CHARACTERISTICS OF FSH PEAKS AND ANTRAL FOLLICULAR WAVE DYNAMICS IN SHEEP

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By

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ABSTRACT

In the ewe, one to three antral follicles emerge or grow from a pool of small antral follicles (1 to 3 mm in diameter) every 3 to 5 days and reach diameters of ≥5 mm before regression or ovulation. Each follicular wave is triggered by a peak in serum concentrations of FSH. It is not clear what characteristics of an FSH peak cause follicular wave emergence and what aspects of development of a follicular wave are regulated by its preceding FSH peak.

In Experiment 1, we found that the amplitude of FSH peaks decreased, while basal serum FSH concentrations increased across the inter-ovulatory interval ($P < 0.05$). However, there were no associated changes in the growth, static or regression phases of follicular waves or the number and size of follicles in a wave. In Experiment 2, using computer-assisted quantitative echotextural analysis, we found that the numerical pixel value (NPV) for the wall of anovulatory follicles emerging in the third wave of the cycle was significantly higher than for waves 1 and 2 at the time of wave emergence but it decreased as follicles reached maximum follicular diameter ($P < 0.05$). A tendency for a similar pattern for the wall of follicles in the last wave of the cycle was also observed ($P = 0.07$).

In Experiment 3, treatment with ovine FSH (oFSH) increased the amplitude of an FSH peak by 5 to 6 fold. This treatment increased estradiol production ($P < 0.05$) but had little effect on other characteristics of the subsequent follicular wave. Daily injections of oFSH (Experiment 4) for four days, resulted in the occurrence of 4 discrete peaks in serum FSH concentrations. Each injection of oFSH resulted in the emergence of a new follicular wave.

In Experiment 5, six cyclic ewes received oFSH (0.1 μg/kg, sc) every 6 h for 42 h, to try to give a gradual increase in the leading slope of an FSH peak. Serum FSH concentrations increased in oFSH treated ewes ($P < 0.05$) resulting in an additional peak between two endogenously driven FSH peaks and therefore, did not give the planned gradual leading slope to an FSH peak. Ovine FSH treatment occurred in the early growth phase of wave 1 of the inter-ovulatory interval and increased the growth rate of growing

follicles in that wave, compared to control ewes ($P < 0.05$). This apparently induced dominance in follicles in wave 1, causing them to suppress wave emergence in response to the injected FSH. In Experiment 6, oFSH was infused constantly (1.98 μg/ewe/h, iv, n = 6) for 60 h. Infusion of oFSH maintained serum FSH concentrations at a level similar to the zenith of a peak. This resulted in a superstimulatory effect with a peak in the mean number of large follicles on Day 2 after the start of FSH infusion ($P < 0.001$).

A hormonal milieu similar to low serum progesterone concentrations was created by treatment of ewes with prostaglandin and medroxyprogesterone acetate (MAP) sponges (Experiment 7). This treatment delayed regression of the penultimate follicular wave of a cycle. However, the delayed follicular atresia was accompanied by a greater degree of apoptosis in somatic cells of follicles growing in the penultimate wave compared to those in the final wave of the cycle, when collected one day before expected ovulation.

In conclusion, trends in basal serum concentrations of FSH and peaks in serum FSH concentrations, across the estrous cycle, are associated with changes in the image attributes of follicles emerging later in the estrous cycle, perhaps reflecting a greater readiness of those follicles for ovulation and formation of CL. The ovine ovary can respond to discrete peaks in serum FSH concentrations with the emergence of new follicular waves on a daily basis. This led us to conclude that follicular dominance is not evident in the ewe and peaks in serum FSH concentrations are likely to be driven by some endogenous rhythm that is unrelated to ovarian follicular secretory products. However, direct dominance can be induced by giving supplemented FSH during the growth phase of a follicle. Extended exposure of ovine ovaries to the serum concentrations of FSH found at the zenith of a peak overrides the mechanisms that recruit follicles into a wave and induces a superovulatory response in cyclic ewes. Finally, an increase in the incidence of apoptosis occurs in antral follicles in sheep that have an extended lifespan, prior to any morphological changes detectable by ultrasonography. This would seem to cause decreased follicular viability and lowered fertility of the oocytes that the follicles contain.

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Dedicated to

My wife, my parents and my brother

for their love, patience and support…

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AUC	Area under the curve
BMP	Bone morphogenetic protein
CL	Corpus luteum
EGF	Epidermal growth factor
FSH	Follicle stimulating hormone
GDF	Growth differentiation factor
GnRH	Gonadotropin releasing hormone
IGF-I	Insulin-like growth factor I
Kg	Kilogram
LH	Luteinizing hormone
MHz	Mega hertz
µg/pg/ng	Microgram/Picogram/Nanogram
MAP	Medroxyprogesterone acetate
mm/mL	Millimetre/Millilitre
mRNA	messenger Ribonucleic Acid
NIH	National institute of health
NPV	Numeric pixel value
oFSH	ovine FSH
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PH	Pixel heterogeneity
SEM	Standard error of mean
TGF	Transforming growth factor

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

The ovary is an important functional unit of the female reproductive system. It is a very dynamic organ system, and undergoes major morphological and functional changes over a relatively short period of time and in a repeated manner. Intricate processes in the ovary, such as follicle formation, oocyte maturation, cellular proliferation, differentiation and apoptosis, ovulation and formation of the corpus luteum occur in a cyclic manner (estrous or menstrual cycle). The physiological mechanisms regulating such phenomena such as the growth of a follicle from 40 μm to 5-6 mm in diameter, the associated cytoplasmic and nuclear maturation of the oocyte, selection of ovulatory follicles(s), growth of the corpus luteum by about 25 fold in 12 days, and timed luteal regression in the absence of pregnancy have been interesting for researchers for many years.

The introduction of real-time ultrasonography led to significant advances in our understanding of ovarian physiology. Moreover, developments in cell and molecular biological techniques have greatly helped to increase our knowledge of the regulation of the growth of ovulatory follicles in several species such as humans, cattle and sheep.

In the research described in this thesis, certain aspects of the regulation of ovarian antral follicular waves were investigated in sheep. Major techniques applied in the experimentations described, included transrectal ultrasonography, computer-assisted image analysis, radioimmunoassay and TUNEL staining. Literature reviewed in this chapter focuses on our current understanding of ovarian follicular development and its

regulation in sheep. Where useful information was lacking for sheep, pertinent references to the literature for other domestic species, laboratory animals and primates have been used.

1.2. Reproductive cyclicity in the ewe

A period of reproductive cyclicity starts after puberty in females of most mammalian species which provides them with repeated opportunities to become pregnant (Senger 2005). In animal species, each reproductive cycle is referred to as an estrous cycle. Estrus is defined as a state of sexual excitability during which the female is sexually receptive for the male to copulate (Senger 2005). In the ewe, estrus lasts for 24 to 48 h, depending on the breed (Land 1970a, Land et al. 1973), and occurs recurrently every 14 to 18 days, with an average cycle length of 17.5 days (Marshall 1904). The length of the ovine estrous cycles is remarkably constant from cycle to cycle (McKenzie and Terrill 1937), with only small differences (≤1 day) among breeds of sheep (McKenzie and Terrill 1937) and little effect of age (Asdell 1946, Hafez 1952). Major variations in the length of estrous cycles are mainly caused by abnormal function of the reproductive system in this species. Longer estrous cycles of about twice the normal length were occasionally reported (McKenzie and Terrill 1937). It was suggested that such cycles reflected a lack of behavioral estrus and/or ovulation in between two estrous cycles (Goodman 1994). The occurrence of long cycles in the ewe can also result from the prolonged lifespan of corpora lutea (CL) (O'Shea et al. 1986). Shorter estrous cycles were mainly reported during the transition from anestrous to the breeding season and during the post-partum period (Bartlewski et al. 1999d, Bartlewski et al. 2000a). The

occurrence of these cycles was related to an inadequate luteal phase, caused by improper formation of a CL or its premature regression (Hunter 1991).

A dominant feature of ovine reproductive cyclicity is a season dependent cessation (anestrus) and restoration (breeding season) of recurrent estrous cycles (Marshall 1904, Hafez 1952). Therefore, the ewe is considered a seasonally polyestrous animal (Hafez 1952). In most breeds of sheep, normal estrus cycles occur during the autumn and winter; however, the length of the breeding season and anestrus vary depending on the breed and geographical latitude (Goodman 1994). In the regions of high latitude, the breeding season is limited to the period between late summer and winter, while in areas closer to the equator, there is no distinct anestrus season and estrous cycles are maintained throughout the year (Robinson 1959, Robinson 1980, Robinson 1988). The duration of exposure to light or photoperiod is a major determinant of the onset of the breeding season or anestrus in sheep (Legan and Karsch 1980). In the ewe, the average length of pregnancy is 150 days (Senger 2005); therefore, the annual rhythm of reproductive activity favors lambing in the spring, when proper environmental conditions support the survival of offspring (Gordon 1997). Although an annual reproductive rhythm drives the occurrence of ovine estrous cycles and ovulation (Goodman 1994), development of ovarian antral follicles appears to continue throughout the anestrus period (Hutchinson and Robertson 1966, Smeaton and Robertson 1971, McNatty et al. 1984, McNeilly 1984, Bartlewski et al. 1998).

The estrous cycle consists of two major phases, the follicular phase and the luteal phase (Senger 2005). The follicular phase (about 20% of the estrous cycle) is characterized by the presence of growing follicles as the primary ovarian structures and secretion of

estradiol as the primary reproductive hormone (Senger 2005). During the luteal phase (about 80% of the estrous cycle), corpora lutea (CL) and progesterone are the main ovarian structures and reproductive hormone, respectively (Senger 2005). The follicular phase can be sub-divided into pro-estrus and estrus (Arthur et al. 1989, Senger 2005). Pro-estrus is characterized by a transition in the estroids secreted by the ovary from progesterone to estradiol, emergence and growth of ovulatory follicle(s) and a marked increase in secretory activity of the entire reproductive system (Arthur et al. 1989, Goodman 1994, Senger 2005). Estrus, the period of sexual receptivity and mating, is characterized by distinct behavioral symptoms (Senger 2005). During estrus, the ewe shows a willingness to accept the ram and 'stands' for him to mount and mate her (Senger 2005). In the ewe, ovulation occurs 24 to 30 h after the onset of estrus, and is a spontaneous process, independent of the act of coitus (McKenzie and Terrill 1937, Robertson 1969, Quirke et al. 1979, Goodman 1994). The luteal phase of the estrous cycle can also be sub-divided into metestrus and diestrus (Arthur et al. 1989, Senger 2005). Metestrus, follows immediately after estrus and is characterized by the formation of a functional corpus luteum and a hormonal transition from secretion of estradiol to progesterone (Senger 2005). Prior to the transformation of the ovulated follicle into a CL, a structure called the corpus hemorrhagicum forms at the site of ovulation due to the rupture of blood vessels in the follicular wall (Senger 2005). Diestrus is characterized by maximum function of the CL and high progesterone secretion (Senger 2005). Reproductive cyclicity is controlled by the neuro-endocrine system, particularly the delicate interactions within the hypothalamic-pituitary-ovarian axis (Goodman 1994).

1.3. Hormonal profiles during the estrous cycle in the ewe

The main neuro-endocrine hormones involved in regulation of reproductive cyclicity include gonadotropin releasing hormone (GnRH) from the hypothalamus and follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin and oxytocin, released by the pituitary gland (Driancourt 2001). Estrogens and inhibins secreted by ovarian antral follicles, progesterone and oxytocin produced by CL, and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secreted by the uterine endometrium are also important (Scaramuzzi et al. 1993).

1.3.1. Secretion of GnRH

The hypothalamus plays a key role in the regulation of the estrous cycle by secretion of GnRH which in turn stimulates the release of the gonadotropins FSH and LH (Arthur et al. 1989, Senger 2005). There are two distinct modes of GnRH release from the hypothalamus (Clarke 2002b, Clarke and Pompolo 2005). One mode consists of GnRH secretion in relatively small pulses lasting about 4 to 5 min (Clarke 2002b). GnRH-pulse frequency is greater during the follicular phase of the estrous cycle (1 to 2 per h), compared to the luteal phase (1 to 2 per 6 h) (Moenter et al. 1991, Clarke and Pompolo 2005, Oakley et al. 2009). This mode of GnRH release is referred to as a tonic/episodic mode (Senger 2005). The ventromedial nucleus and the arcuate nucleus are the two hypothalamic nuclei identified for tonic secretion of GnRH (Senger 2005). The second mode of GnRH release is known as the surge mode (Clarke and Pompolo 2005). The preoptic nucleus, the anterior hypothalamic area and the superchiasmatic nucleus are the hypothalalmic nuclei that compose the surge center and are involved in surge release of

GnRH (Senger 2005). In the absence of progesterone, exposure of the surge center to a threshold level of estrogen results in the release of a large amount of GnRH from terminals of the neurons located in the surge centre (Clarke 1995a). The surge release of GnRH lasts 36 to 48 h and begins prior to and extends beyond the LH surge (Moenter et al. 1991, Caraty et al. 2002). In sheep, hypothalamic-hypophyseal portal GnRH concentrations during each peak are about 40-fold greater than the basal concentrations (Moenter et al. 1991).

1.3.2. Secretion of gonadotropins

Although there is general agreement that GnRH regulates the release of both LH and FSH, discrepancies in the secretory patterns of LH and FSH are noted both in physiologic (Mais et al. 1987, Hall et al. 1992) and pathophysiologic situations (Bishop et al. 1972a, Bishop et al. 1972b, Chappel and Barraclough 1976, Kalra 1976, Strobl and Levine 1988, Normolle et al. 1997). Gonadotrope cells in the anterior pituitary gland are responsible for synthesis and release of the gonadotropins LH and FSH (McNeilly et al. 2003). In sheep, both LH and FSH have been identified in the same gonadotropes and detection of cells expressing only LH or FSH is rare (Taragnat et al. 1998). Release of FSH from the gonadotropes occurs mainly through a constitutive pathway with little storage, while LH is stored in gonadotropes (McNeilly et al. 2003).

Release of LH from the gonadotropes is closely tied to the different modes of GnRH secretion; therefore, two distinct patterns of secretion are also described for LH; tonic/basal release in pulses and a surge release (McNeilly et al. 2003, Senger 2005). The gonadotropes have specific membrane receptors for GnRH (Stojilkovic et al. 1994).

Pulsatile LH release has been detected throughout the ovine estrous cycle by intensive blood sampling, with one pulse every 3 to 4 h (pulse amplitude of 1.6 ng/mL) during the mid-luteal phase or every 20 to 30 min just before the preovulatory LH surge (pulse amplitude of 0.7 ng/mL) (Baird 1978, Rawlings and Cook 1993). The preovulatory LH surge induces ovulation and formation of CL (Goodman 1994). In the ewe, the LH surge occurs around 14 h before ovulation and lasts for 8 to 12 h (Scaramuzzi et al. 1970, Bolt et al. 1971, Arthur et al. 1989, Moenter et al. 1990). In Western White Face ewes the average preovulatory LH surge peak was reported to be about 39 ng/mL (Rawlings and Cook 1993) (Fig 1.1., Top panel).

Based on blood samples collected from the hypothalamic-hypophyseal portal system, it appears that secretion of FSH from the anterior pituitary gland follows an episodic and a basal (constitutive) release pattern (Padmanabhan and McNeilly 2001).The assessment of secretory patterns of FSH in blood samples from peripheral blood vessels is hampered by the long half-life of FSH in circulation (≈ 2 h) and the lack of readily available assays to detect the different isoforms of FSH (Padmanabhan and McNeilly 2001). No pulsatile secretory pattern has been identified for FSH in blood samples collected from peripheral blood vessels (Bister and Paquay 1983, Wheaton et al. 1984, Wallace and McNeilly 1986). The episodic release of FSH seen in the hypothalamic-hypophyseal portal system includes both GnRH-associated and non GnRH-associated pulses of FSH (Padmanabhan and Sharma 2001). The basal/constitutive portion of FSH secretion is by far the major secretory component (Padmanabhan and McNeilly 2001, Padmanabhan and Sharma 2001) and reflects the availability of translatable FSHβ mRNA (Muyan et al. 1994, Farnworth 1995). The occurrence of peaks in serum FSH concentrations with a 4- to 5-d

rhythm has been detected in cyclic, anestrous and pregnant ewes (Bister and Paquay 1983). In the ewe, the duration of each peak in FSH concentrations lasts between 3 to 4 days (Bartlewski et al. 1999a, Duggavathi et al. 2005a). Peaks in serum FSH concentrations are associated with follicular wave emergence (Ginther et al. 1995, Souza et al. 1997, Bartlewski et al. 1998, Bartlewski et al. 2000a, Bartlewski et al. 2000b, Evans et al. 2000) (Fig 1.2.). A preovulatory surge release of FSH has also been described in the ewe (Fig 1.1, Top panel) (Baird et al. 1991, Wheaton et al. 1984). This FSH surge reaches a peak magnitude of 4.36 ± 0.39 ng/mL and occurs simultaneously with the preovulatory surge release of LH (Rawlings and Cook 1993).

1.3.3. Regulation of LH secretion

1.3.3.1. Hypothalamic regulation

Secretion of LH is closely governed by GnRH release. In sheep, LH pulses are invariably preceded or accompanied by GnRH pulses (Levine et al. 1982); this has been verified by measurements of GnRH concentrations in samples taken directly from portal blood or median eminence perfusates (Clarke and Cummins 1982, Levine et al. 1982). The amplitude of LH pulses has also been reported to be correlated with the amplitude of GnRH pulses (Levine et al. 1982). It has been shown that some small GnRH pulses are not followed by corresponding LH pulses (Clarke and Cummins 1982, Levine et al. 1982). It has been suggested that these GnRH pulses are involved in the synthesis of LH for cellular storage. In the ewe, pulsatile release of LH is also maintained during anestrus (Scaramuzzi and Baird 1977, Clarke 1988, Barrell et al. 1992); however, the

frequency and the amplitude of GnRH/LH pulses are decreased in the anestrous season, compared to the breeding season (Thiery et al. 2002, Clarke and Pompolo 2005).

1.3.3.2. *Pituitary regulation*

LH release occurs primarily through a GnRH-induced pathway. GnRH activates and maintains LHβ gene expression (McNeilly et al. 2003, Pawson and McNeilly 2005). However, there is little association between LHβ mRNA concentrations in the anterior pituitary and plasma concentrations of LH (Pawson and McNeilly 2005). In sheep, LHβ transcription even decreases during the follicular phase of the estrous cycle and during the LH surge (Brown and McNeilly 1997). Changes in LH release throughout the estrous cycle appear to be independent of LHβ gene expression (Pawson and McNeilly 2005) and to be mainly associated with the function of intra-cellular stores of LH (McNeilly et al. 2003). Within the gonadotrope, LH is mainly stored in electron-dense granules (Currie and McNeilly 1995) in association with the storage protein secretogranin II which organizes formation of these granules (Crawford and McNeilly 2002, Crawford et al. 2002, Crawford et al. 2004). Exocytosis of the LH granules and the number of gonadotropes that participate in LH release are both regulated by GnRH stimulation (McNeilly et al. 2003). Movement of LH granules within gonadotropes to the cell membrane, a feature referred to as polarization, plays an important role in the coordinated release of LH by these cells (Currie and McNeilly 1995, McNeilly et al. 2003). This effect probably occurs due to increased exposure to estradiol (Thomas and Clarke 1997). Any possible role of LH granules in basal release of LH needs to be investigated (Pawson and McNeilly 2005). Inhibin and activin appear to have no effect on LHβ subunit mRNA expression (Mercer et al. 1987, Gharib et al. 1990).

1.3.3.3. *Regulation by gonadal steroids*

In the ewe, estradiol and progesterone exert both direct and indirect regulatory effects on LH secretion (McNeilly et al. 2003). The amplitude and frequency of LH pulses are regulated by estradiol and progesterone, respectively (Bjersing et al. 1972, Karsch et al. 1979, Goodman and Karsch 1980, Rawlings et al. 1984, Wheaton et al. 1984). With the demise of the corpus luteum at the end of the luteal phase, serum progesterone concentrations decline; this results in an increase in GnRH secretory pulse frequency and accordingly, LH pulse frequency as the negative feedback effect of progesterone is removed (Clarke et al. 1987, Moenter et al. 1991, Clarke 1995a, Clarke 1995b). During the luteal phase of the estrous cycle estradiol, in concert with progesterone, exerts a negative feedback effect, resulting in decreased LH secretion (Moenter et al. 1991). The negative feedback effect of estradiol on LH secretion occurs mainly at the level of the hypothalamus (Goodman and Karsch 1980, Goodman and Karsch 1981, Goodman and Knobil 1981, Clarke et al. 2001, Clarke 2002a, Clarke 2002b). There is also a decreased responsiveness of the pituitary gland to GnRH during the luteal phase of the cycle (Clarke 1995b). However, during the follicular phase of the cycle, when serum estradiol concentrations increase in the absence of progesterone, a 'switch' from negative to positive feedback occurs and the progressive increase in serum estradiol concentrations culminate in the triggering of a preovulatory surge of GnRH and LH secretion (Clarke 2002a). Concurrently, gonadotropes become remarkably responsive to GnRH (Herman and Adams 1990, Clarke 1995a, Clarke 2002a). The preovulatory surge in serum LH concentrations, during the follicular phase of the estrus cycle, is clearly triggered by decreased progesterone and increased estradiol secretion (Scaramuzzi et al. 1970, Bolt et

al. 1971, Baird and Scaramuzzi 1976, Karsch et al. 1980, Jeffcoate et al. 1984, Rawlings et al. 1984, Kaynard et al. 1988, Moenter et al. 1990, Joseph et al. 1992, Pawson and McNeilly 2005). Estradiol failed to induce an LH surge in ovariectomized ewes treated with progesterone (Legan and Karsch 1979); this was due to a lack of high frequency GnRH pulses (Legan and Karsch 1979) and a decreased sensitivity of gonadotropes to estradiol (Koligian and Stormshak 1977).

