in many tissues including the pituitary (Mather *et al.* 1992; Gregory and Kaiser 2004). They have the potential to act in an autocrine and paracrine manner in the local control of FSH production and secretion (Knight and Glister 2001; Padmanabhan and Sharma 2001). Further support for paracrine control of FSH secretion also comes from correlative studies that have shown a negative relationship between the expression pattern of follistatin and FSHβ mRNA, and a positive relationship between activin B and FSHβ mRNA (Dalkin *et al.* 1999).

1.5.3. Gonadal regulation

Some authors (Tsonis *et al.* 1986) have shown that progesterone suppresses the release of FSH from dispersed sheep pituitary cells, while others (Dluzen and Ramirez 1987) have shown that the progesterone infusion had no effect on FSH secretion in estrogen-primed rats. Estradiol, a major regulator of FSH secretion (Baird *et al.* 1991), exerts both positive and negative feedback effects via multiple processes including indirect effects on GnRH secretion (Karsch *et al.* 1993), transcription of FSH subunit mRNAs (McNeilly *et al.* 2003) and FSH heterogeneity (Padmanabhan *et al.* 2002). Baird *et al.* (1991) demonstrated that ovarian inhibin was a potent regulator of FSH secretion. Several lines of evidence for the endocrine role of ovarian inhibins in the regulation of FSH secretion in intact ewes have come from studies involving injection of crude inhibin (e.g. follicular fluid) (Martin *et al.* 1988) and passive immunization against inhibin (Mann *et al.* 1992a). With the advent of specific assays for inhibin A (McConnell *et al.* 1996), circulating concentrations of inhibin A and FSH have been shown to have an inverse relationship in sheep (Knight *et al.* 1998). However, follicular growth to ovulatory diameter in a wave and associated fluctuations in plasma FSH concentrations, have not been shown to have an inverse relationship with inhibin A concentrations in cyclic (Souza *et al.* 1998) or anestrous ewes (Evans *et al.* 2001).

1.6. Transrectal ultrasonography

The application of transrectal ultrasonography has been one of the most profound technological advances in the field of research and clinical reproduction (Pierson and Adams 1995). Transrectal ultrasonography was first used in cattle (Pierson and Ginther 1984), and it took one decade for the adaptation of transrectal ultrasonography to study

ovarian follicular and luteal dynamics in sheep (Schrick *et al.* 1993; Ravindra *et al.* 1994). With the use of ultrasonography, there have been some lines of evidence for (Evans *et al.* 2000) and against (Schrick *et al.* 1993; Ravindra *et al.* 1994) the existence of a "wave" pattern of follicular development in sheep, similar to that seen in cattle (Pierson and Ginther 1984; Pierson and Ginther 1987a; Ginther *et al.* 1996). Some authors noted that the numbers of follicles reaching a maximum diameter of 3 or 4 mm did not vary throughout the ovine estrous cycle (Ginther *et al.* 1995; Bartlewski *et al.* 1999a). However, by limiting the consideration to only those small follicles (2-3 mm in diameter) that reach ovulatory diameter (≥ 5 mm), a clear wave like pattern of follicular development, with a preceding peak in FSH secretion, was demonstrated (Noel *et al.* 1993; Ginther *et al.* 1995; Souza *et al.* 1998; Leyva *et al.* 1998b; Vinoles *et al.* 1999; Gibbons *et al.* 1999; Bartlewski *et al.* 1999a).

Computer-assisted image analysis is a natural extension of the technologic advances in ultrasonography (Singh *et al.* 2003). An ultrasonographic image is composed of thousands of picture elements, or pixels (Pierson and Adams 1995). Each pixel represents a discreet tissue reflector and can assume one of 256 shades of grey (ranging from black to white) in an 8-bit grey-scale image (Pierson and Adams 1995). Computer algorithms have been designed specifically for analysis of ultrasound images, in an effort to provide a quantitative approach to echotextural analysis (Synergyne 2©, Version 1.1, WHIRL, Saskatoon, SK, Canada). These algorithms have been used extensively in studies characterizing the echotexture dynamics of antral follicles and CL at different stages of their growth (Pierson and Adams 1995; Singh *et al.* 1997; Singh *et al.* 1998; Tom *et al.* 1998a; Tom *et al.* 1998b).

1.7. Follicular development

1.7.1. Folliculogenesis

It has been a long standing paradigm that in most mammals, primordial follicles are formed just before or soon after birth, when primary oocytes become surrounded by a squamous layer of somatic cells (pre-granulosa cells; Land 1970b). Populations of primordial (resting pool) and primary (growing pool) ovarian follicles constitute the reserve pool of follicles that ewes utilize during their entire reproductive life (40, 000 to 300, 000 primordial follicles in ewe lambs; (Driancourt *et al.* 1991). Recently, it was demonstrated that follicular renewal continues in adult mouse (Johnson *et al.* 2004) and human (Bukovsky *et al.* 2004) ovaries. Primordial follicles appear to continuously leave the non-growing pool of follicles by being converted into primary follicles (van Wezel and Rodgers 1996). However, this concept needs to be re-examined in light of continued follicle renewal in adult ovaries in some species (Johnson *et al.* 2004; Bukovsky *et al.* 2004). When follicles leave the resting pool, the granulosa cells become cuboidal and begin to express markers of cell proliferation, such as proliferating cell nuclear antigen (Wandji *et al.* 1997; Fortune 2003). When primary follicles become secondary follicles, they have two or three layers of granulosa cells (Driancourt *et al.* 1991; Fortune 2003). Early antral or tertiary follicles are the next stage of follicular development followed by the formation of a complete antrum (Lundy *et al.* 1999). Antral follicles continue to grow under the influence of gonadotropins and acquire steroidogenic capability to form mature Graafian follicles (Hay and Moor 1975b). The estimated lengths of time taken by a follicle, in sheep, to grow from one stage to the other have been reported previously. The period of follicular growth from the

primordial to the preovulatory stage 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 and Mauleon 1981). It takes 24 to 35 more 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).

1.7.2. The early stage of follicular development

Early follicular development, in sheep, refers to the growth of follicles from the primordial to the preantral stage (Cahill and Mauleon 1980). The control of early follicle development is not fully understood. Primordial follicles, in mice, do not express functional LH and FSH receptors (O'Shaughnessy *et al.* 1997). Although there is evidence to support the presence of FSH receptors on granulosa cells as early as the primary follicular stage in sheep (Tisdall *et al.* 1995); however, these receptors may not be coupled to adenylyl cyclase (Fortune *et al.* 1999). The potential effects of LH on the growth of preantral follicles is poorly understood (Fortune 2003). However, pregranulosa cells and primordial follicles respond to activators of the cyclic AMP (cAMP) pathways with expression of aromatase and FSH receptors (McGee and Hsueh 2000). These observations suggest that early antral follicular growth may be independent of gonadotropic hormones (McNatty et al., 1981) and paracrine activators of cAMP, like neurotransmitters, may play a role in early follicular growth (Mayerhofer *et al.* 1997). A role for the oocyte in the initiation of follicular growth has also been suggested (McGee and Hsueh 2000; Eppig 2001). Towards the end of this early stage of

folliculogenesis, follicles become responsive to gonadotropins, which is a prerequisite to subsequent antral follicular growth and maturation (Campbell *et al.* 1995).

1.7.3. Antral follicular waves in sheep and cattle

Sheep and cattle, as other domestic species, show two stages of ovarian antral follicle development (Mihm and Bleach 2003). First, a 'slow growth phase' which is believed to be independent of gonadotropins (Cahill 1981; Lussier *et al.* 1987), second, a 'fast growth phase' that requires gonadotropin support, and is usually described as a follicle wave (Sunderland *et al.* 1994). In evaluating ovarian antral follicular development, a few terms were introduced and became widely used to describe the patterns of antral follicle turnover (Goodman and Hodgen 1983; Evans 2003a). In domestic ruminants, the growing phase is defined as the time taken by the individual antral follicle to grow from emergence, as recorded by transrectal ultrasonography, to its maximum size. The regressing phase is the time taken by this follicle to regress to the minimal recordable size (2 or 3 mm in diameter using ovarian ultrasonography), and the time period between the end of the growing phase and the onset of regression is defined as the static phase (Goodman and Hodgen 1983; Schrick *et al.* 1993; Ravindra *et al.* 1994). Recruitment refers to the synchronized growth of a group of ovarian antral follicles that eventually gain the ability to fully respond to endocrine (gonadotrophic) stimuli. Selection is the process by which only a limited numbers of these cohort of 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 antral follicles from the minimum recordable size, that subsequently ovulate or undergo atresia (Ginther *et al.* 1996).

Ovarian antral follicular dynamics have been most thoroughly studied in cattle (Adams and Pierson 1995). In cows, the gonadotropin-regulated phases of follicular growth (antral follicles) occur in an orderly succession, in a wave-like pattern (Ginther *et al.* 1996). The initial proposition of two or three waves of follicular development (Rajakoski 1960) during the estrous cycle in cattle, with an inter-wave interval of 7-10 days, was confirmed by more advanced studies using ultrasonography (Savio *et al.* 1988; Sirois and Fortune 1988; Pierson and Ginther 1988; Knopf *et al.* 1989). The emergence of a follicular wave, in cattle, is characterized by a significant increase in the number of small antral follicles (6 to 9 follicles in the 4 to 6-mm size range; (Gong *et al.* 1993; Ginther *et al.* 1996)). The emergence of each follicular wave is preceded by a transient peak in plasma FSH concentrations (Adams *et al.* 1992). During the next few days, one of the follicles becomes dominant, and the others (subordinate) become atretic (Ginther *et al.* 1989b). This stage of antral follicular development has recently been termed deviation (Ginther *et al.* 1996). During the growth of the dominant follicle, there is a gradual reduction in the number of small follicles (4 to 6 mm in diameter) (Ginther *et al.* 1996). Initially, all emergent follicles of the wave have the ability to become the dominant follicle (Ginther *et al.* 1996). Approximately 3 days after wave emergence, the dominant follicle reaches a diameter of 8 mm, and it continues to grow at a higher rate compared to the subordinate antral follicles (Ginther *et al.* 1996). The duration of the deviation process in cattle is very short (8 h; (Ginther *et al.* 1999). The ovulatory

follicles (8 to 20 mm in diameter) in cows invariably originate from the last follicle wave of the interovulatory interval (Ginther *et al.* 1996).

There has been a large increase in the understanding of follicular waves in both cyclic and anestrous ewes with use of transrectal ultrasonography (Schrick *et al.* 1993; Ravindra *et al.* 1994; Ginther *et al.* 1995; Souza *et al.* 1998; Leyva *et al.* 1998b; Vinoles *et al.* 1999; Gibbons *et al.* 1999; Bartlewski *et al.* 1999a; Evans *et al.* 2000). Follicular waves have also been documented, based on ultrasonographic observations, in pregnant ewes up to d 26 of pregnancy (Bartlewski *et al.* 2000b). It is noteworthy that no follicle ≥ 3 mm was observed on the CL bearing ovaries in sheep (Bartlewski *et al.* 2000b). This local inhibition of the CL on follicular dynamics is sustained, in the ovary that bore the CL during pregnancy, for up to 4 weeks after parturition (Bartlewski *et al.* 2000b). Many of the studies above did not closely examine changes in the number of follicles among days of the cycle, as per the original definition of waves by Rajakoski (1960). In one study in cyclic sheep, it was shown that there are fluctuations in the number of follicles in different size classes consistent with the definition of follicle waves (Evans *et al.* 2000). However, in many other studies the demonstration of follicular waves during the ovine estrous cycle had to be based on those small follicles (3 mm in diameter) that grew and reached an ovulatory diameter of ≥ 5mm as there were no periodic increases and decreases in the numbers of small follicles with respect to wave emergence (Ginther *et al.* 1995; Souza *et al.* 1997a; Gibbons *et al.* 1999; Bartlewski *et al.* 1999a; Evans 2003b).

The wave-like pattern of antral follicle growth in sheep (Fig. 1.2) differs from the orderly emergence of follicular waves in cattle (Ginther *et al.* 1996) in that emergence

of sequential waves in ewes occurs more frequently and the 1-4 follicles that constitute a wave, all grow to reach an ovulatory diameter (Ginther *et al.* 1995; Bartlewski *et al.* 1999a). In both cyclic and anestrous ewes, 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; Souza *et al.* 1997a; Bartlewski *et al.* 1999a; Evans *et al.* 2000; Vinoles *et al.* 2001). In different breeds of sheep, there are 2 to 4 waves per cycle (Noel *et al.* 1993; Ravindra *et al.* 1994; Ginther *et al.* 1995; Bartlewski *et al.* 1999a; Evans *et al.* 2000). Follicular waves emerge every 4-5 days and emergence of each wave is preceded by a transient increase in serum concentration of FSH in both cyclic and anestrous ewes (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).

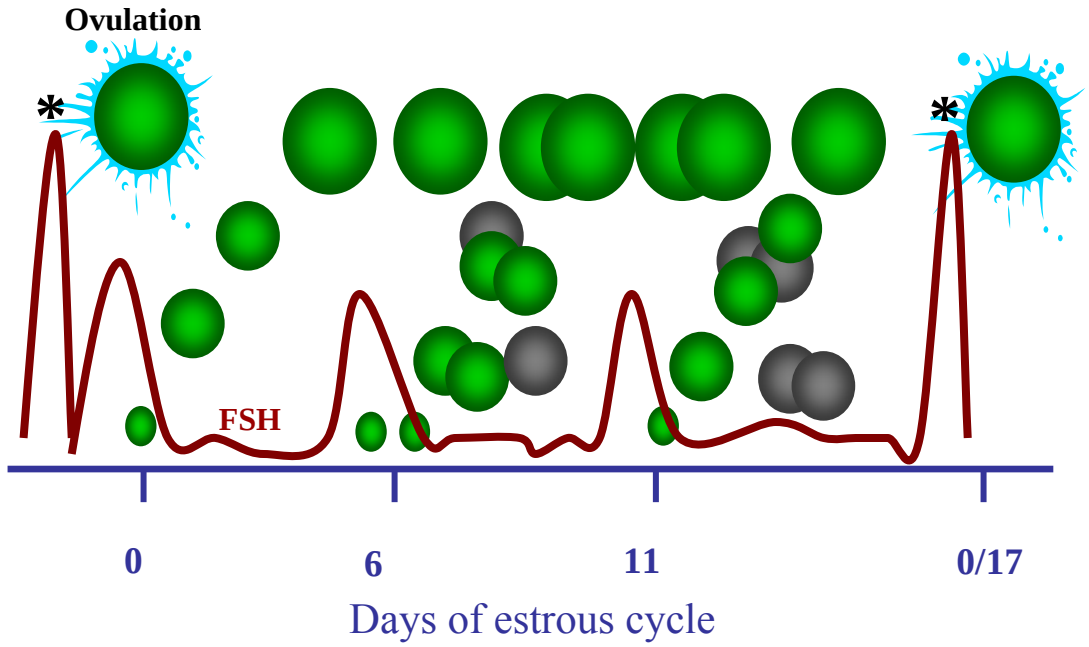

Figure 1.2. Schematic representation of ovarian antral follicular waves in sheep during an estrous cycle. Three follicular waves (defined as 1 or more 3 mm follicles emerging and growing together to reach an ovulatory diameter of ≥ 5 mm, before regression (dark colored spheres) or ovulation) are shown 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).

It has been suggested that the largest, apparently dominant, follicle during the early luteal phase (Vinoles *et al.* 1999) and follicular phase (Ravindra *et al.* 1994) of the ovine estrous cycle may partially suppress the growth of small follicles. However, the fact that more than one follicle acquires the ability to reach an ovulatory diameter in a single wave in sheep suggests that deviation may not occur during the growth of follicles in a wave. There has been some suggestion that in the ewe, the largest follicles in the first wave of the cycle, like those in cattle (Ginther *et al.* 1989a), differ from those in subsequent waves (Bartlewski *et al.* 1999a); the largest follicles in the first wave of the ovine estrous cycle may have a longer lifespan. In many breeds of sheep, ovulatory follicles appear to be similar in maximum diameter to the largest follicles of anovulatory follicular waves of the cycle (Souza *et al.* 1998; Bartlewski *et al.* 1999a). Even though the ovulatory follicles in sheep come from the final follicular wave of the cycle, about 50% of all ovulatory sized follicles in the penultimate wave in prolific Finn sheep are maintained and added to ovulatory follicles of the final wave of the cycle (Bartlewski *et al.* 1999a). The existence of 2 ovulatory waves has also been shown in Rambouille x Booroola ewes (Gibbons *et al.* 1999). In a recent study, such a mechanism of ovulation of follicles in the penultimate wave along with the follicle in the final wave of the cycle was experimentally demonstrated using a treatment with PGF2α and intra-vaginal medroxyprogesterone acetate sponges (Bartlewski *et al.* 2003).

1.8. Hormonal control of folliculogenesis

1.8.1. Gonadotropic hormones

Gonadotropins are the most important promoters of ovarian antral follicular emergence and growth (Baird and McNeilly 1981; Picton *et al.* 1990). In sheep, FSH receptors on

the granulosa cells can be detected at as early as the primary follicle stage (Tisdall *et al.* 1995) and their numbers increase when follicles continue to grow to 2 mm in diameter (Carson *et al.* 1979). LH receptors can be detected in the theca cells of large preantral follicles in sheep (Logan *et al.* 2002). However, during the later stages of follicle growth, when follicles in ovaries of sheep are about 4 mm in diameter, LH receptors appear also in the granulosa cells (Logan *et al.* 2002). During the terminal growth of ovulatory follicles, serum concentrations of FSH were found to be minimal while LH concentrations increased prior to the preovulatory LH surge (Baird and McNeilly 1981). With these observations, it was concluded that early antral follicles are predominantly dependent on FSH and the terminal phases of folliculogenesis are under the control of LH (Campbell *et al.* 1995). FSH alone, but not LH alone, can stimulate the growth of follicles to a preovulatory size in long-term GnRH agonist treated ewes (Picton *et al.* 1990). Withdrawal of FSH in the presence of LH results in the maintenance of preovulatory follicles in 50 to 55 % of GnRH-antagonist treated ewes (Campbell *et al.* 1999). In a recent study (Bartlewski *et al.* 2000a), using frequent blood sampling, the temporal relationship between gonadotropin secretory patterns and the growth of follicular waves during the early luteal phase were investigated. On the day of follicular wave emergence mean serum FSH concentrations were high, and the end of the growth phase of the largest follicle of the first wave of the cycle was associated with an increase in the pulse amplitude of LH secretion. However, whether this increase in LH pulse amplitude was associated with the static phase of the follicle or whether it was associated with decreasing LH pulse frequency in the face of increasing progesterone

concentrations (Hauger *et al.* 1977; Goodman and Karsch 1980; Wheaton *et al.* 1988) was not clear.

In sheep, as in cattle (Adams *et al.* 1992), there is solid agreement that a transient peak in serum FSH concentrations precedes emergence of each follicular wave in both cyclic (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) and anestrous ewes (Bartlewski *et al.* 1998; Evans *et al.* 2001).

1.8.2. Paracrine regulators of follicular growth

Estrogens, acting endocrinologically, may enhance the response of ovarian follicles to gonadotropins in hypophysectomized rats (Richards 1994). This synergistic effect of estradiol is mostly due to the induction by FSH and estradiol of LH receptors in granulosa cells of mature ovarian follicles (Richards *et al.* 2002). Estradiol administration in sheep (Meikle *et al.* 2001) and cattle (Bo *et al.* 1993) has been shown to cause atresia of large antral follicles and induces new follicular wave emergence. From studies in rodents, it has been surmised that estradiol is required for early folliculogenesis (Findlay *et al.* 2000; Richards 2001; Britt and Findlay 2003). It has been surmised in ewes, that the CL acts locally to increase the numbers of all follicles visible on the ovarian surface (Dailey *et al.* 1982). However, in another study, the presence of the CL was shown to locally suppress the numbers of antral follicles not growing beyond 3 mm in diameter in Western White Face ewes (Bartlewski *et al.*

2001b). In that study, it was also shown that there was no inhibitory effect of the CL on the numbers of follicles growing beyond 3 mm in diameter.

Ovarian inhibins and activin (Cahill *et al.* 1985; Findlay 1993), Bone marphogenic proteins (BMPs; Souza *et al.* 2002), transforming growth factor α (TGFα; Teerds and Dorrington 1992), epidermal growth factor (EGF; Skinner *et al.* 1987), and insulin-like growth factors and their binding proteins (Monget and Monniaux 1995; Monget *et al.* 2002), have both autocrine and paracrine effects that can modulate follicular growth (Knight and Glister 2001).

Ovine follicular fluid contains large amounts of inhibins (Tsonis *et al.* 1986) which are secreted by the granulosa cells (Knight and Glister 2001). It was suggested that inhibin mediated the suppressive effects of follicular fluid on FSH production and follicle growth (Mather *et al.* 1992). However, treatment with inhibin-free ovine follicular fluid resulted in equally strong suppression of follicular development and FSH secretion in treated ewes (Campbell *et al.* 1991a), suggesting that other compound(s) present in follicular fluid have similar inhibitory effects. Inhibin enhances gonadotropin-induced steroid secretion by ovine granulosa cells *in vitro*, and in turn stimulates inhibin production by the granulosa cells (Campbell *et al.* 1995). Activin induced proliferation has been demonstrated in rat granulosa cells from both small and large follicles cultured *in vitro* (Miro and Hillier 1996). In the mouse model, targeted deletion of the inhibin α subunit, which results in overproduction of activin, causes granulosa cells to undergo uncontrolled proliferation, leading to ovarian tumor development (Matzuk *et al.* 1992). In contrast, in activin knockout mice, follicle development is arrested at an early antral stage providing direct evidence for a key role for activin in granulosa cell proliferation

(Matzuk *et al.* 1996). Follistatin inhibits the effects of activin on human granulosa luetin cells, but it has no effects in the absence of activin (Cataldo *et al.* 1994).

TGFα (Teerds and Dorrington 1992) and EGF (Skinner *et al.* 1987) are produced by the theca cells and bind to their respective receptors on the granulosa cells in ewes. TGFα was shown to acutely suppress estradiol, inhibin and androstendione production, which caused atresia of large antral follicles (Campbell *et al.* 1994). EGF inhibits estradiol-17β production and consequently, the preovulatory LH surge and behavioral estrus in cyclic ewes (Radford *et al.* 1987). In a study involving direct infusion of EGF into the ovarian artery during the follicular phase of the estrous cycle in sheep, EGF was shown to influence inhibin and estrogen production in granulosa cells (Murray *et al.* 1993).

BMPs are members of TGF-β superfamily of peptides and are produced in the theca layer of follicles (Shimasaki *et al.* 2003) and oocytes (Eppig 2001). The BMPs originating from the theca cells have been shown to influence granulosa cell differentiation in sheep (Souza *et al.* 2002). BMPs from the oocyte have also been shown to influence proliferation and differentiation of granulosa cells in mice (Eppig 2001).

IGF-I is present in high levels in follicular fluid during the growth phase of large (dominant) follicles in sheep (Monget *et al.* 1993). IGF-I is secreted by the granulosa cells (Monget *et al.* 2002) and, in contrast to TGFα and EGF, has been shown to stimulate ovarian follicular development by acting synergistically with FSH in the ovarian dominant follicles in cows (Fortune *et al.* 2001). IGF-I also stimulates granulosa cell proliferation in small antral follicles in sheep (Monget and Monniaux

1995) and stimulates progesterone secretion by the granulosa cells of large (> 5 mm), but not small, ovine antral follicles, i*n vitro* (Monniaux and Pisselet 1992). Direct ovarian infusion of an IGF-I analogue significantly enhanced steroid production in ewes (Cohick *et al.* 1996).

1.9. Follicular steroidogenesis in sheep

The synthesis of steroid hormones by the developing follicle is dependent upon the presence and activities of several key proteins, such as steroidogenic factor 1 (SF-1), steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc), cytochrome P450 17α-hydroxylase (17α-OH), 3β-hydroxysteroid dehydrogenase (3β-HSD), and cytochrome P450 aromatase (P450arom; Bao and Garverick 1998). In sheep, granulosa cells in preantral and antral follicles express FSH receptor (FSH-R) mRNA and those in large antral follicles express P450scc, P450arom, and LH receptor (LH-R) (Juengel *et al.* 2000). Moreover, theca cells of antral follicles express LH-R and the steroidogenic enzyme mRNAs for androgen synthesis (Huet *et al.* 1997; Conley *et al.* 1995). The onset of expression of steroidogenic enzymes in follicles of different size categories dissected out from ovaries collected from the abattoir, was studied in sheep (Logan *et al.* 2002), using a classification system defined by specific morphologic criteria (Lundy *et al.* 1999). According to this study: ovine oocytes express StAR mRNA at all stages of follicular development; the onset of expression cells of StAR, P450scc, 17α-OH, 3β-HSD, and LH-R in theca occurs in large type 4 (large preantral) follicles just before antrum formation; granulosa cells in follicle types 1–3 (primordial to small preantral stage) express 3ß-HSD and SF-1; the granulosa cells of large type 5 (antral) follicles express SF-1, StAR, P450scc, LH-R, and P450arom genes.

However, in some studies in sheep (Conley *et al.* 1995), 3β-HSD expression was restricted to the theca layer of antral follicles and the granulosa cells did not express the enzyme. The reason for this disparity remains unknown, even though it has clearly been demonstrated that follicular fluid of ovine follicles contain progesterone (Evans *et al.* 2000). One important limitation of the removal of follicles from ovaries collected from the slaughterhouse is that the growth or follicular wave status of the follicle is largely unknown, except for histological features. However, classification of bovine follicles based on histological criteria does not correlate well with ultrasound and endocrine measures (Price *et al.* 1995). Another important limitation of post mortem follicle collection is that it is not possible to correlate enzyme expression patterns to the circulating endocrine milieu.

In cattle, the emergence of the first follicular wave of the cycle is associated with the onset of expression of the P450arom enzyme under the influence of a peak in serum FSH concentrations (Bao *et al.* 1997a). The growth of the dominant follicle of both anovulatory (Bao *et al.* 1997b) and ovulatory (Voss and Fortune 1993a; Voss and Fortune 1993b) follicular waves in cattle is associated with a progressive increase in the expression of steroidogenic enzymes such as 3β-HSD and P450arom in the granulosa cells. The expression of all these enzymes is downregulated in the granulosa and theca cells of the follicles collected after the LH surge (Voss and Fortune 1993a; Voss and Fortune 1993b), even in the face of a very high concentrations of progesterone in preovulatory follicular fluid. The mechanism responsible for such a high concentration of progesterone is not known. The expression pattern of steroidogenic enzymes and receptors for endocrine and paracrine regulators of follicular growth, have not been

studied in sheep using follicles collected at specific stages of growth in a wave, as determined by transrectal ultrasonography.

1.10. General objectives

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

The ability to detect ovulation and forming CL, using transrectal ultrasonography, may help in studying luteogenesis and understand the pathophysiology of luteal dysfunction in sheep. In this context, the objective of the first study was to use newer high-resolution ultrasonographic equipment to determine the earliest time, after ovulation, at which the developing ovine CL could be detected by transrectal ultrasonography. A second objective was to see if there were correlations between echotextural characteristics and the histological and functional characteristics of the developing ovine CL. Of particular interest was to see if ovulation could be confirmed earlier than 3-5 days after ovulation as was reported in previous studies (Bartlewski *et al.* 1999b).

Even though follicular waves have been demonstrated, a clear demonstration of an increase in the numbers of small follicles followed by a decline in their numbers in association with the growth of the largest follicle of a wave, as seen in cattle (Ginther *et al.* 1996), is lacking for sheep. The objective of the second study was to see if improving the lower limit for measuring small follicles in the ewe, from 2 to 3 mm down to 1 mm, would reveal changes in numbers of small antral follicles at follicle wave emergence, similar to the increase in numbers of such follicles seen in cattle (Ginther *et al.* 1996).

The individual roles of FSH and LH in the growth of antral follicles in the ewe are not completely understood. In a previous study from our laboratory (Bartlewski *et al.* 2000a), using blood samples collected every 12 min for 6 h on each day from ovulation to d 7 after ovulation, it was observed that serum FSH concentrations were closely associated with antral follicular growth and LH concentrations with CL growth. The potential involvement of LH in the maintenance of ovulatory sized follicles during the static phase of a wave could not be tested directly as the period of intensive blood sampling coincided with the increasing serum progesterone concentrations of the early luteal phase. Thus, the objective of the third study was to investigate involvement of FSH and LH in antral follicular emergence and growth. For this purpose, we utilized intensive blood sampling done between d 7 and 14 after ovulation. The period of blood sampling, from mid to late luteal phase, encompassed the emergence and growth of the penultimate and final follicular waves of the ovine estrous cycle.

Evidence in sheep for the existence of the powerful follicular dominance seen in cattle is equivocal (Scaramuzzi *et al.* 1993; Driancourt 2001; Evans 2003b). In cattle, treatments with physiological or even supraphysiological concentrations of FSH (or eCG), given in the presence of a growing dominant follicle, failed to elicit the emergence of a follicular wave (Pierson and Ginther 1988; Guilbault *et al.* 1991; Adams *et al.* 1993). The objective of the fourth study, in ewes, was to see if the presence of a large growing antral follicle(s) would inhibit the emergence of a new follicular wave in response to a physiological dose of ovine FSH.

The relationship between the characteristics of FSH peaks and those of follicular waves is not well understood for sheep (Driancourt 2001). In addition, it is not known if an

increase in the frequency of FSH peaks using repeated injections of exogenous oFSH would disrupt the rhythmic occurrence of endogenous FSH peaks and the regular emergence of follicular waves in sheep. As injection of ovine FSH induced a follicular wave in both cyclic and anestrous ewes and the induced follicular wave did not disrupt the normal rhythm of FSH peaks and follicular waves (chapter 6), a question arose as to the regulation of the peaks in FSH secretion that precede follicular waves. Could there be an ovarian independent endogenous rhythm in FSH secretion in the ewe?

We designed a series of 3 experiments to examine the relationship between FSH peaks and follicular wave dynamics in Western White Face ewes. Experiment 1 was intended to monitor the effect of a doubling of the FSH-peak amplitude on the growth of antral follicles in the subsequent follicular wave. Experiment 2 was intended to monitor the effect of increasing the frequency of peaks in daily FSH concentrations (peaks at an interval of 2 to 2.5 days as compared to the normal interval of 4 to 5 d) on the rhythmic occurrence of endogenous FSH peaks and the emergence of follicular waves. Experiment 3 was intended to see if periodic peaks in serum FSH concentrations were present in ovariectomized ewes similar to those in intact ewes.

The expression pattern of steroidogenic enzymes has been studied in ovine ovaries collected from the slaughterhouse (Conley *et al.* 1995; Huet *et al.* 1997; McNatty *et al.* 1999; Logan *et al.* 2002; Juengel *et al.* 2002). Although this allows a comparison of the expression of steroidogenic enzymes in follicles of different size categories, it does not allow the correlation of amounts of steroidogenic enzymes with stages of antral follicular growth in a wave during a specific stage of the estrous cycle or the changing endocrine milieu in the circulation. A systematic study of the expression pattern of

steroidogenic enzymes in theca and granulosa compartments, at specific stages of antral follicular growth, in a specific wave of the cycle as defined by ultrasonography and their correlation with circulating endocrine variables, has not been done for sheep. The objectives of the sixth study were: 1) to use transrectal ovarian ultrasonography to facilitate the collection of ovarian antral follicles from cyclic Western White Face ewes when the largest follicle(s) in a follicular wave reached a specific diameter/stage of growth; 2) to detect expression of the steroidogenic enzymes (3β-HSD, 17α-OH and aromatase) in theca and granulosa compartments of antral follicles at specific stages of their growth using standardized immunohistochemistry (IHC) protocols; 3) to quantify the expression pattern of the steroidogenic enzymes by gray-scale densitometric analysis; and 4) to correlate the expression patterns of steroidogenic enzymes in antral follicles with endocrine variables in the circulation.

Chapter 2: HYPOTHESES

1. The developing ovine CL could be detected using high-resolution ultrasound equipment, as early as 12-24 h after ovulation. The echotextural characteristics of the developing ovine CL are closely associated with its morphological (i.e., luteal tissue content) and functional (i.e., progesterone production) development.

2. The numbers of small antral follicles does not vary significantly in relation to the day of follicular wave emergence.

3. FSH secretory patterns are associated with the growth and demise of the follicles in a wave. LH secretory patterns are associated with luteolysis but not with the growth and demise of the follicles in a wave.

4. The ability of a growing (dominant) follicle to suppress the growth and emergence of other follicles is by a direct effect (i.e., it can not be overcome with exogenous oFSH administered during the growth phase of the largest follicle of a wave).

5. Doubling the amplitude of the FSH-peak does not alter the growth pattern of the largest follicle of the treated wave in sheep. Increasing the frequency of FSH peaks with administration of exogenous oFSH, does not disrupt the rhythmic occurrence of endogenous FSH peaks and emergence of follicular waves in the ewe. Ovariectomy does not affect the periodic increases (peaks) in FSH secretion in sheep.

6. The expression of steroidogenic enzymes increases with the growth of the largest follicle in the first wave of the follicle. The expression of steroidogenic enzymes is downregulated in preovulatory follicles after the preovulatory LH surge, except for that of 3β-HSD. The expression profiles of steroidogenic enzymes and follicular growth reflect the endocrine milieu in the circulation.

Chapter 3: DETECTION OF OVULATION BY TRANSRECTAL ULTRASONOGRAPHY AND ECHOTEXTURAL CHARACTERIZATION OF THE FORMING CORPUS LUTEUM IN CYCLIC SHEEP[*]

Duggavathi R, Bartlewski PM, Pierson RA and Rawlings NC.

3.1. Abstract

To date it has not been possible to detect CL by ultrasonography, immediately following ovulation, in the ewe. Early CL detection is essential to be able to relate luteal outcome to the developmental pattern of the ovulated follicle and to confirm ovulation. Image analysis of the CL may be useful in providing a non-invasive picture of CL differentiation and function. This study was designed to use high resolution ultrasonography to monitor, and to correlate the echotextural, histological and functional attributes of the developing ovine CL from d 1 to 3 after ovulation. Ten ewes underwent twice daily transrectal ultrasonography and blood sampling from the day of synchronized estrus. Ewes were ovariectomized at 12-24, 36-48 and 60-72 h after ovulation. Ovaries collected were scanned in a water bath before processing them for histology. Ultrasonographic images of CL were analyzed for echotexture. Histological sections were analyzed for the percentage area of the CL occupied by blood clot or luteal tissue. Serum samples were analyzed for progesterone concentration. Numerical pixel value, heterogeneity and the percentage of the CL occupied by blood clot declined ($P < 0.05$) from 12-24 to 60-72 h after ovulation. Luteal area and serum progesterone concentration increased ($P < 0.05$) from 12-24 to 60-72 h. The results indicated that it

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was possible to visualize developing CL as early as 12-24 h after ovulation in the ewe. Echotexture of the CL was closely associated with its morphological and functional characteristics; image analysis holds promise for the non invasive monitoring of CL differentiation and growth.

3.2. Introduction

The use of *in vivo*, real-time ovarian ultrasonography is one of the most significant advances in the area of reproductive biology and medicine (Pierson and Adams 1995; Kot and Ginther 1999; Checura *et al.* 2002). This technique has been adapted as a research and diagnostic tool in several domestic species (Kot and Ginther 1999; Bartlewski *et al.* 1999a), and for both research (Hess and Pierson 2000) and clinical management (Santolaya-Forgas 1992; Bourne *et al.* 1996) in women. Ultrasonography has allowed for non-invasive, serial visualization of ovarian antral follicles and corpora lutea (CL). This, in turn, paved the way for systematic studies of the dynamic growth and regression of ovarian structures (Pierson and Adams 1995; Adams and Pierson 1995). The technique has also permitted determination of follicular and luteal viability, and has proven useful for studying physiological processes associated with ovarian activity.

In sheep, as in other species, ovulation has been defined as the disappearance of a large ovulatory-sized follicle (e.g., 5-7 mm in diameter in sheep) that had been detected and followed by ultrasonography from before estrus (Ravindra *et al.* 1994). Ovulations have normally been confirmed by ultrasonographic detection of CL 3 to 5 d later (Bartlewski *et al.* 1999b), and the mean day of detection of CL in sheep averaged d 3 after ovulation (Bartlewski *et al.* 1999a; Bartlewski *et al.* 1999b). However, ovulations followed by

short life-span CL have been documented in otherwise normal ovine estrous cycles (Bartlewski *et al.* 1999b). In addition, ovulations without CL formation or resulting in short life-span CL have been documented more frequently in different estrous synchronization/ovulation induction regimens in both cyclic and seasonally anestrous ewes (Bartlewski *et al.* 1999b; Bartlewski *et al.* 2001a; Barrett *et al.* 2002). Therefore, detecting CL immediately after ovulation is important to confirm ovulation and determine ovulation rate, as well as to allow correlation between the developmental pattern of the ovulated follicle and luteal outcome.

There is considerable histological information on the development of the CL and its cellular composition in domestic species including the sheep (Farin *et al.* 1986; Braden *et al.* 1989; Farin *et al.* 1989; Niswender *et al.* 2000). However, correlations among ultrasonographic, morphological and functional attributes of developing CL in domestic animals are particularly scarce; a study documented such correlations for CL at 3 discrete stages of the estrous cycle in heifers, namely d 1-3, 5-9 and 15-19 after ovulation (Singh *et al.* 1997).

Ultrasonographic images are composed of picture elements (pixels) resulting from the refraction or transmission of high frequency sound waves (Pierson and Adams 1995; Singh *et al.* 1997; Kot and Ginther 1999). Each pixel represents the ability of a small, discrete unit of tissue to refract or transmit ultrasound waves, resulting in an image displayed in various shades of gray (Singh *et al.* 1997). Collective numerical pixel values (brightness) and heterogeneity are functions of differences in tissue densities and macromolecular composition (Pierson and Adams 1995; Singh *et al.* 1997; Tom *et al.* 1998a; Kot and Ginther 1999). It has been demonstrated that pixel analysis

(echotextural characteristics) of images of follicles and CL in cattle and women reflect discrete changes in their morphology and secretory function (Pierson and Adams 1995). Such studies do not exist for sheep, which unlike women and cattle, ovulate one or more follicles at the end of each estrous cycle.

Thus, the objective of the present study was to use newer high-resolution ultrasonographic equipment to determine the earliest time at which the developing ovine CL could be detected by transrectal ultrasonography post-ovulation. A second objective was to see if there were correlations between the echotextural characteristics, histological and functional characteristics of the developing ovine CL. The latter would facilitate rapid, non-invasive studies of terminal follicle development and luteogenesis, in relation to hormone secretion, in this animal model. We hypothesized that: 1) the developing ovine CL could be detected using high-resolution ultrasound equipment, as early as 12-24 h after ovulation; 2) the echotextural characteristics of the developing ovine CL are closely associated with its morphological (i.e., luteal tissue content) and functional (i.e., progesterone production) development.

3.3. Materials and methods

The experimental procedures were approved by the University of Saskatchewan Committee on Animal Care Assurance. Ten adult, clinically healthy Western White Face ewes (4 to 6 yr of age, average body weight: 88 ± 7 kg) were used in this experiment conducted during the middle portion of the breeding season (November). All ewes received daily maintenance rations of alfalfa pellets; water, hay and cobalt iodized salt bars were available *ad libitum*. Estrus was synchronized by a 12-day treatment with intravaginal sponges containing medroxyprogesterone acetate (MAP;

Veramix®, Upjohn; 60 mg; Orangeville, ON, Canada). After sponge withdrawal, all ewes were checked for estrus twice daily using a vasectomized, crayon-harnessed ram.

3.3.1. Transrectal ultrasonography and blood sampling

All ewes underwent transrectal ultrasonography twice a day, from the time they were marked by a ram (estrus) until ovariectomy. Transrectal ultrasonography was done with high-resolution, real-time B-mode ultrasonographic equipment (Aloka SSD-900; Aloka Co. Ltd., Japan) connected to a 7.5-MHz transducer (UST-5821; Aloka Co. Ltd., Japan). During each scanning session, the settings of the scanner that affect image attributes, such as overall time-gain, near-field and far-field gains, compensation and beam focus, were kept at pre-determined levels. Images were displayed at 2-times magnification. The number, diameter and relative position of all follicles ≥ 1 mm in diameter and CL were sketched on ovarian charts, and all ovarian images were recorded on high-grade video tapes (Fuji S-VHS, ST-120 N), using a super VHS-VCR (Panasonic AG-1978, Matsushita Electric, Mississauga, ON, Canada) equipped with digital frame memory. Ovulation was regarded as the disappearance of a large ovulatory-sized follicle (e.g., 5-7 mm in diameter) (Schrick *et al.* 1993; Ravindra *et al.* 1994), which had been detected and followed by ultrasonography from just before estrus. Blood samples (10 ml) were collected by jugular venipuncture using vacutainers (Becton Dickinson, Rutherford, NJ, USA) prior to each ultrasonographic examination.

3.3.2. Collection of ovaries, water-bath ultrasonography and histology

Ovaries were removed surgically at 12-24 h (4 ewes), 36-48 h (3 ewes) and 60-72 h (3 ewes) after ovulation detected by transrectal ultrasonography. All ovariectomies were performed within 3 h after the last ultrasonographic examination. General anesthesia

was induced by intravenous injection of 2.5 % thiopental sodium (Pentothal®, Abbott Laboratories Ltd., Quebec, Canada; 25 mg/kg) and maintained by 3-5 % halothane (Halothane®, Halocarbon Laboratories, NJ, USA). Ovaries were exteriorized by mid-ventral laparotomy. Ovaries containing luteal structures were excised, placed immediately in phosphate buffered saline (PBS; 37° C; 0.1 mol phosphate buffer/l, 0.9% (w/v) sodium chloride, pH 7.2-7.4) and transported to the laboratory within 30 min of dissection. The ovaries were placed in a degassed water bath and ultrasound images were collected using an Advanced Technology Laboratories (ALT) ultramark HDI 5000 ultrasound machine (Advanced Technologies Laboratory, Bothell, WA, USA), equipped with a broad-band (5-9 MHz), convex-array transducer designed for transvaginal scanning in humans.

Following water-bath ultrasonography, corpora lutea (12-24 h: n = 11 (4 ewes); 36-48 h: n = 7 (3 ewes); and 60-72 h: n =6 (3 ewes)) were dissected and fixed in Haly's solution for 24 h. Subsequently, fixed tissues were washed in running water for 6 to 8 h. CL were then cut, approximately through the midline (maximum diameter), and placed in PBS until preparation of paraffin blocks. A set of 2-3 sections of 5 μ thickness was obtained for each CL, and stained with hemotoxylin and eosin for routine histology.