1.3.4. Regulation of FSH secretion

1.3.4.1. Hypothalamic regulation

In the ewe, a small amount of FSH is secreted concurrent with the preovulatory GnRH/LH surge (Baird and McNeilly 1981, Baird et al. 1981). Collecting samples from hypophysial portal (Padmanabhan et al. 1997) or cavernous sinus (Clarke 2002b) blood also showed pulses in FSH secretion; however, only a small proportion of GnRH pulses were associated with FSH pulses and there was no trace of FSH pulses in peripheral blood vessels. Moreover, blocking the GnRH input by GnRH antagonist treatment did not interrupt episodic FSH secretion, indicating the existence of GnRH-independent FSH pulses (Padmanabhan et al. 2003). It appears that basal/constitutive FSH secretion which accounts for the major portion of FSH in circulation, occurs independent of pulsatile GnRH stimulation (Padmanabhan et al. 2002). However, a complete blockade of GnRH signals, after GnRH agonist treatment, resulted in decreased serum concentrations of FSH, associated with reduced mRNA expression for FSHβ subunit in the ewe (McNeilly et al. 1991). Moreover, it has been suggested that the frequency of GnRH pulses can affect gonadotropin secretion, with low GnRH pulse frequency

favoring FSH secretion and high frequency pulses increasing LH secretion (Padmanabhan and McNeilly 2001). Therefore, GnRH is essential for FSHβ gene expression, but it has only minimal regulatory effects on pulsatile and constitutive secretion of FSH. The existence of a separate hypothalamic releasing factor for FSH has also been suggested; however, the identity of such a factor is still unknown (Padmanabhan and McNeilly 2001). In the ewe, major fluctuations in serum FSH concentrations occur rhythmically every 3 to 5 days and are referred to as FSH peaks. The mechanisms involved in regulation of the periodicity of FSH peaks needs to be investigated in the ewe.

1.3.4.2. *Pituitary regulation*

It has been shown that activins, follistatin and also inhibins can be produced in the pituitary gland (Mather et al. 1992, Gregory and Kaiser 2004) and act as autocrine or paracrine factors to regulate the production of FSH (Knight and Glister 2001, Padmanabhan and Sharma 2001). Intra-gonadotrope activin stimulates transcription of the FSHβ subunit (McNeilly et al. 2003). This effect of activin is intensified by a synergistic effect with GnRH (Miller et al. 2002, Nicol et al. 2004). Moreover, it has been suggested that activin stabilizes FSHβ mRNA (Attardi and Winters 1993). Follistatin, produced by the pituitary, is an activin binding protein and hence, can negatively affect the action of activin (Farnworth 1995). It has been reported, based on *in vitro* studies, that the bone morphogenetic proteins (BMPs) show an inhibitory effect on the secretion of FSH from ovine pituitary gonadotropes (Faure et al. 2005). It has been suggested that this effect of BMPs involves preventing the activin-mediated

induction of FSH mRNA production and is synergistic with the negative effects of estradiol on FSH secretion (Faure et al. 2005).

1.3.4.3. Gonadal regulation

Estradiol is a key regulator of FSH secretion in the female (Mann et al. 1990, Baird et al. 1991, Clarke 2002a), acting directly at the level of the pituitary by suppressing FSHβ mRNA transcription (Miller et al. 2002). Estrogen effects are through estrogen receptor alpha (ERα) which is present in gonadotropes (Sheng et al. 1998, Tobin et al. 2001). Ovarian inhibin plays an important role in the modulation of the action of activin (Martin et al. 1988, Baird et al. 1991, Mann et al. 1992). Inhibin binds to the activin type II receptor and therefore, blocks the action of activin (Bernard et al. 2001, Bernard et al. 2002). In sheep, an inverse relationship was found between serum concentrations of inhibin A and FSH (Knight et al. 1998). Moreover, immunization against, or injection of inhibin, during the ovine estrous cycle, increased or decreased serum FSH concentrations, respectively; these effects were exerted via changes in FSHβ mRNA levels (Mann et al. 1989, Brooks et al. 1992, Clarke et al. 1993). However, the pattern of serum concentrations of inhibin A and the daily fluctuations in FSH concentrations associated with antral follicular growth and development in either cyclic (Souza et al. 1998) or anestrous ewes (Evans et al. 2001a) do not appear to be inversely related. It appears that inhibin exerts an overall level of negative feedback on FSH secretion but is not involved in the acute regulation of the day-to-day fluctuations in the serum FSH concentrations (Baird et al. 1991). Effect(s) of progesterone on FSH secretion are not clear. While some authors reported no effects of progesterone infusion on FSH secretion

(Dluzen and Ramirez 1987), others reported a suppressive effect of progesterone on FSH release from isolated ovine gonadotropes (Tsonis et al. 1986).

1.3.5. Secretion of estradiol

In the ewe, estradiol is secreted by antral follicles in a pulsatile manner (Baird 1978). There is a temporal association between LH pulses and transient increases in the secretion of estradiol-17β in cyclic (Baird and Scaramuzzi 1976, Baird et al. 1976) and anestrous ewes (Scaramuzzi and Baird 1977). An increase in estradiol secretion can be detected even within 5 min after a pulse of LH (Baird 1978, Martin 1984). Estradiol is mainly produced by large (≥5 mm in diameter), non-atretic ovarian follicles (Bjersing et al. 1972, Evans et al. 2000). Periodic increases in serum estradiol concentrations have also been reported in cyclic ewes, with 3 or 4 peaks (peak amplitude of 4.6 ± 0.6 pg/mL), lasting 3 to 4 days each (Bartlewski et al. 1999a), during each estrous cycle (Scaramuzzi et al. 1970, Cox et al. 1971a, Cox et al. 1971b, Campbell et al. 1995, Bartlewski et al. 1999a). Application of frequent ultrasonographic examination of the ovaries revealed a synchrony between the occurrence of peaks in serum estradiol concentrations and the end of the growth phase of the largest follicle in a follicular wave (Souza et al. 1998, Bartlewski et al. 1999a, Bister et al. 1999) (Fig 1.1., Bottom panel). Binding of LH to its receptor on the follicular theca cells, enhances synthesis of androgens (Senger 2005). Androgens produced in the theca cells, diffuses into the granulosa cells, where androgens are used as substrate for the synthesis of estradiol after activation of the aromatase enzyme by FSH (Carson et al. 1979, Fortune and Quirke 1988, Armstrong et al. 1998). During the follicular phase of the estrous cycle, maturation of the preovulatory follicles, together with increased expression of LH

receptors in both theca (primarily) and granulosa cells, induces increased estradiol secretion (Carson et al. 1979, Armstrong et al. 1981, England et al. 1981, Webb and England 1982). Estradiol production by the preovulatory follicle(s) increases in response to the progressive rise in LH secretion during the LH surge; however, once serum LH concentrations exceed a threshold (about 5 ng/mL), the prevulatory follicle(s) is no longer capable of responding to LH by producing estradiol (Baird 1978). In sheep, serum estradiol concentrations drop within 16 to 24 h of the LH surge and progesterone concentrations increase in the follicular fluid at the time of LH surge (Baird 1978, England et al. 1981, Wheaton et al. 1988, Campbell et al. 1990, Baird et al. 1991).

1.3.6. Secretion of progesterone

Progesterone is produced by the corpus luteum (CL), a temporary endocrine gland which usually forms after ovulation by transformation of the follicular cells into luteal cells (Juengel and Niswender 1999) (Figure 1.2.). The corpus luteum is composed of small and large luteal cells which originate from follicular theca and granulosa cells, respectively (Niswender et al. 2000, Senger 2005). By formation and maturation of the CL, serum progesterone concentrations increase from the day of ovulation to day 11 after ovulation and then drop to a nadir by day 15 after ovulation in the ewe (Edgar and Ronaldson 1958, Bartlewski et al. 1999a) (Fig 1.1., Bottom panel). Secretion of progesterone occurs in a pulsatile manner, with an average of 8 pulses in 24 h during the luteal phase (Alecozay et al. 1988). Although there is no temporal association between the occurrence of progesterone and LH pulses (Baird and Scaramuzzi 1976), LH is the main hormone involved in the regulation of luteal production and secretion of progesterone (Schomberg et al. 1967, Niswender et al. 1986). Treatment of ewes with

LH caused an increase in progesterone secretion (McCracken et al. 1971, Baird and Collett 1973). Furthermore, in hypophysectomized ewes, administration of LH maintained luteal function (Kaltenbach et al. 1968). On the other hand, premature luteal regression has been reported in normal ewes treated with LH antisera (Fuller and Hansel 1970). Small luteal cells respond to LH with increased secretion of progesterone (Niswender et al. 2000). However, large luteal cells, responsible for most progesterone secretion, are not responsive to LH (Goodman 1994).

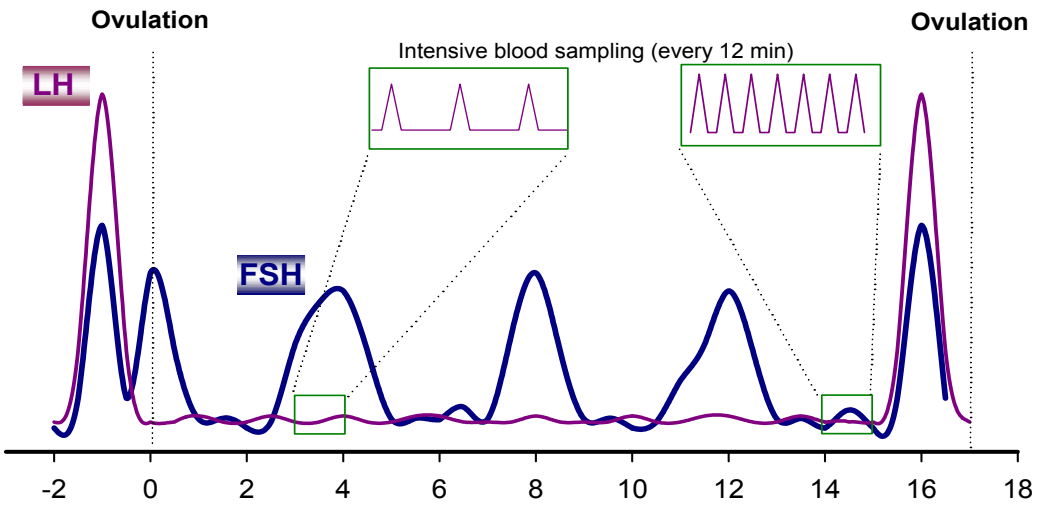
Although serum progesterone concentrations appear to vary among different breeds of sheep, reported observations have been contradictory. Some authors reported a higher serum progesterone concentration in prolific ewes, compared to non-prolific breeds (Quirke et al. 1979, Cahill et al. 1981); however, others (Bartlewski et al. 1999d) have shown a greater serum progesterone concentrations in non-prolific ewes. The latter concept is supported by studies in which sub-luteal serum progesterone concentrations prolonged the lifespan of large antral follicles (Johnson et al. 1996, Vinoles et al. 1999) and resulted in an increase in ovulation rate of non-prolific ewes (Bartlewski et al. 2003).

1.3.7. Secretion of inhibins, activins and follistatin

Inhibins and activins are members of the transforming growth factor β (TGF-β) superfamily which have a disulphide-linked dimeric glycoprotein structure (de Kretser et al. 2000, Knight and Glister 2003). Inhibins are composed of a unique α subunit and either a βA (inhibin A) or a βB (inhibin B) subunit (Knight and Glister 2001). Follistatin is a cysteine-rich monomeric glycoprotein and does not belong to the TGF-β

superfamily (Knight and Glister 2001). Follistatin acts as a binding protein to activin (Knight and Glister 2001). It has been suggested that follistatin neutralizes the biological activities of activin (Mather et al. 1993) and several bone morphogenetic proteins (Iemura et al. 1998). In the ewe, circulating concentrations of follistatin do not change during the estrous cycle and pregnancy (Xia et al. 2003). Expression of mRNA encoding follistatin is similar in ovine granulosa cells of growing preantral, antral and early static phase follicles and at different stages of the estrous cycle, but it is low in granulosa cells of preovulatory follicles (Tisdall et al. 1994).

Encoding genes for both inhibin A and B are expressed in the granulosa cells of ovarian antral follicles in sheep (Tisdall et al. 1994). It appears that large ovarian follicles are the main source of inhibin, since its expression is positively related to the size of follicles and their ability to secret estradiol (Campbell and Baird 2001). In ewes with ovarian autotransplants, significant changes in the circulating concentrations of inhibin A only concurred with the development of the largest follicle of the first follicular wave of a cycle (Souza et al. 1998). In another study no association between the development of follicular waves and fluctuations in serum concentrations of inhibin A was noted (Evans et al. 2001a). Potential effects of inhibin B in regulation of FSH secretion are unclear in sheep (McNeilly et al. 2003). Results from *in vitro* studies have shown that inhibin B is about 10 times less potent in suppression of FSH secretion from ovine gonadotropes when compared to inhibin A (Robertson et al. 1996).


Ovulation
Ovulation
Intensive blood sampling (every 12 min)
LH
FSH
-2
0
2
4
6
8
10
12
14
16
18

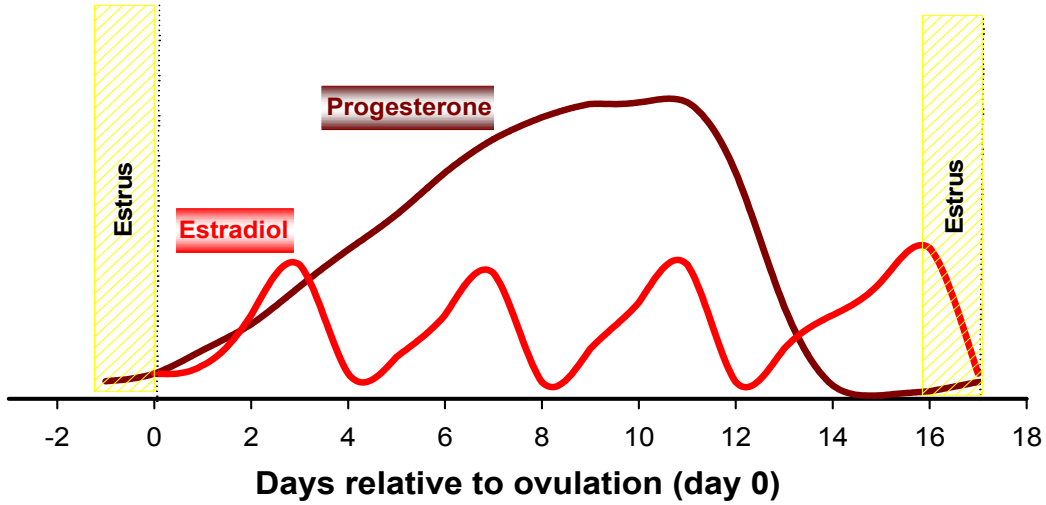

Progesterone
Estradiol
Estrus
Estrus
-2
0
2
4
6
8
10
12
14
16
18
Days relative to ovulation (day 0)

Fig. 1.1. Schematic representation of changes in serum concentrations of FSH, LH (Top panel) and progesterone and estradiol (Bottom panel) during an inter-ovulatory interval in the ewe. Except for high concentrations during preovulatory surge, serum LH concentrations remain at basal level throughout the luteal phase. Pulses of LH secretion can be detected in samples taken frequently from peripheral blood vessels. FSH secretion remains almost non-pulsatile and transient peaks in serum FSH concentrations are detectable every 4 to 5 d during an inter-ovulatory interval. Periodic peaks in serum estradiol concentrations ocurr during each inter-ovulatory interval and those peaks appear to coincide with nadirs in serum concentrations of FSH. Circulating concentrations of progesterone increase from day 0 to day 11 and then reach a nadir by day 15 after ovulation.

1.4. Follicular growth and development

1.4.1. Folliculogenesis

In mammals, follicular development consists of a series of events which begin after conception by development of the ovary, and terminate with ovulation of a mature follicle. Development of the ovary during the prenatal period requires germ cell migration, proliferation, and association with somatic cells. These processes lead to establishment of a finite reserve of primordial follicles from which follicular development occurs over the entire reproductive lifespan (Picton 2001). A primordial follicle is composed of a primary oocyte surrounded by a single layer of squamous somatic cells (pre-granulosa cells) (Land 1970a). Primordial follicles are located in a thin layer in the outermost part of the ovarian cortex (Picton 2001). In ewe lambs, the size of this reserve or resting pool of primordial follicles has been estimated to be between 40,000 to 300,000 (Driancourt 2001). Some authors have suggested that renewal of the resting ovarian follicular pool continues during the postnatal period in mice (Johnson et al. 2004) and humans (Bukovsky et al. 2004). This concept needs to be further investigated. On a near daily basis a small number of quiescent primordial follicles enter into a growing pool and develop into primary follicles (van and Rodgers 1996). A primary follicle is composed of a primary oocyte surrounded by single layer of cuboidal granulosa cells (McNatty et al. 1999, Driancourt 2001). Markers of cell proliferation, such as proliferating cell nuclear antigen, have been identified in the somatic cells of primary follicles (Wandji et al. 1997, Fortune 2003). When primary follicles develop into secondary follicles, they are characterized by having two or three

layers of granulosa cells (Driancourt et al. 1991, Fortune 2003). By further growth, secondary follicles become tertiary or early antral follicles (more than three layers of granulosa cells). Antral follicles have a fluid filled antrum within the granulosa cells (Lundy et al. 1999). Antral follicles grow under the effect of gonadotropins and acquire steroidogenic capability as mature Graafian follicles (Hay and Moor 1975). Development of primordial follicles into preantral and preovulatory stages requires several months and several weeks, respectively (Driancourt 2001). In the ewe, growth of primordial follicles to the early antral stage (180-250 μm in diameter) takes an average of 130 days (Cahill and Mauleon 1980, Cahill and Mauleon 1981, Picton et al. 2008). It takes about 24 to 35 days for preantral follicles to reach 0.5 mm, 5 days to reach 2.2 mm and about 4 days to reach a preovulatory size of 4.5 to 5 mm in diameter (Turnbull et al. 1977, McNeilly 1984).

1.4.2. The early stage of follicular development

Early follicular development covers the period of follicular growth between the primodial and preantral stages (Cahill and Mauleon 1980, Cahill and Mauleon 1981). Initiation of growth of primordial follicles can be described in two distinct phases (Picton 2001). First there is a transformation of the squamous pregranulosa cells into a cuboidal form and their entrance into the cell cycle required for proliferation. The second phase is characterized by an increase in the number of granulosa cells (Picton 2001). Mechanisms involved in the initiation and regulation of early follicular development are unclear. It is thought that early follicular development is a gondotropin-independent stage (McNatty et al. 1981a, McNatty et al. 1981b). In mice, primordial follicles do not express functional receptors for FSH and LH (O'Shaughnessy et al.

1997). Although expression of FSH receptors has been detected on granulosa cells of primary follicles in sheep, their proper functioning through adenylyl cyclase has not been confirmed (Fortune et al. 1999). It has been suggested that paracrine activators of cyclic AMP (cAMP), such as vasoactive intestinal polypeptide and norepinephrine, are involved in the initiation of follicular growth (Mayerhofer et al. 1997). It has been shown that primordial follicles and pregranulosa cells respond to activation of the cyclic AMP (cAMP) pathways with expression of aromatase and FSH receptors (McGee and Hsueh 2000). It is also been suggested by many authors that factors released by the oocyte are involved in initiation of primordial follicle growth and that those factors may moderate the actions of the gonadotropins FSH and LH on preantral and antral follicle growth (McGee and Hsueh 2000, Eppig 2001, Knight and Glister 2006, McNatty et al. 2007, Webb and Campbell 2007, Webb et al. 2007). Major suggested regulators of follicular growth include epidermal growth factor (EGF) (Qu et al. 2000) and its receptor; activin (Hulshof et al. 1997, Telfer et al. 2008); insulin like growth factor I (IGF-I) and its binding proteins (Thomas et al. 2007); members of the transforming growth factor-β (TGF- β) superfamily (Knight and Glister 2006) such as somatic derived anti-Mullerian hormone (AMH), oocyte derived growth differentiation factor-9 (GDF-9) (McGrath et al. 1995, Dong et al. 1996); and the bone morphogenetic proteins (BMPs) especially BMP4, BMP7 and BMP15 (Shimasaki et al. 1999, Otsuka et al. 2001, Shimasaki et al. 2003).

1.4.3. Antral follicular waves in the ewe

Generally, two stages of ovarian antral follicular growth have been identified in most domestic species (Mihm and Bleach 2003). An earlier stage, when antral follicles are

independent of gonadotropins, is referred to as a 'slow growth phase' (Cahill and Mauleon 1981, Lussier et al. 1987). This stage is followed by a 'fast growth phase', during which, antral follicles require gonadotropin support for further growth and development (Sunderland et al. 1994). In sheep, growth of antral follicles up to 2 mm in diameter is believed to occur independently of gonadotropins (Driancourt 2001). The 'fast growth phase' of ovarian antral follicles is described by an established follicular growth model known as the 'wave-like growth pattern'. In the ewe, a follicular wave is defined as the growth of a single follicle, or simultaneous growth of two or more follicles, from 2 to 3 mm in diameter to an ostensibly ovulatory size of ≥5 mm in diameter before regression (anovulatory wave) or ovulation (ovulatory wave) (Duggavathi et al. 2003a, Duggavathi et al. 2004). Follicles in a wave emerge or grow from the pool of small follicles within a period of 24 h (Bartlewski et al. 1998, Bartlewski et al. 1999a, Duggavathi et al. 2003a). Some characteristics of follicular development are well defined and extensively used by researchers to address the pattern of follicular growth and establishment of follicular waves in domestic ruminants (Ravindra et al. 1994, Bartlewski et al. 1998, Ginther et al. 1995, Bartlewski et al. 2000b, Evans et al. 2000, Driancourt 2001, Duggavathi et al. 2004, Adams et al. 2008, Peter et al. 2009). These elements, known as follicular dynamics or follicular wave dynamics mainly include the following parameters:

Follicle emergence or follicular wave emergence is the beginning of the growth of a group of follicles (in sheep: usually 1 to 3 follicles) from the minimum recordable size (2 to 3 mm in sheep) to ≥5 mm in diameter. The *growth phase* of a follicular wave is the period of time between emergence of a follicle (in sheep: from 2 or 3 mm in diameter)

and achievement of maximum size. The *regression phase* refers to the time taken by a follicle to regress from the maximum to its minimal recordable size. The time period between the end of the growth phase and the onset of the regression phase is defined as the *static phase* (Goodman and Hodgen 1983, Schrick et al. 1993, Ravindra et al. 1994, Bartlewski et al. 1999b). In sheep, the *number of follicles in a wave* is defined as the number of follicles growing from 2 or 3 mm to ≥5 mm in diameter in each follicular wave. *The Growth* or *regression rate* of a follicle is the change in size of a follicle during the growth or regression phase divided by the length of that period, respectively. Follicle *recruitment* is characterized by the synchronized growth of a group of ovarian antral follicles that become responsive to gonadotropic stimuli and enter a wave. By a *selection* process, a limited numbers of recruited follicles gain support to continue their growth to an ovulatory size and avoid atresia. Dominance is referred to as the ability of a large selected antral follicle (dominant follicle) of a wave to survive and develop further in an endocrine milieu suppressive to other co-existing follicles (subordinate follicles) (Ginther et al. 1996).