3.3.3. Computer assisted analysis of ultrasound images

Ultrasound images of developing CL taken in the water bath, at half distance from one edge of the CL (maximum diameter), with the HDI 5000 ultrasonographic equipment, were stored directly as digitized graphic images. For transrectal images (Aloka SSD-900 equipment) recorded on videotapes, images were selected at half distance from one edge of the CL. The selected images were digitized at standardized settings, with a

resolution of 640 by 480 pixels and 256 shades of gray, and stored as graphic images. The digitization was done using a digital image acquisition system (Picvision+, Imaging Technology Inc., Woburn, MA, USA). Echotextural analysis of images was done using a Sparc Station 10SX (Sun Microsystems, Mt. View, CA, USA) and a custom-developed algorithm optimized for ultrasound images (Synergyne 1©, Saskatoon, Canada). Quantitative echotexture analysis was performed based upon sequential measurements of numeric pixel values (gray scale values of individual picture elements ranging from 0, absolute black to 255, absolute white). Each image was divided into 4 quadrants and mean numeric pixel values were measured within a computer-generated spot-meter encompassing approximately 25% of each quadrant (Pierson and Adams 1995; Singh *et al.* 1997; Tom *et al.* 1998a). Special care was taken to prevent anechoic central cavities, which were seen in CL of 60-72 h, from falling under the spot meter. The central cavities were distinguished from follicles by the surrounding border of distinct luteal tissue (Pierson and Ginther 1987b). The mean numerical pixel value (brightness) for each image was the mean of the numerical pixel values for the 4 quadrants. Pixel heterogeneity also was calculated; it was defined as the standard deviation of the mean numerical pixel values from the 4 quadrants of each image.

Luteal structures in digitized ultrasound images were outlined and the area within the outline was calculated using the algorithm above (Synergyne 1©, Saskatoon, Canada). In addition, maximal cross-sectional areas of follicles observed at the last transrectal ultrasound scan before ovulation were calculated using the follicle diameters determined with built-in calipers (Aloka SSD-900).

3.3.4. Computer assisted analysis of histological images

One stained image per each CL was digitized at 1.25-times magnification using imaging software designed for use in light microscopy (Northern Eclipse; Empix Imaging Inc., ON, Canada). Luteal tissue and islands of blood clot (areas containing clumps of eosin stained erythrocytes) were outlined on the images at 100 % to 200 % zoom-magnification, and the area falling within each outline was calculated. Areas of central cavities within CL, containing serous transudate (eosin stained), but not blood clot, were also determined. These areas were subtracted from the total area to obtain a corrected luteal area. The total areas of blood clot for each developing CL were expressed as a percentage of the total luteal area.

3.3.5. Hormone assay

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. Circulating concentrations of progesterone were determined by a validated radioimmunoassay (Rawlings *et al.* 1984). The range of the standard curve was from 0.10 to 10 ng/ml. The assay sensitivity, or the minimum concentration of hormone significantly displacing labeled progesterone from the antibody, was 0.03 ng/ml. The intra-assay CV's were 11.3 % or 6.8 % for reference sera with mean progesterone concentrations of 0.19 or 0.78 ng/ml.

3.3.6. Statistical analyses

All statistical analyses were done using SigmaStat® Statistical Software (Version 2.0 for Windows®, 1997; Chicago, IL, USA). The effect of time after ovulation on various parameters was assessed by one-way ANOVA. The effect of time after ovulation the source of ultrasound images and their interactions were assessed by two-way repeated

measures ANOVA. Multiple comparisons were made by the method of Fisher's least significant difference (LSD). Correlation coefficients among luteal areas, numerical pixel values and serum progesterone concentrations were obtained by Pearson Product Moment Correlations. All results are expressed as means ± SEM.

3.4. Results

Twenty three of 24 ovulations (96 %) observed at laparotomy had been detected by transrectal ultrasonography, using the Aloka SSD-900 ultrasound equipment; one doubtful ovulation was subsequently re-confirmed by replaying the video tape containing the image of that ovary. It was found that the doubtful ovulation was that of a 4-mm follicle. The mean diameter and cross-sectional area, for pre-ovulatory follicles, did not differ ($P > 0.05$) among groups of CL collected at 12-24, 36-48 or 60-72 h after ovulation (Table 3.1). The area of the CL at 12-24 h after ovulation did not vary ($P > 0.05$) from that of the follicles from which they were derived (Table 3.1).

Mean luteal areas obtained using both the transrectal ultrasonography and ultrasonography in a water bath, increased ($P < 0.05$) from 12-24 to 60-72 h after ovulation (Table 3.1). There was no difference ($P > 0.05$) between luteal areas calculated in images generated using either type of ultrasonographic equipment at 36-48 and 60-72 h post-ovulation; however, the mean luteal area was smaller in transrectal images as compared to images from the water bath at 12-24 h after ovulation ($P < 0.05$; Table 3.1). There was a strong and positive correlation between luteal areas from the two sources of images ($r = 0.89$; $P < 0.0001$). Serum progesterone concentrations and luteal areas were also positively correlated ($r = 0.88$; $P < 0.0001$).

3.4.1. Descriptive morphology of developing CL

Inspection of ultrasonographic images of CL, generated by water bath scanning using the HDI 5000 equipment, revealed that less echoic luteal tissue and more echoic blood clot could be differentiated even in the images at 12-24 h after ovulation (blood clot is towards the ovarian border within the marked area, and is encapsulated by luteal tissue in Fig. 3.1A). In images at 12-24 h, generated by transrectal ultrasonography *in vivo*, such differentiation was not evident; however, all CL could be seen as hyperechoic areas. By 60-72 h after ovulation, in the images from both HDI 5000 and Aloka SSD-900 equipments, CL exhibited a uniform echotextural pattern. Central cavities, if present, were anechoic, similar to follicular antra. Histologically, folding of the follicular wall after ovulation was observed in all the images of CL of 12-24 h after ovulation. This morphological feature was minimal in CL at 36-48 h and absent in those at 60-72 h following ovulation (Fig. 3.1). A central cavity filled with serous transudate was observed in 33 % of the CL (2 of 6) collected at 60-72 h after ovulation.

3.4.2. Quantitative echotexture analysis of ultrasound images

The mean numerical pixel values of images from the water bath (Fig. 3.2) declined from 12-24 to 60-72 h (136.5 ± 4.2 vs. 91.5 ± 3.7, respectively; $P < 0.05$), but in transrectal images (Fig. 3.2), it only declined from 36-48 to 60-72 h (95.0 ± 7.1 vs. 70.1 ± 3.7, respectively; $P < 0.05$). Mean pixel heterogeneity (Fig. 3.2) did not differ between 12-24 and 36-48 h ($P > 0.05$), but it declined by 60-72 h for images generated from both scanning in the water bath and transrectal ultrasound scanning ($P < 0.05$).

3.4.3. Quantitative histomorphometric analysis

The percentage of the CL occupied by blood clot declined from 12-24 to 60-72 h after ovulation ($P < 0.05$; Fig 3). The mean number of blood clot islets increased from 12-24 to 60-72 h post-ovulation ($P < 0.05$; Fig 3).

3.4.4. Serum progesterone concentrations

Mean serum progesterone concentrations increased ($P < 0.05$) from 12-24 to 60-72 h after ovulation detection (0.05 ± 0.02 and 0.5 ± 0.06 ng/ml, respectively; Table 3.1).

Table 3.1. Follicular and luteal characteristics obtained from ultrasonographic images collected transrectally (Aloka SSD-900 ultrasonographic equipment) or in a water bath (HDI 5000 ultrasonographic equipment) 12-24, 36-48 or 60-72 h after ovulation (d 0 = day of ovulation) in sheep.

End point	Days after detected ovulation		
	12-24 h	36-48 h	60-72 h
Number of ewes	4	3	3
Number of ovulations determined by transrectal ultrasonography	10	7	6
Number of CL detected at surgery	11	7	6
Follicle diameter 12 h before ovulation (mm)	6.0 ± 0.3	5.5 ± 0.2	5.5 ± 0.2
Follicle area 12 h before ovulation (mm^2)	29.6 ± 3.4	23.4 ± 1.8	24.0 ± 1.9
Luteal area from transrectal images (mm^2)	30.5 ± 1.1^{ax}	41.3 ± 1.3^{b}	60.2 ± 3.4^{c}
Luteal area from water bath images (mm^2)	34.2 ± 2.0^{ay}	42.7 ± 2.1^{b}	61.6 ± 3.0^{c}
Serum progesterone concentrations (ng/ml)	0.05 ± 0.02^{a}	0.2 ± 0.01^{b}	0.5 ± 0.1^{c}

[a,b,c] Significant difference ($P < 0.05$) between groups within a row
[xy] Significant difference ($P < 0.05$) between points within a column

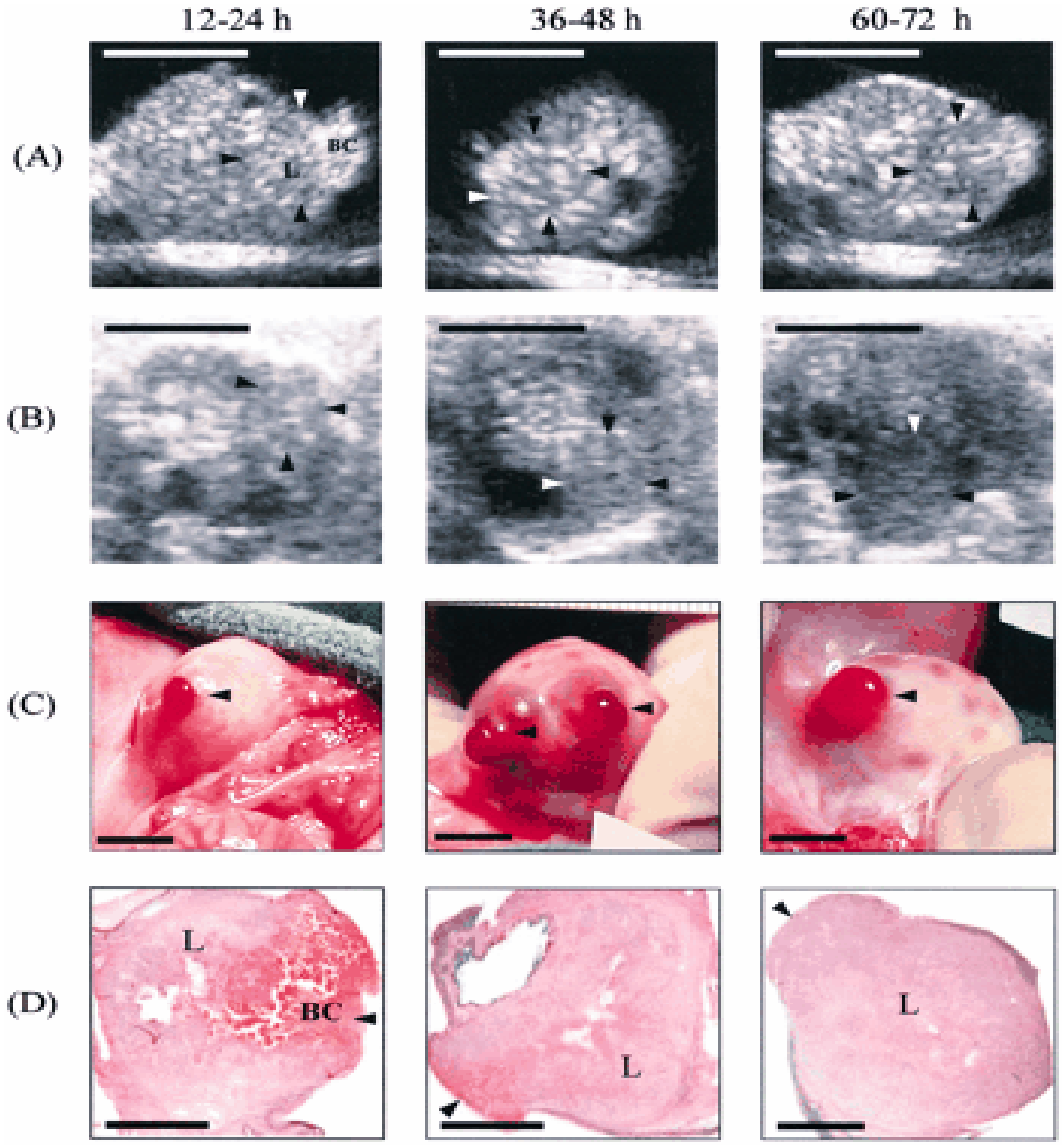

Figure 3.1. Ultrasonograms of luteal images generated in a water bath with the HDI 5000 ultrasonographic equipment (A) and by transrectal ultrasonography using the Aloka SSD-900 ultrasonographic equipment (B), photomicrogaphs of corpora hemorrhagica (C) and histological sections (1.25 X magnification; D) at 12-24, 36-48 and 60-72 h after ovulation detected by transrectal ultrasonography using the Aloka SSD-900 equipment. Arrow heads indicate the margins of CL in ultrasonograms and the ovulation site in the photomicrographs. L – luteal area; BC – blood clot. Solid bar – 10 mm in (A), (B), (C) and 2.5 mm in (D)

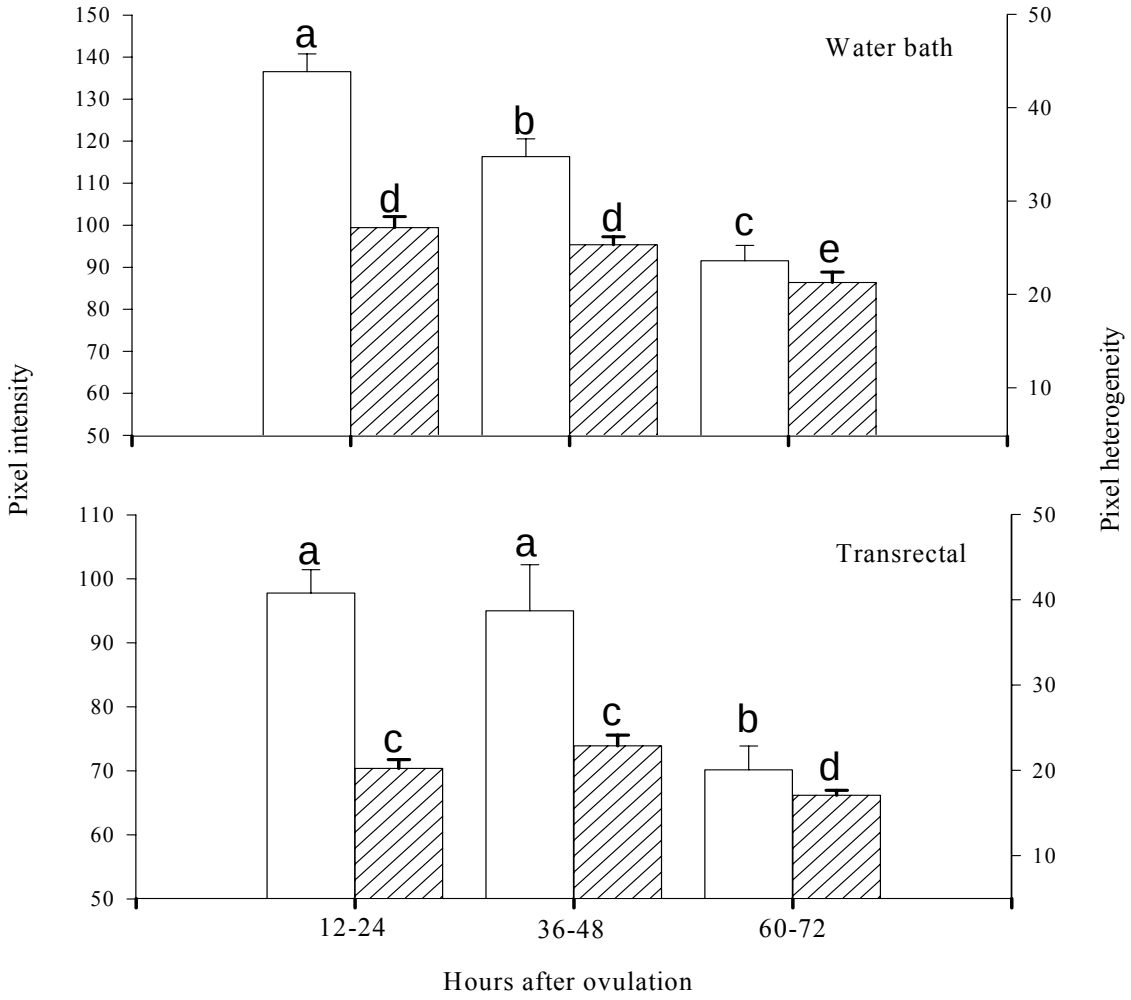

Figure 3.2. Mean numerical pixel values (hollow bars) and heterogeneity (hatched bars) for the images of CL collected at 12-24 (n = 11), 36-48 (n = 7) and 60-72 h (n = 6) after ovulation detected by transrectal ultrasonography. The images were collected by scanning ovaries in a water bath using the HDI 5000 ultrasonographic equipment (top panel) or by transrectal ultrasonography using the Aloka SSD-900 ultrasonographic equipment (lower panel). Letters show significant differences for numerical pixel value or pixel heterogeneity between hours after ovulation, within each panel ($P < 0.05$).

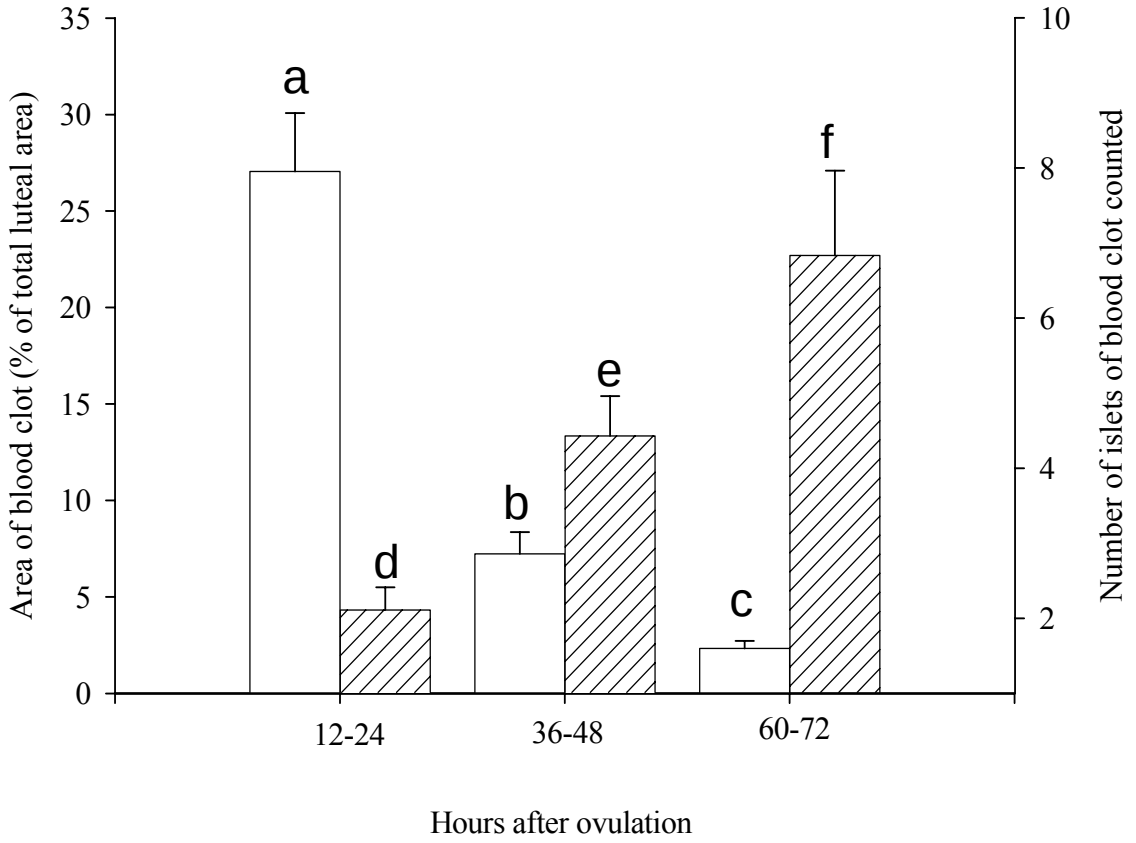

Figure 3.3. Mean area of blood clot expressed as a percentage of the total luteal area (hollow bars) and numbers of discrete islets of blood clot counted per image (hatched bars) for the hematoxylene and eosine stained sections of CL collected at 12-24 (n = 11), 36-48 (n = 7) and 60-72 h (n = 6) after the ovulations detected by transrectal ultrasonography. Letters show significant differences for blood clot area (a, b, c) or numbers of islands of clot (d, e, f) between hours after ovulation ($P < 0.05$).

3.5. Discussion

In the present experiment, a total number of 24 ovulations were detected at laparotomy in 10 ewes. Among those ovulations, 23 ovulations (96%) were identified by transrectal ultrasonography prior to surgery. The one ovulation not detected by transrectal ultrasonography was that of a 4-mm follicle. Observations on ovulation data from all experiments in our laboratory (2002), using transrectal ovarian ultrasonography in cyclic ewes, revealed that all ovulations were from ≥ 5-mm follicles except for only 1.7 % of all (2 of 121) detected ovulations, which were from 4-mm follicles (Duggavathi, Barrett, Bartlewski and Rawlings; unpublished data). We, therefore, concluded that the use of high-resolution ultrasonographic equipment allowed us to accurately detect follicle rupture and to identify the differentiating CL as early as 12-24 h after ovulation in sheep. This added ability to visualize the developing CL was further confirmed by scanning of dissected ovaries in a water bath (Fig. 3.1 A) and by histology (Fig. 3.1 D). Using lower resolution ultrasonographic equipment (Aloka 500-SSD), the CL could only be detected between days 3 to 5 after ovulation in the ewe (Bartlewski *et al.* 1999a; Bartlewski *et al.* 1999b).

The characteristic folding of the follicular wall after ovulation could be observed in all of the images of CL at 12-24 h post-ovulation, which was in agreement with previous histological studies (Smith *et al.* 1994). However, this morphological feature was reduced in CL at 36-48 h and absent in those at 60-72 h after ovulation (Fig. 3.1), suggesting the morphological remodeling of luteal tissue by about 72 h post-ovulation. The morphological remodeling of the CL due to migration of fibroblasts, endothelial

cells and theca interna cells, was presumably facilitated by the folding of the follicular wall (O'Shea *et al.* 1980).

Two quantitative echotextural variables of ultrasound images were utilized in the present study to characterize the formation of CL: the numerical pixel value (brightness) and pixel heterogeneity (Pierson and Adams 1995; Singh *et al.* 1997; Tom *et al.* 1998a; Kot and Ginther 1999; Checura *et al.* 2002). The numerical pixel value and heterogeneity are dependent on differences in tissue densities and macromolecular composition (Pierson and Adams 1995; Singh *et al.* 1997; Tom *et al.* 1998a; Kot and Ginther 1999). Numerical pixel values of CL in the present study reached a minimum at 60-72 h after ovulation (Fig. 3.2), in contrast to approximately 2 d after ovulation in heifers (Kot and Ginther 1999). In mares, high numerical pixel values of CL on d 1 were attributed to an increase in the number of reflective surfaces created by the apposing folds of the collapsed follicular wall (Ginther 1995). In a study in mares, only 50 % of ovulations resulted in transient corpora hemorrhagica (Ginther 1988) and in heifers, the proportion was even lower (2 of 23; Pierson and Ginther 1987). Therefore, hyperechoic fibrin-like strands were not a common finding in luteal cavities in these species. In an ultrasonographic study of atherosclerotic carotid plaques in humans (Lal *et al.* 2002), fibrin and fibrous tissue had the second highest gray scale (pixel) intensity amongst blood, lipid, fibrous tissue and calcium. In the ewes of the present study, all the ovulations resulted in the formation of a blood clot in the former antrum of the collapsed follicle (Fig 1). Thus, it is logical to assume that in addition to the increased reflective surfaces of the collapsed follicular wall, the presence of a blood clot influenced the echotexture of the developing CL in sheep.

The greater resolution of the HDI 5000 ultrasound equipment probably accounted for subtle differences in the pattern of change in numerical pixel values of CL images, from 12-24 to 60-72 h after ovulation, as monitored by the two types of ultrasound scanners used in this study (Fig. 3.2). The borders of luteal tissue could be precisely demarcated from the ovarian stroma even at 12-24 h after ovulation, using the higher resolution ultrasound equipment and water-bath scanning. This precision in outlining the luteal tissue may have contributed to the higher mean luteal areas recorded from images generated in the water bath compared to transrectal images (Table 3.1). However, the slightly reduced precision of outlining the borders of CL in images generated by transrectal ultrasonography was not great enough to undermine our ability to determine the luteal areas, as they were still comparable to the images generated by water bath scanning and showed a similar trend with time (Table 3.1). In addition, the luteal areas in images from both sources correlated positively with serum progesterone concentrations. Finally, luteal areas doubled from 12-24 h to 60-72 h after ovulation (Table 3.1), in agreement with earlier histological studies in cyclic ewes (Reynolds *et al.* 1994; Reynolds and Redmer 1999).

Pixel heterogeneity of CL did not change significantly until 36-48 h after ovulation (Fig. 3.2). Pixel heterogeneity probably reflected existence of interspersed hyper- and hypo-echoic areas in a heterogeneous luteal tissue composition due to the presence of echoic luteal cells of various types and blood clot, and also extruded non-echoic serum in the clot (Berne and Levy 1999). The significant decline in pixel heterogeneity of CL by 60-72 h post-ovulation could be due to the fact that luteal tissue was predominant by ~72 h and islets of blood clots were fewer in number and more diffuse (Figs. 1 and 3),

resulting in more homogeneous echogenic patterns. In addition, luteal cell migration and expansion may have also reduced the number of hyperechoic folds of follicular wall (Ginther 1995) that were predominant at 12-24 h after ovulation (Fig. 3.1; Murphy *et al.* 2001).

A central luteal cavity, containing serous transudate but not blood clot, was observed in 33 % of the CL at 60-72 h during the present study. Such central cavities have been observed in several domestic species including sheep (Bartlewski *et al.* 1999b), cattle (Kastelic *et al.* 1990; Singh *et al.* 1997) and horses (Townson and Ginther 1989). All CL of d 1 had a blood clot (Fig 1) in the former follicular antrum. A few of such clots resulted in central cavities filled with anechoic serous transudate. Such fluid filled cavities probably failed to be breached through and filled in by proliferating luteal cells.

The importance of our ability to identify CL during luteogenesis lies in the fact that it provides a non-invasive technique to look at relationships between various characteristics of ovulatory follicles (growth rate, time from emergence to ovulation, and endocrine milieu at the time of emergence and during the growth phase), and formation and ensuing function of CL. Luteal inadequacy can lead to death of the embryo or the fetus (Reynolds and Redmer 1998), and it has been identified as a major cause of recurrent miscarriages in women (Li *et al.* 2000). Various luteal inadequacies in the ewe, such as short life-span CL and ovulations that do not result in the formation of functional CL have been documented in our laboratory (Bartlewski *et al.* 1999a; Bartlewski *et al.* 1999b; Bartlewski *et al.* 2001a; Barrett *et al.* 2002). In addition, it is unclear whether reduced luteal vascularity is secondary to luteal insufficiency or whether inadequate luteal vascularization is a primary cause of luteal dysfunction

(Reynolds *et al.* 2000). Differences in echotextural characteristics between the 2 ovulatory-sized follicles of the same follicle wave but with different fates (i.e., ovulatory vs. anovulatory follicle) have been shown in women (Pierson and Adams 1995); echotextural characteristics of growing ovarian antral follicles have been documented in heifers (Singh *et al.* 1998; Tom *et al.* 1998b) but not in sheep. Such studies, along with the present ultrasonographic characterization of CL development, hold promise to increase our understanding of normal luteogenesis and etiology of CL dysfunction.

In ultrasonographic imaging-based studies of the ovaries in domestic animals, ovulation has traditionally been defined as disappearance of ovulatory sized follicles (Schrick *et al.* 1993; Ravindra *et al.* 1994). However, with the present demonstration of early CL detection in the ewe, ovulation can now be defined in this species, as the disappearance of ovulatory-sized follicles followed by the detection of forming CL. In addition, recent studies have documented the incidences of several types of abnormal follicular development, failure of ovulation and inadequate luteal function in women (Hess and Pierson 2000). Apart from laboratory mammals, the sheep has evolved as the principal animal model (Baird 1983) for studying physiology of the human reproductive cycle. Thus, the present demonstration of CL visualization in sheep creates a model for studying luteal function in women.

In conclusion, the results of the present study showed that it is possible to detect growing CL in the ewe by transrectal ultrasonography as early as 12-24 h after ovulation, thus permitting future studies of the follicular growth-CL formation inter-phase in this animal model species. Echotextural attributes closely correlated with

morphological and functional changes in the growing CL. Ultrasonography and computer-assisted image analyses may greatly aid in studies of luteogenesis and the etiology of CL dysfunction in the ewe.

Chapter 4: USE OF HIGH-RESOLUTION TRANSRECTAL ULTRASONOGRAPHY TO ASSESS CHANGES IN NUMBERS OF SMALL OVARIAN FOLLICLES AND THEIR RELATIONSHIPS TO THE EMERGENCE OF FOLLICULAR WAVES IN CYCLIC EWES[*]

Duggavathi R, Bartlewski PM, Barrett DMW and Rawlings NC.

4.1. Abstract

Transrectal ovarian ultrasonographic studies have shown that, in cattle, follicular wave emergence is associated with a large increase in the number of small antral follicles (4 to 6 mm in diameter); an analogous association has not been found for small follicles (2 to 3 mm in diameter) in the ewe. In previous studies in ewes, accurate assessment of the number of follicles has been limited to follicles 2 or 3 mm in diameter. Newer, high resolution equipment allowed us to identify follicles ≥ 0.4 mm and to quantify all antral follicles ≥ 1 mm in diameter in 7 cyclic Western White Face ewes. This allowed us to expand the small follicle pool examined, from 1 to 4 follicles/d (2 to 3.5 mm in diameter) in earlier studies, to 8 to 18 follicles/d (1 to 3 mm in diameter). Total numbers of small follicles (≥ 1 mm and ≤ 3 mm in diameter) increased between Days -1 and 0 (Day 0 = day of ovulation), and declined between Days 1 and 3 ($P < 0.05$). There were no significant changes in the number of small or medium (4 mm in diameter) follicles around days of follicle wave emergence (± 2 d). The 1 to 3 follicles in the 2 to 3 mm size range, which constituted a follicle wave (i.e., grew to ≥ 5 mm in diameter before regression or ovulation), were the only small follicles to emerge in an orderly succession during the estrous cycle, approximately every 3 to 5 d. Thus, unlike in cattle,

[*] Theriogenology **60**: 495-610.

there is no apparent increase in numbers of small follicles at follicle wave emergence in cyclic sheep, and little evidence for selection of recruited follicles and follicular dominance.

4.2. Introduction

Studies using transrectal ovarian ultrasonography (Pierson and Ginther 1988; Savio *et al.* 1988; Sirois and Fortune 1988; Ginther *et al.* 1989b) have confirmed that follicular growth in cattle occurs in waves (Knopf *et al.* 1989). Emergence of follicular waves appears to be FSH dependent as it is consistently preceded by an increase in circulating concentrations of FSH (Adams *et al.* 1992; Sunderland *et al.* 1994; Bodensteiner *et al.* 1996). In cattle, there are generally 2 or 3 waves of antral follicle growth per estrous cycle (Ginther *et al.* 1989a). One of the defining features of follicular wave emergence in cattle is a significant increase in the number of small follicles within the ovary (6 to 9 follicles in the 4 to 6-mm size range; (Gong *et al.* 1993; Ginther *et al.* 1996)). Subsequent growth of the dominant follicle of a wave is associated with a decrease in the number of small follicles (Fortune 1994; Driancourt 2001; Garverick *et al.* 2002). Dominant ovulatory or anovulatory follicles reach a maximum size of 8 to 20 mm in diameter in cattle (Ginther *et al.* 1989a).

In sheep, antral follicular dynamics have been studied by laparoscopy and ultrasonography (Noel *et al.* 1993; Ravindra *et al.* 1994; Ginther *et al.* 1995; Souza *et al.* 1997a; Bartlewski *et al.* 1999a; Evans *et al.* 2000; Vinoles *et al.* 2001). Laparoscopic studies have been confined to detecting antral follicles ≥ 2 mm in size and visible on the surface of the ovary (Noel *et al.* 1993). Ultrasonographic technique has made it possible to visualize all ovarian antral follicles ≥ 2 mm in diameter; however, 3

mm has frequently been used as a more accurate cut off point (Ginther *et al.* 1995; Bartlewski *et al.* 1999a). In cyclic ewes, follicular waves also appear to occur, although a wave-like pattern of follicle emergence was not seen in every study (Schrick *et al.* 1993; Lopez-Sebastian *et al.* 1997). In different breeds of sheep, when follicular waves were detected, there were 2 to 4 waves per cycle (Noel *et al.* 1993; Ravindra *et al.* 1994; Ginther *et al.* 1995; Bartlewski *et al.* 1999a; Evans *et al.* 2000), and each wave was preceded by a transient increase in serum concentration of FSH (Ginther *et al.* 1995; Souza *et al.* 1997a; Bartlewski *et al.* 1999a). In ewes, a follicular wave consists of 1 to 3 follicles growing from 2 to 3 mm 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; Souza *et al.* 1997a; Bartlewski *et al.* 1999a; Evans *et al.* 2000; Vinoles *et al.* 2001). It seemed, therefore, reasonable to equate 4 to 6 mm follicles in cattle and 1 to 3 mm follicles in sheep as small follicles, mainly because maximum follicle size in cattle is also approximately twice that seen in the ewe (Noel *et al.* 1993; Ravindra *et al.* 1994; Ginther *et al.* 1995; Souza *et al.* 1997a; Bartlewski *et al.* 1999a; Evans *et al.* 2000; Vinoles *et al.* 2001). Although a previous report by Noel *et al*., (1993) has suggested that a moderate increase in small follicle numbers occurs during each follicle wave in sheep (up to 3 follicles/ewe), there are no studies suggesting that there is a large increase in the number of small follicles (2 to 3 mm in diameter) at wave emergence in the ewe, as seen in cattle (4 to 6 mm follicles; (Gong *et al.* 1993; Ginther *et al.* 1996)). Conversely, it has been demonstrated that there is no significant increase in the number of small ovarian follicles even after superovulation treatments in ewes (Eckery *et al.* 1994).

Newer ultrasonographic equipment allows us to detect antral follicles as small as 0.4 mm in diameter and to enumerate > 1 mm follicles in the ewe (Aloka SSD 900, User manual). The purpose of the present study was to see if improving the lower limit for measuring small follicles in the ewe, from 2 to 3 mm down to 1 mm, would reveal changes in numbers of small antral follicles at follicle wave emergence, similar to the increase in numbers of such follicles seen in cattle. We hypothesized that the numbers of small antral follicles does not vary significantly in relation to the day of follicular wave emergence.

4.3. Materials and methods

4.3.1. Animals and experimental procedures

Seven adult, clinically healthy, non-prolific Western White Faced ewes (4 to 6 yr of age, average body weight: 90 ± 7 kg) were used in this study (November to December). All ewes received daily maintenance rations of alfalfa pellets while water, hay and cobalt iodized salt bars were available ad libitum. Estrus was synchronized by a 12 d treatment with medroxyprogesterone acetate (MAP)-containing sponges (Veramix®, Upjohn; 60 mg; Orangeville, ON, Canada). Ewes were checked for estrus with a vasectomized crayon-harnessed ram and an electronic estrus detector measuring changes in vaginal mucous impedance (Bartlewski *et al.* 1999d). Daily transrectal ultrasonography was done for 1 cycle, beginning ~17 d after the synchronized estrus, and utilized a high-resolution, real-time B-mode echo camera (Aloka SSD-900; Aloka Co. Ltd., Japan) connected to a 7.5 MHz transducer. Images were displayed at image magnification X 2. The number, diameter and relative position of all follicles ≥ 1 mm in diameter and corpora lutea (CL) were sketched on ovarian charts, and all ovarian

images were recorded on high-grade video tapes (Fuji S-VHS, ST-120 N) for retrospective analysis of ovarian data. Blood samples (10 mL) were collected each day before scanning, by jugular venipuncture into vacutainers (Becton Dickinson, Rutherford, NJ, USA).

4.3.2. Hormone assays and data analyses

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. Circulating concentrations of FSH (Currie and Rawlings 1987), progesterone (Rawlings *et al.* 1984) and estradiol (Joseph *et al.* 1992) were determined by validated radioimmunoassays. The ranges of the standard curves were from 0.12 to 16.0 ng/mL, from 0.10 to 10 ng/mL, and from 1 to 50 pg/mL in the FSH, progesterone and estradiol assays, respectively. The sensitivities of assays (defined as the lowest concentration of hormone capable of significantly displacing labelled hormone from the antibody; unpaired t-test, $P < 0.05$) were as follows: FSH, 0.1 ng/mL, progesterone, 0.03 ng/mL, and estradiol, 1 pg/mL. Progesterone and FSH concentrations were analyzed in single assays. The intra-assay CVs were 11.6 % or 2.9 % respectively, for reference sera with mean FSH concentrations of 0.39 ng/mL or 1.50 ng/mL. For reference sera with mean progesterone concentrations of 0.22 ng/mL or 0.63 ng/mL, the intra-assay CVs were 19.1 % or 5.5 % respectively. The intra- and interassay CVs for ovine reference sera with mean estradiol concentrations of 3.90 or 12.00 pg/mL were 11.3 % and 12.7 % or 12.8 % and 12.4 %, respectively.

Peaks in daily serum concentrations of FSH were determined using a cycle-detection computer program (Clifton and Steiner 1983). Daily concentrations of progesterone and estradiol were aligned to the days of ovulation at the beginning and end of the estrous

cycle studied, and analyzed as detailed below for follicular data. Similarly, daily serum concentrations of FSH, progesterone and estradiol were normalized to the days of wave emergence and analyzed for the period from 2 d before to 2 d after the day of emergence, as described below for follicular data.

4.3.3. Follicular data summary

The size and numbers of all follicles ≥1 mm in diameter were determined for each ewe on a daily basis (follicles were counted whether they grew further or not), and the follicles were grouped into 3 different categories: follicles ≥1 mm and ≤3 mm in diameter were categorized as small follicles (because preliminary analyses for the 1, 2 and 3 mm follicles showed similar patterns, the follicles were grouped as a single size-class for subsequent analyses and presentation); follicles 4 mm in diameter as medium follicles; and ostensibly ovulatory-sized follicles (≥5 mm in diameter) as large follicles. This categorization was also planned to be comparable to the previous classification of follicles when the Aloka SSD-500 ultrasonographic equipment was used (Bartlewski *et al.* 1999a). In addition, daily numbers of 2 to 3 mm follicles that subsequently grew to ≥5 mm in diameter (emergent follicles) were noted.

The data were then normalized to the day of ovulation (Day 0) at the beginning of the interovulatory interval studied and analyzed for day effects, from 1 d before to 17 d after ovulation. As preliminary analyses showed intriguing changes during the periovulatory periods, data were re-normalized to the days of ovulation and trends in mean daily numbers of follicles in different size-classes were analyzed for the day of ovulation +5 d (for ovulation at the beginning of the cycle) and the day of ovulation -5 d (for ovulation at the end of the estrous cycle studied).

Finally, the data were examined for temporal associations with follicular wave emergence. As 1 mm follicles were numerous, it was not possible to maintain their individual identity from day to day; however, 74 % and 26 % of the follicle waves in all ewes could be traced back to 2 and 3 mm follicles, respectively. Thus, in the present experiment, a follicular wave was regarded as a follicle or group of follicles that grew from 2 or 3 mm to an ostensibly ovulatory size of ≥ 5 mm in diameter, with emergence restricted to a 24 h period. Preliminary inspection of follicular data revealed that the ewes under study had 2 (n = 1), 3 (n = 4) or 4 (n = 2) follicular waves during their respective estrous cycles. To examine the variation in follicular populations during the periods of follicular wave emergence, the data were aligned to the days of wave emergence (retrospectively identified as the day on which 2 or 3 mm follicles that grew to ≥5 mm diameter before regression or ovulation were first detected), and analyzed for the period from 2 d before to 2 d after the day of emergence. For the purpose of statistical analyses, the follicular waves were grouped according to the mean day of emergence and relative to the day of first ovulation of the estrous cycle studied (Day 0; Wave A: Days -1 to 2, Wave B: Days 3 to 6, Wave C: Days 7 to 10, and Wave D: Days 11 and 13 after ovulation). Letters were assigned to the waves because in individual ewes with 2, 3 or 4 waves over the period of study, these waves did not necessarily emerge at a time that placed them in consecutive groupings as described above.

4.3.4. Statistical analyses

Statistical differences were assessed by one-way ANOVA (SigmaStat® Statistical Software, version 2.0 for Windows® 95, NT and 3.1, 1997; Chicago, IL, USA). Repeated measures-ANOVA (RM-ANOVA) was used where necessary. Two-way

RM-ANOVA was used to determine the main effects of wave (Waves A to D) and day (Days -2 to + 2; Day 0 = day of wave emergence), and their interaction for daily numbers of ovarian follicles and serum FSH concentrations at the time of follicular wave emergence. Comparison of the different groups of follicular waves showed no significant main effect of wave or wave-by-day interaction ($P > 0.05$) for daily numbers of ovarian antral follicles in different size categories or circulating concentrations of FSH, with respect to days of wave emergence; therefore, data for all the waves were combined for presentation. Multiple comparisons were made by the method of Fisher's least significant difference (LSD). All values are means ± SEM.

4.4. Results

4.4.1. General results

The mean duration of the interovulatory interval was 17.5 ± 0.4 d. A total of 22 emerging follicular waves were identified with ultrasonography. Ewes in the present study had 3.1 ± 0.3 follicular waves (range: 2 to 4) and 3.1 ± 0.3 transient peaks of FSH per estrous cycle; the numbers of follicular waves and FSH peaks detected in individual ewes were strongly and positively correlated ($r = 0.77$; $P < 0.05$). When the days of follicle wave emergence were categorized (Waves A to D), they fell into 4 different periods with mean days of emergence on Days 0.0 ± 0.03 (Wave A; n = 7), 5.0 ± 0.3 (Wave B; n = 5), 7.5 ± 0.5 (Wave C; n = 4), and 11.6 ± 0.3 (Wave D; n = 6) after ovulation.

4.4.2. Follicle populations throughout the estrous cycle

When follicle populations were normalized to the day of ovulation (Day 0) at the beginning of the estrous cycle studied and analyzed for Days –1 to 17, there was a

significant day effect ($P < 0.05$) for the mean daily numbers of 2 to 3 mm follicles that subsequently grew to ≥5 mm in size before regression or ovulation (emergent follicles of waves); the greatest numbers of such follicles were recorded on Days -1 to 0, 7 and 12 (Fig. 4.1).

There was a significant main effect of day ($P < 0.05$) for mean daily numbers of 1, 2 and 3 mm follicles counted whether they grew further or not (Fig. 4.2A). For all size-classes above, peak follicle number was recorded on Day 1 after first ovulation of the estrous cycle studied, followed by a decline ($P < 0.05$) between Days 1 and 3 (1 mm follicles) or Days 1 and 5 (2 and 3 mm follicles). There were no changes in numbers of these small follicles (1, 2 and 3 mm in diameter) during diestrus, except for a transient increase ($P < 0.05$) in the number of 2 mm follicles between Days 6 and 10, followed by a decrease ($P < 0.05$) from Days 10 to 15. Follicles in the 1 and 3 mm size-classes ($P < 0.05$) increased in number at the end of the observation period, from Days 16 to 17 (Fig. 4.2A). A numerical, but not significant, increase was also seen for 2 mm follicles, from Days 15 to 17 (Fig. 4.2A). When the data for all small follicles (≥1 mm but ≤3 mm in diameter) were combined, there was a significant overall day effect ($P < 0.05$). Small follicle numbers decreased ($P < 0.05$) from Days 1 to 3, and tended ($P < 0.08$) to increase from Days 16 to 17 (Fig. 4.2B). Mean daily numbers of medium follicles (4 mm in diameter) did not vary ($P > 0.05$) throughout the interovulatory interval studied (Fig. 4.2C). Mean daily numbers of large follicles (≥5 mm in diameter) varied ($P < 0.05$); large follicle numbers increased ($P < 0.05$) from Days 1 to 3 and from Days 6 to 8, followed by a decline between Days 8 to 11, and again from Days 13 to 17 ($P < 0.05$; Fig. 4.2C).