Since ovarian follicular dynamics has been most thoroughly studied in cattle (Adams et al. 1992, Adams and Pierson 1995), it will be beneficial to compare follicular dynamics in sheep to that of cattle. In cattle, the occurrence of either two or three follicular waves in an orderly succession during the estrous cycle is most common (Rajakoski 1960, Ginther et al. 1996), with an inter-wave interval of 7 to 10 days (Pierson and Ginther 1988, Savio et al. 1988, Sirois and Fortune 1988, Knopf et al. 1989). Emergence of each follicular wave in cattle is preceded by a transient peak in circulating FSH concentrations (Adams et al. 1992, Adams et al. 2008). Emergence is marked by an

increase in the number of small antral follicles in the ovary. This has been characterized as 8 to 41 follicles, 3 to 4 mm in diameter (Adams et al. 1992, Adams and Pierson 1995, Adams 1999) or 6 to 9 follicles in the size range of 4 to 6 mm in diameter (Gong et al. 1993, Ginther et al. 1996).

During the next few days, one follicle is selected to continue growth (dominant follicle), whereas the others become atretic and regress (subordinate follicles) (Ginther et al. 1989, Adams et al. 2008). The selection process of a dominant follicle is referred to as deviation (Ginther et al. 1996), which in cattle, occurs over a time period of about 8 h (Ginther et al. 1999). It has been shown in cattle, that all recruited follicles in a wave are capable of becoming the dominant follicle (Ginther et al. 1996). When the dominant follicle is selected, that follicle suppresses the growth of all other subordinate follicles in the existing wave, and prevents the emergence of a new follicular wave (Ginther et al. 1996, Adams et al. 2008). In cows, the dominant follicle of the last follicular wave of the estrous cycle is commonly the ovulatory follicle (8 to 20 mm in diameter) of that cycle (Ginther et al. 1996).

The introduction of real-time ultrasonography and application of frequent transrectal ultrasonographic examination of the ovaries, have led to a breakthrough in our understanding of follicular wave dynamics in both cyclic and anestrous ewes (Ravindra et al. 1994, Schrick et al. 1993,Ginther et al. 1995, Bartlewski et al. 1998, Leyva et al. 1998, Bartlewski et al. 1999a, Gibbons et al. 1999, Vinoles et al. 1999, Evans et al. 2000, Duggavathi et al. 2003a,). In the ewe, antral follicular waves occur every 4 to 5 days during both the breeding season and seasonal anestrus (Ginther et al. 1989, Bartlewski et al. 1998, Bartlewski et al. 2000b, Bartlewski et al. 2000c, Evans et al.

2000). During the breeding season, 3 or 4 follicular waves commonly occur in each estrous cycle (Noel et al. 1993, Ginther et al. 1995, Bartlewski et al. 1999a, Duggavathi et al. 2004). As in cattle, emergence of each follicular wave in the ewe has been shown to be associated with a preceding transient peak in serum FSH concentrations (Noel et al. 1993, Ginther et al. 1995, Bartlewski et al. 1998, Souza et al. 1998, Bartlewski et al. 1999a, Evans et al. 2000, Evans et al. 2001a, Duggavathi et al. 2003a, Duggavathi et al. 2004). Emergence of follicular waves has also been reported up to d 26 of pregnancy in sheep (Bartlewski et al. 2000a). In the ewe, each follicular wave is characterized by emergence of 1 to 3 follicles that grow from 2 to 3 mm to ≥5 mm in diameter before regression or ovulation (Ravindra et al. 1994, Ginther et al. 1995, Bartlewski et al. 1999a, Evans et al. 2000, Vinoles et al. 2001). Unlike in cattle, it has been demonstrated in many studies that emergence of follicular waves in sheep are not associated with a temporal increase in the number of small (2 to 3 mm in diameter) antral follicles in the ovary (Ginther et al. 1995, Gibbons et al. 1999, Evans 2003, Duggavathi et al. 2004). A suppressive effect on the growth of small follicles has been suggested for the largest follicles of a wave emerging in the early luteal phase (Vinoles et al. 1999) or the follicular phase (Ravindra et al. 1994) of the estrous cycle. However, in vitro studies did not show any inhibitory effect of the dominant follicle on cell division in small follicles in the ewe (Driancourt et al. 1991). Emergence and growth of more than one follicle in each follicular wave in sheep indicates that deviation possibly does not take place during the development of follicular waves. In the ewe, ovulatory follicles mainly originate from the final follicular wave of the cycle, as in cattle (Ginther et al. 1995, Bartlewski et al. 1999a); however, some prolific breeds have been shown to ovulate follicles from both the final and penultimate waves of the estrous cycle (Bartlewski et al. 1999a,

Gibbons et al. 1999). Again, in contrast to cattle, the presence of a large healthy follicle at the time of FSH treatment did not influence the consequent ovulation rate in sheep (Driancourt et al. 1991, Gonzalez-Bulnes et al. 2002a); indicating that the mechanism of dominance described in cattle is not as active in sheep (Ginther et al. 1989).

1.5. Hormonal control of antral follicular growth and development

1.5.1. Gonadotropic hormones

Gonadotropins play a central role in the regulation of antral follicular growth and development (Baird and McNeilly 1981, Ireland 1987, Picton et al. 1990). In sheep, expression of FSH receptors has been reported as early as the primary follicle stage (Tisdall et al. 1995). The quantity of FSH receptors increases as a follicle grow to ≥2 mm in diameter (Carson et al. 1979). In large preantral follicles LH receptors can be detected in the theca cells (Logan et al. 2002). In sheep, LH receptors are also expressed in granulosa cells of growing follicles but only when antral follicles are about 4 mm in diameter (Logan et al. 2002). It has been shown that both FSH and estradiol can induce synthesis of LH receptors by the granulosa cells (Uilenbroek and Richards 1979, England et al. 1981). These findings suggest that earlier stages of antral follicular growth are mainly FSH dependent, while terminal stages of antral follicular growth are controlled by LH (Campbell et al. 1995). In GnRH-suppressed ewes, treated with a GnRH agonist, FSH alone, but not LH alone, can stimulate the growth of follicles to a prevulatory size (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). As in cattle, it has been shown by many authors, that emergence

of each follicular wave in the ewe is preceded by a transient peak in serum FSH concentration, during both the breeding season (Ginther et al. 1995, Bartlewski et al. 1999a, Bister et al. 1999, Bartlewski et al. 2000b, Evans et al. 2000, Duggavathi et al. 2005a, Duggavathi et al. 2005b) and seasonal anestrus (Bartlewski et al. 1998, Bartlewski et al. 2000c, Evans et al. 2001a).

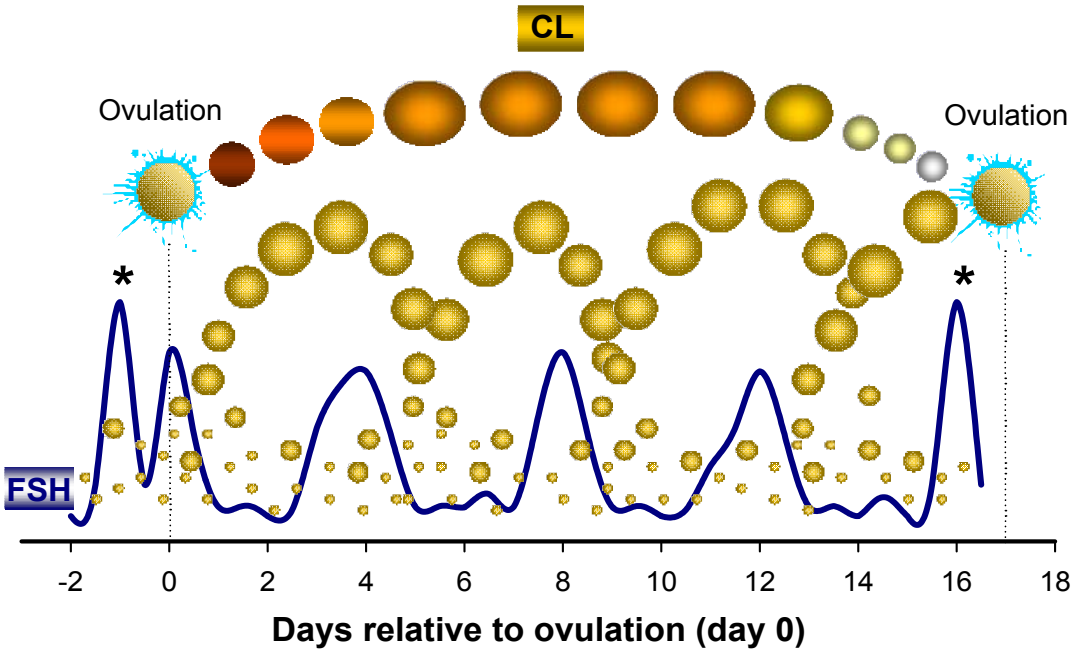


Fig. 1.2. Schematic demonstration of ovarian antral follicular waves and formation of a corpus luteum (CL) during an inter-ovulatory interval. Four follicular waves are shown here. In sheep, a follicular wave is defined as the growth of 1 to 3 follicles from the pool of small follicles (2 to 3 mm in diameter) in the ovary to ≥5 mm in diameter, followed by regression (anovulatory wave) or ovulation (ovulatory wave). Emergence of each follicular wave is associated with a transient peak in serum FSH concentrations (dark blue line) preceding that wave. * indicates preovulatory FSH surge that is coincidental with preovulatory LH surge (not shown). The corpus luteum forms by transformation of follicular cells (after ovulation) into luteal cells. Regression of the CL starts approximately 10 days after ovulation in the ewe and initiates the cascade of events that lead to the next ovulation.

1.5.2. Gonadal steroids

Based on the hypophysectomized rat model, it has been suggested that ovarian estrogens increase the response of ovarian follicles to gonadotropins (Richards 1994). Further studies in rodents confirmed that estradiol is required for the early stages of folliculogenesis (Findlay et al. 2000, Richards 2001, Britt and Findlay 2003). Moreover, treatment with estradiol has been shown to increase synthesis of LH receptors in granulosa cells of mature ovarian follicles in rats, via a synergistic effect with FSH (Richards et al. 2002). Estradiol treatment in sheep (Meikle et al. 2001) and cattle (Bo et al. 1993) induced atresia of large antral follicles and resulted in emergence of a new follicular wave.

Progesterone may alter follicular development in response to gonadotropins in both CL-bearing and non-CL-bearing ovaries (Rexroad and Casida 1977, McLeod and Haresign 1984, Hunter and Armstrong 1987, Scaramuzzi and Downing 1999). It has been shown that subluteal or submaximal concentrations of progesterone prolonged the lifespan of large antral follicles in cyclic ewes (Johnson et al. 1996, Vinoles et al. 1999). Some authors reported a lower circulating serum concentration of progesterone during the luteal phase of the estrous cycle in prolific ewes, compared to non-prolific breeds (Bartlewski et al. 1999a). This was associated with a greater ovulation rate in prolific ewes, with follicles from the penultimate wave have a prolonged lifespan and ovulating with those growing in the final wave of the estrous cycle (Bartlewski et al. 1999a, Gibbons et al. 1999). Whether this effect of progesterone is mediated by changes in LH pulse frequency is not clear (Leyva et al. 1998, Bartlewski et al. 2003).

1.5.3. Paracrine regulators of follicular growth

Some of the more frequently described paracrine/autocrine factors that have been suggested to affect the growth of antral follicles in sheep include ovarian inhibins and activins (Cahill et al. 1985, Findlay 1993); bone monrphgenetic 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).

The granulosa cells of ovine antral follicles secret inhibin (Knight and Glister 2001) and therefore, it can be detected in follicular fluid in high concentrations (Tsonis et al. 1986). In the presence of gonadotropins, inhibin increased the steroid production of ovine granulosa cells *in vitro* (Campbell et al. 1995). It has been shown that activin induces the proliferation of rat granulosa cells *in vitro* (Miro and Hillier 1996). In an activin knockout mice model, follicular development was arrested at an early antral stage (Matzuk et al. 1996). These evidences suggest an important role for activin in regulation of granulosa cell proliferation. In the ewe, BMPs are secreted by follicular theca cells (Liao et al. 2003, Shimasaki et al. 2003) and the oocyte (Eppig 2001). In sheep, BMPs appear to be involved in granulosa cell differentiation (Souza et al. 2002). In vitro studies using immature rat ovaries have shown that follicular theca cells secret TGFα (Teerds and Dorrington 1992) and EGF (Skinner et al. 1987), while their respective receptors can be detected on the follicular granulosa cells in sheep. In ovary autotransplanted ewes, infusion of antral follicles with TGFα acutely suppressed estradiol, inhibin and androstendione production and was followed by atresia of large antral follicles (Campbell et al. 1994). In cyclic ewes, EGF treatment suppressed

estradiol-17β production and hence, inhibited the preovulatory LH surge and behavioral estrus (Radford et al. 1987a, Radford et al. 1987b). Suppressed inhibin and estrogen production by granulosa cells was also observed in sheep when EGF was directly infused into the ovarian artery (Murray et al. 1993). EGF has been shown to sustain the health and viability of activated ovine primordial follicles in vitro (Andrade et al. 2005).

The IGF system is composed of two ligands (IGF-I and II), two receptors and at least six binding proteins (IGFBPs) that control the bioavailability of the ligands (Hunter et al. 2004, Silva et al. 2009). It has been shown that all components of the IGF system are expressed in both healthy and atretic follicles in sheep (Munoz-Gutierrez et al. 2005, Hastie and Haresign 2006). A simultaneous expression of mRNA encoding components of the IGF system has been also detected in the ovine ovary throughout both the breeding and non-breeding seasons (Hastie et al. 2004, Hastie and Haresign 2006). IGF-I is produced by the follicular granulosa cells (Monget et al. 2002) and its concentration in follicular fluid is high during the growth phase of large follicles in sheep (Monget et al. 1993). In sheep, it has been shown that IGF-I mainly stimulates the proliferation of granulosa cells from small follicles, whereas, it increases secretion of progesterone by granulosa cells from large follicles (Monniaux and Pisselet 1992, Monget and Monniaux 1995). Proteolytic degradation of IGFBPs has been also shown to change during the growth and atresia of antral follicles in the ewe (Besnard et al. 1996a, Besnard et al. 1996b). In cattle, IGF-I plays an important role to increase the sensitivity of small antral follicles to gonadotropins and acts synergistically with FSH to stimulate follicular development (Fortune et al. 2001, Monget et al. 2002). An FSH-dependent regulatory mechanism for expression of IGFBPs has been suggested in cattle (Armstrong et al.

1998). It appears that the IGF system mediates the preparation of the selected bovine dominant follicle to survive and develop under conditions of decreasing FSH and increasing LH availability in cattle (Silva et al. 2009). However, more elucidation is required regarding the relevance and function of this system for follicular development in the ewe.

Local effects of the CL on follicular development have been investigated in sheep. It has been suggested that CL increased the number of all visible follicles in the ovine ovary (Dailey et al. 1982). However, other reports showed that the presence of a CL locally suppressed the number of follicles ≤3 mm in diameter, while the number of follicles >3 mm in diameter was not affected (Bartlewski et al. 2001b).

1.6. Transrectal ultrasonography

Introduction of real-time ulatrasonographic imaging of the reproductive tract has profoundly influenced reproductive research and clinical diagnostics (Pierson and Adams 1995). Besides its unique diagnostic applications, ultrasongraphic imaging of the reproductive tract allows repeated and non-invasive observations of the same individual. In animal species, application of transrectal ultrasonographic examination of the reproductive tract was first introduced in the 1980's and initially used in cattle and horses (Ginther 1983, Pierson and Ginther 1984a, Pierson and Ginther 1984b, Adams et al. 1987). A few years later, this technique was adapted to study ovarian follicular and luteal dynamics in sheep (Schrick et al. 1993, Ravindra et al. 1994, Ginther et al. 1995). Some initial attempts failed to show a distinct wave pattern of follicular development in sheep (Schrick et al. 1993). However, application of more specific definitions in sheep

to characterize the small (2 to 3 mm in diameter) or the ovulatory sized (≥5 mm) follicles demonstrated a clear wave-like pattern of follicular development in this species (Ginther et al. 1995, Bartlewski et al. 1998, Leyva et al. 1998, Souza et al. 1998, Gibbons et al. 1999, Duggavathi et al. 2004,).

1.6.1. Computer-assisted image analysis

Computer-assisted analysis of ultrasound images is a valuable extension to ultrasonography (Singh et al. 2003). Ulatrasonography is based upon the ability of body tissues to reflect or transmit high frequency sound waves (Pierson and Adams 1995). An ultrasound image is a two dimensional array of picture elements known as pixels (Ginther 1995). Each pixel represents one of 256 shades of grey ranging between 0 (black) and 255 (white) (Singh et al. 2003). The human eye is capable of distinguishing among only 18 to 20 shades of grey (Singh et al. 2003); therefore, quantitative evaluation of the changes in the ultrasonographic appearance of the tissue (echotexture) is not feasible by the human eye. Complex computer algorithms were designed in order to objectively analyze and quantify the range of 256 shades of grey of pixels in ultrasound image echotexture (SYNERGYNE Version 2.8®, Saskatoon, SK, Canada) (Singh et al. 2003). Image attributes of follicles, representing histomorphology, can be utilized to assess stage of development and aspects of physiological function (Singh et al. 2003). Image analysis provides investigators with greater quantitative information on the echotextural dynamics of antral follicles (Tom et al. 1998b) and CL (Tom et al. 1998a, Duggavathi et al. 2003b,). The validity of image analysis to predict the physiologic status of ovarian follicles has been tested in domestic species, mainly cattle, by evaluating correlations among ultrasound image characteristics and histomorphologic

and functional attributes of follicles and oocyte viability (Kastelic et al. 1990, Singh et al. 2003).

1.7. General objectives

With the background information reviewed above, here is a list of general objctives that were addressed in the research work described in this thesis:

Identification of the various phases of the lifespan of follicles (growing, static and regression phases) is limited to perform serial ultrasonographic examinations of the ovaries on a daily basis. Using computer-assisted image analysis, our first objective was to determine if ultrasonographic image attributes changed during development and regression of antral follicles in follicular waves in the normal cyclic ewe. Since subsequent waves develop at different phases of the cycle and therefore under different endocrine milieus, our second objective was to investigate if image attributes of the follicular wall and antrum, varied amongst antral follicles emerging at different stages of the inter-ovulatory interval.

Peaks in serum FSH concentration are essential for follicular wave emergence. However, it is not clear that what characteristics of FSH peaks (e.g. Height, duration, shape) are essential for wave emergence, growth and function. Our third objective was to see if the peaks in FSH secretion that precede follicular waves change across the inter-ovulatory interval and to see if that is accompanied by alterations in any of the characteristics of the follicular waves that follow each FSH peak. We hoped this would indicate parameters of FSH secretion critical for follicular wave emergence and growth. Our fourth objective was to investigate the effects of a 5 to 6 fold increase in FSH peak

amplitude, compared to control, untreated ewes, on ovarian follicular wave dynamics in cyclic ewes.

The existence of follicular dominance is unclear in the ewe. Therefore, our fifth objective was to test the presence of dominance. This was done by testing whether the presence of a large growing follicle in the ovary would suppress emergence of a new follicular wave in response to injection of FSH. In addition, we examined how frequently follicular waves could be induced by injections of oFSH.

The FSH peaks that precede the emergence of ovarian follicular waves in the ewe appear to vary in characteristics, such as peak height, duration and the shape of the leading and trailing slopes of the peak. However, peaks in serum FSH concentrations, with different characteristics, can trigger emergence of follicular waves when they reach a required threshold. Our sixth objective was to see if a very gradual increase in the leading slope of an FSH peak would be detected by the ovary as a proper signal to stimulate emergence of a new follicular wave.

Peak in serum FSH concentrations could be simply defined as a temporary and gradual rise in basal concentrations to the threshold levels required for emergence of a follicular wave. It is not clear whether a discrete peak is required to signal a follicular wave or merely an increase in basal concentrations of FSH to a threshold value. Our seventh objective was to see if raising basal serum concentrations of FSH to the concentrations seen at the zenith of a peak and maintaining it for several days would allow multiple follicle waves to emerge.

In prolific breeds of ewe, ovulation of follicles recruited from both final and penultimate waves of the cycle has been proposed as the mechanism of increased ovulation rate. The prolonged lifespan of follicles growing in the penultimate wave of the cycle assures their presence at the time of ovulation. Mechanisms controlling the increased survival of penultimate wave follicles in prolific breeds of ewe have not been studied. Using an extended follicular lifespan model in the ewe, introduced by Bartlewski et al. (2003), we investigated the susceptibility of follicles with a longer lifespan to apoptosis. Our eighth objective was to quantify and compare the degree of apoptosis in aged follicles and follicles with a normal lifespan in cyclic ewes.

CHAPTER 2: HYPOTHESES

Ultrasound image attributes of antral follicles will change with the stages of development and regression within a follicular wave and will also differ amongst the different follicular waves in an estrous cycle.

Characteristics of the FSH peaks vary across the inter-ovulatory interval and this affects the growth patterns of follicles in the following follicular waves.

An increase in the endogenous FSH peak amplitude to a supra physiological level by administration of oFSH will affect the number of follicles recruited into and the growth characteristics of the following follicular wave.

The ovine ovary is capable of responding to frequent, even daily peaks in serum FSH concentrations with emergence of a new follicular wave, indicating a lack of follicular dominance.

A new follicular wave with normal follicular dynamics would emerge after an induced FSH peak with a gradual leading slope.

Maintaining elevated basal serum FSH concentrations would induce continuous emergence of new follicular waves in the sheep ovary.

The aged antral follicles of the penultimate wave of the cycle in ewes given prostaglandin and MAP will show a greater degree of follicular apoptosis compared to follicles in the final wave of the cycle.

CHAPTER 3: CHARACTERISTICS OF PEAKS IN SERUM CONCENTRATIONS OF FOLLICLE STIMULATING HORMONE (FSH) AND ESTRADIOL AND FOLLICULAR WAVE DYNAMICS DURING THE INTER-OVULATORY INTERVAL IN CYCLIC EWES[*]

Toosi BM, Seekallu SV, Barrett DMW, Davies KL, Duggavathi R, Bagu ET and Rawlings NC

3.1. Abstract

There are 3 or 4 follicular waves in the inter-ovulatory interval of cyclic ewes. Each follicular wave is preceded by a transient peak in serum FSH concentrations. Serum concentrations of estradiol also increase concurrent to the growth of follicle(s) in each wave. In the present study we investigated the patterns of follicular wave development and characteristics of FSH and estradiol peaks in all of the follicular waves of the inter-ovulatory interval and after induction of a supra-physiologic FSH peak in cyclic ewes. In Experiment 1, nineteen ewes underwent daily ovarian ultrasonography and blood sampling for a complete inter-ovulatory interval. In Experiment 2, seven ewes received two injections of oFSH, 8 h apart (1μg/kg; sc) at the expected time of the endogenous FSH peak preceding the second follicular wave of the inter-ovulatory interval. In Experiment 1, the amplitude of the FSH peaks decreased (up to 50%), while basal serum FSH concentrations increased across the inter-ovulatory interval ($P < 0.05$). Maximum follicular diameter was greater ($P < 0.05$) for wave 1 and 4 (6.0 ± 0.3 and 6.1 ± 0.2 mm, respectively) than in waves 2 and 3 (5.3 ± 0.1 and 5.4 ± 0.3 mm, respectively). Lifespan was greater for follicles in wave 1 compared to other waves ($P < 0.05$). Treatment with ovine FSH increased the amplitude of an FSH peak by 5 to 6 fold. This treatment increased estradiol production ($P < 0.05$) but had little effect on other characteristics of

[*] *Theriogenology (In Press)*

the subsequent follicular wave. We concluded that changes in the amplitude and duration of the peaks in serum concentrations of FSH that precede follicular waves across the inter-ovulatory interval, do not influence the characteristics of the following follicular waves.

3.2. Introduction

In the ewe, development of ovarian antral follicles occurs in a wave-like pattern (Noel et al. 1993, Ravindra et al. 1994, Ginther et al. 1995, Souza et al. 1997, Bartlewski et al. 1999a, Evans et al. 2000, Driancourt 2001). One to three follicles emerge or grow further from a pool of small follicles (1 to 3 mm in diameter) reaching ≥5 mm in diameter before regression (anovulatory wave) or ovulation (ovulatory wave) (Ginther et al. 1995, Bartlewski et al. 1999a, Driancourt 2001). The average inter-wave interval is 3 to 5 days, with 3 or 4 follicular waves in the inter-ovulatory interval of cyclic ewes (Bartlewski et al. 1999a, Ginther et al. 1999). Development of each follicular wave is associated with a transient peak in serum FSH concentrations, lasting 3 to 4 days with the FSH peak zenith occurring within 24 h of wave emergence (Ginther et al. 1995 Bartlewski et al. 1998, Bartlewski et al. 1999a, Bartlewski et al. 2000b, Driancourt 2001). Serum concentrations of estradiol also increase concurrent to the growth of follicle(s) in each wave, with a peak in serum estradiol concentrations occurring around the end of the growth phase of the largest follicle in the wave (Bartlewski et al. 1999a, Bartlewski et al. 2000b, Driancourt 2001).

Peaks in serum FSH concentration are essential for follicular wave emergence (Ginther et al. 1995, Bartlewski et al. 1999a, Bartlewski et al. 2000b, Driancourt 2001).

Truncation of endogenous FSH peaks resulted in disappearance of follicular waves in cyclic ewes (Barrett et al. 2006). Moreover, treatment of ewes with physiologic doses of exogenous oFSH to induce an FSH peak, during the inter-wave interval, resulted in emergence of a new follicular wave without disruption of the normal pattern of FSH peaks and follicular waves (Duggavathi et al. 2004, Duggavathi et al. 2005a). However, the mechanism whereby FSH initiates follicular waves is not known, neither do we know what characteristics of FSH peaks (e.g. Height, duration, shape) are essential for wave emergence, growth and function. Superovulatory doses of FSH stimulate growth of a large number of follicles to ovulatory diameters in sheep (Wright et al. 1981, Bari et al. 2001, Bartlewski et al. 2008); and, the greater the number of small antral follicles present at the time of wave emergence the greater the number of growing follicles after administration of a superovulatory dose of FSH (Gonzalez-Bulnes et al. 2000). However, doubling the amplitude of an endogenous FSH peak, by administration of oFSH did not change the characteristics of the following follicular wave in anestrous ewes (Duggavathi et al. 2005a). Whether variations in peak FSH concentrations over a wider but still physiologic range, could affect follicular wave dynamics in the ewe, is not clear. Investigations in cattle have shown an inverse association between the serum concentrations of FSH and the number of follicles ≥3 mm in diameter during follicular waves (Burns et al. 2005).

Ewes with a history of a high ovulation rate have follicular waves with a shorter growth phase and smaller maximum follicular diameter when compared with ewes with a history of a low ovulation rate (Gibbons et al. 1999). The growth rate of follicles in the penultimate follicular wave of the inter-ovulatory interval in prolific ewes was longer

compared to non-prolific ewes, resulting in an extended lifespan, and ovulation of those follicles with follicles that grew in the final wave of the interval (Bartlewski et al. 1999a). The role of FSH and estradiol in such variation of the growth and function of follicular waves, although poorly understood, may have important implications in the regulation of fertility in sheep (Driancourt 2001, Duggavathi et al. 2004, Duggavathi et al. 2005a, Barrett et al. 2006).

Our objective in Experiment 1 of the present study was to see if the peaks in FSH secretion that precede follicular waves change across the inter-ovulatory interval and to see if that is accompanied by alterations in any of the characteristics of the follicular waves that follow each FSH peak. We hoped this would indicate parameters of FSH secretion critical for follicular wave emergence and growth. Experiment 2 was designed to investigate the effects of a 5 to 6 fold increase in FSH peak amplitude, compared to control, untreated ewes, on ovarian follicular wave dynamics in cyclic ewes. We hypothesized that characteristics of the FSH peaks vary across the inter-ovulatory interval and this affects the growth patterns of follicles in the following follicular waves; also, a supra-physiologic increase in the endogenous FSH peak amplitude by administration of oFSH will affect the number of follicles recruited into and the growth characteristics of the following follicular wave.

3.3. Materials and methods

3.3.1. Experiment 1

3.3.1.1. Animals

All Animal experimentation was performed according to the guidelines of the Canadian Council on Animal Care and was approved by the local animal care committee. Nineteen (5 to 7 years of age), normally cycling, nulliparous Western White Face ewes with an average body weight of 78 ± 5.8 kg were used in this study (October-December). All animals were housed in sheltered dry lots (Saskatoon, SK., Canada; 52 ºN latitude). Animals received daily maintenance rations of alfalfa pellets with water and cobalt iodized salt licks available *ad libitum*. Estrus was detected with three vasectomized crayon-harnessed rams.

3.3.1.2. Ultrasonography

Transrectal ovarian ultrasonography was performed with a B-mode, real-time echo camera (Aloka SSD 900; Aloka Co. Ltd., Tokyo, Japan) equipped with a stiffened 7.5 MHz linear array transducer. All ewes underwent daily ultrasonographic examination for a complete interovulatory interval, starting two days before the expected day of estrus. The day of ovulation was defined as the day on which a large, previously identified ovarian follicle (≥5 mm in diameter) was no longer seen (Ravindra et al. 1994; Bartlewski et al. 1999a). The size and relative position of all follicles ≥1 to 2 mm in diameter were sketched on ovarian charts and also recorded on high grade video tape (Fuji S-VHS, ST-120 N; Fujifilm, Tokyo, Japan), using a compatible VCR (Panasonic,

Super VHS, AG 1970; Matsushita Electronics of Canada Ltd, Mississauga, ON, Canada).

3.3.1.3. *Analysis of follicular data*

Ewes had either three (n = 9) or four (n = 10) follicular waves in their inter-ovulatory interval. In ewes with 3 or 4 follicular waves, the first and second follicular waves and the ovulatory wave (wave 3 of ewes with 3 waves and wave 4 of ewes with 4 waves) were pooled for analysis as in both groups of ewes the follicles emerged on similar days of the inter-ovulatory interval. In all ewes, ovulation occurred from the final follicular wave in the ovulatory interval. For consistency, when ovulation occurred from both final and penultimate follicular waves (two ewes) data for ovulatory follicles from penultimate follicular waves were excluded from analysis. The inter-wave interval, number of follicles emerging in each follicular wave (follicles which grew from 2 to 3 mm to ≥5 mm in diameter) and maximum follicular diameter were calculated. The length of the growth, static and regression phases as well as the growth and regression rates were calculated for all follicles in each follicular wave as defined previously (Bartlewski et al. 1999a, Duggavathi et al. 2004). The number of small (1 to 3 mm in diameter), medium (4 mm in diameter) and large (≥5 mm in diameter) size follicles were counted in each inter-wave interval and were reported as the average per day for each size category. Follicle numbers in each inter-wave interval reflected numbers for the follicular wave emerging at the onset of the interval. For ovulatory waves, the duration between wave emergence and ovulation was calculated.

3.3.1.4. Blood sampling

Blood samples (10 ml) were collected daily into vacutainers (Becton Dickinson, Rutherford, NJ, USA) prior to each ultrasound examination and kept at room temperature for 24 h. Serum was then harvested and stored at -20 ºC until assayed. Concentrations of FSH and estradiol in serum samples collected daily were normalized to the day of ovulation (Day 0) for further analysis.

3.3.1.5. Statistical analysis

Characteristics of follicular waves, FSH and estradiol peaks were compared amongst different waves in the inter-ovulatory interval by one way repeated measures Analysis of Variance (SigmaStat® Statistical Software for Windows, Version 2.03, 1997, SPSS Inc., Chicago, IL, USA). All values are means ± SEM and statistical significance was set as $P < 0.05$.

3.3.2. Experiment 2

3.3.2.1. Animals

Thirteen cyclic (November-December) Western White Face ewes with an average body weight of 83 ± 6.2 kg were randomly divided into treatment (n = 7) and control (n = 6) groups. Estrus was synchronized with a 14-day treatment using progestogen-releasing intravaginal sponge (Medroxyprogesterone Acetate/MAP, 60 mg; Veramix®, Up-John, Orangeville, ON, Canada). The study was conducted in the second cycle after synchronization. Estrus detection and all maintenance conditions were the same as those in Experiment 1.

3.3.2.2. Ultrasonography

From two days prior to the expected day of estrus, all ewes underwent twice daily transrectal ovarian ultrasonography (at 08:00 and 20:00 h) to detect ovulation and a growing 4 mm follicle in the first follicular wave of the inter-ovulatory interval. After detection of the third follicular wave in the inter-ovulatory interval, ultrasonographic examination of the ovaries was done daily until detection of the next ovulation.

3.3.2.3. Ovine FSH preparation and injection

One milligram of the ovine FSH (oFSH) used in this study had a biological potency of 90 x NIH-oFSH-S1 and less than 0.1 x NIH-oLH-S1 (Teri.oFSH/ig.1, Tucker Endocrine Research Institute LLC, Atlanta, GA, USA). The oFSH was prepared in saline with 0.05% BSA (w/v; Sigma Chemical Co., St. Louis, MO, USA) and 50% polyvinylpyrrolidone (w/v; Sigma). Sixty hours after detection of the first growing 4 mm follicle post ovulation (expected time of the next endogenous FSH peak), animals in the treatment group received two injections of oFSH 8-hour apart (1 μg/kg, sc). Control animals were injected with vehicle. With this timing, oFSH or vehicle was given at the expected time of the peak in endogenous FSH concentrations associated with the emergence of the next follicular wave (designated as wave 2 of the inter-ovulatory interval). The dose of exogenous oFSH was intended to increase the amplitude of the second endogenous FSH peak in the inter-ovulatory interval by 5 to 6 fold.

***3.3.2.4**. Blood sampling*

Blood samples (10 ml) were taken daily (prior to each ultrasound examination), and at 08:00, 14:00, 20:00 and 02:00 h from 24 h before to 48 h after treatment, using vacutainers (Becton Dickinson). Sera from blood samples was collected and stored as in Experiment 1. The pattern of concentrations of FSH and estradiol in serum samples, collected daily or every 6 h were normalized to the day of treatment (Day 0).

***3.3.2.5.** Analysis of follicular data*

Follicular wave characteristics (similar to Experiment 1) for the wave emerging after treatment (wave 2 in the inter-ovulatory interval) were calculated for treatment and control ewes and compared between these groups (Table 3.2.).

***3.3.2.6.** Statistical analysis*

Characteristics of the peaks in serum concentrations of FSH were compared between ewes given oFSH and vehicle-treated ewes by student's t-test (SigmaStat® Statistical Software for Windows, Version 2.03, 1997, SPSS Inc.). Using the same statistical method, characteristics of follicular wave 2 (emerging immediately after treatment) and its corresponding peak in serum concentrations of estradiol were compared between the treatment and control groups. All values are means ± SEM and statistical significance was set as $P < 0.05$.

3.3.3. Hormone assays and data analysis for Experiments 1 and 2

Serum concentrations of FSH (Currie and Rawlings 1989) and estradiol (Joseph et al. 1992) were determined by established radioimmunoassays. The sensitivity of the assays

(defined as the lowest concentration of hormone capable of significantly displacing labeled hormone from the antibody) for FSH and estradiol were 0.1 ng/mL and 1 pg/mL, respectively. For reference sera with mean FSH concentrations of 0.45 or 3.28 ng/mL, the intra- and inter-assay CVs were 9.5 or 2.8% and 12.6 or 4.4% respectively. For estradiol, the intra- and inter assay CVs for reference sera with mean concentrations of 7.65 or 23.12 pg/mL were 9.8 or 7.7% and 14.2 or 9.6%, respectively. Peaks in serum concentrations of FSH and estradiol, were detected using a cycle detection computer program (Clifton and Steiner 1983) modified for Windows® XP. Basal serum FSH and estradiol concentrations for each peak (nadir concentration prior to each FSH/estradiol peak), peak concentrations, peak amplitude, and peak duration (duration between the pre- and post-peak nadirs) were calculated for all follicular waves of the ovulatory interval in Experiment 1 and for follicular wave 2 in Experiment 2.

3. 4. Results

3.4.1. Experiment 1

3.4.1.1. Follicular wave characteristics

Comparisons of characteristics of follicular waves amongst different waves in the ovulatory interval are given in Table 3.1. The average inter-ovulatory interval was 17.6 ± 0.2 days. The day of wave emergence for each follicular wave was associated with the corresponding day of the peak in serum FSH concentrations. The average number of medium size follicles recorded daily (4 mm in diameter) increased to wave 3, and declined to the ovulatory wave; numbers were higher in the ovulatory wave than wave 1 ($P < 0.05$). The number of large follicles seen daily (≥5 mm in diameter) was higher in

waves 2, 3 and the ovulatory wave than in wave 1 and in waves 2 and 3 compared to the ovulatory wave ($P < 0.05$). Follicles that emerged in wave 1 and the ovulatory wave had a longer growth phase than follicles that emerged in wave 2 ($P < 0.05$), and they grew to a greater maximum follicular diameter than follicles in waves 2 and 3 ($P < 0.05$). Follicles in the ovulatory wave had a shorter static phase than those in waves 1 and 2 ($P < 0.05$). The duration of the regression phase was greater in wave 1 than that of waves 2 and 3 ($P < 0.05$). The mean length of the follicular lifespan was greater ($P < 0.05$) in wave 1 when compared with other follicular waves in the inter-ovulatory interval and the shortest lifespan was seen for the ovulatory wave ($P < 0.05$). A greater inter-wave interval was seen between waves 1 and 2 when compared to the interval between waves 2 and 3 ($P < 0.05$).

3.4.1.2. *Characteristics of FSH and estradiol peaks*

Characteristics of the FSH peaks preceding follicular waves during the inter-ovulatory interval are shown in Fig. 3.1. The basal serum concentrations of FSH preceding FSH peaks for wave 3 and the ovulatory wave were higher than those of waves 1 and 2 ($P < 0.001$). The amplitude of FSH peaks were greater in waves 1 and 2 in comparison to wave 3 and the ovulatory wave ($P < 0.001$). The duration of FSH peaks preceding wave 2 were longer than for wave 3 and the ovulatory wave, and for wave 1 were longer than for wave 3 ($P < 0.05$). Comparisons of characteristics of estradiol peaks amongst different follicular waves in the inter-ovulatory interval are given in Fig. 3.1. Peak serum concentrations of estradiol and peak amplitude were higher in the ovulatory wave in comparison to the anovulatory waves of the inter-ovulatory interval.

Table 3.1. Comparison of characteristics (Mean ± SEM) of different follicular waves in the inter-ovulatory interval of the nineteen cyclic Western White Face ewes in Experiment 1.

[a, b, c] Significant difference ($P < 0.05$) for a parameter among different waves within a row. * Day 0 = Day of ovulation

	Wave 1	Wave 2	Wave 3	Ovulatory wave
Day of wave emergence*	0.0 ± 0.1	5.0 ± 0.2	8.1 ± 0.2	11.3 ± 0.3
Day of FSH peak*	-0.1 ± 0.1	5.0 ± 0.2	8.1 ± 0.2	11.4 ± 0.3
Day of corresponding peak in estradiol concentration*	2.3 ± 0.2	7.0 ± 0.3	11.1 ± 0.4	14.8 ± 0.2
No. follicles in the wave	1.9 ± 0.8	1.9 ± 0.8	1.4 ± 0.7	2.1 ± 0.9
No. small (1 to 3 mm in diameter) follicles in the inter-wave interval (follicles/day)	13.3 ± 0.5	13.6 ± 0.8	15.2 ± 1.8	13.3 ± 1.3
No. medium size (4 mm in diameter) follicles in the inter-wave interval (follicles/day)	0.8 ± 0.1[a]	1.1 ± 0.2[ac]	1.7 ± 0.2[b]	1.3 ± 0.1[c]
No. large (≥5 mm in diameter) follicles in the inter-wave interval (follicles/day)	1.1 ± 0.1[a]	2.0 ± 0.1[b]	2.1 ± 0.2[b]	1.8 ± 0.2[c]
Growth phase (d)	4.1 ± 0.4[a]	2.9 ± 0.3[b]	3.3 ± 0.5[ab]	3.9 ± 0.4[a]
Static phase (d)	1.9 ± 0.4[a]	2.3 ± 0.4[a]	1.3 ± 0.5[ab]	1.1 ± 0.2[b]
Regression phase (d)	3.5 ± 0.3[a]	2.7 ± 0.3[b]	2.6 ± 0.4[b]	-
Growth rate (mm/day)	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
Regression rate (mm/day)	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.2	-
Maximum follicular diameter (mm)	6.0 ± 0.3[a]	5.3 ± 0.1[b]	5.4 ± 0.3[b]	6.1 ± 0.2[a]
Follicular lifespan (d)	9.5 ± 0.4[a]	8.0 ± 0.4[b]	7.3 ± 0.6[b]	5.0 ± 0.3[c]
Inter-wave interval (d)	W1 to W2: 5.2 ± 0.2 [a]	W2 to W3: 4.3 ± 0.3 [b]	W3 to W4: 4.4 ± 1.1[ab]	

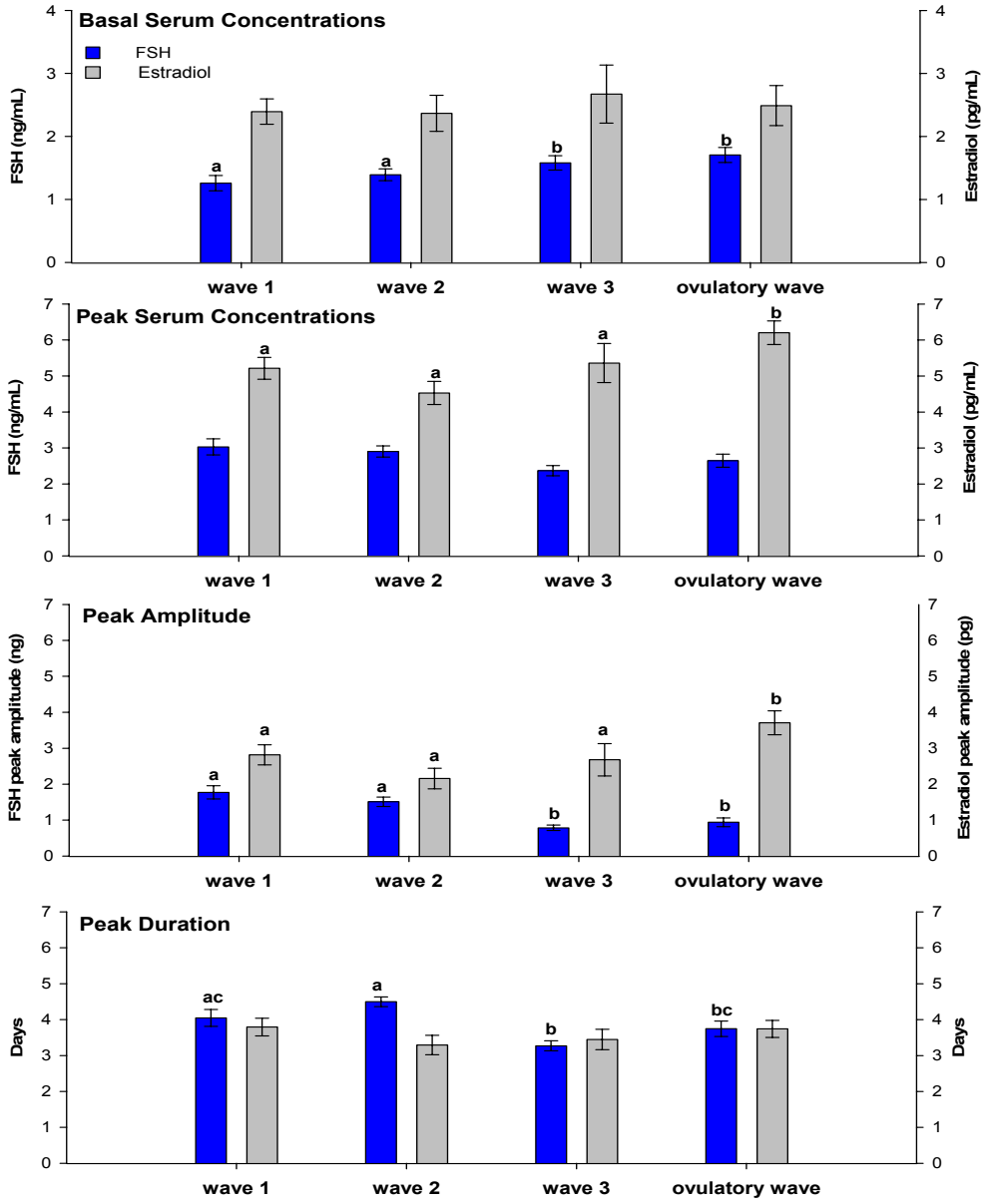


Fig. 3.1. Comparisons of characteristics of peaks in serum concentrations of FSH (black bars) and estradiol (grey bars) amongst different follicular waves in the inter-ovulatory interval of the nineteen cyclic, Western White Face ewes in Experiment 1. Data for follicular waves 1 and 2 and the ovulatory wave in ewes with three (n = 9) and four (n = 10) follicular waves in the inter-ovulatory interval were pooled for analysis. Data for the third follicular wave represent only those waves in animals with four follicular waves in the inter-ovulatory interval.
[a, b, c] Significant differences ($P < 0.05$) between waves.