4.4.3. Follicle populations, progesterone and estradiol during first 5 d of estrous cycle

Follicle populations were normalized to the day of ovulation (Day 0) at the beginning of the estrous cycle studied, and analyzed for Days 0 to 5. Mean daily numbers of small follicles decreased ($P < 0.05$) from Days 1 to 3 (Fig. 4.3A). Mean daily numbers of medium and large follicles increased ($P < 0.05$) from Days 0 to 2, and from Days 0 to 3 (Fig. 4.3A), respectively.

Similarly, serum concentrations of progesterone and estradiol were normalized to the day of ovulation (Day 0) at the beginning of the estrous cycle studied, and analyzed for Days 0 to 5. There was a significant effect of day for mean serum concentrations of progesterone ($P < 0.001$) and estradiol ($P < 0.001$; Fig. 4.3B). Progesterone concentrations increased ($P < 0.05$) from Days 0 to 3, and again between Days 3 and 5. Circulating concentrations of estradiol reached a peak ($P < 0.05$) on Day 3 and subsequently declined ($P < 0.05$) by Day 5 after the first ovulation of the estrous cycle studied.

4.4.4. Follicle populations, progesterone and estradiol during last 5 d of estrous cycle

Follicle populations were normalized to the day of ovulation (Day 0) at the end of the estrous cycle studied, and analyzed for Days -5 to 0. Mean daily numbers of small follicles increased ($P < 0.05$) from Days -2 to 0 (Fig. 4.4A). Mean daily numbers of medium follicles did not vary ($P > 0.05$). Mean daily numbers of large follicles decreased ($P < 0.05$) from Days -5 to 0 (Fig. 4.4A).

Similarly, serum concentrations of progesterone and estradiol were normalized to the day of ovulation (Day 0) at the end of the estrous cycle studied, and analyzed for Days -

5 to 0. Mean daily concentrations of progesterone decreased ($P < 0.05$) to a basal level at Day -2 before the last ovulation of the interovulatory interval studied (Fig. 4.4B). Estradiol concentrations rose between Days -5 and -3, and then declined on the day of ovulation (Day 0; $P < 0.05$; Fig. 4.4B).

4.4.5. Follicle populations, FSH, progesterone and estradiol at follicle wave emergence

Follicle populations and serum concentrations of FSH, progesterone and estradiol were normalized to days of wave emergence (Day 0), and analyzed for the period from 2 d before to 2 d after the day of wave emergence. There were no significant main effects of wave (Waves A to D) or day (Days -2 to + 2; Day 0 = day of wave emergence), and the interaction of these terms was not significant ($P > 0.05$) for mean daily numbers of all 1, 2 and 3 mm follicles at follicular wave emergence (Fig. 4.5A). There was no significant main effect of wave or day nor the day-by-wave interaction ($P > 0.05$) for mean daily numbers of small (1 to 3 mm in diameter), medium or large follicles, from 2 d before to 2 d after the day of wave emergence (data for all waves were combined for presentation; Fig. 4.5B). Mean daily concentrations of FSH increased significantly from 1.46 ± 0.16 ng/mL on Day -2 to 1.88 ± 0.15 ng/mL on Day 0, and then decreased ($P < 0.05$) to 1.47 ± 0.14 ng/mL on Day 2 after wave emergence.

Changes in circulating concentrations of progesterone and estradiol during the periods encompassing the emergence of successive follicular waves (± 2 d) are shown in Figure 6. There was a significant main effect of wave (Waves A to D; $P < 0.001$), but not of day ($P > 0.05$), and the wave-by-day interaction was significant ($P < 0.01$) for mean serum concentrations of progesterone. Progesterone concentrations were higher around

emergence of Waves C and D as compared to Waves A and B, and were higher for Wave B than Wave A ($P < 0.05$). Mean daily progesterone concentrations did not vary from 2 d before to 2 d after emergence of Waves A and C ($P > 0.05$). At the emergence of Wave B, progesterone concentrations increased between Days -2 and 0, and again between Days 0 and 2 ($P < 0.05$). Serum concentrations of progesterone were lower ($P < 0.05$) 2 d after the emergence of Wave D follicles, as compared to Days -2 to 1 relative to wave emergence.

There was a significant main effect of wave ($P < 0.001$), but the main effect of day and wave-by-day interaction were not significant ($P>0.05$) for mean serum concentrations of estradiol at follicle wave emergence. Circulating concentrations of estradiol were higher ($P < 0.05$) at the time of emergence of Waves A and B compared to Waves C and D. Estradiol concentrations did not differ ($P > 0.05$) between Waves A and B nor between Waves C and D.

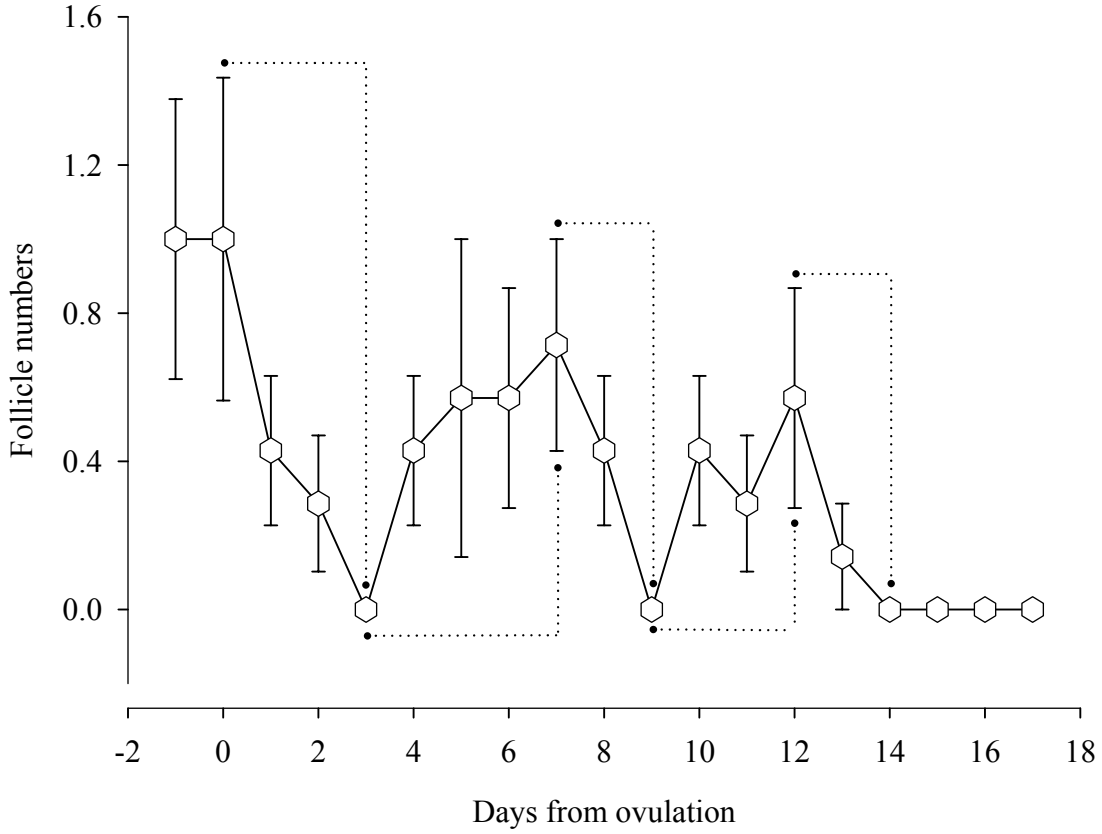

Figure 4.1. Mean daily numbers of 2 to 3 mm antral follicles that subsequently grew to ovulatory size (≥ 5 mm in diameter; follicular waves), from 1 d before to 17 d after ovulation (Day 0 = day of ovulation at the beginning of the estrous cycle studied), in 7 cyclic Western White Face ewes monitored by high-resolution transrectal ovarian ultrasonography (November-December). Dotted lines denote significant differences between points ($P < 0.05$).

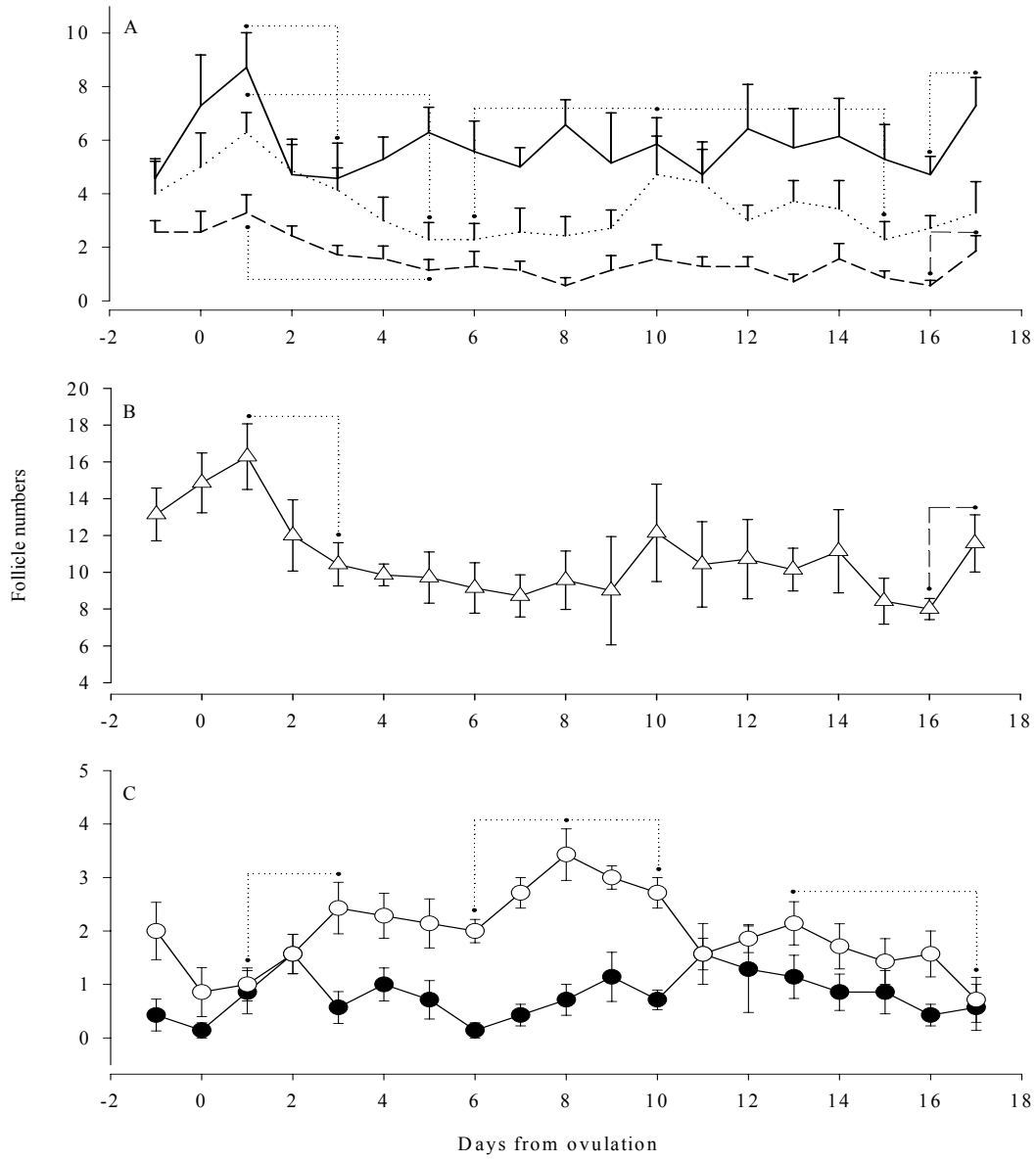

Figure 4.2. Mean daily numbers of 1 (solid line), 2 (dotted line) and 3 mm (dashed line) follicles (A), of all small antral follicles (≥ 1 mm but ≤ 3 mm in diameter; B), and of medium-sized (4 mm in diameter;) and large (≥ 5 mm in diameter;) antral follicles (C), from 1 d before to 17 d after ovulation (Day 0 = day of ovulation at the beginning of the estrous cycle), in 7 cyclic Western White Face ewes monitored by high-resolution transrectal ovarian ultrasonography (November-December). Lines denote significant differences between points (dotted: $P < 0.05$; dashed: $P = 0.08$). See text for additional statistical descriptions.

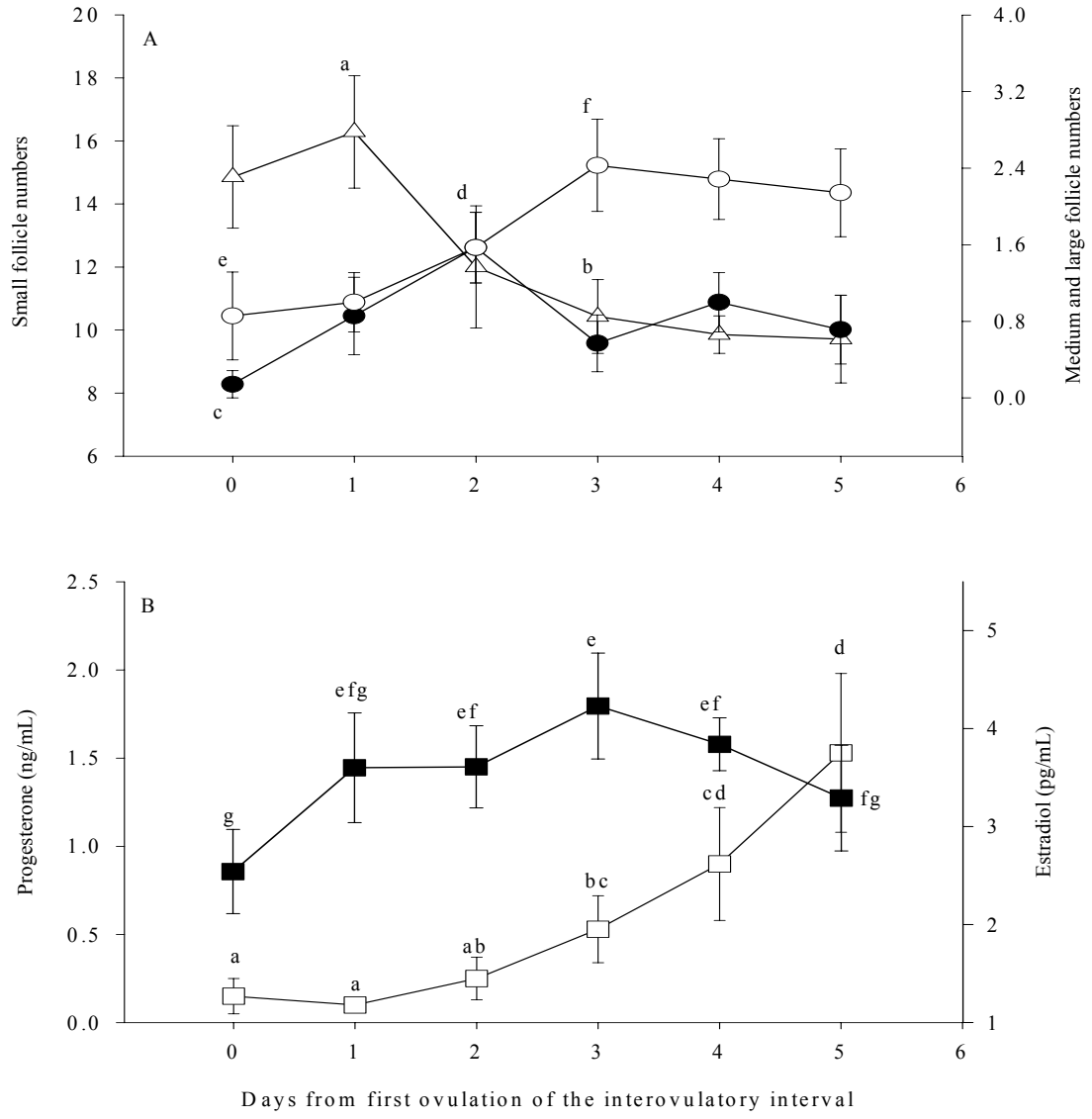

Figure 4.3. Mean daily numbers of small (≥ 1 mm but ≤ 3 mm in diameter; Δ), medium (4 mm in diameter; ●) and large (≥ 5 mm in diameter; ○) ovarian antral follicles (A) with accompanying serum concentrations of progesterone (□) and estradiol (■; B). Data were normalized to the day of ovulation and analyzed from Days 0 to 5 (Day 0 = day of the first ovulation of the estrous cycle studied). Follicles were recorded in 7 Western White Face ewes monitored daily by high-resolution transrectal ultrasonography during 1 estrous cycle (November-December). Significant differences ($P < 0.05$) are shown separately for individual follicle size-classes (Fig. 4.3A): ^{ab} for small follicles, ^{cd} for medium follicles, and ^{ef} for large follicles. Letters denote significant ($P < 0.05$) differences between daily means (Fig. 4.3B): ^{a,b,c,d} for progesterone concentrations and ^{e,f,g} for estradiol concentrations.

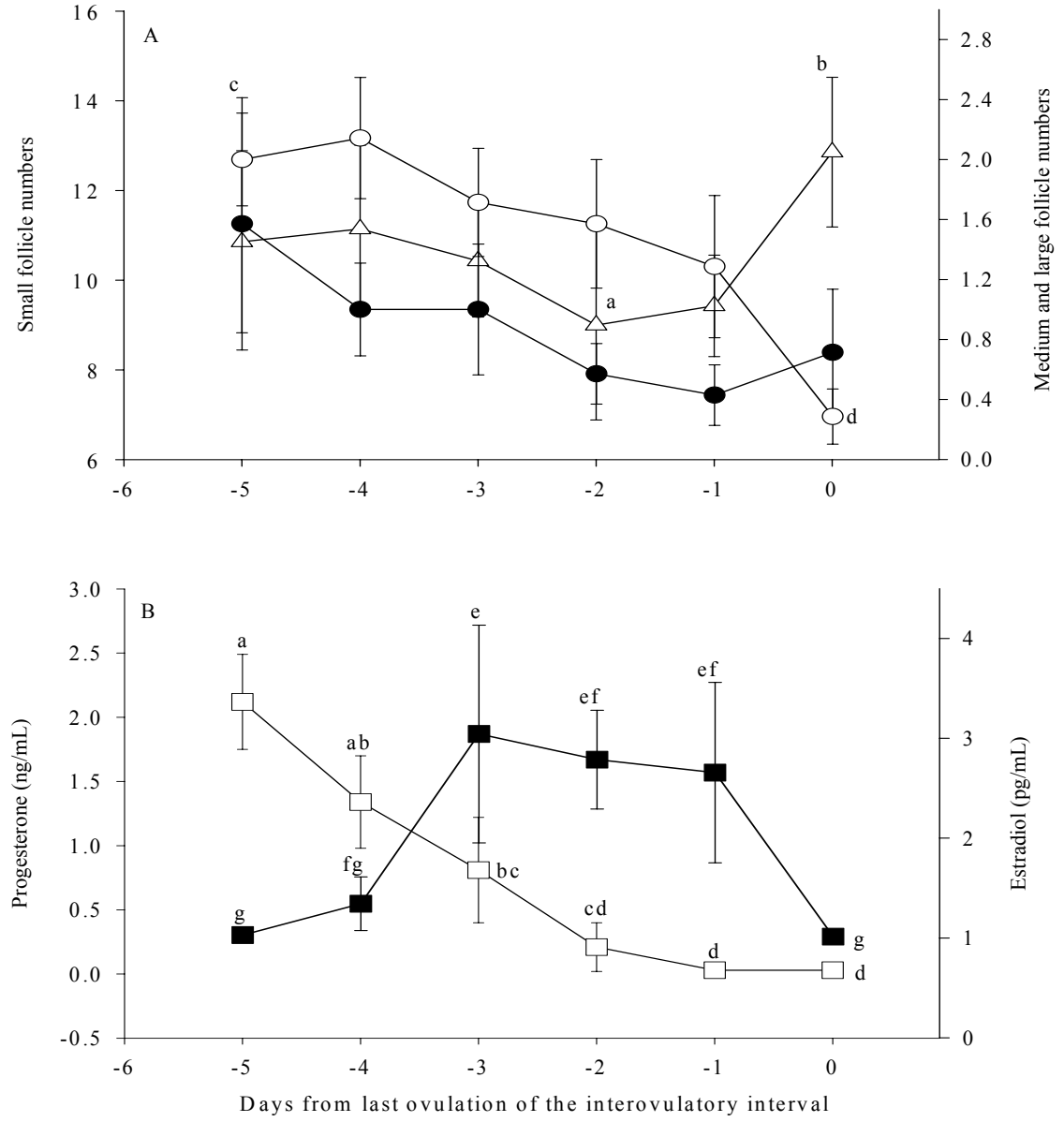

Figure 4.4. Mean daily numbers of small (≥ 1 mm but ≤ 3 mm in diameter; Δ), medium (4 mm in diameter; ●) and large (≥ 5 mm in diameter; ○) ovarian antral follicles (A) with accompanying serum concentrations of progesterone (□) and estradiol (■; B). Data were normalized to the day of ovulation and analyzed from Days -5 to 0 (Day 0 = day of the last ovulation of the estrous cycle studied). Follicles were recorded in 7 Western White Face ewes monitored daily by high-resolution transrectal ultrasonography during 1 estrous cycle (November-December). Significant differences ($P < 0.05$) are shown separately for individual follicle size-classes (Fig. 4.4A): ^{a,b} for small follicles, ^{c,d} for large follicles. Letters denote significant ($P < 0.05$) differences between daily means (Fig. 4.4B): ^{a,b,c,d} for progesterone concentrations and ^{e,f,g} for estradiol concentrations.

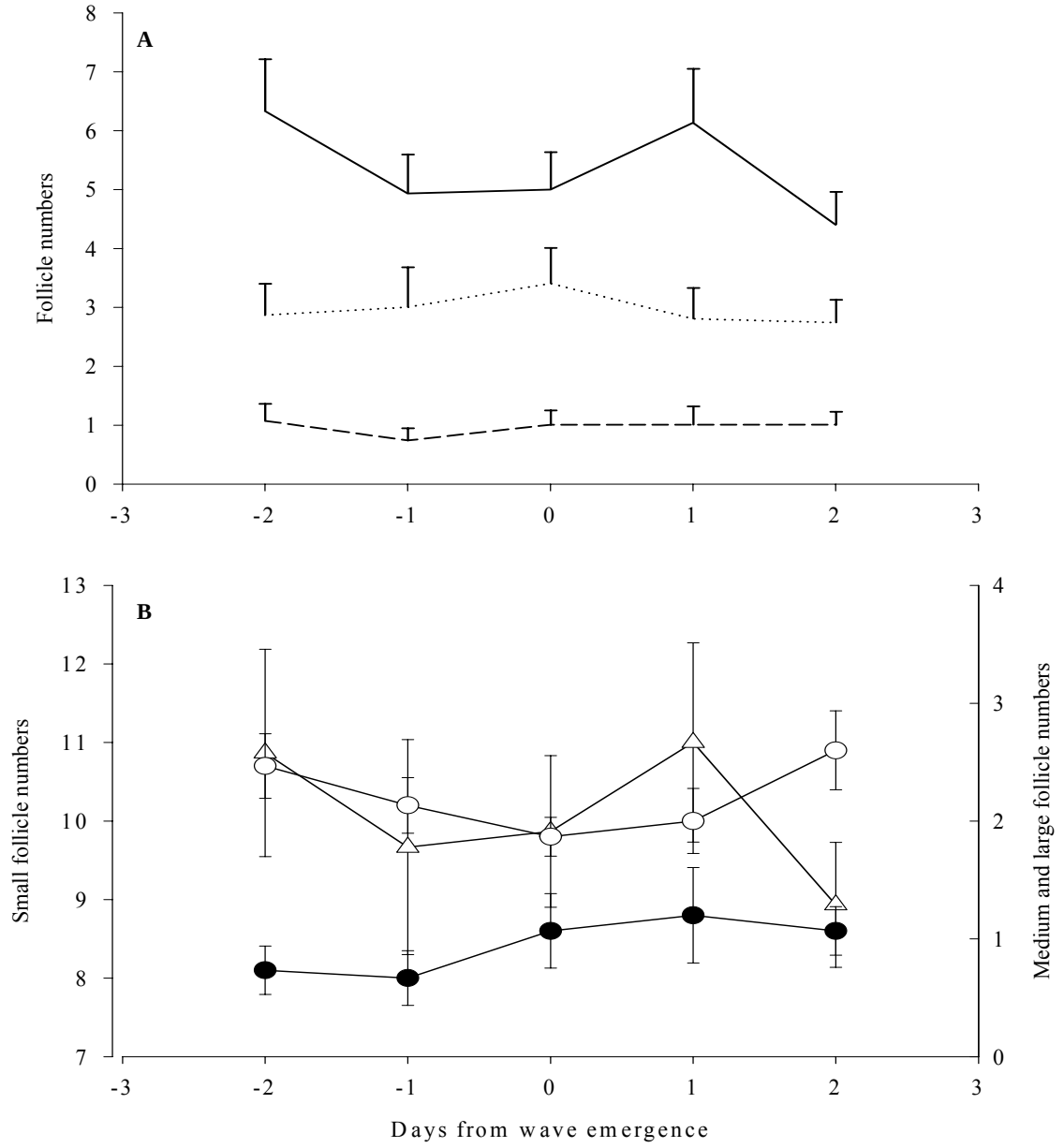

Figure 4.5. Mean daily numbers of 1 mm (solid line), 2 mm (dotted line) and 3 mm (dashed line) follicles (A), and of small (≥ 1 mm but ≤ 3 mm in diameter; Δ), medium (4 mm in diameter; ●) and large (≥ 5 mm in diameter; ○) antral follicles (B), from 2 d before to 2 d after the days of wave emergence (Day 0). Data were compiled for 22 follicular waves detected with high-resolution transrectal ultrasonography in 7 Western White Face ewes studied during 1 estrous cycle (November-December). See text for the details of statistical analyses.

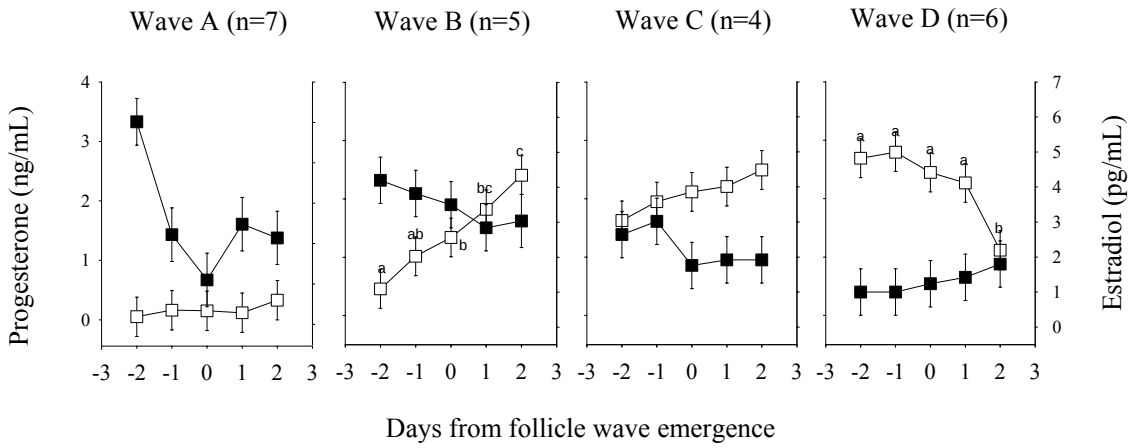

Figure 4.6. Mean daily concentrations of progesterone (□) and estradiol (■) from 2 d before to 2 d after the day of follicular wave emergence. A total of 22 follicular waves detected with high-resolution ultrasonography in 7 cyclic Western white-faced ewes (November-December) were categorized according to the mean day of emergence (Wave A: Days -1 to 2, Wave B: Days 3 to 6, Wave C: Days 7 to 10; and Wave D: Days 11 and 13 after the first observed ovulation). Superscript letters ^{a,b,c} indicate significant differences ($P < 0.05$) between mean daily concentrations of progesterone. See text for additional details and statistical descriptions.

4.5. Discussion

The results of the present study with regards to estrous cycle length, mean number of follicular waves and their accompanying FSH peaks, as well as temporal associations between wave emergence and the FSH peaks, are in agreement with previous studies in cyclic ewes (Ginther *et al.* 1995; Bartlewski *et al.* 1999a; Evans *et al.* 2000). However, in the present study, all detected follicular waves were seen to emerge within a 24 h period. Clearly, the use of a newer high-resolution echo camera allowed us to accurately determine the sizes and positions of antral follicles ≥1 mm in diameter (ALOKA SSD-900 user manual). This resulted in increased precision in mapping the progression of small follicle growth (follicles >1 mm in diameter) on a daily basis. Consequently, the wave emergence window could be shortened from 48 h in earlier studies (Ginther *et al.* 1995; Bartlewski *et al.* 1999a) to 24 h in the present experiment.

In cattle, follicular wave emergence is associated with a transient increase in the numbers of small antral follicles (6 to 9 follicles in the 4 to 6 mm size-range) in the ovary (Gong *et al.* 1993; Ginther *et al.* 1996). The recruited follicles then continue to grow together for 2 to 3 d before one follicle is selected to establish functional dominance, hence suppressing the further growth of other smaller follicles (Gong *et al.* 1993; Fortune 1994; Driancourt 2001). The dominant follicle reaches a maximum size of 8 to 20 mm in diameter (Ginther *et al.* 1989a). In sheep, 1 to 3 follicles of 2 to 3 mm in diameter emerge and grow to a maximum diameter of 4 to 12 mm in an orderly succession throughout the ovulatory cycle (Noel *et al.* 1993; Ravindra *et al.* 1994; Ginther *et al.* 1995; Souza *et al.* 1997a; Bartlewski *et al.* 1999a; Evans *et al.* 2000; Vinoles *et al.* 2001). In most of these earlier studies, there was no clear indication of a

large change in the numbers of small antral follicles at follicle wave emergence in sheep (Ravindra *et al.* 1994; Ginther *et al.* 1995; Souza *et al.* 1997a; Bartlewski *et al.* 1999a; Vinoles *et al.* 2001). In some studies, however, a wave-like pattern in small follicle numbers was reported (Noel *et al.* 1993; Evans *et al.* 2000). When 2 to 3.5 mm follicles were enumerated by laparoscopy (Noel *et al.* 1993), the number of follicles identified increased from 0 to approximately 1 to 3 at the time of emergence of each follicular wave; all of these follicles appeared to grow to ovulatory diameters or to constitute the follicle(s) of sequential waves. Using ultrasonography, increases in numbers of 2 to 3 mm follicles during the ewe's estrous cycle were shown (Evans *et al.* 2000), but the peaks in numbers of small follicles did not consistently occur, unlike in cattle, at or around the time of follicle wave emergence. There are no reports in sheep of a large increase in the number of small follicles at follicle wave emergence, which would be similar to that consistently seen in cattle (Gong *et al.* 1993).

The question remained whether changes in small follicle populations in sheep simply involved smaller follicles (< 2 mm diameter). In enumerating follicles in the 1 to 3 mm size-range in the present study, we were able to expand the daily-measured small follicle pool, as seen during the ewe's estrous cycle, from 1 to 4 follicles in the 2 to 3 mm size-range determined previously (Noel *et al.* 1993; Ginther *et al.* 1995; Bartlewski *et al.* 1999a; Evans *et al.* 2000), up to 8 to 18 follicles (Figs. 2 to 5). Even after expanding the measured small follicle pool to 1 mm follicles, we observed no increase in the total number of small follicles (1 to 3 mm size range) at follicle wave emergence (Fig. 4.5); changes were seen only during the periovulatory periods (Figs. 3 and 4). Moreover, the total numbers of 1, 2 or 3 mm follicles analyzed separately did not show

any significant changes in relation to the days of follicular wave emergence, at any stage of the interovulatory period studied. Differences in circulating concentrations of progesterone and estradiol among different stages of the estrous cycle in which follicular waves emerged (Fig. 4.6), did not appear to affect the numbers of small follicles present at follicle wave emergence (Fig. 4.3B and 4.4B).

Therefore, based on the present observations, there does not appear to be recruitment of a large number of small follicles at the emergence of a new follicle wave in the ewe nor suppression of small follicle numbers during the growth phase of a wave (Fig. 4.5). Total small follicle numbers do not appear to change prior to or after wave emergence. Of this pool of small follicles, only 1 to 3 follicles, in the 2 to 3 mm size-range, begin to grow further in each follicular wave, approximately every 3 to 5 d throughout the interovulatory interval (Fig. 4.1). This finding is in agreement with earlier studies (Noel *et al.* 1993; Ginther *et al.* 1995; Bartlewski *et al.* 1999a; Evans *et al.* 2000). These 1 to 3 follicles clearly constitute the follicular wave. Unlike in cattle (Ginther *et al.* 1996; Driancourt 2001), there would appear to be little or no selection of follicles recruited into a wave, and no unequivocal evidence for follicular dominance. The lack in sheep of powerful follicular dominance, as seen in cattle, has been suggested previously (Driancourt 2001).

In cattle, the number of small follicles (4 to 6 mm in diameter) which give rise to the first follicle wave of the estrous cycle increases around the day of ovulation (Pierson and Ginther 1987a; Ginther *et al.* 1989a; Ginther *et al.* 2001a). In the ewes in the present study, the periovulatory increase in total numbers of small follicles occurred during the period encompassing the emergence of the first follicular wave of the cycle.

However, there was no significant day effect for mean daily numbers of small follicles upon normalization to the day of wave emergence, including the first wave of the estrous cycle studied. The increase in the total number of small follicles during the periovulatory period in sheep may be caused, at least in part, by a factor(s) other than that which triggers the emergence of a follicle wave. One such factor could be the demise of the corpus luteum (CL). This assumption seems logical as the ovine CL locally suppresses numbers of small follicles (Bartlewski *et al.* 2001b). In the present study, the total number of small follicles began to increase from Day -2 (i.e., 2 d before ovulation and after luteolysis; (Baird and Scaramuzzi 1975; Bartlewski *et al.* 1999a)), and reached a peak on Day 1, beyond the mean day of first wave emergence (Day 0.0 ± 0.03; Day 0 = ovulation) and just before the CL becomes functionally active (Fig. 4.3B). This periovulatory increase in the total number of small follicles may also have resulted from changes in the pattern of LH secretion after luteolysis (Rawlings and Cook 1993). A periovulatory increase in follicle numbers has been previously reported in the ewe (Vinoles *et al.* 2001); however, this was in total follicle numbers, not just small antral follicles.

In summary, the present results indicate that, even though the emergence of 2 to 3 mm follicles that attained an ovulatory size of ≥ 5 mm in diameter before regression or ovulation exhibited a distinct wave-like pattern, the total number of small follicles (1 to 3 mm in diameter) did not change throughout the estrous cycle, except for an increase during the periovulatory period. We conclude that follicle wave emergence in sheep involves the recruitment of 1 to 3 follicles from the relatively stable small follicle pool, in contrast to the 6 to 9 follicles from the analogous follicle pool in cattle. Again, in

contrast to cattle (Ginther *et al.* 1996), there appears to be little selection of recruited follicles and no evidence for follicular dominance in cyclic sheep.

Chapter 5: THE TEMPORAL RELATIONSHIP BETWEEN PATTERNS OF LH AND FSH SECRETION, AND DEVELOPMENT OF OVULATORY-SIZED FOLLICLES DURING THE MID TO LATE LUTEAL PHASE OF SHEEP[*]

Duggavathi R, Bartlewski PM, Barrett DMW and Rawlings NC.

5.1. Abstract

The aim of the present study was to investigate the temporal relationship between the secretory pattern of LH and FSH and ovarian antral follicular waves during the luteal phase of the estrous cycle in sheep. The growth pattern of ovarian antral follicles and CL were monitored by transrectal ultrasonography and gonadotropin concentrations were measured in blood samples collected every 12 min for 6 h a day from d 7 to d 14 after ovulation. There were 2 follicular waves (penultimate and final waves of the cycle) emerging and growing during the period of intensive blood sampling. Mean and basal LH concentrations and LH pulse frequency increased ($P < 0.001$) with decreasing progesterone concentration at the end of the cycle. Mean and basal FSH concentrations reached a peak ($P < 0.01$) on the day of follicular wave emergence before declining to a nadir by 2 d after the emergence. None of the parameters of pulsatile LH secretion varied significantly with either the emergence of the final follicular wave or with the end of the growth phase of the largest follicle of the penultimate wave of the cycle. However, mean and basal LH concentrations did increase ($P < 0.05$) after the end of the growth phase of the largest follicle of the final follicular wave of the cycle; but, the end of the growth phase of the largest follicle of the final wave coincided with functional luteolysis. In summary, there was no abrupt or short-term change in pulsatile LH

[*] Theriogenology (in press).

secretion in association with the emergence or growth of the largest follicle of a wave. We concluded that the emergence and growth of ovarian antral follicles in follicular waves does not require changes in LH secretion and may perhaps involve changes in the follicular sensitivity to LH.

5.2. Introduction

Ovarian antral follicles in sheep emerge from a pool of 1 to 3 mm diameter follicles and grow in a wave-like pattern with 3 to 4 follicular waves during each 17-d estrous cycle (Bartlewski *et al.* 1999a). A follicle(s) from the last wave of the cycle ovulates and forms a CL (Bartlewski *et al.* 1999a). The role of a transient peak in serum FSH concentration in the emergence of follicular waves during both breeding and non-breeding seasons, and during transition periods has been demonstrated unequivocally (Ginther *et al.* 1995; Bartlewski *et al.* 1998; Souza *et al.* 1998; Bister *et al.* 1999; Bartlewski *et al.* 1999a; Bartlewski *et al.* 1999b; Evans *et al.* 2000; Evans *et al.* 2001; Duggavathi *et al.* 2003a; Duggavathi *et al.* 2004). The first and second follicular waves of the ovine estrous cycle emerge in an environment of increasing progesterone concentrations (Ginther *et al.* 1995; Bartlewski *et al.* 1999a; Evans *et al.* 2000), and the growth of the follicles in the first wave occurs when the LH pulse frequency is decreasing (Bartlewski *et al.* 2000a). The penultimate and final follicular waves emerge during a period in which the CL maintains maximal function and there are constant serum concentrations of progesterone (Ginther *et al.* 1995; Bartlewski *et al.* 1999a; Evans *et al.* 2000). The growth of antral follicles in the final follicular wave occurs when FSH concentrations are falling and LH pulse frequency is increasing following luteolysis (Baird 1983; Scaramuzzi *et al.* 1993). Although differing progesterone

environments alter gonadotropin secretory patterns, follicular waves at all stages of the cycle in the ewe are similar in duration and maximal follicle size (Bartlewski *et al.* 1999a; Duggavathi *et al.* 2003a).

The individual roles of FSH and LH in the growth of antral follicles in the ewe are not completely understood (Campbell *et al.* 1999). FSH alone, but not LH alone, can stimulate the growth of follicles to a preovulatory size in long-term GnRH agonist treated ewes (Picton *et al.* 1990). Experiments that used follicular fluid (McNeilly 1984; Campbell *et al.* 1991a) or inhibin (Campbell and Scaramuzzi 1996) to depress FSH during the follicular phase of sheep were confounded by potential local actions of inhibin and other components of follicular fluid (Campbell *et al.* 1999). Withdrawal of FSH in the presence of LH resulted in the maintenance of preovulatory follicles in 50 to 55 % of GnRH-antagonist treated ewes (Campbell *et al.* 1999).

In a previous study from our laboratory (Bartlewski *et al.* 2000a), using blood samples collected every 12 min for 6 h from each day from ovulation to d 7 after ovulation, it was observed that the FSH concentrations were closely associated with antral follicular growth and LH concentrations with CL growth. The potential involvement of LH in the maintenance of the ovulatory sized follicles during the static phase of a wave could not be tested directly as the period of intensive blood sampling coincided with the increasing serum progesterone concentrations of the early luteal phase. Increasing progesterone secretion suppresses LH pulse frequency (Hauger *et al.* 1977; Karsch *et al.* 1979; Goodman and Karsch 1980; Wheaton *et al.* 1984).

In a previous study in heifers (Ginther *et al.* 1998), similar intensive blood sampling did not reveal a change in FSH concentrations or LH pulsatility that could be associated with the deviation of dominant follicular growth. Nevertheless, this study, like our previous study above, was confounded by the fact that the period of intensive blood sampling involved concurrent growth of the CL.

The purpose of the present study was to investigate involvement of FSH and LH in antral follicular emergence and growth. For this purpose, we utilized intensive blood sampling done between d 7 and 14 after ovulation. This period of blood sampling, from mid to late luteal phase, encompassed the emergence and growth of the penultimate and final follicular waves of the ovine estrous cycle. It was hoped that this study would confirm the relationship of FSH secretory patterns with antral follicular emergence and growth in the ewe, and allow us to look at relationships between LH secretion and antral follicular emergence and growth at a time when progesterone secretion would be largely stable. We hypothesized that: 1) FSH secretory patterns are associated with the growth and demise of the follicles in a wave; and 2) LH secretory patterns are associated with the luteolysis, but not with the growth and demise of the follicles in a wave.

5.3. Materials and methods

5.3.1. Animals and intensive blood sampling

The 7 sexually mature Western White Face cross bred ewes employed in the present study (November-December) were housed in outdoor paddocks and fed daily maintenance rations of alfalfa pellets, with hay, water and mineralized salt licks available *ad libitum*. Estrous cycles were synchronized with 2 injections of $PGF_{2\alpha}$, as dinoprost tromethamine (Lutalyse®, 15 mg i.m.; Upjohn, Orangeville, ON, Canada),

given 9 d apart. Estrus was detected with 2 vasectomized crayon-harnessed rams. Blood samples (5 mL) were collected every 12 min for 6 h, for 8 consecutive days (between 9 am and 3 pm) via indwelling jugular catheters (vinyl tubing; 1.00 mm i.d. X 1.50 mm o.d.; 530070, Biocorp Australia Propriety Ltd., Huntingdale, VIC, Australia), commencing 7 d after ovulation. Cannulae were inserted 24 h before the first intensive bleed and were filled with heparinized saline between the bleeds (1000 U.S.P. units of heparin sodium per 1 L of saline; Hepalean, Organon Teknika Inc., Toronto, ON, Canada). During sampling, ewes were kept in pens in a barn with large windows to ensure ambient light exposure. Blood samples were allowed to clot overnight at room temperature; serum was harvested and stored at -20º C until assayed. Hematocrits were checked each day, and there was no more than a 9.5% decrease in packed cell volume (PCV) from the beginning to the end of the 8-day period of blood sampling.

5.3.2. Transrectal ultrasonography

Ovaries were visualized with a B-mode scanner (Aloka SSD 900 ultrasound equipment, Aloka Co. Ltd., Tokyo, Japan) connected to a stiffened 7.5-MHz transducer, as described previously (Duggavathi *et al.* 2003a). Daily scanning was performed between 11 am and 12 pm from the day of estrus until 1 d after the ovulation at the end of the estrous cycle, to record all ovarian follicles ≥1 mm in diameter and CL. Sizes and relative positions of follicles and CL were sketched on ovarian charts. All images were recorded on S-VHS video tapes, using a compatible VCR for later image analysis. Ovulation was confirmed by the disappearance of large antral follicles (≥ 5 mm in diameter) and detection of corpora hemorrhagica (Duggavathi *et al.* 2003b) as determined by transrectal ultrasonography.