3.4.2. Experiment 2

3.4.2.1. Characteristics of FSH and estradiol peaks

By giving two injections of oFSH, 8-h apart at the expected time of the second endogenous FSH peak in the inter-ovulatory interval, the FSH peak amplitude was about 6-fold greater in the treatment group compared to that of the control group (8.45 and 1.34 ng, respectively; $P < 0.05$; Fig. 3.2). Basal serum concentrations of the FSH peak (nadir concentrations prior to the treatment) were 1.03 ± 0.22 and 1.29 ± 0.26 ng/mL in ewes treated with oFSH and vehicle respectively ($P > 0.05$). There was no significant difference in the duration of the FSH peak between the oFSH- and vehicle-treated ewes (3.9 ± 0.3 and 3.8 ± 0.2 d, respectively). The amplitude of the estradiol peaks associated with the second follicular wave of the inter-ovulatory interval was greater after treatment with oFSH than that in control ewes (3.21 ± 0.42 and 1.90 ± 0.45 pg, respectively; $P < 0.05$). However, basal and peak concentrations as well as duration of the estradiol peaks did not differ significantly after treatment with oFSH (2.74 ± 0.74 ng/mL, 5.95 ± 0.42 ng/mL and 3.3 ± 0.4 d, respectively) and vehicle (2.78 ± 0.74 ng/mL, 4.68 ± 0.62 ng/mL and 2.5 ± 0.3 d, respectively).

3.4.2.2. Follicular wave characteristics

Characteristics of the follicular waves emerging after treatment with oFSH or vehicle are compared in Table 3.2. Increasing the amplitude of the FSH peak prior to wave 2 of the inter-ovulatory interval resulted in fewer small follicles in the wave ($P < 0.05$), and a lower growth rate of follicles compared to control ewes ($P < 0.05$).

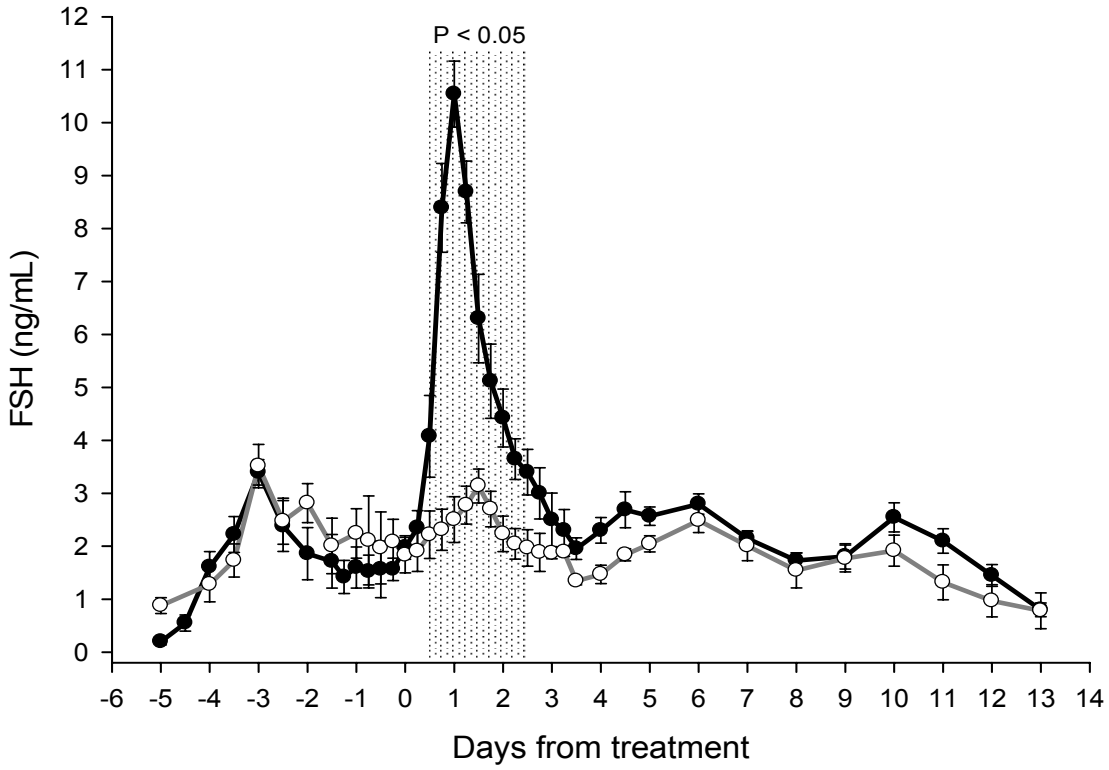


Fig. 3.2. Mean (± SEM) serum concentrations of FSH in oFSH-treated (●; n = 7) and control (○; n = 6) cyclic, Western White Face ewes from 6 days before to 13 days after treatment. FSH peaks for all ewes were normalized to the mean day of occurrence of the zenith of the FSH peak for each wave relative to the day of treatment (day 0). Treatment included two injections of oFSH (1μg/kg, sc) or vehicle, 8-hour apart, with the first injection given 60 h after detection of a growing 4 mm follicle in the first follicular wave of the inter-ovulatory interval. This placed the oFSH treatment at the time of endogenous FSH peak preceding the second wave of the inter-ovulatory interval. Serum FSH concentrations were significantly different ($P < 0.05$) between the two groups of ewes in the samples taken during the period outlined with shading.

Table 3.2. Comparison of the characteristics (Mean ± SEM) of follicular waves emerging after treatment with oFSH (treatment; n = 7) or vehicle (control; n = 6). Treatment included two injections of oFSH (1μg/kg, sc) or vehicle, 8-hour apart, with the first injection given 60 h after detection of a growing 4 mm follicle in the first follicular wave of the inter-ovulatory interval. This placed the oFSH treatment at the time of endogenous FSH peak preceding the second wave of the inter-ovulatory interval in the cyclic Western White Face ewes in Exp. 2.

	Wave 2	
	Treatment	**Control**
Day of wave emergence*	0.8 ± 0.2	1.5 ± 0.3
Day of FSH peak*	0.9 ± 0.1	0.9 ± 0.4
Day of corresponding peak in estradiol concentrations*	2.4 ± 0.7	2.7 ± 0.8
No. follicles in the wave	1.6 ± 0.3	2.0 ± 0.4
No. small (1 to 3 mm in diameter) follicles in the inter-wave interval (follicles/day)	13.6 ± 0.4[a]	15.6 ± 0.3[b]
No. medium size (4 mm in diameter) follicles in the inter-wave interval (follicles/day)	2.3 ± 0.4	1.7 ± 0.3
No. large (≥5 mm in diameter) follicles in the inter-wave interval (follicles/day)	2.1 ± 0.2	1.8 ± 0.2
Growth phase (d)	2.5 ± 0.2	2.1 ± 0.8
Static phase (d)	1.6 ± 0.4	1.8 ± 0.5
Regression phase (d)	2.5 ± 0.2	2.1 ± 0.3
Growth rate (mm/day)	0.9 ± 0.1[a]	1.3 ± 0.1[b]
Regression rate (mm/day)	0.8 ± 0.1	0.9 ± 0.1
Maximum follicular diameter (mm)	5.1 ± 0.1	5.4 ± 0.2
Follicular lifespan (d)	6.9 ± 0.7	6.6 ± 0.7
Inter-wave interval (d)	3.7 ± 0.4	4.6 ± 0.6

[a, b] Significant differences ($P < 0.05$) between treatment and control groups.

* Day 0 = Day of treatment

3.5. Discussion

It was interesting that in Experiment 1 the amplitude of the FSH peaks that precede the emergence of follicular waves decreased across the inter-ovulatory interval. Although not as clear a trend, peak duration also declined suggesting that peaks of greater amplitude are also of longer duration. As peak amplitude declined across the period of study basal serum FSH concentrations increased, perhaps indicating greater FSH concentrations available in the pituitary for basal secretion. Follicle stimulating hormone is synthesized and constitutively secreted from gonadotropes in the pituitary gland (Padmanabhan and Sharma 2001, Crawford and McNeilly 2002). It has been suggested that changes in GnRH pulse frequency can selectively regulate production of FSH and LH, with slower frequencies, as seen during the luteal phase of an estrous cycle, favoring FSH-mRNA expression levels (Haisenleder et al. 1990, Haisenleder et al. 1991). This may explain the higher basal FSH concentrations preceding those peaks for the follicular waves emerging during the luteal phase of the estrous cycle (waves 3 and 4).

The most obvious reason for the decline in FSH peak amplitude across the inter-ovulatory interval would be the cumulative exposure of the hypothalamic pituitary axis to progesterone and estradiol-17β during the luteal phase of the inter-ovulatory interval (Nett et al. 2002, McNeilly et al. 2003). It is intriguing that FSH peaks can decline in amplitude by up to 50% across the inter-ovulatory interval and yet still induce follicular waves. However, peak concentrations of FSH remained unchanged across the inter-ovulatory interval in Experiment 1. In cyclic ewes given estradiol, truncation of FSH peak concentrations to 45% of control values, without changes in basal and mean FSH

concentrations, interrupted the emergence of antral follicular waves (Barrett et al. 2006). These data suggest the existence of a threshold for serum concentrations of FSH to induce the emergence of a follicular wave (Picton and McNeilly 1991, Driancourt 2001, Barrett et al. 2006). Superovulatory doses of FSH stimulate the growth of numerous ovulatory-sized follicles in sheep (Wright et al. 1981, Bari et al. 2001, Riesenberg et al. 2001a, Boscos et al. 2002, Gonzalez-Bulnes et al. 2002a). However, the effects of superovulatory doses of FSH are not physiological. In a previous study, giving exogenous oFSH to create a 2-fold increase in the amplitude of the FSH peak preceding a follicular wave, did not change the characteristics of the ensuing follicular wave in anestrous Western White Face ewes (Duggavathi et al. 2005a). This observation and the results of Experiment 2 of the present study would lead us to conclude that increasing FSH peak concentrations above a critical threshold, but within a physiological range, has little effect on the growth, lifespan and size of follicles in a wave.

In Experiment 1, the maximum follicular diameter of the follicles in follicular waves was greater for wave 1 and 4 but these waves emerged and grew under different FSH backgrounds as described above. Together with the results from Experiment 2 it can be speculated that the characteristics of an FSH peak do not affect the maximum follicular diameter in the following follicular wave. In a previous study it was noted that the characteristics of FSH peaks were not associated with significant differences in the diameter of large antral follicles in ewes differ in prolificacy (Bartlewski et al. 1999a, Gibbons et al. 1999). In the present study, it is clear that waves 1 and 4 would have grown prior to and after the luteal phase of the inter-ovulatory interval when LH pulse frequency would be high; it is lower during the luteal phase (Bartlewski et al. 2000b,

Driancourt 2001). It has been established that large antral follicles can become LH dependent and less dependent on FSH (Ginther et al. 1989, Ginther and Kot 1994). The follicles in the waves above would have received enhanced support from LH and achieved a greater maximum size. The greater maximum follicular diameter of follicles in wave 1 and the ovulatory wave compared to other waves in the luteal phase of the estrous cycle, have also been reported in goats and cattle (Ginther et al. 1989, Ginther and Kot 1994).

Although, the peak in serum concentrations of esradiol-17β was greater for the ovulatory wave of the inter-ovulatory interval compared to other waves, this difference was not great. This emphasizes the role of progesterone in blocking the stimulation of a pre-ovulatory surge release of LH by estradiol in waves 1 through 3. At the end of the luteal phase enhanced LH pulse frequency can stimulate estrogen production by the ovulatory follicle (Bartlewski et al. 2000b). It is unclear why the greater LH pulse frequency seen early in the inter-ovulatory interval did not give greater estrogen production from follicles in wave 1; perhaps this is because pulsed LH secretion would decline (Ginther 1995, Bartlewski et al. 2000b, Evans et al. 2000) as progesterone secretion increased in the early luteal phase concurrent with the growth of follicles in the first wave (Souza et al. 1998, Bartlewski et al. 1999a, Bartlewski et al. 1999c, Evans et al. 2002). In Experiment 2, enhancing the FSH peak amplitude to the upper end of the physiological range did enhance estrogen production from wave 2 of the inter-ovulatory interval.

In Experiment 1 follicular lifespan was greater for wave 1 and shortest for the ovulatory wave. The longer lifespan for follicles in wave 1 was reflected in the long interval from wave 1 to wave 2 compared to intervals between other waves. Again, greater LH support

was available for waves 1 and 4 giving longer growth phases than for other waves (Bartlewski et al. 1999a). It is interesting that the enhanced lifespan for wave 1 involved a longer regression phase as well as a longer growth phase. The length of the regression phase and FSH peak amplitude both declined across the inter-ovulatory interval. However, the major decline in length of the regression phase occurred between wave 1 and 2. This was most likely as a result of enhanced LH support of wave 1 (greater maximum follicle diameter) but not wave 2. The shortened static phase and lifespan for ovulatory follicles (wave 4) would obviously reflect the truncation of lifespan by ovulation. A possible explanation for the greater average daily number of medium and large sized follicles in wave 3 could be the presence of static or regressing follicles from waves 1 and 2 (with mean follicular lifespan of 9.5 ± 0.4 and 8.0 ± 0.4 days, respectively) simultaneous with development of the third follicular wave.

In summary, although the ovulatory follicles growing at the end of the inter-ovulatory interval do result in greater serum concentrations of estradiol, compared to follicles in other follicular waves the difference was minimal. This emphasizes the role of progesterone in restraining the ability of estradiol to induce an LH surge at other times in the inter-ovulatory interval. In Experiment 2, increasing the serum FSH peak concentration by 6 fold compared to control ewes had surprisingly little effect on the follicles growing in the subsequent follicular wave; although estradiol production was enhanced. The results of both Experiments 1 and 2 would lead us to conclude that although peaks in serum concentrations of FSH are required to trigger ovarian antral follicular waves in the ewe, variation in peak amplitude and duration and basal serum

concentrations of FSH, across the inter-ovulatory interval, do not have a marked influence on the characteristics of follicles in those waves.

CHAPTER 4: EVALUATION OF THE ULTRASOUND IMAGE ATTRIBUTES OF DEVELOPING OVARIAN FOLLICLES IN THE FOUR FOLLICULAR WAVES OF THE INTER-OVULATORY INTERVAL IN EWES[†]

Toosi BM, Seekallu SV, Pierson RA and Rawlings NC

4.1 Abstract

Computer-assisted quantitative echotextural analysis was applied to ultrasound images of antral follicles in the follicular waves of an inter-ovulatory interval in sheep. The ewe has 3 or 4 waves per cycle. Seven healthy, cyclic Western White Faced ewes underwent daily, transrectal, ovarian ultrasonography for an inter-ovulatory interval. Follicles in the third wave of the ovulatory interval had a longer static phase than those in waves 1 and 2 ($P < 0.05$). The numeric pixel value for the wall of anovulatory follicles emerging in the third wave of the cycle was significantly higher than for waves 1 and 2 at the time of emergence (156.7 ± 8.09, 101.6 ± 3.72 and 116.5 ± 13.93 respectively) and it decreased as follicles in wave 3 reached maximum follicular diameter ($P < 0.05$). The numeric pixel value of the antrum in the ovulatory follicles decreased as follicular diameter increased to ≥5 mm in diameter ($P < 0.05$). The pixel heterogeneity of the follicular antrum in wave 1 increased from the end of the growth phase to the end of the regression phase for follicles in that wave ($P < 0.05$). The total area for the wall and antrum of the follicles studied, were correlated with follicular diameter in all follicular waves ($r = 0.938$, $P < 0.01$ and $r = 0.941$, $P < 0.01$ for the wall and antrum respectively). Changes in image attributes of the follicular wall and antrum indicate potential morphological and

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functional differences amongst antral follicles emerging at different stages of the inter-ovulatory interval in cyclic ewes.

4.2. Introduction

Ultrasonographic imaging of the reproductive tract is well-developed in different domestic animal species (Pierson and Ginther 1986, Adams et al. 1989, Schrick et al. 1993, Ravindra et al. 1994, Ginther et al. 1995, Souza et al. 1997, Kulick et al. 1999). Particular emphasis has been placed on enhancing our understanding of ovarian function (Schrick et al. 1993, Ravindra et al. 1994, Ginther et al. 1995, Pierson and Adams 1995, Souza et al. 1997, Bartlewski et al. 2000b). Imaging allows repeated, non-invasive, visual assessment of changes in ovarian structures with time (Singh et al. 2003). Using ultrasonography, wave-like patterns of follicular development were reported in sheep (Ravindra et al. 1994, Ginther et al. 1995, Souza et al. 1997). An antral follicular wave in the ewe is defined as the emergence or growth of 1 to 3 follicles from a pool of small follicles (1 to 3 mm in diameter); the follicles attain diameters of ≥5 mm before regression (anovulatory wave) or ovulation (ovulatory wave) (Bartlewski et al. 1999a, Duggavathi et al. 2003a). The average inter-wave interval is 4 to 5 days and 3 or 4 follicular waves emerge in each inter-ovulatory interval (Bartlewski et al. 1999a, Duggavathi et al. 2003a).

Ultrasonography is based upon the ability of body tissues to reflect or transmit high frequency sound waves (Pierson and Adams 1995, Aldrich 2007). Reflection or echo of the ultrasound beams depends on the relative density and micro-structural organization of the tissue (Pierson and Adams 1995, Staren 1996). An ultrasound image is a two

dimensional array of picture elements called pixels (Baxes 1994). Each pixel represents one of 256 shades of grey ranging between 0 (black) and 255 (white) (Baxes 1994, Singh et al. 2003). The human eye is capable of distinguishing among only 18 to 20 shades of grey (Baxes 1994); therefore, quantitative evaluation of the changes in the ultrasonographic appearance of the tissue (echotexture) is not feasible by the human eye (Singh et al. 2003). Complex computer algorithms were designed in order to objectively analyze and quantify the range of 256 shades of grey of pixels in ultrasound image echotexture (Pierson and Adams 1995, Singh et al. 2003).

Identification of the various phases of the lifespan of follicles (growing, static and regression phases) is only feasible with retrospective examination of ultrasonographic data collected daily (Singh et al. 1998, Pierson and Adams 1995, Singh et al. 2003). The phases of the lifespan of antral follicles and their physiological function have been shown to be associated with histomorphologic characteristics of follicles (Singh et al. 1998, Tom et al. 1998b, Singh and Adams 2000, Vassena et al. 2003a). Therefore, image attributes of follicles, representing histomorphology, can be utilized to assess stage of development and aspects of physiological function (Singh et al. 1998, Tom et al. 1998b). The validity of image analysis to predict the physiologic status of ovarian follicles has been tested in domestic species, mainly cattle, by evaluating correlations among ultrasound image characteristics and histomorphologic and functional attributes of follicles and oocyte viability (Singh et al. 1998, Tom et al. 1998a, Tom et al. 1998b, Vassena et al. 2003b, Davies et al. 2006, Duggavathi et al. 2006). Image attributes of antral follicles in cattle were found to be related to the cellular and vascular composition of the follicular wall and to some aspects of secretory activity of the follicle (Singh et al.

1998). Image analysis may eventually allow prediction of the viability of an antral follicle and the oocyte it contains (Pierson and Adams 1995, Singh et al. 2003, Vassena et al. 2003b).

Our objective was to determine if ultrasonographic image attributes would be indicative of changes during development and regression of antral follicles in follicular waves in the normal cyclic ewe. Since subsequent waves develop at different phases of the cycle and therefore under different endocrine milieus, we hypothesized that ultrasound image attributes of antral follicles would change with the stages of development and regression within a follicular wave and would also differ amongst the different follicular waves in an estrous cycle.

4.3. Materials and methods

4.3.1. Animals

All animal experimentation was performed in compliance with the guidelines of the Canadian Council on Animal Care and was approved by the local animal care committee. Seven healthy, normally cycling, nulliparous, Western White Face ewes, 6 to 7 years of age (average body weight 79.9 ± 4.12 kg), were used in this study (November-December). All ewes were housed indoors with lighting set to simulate the natural light/dark cycle.

Animals received daily maintenance rations of alfalfa pellets with water, hay and cobalt iodized salt licks available *ad libitum*. Estrus was synchronized by application of a progestogen-releasing intravaginal sponge for 14 days (Medroxyprogesterone Acetate,

60 mg; Veramix®, Pharmacia & Upjohn Animal Health, Orangeville, ON, Canada). Estrus was detected with two vasectomized crayon-harnessed rams. The study was conducted in the second cycle after synchronization.