Ovarian data were combined for the 2 ovaries of each ewe. A follicular wave was defined as one or more antral follicles growing from 2-3 mm to ≥ 5 mm in diameter before regression or ovulation (Duggavathi *et al.* 2003a). The growing phase of the largest follicle of the wave was the time taken by that follicle to grow from 2-3 mm to its maximum diameter, and the static phase was regarded as the time during which that follicle was seen to maintain its maximum diameter. If more than 1 follicle attained the same maximum size, the follicle that reached the maximum diameter first and/or remained at its maximum size for the longest period of time was regarded as the largest follicle of the wave.

5.3.3. Hormone assays and analyses

The double antibody radioimmunoassays (RIA) used to determine serum concentrations of gonadotropic hormones and steroids have been described elsewhere (FSH, LH, estradiol-17β and progesterone (Rawlings *et al.* 1984; Rawlings and Howell 1988; Currie and Rawlings 1989; Joseph *et al.* 1992)). Serum concentrations of LH and FSH are given in terms of a NIAMDD-oLH-24 and a NIDKK-oFSH-RP1, respectively. The sensitivities of the assays, defined as the lowest concentrations of standard capable of significantly displacing labeled hormones from the antiserum (unpaired t-test), were as follows: LH and FSH, 0.1 ng/ml; progesterone, 0.03 ng/ml; and estradiol, 1 pg/ml. The ranges of standards used in the LH, FSH, progesterone and estradiol assays were 0.06 to 8.0 ng/mL, 0.13 to 16.0 ng/mL, 0.10 to 10 ng/mL and 1–50 pg/ml, respectively. For reference sera with mean LH concentrations of 0.13 or 0.99 ng/mL, the intra- and inter assay coefficients of variation (CVs) were 10.3% and 8.3% or 11.1% and 10.0%, respectively. For FSH reference sera with mean concentration of 1.27 or 3.43 ng/mL,

the intra- and inter assay CVs were 9.6% and 8.3% or 5.9% and 8.8%, respectively. For reference sera with mean progesterone concentrations of 0.22 or 0.63 ng/ml, the intra-assay CVs were 9.1 or 5.5%, respectively. The intra- and inter-assay CVs for ovine reference sera with mean estradiol concentrations of 3.9 or 12.0 pg/ml were 11.3 and 12.7% or 12.8 and 12.4%, respectively.

The PC-PULSAR program ((Merriam 1987), which has been modified for use on an IBM-PC by J.F. Gitzen and V.D. Ramirez, Urbana, IL, USA) was used to estimate LH/FSH pulse frequency and amplitude as well as mean and basal serum concentration of the hormones. Serum FSH concentrations did not show pulsatile pattern in 5 of 7 ewes of the present study. Thus, analyses for pulse frequency and pulse amplitude were not done for serum FSH concentrations. The basal serum level ("smoothed series") was generated after the removal of short-term variation in hormone concentrations, including possible pulses. Standard deviation criteria (G and Baxter parameters) were used for pulse detection.

Parameters of gonadotropin concentrations as determined using the PULSAR program were converted into the percentage of the maximal value recorded during the period from d 7 to 14 after ovulation in each ewe (Bartlewski *et al.* 2000a); this transformation was done to remove inter-animal variation in serum gonadotropin concentrations. Data were aligned as follows:

1) d -8 to -1 from functional luteolysis (defined as the day on which the serum progesterone concentration reached < 0.5 ng/ml (Wheaton *et al.* 1988)). Functional luteolysis occurred on d 15 (5 ewes), d 14 (1 ewe) and d 16 (1 ewe) after ovulation; with a mean of 14.7 ± 0.4 d post-ovulation. For data analysis, d 15 was taken as the

mean day of functional luteolysis and as the 7-d intensive blood sampling ran from d 7 to d 14 after ovulation, data was first analyzed from d –8 to d –1 from functional luteolysis;

2) day of emergence of the final follicular wave ± 2 d. Only data for the final follicular wave of the cycle were included for this analysis because the penultimate waves emerged on d 7 (mean 7.0 ± 0. 4) after ovulation in most ewes; data for both d –2 and d –1 were available for only one ewe (the penultimate wave emerged on d 9 of the cycle in this ewe);

3) the end of the growth phase (maximal follicle size or onset of the static phase) of the largest follicle of the wave ± 2 d. For this analysis, the data (within the period of the 7-d intensive blood sampling) were available for penultimate waves in 4 ewes and for final follicular waves in 5 ewes. The FSH and estradiol data for penultimate and final waves were combined because there were no differences between waves for daily serum estradiol, and mean and basal serum FSH concentrations ($P > 0.05$; data not shown). However, LH data were analyzed for the 2 waves separately as there were significant differences between the two follicular waves in terms of mean and basal serum LH concentrations.

5.3.4. Statistical analyses

The effects of day on the endpoints mentioned above were analyzed by 1-way ANOVA with repeated measures (Sigma Stat® Statistical Software, version 2.0 for Windows® 95, NT and 3.1, 1997; Chicago, IL, USA). When an overall analysis was significant ($P < 0.05$), the Fisher's LSD test was done to determine differences between individual (daily) means. All results are expressed as mean ± SEM.

5.4. Results

All of the 7 ewes studied were marked by rams within a 48-h period and the transrectal ultrasonographic examination of the ovaries revealed that they ovulated between 24 and 48 h after the onset of estrus. There were 2 to 4 antral follicular waves emerging during the interovulatory period in the ewes of the present study.

Each ewe studied had 2 follicular waves emerging over the 8-d period of blood sampling between d 7 and d 14 after ovulation. The penultimate and the final follicular waves of the cycle emerged on d 7 and 10 after ovulation, respectively. The characteristics of the penultimate and final follicular waves are shown in Table 5.1.

5.4.1. Data centralized to 1 day before functional luteolysis

Diameter profiles of the CL and the largest follicle of the penultimate and final follicular waves of the cycle are overlaid on the area plot of the mean daily concentrations of progesterone in Figure 1. The mean diameter of the CL did not show any significant pattern throughout the period from d -8 to d -1 before functional luteolysis ($P > 0.05$). Mean daily serum concentrations of progesterone did not change from d -8 and -2 but there was a significant decline between d -2 and d -1 before functional luteolysis. The penultimate follicular wave emerged on d -8 before functional luteolysis (corresponding to d 7 after ovulation) and reached an ovulatory diameter of ≥ 5 mm on d -5 before functional luteolysis. The final follicular wave emerged on d -5 before functional luteolysis (corresponding to d 10 after ovulation) and reached an ovulatory diameter of ≥ 5 mm on d -2 before functional luteolysis.

There was a significant effect of day for mean and basal LH concentrations ($P < 0.001$), LH pulse frequency ($P < 0.001$) and amplitude ($P < 0.05$), and mean and basal FSH concentrations ($P < 0.05$; Fig. 5.2). The mean and basal concentrations of LH and LH pulse frequency were low from d -8 to d -3 before functional luteolysis and increased ($P < 0.001$) between d -2 and d -1 before functional luteolysis. LH pulse amplitude increased ($P < 0.05$) from d -7 to d -6 and remained elevated until d -2 before it tended to decline ($P = 0.08$) to d -1 before functional luteolysis. The mean and basal concentrations of FSH increased ($P < 0.01$) from d -8 to d -6 and remained elevated until d -3 then declined to d -1 ($P < 0.01$) before functional luteolysis.

5.4.2. Hormonal data centralized to the day of emergence of the follicular wave

Mean daily serum concentrations of estradiol, mean serum concentration of FSH, and basal concentration of FSH and LH from 2 days before (d -2) to 2 days after (d 2) the day of follicular wave emergence (d 0) are shown in Figure 3. There was a significant day effect for mean daily concentrations of estradiol ($P < 0.05$), mean and basal FSH concentrations ($P < 0.01$), and there was a tendency for an effect of day for basal LH concentrations ($P = 0.07$). However, there were no significant patterns ($P > 0.05$) for mean LH concentration, and LH pulse frequency and amplitude. Estradiol concentrations increased ($P < 0.05$) from d 0 (48.4 ± 17 % maximum) to d 2 (76.1 ± 15 % maximum) after the day of emergence. Mean and basal concentrations of FSH increased ($P < 0.01$) from d -2 to d 0 before declining to a nadir on d 2 ($P < 0.05$) after the day of follicular wave emergence. Basal LH concentrations tended to increase from d 0 to d 2 of wave emergence ($P = 0.07$).

5.4.3. Hormonal data centralized to the last day of the growth phase of the largest follicle of a wave

Mean and basal concentrations of FSH and LH overlaid on mean daily concentrations of estradiol from 2 days before (d -2) to 2 days after (d 2) the last day of the growth phase (d 0) of the largest follicle of a follicular wave are shown in Figure 4. There was no significant effect of wave (penultimate or final), but there was a significant effect of day for mean and basal concentrations of FSH ($P < 0.05$). There was a significant effect of wave, day and a wave x day interaction for mean and basal concentrations of LH ($P < 0.01$), but no significant effects ($P > 0.05$) for mean daily concentrations of estradiol and LH pulse frequency and amplitude. Mean and basal FSH concentrations increased ($P < 0.02$) from a nadir on d -1 to a peak on d 2 after the end of growth phase of the largest follicle of both penultimate and final waves (data combined). Mean and basal LH concentrations increased ($P < 0.01$) from d 0 to d 2 after the end of growth phase of the largest follicle of the largest follicle of the final wave. However, there was no significant trend for LH pulse characteristics in relation to the end of the growth phase of the largest follicle of the penultimate wave.

Table 5.1. Characteristics of penultimate and final follicular waves in 7 Western White Faced ewes. Follicular waves emerged during the period of intensive blood sampling, with samples taken every 12 min for 6 h a day from d 7 to d 14 after ovulation.

Parameter	Penultimate wave	Final wave
Mean day of emergence after ovulation	7.0 ± 0.4^{a}	10.4 ± 0.7^{b}
Number of follicles per wave	1.4 ± 0.2	1.2 ± 0.2
Largest diameter of the follicle (mm)	5.1 ± 0.2	5.4 ± 0.2
Growth rate of the follicle of the wave (mm/d)	1.1 ± 0.1	1.2 ± 0.1

Note: a, b indicate significant difference ($P < 0.001$) within a column.

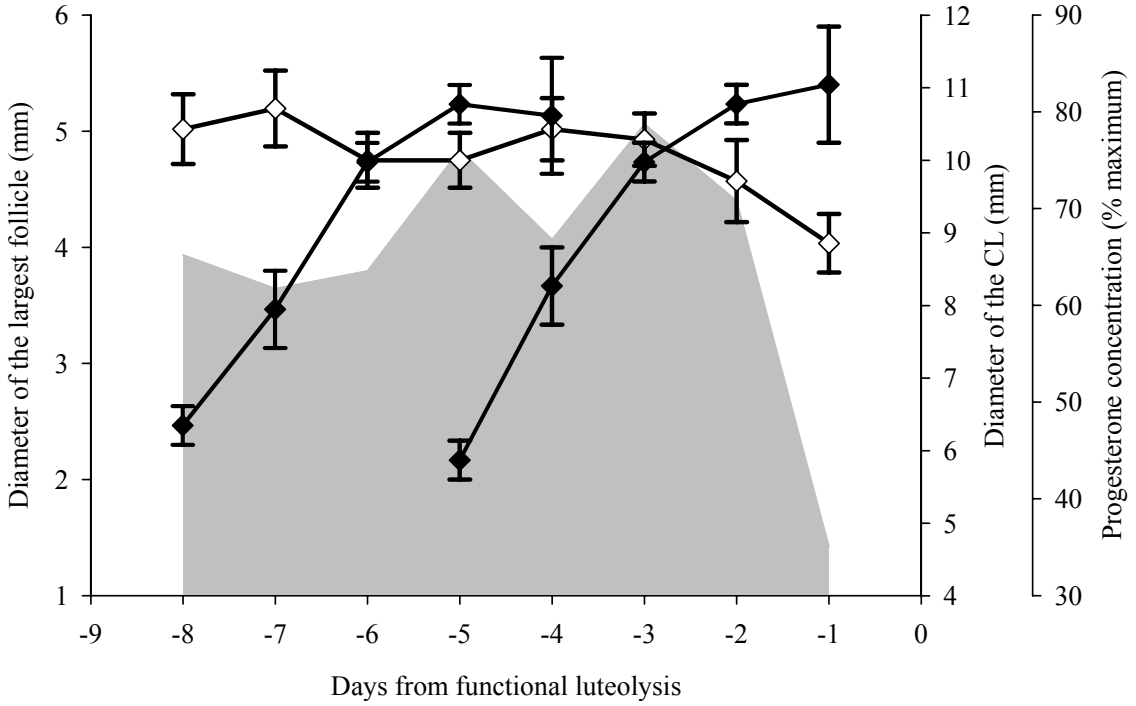

Figure 5.1. Mean daily diameter profiles (mm) of the CL(◊)and the largest follicle (♦) of the penultimate and final follicular waves of the cycle overlaid on the mean daily concentrations (percentage of the maximum value for each ewe) of progesterone (shaded area) in Western White Face ewes (n = 7). The progesterone concentrations were measured in daily blood samples collected from d 7 to 14 after ovulation. The ewes underwent daily transrectal ultrasonography throughout one estrous cycle. The data were centralized to the day of functional luteolysis (see text for the definition) and are presented for the period between d -8 to d -1 before functional luteolysis.

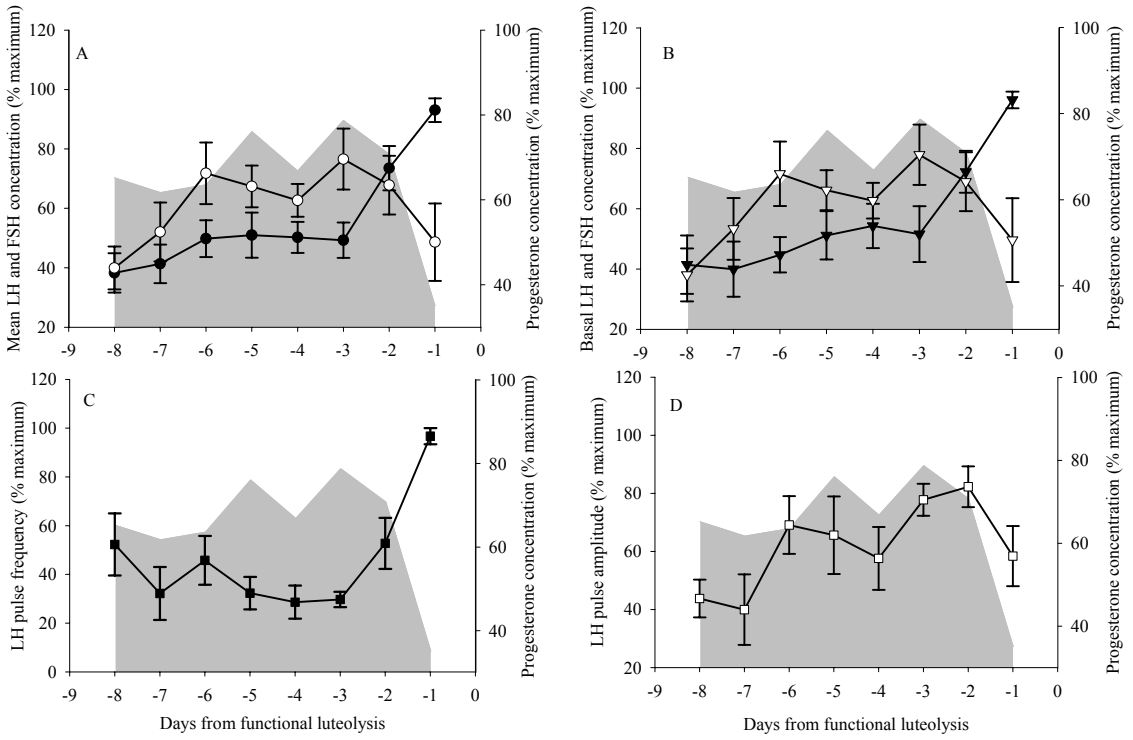

Figure 5.2. Mean (Panel A) and basal (Panel B) concentrations of FSH (○,∇, respectively) and LH (●, ▼, respectively) and LH pulse frequency (■; Panel C) and amplitude (□; Panel D) overlaid on the mean daily concentrations of progesterone for Western White Face ewes (n = 7). The gonadotropin concentrations were measured in blood samples collected every 12 min for 6 h per day from d 7 to 14 after ovulation. The progesterone concentrations were measured in daily blood samples collected from d 7 to 14 after ovulation. Hormonal data were expressed as percentage of the maximum value for each ewe. The ewes underwent daily transrectal ultrasonography throughout one estrous cycle. The data were centralized to the day of functional luteolysis (see text for the definition) and are presented for the period between d -8 to d -1 before functional luteolysis.

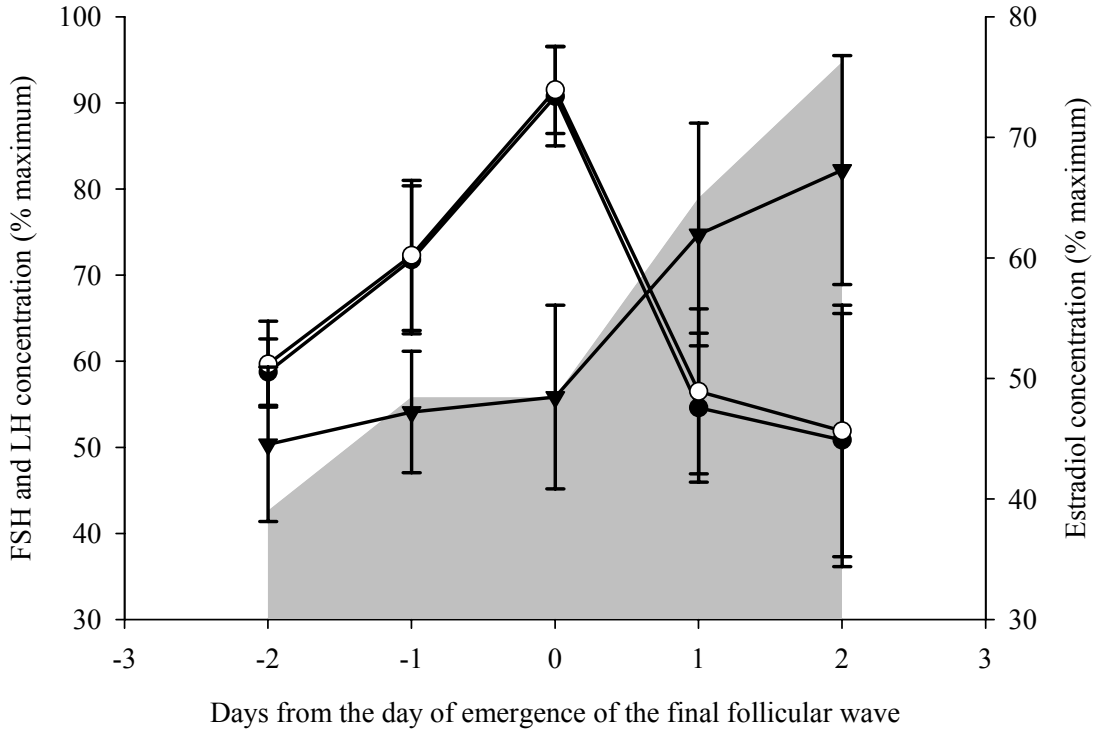

Figure 5.3. Mean (●) and basal (○) concentrations of FSH and basal concentrations of LH (▼) overlaid on the mean daily concentrations of estradiol for Western White Face ewes (n = 7). The gonadotropin concentrations were measured in blood samples collected every 12 min for 6 h per day from d 7 to 14 after ovulation. The estradiol concentrations were measured in daily blood samples collected from d 7 to 14 after ovulation. Hormonal data were expressed as percentage of the maximum value for each ewe and are presented for the period from 2 d before to 2 d after the day of emergence of the final follicular wave (d 0). * indicates significant difference ($P < 0.05$).

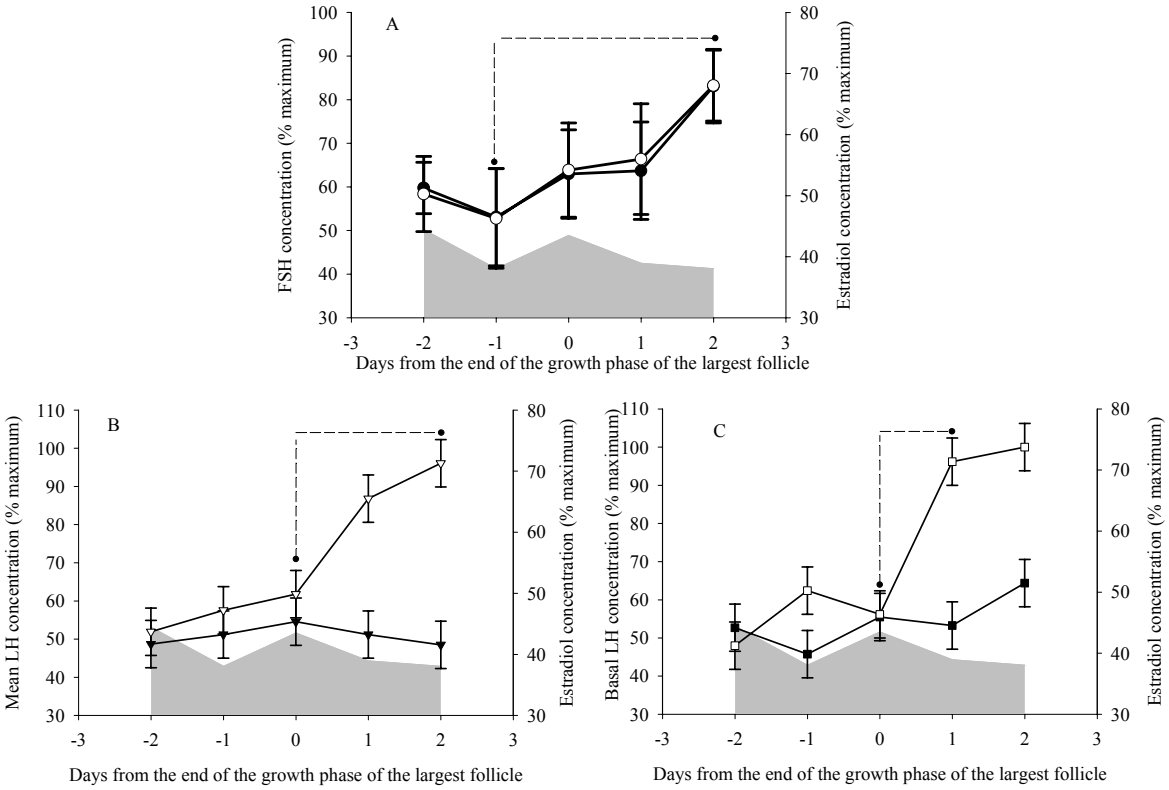

Figure 5.4. Panel A: Mean and basal concentrations of FSH (●, ○, respectively; n = 7) combined for the penultimate and final waves; Panel B: mean concentrations of LH (n = 4-5) for the penultimate and final waves (▼,∇, respectively) and Panel C: basal concentrations of LH (n = 4-5) for the penultimate and final waves (■, □, respectively). The gonadotropin data were overlaid on the mean daily concentration of estradiol for Western White Face ewes. The gonadotropin concentrations were measured in blood samples collected every 12 min for 6 h per day from d 7 to 14 after ovulation. The estradiol concentrations were measured in daily blood samples collected from d 7 to 14 after ovulation. Hormonal data were expressed as percentage of the maximum value for each ewe and are presented for the period from 2 d before to 2 d after the last day of the growth phase of the largest follicle of the wave. Dashed line indicates significant difference ($P < 0.01$) between the days from the end of the growth phase of the largest follicle of a wave.

5.5. Discussion

The patterns of FSH and LH secretion from ovulation to the mid-luteal phase in relation to the emergence and growth of the first 2 follicular waves of the estrous cycle in sheep were investigated in a previous study from our laboratory (Bartlewski *et al.* 2000a). It was shown that FSH concentrations were associated with follicular growth and regression, whereas LH concentrations were associated with CL development. Nonetheless, LH pulse amplitude increased during the static phase of the first follicular wave of the cycle. It was not clear whether this increase in LH pulse amplitude was associated with the static phase of the follicle or whether it was associated with decreasing LH pulse frequency in the face of increasing progesterone concentrations (Hauger *et al.* 1977; Goodman and Karsch 1980; Wheaton *et al.* 1988). The present study investigated the characteristics of LH and FSH in relation to growth of follicular waves emerging in the mid- to late-luteal phase when serum progesterone concentrations were largely in a plateau phase.

In the present study, the diameter of the CL did not vary during the period analyzed (d -8 to d -1 before functional luteolysis) even though progesterone concentrations declined precipitously from d-2 to d-1 (Fig. 5.1). This indicated, in agreement with the observations in heifers (Erb *et al.* 1971; Kastelic *et al.* 1990), that functional luteolysis began well before morphological changes could be observed by ultrasonography. In the present study, considering the data aligned to functional luteolysis, the significant increase in LH pulse frequency and mean and basal concentrations of LH in association with functional luteolysis indicated an inverse relationship between serum concentrations of LH and progesterone. Such a relationship between progesterone and

pulsatile release of LH during the follicular phase of the ovine estrous cycle has been shown in previous studies (Hauger *et al.* 1977; Baird 1978; Wheaton *et al.* 1984; Rawlings and Cook 1993). The increased LH pulse amplitude between d -6 to d -2 before functional luteolysis (Fig. 5.2D) probably reflected the progesterone-driven decrease in LH pulse frequency (Goodman and Karsch 1980). A reciprocal relationship between LH pulse amplitude and frequency has been demonstrated in previous studies (Hauger *et al.* 1977; Karsch *et al.* 1979; Wheaton *et al.* 1984; Wheaton *et al.* 1988; Karsch *et al.* 1993; Bartlewski *et al.* 2000a). The patterns of mean and basal concentrations of FSH in the present study were more likely associated with follicular wave emergences which occurred during the luteal phase (penultimate and final follicular waves; Table 5.1). This observation confirms the lack of effects of progesterone on FSH concentrations demonstrated previously (McNatty *et al.* 1989b).

In the present study, mean and basal serum concentrations of FSH reached a peak on the day of follicular wave emergence (Fig. 5.3). A temporal relationship between an FSH peak and follicular wave emergence has been demonstrated in several studies in sheep (Ginther *et al.* 1995; 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). Picton *et al*., (1990) demonstrated that FSH stimulated follicular growth to ovulatory diameter in GnRH-agonist treated ewes. In a previous study from our laboratory (Duggavathi *et al.* 2004), we observed that treatment with exogenous oFSH to create a physiological peak in FSH concentrations stimulated the emergence of a new follicular wave in sheep; this unequivocally demonstrated a

functional relationship between peaks in daily serum FSH concentration and follicular wave emergence.

In the present study, the decline in mean and basal serum FSH concentrations in association with follicular growth and the concomitant increase in estradiol concentrations (Fig. 5.3) suggested a negative feedback effect of estradiol on FSH secretion. The negative feedback regulation of FSH by estradiol has been proposed by several authors (Mann *et al.* 1992a; Bartlewski *et al.* 1999a; Barrett *et al.* 2003). Inhibin has also been implicated in the regulation of FSH secretion (Rohan *et al.* 1991; Thomas *et al.* 1995; Souza *et al.* 1998; Knight *et al.* 1998; Findlay *et al.* 2000; Campbell and Baird 2001; Evans *et al.* 2002). Immunization against inhibin caused a greater increase in FSH concentrations than immunization against estradiol-17β (Mann *et al.* 1993). Also, unlike estradiol which is primarily produced by large estrogenic follicles (Scaramuzzi *et al.* 1970; Cox *et al.* 1971; Bjersing *et al.* 1972), inhibin is thought to be produced by a range of growing follicles (Mann *et al.* 1992a; Mann *et al.* 1992b). However, the ablation of either a large follicle or all visible follicles resulted in a similar decline in serum inhibin A concentrations (Evans *et al.* 2002). However, in a recent study (Duggavathi *et al.* 2004), a follicular wave induced by exogenous ovine FSH injection did not postpone the time of occurrence of the next endogenous FSH peak as compared to the vehicle treated ewes. The induced follicular wave was associated with a peak in estradiol concentration comparable to all other follicular waves (Duggavathi *et al.* 2004). In addition, treatment with estradiol-17β-implants, which increased mean daily serum estradiol concentration in treated ewes by 1.4 fold in relation to control ewes, did not alter FSH concentrations and follicular wave emergence (Barrett *et al.*

2003). These discrepancies clearly indicate that the roles of antral follicular estradiol and inhibin in the regulation of FSH are still equivocal and need to be clarified.

In addition to FSH, we were also interested in possible changes in the pulsatility of LH in relation to follicular growth. The basal LH concentrations tended to increase (P = 0.07) from the day of wave emergence (Fig. 5.3). However, the continued increase in basal LH concentrations in association with the end of the growth phase of the largest follicle could be demonstrated only for the data of the final follicular wave, not for the data of the penultimate wave (Fig. 5.4). These observations of the present study suggested that LH did not seem to be associated with the growth of the largest follicle of the wave, in agreement with our previous study (Bartlewski *et al.* 2000a). Even though the mean and basal concentrations of LH were associated with the static phase of the largest follicle of the final follicular wave of the cycle (but not of the penultimate wave; Fig. 5.4), the growth patterns of the largest follicles of the penultimate and final waves did not differ (Table 5.1). Thus, it is likely that the significant increase in LH concentrations associated with the static phase of the largest follicle of the final wave might be due to the coincidental decline in serum progesterone concentrations due to luteolysis. The end of the growth phase of the largest follicle of the final wave occurred around the time of the beginning of functional luteolysis (Fig. 5.1).

There have been several studies suggesting a role for LH in follicular growth in sheep (McNeilly *et al.* 1992; Picazo *et al.* 1996; Dobson *et al.* 1997; Abdennebi *et al.* 1999; Campbell *et al.* 1999). Withdrawal of FSH in the absence of LH resulted in atresia of follicles indicating a role for LH in continued follicular growth (Campbell *et al.* 1999). Increased LH frequency caused by a decrease in the serum progesterone concentration

resulted in the prolongation of the life span of the largest follicle of the first wave of the cycle in ewes (Vinoles *et al.* 1999). The secretion of both androstenedione and estradiol began to increase within 5 min of a pulse of LH, and elevated levels were maintained for about 2 h (Baird 1978; Martin 1984). However, a temporal association of an abrupt or short-term change in LH pulsatility in association with the growth of the largest follicle of a follicular wave has not been demonstrated in sheep during the first half of the cycle (Bartlewski *et al.* 2000a); or even in cattle (Ginther *et al.* 1998), where clear follicular selection, deviation/dominance have been demonstrated (Ginther *et al.* 1996).

In our previous study (Bartlewski *et al.* 2000a), an increase in LH pulse amplitude was shown to occur with the onset of the static phase of the first wave of the cycle in sheep. The confirmation of the significance of this increase for follicular growth was not then possible because of the increasing progesterone concentration associated with early luteal phase. In the present study, the lack of a significant increase in serum LH concentrations temporally associated with the end of the growth phase of the largest follicle in the penultimate wave suggest that increasing LH concentrations are not required for antral follicular growth. Nevertheless, there are reports that suggest the dependence of follicles on LH. In cattle, a follicle survives the declining phase of an FSH peak by changing its dependency to LH (Ginther *et al.* 1996). LH receptors are expressed in granulosa cells of ovine ovarian follicles in the diameter range of 4-5 mm (Logan *et al.* 2002), which constitute the stage of growth of follicles to an ovulatory diameter in a follicular wave (Bartlewski *et al.* 1999a; Duggavathi *et al.* 2003a). In addition, subluteal concentrations of progesterone associated with increased LH pulse frequency prolonged the lifespan of the largest follicle of the first wave of the ovine

estrous cycle (Taft *et al.* 1996; Vinoles *et al.* 1999). In heifers, LH had a positive effect on the diameter of the dominant follicle and influenced estradiol secretion by the dominant follicle (Ginther *et al.* 2001b). Therefore, it appears that although some LH treatments can influence follicular growth, there is no clear temporal association between LH secretory pattern and antral follicular growth in sheep (Bartlewski *et al.* 2000a). It appears that only basal concentrations of LH are required to support antral follicular growth.

In conclusion, changes in serum concentrations of FSH and LH in relation to follicular growth were studied in the ewe during a period of relatively constant progesterone concentration, from d 7 to d 14 after ovulation. There was a peak in mean and basal FSH concentrations on the day of follicular wave emergence of the final follicular wave during the luteal phase. This indicated, in agreement with previous studies, that FSH secretion is clearly associated with follicular growth. A lack of change in LH pulsatility in association with the emergence or growth of the largest follicle of a wave suggested that the LH-dependent survival of a follicle did not require changes in LH secretion and may involve changes in the sensitivity of the growing follicle to LH.

Chapter 6: PATTERNS OF ANTRAL FOLLICULAR WAVE DYNAMICS AND ACOMPANYING ENDOCRINE CHANGES IN CYCLIC AND SEASONALLY ANESTRSOUS EWES TREATED WITH EXOGENOUS OVINE FSH (oFSH) DURING THE INTER-WAVE INTERVAL*

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6.1. Abstract

In the ewe, ovarian follicular waves emerge every 4 to 5 d and are preceded by a peak in FSH secretion. It is unclear whether large antral follicle(s) in a wave suppress the growth of other smaller follicles during the inter-wave interval, as seen in cattle. In this study, anoestrous (N = 6; Experiment 1) and cyclic (N = 5; Experiment 2) ewes were given oFSH (0.5 µg/kg; 2 s.c. injections, 8 h apart) during the growth phase (based on ultrasonography) of a follicular wave (Wave 1). Control ewes (N = 5 and 6, respectively) received vehicle. In oFSH-treated ewes, serum FSH concentrations reached a peak ($P < 0.05$) by 12 h after oFSH treatment and this induced FSH peak did not differ ($P > 0.05$) from the endogenous FSH peaks. In all ewes, emergence of follicular Waves 1 and 2 was seen ($P > 0.05$). However, in oFSH-treated ewes, an additional follicular wave emerged ~0.5 d after treatment; during the inter-wave interval of Waves 1 and 2 without delaying the emergence of Wave 2. The growth characteristics and serum estradiol concentrations did not differ ($P > 0.05$) between oFSH-induced waves and waves induced by endogenous FSH peaks. We concluded that, unlike in cattle, the largest follicle of a wave in sheep has limited direct effect on the growth of other follicles induced by exogenous oFSH. In addition, the largest follicle of a wave may possibly not influence the rhythmicity of follicular wave emergence, as it does in cattle.

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6.2. Introduction

In cattle, ovarian antral follicles grow in a wave-like pattern, with 2 to 3 waves emerging from the pool of small follicles in the ovary during each estrous cycle (Ginther *et al.* 2003). During each wave, around 7 to 11 small follicles (4 mm in diameter) enter a common-growth phase of about 3 days (Ginther *et al.* 2003). Following the common-growth period, one follicle of the cohort grows rapidly to attain an ovulatory diameter (dominant follicle; Ginther *et al.* 2003), suppressing the growth of other follicles (subordinate follicles) and preventing emergence of a new follicular wave (Armstrong and Webb 1997). Each antral follicular wave is stimulated by an increase in FSH secretion (Adams *et al.* 1992) caused by the regression of the dominant follicle of the previous wave (Ginther *et al.* 2002). The dominant follicle subsequently acquires LH dependency for its own continued growth and suppresses FSH secretion starving subordinate follicles of sufficient FSH support (Pierson and Ginther 1988; Armstrong and Webb 1997; Austin *et al.* 2002; Ginther *et al.* 2003). However, there is evidence of a direct inhibitory effect of the dominant follicle on the remaining follicles (Armstrong and Webb 1997). Superovulatory treatments started in the presence of the dominant follicle in cows are less successful than in the absence of such a follicle (Maciel *et al.* 1995; Kafi and McGowan 1997). It has also been shown that treatments with physiological or even superphysiological concentrations of FSH given in the presence of a growing dominant follicle in cattle, fail to elicit the emergence of a follicular wave (Guilbault *et al.* 1991; Adams *et al.* 1993).

Ovarian antral follicles reaching ovulatory diameters of 5-7 mm in diameter grow in a wave-like pattern in cyclic (Souza *et al.* 1998; Vinoles *et al.* 1999; Bartlewski *et al.* 1999a; Evans *et al.* 2000; Duggavathi *et al.* 2003a) and seasonally anestrous (Souza *et al.*

1996; Bartlewski *et al.* 1998; Evans *et al.* 2001) sheep. In the ewe, a follicular wave has been defined as one or more follicles that emerge from a pool of small (2-3 mm in size) antral follicles and grow to ≥ 5-mm in diameter before regression or ovulation (Bartlewski *et al.* 1998; Bartlewski *et al.* 1999a; Duggavathi *et al.* 2003a). There are typically 3 to 4 waves during each ovine estrous cycle with waves emerging every 3-5 days (Bartlewski *et al.* 1999a; Duggavathi *et al.* 2003a). Transient peaks in mean serum FSH concentrations have been shown to proceed wave emergence (Bartlewski *et al.* 1998; Bartlewski *et al.* 1999a; Evans *et al.* 2000; Evans *et al.* 2001; Duggavathi *et al.* 2003a).

Evidence in sheep for the existence of the powerful follicular dominance seen in cattle is equivocal (Scaramuzzi *et al.* 1993; Driancourt 2001; Evans 2003b). There was no increase in the number of small (1-3 mm in diameter) antral follicles at the onset of a follicular wave in ewes and no obvious subordinate follicles (Duggavathi *et al.* 2003a). Co-culturing large and small ovine antral follicles did not result in atresia of the small follicles (Driancourt *et al.* 1991). In the ewe, two or more follicles have been shown to grow in a wave and antral follicles from the final and penultimate waves of the estrous cycle have been shown to ovulate at the same time (Bartlewski *et al.* 1999a; Evans 2003b; Bartlewski *et al.* 2003). However, when cycling ewes were treated with low levels of progesterone for several days, the lifespan of large antral follicles was prolonged and emergence of follicular waves was suppressed (Vinoles *et al.* 1999). Increased LH secretory pulsatility during the luteal phase of the estrous cycle also prolonged the lifespan of the largest antral follicles in ewes (Johnson *et al.* 1996; Dobson *et al.* 1997; Vinoles *et al.* 1999). Ablation of the largest antral follicles in the ewe is followed by a

peak in FSH secretion, but not by synchronous emergence of a new follicular wave (Evans *et al.* 2002). Superovulatory treatment with FSH in ewes, when a large antral follicle was present, resulted in a reduced ovulatory response compared with treatment applied in the absence of a large follicle; however, the presence of a large follicle did not reduce the number of large follicles growing as a result of treatment (Rubianes *et al.* 1997).

The objective of the present study in ewes was to see if, as demonstrated in cattle (Adams *et al.* 1993), the presence of a large growing antral follicle(s) would inhibit the emergence of a new follicular wave in response to a physiological dose of oFSH. We hypothesized that, as appears to be the case in cattle (Adams *et al.* 1993), at least part of the ability of a growing (dominant) follicle to suppress the growth and emergence of other follicles is by a direct effect (i.e., it can not be overcome with exogenous oFSH administered during the growth phase of the largest follicle of a wave). In anestrous ewes, estradiol and presumably inhibin production by large antral follicles are lower compared with cyclic ewes (Yuthasastrakosol *et al.* 1975), which may influence the occurrence and/or degree of follicular dominance (Evans 2003b). Therefore, the present experiment was performed on both cyclic and seasonally anestrous ewes.

6.3. Materials and methods

6.3.1. Experiment 1 (anestrus)

The experimental procedures were performed according the guidelines of the Canadian Council on Animal Care. Eleven adult, anestrous Western White Face ewes (mean body weight: 75.3 ± 1.9 kg) were used in the present study during mid-anestrus (June-July).

Ewes were kept in sheltered pens and fed a maintenance diet of hay, with water and cobalt iodized salt bars available *ad libitum*.

Hormone preparation

The oFSH used was NIDDK-oFSH-18. Each 1 mg of oFSH has a biological potency of FSH equivalent of 65.6 × NIH-oFSH-S1 or 1640 IU and biological potency of LH equivalent of 0.1 × NIH-oLH-S1 or 106 IU. The oFSH injections were prepared in saline with 0.05% BSA (w/v; Sigma®, St. Louis, USA) and 50% Polyvinyl-pyrrolidone (w/v; Sigma®, St. Louis, USA).

Treatment administration

Treatment involved two subcutaneous injections of either oFSH (0.5 μg/kg) or vehicle given 8 h apart. This treatment regimen was based on preliminary trials (involved different doses of oFSH and different time intervals between two injections) and was designed to result in an FSH peak of physiological amplitude (Bartlewski *et al.* 1999a; Bartlewski *et al.* 1999b). In preliminary trials oFSH injections did not result in any increase in serum LH concentrations ($P > 0.05$). Six ewes were injected with oFSH (oFSH-treated group) and 5 control ewes were injected with vehicle only.

A 4-mm follicle, which grew from the pool of 2-3 mm follicles, was detected with ultrasonography and it was designated as a Wave 1 follicle. Such a follicle had to have emerged 3-5 d after the emergence of the previous follicular wave (i.e., at the normal time interval for waves in ewes; (Bartlewski *et al.* 1999a)). The first injection of oFSH or vehicle was given 24 h after the detection of the 4-mm follicle of Wave 1, but only if this

follicle remained at 4 mm or grew further. The second injection was given 8 h after the first. Timing of the treatment was designed to give oFSH injections during the growth phase of the largest follicle of Wave 1 and the expected time of the inter-wave nadir in endogenous FSH secretion.

6.3.2. Experiment 2 (breeding season)

Eleven adult, cyclic Western White Face ewes (mean body weight = 81.2 ± 2.3 kg; maintained as explained in Experiment 1) were used in the present study during the breeding season (October-November). Estrus detection was done twice daily (0800 and 2000 h) using a crayon harnessed vasectomized rams.

Treatment administration

Treatment involved two subcutaneous injections of either oFSH (0.5 μg/kg) or vehicle given 8 h apart (see Experiment 1 for details). Five ewes were injected with oFSH (oFSH-treated group) and 6 control ewes were injected with only vehicle.

A 4-mm follicle, which grew from the pool of 2-3 mm follicles, was detected with ultrasonography and it was designated as a Wave 1 of the estrous cycle. Such a follicle had to have emerged around the day of ovulation (i.e., mean day of emergence of the first wave of the cycle (Bartlewski *et al.* 1999a; Duggavathi *et al.* 2003a)). The first injection of oFSH or vehicle was given 12 h after the detection of the 4-mm follicle of Wave 1, but only if this follicle remained at 4 mm or grew further. The second injection was given 8 h after the first. The timing of the oFSH injection in Experiment 2 (cyclic ewes) was advanced by 12 h as compared to Experiment 1 (anestrous ewes) to create an FSH peak

more precisely during the period corresponding to the nadir in endogenous FSH secretion and during middle of the growth phase of the largest follicle of a wave (Wave 1).

6.3.3. Transrectal ovarian ultrasonography and blood sampling

Ewes, in both Experiments 1 and 2, were scanned twice daily (0800 and 2000 h), using a high-resolution real time B-mode ultrasound equipment (Aloka SSD-900; Aloka Co. Ltd., Japan) connected to a 7.5-MHz transducer, until a follicular wave emerging after treatment was identified (i.e. when a new follicle that could be tracked back to 2-3-mm diameter began to grow and reached ≥5 mm in diameter after treatment). Subsequently, ewes were scanned daily until the largest follicle of the wave emerging after treatment regressed to 2-3 mm in diameter (i.e., end of the follicular regression phase). During each scanning session, relative position and diameter of all follicles ≥1 mm in diameter and corpora lutea were sketched on ovarian charts. In addition, all ovarian images were recorded on high-grade video tapes (Fuji S-VHS, ST-120 N) for retrospective analysis of ovarian data. Blood samples (10 ml) were collected from all ewes by jugular venipuncture into vacutainers (Becton Dickinson, Rutherford, NJ, USA), just before each scanning session.

6.3.4. Hormone assays

Blood samples were allowed to clot for 18-24 h at room temperature and serum was harvested and stored at –20 °C until assayed. Serum samples were analyzed by validated radioimmunoassay for circulating concentrations of FSH (Currie and Rawlings 1989) and estradiol (Joseph *et al.* 1992). The ranges of the standard curves were: 0.12 to16 ng/ml for FSH and 1.0 to 50 pg/ml for estradiol. The sensitivities of assays (defined as the lowest concentration of hormone capable of significantly displacing labelled hormone

from the antibody; unpaired *t*-test, $P < 0.05$) were: 0.1 ng/ml and 1 pg/ml for FSH and estradiol respectively. The intra-assay coefficients of variation (all samples were analyzed in a single assay) were 11.6% or 2.9% respectively, for reference sera with mean FSH concentration of 0.39 ng/ml or 1.50 ng/ml. The intra- and inter-assay of coefficients of variation were 16.5 and 7.8% or 14.5 and 8.9%, respectively for sera with mean estradiol concentration of 3.5 or 12.0 pg/ml. Peaks in daily serum concentrations of FSH and estradiol were determined using the cycle-detection program (Clifton and Steiner 1983); periods of every 12 or 24 h blood sampling were analyzed separately to avoid false positive peaks because of infrequent sampling.

6.3.5. Data analysis

A follicular wave consists of a follicle or a group of follicles that emerge and grow from 2 or 3 mm in diameter to ≥5 mm (growth phase) and remain at their maximum diameter (static phase) before regression to 2 or 3 mm in diameter (regression phase; Bartlewski *et al.* 1999a). Emergence was restricted to a 24-h period (Duggavathi *et al.* 2003a). The follicular wave during whose growth phase the treatment was administered was designated as Wave 1. Any follicular wave induced by treatment was designated as Wave A. The follicular wave emerging after the induced wave (oFSH treated ewes) or the follicular wave emerging after 4 to 5 d after the emergence of Wave 1 (control ewes) was designated as Wave 2. The time of follicular wave emergence was determined in relation to time of first injection of treatments.

The inter-wave interval was 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) of two consecutive follicular waves. The length of the growth, static and regression phases were

considered for Waves 1 and A, but only the growth phase for Wave 2; not all ewes had a regressing follicle in Wave 2 on the last day of the experiments. The mean daily numbers of small follicles (≥1 mm but ≤ 3 mm in diameter) were centralized to the time of first injection of the treatment and analyzed for the period from 2 d before to 2 d after the time of injection.

Mean serum concentrations of FSH were aligned to the time of first injection of oFSH or vehicle and analyzed for the period from 2.5 d before to 7 d after the treatment. The peak serum concentrations of estradiol following emergence of each follicular wave and the interval between the time of follicular wave emergence and the time of the peak in serum estradiol concentrations were calculated for all ewes.

6.3.6. Statistical analyses

Serum FSH concentrations and daily numbers of small follicles were analyzed, for effects of Time, Group and Time x Group, by two-way repeated measures analysis of variance (RM ANOVA; SigmaStat® Statistical Software, version 2.0 for Windows® 95, NT and 3.1, 1997; Chicago, IL, USA). The characteristics of estradiol peaks and various follicular parameters for Waves 1 and 2 were compared between oFSH-treated and control ewes by two-way RM ANOVA. Additionally, comparisons of Waves1, A and 2 detected in oFSH-treated ewes were made by one-way RM ANOVA. Multiple comparisons were made by the method of Fisher's least significant difference. Results are reported as least square means ± SEM. Statistical significance was defined as $P < 0.05$.

6.4. Results

6.4.1. Experiment 1

Administration of exogenous oFSH

Injections of oFSH or vehicle were given to oFSH-treated and control ewes at 1.7 ± 0.2 and 2.1 ± 0.1 d after emergence, respectively ($P > 0.05$), and at 0.9 ± 0.4 and 0.4 ± 0.2 d before the end of the growth phase of the largest follicle of Wave 1, respectively ($P > 0.05$). At the time of treatment, oFSH-treated and control ewes had basal serum FSH concentrations of 1.6 ± 0.2 and 1.4 ± 0.2 ng/ml, respectively ($P > 0.05$; Fig. 6.1A). Analysis of serum FSH concentrations from 2.5 d before to 7 d after the first injection of oFSH or vehicle (time 0) showed no main effect of Group ($P > 0.05$), but a significant effect of Time and a Group x Time interaction ($P < 0.05$). Serum concentrations of FSH were higher ($P < 0.05$) in oFSH-treated compared to control ewes at 12 and 24 h after the first injection (Fig. 6.1A). The mean concentration of induced FSH peak (2.8 ± 0.2 ng/ml) in oFSH-treated ewes did not differ ($P > 0.05$) from that of the endogenous FSH peak (2.4 ± 0.2 ng/ml), which immediately preceded the emergence of Wave 1. The mean duration of the induced FSH peak (nadir-to-nadir) was 1.6 ± 0.1 d. The cycle detection analyses revealed 2 peaks in serum FSH concentrations in both oFSH-treated and control ewes; these peaks occurred at the same time in the ewes of both groups (Fig. 6.1A) and preceded the emergence of Waves 1 and 2, respectively (Table 6.1). An additional peak (preceding Wave A) was seen at 0.5 ± 0.0 d after the first injection of oFSH only in oFSH-treated ewes (Table 6.1; Fig. 6.1A).

Follicular wave emergence

The mean time of wave emergence, in relation to the time of treatment (d 0), for Waves 1 and 2 did not differ between oFSH-treated and control ewes ($P > 0.05$; Table 6.1; Fig. 6.2A). An additional wave (Wave A) emerged at 0.6 ± 0.2 d after the first injection of oFSH in oFSH-treated ewes (Table 6.1; Fig. 6.2A). The mean day of emergence for Wave A was 2.2 ± 0.2 d after emergence of Wave 1 and 0.5 ± 0.2 d before the end of the growth phase of the largest follicles in this wave in oFSH-treated ewes.

Estradiol concentrations

There was no significant effect ($P > 0.05$) of Wave, Group or a Wave x Group interaction for the mean interval between the day of follicular wave emergence and the day on which peak serum estradiol concentrations occurred, or the mean peak estradiol concentrations, following the emergence of follicular Waves 1 and 2, in both oFSH-treated and control ewes (Table 6.1). There were no significant differences ($P > 0.05$) for the parameters above between follicular Waves 1, A and 2 in oFSH-treated ewes (Table 6.1).

Characteristics of the largest follicle of the wave

There was no significant effect ($P > 0.05$) of Wave, Group or a Wave x Group interaction for the length of the growth, static and regression phases, or for mean growth rates and maximum diameter of the largest follicles of Waves 1 and 2, in both oFSH-treated and control ewes (Table 6.2). There was no significant effect ($P > 0.05$) of Wave for the parameters above, for Waves 1, A and 2 in oFSH-treated ewes (Table 6.2).

Numbers of small antral follicles

There was no significant effect of Time, Group or a Time x Group interaction ($P > 0.05$) for mean numbers of small follicles (≥1 mm but ≤3 mm in diameter) analyzed for the period from 2 d before to 2 d after the first injection of oFSH or vehicle (d 0; Fig. 6.2A). The numbers of small follicles at any time point ranged from 11 to 13 in oFSH-treated ewes and from 10 to 13 in control ewes.

6.4.2. Experiment 2

Administration of exogenous oFSH

Injections of oFSH or vehicle were given to oFSH-treated and control ewes at 1.2 ± 0.1 and 1.3 ± 0.1 d after emergence, respectively ($P > 0.05$) and 1.5 ± 0.2 and 1.8 ± 0.3 d before the end of growth phase of the largest follicle of Wave 1 of the cycle, respectively ($P > 0.05$). At the time of treatment, oFSH-treated and control ewes had basal serum FSH concentrations of 2.4 ± 0.3 and 2.1 ± 0.4 ng/ml, respectively ($P > 0.05$; Fig. 6.1B). Analysis of serum FSH concentrations from 2.5 d before to 7 d after the first injection of oFSH or vehicle (time 0) showed a significant main effect of Group and Time, and a Group x Time interaction ($P < 0.05$). Serum concentrations of FSH were higher ($P < 0.05$) in oFSH-treated ewes compared to control ewes at 12, 24 and 36 h after the first injection (Fig. 6.1B). The mean concentrations of induced FSH peaks (3.6 ± 0.3 ng/ml) in oFSH-treated ewes did not differ ($P > 0.05$) from the endogenous FSH peak (3.9 ± 0.3 ng/ml), which immediately preceded the emergence of Wave 1 of the cycle. The mean duration of the induced FSH peak (nadir-to-nadir) was 1.7 ± 0.1 d. The cycle detection analyses revealed 2 peaks in serum FSH concentrations in both oFSH-treated and control

ewes; these peaks occurred at the same time in the ewes of both groups (Fig. 6.1B) and preceded the emergence of Waves 1 and 2, respectively (Table 6.3). An additional peak (preceding Wave A) was seen at 0.5 ± 0.0 d after the first injection of oFSH only in oFSH-treated ewes (Table 6.3; Fig. 6.1B).

Follicular wave emergence

The mean time of wave emergence, in relation to the time of treatment (d 0), for Waves 1 and 2 did not differ between oFSH-treated and control ewes ($P > 0.05$; Table 6.3; Fig. 6.2B). There was an additional wave (Wave A) that emerged at 0.5 ± 0.0 d after the first injection of oFSH only in oFSH-treated ewes ($P < 0.05$; Table 6.3; Fig. 6.2B). The mean day of emergence of Wave A was 1.8 ± 0.1 d after the emergence of Wave 1 and 1.0 ± 0.2 d before the end of the growth phase of the largest follicles of this wave in oFSH-treated ewes.

Estradiol concentrations

There was no significant effect ($P > 0.05$) of Wave, Group or a Wave x Group interaction for the interval between the day of follicular wave emergence and the day of peak serum estradiol concentrations, or for peak estradiol concentrations following the emergence of Waves 1 and 2, in both oFSH-treated and control ewes (Table 6.3). There was no significant effect ($P > 0.05$) of Wave on the parameters above for follicular Waves 1, A and 2 in oFSH-treated ewes (Table 6.3).

Characteristics of the largest follicle of the wave

There was no significant effect ($P > 0.05$) of Wave, Group or a Wave x Group interaction for the length of the growth, static and regression phases, or for mean growth rate and maximum diameter of the largest follicles of Waves 1 and 2, in both oFSH-treated and control ewes (Table 6.4). There was no significant effect ($P > 0.05$) of Wave for the parameters above, for Waves 1, A and 2 in oFSH-treated ewes (Table 6.4).

Numbers of small follicles

There was no significant effect ($P > 0.05$) of Time, Group or a Time x Group interaction for mean numbers of small follicles (≥1 mm but ≤3 mm in diameter) analyzed for the period from 2 d before to 2 d after the first injection of oFSH or vehicle (d 0; Fig. 6.2B). The numbers of small follicles at any time point ranged from 9 to 13 in oFSH-treated ewes and from 11 to 15 in control ewes.

Table 6.1. FSH peaks and characteristics of follicle waves that emerged before (Wave 1) and after (Waves A and 2) treatment with either a physiological dose of oFSH or vehicle, administered 24 h after the largest follicle of a wave (Wave 1) reached 4 mm in diameter, in oFSH-treated (N = 6) and control ewes (N = 5) during anestrus (Experiment 1).

Parameter	**Group**	**Wave 1**	**Wave A**	**Wave 2**
Day of FSH peak as detected by cycle detector (d 0 = day of treatment)	FSH-treated	-2.0 ± 0.2^{aA}	0.5 ± 0.0^{b}	2.7 ± 0.2^{cB}
	Control	-2.0 ± 0.2^{aA}	No peak	3.0 ± 0.2^{bB}
Day of follicle wave emergence (d 0 = day of treatment)	FSH-treated	-1.9 ± 0.2^{aA}	0.6 ± 0.2^{b}	3.0 ± 0.2^{cB}
	Control	-2.2 ± 0.3^{aA}	Did not emerge	2.8 ± 0.3^{bB}
Interval between the day of wave emergence and day of estradiol peak	FSH-treated	1.9 ± 0.1	1.9 ± 0.2	2.3 ± 0.2
	Control	2.2 ± 0.1	No Wave A	2.3 ± 0.1
Estradiol concentration at peak after wave emergence	FSH-treated	4.8 ± 0.4	4.9 ± 0.4	4.6 ± 0.2
	Control	5.0 ± 0.5	No peak	4.9 ± 0.3

$^{a, b, c}$ Denote significant difference ($P < 0.05$) for a parameter among different waves within a row.
$^{A, B}$ Denote significant difference ($P < 0.05$) for a parameter between two groups within a column.

Table 6.2. Characteristics of the largest follicle of the waves that emerged before (Wave 1) and after (Waves A and 2) treatment with either a physiological dose of oFSH or vehicle, administered 24 h after the largest follicle of a wave (Wave 1) reached 4 mm in diameter, in oFSH-treated (N = 6) and control ewes (N = 5) during anestrus (Experiment 1).

Parameter	**Group**	**Wave 1**	**Wave A**	**Wave 2**
Growth phase (days)	FSH-treated	2.7 ± 0.2	2.1 ± 0.2	2.3 ± 0.2
	Control	2.2 ± 0.3	No Wave A	2.5 ± 0.3
Static phase (days)	FSH-treated	1.8 ± 0.4	2.6 ± 0.4	Not Determined
	Control	3.2 ± 0.4	No Wave A	3.5 ± 0.4
Regression phase (days)	FSH-treated	2.9 ± 0.3	2.9 ± 0.3	Not Determined
	Control	2.1 ± 0.3	No Wave A	3.0 ± 0.3
Maximum diameter (mm)	FSH-treated	5.2 ± 0.3	5.2 ± 0.3	5.6 ± 0.3
	Control	5.1 ± 0.3	No Wave A	5.1 ± 0.4
Growth rate (mm/day)	FSH-treated	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1
	Control	1.3 ± 0.2	No Wave A	1.2 ± 0.2

Table 6.3. FSH peaks and characteristics of follicle waves that emerged before (Wave 1) and after (Waves A and 2) treatment with either a physiological dose of oFSH or vehicle, administered 12 h after the largest follicle of Wave 1 of the estrous cycle reached 4 mm in diameter, in oFSH-treated (N = 5) and control ewes (N = 6) during breeding season (Experiment 2).

Parameter	**Group**	**Wave 1**	**Wave A**	**Wave 2**
Day of FSH peak as detected by cycle detector (d 0 = day of treatment)	FSH-treated	-1.7 ± 0.2^{aA}	0.5 ± 0.0^{b}	3.4 ± 0.2^{cB}
	Control	-1.5 ± 0.1^{aA}	No peak	3.7 ± 0.2^{bB}
Day of follicle wave emergence (d 0 = day of treatment)	FSH-treated	-1.2 ± 0.2^{aA}	0.5 ± 0.0^{b}	3.7 ± 0.3^{cB}
	Control	-1.2 ± 0.1^{aA}	Did not emerge	3.9 ± 0.2^{bB}
Interval between the day of wave emergence and day of estradiol peak	FSH-treated	2.3 ± 0.1	2.8 ± 0.2	2.7 ± 0.3
	Control	2.8 ± 0.3	No Wave A	2.7 ± 0.1
Estradiol concentration at peak after wave emergence	FSH-treated	5.5 ± 1.5	5.8 ± 1.7	6.3 ± 1.5
	Control	5.3 ± 0.5	No peak	5.5 ± 0.4

$^{a, b, c}$ Denote significant difference ($P < 0.05$) for a parameter among different waves within a row.
$^{A, B}$ Denote significant difference ($P < 0.05$) for a parameter between two groups within a column.

Table 6.4. Characteristics of the largest follicle of the waves that emerged before (Wave 1) and after (Waves A and 2) treatment with either a physiological dose of oFSH or vehicle, administered 12 h after the largest follicle of Wave 1 of the estrous cycle reached 4 mm in diameter, in oFSH-treated (N = 5) and control ewes (N = 6) during breeding season (Experiment 2).

Parameter	**Group**	**Wave 1**	**Wave A**	**Wave 2**
Growth phase (days)	FSH-treated	2.5 ± 0.2	2.5 ± 0.2	2.8 ± 0. 3
	Control	2.8 ± 0.3	No Wave A	3.1 ± 0.2
Static phase (days)	FSH-treated	3.1 ± 0.4	3.2 ± 0.4	Not Determined
	Control	3.0 ± 0.1	No Wave A	Not Determined
Regression phase (days)	FSH-treated	3.4 ± 0.1	3.9 ± 0.4	Not Determined
	Control	3.1 ± 0.3	No Wave A	Not Determined
Maximum diameter (mm)	FSH-treated	5.9 ± 0.4	5.7 ± 0.4	5.3 ± 0.4
	Control	5.7 ± 0.5	No Wave A	5.6 ± 0.5
Growth rate (mm/day)	FSH-treated	1.2 ± 0.2	1.1 ± 0.2	1.3 ± 0.2
	Control	1.2 ± 0.2	No Wave A	1.2 ± 0.1

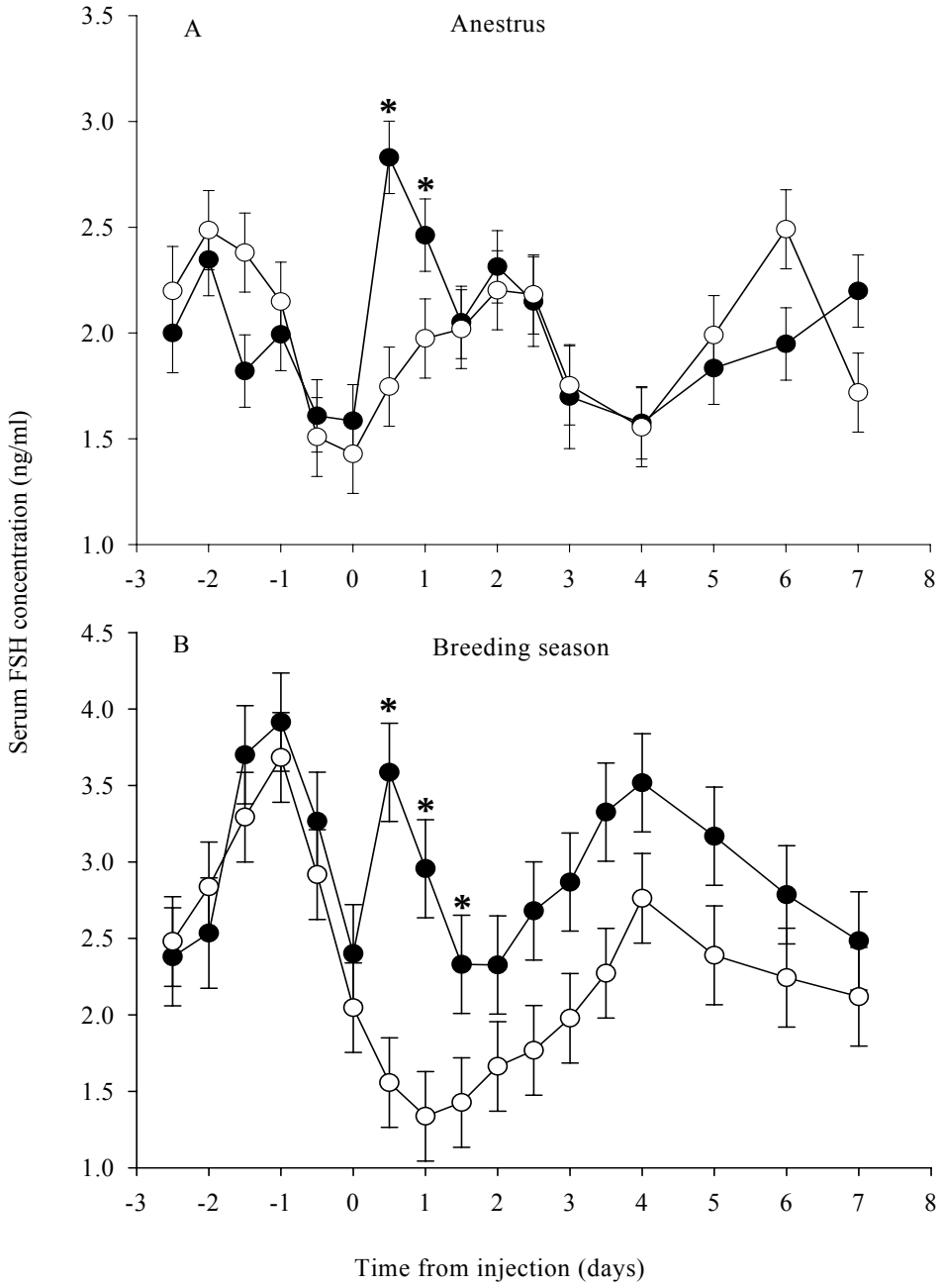

Figure 6.1. Mean circulating concentrations of FSH in oFSH-treated (•) and control (o) ewes. FSH concentrations were centralized to the time of the first injection (d 0) of the two injections oFSH or vehicle given 8 h apart, and were considered from 2.5 d before to 7 d after the first injection. First injections were given 24 h (A; non-breeding season; Experiment 1) or 12 h (B; breeding season; Experiment 2) after the largest follicle of a wave (non-breeding season) or the first wave of the estrous cycle (breeding season) reached ≥4 mm in diameter (expected time of nadir in endogenous FSH concentrations). * Significant difference between the two groups ($P<0.05$).

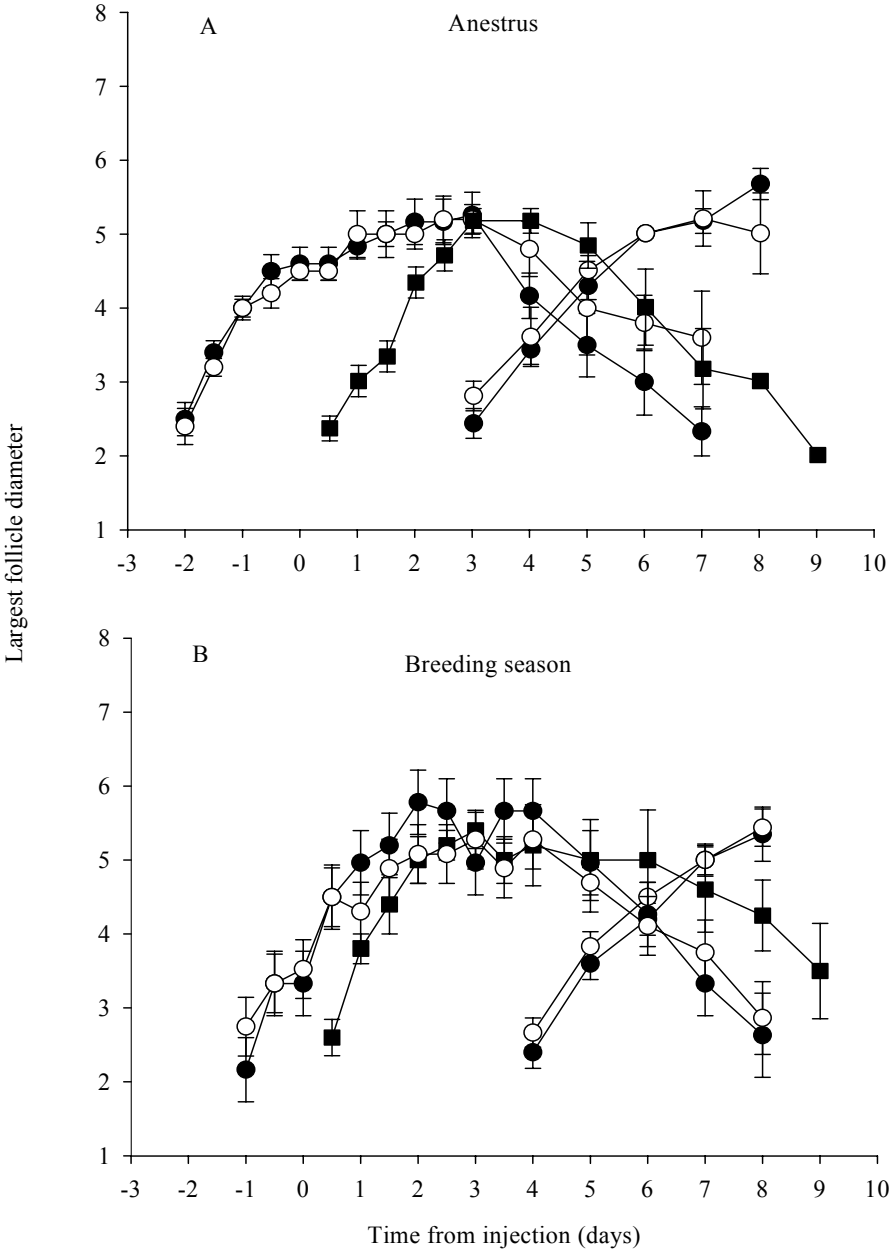

Figure 6.2. Mean diameter profiles of the largest follicle of Waves 1 (●), 2 (●) (normal endogenous waves) and A (■) (oFSH-induced wave) in oFSH-treated ewes and Waves 1 (o) and 2 (o) in control ewes. The diameters of the largest follicle of all waves were centralized to the time of the first injection (d 0) of the two injections of oFSH or vehicle given 8 h apart. The mean diameter profiles are shown from the mean day of emergence to the mean day of regression of follicular waves in relation to the time of first injection. First injections were given 24 h (A; non-breeding season; Experiment 1) or 12 h (B; breeding season; Experiment 2) after the largest follicle of a wave (non-breeding season) or the first wave of the estrous cycle (breeding season) reached ≥4 mm in diameter (expected time of nadir in endogenous FSH concentrations).

6.5. Discussion

In both experiments, the induced-FSH peak occurred before the next endogenous FSH peak in both oFSH-treated and control ewes (Fig. 6.1A and B). The FSH peak created by the treatment regimen, in both Experiment 1 and 2, was similar in amplitude to endogenous FSH peaks (Fig. 6.1A and B). The duration of the created-peak (nadir-to-nadir) was about 1.5 d, which was shorter than the duration of endogenous FSH peaks for both cyclic (Bartlewski *et al.* 1999a) and anestrous (Bartlewski *et al.* 1998) ewes. The mean duration of FSH fluctuations during the breeding season and anestrus in Western White Face ewes is 3 to 4 d (Bartlewski *et al.* 1998; Bartlewski *et al.* 1999a). However, the FSH peaks created in the present study consistently resulted in the emergence of new follicular waves, which appeared to contain physiologically normal follicles, in terms of their growth characteristics and estradiol secretion (Tables 6.2 and 6.4). These observations and the recent finding in our laboratory that truncation of amplitude of FSH peaks by supraphysiological doses of estradiol blocked follicular wave emergence in cyclic ewes (Barrett *et al.* 2003), suggest the importance of FSH peak amplitude, but not duration, for follicular wave emergence in the ewe.

In both present experiments, treatment with oFSH induced the emergence of a new follicular wave (Fig. 6.2) in the presence of a large growing follicle of a previous wave (i.e., the wave in which treatment was applied; Wave 1). In cattle, injection of a superovulatory dose of recombinant bovine FSH (rbFSH) on d 5 of the estrous cycle (i.e., after the selection of the dominant follicle of the first wave of the cycle) did not rescue subordinate follicles from atresia nor did it stimulate the emergence of a new follicular wave (Adams *et al.* 1993). Although there is much evidence that ovarian follicular

dominance in cattle involves suppression of FSH secretion (Ginther *et al.* 2002), the findings of Adams *et al.* (1993) described above do not allow us to eliminate a direct effect of the dominant follicle on smaller, or subordinate follicles. It has been suggested that ovarian follicular dominance could involve local inhibitory or regulatory factors (Armstrong and Webb 1997; Ginther *et al.* 2002). Based on the present results, however, the growing follicle of a wave in the ewe did not appear to directly inhibit the emergence and growth of smaller follicles stimulated by exogenous oFSH.

Several authors have postulated the existence of follicular dominance in cyclic ewes (Scaramuzzi *et al.* 1993; Lopez-Sebastian *et al.* 1997; Campbell *et al.* 1999; Evans *et al.* 2000; Gonzalez-Bulnes *et al.* 2001; Evans 2003b), however, other, especially more recent, evidence would bring this postulate into question. Follicular waves have been frequently found to emerge in the presence of growing ovulatory-sized follicles from a previous wave in cyclic (Johnson *et al.* 1996; Leyva *et al.* 1998b; Bartlewski *et al.* 1999a; Flynn *et al.* 2000) and anestrous ewes (Evans *et al.* 2001). Several reports have indicated a lack of any temporal relationship between follicular wave emergence and estradiol secretion during the anestrous period in sheep (Bartlewski *et al.* 1998; Evans *et al.* 2001), although follicular wave emergence was maintained and associated with rhythmic FSH peaks across anestrus (Bartlewski *et al.* 1998; Evans *et al.* 2001). A large (presumably dominant) follicle did not inhibit eCG-induced growth or function of other follicles in ewes (Driancourt *et al.* 1991). Co-culturing small follicles with large follicle, in sheep, in a closed system did not decrease thymidine incorporation by granulosa cells of the small follicles as compared to those of small follicles cultured alone (Driancourt *et al.* 1991). Recently, it has been demonstrated that follicles from the final and penultimate waves of

the estrous cycle in sheep can ovulate together and form healthy corpora lutea (Bartlewski *et al.* 1999a; Evans 2003b;Bartlewski *et al.* 2003). Therefore, the largest follicles of waves in the ewe may not exert the same functional dominance as is seen in cattle.

Injection of oFSH in the present study did not alter the number of small antral follicles. The presence of experimentally induced large follicles in sheep has been shown to reduce the number of small follicles and block the emergence of follicular waves (Vinoles *et al.* 1999). However, in that experimental approach (Vinoles *et al.* 1999), the characteristics of the follicles with prolonged lifespan, were essentially non-physiological in that they grew to unusually large diameters and did not produce estradiol throughout the period of their apparent dominance. In addition, in a recent ultrasonographic study, there was no increase in the numbers of small follicles during periods encompassing follicular wave emergence in cyclic ewes (Duggavathi *et al.* 2003a). Follicular wave emergence in cattle is clearly associated with a transient increase in numbers of small, 3-4-mm follicles, from which the dominant follicle is selected (Gong *et al.* 1993; Ginther *et al.* 1996); this would not appear to be the case in sheep.

In addition, injection of oFSH in the present study did not affect the development of Wave 1 follicles, as neither the growth of the largest follicle or estradiol secretion during the growth of Wave 1 was affected by the treatment (Fig. 6.2A and B; Table 6.1-6.4). The lack of effect of FSH on large growing follicles has been previously demonstrated in sheep (Rubianes *et al.* 1997; Driancourt 2001) and in cattle (Adams *et al.* 1993).

In cattle, the FSH peak that stimulates the emergence of a follicular wave occurred late in the static phase of the dominant follicle of the previous wave (Adams *et al.* 1992; Gibbons *et al.* 1997) when it was undergoing atresia (Singh and Adams 2000). In sheep, a follicular wave emerged at the end of the static phase or early regression phase of the largest follicle from the previous wave (Evans *et al.* 2000). If an apparent dominant follicle, in sheep, strictly regulated the inter-wave interval by direct (follicle to follicle) or indirect (by regulating FSH secretion) means, then follicular waves should not be able to emerge at different times in the life span of a previous follicular wave. However, in the present study, the follicular wave induced by exogenous oFSH (Wave A) emerged during the growth phase of the previous wave (Wave 1), and did not disrupt the rhythmic pattern of occurrence of the next endogenous FSH peaks (Tables 6.1 and 6.3) and the emergence of follicular wave (Wave 2; Tables 6.1 and 6.3; Fig. 6.2A and B). In addition, it is interesting that the second follicular waves (Waves 2) stimulated by endogenous FSH peak emerged at the end of growth phase of the oFSH-induced waves (Wave A; Fig. 6.2A and B), the period at which its estradiol secretory ability is expected to be maximal (Cox *et al.* 1971; Bjersing *et al.* 1972; Baird *et al.* 1976a; Scaramuzzi and Baird 1977; Souza *et al.* 1996; Evans *et al.* 2000). This again brings into question the importance of follicular dominance as a regulator of follicular dynamics in the ewe.

In summary, in cyclic and anestrous ewes, the largest growing follicle of a wave failed to inhibit the stimulation of emergence of a new follicular wave by a physiological dose of oFSH given at the expected time of the endogenous inter-wave FSH nadir. The largest follicle of the oFSH-induced wave did not disrupt the rhythmic pattern of endogenous FSH secretion or emergence of the next follicular wave. The largest follicle of the oFSH-

induced wave did not differ from those of follicular waves stimulated by endogenous FSH peak, in its morphological attributes and estradiol secretory ability. The injection of a physiological dose of oFSH did not alter the number of small antral follicles in ewes. Thus, we conclude that, unlike in cattle, the large growing follicle of follicular waves in ewes has limited direct effect on the emergence of other antral follicles induced by physiological concentrations of oFSH. In addition, the large growing follicle of a wave may possibly not influence the rhythmicity of endogenous FSH secretion and follicular wave emergence, as it does in cattle.

Chapter 7: THE EFFECT OF THE MANIPULATION OF FSH-PEAK CHARACTERISTICS ON FOLLICULAR WAVE DYNAMICS IN ANESTROUS SHEEP: DOES AN OVARIAN INDEPENDENT ENDOGENOUS RHYTHM IN FSH SECRETION EXIST?*

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7.1. Abstract

It is well accepted that the emergence of follicular waves, in sheep, is preceded by peaks in serum FSH concentrations. There seems to be no evidence for a relationship between the amplitude of FSH peaks and the number of follicles growing in a wave; even though, superovulatory doses of FSH result in multiple ovulations. In a recent study in both cyclic and anestrous ewes, we demonstrated that the largest follicle of a wave, induced by exogenous oFSH, did not postpone the occurrence of the next endogenous FSH peak and follicular wave emergence. We designed 3 experiments to examine the relationship between FSH peaks and follicular wave emergence in Western White Face sheep. In experiment 1, anestrous ewes were treated with oFSH or vehicle (n = 6 per group), at the expected time of a peak in endogenous serum FSH concentrations, so as to double the amplitude of the FSH peak in FSH-treated ewes. In experiment 2, anestrous ewes were treated with either oFSH or vehicle (n = 6 per group) at the expected time of 2 consecutive inter-peak nadirs such that the treated ewes had 5 FSH peaks in the time-frame of 3 FSH peaks in control ewes. All the ewes in experiments 1 and 2 underwent daily transrectal ultrasonography of ovaries and blood sampling. In experiment 3, 5 ewes were ovariectomized on d 2-3 after ovulation of an estrous cycle. Daily blood samples

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were collected for a control period during the estrous cycle before ovariectomy and then for 3 17-d periods, one immediately after ovariectomy, one 2 months after ovariectomy and one during the following anestrus. Daily blood samples were also collected from another group of 8 ovariectomized ewes, which were treated with estradiol-implants and medroxyprogesterone acetate releasing intra-vaginal sponges for 10 days. Doubling the amplitude of the FSH-peak that preceded follicular wave emergence did not alter the characteristics of the following follicular wave. Creation of physiological peaks of serum FSH concentrations every 2-2.5 d stimulated the emergence of additional follicular waves but did not alter the rhythmic occurrence of FSH peaks and follicular wave emergence. Follicular waves resulting from endogenous peaks in FSH secretion emerged and grew in the presence of the growing largest follicle of the follicular waves induced by exogenous oFSH. Finally based on the observation of serum FSH concentrations in ovariectomized ewes, it appeared that there was an endogenous rhythm for peaks in daily serum FSH concentrations, which was, at least in part, independent of regulation by ovarian follicular growth patterns.

7.2. Introduction

Ovarian antral follicular growth occurs in a wave-like pattern in sheep, both during the breeding season (Noel *et al.* 1993; Ginther *et al.* 1995; Souza *et al.* 1998; Leyva *et al.* 1998b; Vinoles *et al.* 1999; Gibbons *et al.* 1999; Bartlewski *et al.* 1999a; Evans *et al.* 2000) and anestrus (Bartlewski *et al.* 1998; Evans *et al.* 2001). The emergence of follicular waves is preceded by a transient peak in circulating FSH concentrations (Ginther *et al.* 1995; Bartlewski *et al.* 1998; Souza *et al.* 1998; Bister *et al.* 1999; Bartlewski *et al.* 1999a; Bartlewski *et al.* 1999b; Evans *et al.* 2000; Evans *et al.* 2001;

Duggavathi *et al.* 2003a; Duggavathi *et al.* 2004). However, the relationship between the characteristics of FSH peaks and those of follicular waves has not been studied (Driancourt 2001). Treatment of ewes with a superovulatory dose of FSH results in increased numbers of follicles growing to an ovulatory diameter (Riesenberg *et al.* 2001; Boscos *et al.* 2002; Gonzalez-Bulnes *et al.* 2002; Gonzalez-Bulnes *et al.* 2003); however, the effects of changes in the amplitude of FSH peaks within a physiological range have not been tested. In a recent study from our laboratory (Duggavathi *et al.* 2004), we demonstrated that the presence of the largest follicle of a wave, induced by a treatment with ovine FSH (oFSH), did not postpone the occurrence of the next endogenous FSH peak. However, it is not known, if an increase in the frequency of FSH peaks, using repeated injections of FSH, would disrupt the rhythmic occurrence of endogenous FSH peaks and the regular emergence of follicular waves. As injection of oFSH induced a follicular wave that did not disrupt the normal rhythm of FSH peaks and follicular waves (Duggavathi *et al.* 2004), a question arose as to the regulation of the peaks in FSH secretion that precede follicular waves. Could there be an ovarian independent endogenous rhythm in FSH secretion in the ewe?

We designed a series of 3 experiments to examine the relationship between FSH peaks and follicular wave dynamics in Western White Face ewes. Experiment 1 was intended to monitor the effect of a doubling of the FSH-peak amplitude on the growth of antral follicles in the subsequent follicular wave. Experiment 2 was intended to monitor the effect of increasing the frequency of peaks in daily FSH concentrations (peaks at an interval of 2 to 2.5 days as compared to the normal interval of 4 to 5 d) on the rhythmic occurrence of endogenous FSH peaks and the emergence of follicular waves. Experiment

3 was intended to see if periodic peaks in serum FSH concentrations were present in ovariectomized ewes similar to those in intact ewes. We hypothesized that: 1) doubling the amplitude of the FSH-peak does not alter the growth pattern of the largest follicle of the treated wave in sheep; 2) increasing the frequency of FSH peaks with administration of exogenous oFSH, does not disrupt the rhythmic occurrence of endogenous FSH peaks and emergence of follicular waves in the ewe; and 3) ovariectomy does not affect the periodic increases (peaks) in FSH secretion in sheep.

7.3. Materials and Methods

7.3.1. General methodology

Experimental procedures were performed according the guidelines of the Canadian Council on Animal Care.

7.3.2. Hormone preparation

One mg of the oFSH preparation (NIDDK-oFSH-18) used in the present study has a biological potency of 65.6 x NIH-oFSH-S1 or 1640 IU and 0.1 x NIH-oLH-S1 or 106 IU. The oFSH for injection was prepared in saline with 0.05% BSA (w/v; Sigma, St. Louis, MO) and 50% polyvinylpyrrolidone (w/v; Sigma, St. Louis, MO; Duggavathi *et al.* 2004).

7.3.3. Transrectal Ovarian Ultrasonography and Blood Sampling

The anestrous ewes, in experiments 1 and 2, were scanned twice daily (0800 and 2000 h) until the emergence of the third follicular wave was identified (see below for details), using high-resolution real time B-mode ultrasound equipment (Aloka SSD-900; Aloka Co., Ltd., Tokyo, Japan) connected to a 7.5-MHz transducer. During each scanning

session, the relative position and diameter of all follicles ≥ 1-mm diameter and corpora lutea were sketched on to ovarian charts. In addition, all ovarian images were recorded on high-grade video tapes (Fuji S-VHS, ST-120 N; Fujifilm, Tokyo, Japan) for retrospective analysis of ovarian data. Blood samples (10 ml) were collected from all ewes by jugular venipuncture into vacutainers (Becton Dickinson, Rutherford, NJ) just before each scanning session.

7.3.4. Hormone analyses

Blood samples were allowed to clot for about 24 h at room temperature and the serum harvested was stored at -20ºC until assayed. Serum concentrations of FSH (Currie and Rawlings 1989) and estradiol (Joseph *et al.* 1992) were determined by established radioimmunoassays. The ranges of the standards for FSH and estradiol assays were 0.12 to 16.0 ng/ml and 1 to 100 pg/ml, respectively. The sensitivities of the assay (defined as the lowest concentration of hormone capable of significantly displacing labelled hormone from the antibody) were 0.1 ng/ml for FSH assays and 1 pg/ml for estradiol assays. The intra- and inter-assay coefficients of variation (CVs) for FSH assays for sera with a concentration of 0.46 ng/ml or 1.37 ng/ml were 6.5% and 9.1% or 6.2% and 8.8%, respectively, and for estradiol assays for sera with a concentration of 3.5 pg/ml or 12.0 pg/ml were 16.5% and 7.1% or 14.2% and 8.9%, respectively.

7.3.5. Experiment 1

Animals

Twelve adult, anestrous (June-July) Western White-Face ewes (age: 2-3 years; mean body weight: 90.0 ± 5.6 kg) were used in this experiment. The ewes were kept outside in

sheltered pens and were fed daily maintenance rations of alfalfa hay, with water available *ad libitum*.

Treatment administration

Treatment involved two subcutaneous injections of either oFSH (0.5 μg/kg) or vehicle given 8 h apart. This treatment regimen was based on preliminary trials and in which treatment was designed to result in an FSH peak of physiological amplitude (Duggavathi *et al.* 2004). Six ewes were injected with oFSH (oFSH-treated group), and six control ewes were injected with vehicle only.

A 4-mm follicle, which grew from the pool of 2- to 3-mm follicles, was detected with ultrasonography, and it was designated as the wave 1 follicle. Such a follicle had to have emerged (and grown beyond the 2- to 3-mm stage) 3–5 days after the emergence of the previous follicular wave (i.e., at the normal time interval for waves in ewes) (Bartlewski *et al.* 1998). The first injection of the treatment with either oFSH or vehicle was given 60 h after the detection of the 4-mm follicle, but only if the 4 mm follicle had grown to ≥ 5 mm to establish a follicular wave; the second injection of oFSH was given 8 h later. Timing of the treatment was designed to give oFSH or vehicle injections at the expected time of the peak in endogenous FSH concentration associated with the emergence of a follicle wave (designated as wave 2). This treatment regimen was expected to result in a doubling of the amplitude (defined as the difference between the nadir and the peak concentrations) of the endogenous FSH peak in FSH-treated ewes.