4.3.2. Ultrasonography

All ewes underwent daily transrectal ovarian ultrasonography with an instrument (Aloka SSD 900; Aloka Co. Ltd., Tokyo, Japan) equipped with a stiffened 7.5 MHz linear array transducer. Ultrasonographic examination of the ovaries began two days before the expected day of estrus and continued for a complete inter-ovulatory interval. The day of ovulation was defined as the day on which an ovarian follicle ≥5 mm in diameter, which had been previously identified, was no longer seen (Bartlewski et al. 1999a). Images of all ovarian follicles ≥3 mm in diameter were recorded on high grade video tape (Fuji S-VHS, ST-120 N; Fujifilm, Tokyo, Japan), using a compatible VCR (Panasonic, Super VHS, AG 1970; Matsushita Electronics of Canada Ltd, Mississauga, ON, Canada), for image analysis. All equipment and machine settings (near-field, far-field, and overall gain) were standardized for optimal ovarian imaging and the settings maintained for the study. The diameter and relative position of all follicles ≥3 mm in diameter were also sketched on ovarian charts to identify different follicular waves and map the patterns of growth and regression of follicles in the waves. The length of the growth, static and regression phases for anovulatory follicular waves were calculated as previously defined (Bartlewski et al. 1999a).

4.3.3. Image acquisition and analysis

Image analysis was performed for one follicle per wave (growing from 3 mm to ≥5 mm in diameter). For consistency, when there was more than one follicle in a wave, the follicle that emerged first was analyzed. Images of follicles collected daily from emergence (3 mm in diameter) until regression (3 mm in diameter) or ovulation, which had the greatest cross-sectional diameter, were digitized at standardized settings. The digitized images (resolution of 640×480 pixels and 256 shades of grey) were then analyzed with a series of custom-developed computer algorithms optimized for ultrasonography (SYNERGYNE Version 2.8©, Saskatoon, SK, Canada).

4.3.3.1. *Image analysis of follicular wall and antrum*

The follicular antrum was outlined at the inner boundary of the follicular wall to evaluate its area. The area encompassing the follicular wall was then outlined and the area located between the outer and inner boundaries was analyzed. This procedure was performed by a single trained individual for all follicles to minimize subjective deviation. The numerical pixel value (NPV) was calculated. This metric is the mean pixel value (MPV) of the grey-scale values of all the pixels within the outlined area. Pixel heterogeneity (PH) was also calculated; this is defined as the standard deviation of the values of the selected pixels within an area, from the mean pixel value calculated for that area. Area under the curve (AUC) was defined as the area of the sampled region in standard scale-bar units.

4.3.4. Blood sampling and hormone assays

Blood samples were collected daily (10 mL) into vacutainers (Becton Dickinson, Rutherford, NJ, USA) prior to each ultrasound examination and kept at room temperature for 24 h. Serum was then separated and stored at -20 ºC until assayed. Serum concentrations of FSH (Currie and Rawlings 1989) and progesterone (Rawlings et al. 1984) were determined by established radioimmunoassays. The sensitivity of the assays (defined as the lowest concentration of hormone capable of significantly displacing labeled hormone from the antibody) for FSH and progesterone were 0.1 and 0.03 ng/mL, respectively. For reference sera with mean FSH concentrations of 0.93 or 3.76 ng/mL, the intra- and inter-assay CVs were 6.1 or 3.4% and 6.1 or 3.2%, respectively. The intra- and inter assay CVs were 13.2 or 6.3% and 12.1 or 5.9%, respectively, for reference sera with mean progesterone concentrations of 0.26 or 1.06 ng/mL. Peaks in serum concentrations of FSH, in samples taken daily, were detected using a cycle detection computer program (Clifton and Steiner 1983).

4.3.5. Statistical analysis

Follicular wave characteristics (Table 4.1.) were compared among different waves by one way repeated measures Analysis of Variance (SigmaStat® Statistical Software for Windows, Version 2.03, 1997, SPSS Inc., Chicago, IL, USA). To investigate daily changes of image attributes over the follicular lifespan within each wave and compare these attributes among different waves, data were normalized to the first day of follicular lifespan and analyzed by two way repeated measures Analysis of Variance (SigmaStat® Statistical Software for Windows, Version 2.03, 1997, SPSS Inc.). Main effects were

day in follicular lifespan and wave in the inter-ovulatory interval, with day by wave interaction. Multiple comparisons were made by Fisher's least significant difference (LSD). Correlations between follicular diameter and the area under the curve for the follicular wall and antrum, as well as correlations among the serum progesterone concentrations and image attributes of the follicular wall and antrum were analyzed using Pearson's correlation (SigmaStat® Statistical Software for Windows, Version 2.03, 1997, SPSS Inc.). All values are means ± SEM.

4.4. Results

4.4.1. General results

The mean length of the inter-ovulatory interval was 18.3 ± 0.32 days. In six ewes, four distinct follicular waves were detected during the ovulatory interval, while one animal had three waves. Emergence of each follicular wave was associated with a transient peak in circulating FSH concentrations (Fig. 4.1.). Follicular waves 1 and 3 in the ewe with 3 waves emerged on days 1 and 12 of the inter-ovulatory interval respectively. These days were the same as the days of emergence for waves 1 and 4 in the rest of the animals; therefore, data were pooled for analysis. In the ewe with 3 waves, wave 2 emerged about two days later (Day 6) than wave 2 in the rest of the ewes, and therefore, was excluded from analysis. There was no difference in the length of the growth (2.7 ± 0.25 d), and regression (2.8 ± 0.23 d) phases between different waves ($P > 0.05$; Table 4.1.; Fig. 4.1.). The mean length of the static phase was greater in wave 2 ($P < 0.05$) compared to other waves (Table 4.1.; Fig. 4.1.).

Table 4.1. Comparison of wave characteristics (Mean ± SEM) for the follicular waves seen during the inter-ovulatory interval in the seven normal cyclic ewes used in this study.

	Follicular waves			
	Wave 1	**Wave 2**	**Wave 3**	**Ovulatory wave**
No. follicles included in analysis	7	6	7	7
No. follicles in the wave	1.3 ± 0.23	1.1 ± 0.11	1.2 ± 0.22	1.1 ± 0.09
Day of emergence after ovulation (day 0)	0.6 ± 0.49[a]	4.5 ± 0.22[b]	8.1 ± 0.22[c]	11.6 ± 0.24[d]
Growth phase (d)	2.9 ± 0.48	2.5 ± 0.31	2.7 ± 0.57	3.9 ± 0.52
Static phase (d)	1.9 ± 0.33[a]	3.0 ± 0.42[b]	1.9 ± 0.63[a]	1.6 ± 0.38[a]
Regression phase (d)	2.9 ± 0.48	2.7 ± 0.61	2.7 ± 0.22	-
Growth rate (mm/day)	1.0 ± 0.12	0.9 ± 0.11	1.1 ± 0.19	0.9 ± 0.12
Regression rate (mm/day)	1.0 ± 0.22	1.0 ± 0.21	0.9 ± 0.13	-
Maximum follicular diameter (mm)	5.8 ± 0.25	5.2 ± 0.17	5.3 ± 0.18	5.7 ± 0.33
Inter-wave interval (d)	W1 to W2: 3.9 ± 0.12	W2 to W3: 3.6 ± 0.24	W3 to W4: 3.5 ± 0.33	

[abcd] $P < 0.05$

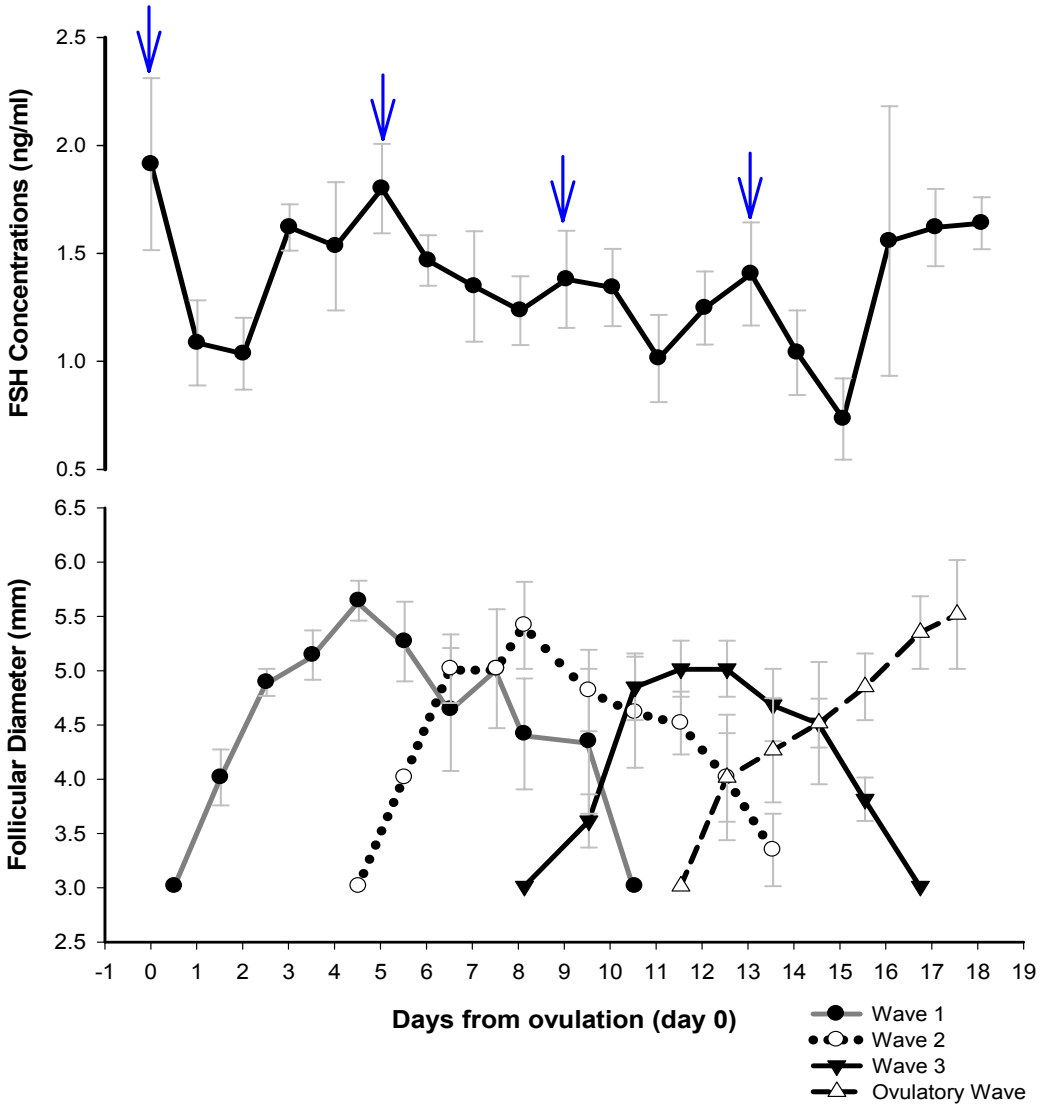

Fig. 4.1. Top panel: Mean (± SEM) serum FSH concentrations in cyclic Western White Face ewes. Daily serum concentrations of FSH were normalized to the day of ovulation (day 0). Arrows indicate peaks in serum concentrations of FSH detected by the cycle detection computer program. **Bottom panel: Mean (± SEM) follicular diameter of follicles emerging in different follicular waves during the inter-ovulatory interval in normal cyclic ewes.** Data are normalized to the corresponding mean day of wave emergence for each follicular wave after ovulation (day 0).

4.4.2. Numerical pixel value (NPV) of the follicular wall and antrum

No changes were observed in the NPV of the follicular wall in the first and second waves of the ovulatory interval as the follicles grew from 3 mm to ≥5 mm and then regressed to 3 mm in diameter ($P > 0.05$; Fig. 4.2). The mean NPV for the wall of anovulatory follicles, emerging in the third wave of the inter-ovulatory interval, was higher than for wave 1 and 2 at the time of emergence ($P < 0.01$; Fig. 4.2). For wave 3, the mean NPV for the wall decreased to a minimum as the follicles reached their maximum follicular diameter around day 3 after emergence ($P < 0.05$). The NPV of the wall for follicles in the ovulatory wave (last wave of the inter-ovulatory interval) tended to decrease as they gained maximum follicular diameter ($P = 0.07$). The mean NPV for the follicular antrum in the first, second and third waves of the inter-ovulatory interval did not change as the follicular diameter increased from 3 mm to ≥5 mm and then decreased to 3 mm by the end of regression ($P > 0.05$; Fig. 4.2). The mean NPV of the antrum for the ovulatory follicles decreased while the follicular diameter increased from 3 mm to its maximum diameter ($P < 0.05$).

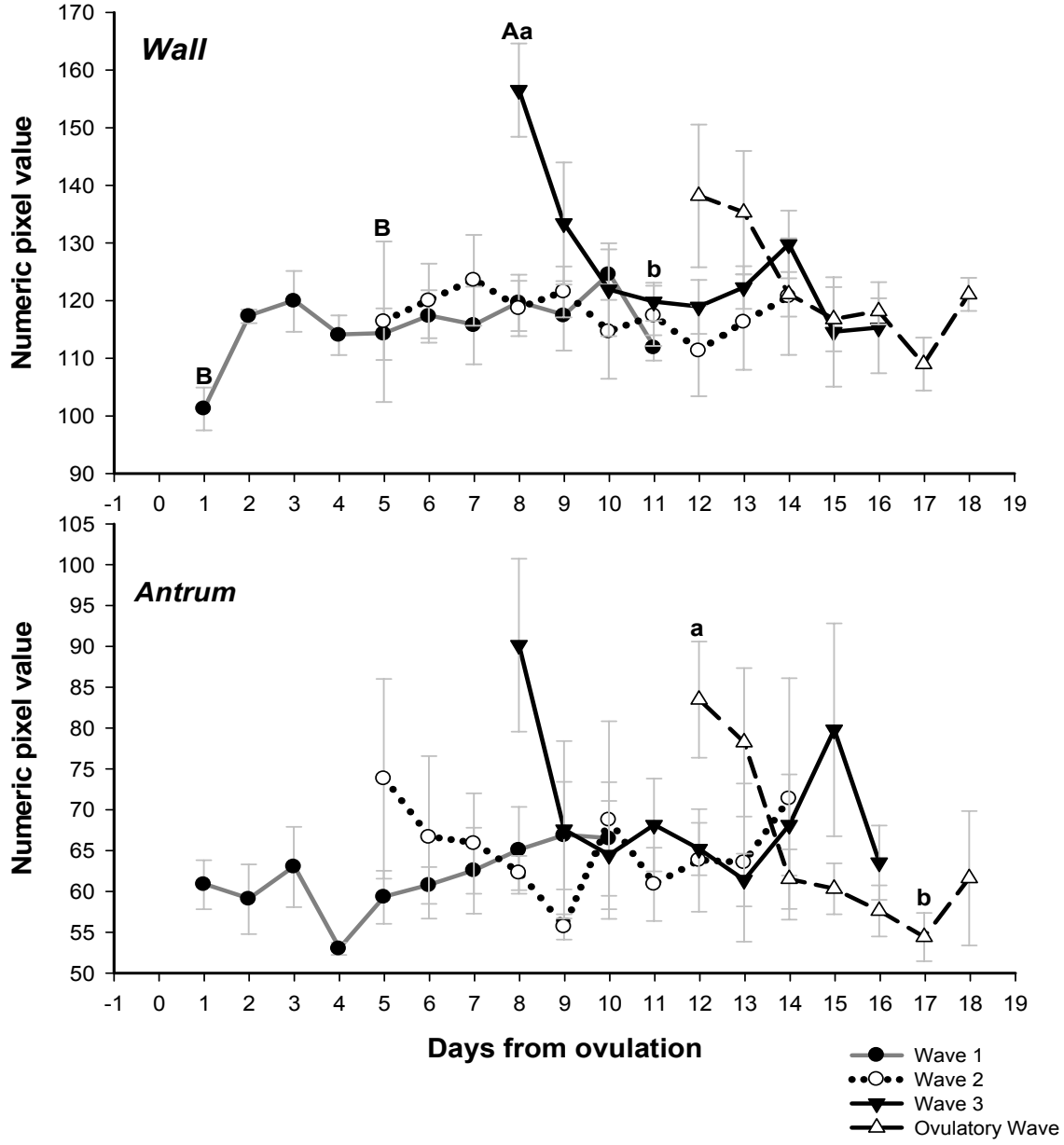

Fig. 4.2. Changes in the mean (± SEM) numeric pixel value (NPV) of the follicular wall and antrum (top and bottom panels respectively) for follicular waves during the inter-ovulatory interval in normal cyclic ewes. Data are normalized to the corresponding mean day of wave emergence for each follicular wave after ovulation (day 0). ^{ab} denote significant difference ($P<0.05$) within days of follicular lifespan. ^{AB} denote significant difference ($P<0.05$) at the time of emergence amongst different waves.

4.4.3. Pixel heterogeneity (PH) of the follicular wall and antarum

The PH of the follicular wall did not change with growth and regression of follicles in each follicular wave and did not differ among different waves in the inter-ovulatory interval ($P > 0.05$; Fig. 4.3.). Pixel heterogeneity for the follicular antrum in the first follicular wave of the inter-ovulatory interval increased between the end of the growth phase and the late regression phase of follicular development ($P < 0.05$); however, no significant pattern was observed for changes in pixel heterogeneity for the follicular antrum in other follicular waves (Fig. 4.3.).

4.4.4. Area under the curve for the follicular wall and antrum

The area under the curve for both the follicular wall and antrum were correlated with follicular diameter in all follicular waves ($r = 0.938$, $P < 0.01$ and $r = 0.941$, $P < 0.01$ for the wall and antrum, respectively). The mean area under the curve for the follicular wall and antrum increased in all waves as follicular diameter increased and then decreased in anovulatory waves while follicles regressed ($P < 0.01$; Fig. 4.4.).

4.4.5. Serum progesterone concentrations

Mean serum progesterone concentrations increased gradually between day 2 and 11 after ovulation as the corpora lutea formed. Mean serum progesterone concentrations reached a peak of 2.2 ± 0.45 ng/mL on day 12 after ovulation ($P < 0.01$). Luteolysis resulted in a dramatic drop in serum progesterone concentrations to basal levels on day 16 after ovulation ($P < 0.01$). Serum progesterone concentrations were not correlated with

changes in the numeric pixel value and pixel heterogeneity of the follicular wall and antrum in any follicular wave ($P > 0.05$).

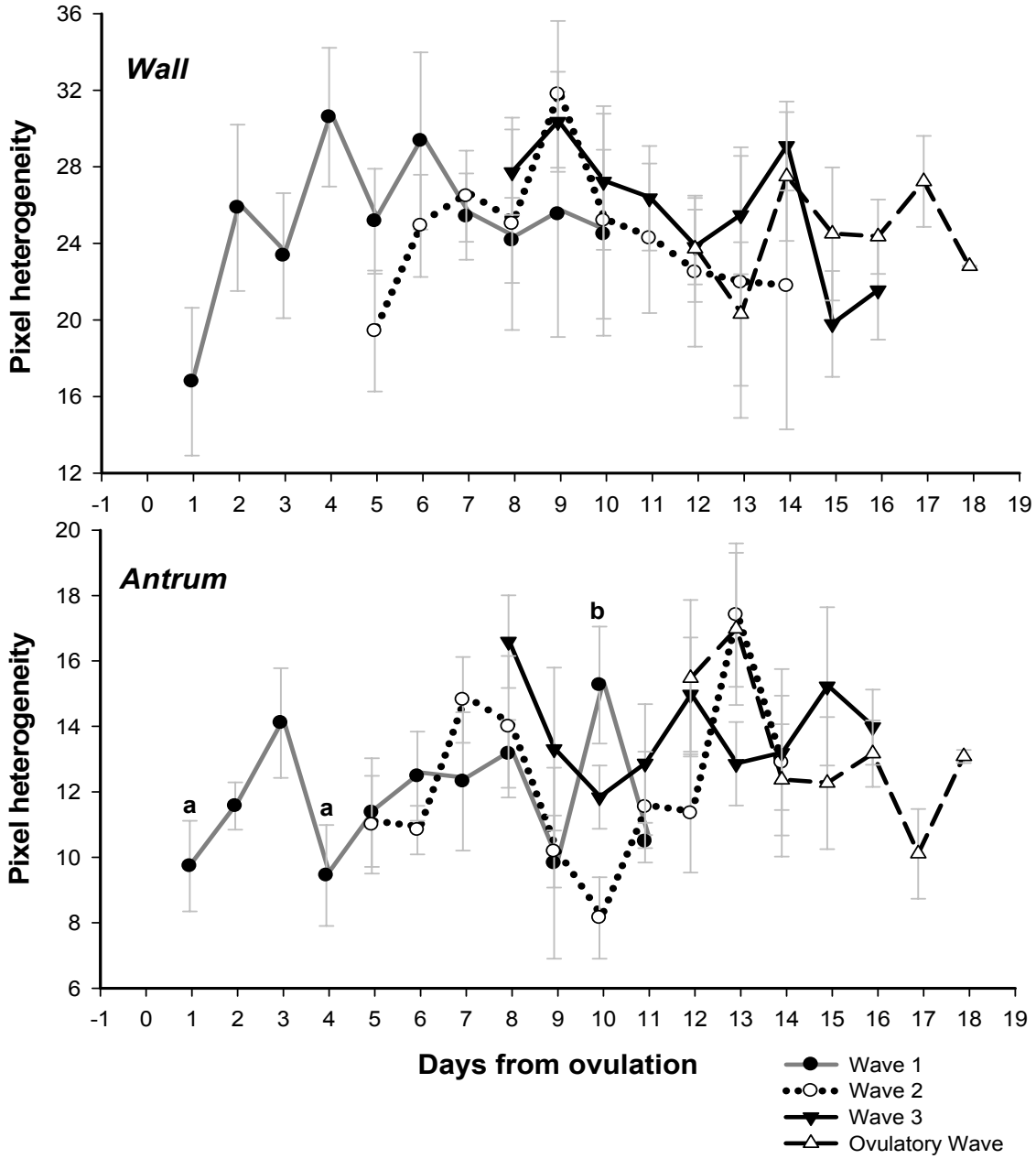

Fig. 4.3. Changes in the mean (± SEM) pixel heterogeneity (PH) for the follicular wall and antrum (top and bottom panels respectively) for follicular waves during the inter-ovulatory interval in normal cyclic ewes. Data are normalized to the corresponding mean day of wave emergence for each follicular wave after ovulation (day 0). ^{ab} denote significant difference ($P<0.05$) within days of follicular lifespan.

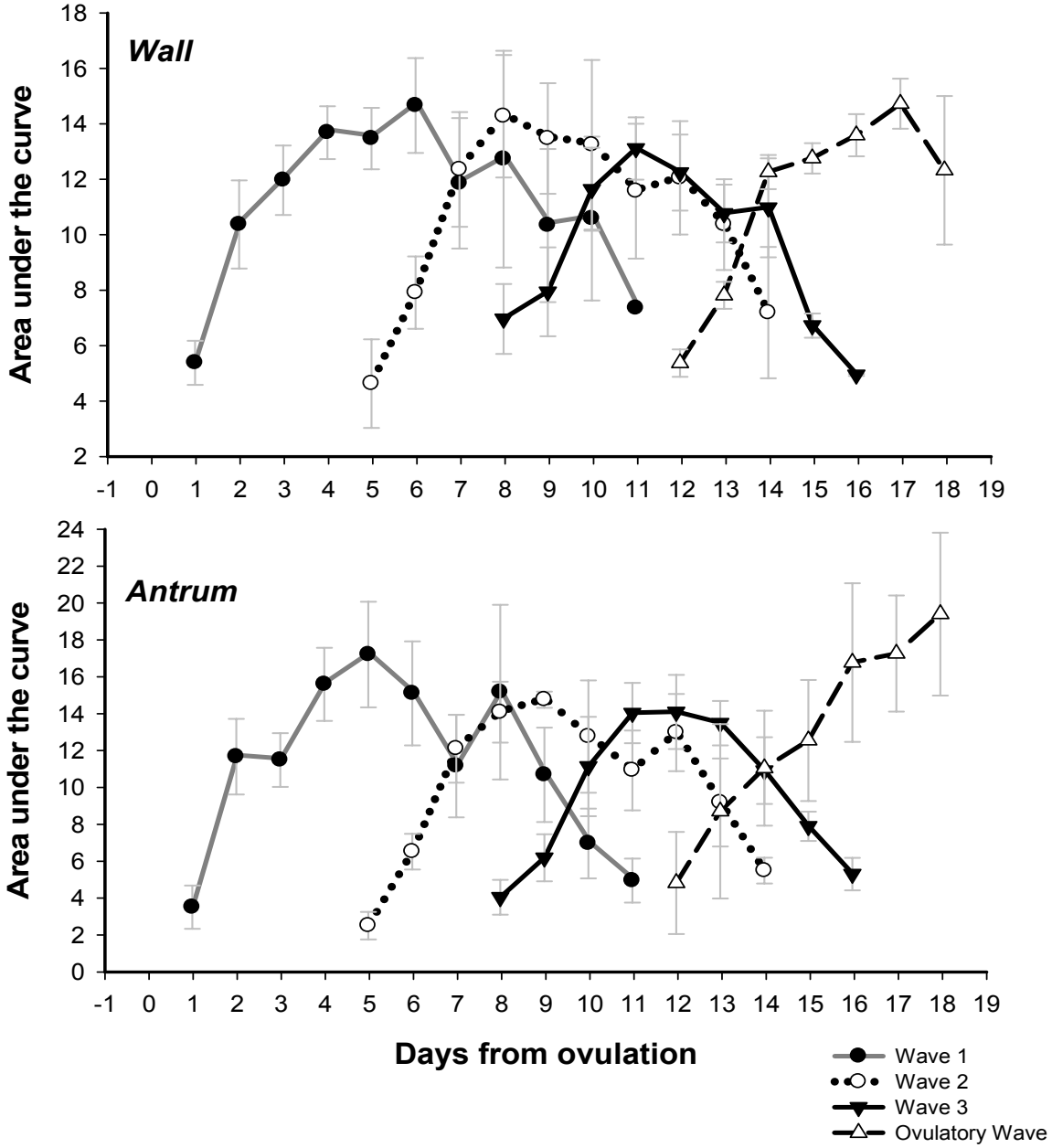

Fig. 4.4. Changes in the mean (± SEM) area under the curve (AUC) for the follicular wall and antrum (top and bottom panels respectively) for follicular waves during the inter-ovulatory interval in normal cyclic ewes. Data are normalized to the corresponding mean day of wave emergence for each follicular wave after ovulation (day 0).

4.5. Discussion

Our hypothesis that ultrasound image attributes of antral follicles reflect the phases of follicle development and regression over an inter-ovulatory interval was partially supported. We observed a wave-dependent alteration in the image attributes of antral follicles. The mean pixel value of the follicular wall in wave 3 was higher than previous anovulatory waves of the ovulatory interval (waves 1 and 2) at the time of emergence and the wall of the follicles in wave 3 became darker (decreased NPV) as follicular diameter increased. A similar tendency was also seen for ovulatory follicles (Wave 4; P = 0.07). No trend in NPV was seen for waves 1 and 2. Echotexture of the follicular wall is dependent on the thickness of the wall, degree of vascularization in the theca layer, vascular blood flow and the amount of lipid in steroid-producing granulosa and theca cells (Singh et al. 1998, Tom et al. 1998b, Singh and Adams 2000, Liu et al. 2007b). Investigations using cattle revealed that hypertrophy and proliferation of granulosa and theca cells, as well as development of the dense connective tissue of vascular walls, were reflected as brighter images with increased NPV (Singh et al. 1998, Tom et al. 1998b, Singh and Adams 2000, Liu et al. 2007b). However, increased blood flow alters the proportion of vascular tissue to blood, and results in a lower NPV in the follicular wall (Tom et al. 1998b). These changes may reflect alterations in the biological function of follicles (Singh et al. 1998, Tom et al. 1998b, Liu et al. 2007a, Liu et al. 2007b, Liu et al. 2008). The reason(s) for the higher NPV of the follicular wall in wave 3 at emergence, as observed in this study, is not clear and requires histomorphological investigation.

The decreasing NPV of the wall in follicles emerging after the mid-interovulatory interval may be related to increased blood flow, as growing follicles develop to become mature ovulatory sized structures. Tom et. al. (1998b) reported a rapid decrease in NPV of the follicular wall during the growth of ovulatory follicles and during the transition into the static phase of dominant anovulatory follicles in cattle. Histological evaluation in the same study revealed high vascularity in the follicular wall. In another investigation in cattle, the wall of preovulatory follicles had low granulosa cell density and high vascularity and edema within the theca interna (Singh and Adams 2000). Preovulatory follicles destined to develop functional corpora lutea in cattle and humans had a lower NPV (darker) for the follicular wall than atretic follicles (Martinuk et al. 1992, Singh et al. 1998, Tom et al. 1998b). It has been suggested that echotextural characteristics of the follicular wall are indicative of the steroidogenic activity of the theca and granulosa cells in cattle (Singh et al. 1998). In ewes given equine chorionic gonadotropin (eCG), high NPV for the follicular wall was associated with higher serum progesterone concentrations after ovulation and corpus luteum formation (Liu et al. 2007b). Based on the present echotextural analysis of the follicular wall in cyclic ewes, we speculated that follicles emerging after the middle of the inter-ovulatory interval may be more adapted for ovulation and corpus luteum formation. In normal cyclic ewes, ovulation normally occurs from the last wave of the cycle; however, in some prolific breeds of ewes, follicles from the penultimate wave of the cycle can ovulate (Bartlewski et al. 1999a). Short-term treatment with medroxyprogesterone acetate (MAP) releasing intravaginal sponges at midcycle, after induction of luteolysis with $PGF_{2\alpha}$, can induce follicles from both waves to ovulate in Western White Face sheep (Bartlewski et al.

2003, Liu et al. 2007a). It is exciting to note that the NPV of the walls of follicles emerging after middle of the inter-ovulatory interval differed from the walls of follicles of waves 1 and 2 of the ovulatory cycle. The reasons for this contrast could be investigated further by parallel histomorphological and functional evaluation of follicles at different time points of the inter-ovulatory interval.

Granulosa cells are sloughed into the antrum individually or as clusters of cells during the later stages of follicular development (Singh et al. 1998, Singh and Adams 2000). This may cause an increase in the NPV of the antrum, especially near the follicular wall. In the present study, echotextural analysis of the whole follicular antrum did not reveal significant changes in pixel values in waves 1, 2 and 3 of the inter-ovulatory interval. This observation is in agreement with results of another report that the mean pixel values of the antrum in bovine anovulatory dominant follicles did not vary significantly during the follicular lifespan (Tom et al. 1998b). However, other studies in cattle showed brighter (higher NPV) follicular antra during the late-static and regression phases of anovulatory follicles (Vassena et al. 2003a). In the current study there was a decrease in the NPV of the follicular antrum in ovulatory follicles as they reached their maximum diameter. A tendency for a similar drop in the NPV as the diameter of the follicular antrum increased, was also noted for waves 2 and 3 of the inter-ovulatory interval ($P = 0.09$ and $P = 0.07$, respectively; Fig. 2). This trend might be related to the growth of follicles and the increase in antral fluid volume with low echogenicity. The drop in NPV occurred as follicles attained their maximum steroidogeneic potential. For ovulatory follicles, the more obvious drop in NPV of the follicular antrum as the follicles grew, may represent less cellular debris in the antrum compared to earlier waves in the inter-

ovulatory interval. In the ewe, the formation of short-lived corpora lutea following multiple GnRH injections was related to a greater NPV of the central antrum prior to GnRH treatment (Liu et al. 2007b). This was interpreted to suggest that follicles forming into optimal luteal structures shed greater numbers of granulosa cells into their antra in comparison to follicles forming normal corpora lutea (Liu et al. 2007b).

A significant alteration in PH of the follicular antrum occurred only in the first wave of the inter-ovulatory interval in the present study. The increase in PH of the follicular antrum in wave 1 of the inter-ovulatory interval started from the beginning of the static phase and continued through the regression phase of the follicular lifespan (Fig. 4.3.). This could be explained by the accumulation of echoic cellular debris and macromolecules within the follicular antrum (Pierson and Adams 1995, Singh et al. 1998, Tom et al. 1998b); however, this pattern was not observed in the other anovulatory waves of the inter-ovulatory interval. The follicular wall in preovulatory bovine follicles showed low values for PH (Tom et al. 1998b); however, in the present study, the PH of the follicular wall in the ovulatory wave did not change as the follicles grew to ovulatory diameters. Ovarian antral follicles are much smaller in the ewe than in cattle or humans and this may account for the high variation in the gray-scale pixel values that we observed. Utilizing regional echotextural analysis requires selection of the whole area of the follicular wall and/or antrum for analysis (Pierson and Adams 1995, Birtch et al. 2005). Due to irregularities at the follicular wall-antrum interface and the small size of ovine ovarian follicles, it is inevitable that some pixels from each region (wall or antrum) enter into the analysis of the other region, resulting in greater variation or heterogeneity in pixel values.

In the present study, the length of the static phase was longer for the follicles emerging in the second wave of the inter-ovulatory interval. Studying the same breed of ewes, Bartlewski et. al. (1999a) reported an extended static phase for the follicles emerging in wave 1 and a longer growing phase for the follicles in wave 4 of an inter-ovulatory interval, while other wave dynamics remained unchanged. These results lead us to conclude that the pattern of growth and regression of follicles do not vary consistently among the waves of the estrous cycle in the ewe. This is interesting as the first and last waves would develop against a background of increasing and decreasing serum progesterone concentration, respectively, while waves 2 and 3 develop largely in an environment of high serum progesterone concentration.

Quantitative assessment of the area under the curve (AUC), combined with follicular diameter data may assist in interpretation of echotextural data, especially for the follicular wall. An increase or decrease in follicular diameter, with no change in AUC for the follicular wall suggests a decrease or increase in the thickness of follicular wall respectively. Variation in the AUC for both follicular wall and antrum were highly correlated with changes in the follicular diameter. Therefore, it can be speculated that the changes in the image attributes of the follicular wall are mainly due to alterations in follicular size rather than the wall thickness. However, this assumption must be verified by measurements of follicular wall thickness using histological sections or computer-assisted programs.

In summary, results from this study partially supported the hypothesis that follicular image attributes change during the lifespan of follicles within a follicular wave, reflecting the developmental stage of the follicle. Interestingly, the changes were

dependent on the time of wave emergence during the inter-ovulatory interval. A greater alteration in NPV of the follicular wall was observed in follicular waves emerging after middle of the inter-ovulatory interval, suggesting morphological changes in the wall, related to the potential for ovulation of those follicles. The NPV of the antrum in the 4th wave of the inter-ovulatory interval also decreased as these ovulatory follicles grew to ≥5 mm in diameter. This might be indicative of less atretic granulosa cells and other debris in the follicular antrum, suggesting a different morphological and functional status of follicles destined for ovulation and formation of corpora lutea. Pixel heterogeneity of the antrum in the first wave of the inter-ovulatory interval increased during the static and regression phases of the follicular lifespan. There are potential morphological and functional differences amongst antral follicles emerging at different stages of the inter-ovulatory interval in cyclic ewes. These findings also suggest predictive potential for image analysis as a diagnostic tool to evaluate follicular physiologic status in sheep.

CHAPTER 5: OVARIAN FOLLICULAR DOMINANCE IN THE EWE AND THE INDUCTION OF DAILY FOLLICULAR WAVES

Toosi BM, Seekallu SV, Zeigler AC, Barrett DMW and Rawlings NC

5.1. Abstract

In the ewe, large antral follicles emerge and grow in waves every 4 to 5 days both during the breeding season and seasonal anestrus. Emergence of each wave is preceded by a transient peak in serum FSH concentrations. The existence of follicular dominance, or the ability of follicles in a wave to block the development of other large antral follicles, in the ewe is unclear. This experiment was designed to see if emergence of follicular waves could be induced on a daily basis with injection of ovine FSH. Six anestrus ewes were treated with two daily injections of oFSH (0.35 μg/kg; sc; 8 h apart) for 4 days, starting 24 h after the expected time of an endogenously driven FSH peak. Six anestrous ewes were treated with vehicle. Ultrasonography was done twice daily and blood samples were collected ever 6 h. Injection of oFSH resulted in the occurrence of 4 discrete peaks in serum FSH concentrations. Each injection of oFSH resulted in the emergence of a new follicular wave. The mean number of small follicles decreased from the first day of treatment to a nadir on day 4 after the start of treatment (16 ± 1.9), compared to control ewes (25 ± 3.8; $P < 0.05$). We conclude that the ovine ovary is able to respond on a daily basis to physiologic peaks in serum FSH concentrations with the emergence of new follicular waves. The existence of direct follicular dominance in the ewe is questionable and the mechanisms that control the rhythm of follicular waves and the replenishment of the pool of small follicles, from which waves originate, are unclear.

5.2. Introduction

In the ewe, development of ovarian antral follicles occurs in a wave-like pattern (Noel et al. 1993, Ravindra et al. 1994, Ginther et al. 1995, Souza et al. 1997, Bartlewski et al. 1999a, Bartlewski et al. 2000b, Evans et al. 2000, Driancourt 2001). A follicular wave is defined as the emergence or growth of 1 to 3 follicles from a pool of small follicles (2 to 3 mm in diameter) in the ovary and their growth to ≥5 mm in diameter before regression (anovulatory wave) or ovulation (ovulatory wave) (Bartlewski et al. 1998, Bartlewski et al. 1999a, Bartlewski et al. 2000b, Duggavathi et al. 2003a). Follicular waves emerge every 4 to 5 days both during the breeding season and seasonal anestrus (Ginther et al. 1995, Bartlewski et al. 1998, Bartlewski et al. 1999a, Bartlewski et al. 2000b). Emergence of each follicular wave is associated with a transient peak in serum FSH concentrations; this peak appears to be essential for follicle wave emergence and each peak lasts 3 to 4 days (Ginther et al. 1995, Bartlewski et al. 1998, Bartlewski et al. 1999a, Bartlewski et al. 2000b, Driancourt 2001,).

In cattle, two or three follicular waves emerge in each estrous cycle (Ginther et al. 2003, Adams et al. 2008, Jaiswal et al. 2009). Each wave is characterized by the initial emergence or growth of 7 to 11 small follicles (4 mm in diameter) followed by the rapid growth of one follicle in this cohort to an ovulatory diameter (dominant follicle) (Ginther et al. 2003). This follicle suppresses the growth of other follicles in the cohort (subordinate follicles) and prevents emergence of a new follicular wave (Armstrong and Webb 1997, Adams et al. 2008). Treatment of cattle with physiological or supraphysiological concentrations of FSH, in the presence of a growing dominant follicle, failed to increase the mean number of major waves with emergence of

additional follicular waves, supporting the concept of direct follicle to follicle dominance (Ginther et al. 2002). In cattle, it has been suggested that this direct dominant effect of a growing follicle in a wave is exerted only when that follicle is in the active growth phase (Adams 1999, Ginther et al. 2002, Adams et al. 2008).

In contrast to cattle, the number of small antral follicles (1 to 3 mm in diameter) did not increase at wave emergence in sheep, except during the periovulatory period (Duggavathi et al. 2003a). In sheep, follicles originating from the penultimate wave of the estrous cycle can ovulate with follicles from the final wave (Bartlewski et al. 1999a, Bartlewski et al. 2003, Evans 2003). In addition, wave overlap has also been noted in ewes treated with exogenous progesterone (Johnson et al. 1996, Leyva et al. 1998, Flynn et al. 2000). However, in those studies, emergence of new follicular waves occurred in the late-growth, static or early regression phase of a large antral follicle, when the ability of that follicle to exert functional dominance would have been questionable. However, emergence of a new follicular wave in the presence of a large growing follicle(s) from a previous wave has been reported in sheep (Duggavathi et al. 2004, Duggavathi et al. 2005a). In one study, ovine FSH was injected to create a peak in serum concentrations of FSH in an interwave interval in the ewe and that peak resulted in emergence of a new follicular wave (Duggavathi et al. 2004). Mechanisms involved in the recruitment of follicles for emergence in each follicular wave are not clear in the ewe (Baird and Campbell 1998, Hunter et al. 2004).

If direct dominance is not as evident in sheep as it is in cattle, the creation of frequent peaks in serum FSH concentrations in the ewe should result in emergence of recurrent follicular waves. The objective of the present study was to see if the ovine ovary

responds to daily injections of oFSH with frequent waves of follicular growth. We hypothesized that direct dominance is not evident in the ewe and the ovine ovary is capable of responding to frequent, even daily peaks in serum FSH concentrations with emergence of a new follicular wave.

5.3. Materials and methods

All Animal experimentation was performed according to the guidelines of the Canadian Council on Animal Care and was approved by the University of Saskatchewan animal care committee.

5.3.1. Animals

Twelve healthy, anestrus (May-June), Western White Face ewes (5 to 7 years of age, average body weight of 82.2 ± 4.3 kg) were randomly divided into treatment (n = 6) and control (n = 6) groups. All animals were housed in sheltered dry lots (Saskatoon, SK., Canada; 52 ºN latitude). Animals received daily maintenance rations of alfalfa pellets with water, and cobalt iodized salt licks available *ad libitum.*

5.3.2. Ultrasonography

All of the anestrous ewes underwent daily ultrasonography, starting at 08:00 h each day to determine the rhythmicity of follicular waves. Scanning was then done twice daily starting at 08:00 and 20:00 h to detect the first appearance of a 4 mm follicle of a new wave. Twice daily ultrasonography continued until 72 h after the last treatment after which observations were reduced to daily for a further 6 days.

Transrectal ultrasonography of ovaries was performed using a high-resolution, real-time B-mode echo camera (Aloka SSD-900; Aloka Co. Ltd., Tokoyo, Japan) connected to a 7.5 MHz transducer. The number, diameter and relative position of all follicles ≥1 mm in diameter were sketched onto ovarian charts, and all ovarian images were recorded on high-grade video tapes (Fuji S-VHS, ST-120 N; Fujifilm, Tokoyo, Japan), using a compatible VCR (Panasonic, AG 1978; Matsushita Electronics, Mississauga, ON, Canada), for retrospective analysis of ovarian data.

5.3.3. Experiment procedures and hormone preparation

Treatment with oFSH was designed to start at 24 h after a peak in serum concentrations of FSH that preceded a follicular wave. Based on previous observations we know this peak occurs 60 h after the first detection of a 4 mm follicle in the previous wave (Duggavathi et al. 2005a, Duggavathi et al. 2004). To create physiological peaks in serum FSH concentrations, each daily treatment consisted of two injections of oFSH 8 h apart (0.35 μg/kg, sc). Control ewes received two injections of vehicle 8 h apart. The Experimental regimen was designed to provide 4 daily treatments of oFSH or vehicle within the period between two endogenously generated peaks in serum concentrations of FSH (Fig. 5.1.).

One milligram of the oFSH used in this experiment (Teri.oFSH/ig.1, Tucker Endocrine Research Institute LLC, Atlanta, GA, USA) had a biological potency of FSH equivalent of 90 x NIH-oFSH-S1 and biological potency of LH less than 0.1 x NIH-oLH-S1. Ovine FSH for injection was prepared in saline with 0.05% bovine serum albumin (BSA; w/v; St. Louis, MO) and 50% polyvinylpyrrolidone (PVP; w/v; Sigma).

5.3.4. Blood sampling and hormone assays

Blood samples (10 ml) were taken in vacutainers (Becton Dickinson, Rutherford, NJ, USA) daily (08:00 h), and at 02:00, 08:00, 14:00 and 20:00 h from 24 h before the first injection to 48 h after the last treatment (total of 6 days). Blood samples were allowed to clot for 18 to 24 h at room temperature, and serum was harvested and stored at -20 ^{o}C until assayed.

All serum samples were analyzed for circulating concentrations of FSH and estradiol by validated radioimmunoassays (Rawlings et al. 1984, Currie and Rawlings 1989). The range of the standard curves was from 0.12 to 16.0 ng/mL and 1.0 to 50 pg/mL for FSH and estradiol, respectively. The sensitivities of assays (defined as the lowest concentration of hormone capable of significantly displacing labeled hormone from the antibody; unpaired t-test, $P < 0.05$) were as follows: FSH, 0.1 ng/mL and estradiol, 1 pg/mL. For reference sera with mean FSH concentrations of 0.45 or 3.28 ng/mL, the intra- and inter-assay CVs were 7.4 or 3.8% and 10.7 or 4.6% respectively. For estradiol, the intra- and inter assay CVs for reference sera with mean concentrations of 7.02 or 22.14 pg/mL were 8.8 or 6.5% and 13.3 or 8.4%, respectively.

Peaks in serum concentrations of FSH, in samples taken twice daily, were determined using the cycle-detection computer program (Clifton and Steiner 1983). Within each peak of FSH secretion, data for all ewes in a group were aligned temporally to the zenith of the peak. To graph the data for each group, the zenith of each peak was normalized to the first day of treatment (Day 0). Serum estradiol concentrations were normalized to the first day of treatment.