7.3.6. Experiment 2

Animals

Twelve adult, anestrous (May-June) Western White-Face ewes (age: 2 to 3 years; mean body weight: of 86.1 ± 1.8 kg) were used for this experiment.

Treatment administration

Treatment, as in experiment 1, involved two subcutaneous injections of either oFSH (0.5 µg per kg) or vehicle given 8 h apart. Six ewes were injected with oFSH (oFSH-treated group), and six control ewes were injected with vehicle only.

A 4-mm follicle, which grew from the pool of 2- to 3-mm follicles, was detected with ultrasonography, and it was designated as the wave 1 follicle. The first injection of the first treatment with oFSH or vehicle was given 12 h after the detection of the 4-mm follicle of wave 1, but only if this follicle remained at 4 mm or grew further. The second injection was given 8 h later. The follicular wave induced in the FSH-treated ewes by exogenous FSH was designated wave A. Based on our previous study (Duggavathi *et al.* 2004), the following wave, induced by endogenous FSH secretion and designated as wave 2, was expected to emerge at a normal inter-wave interval of 4 to 5 days after the day of emergence of wave 1. Once the largest follicle of wave 2 was first identified at 4 mm in diameter, the second treatment with oFSH or vehicle was begun, but only if this follicle remained at 4 mm or grew further. The first injection of the second treatment with oFSH or vehicle was given 12 h after the detection of the 4-mm follicle of wave 2. The second injection was given 8 h later. Timing of the treatments was designed to give oFSH injections during the growth phase of the largest follicle of wave 1 and 2, and the

expected time of the 2 consecutive inter-wave nadirs (between waves 1 and 2, and waves 2 and 3) in endogenous FSH secretion. This regimen gave 5 peaks in serum FSH concentrations over a time period when only 3 peaks should have occurred.

7.3.7. Experiment 3

Five cyclic Suffolk and 8 Western White Face ewes were used in this experiment. Blood samples were collected daily for one estrous cycle (October-November) from 5 of these ewes (period A). Ovariectomy was performed on all ewes on d 2-3 after ovulation in the following cycle. Blood samples were collected daily from 5 of the ovariectomized ewes for 3 separate 17-d periods; first, immediately following ovariectomy (November; period B), second, 2 months after ovariectomy (January; period C) and third, during the following anestrus period (June; period D). Following ovariectomy, the other group of 8 ewes was rested for a 17-d period, without any blood sampling, to allow for the post-ovariectomy increase and stabilization in serum FSH concentrations. Following the rest period, sponges containing medroxy progesterone acetate (Veramix®, Upjohn; 60 mg; Orangeville, Ont., Canada) were inserted. On d 4 following sponge insertion, each of the 8 ewes was fitted with a 5 cm silastic rubber implant (Dow Corning, Midland, MI) containing estradiol-17β (10% w/w; Sigma Chemical Company, St Louis, MO) (Joseph *et al.* 1992), which remained in place for 10 d. On d 10, both the sponge and implant were removed from the ewes. Blood samples were collected daily from the day of estradiol-implant insertion (d 0) to 1 d following sponge and implant removal (d 11; period E).

7.3.8. Follicular Data Analyses

Follicular data were analyzed for experiments 1 and 2. A follicular wave consisted of a follicle or a group of follicles that emerged and grew from 2 or 3 mm in diameter to ≥ 5 mm (growth phase) and remained at their maximum diameter (static phase) before regression to 2 or 3 mm in diameter (regression phase; Bartlewski *et al.* 1999a). Emergence was restricted to a 24-h period (Duggavathi *et al.* 2003a). The following follicular characteristics were analyzed: (1) time of emergence of the follicular waves before and after treatment (expressed in relation to the day of treatment); (2) interval between emergence of two follicular waves (inter-wave intervals); (3) number of follicles growing from 2-3 mm to ≥ 5 mm diameter per wave; (4) Maximum diameter attained by the largest follicle of a follicular wave; and (5) lengths of growing, static and regression phases for the largest follicle of a wave.

7.3.9. Hormone data analysis

Serum FSH concentrations were centralized to the day of treatment (d 0) and analyzed for the periods from 6 d before to 3 d after the treatment for experiment 1 and from 2 d before to 7.5 d after the first treatment (d 0) for experiment 2. For experiment 3, serum FSH concentrations in intact ewes (period A) were centralized to the day of ovulation (d 0), while, serum FSH concentrations in ovariectomized ewes were centralized to the first day of blood sampling (d 0; periods B, C, D and E). Serum FSH concentrations in experiment 3 were analyzed for the period from d 0 to d 17 of blood sampling. Peaks in daily serum concentrations of FSH, in all the 3 experiments, were determined using the cycle-detection program (Clifton and Steiner 1983). As expected (Joseph *et al.* 1992;

Mann *et al.* 1992b) the overall mean serum concentrations of FSH increased after ovariectomy (7 to 20 ng/ml depending on the time after ovariectomy).

The cycle detection program uses the mean FSH concentration for the data set and the coefficient of variation of the FSH assay to determine peaks in serum FSH concentrations (Clifton and Steiner 1983). The CV's of our assays for experiment 3 ranged from 3 to 5 %. The post-ovariectomy basal FSH concentrations had increased by 350 % during the period B and by 500 % during the period C of blood sampling as compared to intact ewes during the period A of blood sampling. The amplitude of FSH peaks have been reported to be about 1 to 2 ng/ml (Ginther *et al.* 1995; Bartlewski *et al.* 1999a; Evans *et al.* 2000). Applied to the data of the present experiment (experiment 3), the cycle detection program did not identify many apparent peaks in serum FSH concentrations in the post-ovariectomy ewes; while it identified all the peaks in serum FSH concentrations in the intact ewes. To ensure that the program detected peaks in daily serum FSH concentrations, of the magnitude seen in normal intact ewes, in the face of very high basal concentrations, the daily serum concentrations of FSH in all the ewes (including intact ewes of period A) in experiment 3 were transformed. The transformation of FSH data was intended to correct the high mean FSH values in ovariectomized ewes to those seen in intact ewes. To achieve this, a constant (k) value was subtracted from each FSH value in a data set of daily FSH concentrations for a ewe. The constant, k, for each FSH data set was determined by using the formula k = FSHmean – 1.9; where FSHmean is the mean serum FSH concentration for a ewe during a period of blood sampling and 1.9 is the overall mean FSH concentration for intact ewes (period A; Fig. 7.5 panel A). This transformation effectively removed the high basal values for FSH concentrations but

retained the variation due to peaks in daily concentrations. The transformed FSH data set was then subjected to cycle detection program. The following parameters for serum FSH concentrations were analyzed for experiment 3: (1) overall mean concentrations of FSH (non-transformed data) for each period of blood sampling; (2) number of FSH peaks, in the transformed data, identified by the cycle detection program; (3) amplitude of FSH peaks, in the transformed data, identified by the cycle detection program; (4) inter-peak interval, in the transformed data, for each period of blood sampling. Serum estradiol concentrations in the ovariectomized ewes fitted with estradiol-implants were centralized to the day of implant insertion and analyzed for the period from the day of implant insertion (d 0) to d 11.

7.3.10. Statistical analyses

Statistical differences were assessed by one- or two-way ANOVA (Sigma Stat® Statistical Software, version 2.0 for Windows® 95, NT and 3.1, 1997; Chicago, IL, USA). Repeated measures ANOVA was used when there was data collected repeatedly over a period of time. Multiple comparisons were made by the method of Fisher's least significant difference. Results are reported as least square means and SEM. Statistical significance was defined as $P < 0.05$.

7.4. Results

7.4.1. Experiment 1

Administration of exogenous oFSH

The mean serum FSH concentrations in FSH-treated and control ewes from 6 d before to 3 d after the injection of oFSH or vehicle (d 0) are illustrated in Figure 1. There was a

peak in serum FSH concentrations on d -4 in both FSH-treated and control ewes ($P > 0.05$) and it was associated with the emergence of follicular wave 1 in both groups (Table 7.1). The next FSH-peak in control ewes was on d 0.5 after treatment (2.8 ± 0.3 ng/ml; Fig. 7.1). In FSH-treated ewes, there was a peak in FSH concentration on d 1 after oFSH injection (5.1 ± 0.2 ng/ml). The FSH concentrations in FSH-treated ewes were higher ($P < 0.001$) from d 0.5 to d 1.5 after the treatment as compared to control ewes (Fig. 7.1). The FSH-peak in FSH-treated ewes was approximately 2 times higher than that in control ewes, as expected.

Emergence of follicular waves

The days of emergence of follicular waves (waves 1, 2 and 3), in relation to the day of treatment (d 0) with oFSH or vehicle are given in Table 7.1 and they did not differ significantly between the groups ($P > 0.05$). The inter-wave interval between waves 1 and 2 did not differ between FSH-treated and control ewes ($P > 0.05$; 4.2 ± 0.3 d vs. 4.5 ± 0.2 d, respectively). Likewise, the inter-wave interval between wave 2 that emerged immediately after treatment and the following wave 3 did not differ between the two groups of ewes ($P > 0.05$; 5.2 ± 1.0 d in FSH-treated ewes vs. 5.3 ± 2.0 d in control ewes). The mean daily numbers of small follicles (≥1 mm and ≤ 3mm in diameter) did not vary ($P > 0.05$) with time from 6 d before to 3 d after treatment and did not differ between the 2 groups of ewes ($P > 0.05$). The overall mean numbers of small follicles for the period of analysis (which encompassed the emergence of waves 1 and 2; Table 7.1), were 13.9 ± 1.5 (range: 11 to 16) and 14.1 ± 1.6 (range: 11 to 16) in FSH-treated and control ewes, respectively.

Characteristics of follicular waves

The growth characteristics of the largest follicle of waves 1, 2 and 3 are given in Table 7.1. There was no difference ($P > 0.05$) in the length of the growth and static phases, maximum diameter and growth rate of the largest follicle in waves 1, 2 and 3 between FSH-treated and control ewes. Likewise, the number of follicles that grew together from 2-3 mm to ≥ 5 mm to constitute a follicular wave did not differ ($P > 0.05$) between the 2 groups of ewes and between follicular waves within each group of ewes (Table 7.1).

7.4.2. Experiment 2

Administration of exogenous oFSH

The mean serum concentrations of FSH from 2 d before to 7.5 d after the first treatment with oFSH or vehicle (d 0) in FSH-treated and control ewes are given in Figure 2. The first treatment was administered on d 0 in both the groups of ewes and the second treatment was administered on d 4.3 ± 0.5 after the first treatment. There were peaks in FSH concentration in FSH-treated ewes on d 0.5 and d 5.0 ($P < 0.01$; 3.5 ± 0.3 ng/ml and 3.7 ± 0.3 ng/ml, respectively; Fig. 7.2) as compared to nadirs in FSH concentrations in control ewes on d 0.5 and d 5.0 (1.7 ± 0.3 ng/ml and 2.0 ± 0.3 ng/ml, respectively; Fig. 7.2).

Days of FSH-peak and the emergence of follicular waves

The mean days on which peaks in FSH concentrations were detected using the cycle detection program and mean days of follicular wave emergence are given in Table 7.2. The interval between emergence of successive follicular waves (waves 1, A, 2, B and 3 in

FSH-treated ewes and waves 1, 2 and 3 in control ewes) was shorter in FSH treated ewes (2.21 ± 0.1 d; $P < 0.001$) as compared to control ewes (4.1 ± 0.2 d). However, if only the waves associated with endogenous FSH-peaks (waves 1, 2 and 3) were considered in both the groups of ewes, the inter-wave interval did not differ ($P > 0.05$) between FSH-treated and control ewes. The inter-wave intervals between waves 1 and 2, and waves 2 and 3 were 4.4 ± 0.5 d and 4.5 ± 0.5 d and 4.4 ± 0.3 d and 4.2 ± 0.3 d in FSH treated and control ewes, respectively. The mean daily numbers of small follicles (≥1 mm and ≤ 3mm in diameter) did not vary ($P > 0.05$) with time from 2 d before to 7.5 d after the first treatment and did not differ between the 2 groups of ewes ($P > 0.05$). The overall mean numbers of small follicles for the period of analysis (which encompassed the emergence of waves 1, A, 2, B and 3 in FSH treated ewes and waves 1, 2 and 3 in control ewes; Table 7.2), were 14.9 ± 2.5 (range: 12 to 18) and 15.6 ± 1.9 (range: 12 to 17) in FSH-treated and control ewes, respectively.

Characteristics of follicular waves

The growth characteristics of the largest follicle of waves 1, A, 2, B and 3 in FSH-treated ewes and waves 1, 2 and 3 in control ewes are given in Table 7.3. Diameter profiles of the largest follicle during the growth phase of waves are shown in Figure 3. There was no difference ($P > 0.05$) in the length of the growth and static phases, maximum diameter and growth rate of the largest follicle in waves 1, 2 and 3 between FSH-treated and control ewes, and between endogenous (waves 1, 2 and 3) and induced (waves A and B) follicular waves in FSH-treated ewes. Likewise, the number of follicles that grew together from 2-3 mm to ≥ 5 mm to constitute a follicular wave did not differ ($P > 0.05$)

between the 2 groups of ewes and between follicular waves within each group of ewes (Table 7.3).

7.4.3. Experiment 3

Transformed values of daily serum FSH concentrations in representative ewes for each blood-sampling period are shown in Figure 4. Mean serum estradiol concentrations during the period of treatment with estradiol-implants in ovariectomized ewes are overlaid on daily serum FSH concentrations in a representative ewe (Fig. 7.4E). The overall mean concentrations of FSH during each period of blood sampling were significantly higher ($P < 0.05$) in ovariectomized ewes as compared to intact ewes (Fig. 7.5A). Mean FSH concentration increased with time after ovariectomy as indicated by the significant difference ($P < 0.05$) between the control period (period A) and periods immediately following ovariectomy (period B), 2 months after ovariectomy (period C) and during the following anestrus (period D) and period during estradiol-implant treatment (period E). There was no significant difference ($P > 0.05$) in the number of FSH peaks detected (Fig. 7.5B) using the cycle detection program, the amplitude of FSH peaks (Fig. 7.5C) or inter-peak intervals (Fig. 7.5D) amongst the periods of blood sampling.

Table 7.1. Mean days of wave emergence, the number of follicles per wave[a] and characteristics of the largest follicle of the waves that emerged before (wave 1) and after (waves 2 and 3) treatment, in oFSH-treated and control ewes during anestrus (experiment 1). Days of wave emergence are expressed in relationship to the day of treatment (d 0). The ewes (n = 6 per group) were treated with either oFSH or vehicle at the expected time of the peak in serum FSH concentrations which was associated with the emergence of wave 2.

Parameter	**Group**	**Wave 1**	**Wave 2**	**Wave 3**
Day of wave emergence	Treatment	-3.4 ± 0.3	0.5 ± 0.0	5.7 ± 1.0
	Control	-3.5 ± 0.0	0.7 ± 0.2	5.8 ± 1.4
Length of growth phase (days)	FSH-treated	2.7 ± 0. 2	2.9 ± 0.2	2.7 ± 0.2
	Control	2.5 ± 0.2	2.9 ± 0.2	3.0 ± 0.2
Length of static phase (days)	FSH-treated	3.0 ± 0.4	2.3 ± 0.4	Not determined
	Control	2.5 ± 0.1	2.9 ± 0.1	Not determined
Maximum diameter (mm)	FSH-treated	5.7 ± 0.4	5.9 ± 0.4	5.7 ± 0.4
	Control	5.9 ± 0.5	6.0 ± 0.5	5.5 ± 0.5
Growth rate (mm/day)	FSH-treated	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
	Control	1.2 ± 0.1	0.9 ± 0.1	1.2 ± 0.1
Number of follicles per wave [a]	FSH-treated	2.4 ± 0.2	1.9 ± 0.1	2.0 ± 0.1
	Control	2.1 ± 0.2	2.0 ± 0.1	2.2 ± 0.1

a Number of follicles that emerged at 2-3 mm in diameter and grew together to reach an ovulatory diameter of ≥ 5 mm

Table 7.2. Mean days on which peaks in serum FSH concentrations were detected using the cycle detection program and mean days of emergence of follicular waves in FSH-treated and control ewes (n = 6 per group; experiment 2), in relation to the day of first treatment (d 0). Values in parentheses are the mean days in relation to the day of the second treatment. The anestrous ewes were treated with oFSH or vehicle during the growth phase of the largest follicle in waves 1 and 2.

	Group	**Peak 1**	**Peak A**	**Peak 2**	**Peak B**	**Peak 3**
Day of FSH-peak	FSH-treated	-1.5 ± 0.1	0.5 ± 0.0	3.0 ± 0.2 (-1.2 ± 0.2)	5.0 ± 0.0 (0.5 ± 0.0)	7.4 ± 0.1 (3.0 ± 0.3)
	Control	-1.5 ± 0.2	No peak A	2.9 ± 0.2 (-1.5 ± 0.2)	No peak B	7.3 ± 0.5 (2.5 ± 0.6)
		Wave 1	**Wave A**	**Wave 2**	**Wave B**	**Wave 3**
Day of wave emergence	FSH-treated	-1.3 ± 0.1	0.6±0.1	2.9±0.2 (-1.4±0.2)	4.8 ± 0.2 (0.7±0.1)	7.4 ± 0.1 (3.0 ± 0.3)
	Control	-1.4 ± 0.2	No wave A	2.9 ± 0.1 (-1.4 ± 0.1)	No wave B	7.3 ± 0.5 (2.8 ± 0.6)

Table 7.3. The number of follicles per wave[a] and characteristics of the largest follicle of the waves that emerged before and after treatment in oFSH-treated and control ewes during anestrus (experiment 2). The anestrous ewes were treated with oFSH or vehicle during the growth phase of the largest follicle in waves 1 and 2.

Parameter	**Group**	**Wave 1**	**Wave A**	**Wave 2**	**Wave B**	**Wave 3**
Length of growth phase (days)	FSH-treated	2.6 ± 0.2	2.9 ± 0.1	2.8 ± 0.1	2.6 ± 0.2	2.7 ± 0. 2
	Control	2.8 ± 0.2	No Wave A	3.1 ± 0.1	No Wave B	2.5 ± 0.2
Length of static phase (days)	FSH-treated	3.1 ± 0.4	3.2 ± 0.4	3.1 ± 0.4	3.2 ± 0.4	Not Determined
	Control	3.0 ± 0.1	No Wave A	3.0 ± 0.1	No Wave B	Not Determined
Maximum diameter (mm)	FSH-treated	5.5 ± 0.4	5.9 ± 0.4	5.2 ± 0.4	5.6 ± 0.4	5.7 ± 0.4
	Control	5.6 ± 0.5	No Wave A	5.7 ± 0.5	No Wave B	5.9 ± 0.5
Growth rate (mm/day)	FSH-treated	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1
	Control	1.2 ± 0.1	No Wave A	1.2 ± 0.1	No Wave A	1.2 ± 0.1
Number of follicles per wave [a]	FSH-treated	2.4 ± 0.2	1.9 ± 0.1	2.9 ± 0.1	1.6 ± 0.1	1.9 ± 0.3
	Control	2.1 ± 0.2	No Wave A	2.7 ± 0.1	No Wave B	1.3 ± 0.3

a Number of follicles that emerged at 2-3 mm in diameter and grew together to reach an ovulatory diameter of ≥ 5 mm

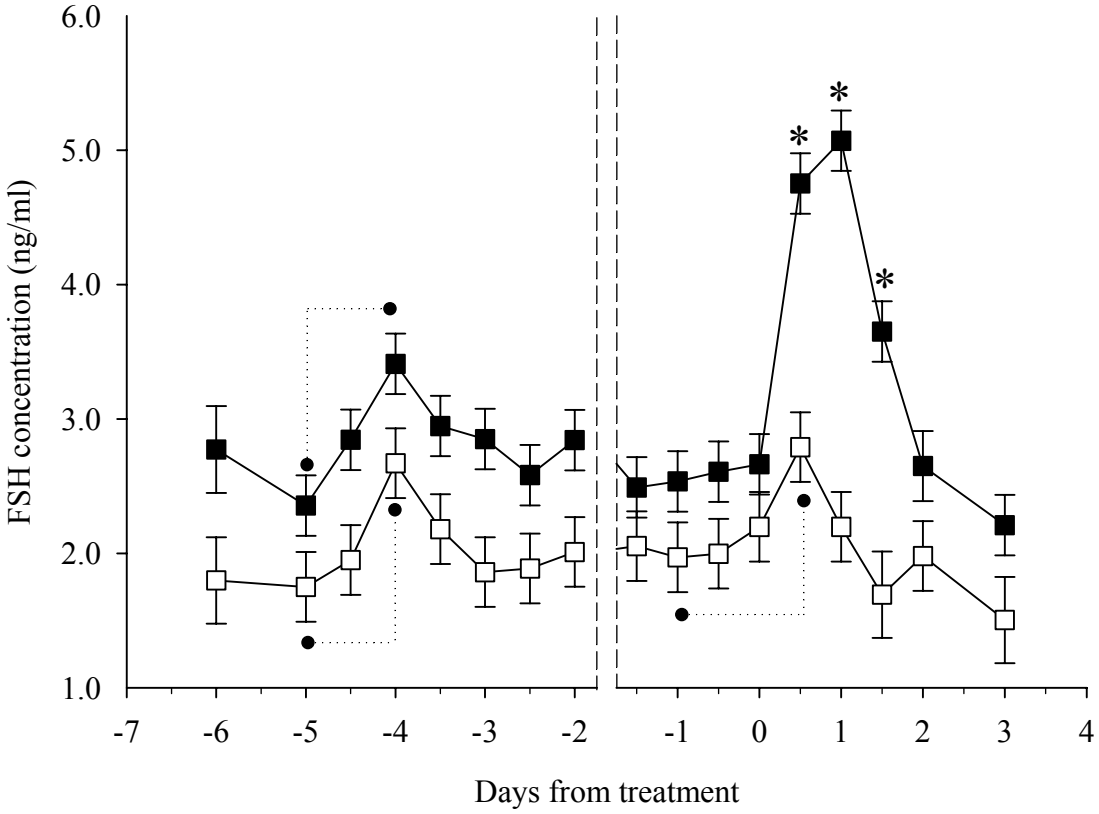

Figure 7.1. Mean daily serum FSH concentrations in FSH-treated (■) and control (□) ewes (n = 6 per group) from 6 d before to 3 d after treatment (d 0) with oFSH or vehicle. The ewes were treated 60 h after the detection of the largest follicle of wave 1, at 4 mm in diameter, by transrectal ultrasonography. The treatment was targeted to be administered at the expected time of a peak in endogenous FSH concentrations. FSH concentrations were centralized to the days of FSH peaks, which were d – 4 (left box) and d 0.5 (right box) in relation to the day of treatment (d 0) and are shown for d – 6 to d – 2 for the first peak and d -1.5 to d 3 for the second peak. Dotted lines denote significant differences ($P < 0.05$) between the indicated points within a group.

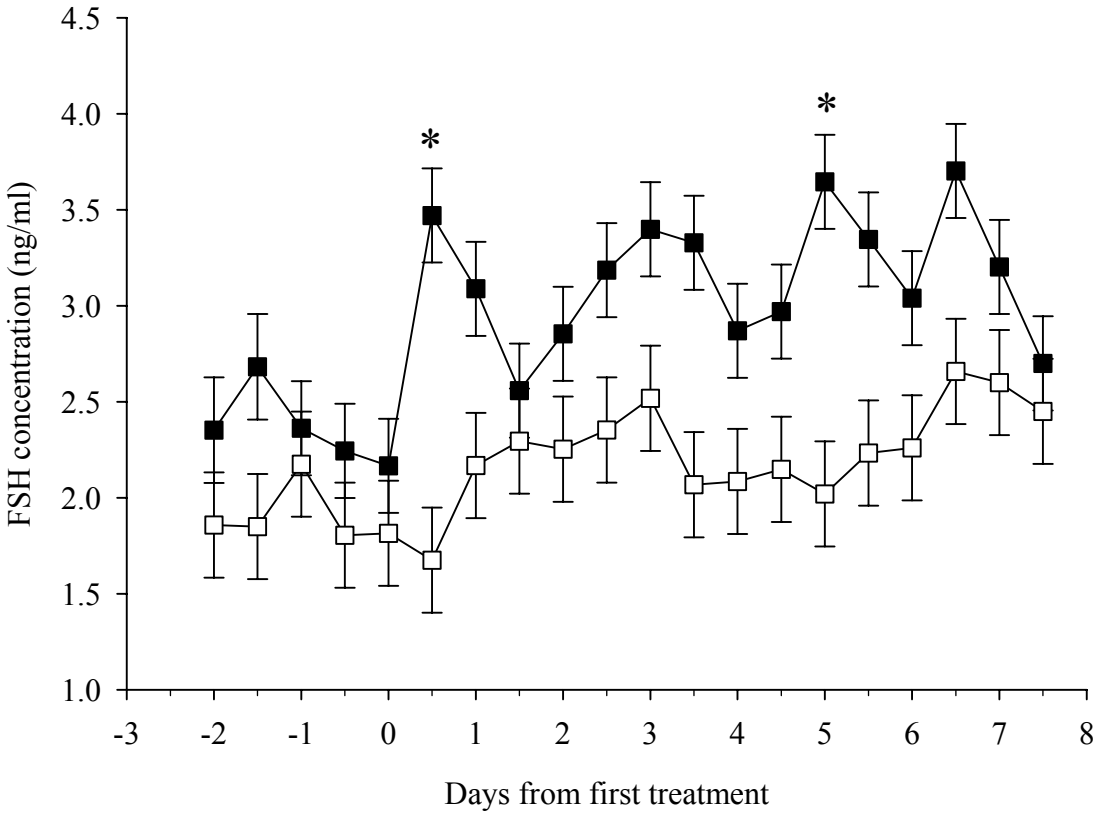

Figure 7.2. Mean daily serum FSH concentrations in FSH-treated (■) and control (□) ewes (n = 6 per group) from 2 d before to 7.5 d after first treatment (d 0) with oFSH or vehicle. The ewes were treated 12 h after the detection of the largest follicle of waves 1 and 2, at 4 mm, by transrectal ultrasonography. The treatments were targeted to be administered at the expected times of 2 consecutive nadirs in endogenous FSH concentrations.

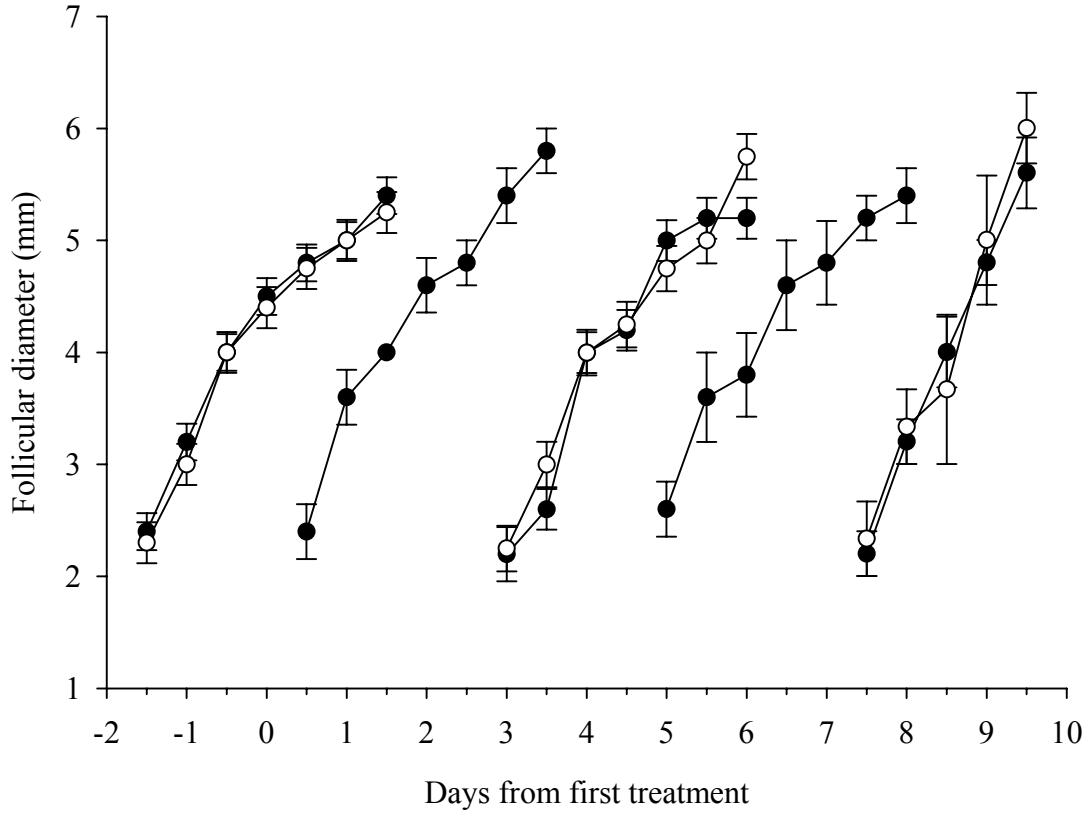

Figure 7.3. Diameter profiles, during the growth phases, of the largest follicle of waves 1, A, 2, B and 3 in FSH-treated ewes (●; n = 6) and waves 1, 2 and 3 in control ewes (○; n = 6). The diameter profiles were centralized to the mean day of emergence of individual waves.

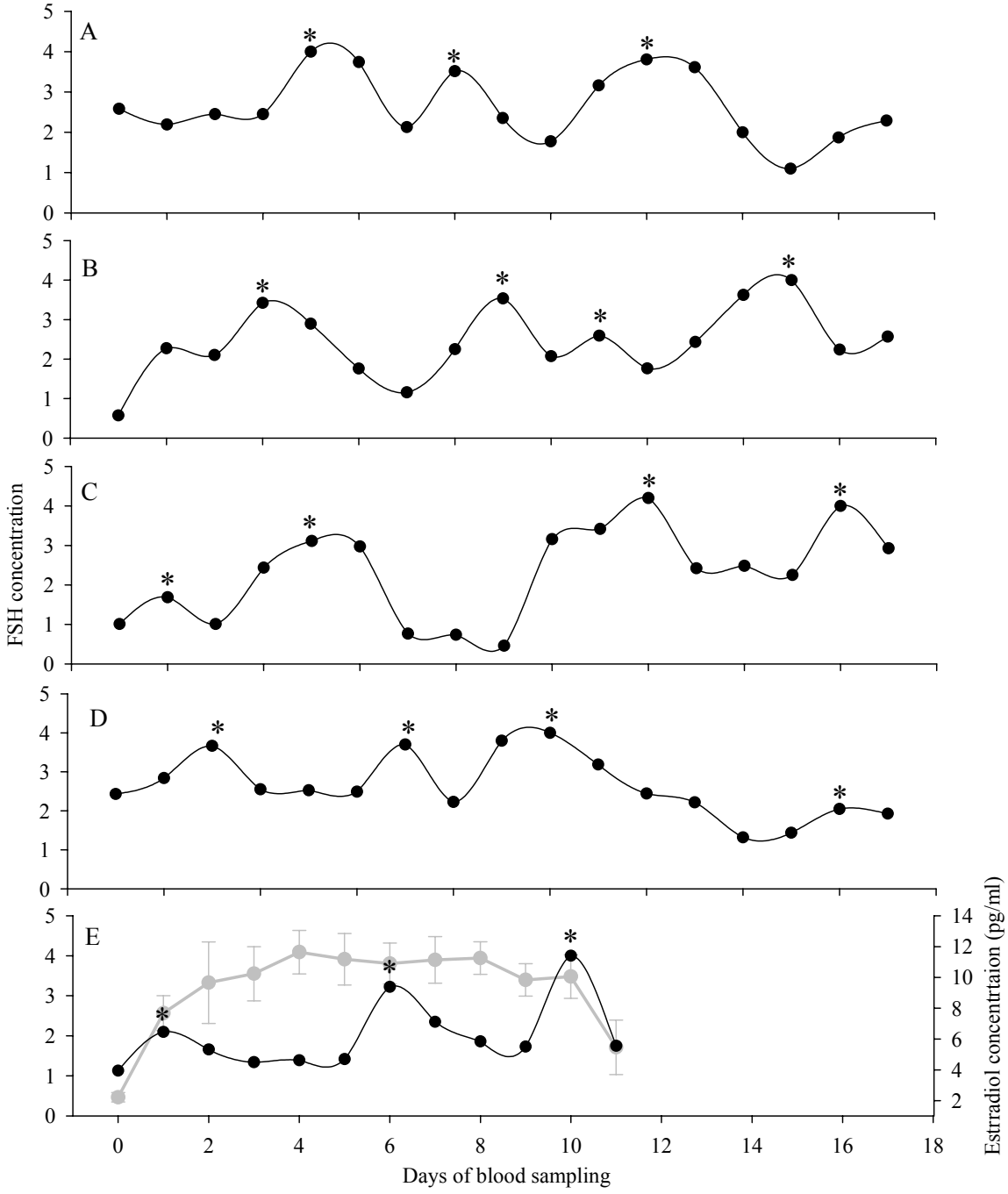

Figure 7.4. Daily FSH concentrations in representative ewes from all periods of bleeding including the control period (intact ewes; period A), immediately after ovariectomy (period B), 2 months after ovariectomy (period C), the following anestrus (period D) and a period of treatment with estradiol implants (separate group of ewes; period E). Mean daily concentrations of estradiol in the group of ewes treated with estradiol-implants are overlaid on the daily FSH concentrations of a representative ewe (E).

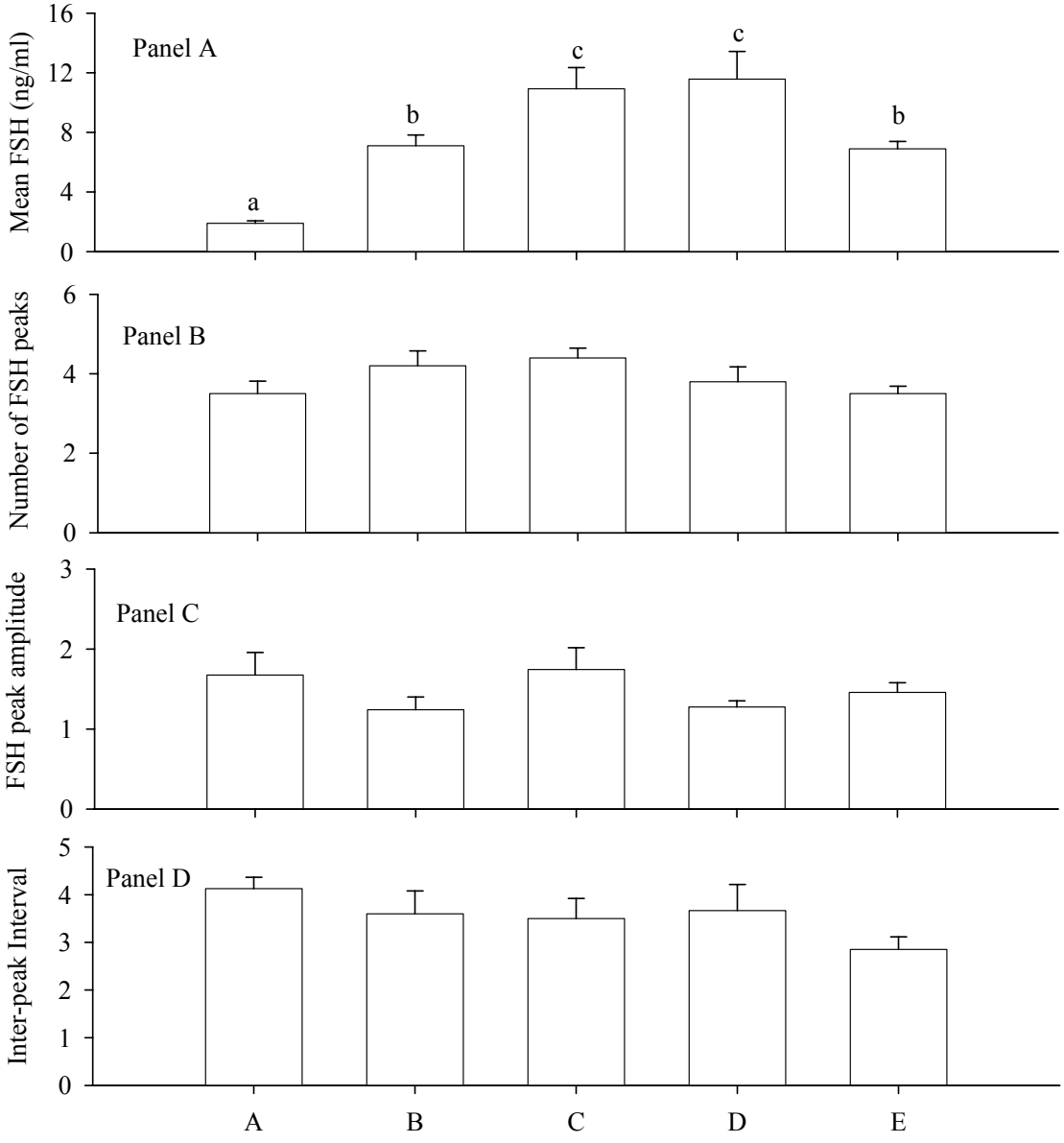

Figure 7.5. Overall mean concentration of FSH (untransformed data; panel A), number of FSH peaks (panel B), FSH peak amplitude (panel C) and inter-peak interval (panel D) for the 17-d periods of blood sampling (periods A, B, C, D and E; n = 5-8 ewes). Daily blood samples were collected from a group of 5 ewes for one estrous cycle and these ewes were ovariectomized on d 2-3 after ovulation in the following cycle. Daily blood samples were also collected for 17-d periods immediately after ovariectomy (period B), 2 months after ovariectomy (period C) and the following anestrus (period D). A separate group of 8 ovariectomized ewes was treated with estradiol implants and daily blood samples were collected for a 10-d period (period E). a,b,c, denote significant difference ($P < 0.05$) in overall mean FSH concentrations between groups of ewes.

7.5. Discussion

Our regimen of oFSH administration in experiment 1 resulted, as expected, in the doubling of the amplitude of the endogenous FSH peak preceding a follicular wave in anestrous ewes (Fig. 7.1). However, none of the growth characteristics of the largest follicles of the FSH-treated-wave (FSH-treated ewes) differed in comparison to the largest follicles of the vehicle-treated-wave (control ewes; Table 7.1).

Suppression of the secondary surge in FSH secretion, after the preovulatory gonadotropin surge, blocks the emergence of the first follicular wave of the cycle in heifers (Turzillo and Fortune 1990). Injection of a superstimulatory dose of FSH before the selection of a dominant follicle, in heifers, increases the diameter of the subordinate follicles while it decreases the diameter of the dominant follicle (Adams *et al.* 1993). A superovulatory dose of FSH stimulates the growth of multiple ovulatory sized follicles in sheep (Riesenberg *et al.* 2001; Boscos *et al.* 2002; Gonzalez-Bulnes *et al.* 2002; Gonzalez-Bulnes *et al.* 2003). However the effects of superovulatory doses of FSH are not physiological. There is a general agreement that FSH is the key regulator of recruitment of follicles into waves in most species and in the ewe there is a threshold of FSH concentration below which recruitment cannot proceed (Driancourt 2001). This threshold appears to vary between ewes (Picton and McNeilly 1991) and between follicles within a specific ewe (Fry and Driancourt 1996). Based on the results of the present study, within a physiological range, doubling the concentration of FSH in the peaks that precede follicular waves does not affect either the small non-recruited follicles or FSH-dependent follicles recruited into a wave. Thus, it is logical to argue that serum FSH concentrations within a physiological range do not necessarily correlate with the number of ovulatory

sized follicles growing in a wave. This argument is supported by several lines of evidence. No study, as yet, has shown a correlation between serum FSH concentrations and the number of follicles recruited (Driancourt and Fry 1988; Driancourt 2001). Previous studies (Driancourt *et al.* 1988; Campbell *et al.* 1991b; Boulton *et al.* 1995; Fry and Driancourt 1996) have reported that there were no differences either in mean circulating FSH concentrations or in the concentration and duration of the FSH peaks that precede follicular waves (Gibbons *et al.* 1999; Bartlewski *et al.* 1999a) between prolific and non-prolific breeds of sheep. In the homozygous Boorola FecB gene carrier ewes, the serum FSH concentrations have been show to be higher in comparison to non-carrier ewes (McNatty *et al.* 1987; McNatty *et al.* 1989a; Hudson *et al.* 1999). However, it has been suggested that the FecB gene has its effects at the ovarian level by increasing the follicular sensitivity to FSH rather than by increasing pituitary FSH secretion (Fry *et al.* 1987; Campbell *et al.* 2003). It would be of interest to look at the effects of the FecB gene on the characteristics of peaks in serum FSH concentrations that precede follicular wave emergence.

In cattle, cauterization of the dominant follicle of the first wave of the cycle advances the emergence of the second wave of the cycle (Ko *et al.* 1991). This indicates that the lifespan of the dominant follicle, in cattle, influences the time of occurrence of the next endogenous FSH peak (Ginther *et al.* 2002). Such a concept has been suggested for sheep (Lopez-Sebastian *et al.* 1997; Gonzalez-Bulnes *et al.* 2001; Evans *et al.* 2002). However, this concept was not supported in the sheep by a recent study (Duggavathi *et al.* 2004), wherein the largest follicle (apparent dominant follicle) of a wave induced by exogenous oFSH did not postpone the time of the next endogenous FSH peak and emergence of a

follicular wave. In the present study in experiment 2, the emergence of waves A and B did not alter the time of occurrence of the endogenous FSH peaks that preceded the emergence of waves 2 and 3 (Fig. 7.2 and Table 7.2). Waves 2 and 3 clearly emerged during the growth phases of waves A and B. The results of experiment 2 support and extend the idea that serum FSH concentrations in sheep may not be under the stringent control of growing follicles.

In studies on superovulation in sheep, the presence of a large follicle may (Gonzalez-Bulnes *et al.* 2003) or may not affect the sheep's response (Driancourt *et al.* 1991; Gonzalez-Bulnes *et al.* 2000) to a superovulatory treatment. Similarly, in cattle, there are several lines of evidence (Kafi and McGowan 1997) that support (Guilbault *et al.* 1991; Huhtinen *et al.* 1992; Bungartz and Niemann 1994; Mapletoft *et al.* 2002) or fail to support (Wilson *et al.* 1990; Gray *et al.* 1992; Rajamahendran and Calder 1993; Maciel *et al.* 1995) the effects of the presence of a dominant follicle on the superovulatory response. In experiment 2, it could be argued that the induction of extra follicular waves (waves A and B) by oFSH treatment overrode the dominance of the largest follicles of the non-induced follicular waves (waves 1, 2 and 3). However, the non-induced follicular waves (waves 2 and 3), in FSH-treated ewes, emerged during the growth phase of the largest follicles of waves A and B (Fig. 7.3). The growth characteristics of the largest follicles of the induced follicular waves, in the present study, did not differ from those of the largest follicles of the non-induced follicular waves in both FSH-treated and control ewes (Table 7.3). Previously, we have shown that the largest follicles of the oFSH-induced follicular waves were associated with normal peaks in estradiol concentrations in comparison with endogenous follicular waves in both FSH-treated and control ewes

(Duggavathi *et al.* 2004). These observations indicate that the apparent dominant follicles of waves A and B were not effective in postponing the occurrence of the next endogenous FSH-peaks and follicular waves. The uninterrupted emergence of 5 follicular waves in FSH-treated ewes in the timeframe of the emergence of 3 follicular waves in control ewes suggested that there are small FSH-sensitive follicles available daily to enter a wave in response to a physiological FSH stimulus. This supposition is supported by our previous observation that the pool of small follicles (1 to 3 mm in diameter) remains constant except for the periovulatory period, despite the emergence of 3 to 4 follicular wave in each ovine estrous cycle (Duggavathi *et al.* 2003a). The concept of follicular dominance does not appear to be as convincing in the ewe as it is in cattle (Driancourt *et al.* 1991; Ravindra *et al.* 1994; Fry and Driancourt 1996; Duggavathi *et al.* 2003a); at least in breeds of sheep, which are strictly monovulators (Lopez-Sebastian *et al.* 1997; Gonzalez-Bulnes *et al.* 2001). Taken together, it appears that the large ovulatory sized follicles in a wave, in sheep, do not exert dominance to the extent seen in cattle.

There is a great deal of evidence that FSH secretion is regulated by factors produced by growing antral follicles such as, estradiol (Salamonsen *et al.* 1973; Jonas *et al.* 1973; Joseph *et al.* 1992; Joseph *et al.* 1995; Meikle *et al.* 2001) and inhibin (Mann *et al.* 1992a; Findlay *et al.* 2000). The studies showing negative effects of estradiol treatment on pituitary FSH secretion in intact (Meikle *et al.* 2001) or ovariectomized (Joseph *et al.* 1992) ewes have largely used high doses of estradiol. At physiological levels an inverse temporal relationship between serum concentrations of estradiol and FSH, in association with the growth of follicular waves, have been demonstrated during the breeding season (Souza *et al.* 1997a; Bartlewski *et al.* 1999a) but not during anestrus (Souza *et al.* 1996;

Bartlewski *et al.* 1998; Bartlewski *et al.* 2000c; Evans *et al.* 2001). However, follicular waves and associated peaks in FSH concentrations have been demonstrated during both the breeding and non-breeding seasons (Noel *et al.* 1993; Souza *et al.* 1996; Bartlewski *et al.* 1998; Leyva *et al.* 1998a; Evans *et al.* 2001). The studies showing a negative effect of inhibin have used various preparations, such as follicular fluid (crude inhibin) (McNeilly 1985), human recombinant inhibin A (Tilbrook *et al.* 1999) or immunization against inhibin (Mann *et al.* 1992a). There do not appear to be negative relationships between circulating concentrations of inhibin and FSH in physiologically uncompromised ewes (Souza *et al.* 1998; Evans *et al.* 2001). In addition, there have been reports that additional follicular factors, other than estradiol and inhibin, can suppress FSH and follicular growth (Law *et al.* 1992; Wood *et al.* 1993). In this regard, a steroid depleted fraction of follicular fluid suppressed follicle development in cattle even though the injected follicular fluid was > 95 % free of inhibin (Law *et al.* 1992). In one other study (Wood *et al.* 1993) in heifers, follicular fluid suppressed both FSH secretion and follicular growth despite the injection of an inhibin antiserum with the follicular fluid.

In a previous study, there were rhythmic peaks in serum FSH concentrations in spite of the absence of regular emergence of follicular wave during transition from breeding season to anestrus in Western White Face ewes (Bartlewski *et al.* 1999c). A 10-d treatment with estradiol-implants to raise the serum estradiol concentrations to 2.4-fold that in control ewes, resulted in the absence of follicular wave emergence in cyclic ewes; nevertheless, there were rhythmic but truncated peaks in serum FSH concentrations during the period of treatment as compared to the control ewes (Barrett *et al.* 2003). A 28-d intravaginal-treatment with oral contraceptives resulted in the absence of follicular

waves and follicular growth beyond 2 mm in diameter; however, there were rhythmic peaks in serum FSH concentrations similar to those in the control ewes (Bartlewski, Duggavathi and Rawlings, Unpublished data). Based on the observations above and from the present results from experiments 1 and 2, we hypothesized that there is an endogenous rhythm of peaks in daily serum concentrations of FSH which is, at least in part, independent of regulation by the ovarian follicular growth pattern. This hypothesis is supported by the results of experiment 3. In experiment 3, we observed peaks in serum FSH concentrations in serum samples collected daily in ovariectomized ewes (Fig. 7.4). These peaks were similar to those in intact ewes in terms of peak amplitude and inter-peak interval, even in the face of elevated basal FSH concentrations (Fig. 7.5).

In summary, doubling the amplitude of the FSH-peak that precedes emergence of a follicular wave did not alter the characteristics of the following follicular wave. Creation of physiological peaks of serum FSH concentrations every 2-2.5 d stimulated the emergence of additional follicular waves but did not alter the rhythmic occurrence of FSH peaks and follicular wave emergence. Follicular waves resulting from endogenous peaks in FSH secretion emerged and grew in the presence of the growing largest follicle of the follicular waves induced by exogenous oFSH, bringing the concept of follicular dominance in the ewe into question. The ovine ovary always appears to have small follicles (2 to 3 mm in diameter) capable of responding to a physiological increase in FSH secretion to produce a follicular wave. Finally, it seems that there is an endogenous rhythm of peaks in daily serum FSH concentrations, which is, at least in part, independent of regulation by ovarian follicular growth patterns.

Chapter 8: PATTERNS OF EXPRESSION OF STEROIDOGENIC ENZYMES IN OVARIAN ANTRAL FOLLICLES DURING THE FIRST WAVE OF THE CYCLE AS COMPARED TO THE PREOVULATORY FOLLICLE[*]

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8.1. Abstract

A systematic study of the expression patterns of steroidogenic enzymes in ovarian antral follicles at various stages of growth in a follicular wave as determined by ultrasonography and their correlation with circulating endocrine variables has not been reported for sheep. Ovaries were collected from ewes (n = 4-5 per group) when the largest follicle(s) of the first wave of the cycle reached i) 3 mm, ii) 4 mm, iii) ≥ 5 mm in diameter or when there was a single preovulatory follicle in the last wave of the cycle, 12 h after estrous detection. The expression pattern of steroidogenic enzymes was determined using immunohistochemistry and grey-scale densitometry. The expression of P450 aromatase in the granulosa cells and 3β-HSD and P450 17α-OH in the theca cells increased progressively from 3-mm follicles to follicles ≥ 5-mm in diameter in the first wave of the cycle and was lower in the preovulatory follicle after the LH surge compared to follicles ≥ 5-mm in diameter. However, the expression of 3β-HSD in granulosa cells increased progressively from 3-mm follicles to follicles ≥ 5-mm in diameter and was maintained at a high level in the preovulatory follicle after the LH surge. The amount of aromatase in the granulosa cells of the growing antral follicles correlated positively with the serum estradiol concentrations. We concluded that the

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expression pattern of steroidogenic enzymes in theca and granulosa compartments of the follicles growing in each wave in the ewe, paralleled with serum estradiol concentrations, with the exception that concentrations of 3β-HSD in granulosa cells increased continuously from follicles 3 mm in diameter to the preovulatory follicle after the LH surge.

8.2. Introduction

Ovarian antral follicles emerge and grow in a wave like pattern in sheep (Ginther *et al.* 1995; Souza *et al.* 1998; Bartlewski *et al.* 1999a; Evans *et al.* 2000) and the emergence of each follicular wave is associated with a transient peak in serum FSH concentrations (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). A peak in serum estradiol concentrations is associated with the attainment of maximum diameter by the largest follicle of a wave (Souza *et al.* 1998; Bartlewski *et al.* 1999a; Evans *et al.* 2000). In sheep, granulosa cells in preantral and antral follicles express FSH receptor (FSH-R) mRNA and those in large antral follicles express cytochrome P450 side chain cleavage (P450scc), cytochrome P450 aromatase (P450arom), and LH receptor (LH-R) (Huet *et al.* 1997; McNatty *et al.* 1999; Logan *et al.* 2002; Juengel *et al.* 2002).

In cattle, the growth of the dominant follicle of a wave is associated with an increase in the mRNA expression of cytochrome P450scc and P450arom in granulosa cells (Bao *et al.* 1997a) and the mRNA expression of P450scc, cytochrome P450 17α-hydroxylase (P450 17α-OH) and 3β-hydroxysteroid dehydrogenase (3β-HSD) in theca cells (Bao *et al.* 1997a,b). The LH surge downregulates the expression of the mRNA for

steroidogenic enzymes in bovine preovulatory follicles (Voss and Fortune 1993a; Voss and Fortune 1993b). However, in the face of low steroidogenic enzyme expression, especially 3β-HSD, progesterone concentrations in follicular fluid are high (Voss and Fortune 1993a). There have been, to our knowledge, no studies that have definitively explained the discrepancy between the increased capacity to produce progesterone and the decline in the expression of 3β-HSD. Similarly, in sows, growth of ovulatory follicles is associated with an increase in the mRNA expression of steroidogenic enzymes, which declines after the preovulatory LH surge (Liu *et al.* 2000).

The expression pattern of steroidogenic enzymes, in sheep, has been studied in ovaries collected from the slaughterhouse (Conley *et al.* 1995; Huet *et al.* 1997; McNatty *et al.* 1999; Logan *et al.* 2002; Juengel *et al.* 2002). Although this allows a comparison of the expression of steroidogenic enzymes in follicles of different size categories, it does not allow the correlation of amounts of steroidogenic enzymes with stages of antral follicular growth in a wave during a specific stage of the estrous cycle or the changing endocrine milieu in the circulation. A systematic study of the expression pattern of steroidogenic enzymes in theca and granulosa compartments, at specific stages of antral follicular growth, in a specific wave of the cycle as defined by ultrasonography and their correlation with circulating endocrine variables, has not been done for sheep. Transrectal ultrasonography has been used for the non-invasive monitoring of growth and ovulation or regression of ovarian antral follicles in many species including sheep (Pierson and Ginther 1987b; Ginther 1988; Ravindra *et al.* 1994; Song *et al.* 1996; Bartlewski *et al.* 1998; Souza *et al.* 1998; Singh *et al.* 2003; Duggavathi *et al.* 2003a). With ultrasonography, it is now possible to remove follicles from an animal at specific

stage of the ovarian follicular wave (Bao *et al.* 1997a; Bao *et al.* 1997b; Liu *et al.* 2000; Singh and Adams 2000).

We hypothesized that, in sheep, 1) the expression of steroidogenic enzymes increases with the growth of the largest follicle in the first wave of the follicle, 2) the expression of steroidogenic enzymes is downregulated in preovulatory follicles after the preovulatory LH surge, except for that of 3β-HSD, 3) the expression profiles of steroidogenic enzymes and follicular growth reflect the endocrine milieu in the circulation. To test these hypotheses, we used transrectal ovarian ultrasonography to facilitate the collection of ovarian antral follicles from cyclic Western White Face ewes when the largest follicle(s) in a follicular wave reached a specific diameter/stage of growth. Here, we report the expression patterns of 3 steroidogenic enzymes (3β-HSD, 17α-OH and aromatase) in theca and granulosa compartments of antral follicles at specific stages of their growth and the relationships with endocrine variables in the circulation. We detected expression of the steroidogenic enzymes using standardized immunohistochemistry (IHC) protocols.

8.3. Materials and Methods

8.3.1. Animals

Nineteen clinically healthy, cyclic (December – January) Western White Face ewes (age: 2 to 4 years; body weight: 92.0 ± 7.1 kg) were used in this experiment. The ewes were kept outside in sheltered pens and were fed daily maintenance rations of alfalfa hay, with water available *ad libitum*. The experimental procedures were performed according to the guidelines of the Canadian Council on Animal Care.

8.3.2. Transrectal ultrasonography and blood sampling

Ewes were checked for estrus twice daily (0800 and 2000 h) with cauda-epididectomized, crayon-harnessed rams. All ewes underwent transrectal ultrasonography twice a day from the time they were marked by a ram (estrus) until ovariectomy. Transrectal ultrasonography was done with real-time, B-mode ultrasonographic equipment (Aloka SSD-900; Aloka Co. Ltd., Tokyo, Japan) connected to a 7.5-MHz transducer (UST-5821; Aloka). During each scanning session, the number, diameter, and relative position of all follicles ≥ 1 mm in diameter were sketched on ovarian charts, and all ovarian images were recorded on high-grade videotapes using a super VHS-VCR (Panasonic AG-1978; Matsushita Electric, Mississauga, ON, Canada). Ovulation was defined as the disappearance of a large, ovulatory-sized follicle (e.g., 5–7 mm in diameter) which was confirmed by the detection of a corpus hemorrhagicum (Duggavathi *et al.* 2003b). Blood samples (10 ml) were collected by jugular venipuncture using vacutainers (Becton Dickinson, Rutherford, NJ, USA) immediately before each ultrasound examination.

8.3.3. Collection of follicles at specific stages of growth

Both ovaries from separate groups of ewes were collected by ovariectomy when the largest follicle(s) of the first wave of the cycle reached a diameter of 3 mm (n = 4 ewes; small follicle group), 4 mm (n = 5 ewes; medium follicle group) and ≥ 5 mm (n = 5 ewes; large follicle group) as determined by transrectal ultrasonography. Ovaries from an additional group of ewes (n = 5 ewes; preovulatory follicle group) were also collected when a follicle from the final wave of the cycle entered the preovulatory stage. Ewes that had only *one* large (≥ 5 mm in diameter) follicle during the estrus

period (12 h after estrus detection) were chosen for the preovulatory follicle group; this was done to ensure the collection of follicles that were destined to ovulate (Bartlewski *et al.* 1999a). Ovariectomies were performed on all ewes (Duggavathi *et al.* 2003b) by mid-ventral laparotomy under general anesthesia induced by intravenous injection of 2.5 % thiopental sodium (Pentothal®, Abbott Laboratories Ltd., Quebec, Canada; 25 mg/kg) and maintained by 3-5 % halothane (Halothane®, Halocarbon Laboratories, NJ, USA). Both ovaries, from each ewe, were exteriorized, excised and snap-frozen in liquid nitrogen immediately after their removal from the body and were stored at –80 ºC until further processing.

8.3.4. Selection and identification of follicles for cryosectioning

Thirty-eight ovaries were collected from 4 groups of ewes. The largest diameter follicle(s) in each ewe (e.g., 3 mm follicle(s) in the ewes in the 3-mm-follicle group) was selected to represent the growth of follicles in the first wave of the cycle in comparison with preovulatory follicles (the most mature follicles). Forty-three follicles were collected from the 19 ewes. To ensure the unbiased analysis of the follicles, all the selected follicles were identified by serial numbers (1 to 43) without revealing their diameter, the ewe number or the group. From then onwards, all follicles were identified by their identification numbers for further analyses until the specific-absorptive indices were determined for each follicle.

8.3.5. Cryosectioning

For cryosectioning, the part of the ovary containing the follicle of interest was cut into a block and was embedded in optimum cutting temperature (OCT; Tissue-Tek®, Catalogue number: 4583, Sakura Finetechnical Co., Ltd., Tokyo, Japan) medium and

trimmed by 30-50 μ thick sections until the follicle of interest was exposed at its maximum diameter (Fig. 8.1A). If two follicles were adjacent to each other in an ovary, then both follicles were cut as a single block. Frozen sections (10 μ thick) were obtained from each of the follicles with a cryostat microtome (Leica CM3050 S, Leica Microsystems, Nussloch, Germany) and mounted on poly-lysine coated glass slides (2 sections per slide). All slides were stored at –30 °C until further use.

8.3.6. Hematoxylin and eosin staining

Hematoxylin and eosin stained sections were utilized to identify granulosa and theca layers in each follicle. Several trials revealed that alcohol caused shrinkage and clumping of granulosa cells in the frozen sections. Therefore, slides were mounted using an aqueous mounting medium (Glycerin jelly: Gelatin 10 g, glycerin 70 ml, phenol crystals 0.25 g and distilled water 60 ml) after staining with diluted hematoxylin (1:1 in distilled water) and aqueous eosin (1% eosin-Y) were used for staining.

8.3.7. Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was used to detect steroidogenic enzymes (3β-HSD, 17α-OH and aromatase) in granulosa and theca layers of the follicles and staining intensities were quantified by gray-scale densitometric analysis (Singh and Adams 1998). Staining was done in 2 batches, to minimize variation in staining among the slides. Reference slides (n = 3 per batch) were used to determine the coefficient of variation (CV) for staining intensity across the series of slides, within and between the two batches. On each slide, one section received a primary antibody while the other a negative-reference serum (normal rabbit serum or normal mouse IgG).

For IHC, frozen sections were allowed to thaw and all incubations were performed at room temperature. The endogenous peroxidase activity was blocked with freshly prepared 1 % hydrogen peroxide (in phosphate buffered saline (PBS) with 1 % Sodium azide) for 30 min and non-specific binding was blocked with 5 % goat serum in PBS with 1 % bovine serum albumin (BSA). Sections were then incubated with primary antibodies or their negative-reference serum for 1 h in a humidified chamber. The primary antibodies were 17α-OH (cytochrome $P450_{C17}$; mouse monoclonal antibody against human CYP17A1 developed and kindly provided by Dr. C.R. Parker Jr., University of Alabama at Birmingham, Birmingham, AL); 3β-HSD (3 β-hydroxysteroid dehydrogenase; rabbit polyclonal antibody against recombinant human type II 3β-HSD, kindly provided by Dr J.I. Mason, University of Edinburgh, Edinburgh, UK); and aromatase (cytochrome $P450_{arom}$; mouse monoclonal antibody against human CYP19A1; MCA2077T; Cedarlane Laboratories Ltd., Hornby, ON, Canada). Negative controls were normal mouse IgG for 17α-OH and aromatase, and normal rabbit serum for 3β-HSD. The dilutions of primary antibodies and negative controls were 1:25 for 17α-OH and aromatase, and 1:500 for 3β-HSD. Following a PBS-wash, both sections of each slide were incubated with horse radish peroxidase (HRP) conjugated secondary antibodies (1:100 dilution; goat anti mouse/rabbit immunoglobulins, DakoCytomation Denmark, Glostrup, Denmark) for 30 min. Immunoreactivity was detected by incubation of the sections with a peroxidase substrate kit for 10 min (Vector® VIP, Vector Laboratories Inc., Burlingame, CA). Finally, the slides were mounted with an aqueous mounting medium. Sections were not counterstained to avoid interference with densitometric procedure (Singh and Adams 1998).

8.3.8. Quantification of IHC data

Quantification of the expression pattern of steroidogenic enzymes (3β-HSD, 17α-OH, and aromatase) in the granulosa and theca layers of the follicle wall was done by gray-scale densitometric analysis (Singh and Adams 1998) using a high-resolution microscope digital camera (DVC; Digital Video Camera Company, Austin, TX) and image analysis software (Northern Eclipes 6.0; Empix Imaging, Inc., Mississauga, ON, Canada). Microscope illumination, condenser position, diaphragm control, objective lens, projective lens, and camera contrast enhancement and offset controls were standardized and remained unchanged throughout the analysis period. A 40 X objective lens and a 0.63 X camera lens were used to obtain a 16-bit (1280 X 1024 pixels) RGB image of follicular wall, covering an area of 0.09 mm^2 of the follicle (Fig. 8.1B). The background subtraction technique was used to account for any non-uniformity of illumination. Moreover, the position of the measuring box (Fig. 8.1B) was kept constant in relation to the microscopic field of view, so that the same part of the field was measured for all images. Mean gray-scale value (transmittance) was measured on a scale ranging from 0 (pure black) to 255 (pure white), within a rectangular-box (Fig. 8.1B), representing a 3000 μ^2 area of the granulosa layer or a 1500 μ^2 area of the theca layer.

Transmittance was recorded at 4 predetermined locations selected based on the uniform random sampling technique (Gundersen and Jensen 1987) within the granulosa and/or theca layer that exhibited a positive reaction (3, 6, 9 and 12 O' clock positions) and 4 areas of the ovarian stroma surrounding the follicle that did not exhibit a positive reaction. Transmittance was also obtained from similar locations on the adjacent

negative control section. A blank slide was used with or without illumination to collect 100 and 0 % transmittance values, respectively. The mean of such 4 gray-scale values, recorded for each analysis, represented 0 or 100 % transmittance values, which ranged from 5 to 16 or 253 to 255, respectively.

The transmittance values obtained, as a mean of 4 values for each antral follicle (both positive and negative), were converted to percentage transmittance using the formula, $Tpc = ((T_R - T_0) * 100))/(T_{100} - T_0)$, where Tpc = percentage transmittance, T_R = raw transmittance from a sample slide, T_0 = 0 % transmittance from the blank slide, T_{100} = 100 % transmittance from the blank slide. The percentage transmittance values were then transformed to absorbance (A = - log10 (Tpc/100) (Fritz *et al.* 1992)), and the absorptive index was calculated by obtaining the ratio of absorbance of reactive to nonreactive locations (the ovarian stroma was used as a nonreactive location). This method of reporting the results provided considerable advantage over other methods because the absorptive index value is independent of section thickness (Gundersen and Jensen 1987).

The *specific absorptive index* for each of the follicles was calculated by subtracting the absorptive index of the section treated with the primary antibody from the absorptive index of sections treated with the negative control for the primary antibody. Intra- and inter-batch coefficients of variation (CV) for gray-scale densitometry for 3β-HSD, 17α-OH and aromatase enzymes were 4.5 % and 5.1 %, 6.1 % and 7.3%, and 4.4 % and 8.6 %, respectively. The specific absorptive index thus calculated was considered to be an indicator of the relative amount (concentration) of the steroidogenic enzyme in the specific cellular layer in the follicle.

8.3.9. TUNEL labeling for apoptosis

A terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) method was used to identify apoptotic cells in the granulosa layer. The level of apoptosis was quantified by cell counts and was used to confirm that the follicles collected for IHC were healthy (Dhar *et al.* 2001). The TUNEL labeling was performed as per the kit manufacturer's instructions (*In situ* cell death detection kit, POD; Roche Molecular Biochemicals, Penzberg, Germany) except that sections were incubated with TUNEL mix or labeling solution at 1:5 dilution. A total of 300 granulosa cells were counted, starting at a random location in the granulosa layer, using a 100 X objective lens of the microscope (Olympus BH-2; Olympus Corporation, Tokyo, Japan). The number of TUNEL-positive cells was expressed as a percentage of total number of cells counted for each follicle studied.

8.3.10. Hormone analysis

Blood samples were allowed to clot overnight at room temperature and the serum was harvested and stored at -20ºC until assayed. Blood samples collected immediately before the last ultrasound examination on the day of ovariectomy were used for the determination of serum concentrations of LH, FSH and estradiol. In the preovulatory follicle group, samples collected immediately before the penultimate ultrasound examination were also used for the determination of serum LH concentrations. This was done to check if there were high concentrations of LH representing the preovulatory surge and to confirm that the follicles collected from the preovulatory group were destined to ovulate. Serum concentrations of estradiol (Joseph *et al.* 1992), FSH (Currie and Rawlings 1989) and LH (Rawlings *et al.* 1984) were determined by

radioimmunoassay. The ranges of the standards were 0.06 to 8.0 ng/ml, 0.12 to 16.0 ng/ml and 1 to 50 pg/ml for the LH, FSH and estradiol assays, respectively. The sensitivities of the assays (defined as the lowest concentration of hormone capable of significantly displacing labeled hormone from the antibody) for LH, FSH and estradiol assays were 0.1 ng/ml, 0.1 ng/ml and 1.0 pg/ml, respectively. The concentrations in low and high reference sera analyzed for every assay were as follows: LH assay – 0.2 and 3.2 ng/ml, respectively; FSH assay – 0.4 and 1.5 ng/ml, respectively; estradiol assay – 5.3 and 12 pg/ml, respectively. The intra- and inter-assay CVs for low or high ovine reference sera analyzed for LH were 9.0% and 3.8% or 6.4% and 1.8%, respectively; for FSH were 4.3% and 3.1% or 6.2% and 8.8%, respectively and for estradiol, 11.3% and 7.3% or 8.3% and 10.2%, respectively.

8.3.11. Statistical analyses

Statistical differences were assessed by one-way ANOVA (Sigma Stat® Statistical Software, version 2.0 for Windows® 95, NT and 3.1, 1997; Chicago, IL, USA). Multiple comparisons were made using Tukey's test. Results are reported as least square means and SEM. Statistical significance was defined as $P < 0.05$.

8.4. Results

8.4.1. General results

All the ewes of the present study were marked by the crayon-harnessed rams. Ovulations in ewes of all groups ($P > 0.05$), except the preovulatory follicle group, were detected at a mean of 0.9 ± 0.1 d after observed estrus. None of the ewes in the preovulatory follicle group had ovulated by the time of ovariectomy, confirming that the collected follicles were preovulatory. Ovariectomy was performed on the ewes of

the 3 mm, 4 mm and ≥ 5 mm follicle groups on days 0.75 ± 0.1, 1.5 ± 0.2 and 2.0 ± 0.0 after the day of ovulation (d 0). Ovariectomy was performed on the ewes of the preovulatory follicle group on day 0.5 ± 0.0 after observed estrus. There were 4.3 ± 1.0, 2.0 ± 0.5, 2.2 ± 0.4 and 1.0 ± 0.0 follicles per ewe collected from the 3-mm, 4-mm, ≥ 5 mm and preovulatory follicle groups, respectively.

8.4.2. Diameters of the follicles collected

The mean diameters of the largest follicles collected in ewes of the 4 groups are shown in Fig. 8.3 (top panel). The diameter of the preovulatory follicle did not differ ($P > 0.05$) from the diameter of the largest follicle in the ≥ 5-mm-follicle group. The growth profile of the largest follicle in the ewes of the ≥ 5-mm-follicle group is overlaid on the mean diameters of the largest follicles of the groups of ewes from which the follicles of the first wave of the cycle were collected. This growth profile did not differ from the profile of mean diameter of follicles collected from 3-mm and 4-mm follicle groups.

8.4.3. Serum estradiol concentration

The mean serum concentrations of estradiol, on the day of ovariectomy, increased significantly ($P < 0.001$) from the 4-mm-follicle group to the ≥ 5-mm-follicle group, before declining in the preovulatory follicle group ($P < 0.02$; Fig. 8.3 bottom panel).

8.4.4. Serum LH and FSH concentrations

The mean serum concentrations of FSH, on the day of ovariectomy, declined ($P < 0.01$) from the 3-mm-follicle group to the ≥ 5-mm-follicle group (Fig. 8.4). The FSH concentrations increased ($P < 0.01$) from the ≥ 5-mm-follicle group to the preovulatory follicle group (Fig. 8.4). The mean serum concentrations of LH on the day of

ovariectomy did not differ ($P > 0.05$) between the groups of ewes. However, in the ewes of the preovulatory follicle group, the serum LH concentrations at 12 h before the last ultrasound examination were higher ($P < 0.01$) as compared to that on the day of ovariectomy (Fig. 8.4).

8.4.5. Thickness of the granulosa layer

Granulosa layer thickness in follicles of the 3-mm, 4-mm, ≥ 5-mm and preovulatory follicle groups was 45.9 ± 3.5 µm, 46.2 ± 2.1 µm, 53.0 ± 5.4 µm and 54.3 ± 3.8µm, respectively and did not differ ($P > 0.05$) between the groups.

8.4.6. TUNEL labeling of granulosa cells

There was no difference ($P > 0.05$) between the follicle groups for the number of apoptotic granulosa cells (TUNEL positive) expressed as the percentage of total granulosa counted (Fig. 8.5A). The overall mean percentage of TUNEL positive cells in all follicles studied was 1.5 ± 0.5 %.

8.4.7. 3 β hydroxysteroid dehydrogenase

The 3ß-HSD enzyme was localized in both granulosa and theca cells of all the follicles studied in the current study (Fig. 8.2 top panel). Fig. 8.5B shows the specific absorptive index due to 3ß-HSD localization in theca and granulosa cells separately. The amount of the enzyme in the theca layer increased ($P < 0.05$) from the 3-mm follicle group to the ≥ 5-mm-follicle group and then declined ($P < 0.001$) in the theca cells of the preovulatory follicles (Fig. 8.5B). The amount of the enzyme in the granulosa layer increased ($P < 0.05$) from the 3-mm follicle group to the ≥ 5-mm-follicle group and

there was no difference between the granulosa layer of the follicles of the ≥ 5-mm and preovulatory follicle groups (Fig. 8.5B).

8.4.8. 17 α hydroxylase

The 17 α-OH enzyme was localized exclusively in the theca cells of all the follicles studied in the current study (Fig. 8.2 middle panel). Fig. 8.5C shows the specific absorptive index due to 17 α-OH localization in theca cells. The amount of the enzyme in the theca layer increased ($P < 0.01$) from the 3-mm follicle group to the ≥ 5-mm-follicle group and then declined ($P < 0.001$) in the theca cells of the preovulatory follicles (Fig. 8.5C).

8.4.9. Aromatase

The aromatase enzyme was localized exclusively in granulosa cells of all the follicles in the current study (Fig. 8.2 bottom panel). Fig. 8.5D shows the specific absorptive index due to aromatase localization in granulosa cells. The amount of the enzyme in the granulosa layer increased ($P < 0.01$) from the 3-mm follicle group to the 4-mm follicle group and then to the ≥ 5-mm-follicle group. The amount of the enzyme declined ($P < 0.01$) in the granulosa cells of the preovulatory follicles (Fig. 8.5D). The amount of aromatase in the granulosa cells had a positive correlation with the serum estradiol concentrations ($r = 0.5$; $P < 0.03$).

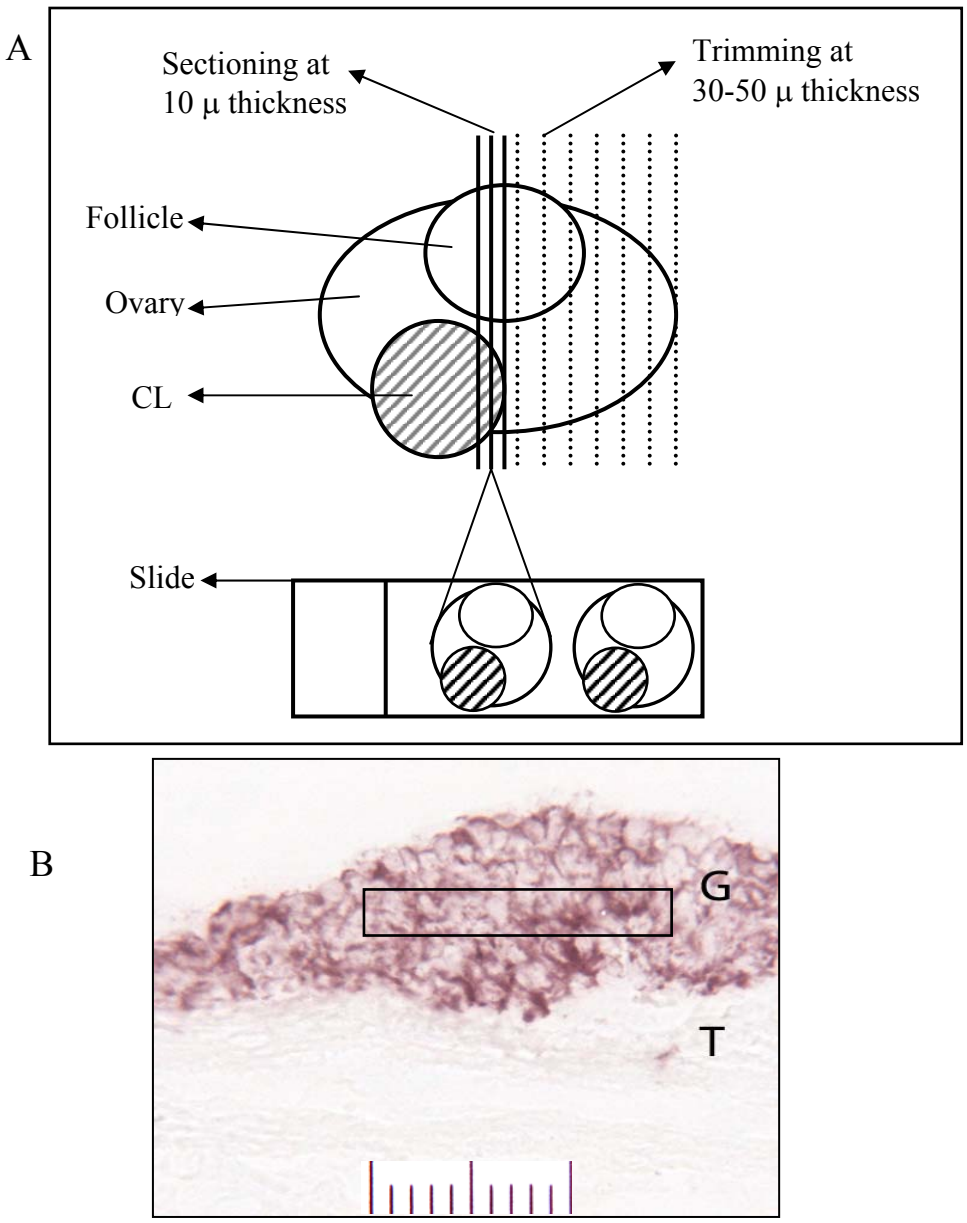

Figure 8.1. A) Schematic representation of the method of obtaining serial sections of a follicle in the plane closest to the central axis of the follicle. An OCT-embedded follicle was first trimmed with 30-50 μ thick sections until the plane closest to the central axis of the follicle was exposed. Minimal or no change in the diameter of the follicle in sequential sections indicated the plane closest to the central axis of the follicle. Serial sections of 10 μ were then cut. Placing 2 sections per slide helped in comparing the staining with a specific antibody and its negative control. B) A representative photomicrograph that was used to obtain a transmittance value within the rectangular box. The image, representing a 0.09 mm^2 area of the follicular, was obtained using a 40X objective lens and a 0.63X digital camera lens, and displayed at 50 % zoom. The scale bar is 100 μm in length. G – granulosa layer; T – theca layer.

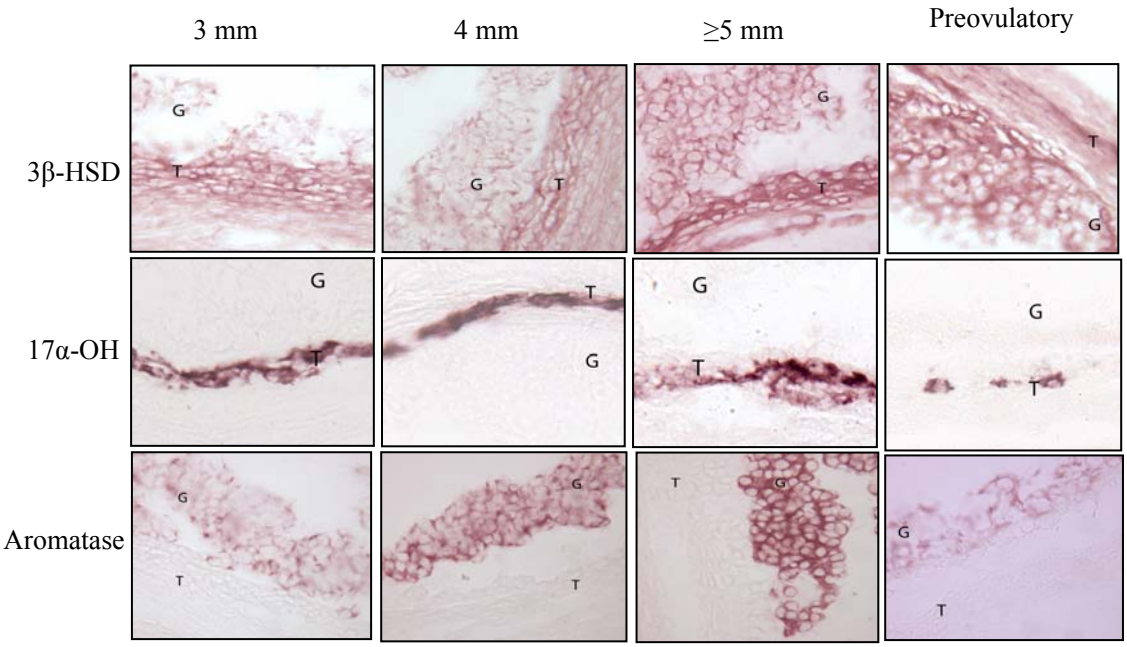

Figure 8.2. Photomicrographs of representative sections of follicles, at a specific stage of growth (3 mm, 4 mm, ≥ 5 mm and preovulatory), stained with antibodies against steroidogenic enzymes (3β-HSD – top panel; 17α-OH – middle panel; and aromatase – bottom panel). The detection of the steroidogenic enzymes was done using IHC (see Materials and methods for details). G – granulosa layer; T – theca layer.

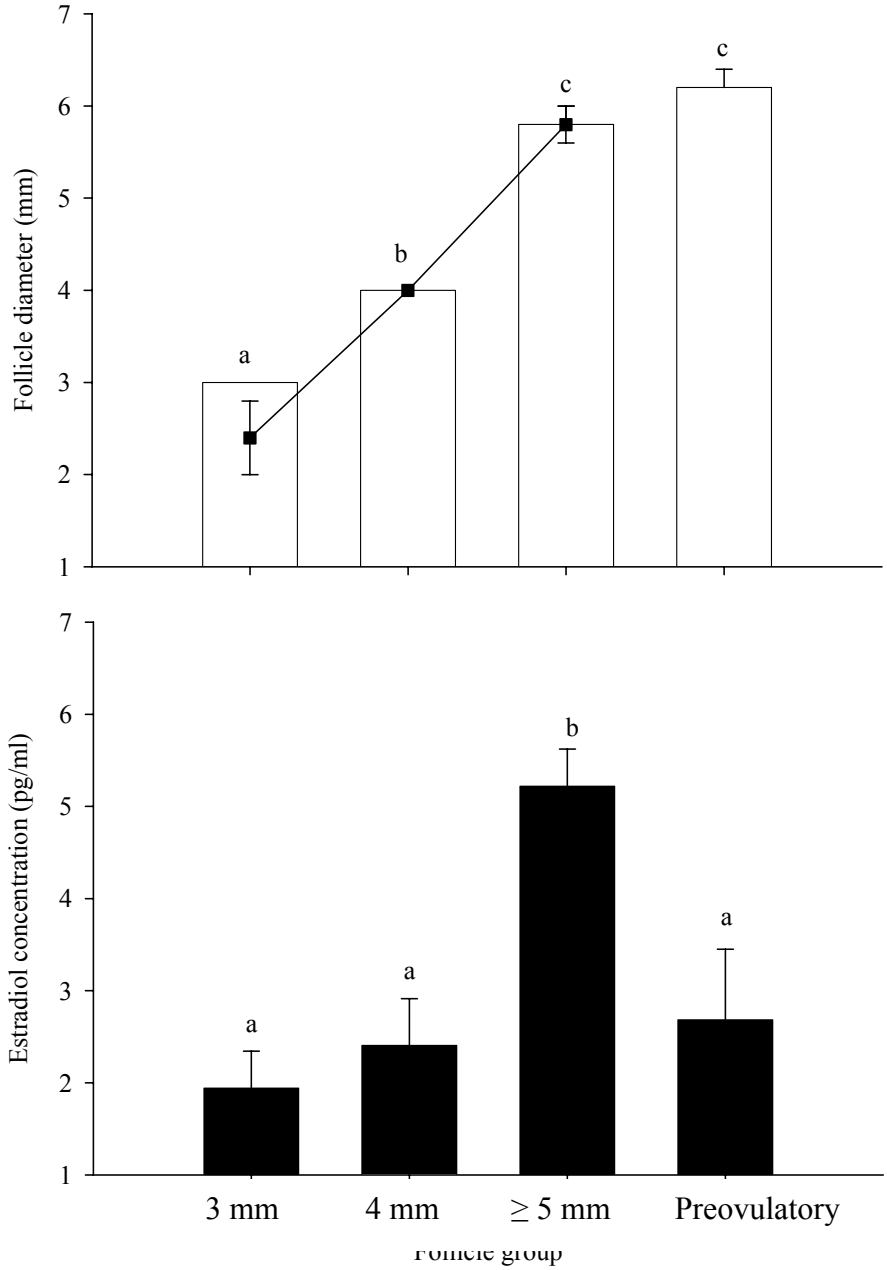

Figure 8.3. Top panel – Mean diameter (white bars; n = 4-5) ± SEM of the follicles collected at a predetermined stage of growth (3 mm, 4 mm, ≥ 5 mm and preovulatory). The diameter (Mean ± SEM; n = 5) profile (♦) of the largest follicle collected from ewes in the ≥ 5-mm-follicle group at the time corresponding to the collection of the follicles in the first wave of the ovine estrous cycle. Bottom panel – Serum concentration of estradiol (mean ± SEM; n = 4-5; black bars) on the day of ovariectomy. Follicles at specific stages of growth in a wave were collected by ovariectomy (3 mm, 4 mm, ≥ 5 mm and preovulatory follicle groups). a, b, c denote significant difference ($P < 0.05$).

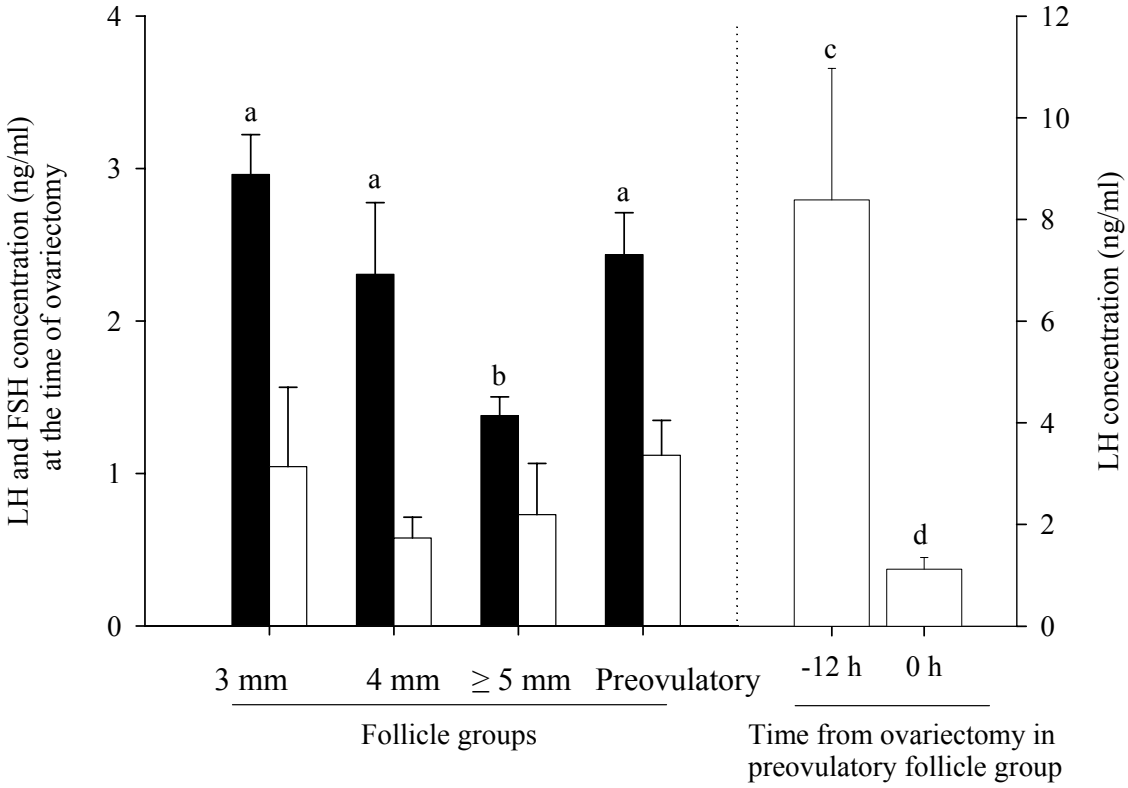

Figure 8.4. Serum concentrations of FSH (mean ± SEM; n = 4-5; black bars) and LH (white bars) on the day of ovariectomy. Follicles at specific stages of growth in a wave were collected by ovariectomy (3 mm, 4 mm, ≥ 5 mm and preovulatory follicle groups). Right panel - Mean (± SEM; n = 5) serum concentration of LH (empty bars) at 12 h before and at the time of ovariectomy in the ewes of the preovulatory follicle group. a, b, c denote significant difference ($P < 0.05$).

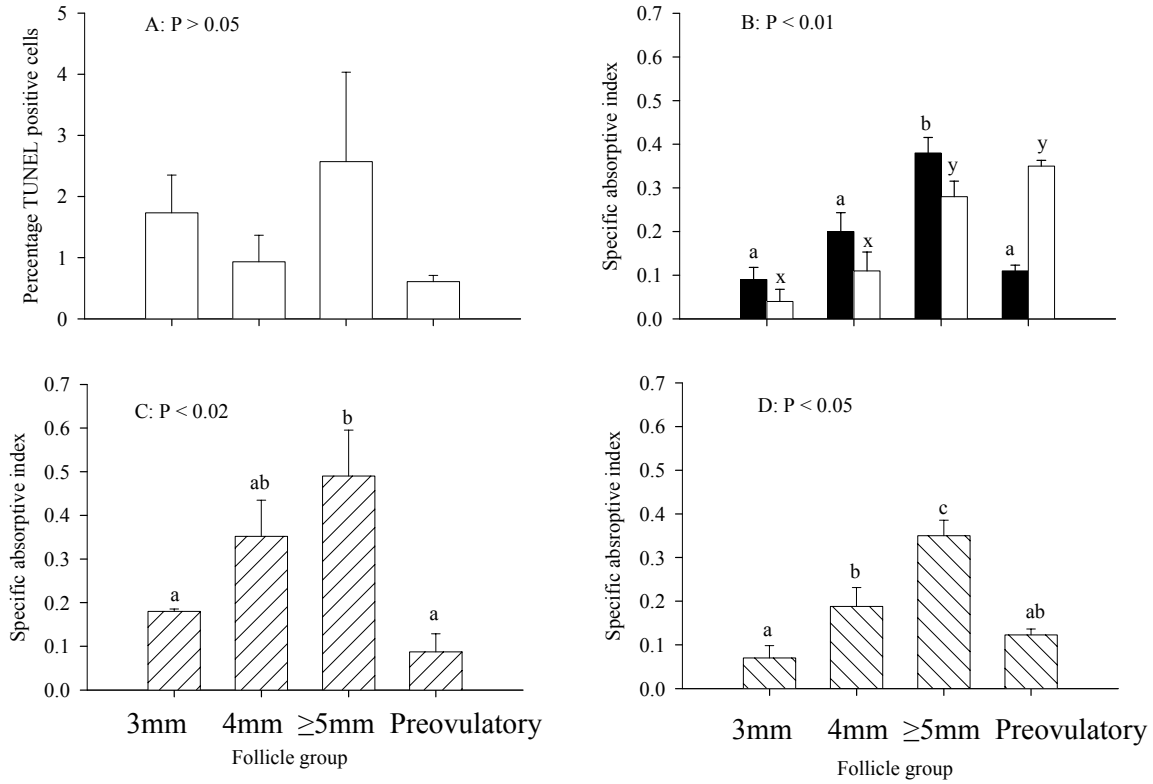

Figure 8.5. Percentage of TUNEL positive cells (mean ± SEM; n = 4-5; white bars) in the granulosa layer (A) and mean (± SEM; n = 4-5) specific absorptive index values due to staining for 3β-HSD (B) in theca (black bars) and granulosa cells (white bars), 17α-OH in theca cells (C; left hatched bars) and aromatase in granulosa cells (D; right hatched bars) in the follicles collected from ewes (3 mm, 4 mm, ≥ 5 mm and preovulatory follicle groups). a, b, c denote significant difference (P < 0.05).

8.5. Discussion

The use of serial transrectal ovarian ultrasonography allowed us to track and collect ovarian antral follicles at specific growth stages in a follicular wave, rather than simply collect follicles of different sizes but unknown follicular-wave status at sacrifice of ewes. This allowed us to assess steroidogenic enzymes concentrations at defined stages of follicular wave development and to correlate these to circulating hormone concentrations. The percentage of apoptotic cells in the granulosa layer of follicles in all the groups of ewes was minimal (< 5%) confirming that the follicle we selected based on the serial transrectal ultrasound examinations were healthy (Dhar *et al.* 2001).

The peak in FSH concentrations on the day of collection of 3-mm follicles (Fig. 8.4) is in agreement with previous studies (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), which demonstrated an FSH peak on the day of follicle wave emergence. The high serum concentrations of FSH in the preovulatory group (Fig. 8.4) could be a part of either the preovulatory surge (Pant *et al.* 1977; Goodman and Karsch 1981; Rawlings and Cook 1993) or the secondary surge in serum FSH concentrations (Pant *et al.* 1977; Bister and Paquay 1983; Wheaton *et al.* 1984); this distinction was not possible as frequent blood samples were not collected in the present study. The functional development of the antral follicles in the first wave of the cycle, in the present study, was indicated by the increase in serum estradiol concentrations from the 3-mm group to the ≥ 5-mm and ovulatory follicle groups (Fig. 8.3 bottom panel). A peak value of estradiol has been shown to occur on days 3-4 after estrus (Cox *et al.* 1971), which corresponds to d 2

after ovulation in the present study. Such an increase in estradiol concentrations during the early luteal phase has been shown in several previous studies (Pant *et al.* 1977; Karsch *et al.* 1979; Campbell *et al.* 1990; Bartlewski *et al.* 1999a; Evans *et al.* 2000; Duggavathi *et al.* 2003a) and has been associated with the culmination of the growth of the largest follicle in the first wave of the cycle (Souza *et al.* 1998; Bartlewski *et al.* 1999a). The follicles collected from the preovulatory follicle group represented fully mature preovulatory follicles. This was supported by the fact that follicles were collected after estrus but before ovulation from ewes which had only one ovulatory sized follicle during this period. In addition, the serum concentrations of LH at 12 h before ovariectomy were high as compared to the LH concentrations on the day of ovariectomy (Fig. 8.4). Serum concentrations of LH at 12 h before ovariectomy, in fact, were comparable to the concentrations of LH during the declining phase of the preovulatory LH surge (Baird 1978; Goodman and Karsch 1981; Rawlings and Cook 1993).

3β-HSD enzyme expression has been demonstrated in ovine follicles by other groups (Hay and Moor 1975a; Conley *et al.* 1995; Quirke *et al.* 2001; Logan *et al.* 2002). Some workers (Hay and Moor 1975a; Conley *et al.* 1995) have reported that localization of 3ß-HSD in ovine follicles was restricted to theca cells of antral follicles, while others (Logan *et al.* 2002) have demonstrated a variable localization of 3β-HSD in the granulosa cells, in addition to the theca layer, of follicles of various sizes. The results of the present study demonstrated the localization of and a progressive increase in the amount of 3β-HSD in both the granulosa and theca layers of the antral follicles growing in the first follicular wave of the ovine estrous cycle (Fig 2 and 5B). The expression of

17α-OH enzyme was localized exclusively to the theca cells of the antral follicles in the present study (Fig. 8.2). This is in agreement with previous reports (Conley *et al.* 1995; Huet *et al.* 1997; Logan *et al.* 2002), which demonstrated localization of the enzyme to a thin layer of cells close to the basement membrane. In the present study, we confirmed these findings and demonstrated that there was a progressive increase in the amount of 17α-OH in the antral follicles growing in the first wave of the ovine estrous cycle (Fig. 8.5C). In a previous study (Logan *et al.* 2002), aromatase mRNA and protein were localized exclusively in granulosa cells of follicles >3 mm in diameter and in another study (Huet *et al.* 1997) aromatase protein was localized to the granulosa layer of healthy follicles > 3.5 mm in diameter. In the present study, we extended these data by showing a 5-fold increase in the amount of protein present in the granulosa layer of follicles ≥ 5-mm in diameter as compared to the 3-mm follicles of the first follicular wave of the cycle (Fig. 8.2 and 5D). This observation is in agreement with a previous study (Tsonis *et al.* 1984), where an 8-10-fold difference in aromatase activity was found *in vitro* between small and large follicles.

In the present study, the progressive increase in the amount of the 3 steroidogenic enzymes (Fig 5B, C and D), in specific cellular layers of the growing antral follicles, appeared to be due to an increase in the amount of enzyme per cell rather than an increase in the number of granulosa or theca cells. This argument is supported by the fact that there was no significant difference in granulosa layer thickness between the follicular groups and enzyme concentrations were measured in a unit area in all follicles in the present study. Similar observations in cattle have been reported previously (Singh and Adams 2000), where the thickness of the granulosa layer and theca interna, and the

number of granulosa layers did not change during the growing and early static phases of the dominant follicle of the first wave of the cycle. Moreover, follicular growth, in both cattle and sheep, from class 5 (small antral follicles) onwards appears to result from antrum development rather than an increase in the number of granulosa cells (Turnbull *et al.* 1977; Cahill and Mauleon 1980; Lussier *et al.* 1987).

In the present study, there was a positive correlation ($P < 0.05$) between serum concentrations of estradiol and the amount of aromatase in the granulosa cells of the antral follicles, which grew from 3 mm to ≥ 5 mm in association with a peak in serum FSH concentrations. Ovine antral follicles smaller than 3 mm in diameter do not express aromatase enzyme in the granulosa layer (Huet *et al.* 1997; Logan *et al.* 2002). These findings are in line with the findings in cattle (Bao and Garverick 1998) and sheep (Driancourt *et al.* 1996), that follicular recruitment is associated with an FSH-driven initiation of expression of P450scc and P450arom mRNA in granulosa cells of the recruited cohort of antral follicles in a wave. In parallel to the increase in the expression of aromatase, follicles in the ≥ 5 mm follicle group, in the present study, had the highest amount of 3β-HSD and 17α-OH in the theca cells. Pregnenolone is converted to androstenedione in the theca layer by 3β-HSD and 17α-OH enzymes (Hanukoglu 1992) and androstenedione is aromatized to estrogens by aromatase enzyme in the granulosa layer (Hanukoglu 1992). These findings support previous studies, in sheep, that the large growing follicles are the major source of estradiol (Carson *et al.* 1981; Souza *et al.* 1998; Bartlewski *et al.* 1999a; Evans *et al.* 2000).

Another interesting observation, in the present study, was that the amount of 3β-HSD in the granulosa cells reached its highest levels in ≥ 5 mm follicles of the first wave (Fig.

8.5B). This would imply that the largest follicle of a wave, at its maximal growth, produces high quantities of progesterone, as 3β-HSD converts pregnenolone to progesterone (Hanukoglu 1992) and granulosa cells do not express the 17α-OH enzyme (Fig. 8.2 and (Logan *et al.* 2002)). This implication is supported by previous studies (Bartlewski *et al.* 1998; Bartlewski *et al.* 2000c), where a peak in serum progesterone concentrations was associated with the maximum diameter of the largest follicle of the wave in anestrous ewes. Such a correlation could not be confirmed during the estrous cycle, in the face of high levels of progesterone secreted by the CL. In another study (Evans *et al.* 2000), follicular fluid from the largest follicles of anovulatory waves had progesterone concentrations similar to that of estradiol-17β. Taken together, these observations would imply that follicles growing in a wave, in addition to producing estradiol, may also prepare for luteinization by beginning to produce progesterone in all phases of the ovine estrous cycle.

A key finding in the present study was the maintenance of the 3β-HSD enzyme concentration in the granulosa cells of the preovulatory follicle at high levels not different ($P > 0.05$) from that in the largest follicle of the first wave of the cycle (Fig. 8.5B). This is the first time, to our knowledge, that such a high level of 3β-HSD expression has been demonstrated in granulosa cells after the preovulatory LH surge in an untreated, physiologically uncompromised ovine estrous cycle. In fact, the expression of 3β-HSD mRNA has been shown to decline in both theca and granulosa cells in bovine preovulatory follicles following the LH surge (Voss and Fortune 1993a). However, in previous studies in both sheep (Spicer and Enright 1991; Spicer *et al.* 1995; Jolly *et al.* 1997; Evans *et al.* 2000) and cattle (Fortune and Hansel 1985; Spicer

and Enright 1991), ovulatory sized follicles before the LH surge had higher follicular-fluid estradiol concentrations than progesterone; whereas, the preovulatory follicle after the LH surge had higher concentrations of progesterone than estradiol. The results of the present study confirm that the LH surge is followed by maintenance, if not an increase, in the expression of 3β-HSD in the granulosa cells of the ovine preovulatory follicle and this is likely responsible for the increased progesterone concentration in the follicular fluid of preovulatory follicles in sheep (England *et al.* 1981; Murdoch and Dunn 1982). In the present study, there was a decline in the expression of aromatase in the granulosa layer, and 3β-HSD and 17α-OH in the theca layer of the preovulatory follicles compared to the ≥ 5-mm follicles (Fig 2 and 5). This indicated that the increased concentrations of progesterone in the follicular fluid of the preovulatory follicle are due to active synthesis of progesterone by the granulosa cells rather than a passive accumulation due to the loss of capability of the theca cells to metabolize pregnenolone to androgens via 17α-OH (Voss and Fortune 1993b). This also agrees with the idea that in the CL luteal cells of granulosa origin are the major source of progesterone (Schwall *et al.* 1986) and those of theca origin do not show the morphology of secretory cells (Niswender *et al.* 2000).

In summary, the quantitative IHC described in the present study was sensitive enough to show the differential expression of steroidogenic enzymes in theca and granulosa layers of healthy antral follicles in sheep. The expression of aromatase in the granulosa cells and 3β-HSD and 17α-OH in the theca cells increased progressively from 3-mm follicles to ≥ 5-mm follicles in the first wave of the ovine estrous cycle and declined dramatically in the preovulatory follicle after the LH surge. However, the expression of

3β-HSD in the granulosa cells increased progressively from 3-mm follicles to ≥ 5-mm follicles in the first wave of the ovine estrous cycle but was maintained at a high level in the preovulatory follicle after the LH surge. High concentrations of 3β-HSD in the granulosa cells of the preovulatory follicles, after the LH surge, are in fact responsible for the increased follicular fluid concentrations of progesterone, rather than the decreased ability of the theca cells to convert pregnenolone to androgens. Collectively, these observations indicated that follicles growing in each wave differentiate to the same extent in all phases of the cycle and after the LH surge, estradiol and androgen synthesis is shut off while progesterone synthesis is maintained. Finally, the results of the present study show that the present model of follicle collection holds promise for the study of the mechanisms of regulation of follicular growth in discrete follicular waves in the ewe.

Chapter 9: GENERAL DISCUSSION AND FUTURE DIRECTIONS

9.1. General discussion

The results of experiments described in this thesis furthered our understanding of ovarian antral follicular waves and their regulation in the ewe. Transrectal ultrasonography, using high-resolution ultrasound equipment, enabled us to monitor antral follicular growth and ovulation or regression in further detail. In previous studies (Schrick *et al.* 1993; Ravindra *et al.* 1994; Lopez-Sebastian *et al.* 1997; Souza *et al.* 1997a; Bartlewski *et al.* 1999a; Bartlewski *et al.* 1999b; Evans *et al.* 2000; Gonzalez-Bulnes *et al.* 2001), ovulations were defined as the disappearance of large ovulatory sized follicles between 2 consecutive ultrasound examinations and ovulations were confirmed by the detection of growing corpora lutea 3-5 d after ovulation. In the present study (chapter 3), *we demonstrated that it is possible to accurately detect follicle rupture and to identify the differentiating CL as early as 12-24 h after ovulation in sheep. Further, computer assisted ultrasound-image analysis revealed that echotextural characteristics correlated with morphological and functional organization of the forming CL. With this ability to visualize the ovulatory follicle as it ovulates and forms a CL, ultrasonography and image analysis offer a tool to study luteogenesis and the pathophysiology of luteal dysfunction in sheep, as well as in other species.*

With the use of high-resolution ultrasound equipment, we were also able to document the dynamics of small follicles (≥ 1 mm but ≤ 3 mm in diameter) in relation to follicular wave emergence in cyclic ewes (chapter 4). In cattle, wave-like developmental

dynamics of ovarian antral follicles (≥ 4 mm in diameter) has been demonstrated unequivocally with the use of serial ultrasonography (Pierson and Ginther 1987a;Sirois and Fortune 1988; Pierson and Ginther 1988); the emergence of each wave is characterized by a significant increase in the number of small follicles (6 to 9 follicles in the 4 to 6-mm size range; (Gong *et al.* 1993; Ginther *et al.* 1996)) and a peak in serum FSH concentrations. Subsequent growth of the dominant follicle is associated with a decrease (within 1to 2 days) in the number of small (4 to 6-mm) follicles (Ginther *et al.* 1989a; Fortune 1994; Driancourt 2001; Garverick *et al.* 2002). Many earlier studies (Lahlou-Kassi and Mariana 1984) and recent studies that used transrectal ovarian ultrasonography (Schrick *et al.* 1993; Ravindra *et al.* 1994; Lopez-Sebastian *et al.* 1997), failed to support the hypothesis of follicular waves in the ewe. However, follicular waves were documented in both cyclic (Noel *et al.* 1993; Ginther *et al.* 1995; Souza *et al.* 1998; Leyva *et al.* 1998b; Vinoles *et al.* 1999; Gibbons *et al.* 1999; Bartlewski *et al.* 1999a; Evans *et al.* 2000) and anestrous (Bartlewski *et al.* 1998) ewes by considering only those follicles that grew from 3 mm to ≥ 5 mm in diameter. The number of follicles that grew to 3 or 4 mm in diameter, but no further, did not show a wave-like pattern (Ginther *et al.* 1995) nor did the total number of follicles 3 or 4 mm in diameter (Bartlewski *et al.* 1999a).

In the present study (chapter 4), even with our ability to visualize follicles as small as 1 mm in diameter, we were unable to support the hypothesis that the pool of small follicles, from which the follicular waves emerge, increases at wave emergence and decreases with the growth of the largest follicle of the wave, as seen in cattle. However, follicles growing to ≥ 5 mm in diameter did show a wave-like pattern and the

emergence of each such follicular wave was preceded by a peak in serum FSH concentrations. This constant pool of small follicles remained unchanged even in the face of administration of exogenous oFSH at the time of either the nadir (chapters 6 and 7) or peak (chapter 7) in endogenous serum FSH concentrations. *Based on the observations above, it is possible to conclude that in the ewe, follicles reaching ovulatory diameter (≥ 5 mm) do emerge and grow in a wave-like pattern but without variations in number of small follicles (1-3 mm in diameter).* In contrast to cattle, it would make biological sense for sheep to have a constant number of small follicles, to provide a constant pool from which frequent recruitment of follicles to grow to ovulatory diameter can occur.

It is possible that the absence of statistical evidence for a wave-like pattern of change in numbers of small follicles in the ewe could be due to the greater number and speed of change-over of follicles, and the decreased inter-wave interval seen in sheep, as compared to cattle (Evans 2003a,b). The absence of statistical evidence for a wave-like pattern of change in numbers of small follicles in the present study (chapter 4) may also have been due to the fact that ewes employed in the study had 2, 3 or 4 follicular waves per cycle. The data for numbers of small follicles were centralized to the day of follicular wave emergence in the present study (chapter 4). However, the variation in the magnitude of day-to-day changes in the numbers of small follicles, due to different inter-wave intervals in ewes with 2, 3 or 4 waves per cycle, may have obscured statistically significant increases and decreases in the numbers of small follicles. To rule out this possibility, ewes with similar numbers of follicular waves per cycle (a

homogeneous group of ewes) should be examined for dynamics of small follicles in relation to the emergence of follicular waves.

Based on frequent blood sampling (every 12 min for 6 h) in cyclic ewes (chapter 5), it was found that mean and basal serum LH concentrations increased after the end of the growth phase of the largest follicle of the final follicular wave of the cycle but not after the end of the growth phase of that of the penultimate wave of the cycle. The period after the end of the growth phase of the largest follicle of the final wave was temporally coincidental with the time of luteal regression and the precipitous decline in serum progesterone concentrations. In a previous study (Bartlewski *et al.* 2000a), the amplitude of LH pulses increased at the end of the growth phase of the largest follicle of the first wave of the ovine estrous cycle; but this period was temporally coincidental with an increase in serum progesterone concentrations.

In sheep, the first follicular wave of the cycle emerges and grows in an environment of increasing progesterone concentrations when the LH pulse frequency is decreasing (Ginther *et al.* 1995; Bartlewski *et al.* 1999a; Bartlewski *et al.* 2000a; Evans *et al.* 2000). The penultimate and final follicular waves emerge during a period in which the CL maintains maximal function and there are constant serum concentrations of progesterone (Ginther *et al.* 1995; Bartlewski *et al.* 1999a; Evans *et al.* 2000). The growth of antral follicles in the final wave occurs when FSH concentrations are falling and LH pulse frequency is increasing following luteolysis (Baird 1983; Scaramuzzi *et al.* 1993). Although differing progesterone environments alter gonadotropin secretory patterns, follicular waves at all stages of the cycle in the ewe are similar in growth and maximal follicle size, with the exception that the largest follicle of the first wave has a

longer lifespan (Bartlewski *et al.* 1999a). *Based on the observations above, it is logical to conclude that the emergence and growth of ovarian antral follicles in follicular waves does not require changes in LH secretion and may perhaps involve changes in the follicular sensitivity to LH.*

In chapter 4, the follicular waves (defined as group of follicles growing from 3-mm to ≥ 5 mm in diameter; Ginther *et al.* 1995; Bartlewski *et al.* 1999a) emerged from a constant pool of small follicles (1-3 mm in diameter). It was concluded that there was a lack of evidence for selection of recruited antral follicles, in contrast to what is seen in cattle (Ginther *et al.* 1996). This hypothesis was supported by observations in chapter 7, where there was no significant difference in the number of follicles 4 mm and ≥ 5 mm in diameter collected from the ewes of the 4 mm and ≥ 5 mm groups. There were 4.3 ± 1.0, 2.0 ± 0.5 and 2.2 ± 0.4 follicles per ewe collected from the 3-mm, 4-mm and ≥ 5 mm follicle groups, respectively. These observations support the conclusion that *all those follicles that are recruited, in association with a peak in serum FSH concentrations, to grow from 2-3-mm in diameter to 4-mm diameter in a wave, succeed in reaching an ovulatory diameter of ≥ 5 mm.* The number of follicles recruited in each follicular wave in sheep has been reported to be in the range of 1-4 follicles (Souza *et al.* 1998; Bartlewski *et al.* 1999a; Evans *et al.* 2000; Duggavathi *et al.* 2003a). This is in contrast to cattle, where 6 to 9 follicles in the 4-6 mm size range are recruited into a wave, but only 1 follicle is selected to become dominant and reach an ovulatory diameter (Ginther *et al.* 1989b; Jaiswal *et al.* 2004).

The lack of selection of recruited follicles brings into question the existence of follicular dominance in sheep. The existence of follicular dominance in sheep is controversial

(Driancourt 1991; Driancourt 1994; Webb *et al.* 1999). Functional dominance by an ovulatory sized follicle has been defined as the process by which a dominant follicle inhibits the development of other competing follicles within both ovaries while continuing to thrive itself (Goodman and Hodgen 1983; Fortune 1994). In cattle, there is a hierarchy within a follicle wave not only in terms of diameter but also in terms of steroid production (Ginther *et al.* 1996). In fact, such a hierarchy in sheep has been demonstrated in some studies (Lopez-Sebastian *et al.* 1997; Evans *et al.* 2000). However, many other studies (Ginther *et al.* 1995; Gibbons *et al.* 1999; Bartlewski *et al.* 1999a; Bartlewski *et al.* 1999c) failed to demonstrate such a hierarchy, including the findings of chapter 4 of the present study. In the present study (chapter 4), the follicles recruited into a wave grew to reach an ovulatory diameter. The attainment of ovulatory diameter by the largest follicle of a wave was associated with a peak in estradiol concentrations (Ginther *et al.* 1995; Gibbons *et al.* 1999; Bartlewski *et al.* 1999a; Bartlewski *et al.* 1999c). However, the growth of the largest follicle was not associated with a decline in the numbers of small follicles, indicating the absence of functional dominance by the largest follicle of a wave in the ewe (chapter 4).

In sheep, some studies reported that the ovulatory follicle(s) exerts dominance over other follicles during the follicular phase (Ravindra *et al.* 1994; Lopez-Sebastian *et al.* 1997; Evans *et al.* 2000; Gonzalez-Bulnes *et al.* 2001). However, the studies described in this thesis would lead us to argue against the existence of follicular dominance in sheep (chapters 4, 6 and 7). The growth of the largest follicle in a wave was not associated with a decline in the numbers of small follicles (chapter 4). The largest follicle of a follicular wave, in both cyclic and anestrous ewes (chapter 6), did not

inhibit the emergence of a new follicular wave stimulated by exogenous oFSH administered at the expected time of the inter-peak nadir in serum FSH concentrations. The largest follicle of the induced follicular wave was similar to those in other follicular waves in FSH-treated and control ewes in terms of both maximum diameter and estradiol secretion (chapter 6). In addition, the largest follicle of the induced follicular wave failed to postpone the next endogenous FSH-peak and follicular wave emergence (chapter 6). This observation led us to examine the effects of exogenous oFSH administered at 2 consecutive inter-peak nadirs in serum FSH concentrations (chapter 7). This treatment induced additional follicular waves but failed to disrupt the rhythmic occurrence of endogenous FSH peaks and follicular wave emergence. In both studies (chapters 6 and 7) the number of small antral follicles (≥ 1 mm but ≤ 3 mm in diameter) did not change throughout the period encompassing treatment with oFSH and follicular emergence. *Based on these observations, we suggest that the largest follicle of a wave, in sheep, appears to have limited effects on other small follicles and on the time of emergence of the next follicular wave.*

In previous studies, new follicular waves have been shown to emerge in the presence of the estrogen-active largest follicle of the previous follicular wave (Johnson *et al.* 1996; Leyva *et al.* 1998a; Flynn *et al.* 2000; Evans *et al.* 2001). In addition, follicles emerging from two different follicular waves can ovulate together to increase ovulation rate in prolific sheep (Bartlewski *et al.* 1999a). Ovulation of follicles from 2 different waves has also been induced artificially, in non-prolific breeds of sheep (Bartlewski *et al.* 2003). In addition, a large (apparently dominant) follicle failed to inhibit eCG-induced growth or function of other follicles, suggesting that there is no direct effect of the large

(dominant) follicle on the growth of other follicles (Driancourt *et al.* 1991). *Thus, functional dominance, as is present in cattle (Fortune 1994; Ginther et al. 1996), may be absent in sheep.*

The absence of follicular dominance in sheep implies that responses to superovulatory treatments may not be influenced by the presence of a large follicle at the time of superstimulation, in contrast to the observations in cattle (Mapletoft *et al.* 2002). In agreement with this argument, in studies on superovulation in sheep, the presence of a large follicle has been shown to affect (Gonzalez-Bulnes *et al.* 2003) or fail to affect the sheep's response (Driancourt *et al.* 1991; Gonzalez-Bulnes *et al.* 2000) to a superovulatory treatment. The large variations in responses to superovulatory treatments in sheep could be due to other factors such as dose and purity of the stimulant used, stage of the breeding season, breed, nutrition and age (Gonzlez-Bulnes *et al.* 2004).

The failure of the largest follicle of a wave to influence the time of occurrence of the FSH-peak that precedes follicular wave emergence in both cyclic and anestrous ewes (chapters 6 and 7) led us to test the hypothesis of the existence of an endogenous rhythm for periodic peaks in serum FSH concentrations that was independent of ovarian follicular dynamics (chapter 7). The demonstration of regular peaks in serum FSH concentrations, occurring every 4 to 5 days (chapter 7) in ovariectomized ewes during 3 consecutive periods supported our hypothesis. Such an endogenous rhythm was previously suggested by others (Cahill *et al.* 1981; Bister and Paquay 1983). This hypothesis seems to be reasonable for sheep from an evolutionary point of view. Sheep vary in ovulation rate and therefore have to have a mechanism of follicular growth that allows ovulation of more than 1 follicle. We have shown that one such mechanism is

the ovulation of follicles from the penultimate wave of the estrous cycle (Bartlewski *et al.* 1999a), in addition to follicles from the final wave. The largest follicle of the penultimate wave obviously did not inhibit the FSH-peak that stimulated the emergence of the final follicular wave of the cycle (Bartlewski *et al.* 1999a). In addition, other evidence supports an endogenous rhythm of FSH secretion that is independent of ovarian follicular growth and demise. Regular periodic FSH peaks occur during the transition from the breeding season to anestrus, even though some of the peaks are not followed by follicular wave emergence (Bartlewski *et al.* 1999c). Based on the observations above, it is plausible to suggest that *an endogenous rhythm for periodic peaks in serum FSH concentrations that is independent of ovarian follicular dynamics exists, in sheep.*

Growth of follicles in a follicular wave is associated with an increase in the ability of the follicle to secrete estradiol (Karsch *et al.* 1979; Campbell *et al.* 1990; Souza *et al.* 1998; Bartlewski *et al.* 1999a; Evans *et al.* 2000; Driancourt 2001). In this regard, in the present study (chapter 8), we demonstrated a progressive increase in the amount of 3 steroidogenic enzymes (3β-HSD, 17α-OH and aromatase) in the follicles growing from 3 mm to ≥ 5 mm in diameter in the first wave of the ovine estrous cycle, as determined by transrectal ultrasonography. Collection of follicles at specific stages of their growth, based on ultrasonography, allows for the correlation of expression patterns of steroidogenic enzymes with stages of antral follicular growth in a wave during a specific stage of the estrous cycle, and also with the changing endocrine milieu in the circulation. The concentration of 17α-OH in the theca cells and of aromatase in the granulosa cells showed a progressive increase in follicles growing in a wave (3 mm to ≥

5 mm in diameter) and declined in the preovulatory follicles collected after the LH surge. In addition, the concentration of aromatase in the granulosa cells correlated positively with serum concentrations of estradiol.

In this study, we demonstrated a progressive increase in concentration of 3β-HSD in the granulosa cells of 3 mm, 4 mm, ≥ 5 mm diameter follicles and a sustained high concentration in the preovulatory follicles, collected after the LH surge. This is in contrast with the observations that the concentration of the mRNA for 3β-HSD declined in the granulosa cells of preovulatory follicles in cattle (Voss and Fortune 1993a). Several studies in sheep (England *et al.* 1981; Murdoch and Dunn 1982) and cattle (Voss and Fortune 1993a,b) have shown increased ability of the granulosa cells of the preovulatory follicles to synthesize progesterone even before ovulation. In this regard, our observation in chapter 8 implies that the increased concentration of 3β-HSD in the granulosa cells is responsible for this increased ability of preovulatory follicles to synthesize progesterone. High follicular fluid concentrations of progesterone in the largest follicle of the first wave of the ovine estrous cycle have been demonstrated previously (Evans *et al.* 2000). In the present study (chapter 8), there was a progressive increase in the concentration of 3β-HSD in the granulosa cells of growing follicles in a wave (3 mm to ≥ 5 mm in diameter); this again is in contrast with previous observations (Conley *et al.* 1995), which failed to demonstrate 3β-HSD expression in the granulosa cells of ovine antral follicles. These observations, taken together, would imply that the granulosa cells of the largest follicle of an anovulatory follicular wave in sheep, like that of the ovulatory wave of the cycle, acquire the ability to produce progesterone. It is, therefore, reasonable to assume that *the largest follicle of any follicular wave, in the*

ewe, irrespective of the stage of the cycle, is mature enough to ovulate if a gonadotropin surge is provided. The acquisition of the ability of the granulosa cells to produce progesterone seems to be LH-driven (Bao and Garverick 1998). Any such dependency in sheep appeared to be a function of changes in follicular sensitivity to LH rather than any temporal changes in the secretory pattern of LH (chapter 5). In addition, the results of the present study (chapter 8), demonstrate that, in sheep, it is possible to collect healthy growing follicles at a specific stage of the follicular wave and at a specific stage of the estrous cycle. This collection regimen provides a basis for studies investigating the expression profiles of paracrine regulators of follicular growth.

Finally, based on the results of the studies described in this thesis, it is reasonable to propose a model for the regulation of follicular waves in sheep (Fig. 9.1). The FSH-independent growth of follicles up to 2-3 mm in diameter occurs everyday, resulting in a constant pool of small follicles available for emergence into a follicular wave. Further FSH-dependent growth of antral follicles to reach ovulatory diameter (≥ 5 mm) is stimulated by a peak in serum FSH concentrations. As the follicles grow in a wave, they acquire steroidogenic capability and secrete estradiol under the influence of both FSH and LH. LH dependency of steroidogenesis does not involve any temporal changes in LH concentrations. The emergence of follicular waves in an orderly succession appears to be due to an endogenous rhythm of peaks in serum FSH concentrations. These peaks in serum FSH concentrations are largely independent of the pattern of growth and demise of the largest follicle of a wave. The increase in serum estradiol concentrations associated with follicular growth in a wave correlates positively with the expression pattern of aromatase in the granulosa cells. It is of interest that 3β-HSD expression

increases in the granulosa cells of antral follicles growing in a wave and continues beyond the LH surge in preovulatory follicles; follicles of all waves of the cycle appear to develop to a similar size and steroidogenic capacity.

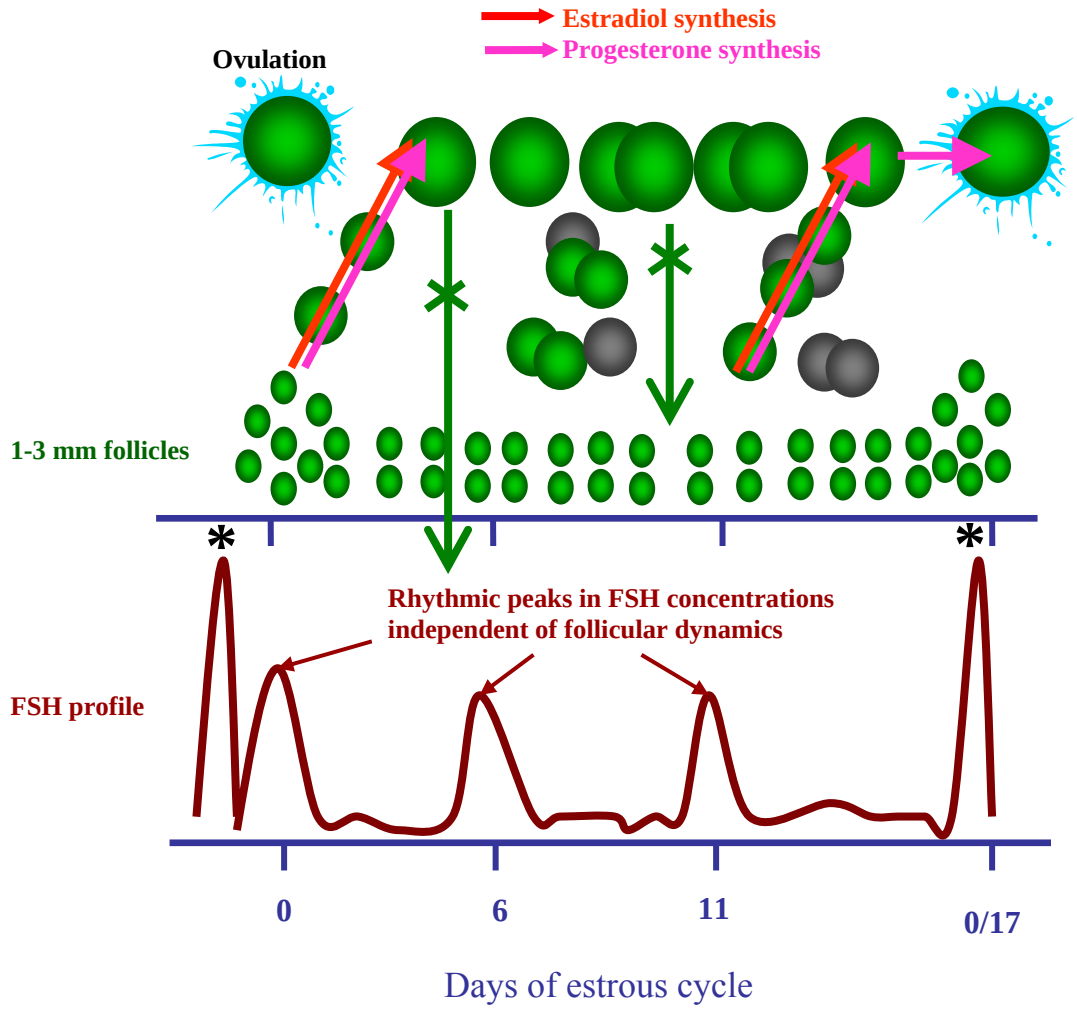

Figure 9.1. A schematic representation of ovarian antral follicular waves (2-3 mm follicles emerging and growing together to reach an ovulatory diameter of ≥ 5 mm, before regression (dark colored spheres) or ovulation). There is a constant pool of FSH-independent small follicles (1-3 mm in diameter) available for emergence into a follicular wave. FSH-dependent growth of antral follicles to reach ovulatory diameter is stimulated by a peak in serum FSH concentrations. As the follicles grow in a wave, they acquire steroidogenic capability (red and pink arrows) under the influence of both FSH and LH. LH dependency of steroidogenesis does not involve any temporal changes in LH concentrations (not shown). The growth of follicle to 2-3 mm in diameter and peaks in serum FSH concentrations, are largely independent of the pattern of growth and demise of the largest follicle of a wave (crossed green arrows). 3β-HSD expression, responsible for progesterone synthesis, increases in the granulosa cells of antral follicles growing in a wave and continues beyond the LH surge in preovulatory follicles; follicles of all waves of the cycle appear to develop to a similar size and steroidogenic capacity. * Preovulatory FSH surge that is coincidental with the preovulatory LH surge.

9.2. Future directions

1. The probable absence of follicular dominance in sheep was supported by induction of follicular waves by exogenous FSH and by the absence of a decline in the number of small follicles following the emergence of a wave in the studies described in this thesis. However, the absence of dominance in sheep needs to be proved by way of ablation of the largest follicle of a wave during its growth phase. Ultrasound guided follicle ablation at a known growth stage of the dominant follicle has been achieved in cattle (Ko *et al.* 1991). We have begun standardization of this procedure in sheep.

2. The failure to demonstrate temporal changes in the number of small follicles in relation to the emergence of follicular waves could be due to the high frequency of wave emergence in sheep (Evans 2003b) or due to the use of groups of ewes with 2, 3 or 4 follicular waves per cycle. In this regard, we plan to study dynamics of small follicles (1-3 mm in diameter), in relation to follicular wave emergence, using 2 separate groups of ewes with either 3 or 4 waves per cycle.

3. The demonstration of statistically significant changes in the numbers of follicles around follicular wave emergence may be possible if we include follicles of smaller diameter (< 1 mm). In cattle, even antral follicles < 1 mm have been shown to follow a wave-like pattern (Jaiswal *et al.* 2004). With the available ultrasound equipment (Aloka SSD 900), it is difficult to count follicles < 1 mm in diameter via transrectal ultrasonography. However, counting such follicles should be possible if ovaries are scanned in a waterbath. We plan to collect

ovaries from separate groups of cyclic ewes, when the largest follicle of the first wave of the cycle reaches 3 mm, 4 mm or ≥ 5 mm in diameter, and 24 h after the largest follicle reaches ≥ 5 mm in diameter. This time line is intended to represent the growth of the largest follicle of the wave through its growth phase and early static phase. Upon collection, all ovaries will be scanned in a water bath and follicles 0.5 to 3 mm in diameter will be counted. With this approach, it should be possible to demonstrate any increase in follicle numbers at wave emergence and any decrease in the number of small follicles as the largest follicle of the first wave reaches its largest diameter and enters the static phase of it lifespan.

4. Induction of a new follicular wave with a physiological dose of oFSH offers an excellent model to study regulation of follicular growth and oocyte maturation. Molecular characteristics of oocyte development during the growth of a follicle in a wave can be studied by inducing follicular wave emergence and collecting the follicles at different time points after treatment, in both cyclic and anestrous ewes.

5. As a follow up of the study described in chapter 8, we plan to detect the expression pattern of cytochrome P450 side chain cleavage and steroidogenic acute regulatory protein in follicles growing in the first wave of the cycle as compared to the preovulatory follicle. These 2 enzymes are very important for steroidogenesis (Logan *et al.* 2002). We also plan to study the expression pattern of cyclin D2 in growing follicles. Cyclin D2 is a cell cycle regulatory protein, implicated in granulosa cell proliferation (Robker and Richards 1998).

6. The growth of antral follicles during the declining phase of a peak in serum FSH concentrations has been suggested to be dependent on the IGF-system (Monniaux and Pisselet 1992). It would be interesting to study the expression pattern of IGFs, their receptors and binding proteins in follicles growing in a wave. For such a study, our protocol of follicle collection described in chapter 7 offers an excellent model.

7. Inhibins are implicated in endocrine regulation of FSH secretion and local regulation of granulosa cell steroidogenesis. It would be interesting to study the expression pattern of inhibins, activins and follistatin in follicles growing in a wave.

8. BMPs are implicated in the paracrine regulation of granulosa cell proliferation, differentiation and apoptosis (Souza *et al.* 2002; Richards *et al.* 2002). BMPs are secreted by both the theca cells (Souza *et al.* 2002) and the oocyte (Eppig 2001). Our protocols of follicular wave induction by exogenous oFSH and collection of follicles at specific stages of growth would provide an excellent model for investigating the individual roles of the theca cells and the oocyte in granulosa cell function.

PUBLICATION RECORD

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3. **Duggavathi R**, Bartlewski PM, Barrett DMW, Gratton C, Bagu ET, and Rawlings NC. 2004. Patterns of antral follicular wave dynamics and accompanying endocrine changes in cyclic and seasonally anestrous ewes treated with exogenous ovine FSH (oFSH) during the inter-wave interval. *Biology of Reproduction* **70**: 821-827**.**

4. **Duggavathi R**, Bartlewski PM, Pierson RA and Rawlings NC. 2003. Luteogenesis in cyclic ewes: Echotextural, histological and functional correlates. *Biology of Reproduction* **69**: 634-9.

5. Bartlewski PM, **Duggavathi R**, Aravindakshan J, Barrett DMW, Cook SJ and Rawlings NC. 2003. Effects of a 6-day treatment with medroxyprogesterone acetate (MAP) after prostaglandin F_2 (PgF_2)-induced luteolysis at mid-cycle on antral follicular development and ovulation rate in non-prolific Western white-faced ewes. *Biology of Reproduction* **68:** 403–1412.

6. **Duggavathi R**, Bartlewski PM, Barrett DMW and Rawlings NC. 2003. Use of high-resolution transrectal ultrasonography to assess changes in numbers of small ovarian antral follicles and their relationships to the emergence of follicular waves in cyclic ewes. *Theriogenology* **60**: 495-610.

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