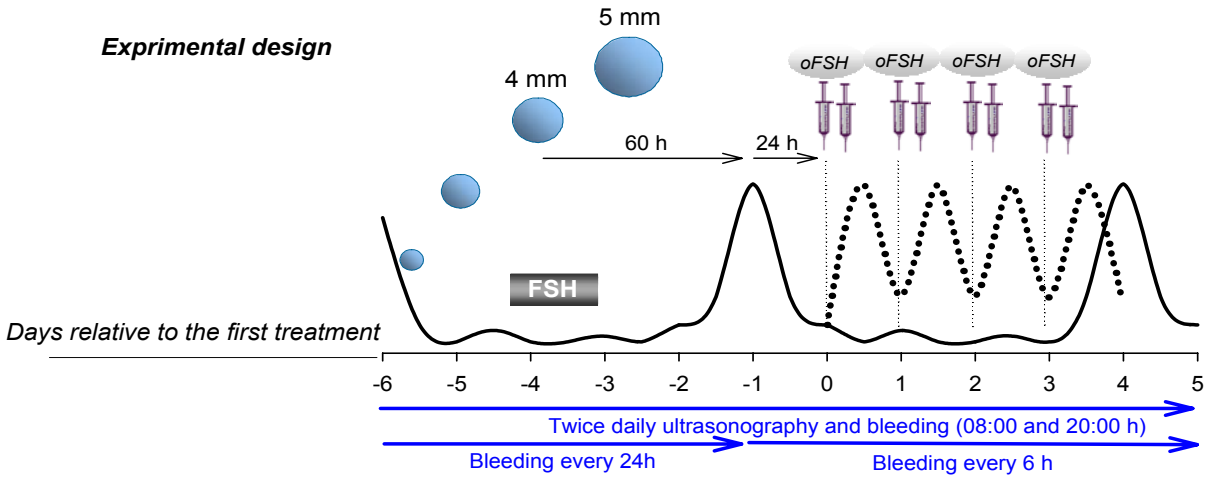

Fig. 5.1. Schematic representation of the experimental design in the present study. Six anestrous ewes were treated with two injections of oFSH 8 h apart (0.35 µg/kg, sc, dotted line) every day for 4 days in the period between two endogenously generated peaks in serum concentrations of FSH (solid line). Control ewes (n = 6) received vehicle only. The first treatment was timed to occur 24 h after the peak in serum concentrations of FSH that preceded a follicular wave. Based on previous observations we know that FSH peaks occur 60 h after the first detection of a 4 mm follicle in the previous follicular wave (Duggavathi et al. 2004). See text for detail and rationale for experimental design.

5.3.5. Follicular data analysis

A follicular wave is regarded as a follicle or group of follicles that emerge or grow from 2 or 3 mm in diameter to an ovulatory size of ≥5 mm in diameter, with emergence restricted to a 24 h period (Bartlewski et al. 1999a, Duggavathi et al. 2004). For consistency, the day of emergence for each growing follicle in a follicular wave was considered when that follicle was first detected at 3 mm in diameter.

Follicular waves emerging within 24 h after each daily treatment with oFSH were considered to be associated with the peak in serum FSH concentrations created by that treatment. With scanning done twice daily, it was possible to accurately map follicular waves emerging on a daily basis over the period of treatment. Follicular waves induced by injection of oFSH every day for 4 days were designated waves A, B, C and D. The wave immediately preceding treatment was designated wave 1 and following treatment in control ewes was wave 2. Characteristics of the follicular waves such as the number of follicles in the wave (follicles growing to ≥5 mm in diameter), maximum follicular diameter, length of growth, static and regression phases (days) as well as growth and regression rates of the largest follicle of a wave were calculated. The number of small (1 to 3 mm in diameter) follicles seen on a daily basis over the experiment was also calculated.

5.3.6. Statistical analysis

Changes in mean serum concentrations of FSH and estradiol over the period of the experiment as well as daily changes in the number of small size follicles were analyzed

by two-way repeated measures Analysis of Variance (SigmaStat® Statistical Software for Windows Version 2.3, 1997, SPSS Inc., Chicago, IL, USA). Comparison of follicular wave characteristics amongst different follicular waves within treatment groups were made by one-way repeated measures Analysis of Variance (SigmaStat® Statistical Software for Windows Version 2.3, 1997, SPSS Inc., Chicago, IL, USA). Multiple comparisons were made by the method of Fisher's least significant difference (LSD). All values are means ± SEM and statistical significance was set as $P < 0.05$.

5.4. Results

5.4.1. Serum concentrations of FSH

After four daily treatments with oFSH in the period between two consecutive endogenously driven peaks in serum concentrations of FSH, discrete peaks were detected by the cycle detection program on days 0.4 ± 0.1, 1.6 ± 0.1, 2.5 ± 0.0, 3.4 ± 0.1 relative to the day of first treatment (Day 0). A significant peak in serum FSH concentrations after each injection was also shown by ANOVA (Fig. 5.2.). Basal serum concentrations of FSH were significantly greater in ewes given oFSH (2.6 ± 0.2 ng/mL) than in control ewes (1.1 ± 0.1 ng/mL) from day 1 to 6 after the first injection of oFSH (day 0). The endogenously driven peak in serum concentrations of FSH on day 5 after onset of treatment in control ewes appeared to be masked by treatment in ewes given oFSH (Fig. 5.2.). However, in ewes given oFSH or vehicle, a peak in serum concentrations of FSH was seen 8.3 ± 0.3 or 8.6 ± 0.3 days after the first injection, respectively (Fig. 5.2.).

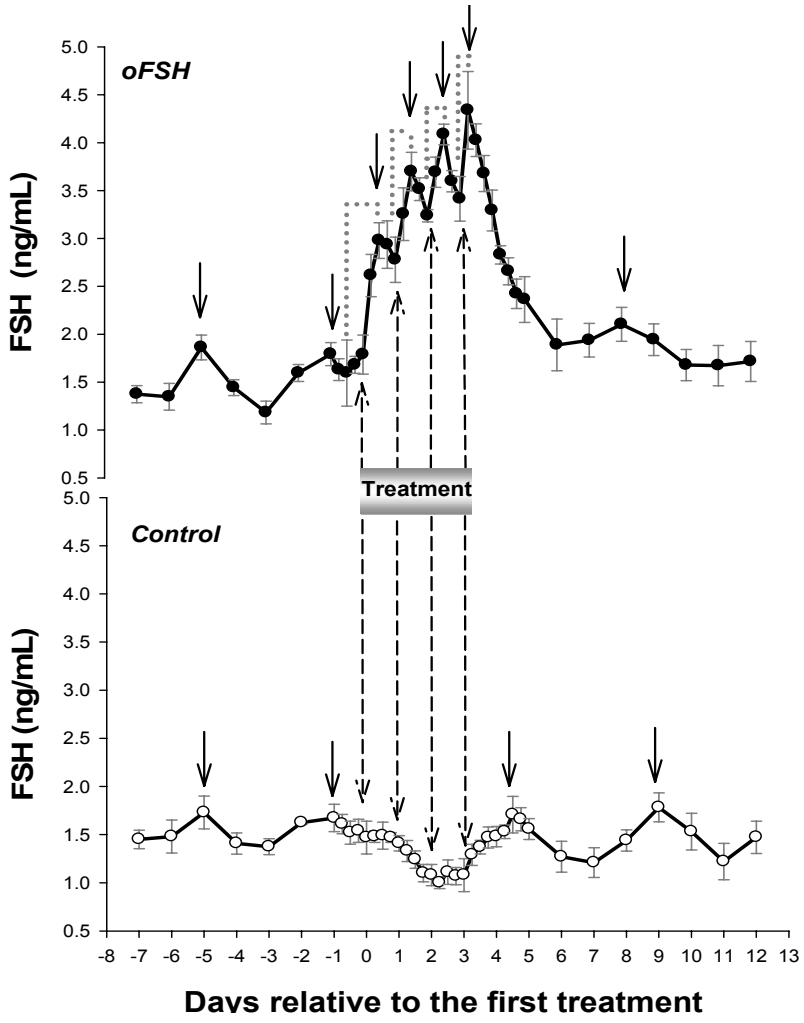

Fig. 5.2. Mean (±SEM) serum concentrations of FSH in oFSH-treated (n = 6; top panel, ●) and control (n = 6; bottom panel, ○) ewes. Treatment included two injections of oFSH (0.35 µg/kg, sc) or vehicle, 8 h apart, given daily for four successive days (represented by dash-lined arrows), starting 24 h after the expected day of an endogenously driven peak in serum FSH concentrations. Within each peak of FSH secretion, data for all ewes in a group were aligned temporally to the zenith of the peak. To graph the data for each group, the zenith of each peak was normalized to the mean day relative to the first treatment when that peak occurred (Day 0 = day of first treatment). The solid arrows denote peaks in serum FSH concentrations identified by the cycle detection program. Dotted lines denote significant increases in serum FSH concentrations after giving each oFSH treatment, compared to basal serum FSH concentrations.

5.4.2. Follicular wave emergence

In ewes given oFSH on days 0, 1, 2 and 3 (Day 0 = day of first treatment), follicular waves emerged on days 0.6 ± 0.1, 1.7 ± 0.1, 2.6 ± 0.1 and 3.6 ± 0.1, respectively (waves A, B, C and D; Fig. 5.3.). Within oFSH treated ewes, the number of follicles that emerged within the first 12 h after the first, second, third and fourth treatment with oFSH, given daily (1.3 ± 0.4, 1.4 ± 0.3, 2.5 ± 0.8 and 1.8 ± 0.6, respectively) was greater than the number of emerging follicles during the second 12 h after each treatment ($P < 0.05$; 0.3 ± 0.2, 0.8 ± 0.2, 0.6 ± 0.3, 0.3 ± 0.2, respectively). Emergence of endogenously driven wave 1 occurred on day -0.6 ± 0.2 and -0.7 ± 0.1 in ewes given oFSH or vehicle, respectively ($P > 0.05$). In control ewes, the mean day of emergence of the next endogenously driven wave (wave 2) was on day 4.2 ± 0.4.

5.4.3. Number of small follicles and serum estradiol concentrations

The mean number of small follicles (1 to 3 mm in diameter) decreased after giving oFSH, reaching a nadir on day 4 after first treatment (Fig. 5.4.; $P < 0.05$). Mean serum estradiol concentrations did not differ between ewes given oFSH and control ewes (3.2 ± 0.3 and 3.3 ± 0.3 pg/mL, respectively; $P > 0.05$).

5.4.4. Follicular wave characteristics

Antral follicular wave characteristics (length of the growth, static and regression phases, growth and regression rates, and maximum follicular diameter) of the largest follicle of a wave did not differ among waves or treatment groups ($P > 0.05$).

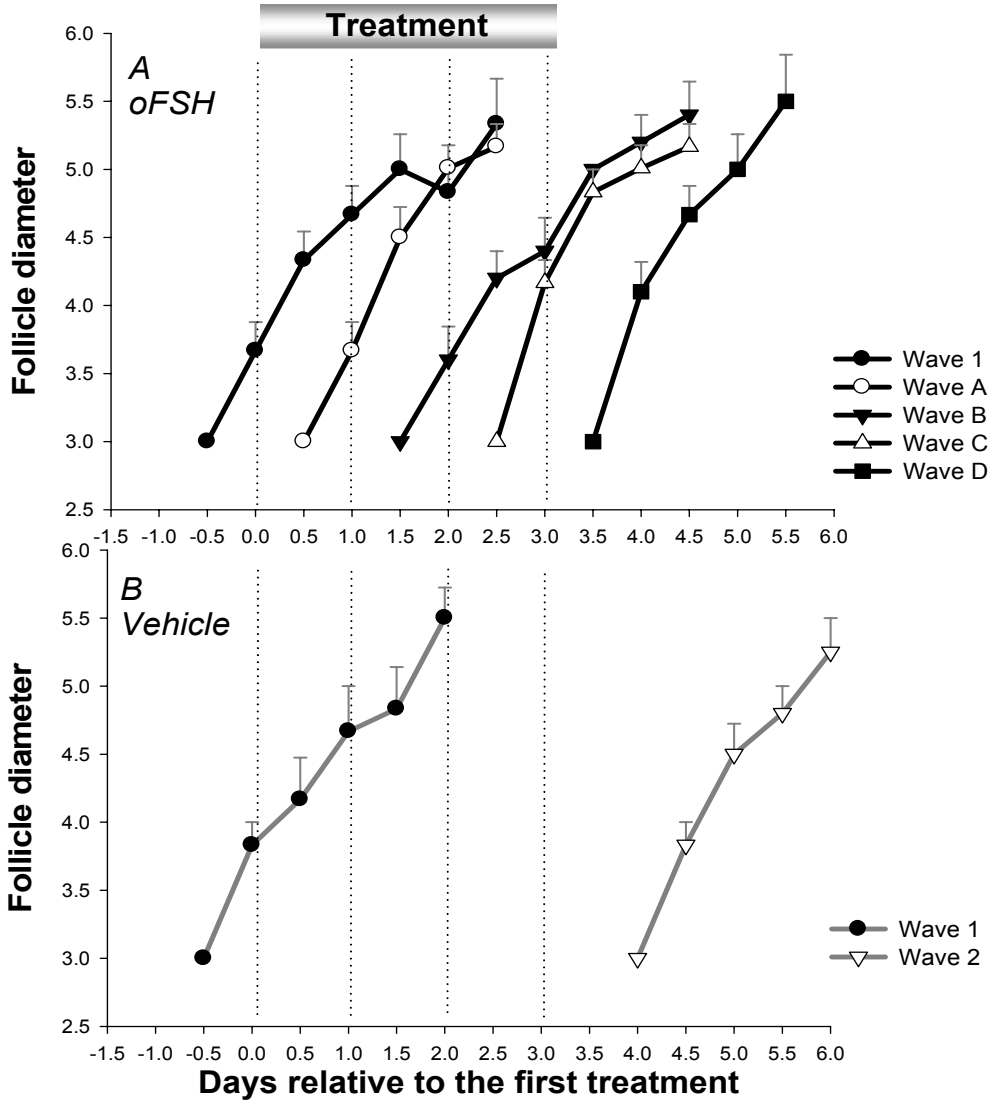
Treatment
A
oFSH
Follicle diameter
Wave 1
Wave A
Wave B
Wave C
Wave D
B
Vehicle
Follicle diameter
Wave 1
Wave 2
Days relative to the first treatment

Fig. 5.3. Panel A: Mean (±SEM) diameter profiles of follicles in waves 1, A, B, C and D emerging between days -1 to 0, 0.5 to 1, 1.5 to 2, 2.5 to 3 and 3.5 to 4, respectively, in oFSH-treated ewes. Panel **B:** Mean (±SEM) diameter profiles of follicles of waves 1 and 2 in control ewes. In panel A and B, data for each follicular wave are graphed from the mean day of wave emergence for that wave, normalized to the day of first treatment. Treatment included two injections of oFSH (0.35 µg/kg, sc) or vehicle, 8 h apart, given daily for four successive days (represented with dash-lines), starting 24 h after the expected day of an endogenously driven peak in serum FSH concentrations (Day 0 = day of first treatment).

* indicates significant difference in number of growing follicles between oFSH-treated and control groups ($P < 0.05$)

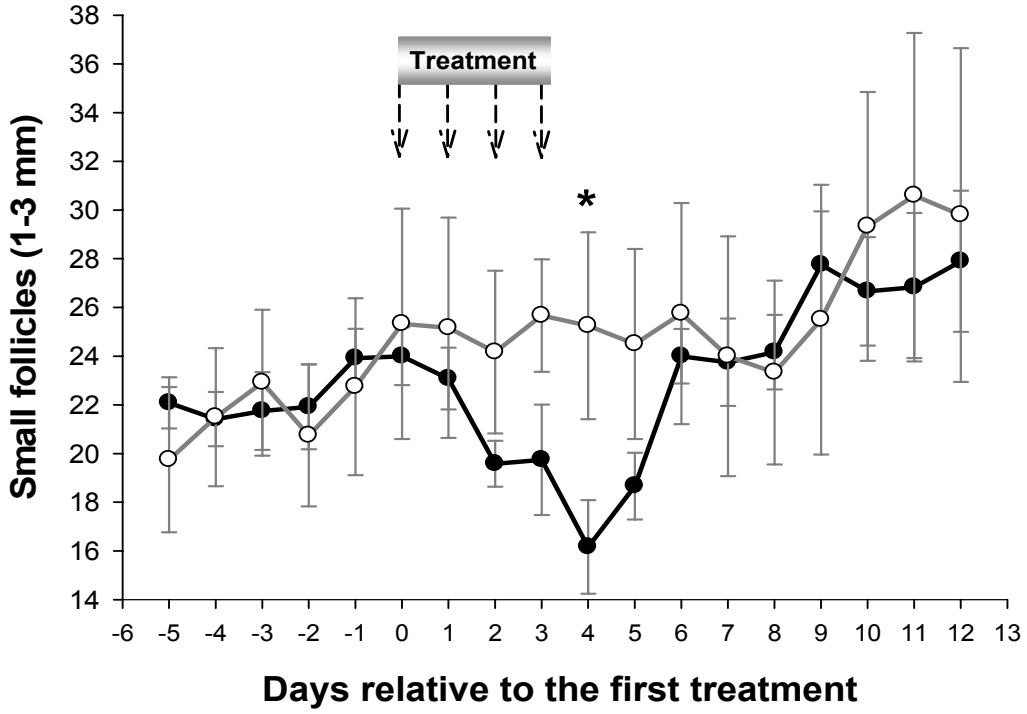

Fig. 5.4. Mean (±SEM) daily (08:00 h) number of small follicles (1-3 mm in diameter) in oFSH-treated (n = 6; ●) and control (n = 6; ○) ewes. Treatment included two injections of oFSH (0.35 µg/kg, sc) or vehicle, 8 h apart, given daily for four successive days (represented with dash-lined arrows), starting 24 h after the expected day of an endogenously peak in serum FSH concentrations. The mean number of small follicles were normalized to the day of the first injection (Day 0) of oFSH or vehicle.
* indicates significant difference in number of small follicles between oFSH-treated and control groups ($P < 0.05$).

5.5. Discussion

In the present study, treatment of anestrous ewes with oFSH on a daily basis successfully created four distinct peaks in serum FSH concentrations. In all oFSH treated ewes, there was at least one follicle emerging in response to each treatment. This confirms that in sheep there is no direct dominance effect from growing follicle(s) of a follicular wave to suppress emergence and growth of a new follicular wave in response to a peak in serum FSH concentrations. This is in agreement with previous reports from our laboratory when emergence of a new follicular wave was induced in the growth phase of an existing follicular wave by giving exogenous FSH (Duggavathi et al. 2004, Davies 2005, Duggavathi et al. 2005a).

In the ewe, it has been suggested that serum concentrations of FSH need to reach a threshold to stimulate emergence and growth of an antral follicular wave (Driancourt 2001). In the present study, oFSH treatment on a daily basis increased the basal serum FSH concentrations during the treatment period. It could be argued that the increased serum concentrations of FSH, probably over the required threshold for follicle stimulation, resulted in a continuous emergence of ovarian antral follicles to ovulatory diameters. Although a great number of follicles grew to ovulatory diameters in response to the oFSH treatments, the emergence of those follicles were distributed mainly within the first 12 h after each oFSH treatments (76.5%), given every 24 h for 4 days. These results indicate that the occurrence of daily, discrete FSH peaks, over increased basal circulating concentrations of FSH, induced the emergence and growth of discrete antral follicular waves.

The last oFSH treatment appeared to mask the occurrence of the next endogenously driven peak in serum FSH concentrations. Interestingly, the occurrence of the first clear endogenously driven FSH peak after oFSH treatment was detected simultaneously with the second peak in serum FSH concentrations after treatment, in control ewes. This emphasizes that giving oFSH on a daily basis did not interrupt the rhythmic occurrence of the endogenously driven peaks in serum FSH concentration. If dominance is unclear in sheep then some other endogenous rhythm would be needed to drive the regular rhythm of peaks in FSH secretion and the associated follicular waves (Duggavathi et al. 2005b). Rhythmic peaks in serum FSH concentrations have been reported in ovariectomized ewes with a similar inter-peak interval and peak amplitudes as those in ovary-intact ewes (Duggavathi et al. 2005b). In another study, a 10-day application of estradiol-releasing implants in cyclic ewes resulted in truncation of peaks in serum FSH concentrations and interruption of follicular wave emergence; however, those truncated peaks showed a rhythmic occurrence during the treatment period (Barrett et al. 2006).

In the present study, 5 follicular waves emerged in ewes treated with oFSH in the time frame of the emergence of 2 follicular waves in control ewes. This suggests that small FSH-sensitive follicles are available on a daily basis to enter a wave in response to a physiological FSH stimulus. In cattle, follicular wave emergence is associated with a transient increase in the numbers of small antral follicles (7 to 11 follicles of about 4 mm in diameter) in the ovary (Ginther et al. 2001, Ginther et al. 2003). However, no significant day effect for the number of small follicles has been reported in ewes except around the time of ovulation (Duggavathi et al. 2003a). In contrast to normal follicular waves in cattle, the number of small follicles (1 to 3 mm in diameter) in the ovary, in the

present study in sheep, declined towards the end of the treatment period. These results are in agreement with other observations in sheep indicating a lack of recruitment of a large number of small follicles at the emergence of a new follicle wave or follicle-to-follicle suppression of small follicles during the growth phase of a wave (Noel et al. 1993, Ginther et al. 1995, Bartlewski et al. 1999a, Evans et al. 2000, Duggavathi et al. 2003a). Moreover, the drop in the number of small follicles in this study indicated that the growth of follicles into larger size categories (4 mm and ≥5 mm in diameter) in response to frequent oFSH treatments caused depletion of the small follicle pool. This also probably confirms the gonadotropin independent growth of antral follicles which are less than 2 mm in diameter in sheep (Driancourt 2001). Based on the present results, it is interesting to speculate that small follicles in the ovary remain responsive to physiological peaks in serum FSH concentrations for some time; however, only a few of them ever emerge into follicular waves in response to peaks in serum FSH concentrations. It has been suggested that selection of 1 to 3 small follicles to emerge and grow in each follicular wave is associated with an intricate relationship between gonadotropins and local factors in the ovary (Campbell et al. 1999, Webb et al. 2007). However, the mechanism for recruitment of small follicles into a follicular wave is unclear as is the regulation of the rate of replenishment of small follicles in the ovary.

In summary, daily injections of oFSH for four days resulted in emergence of new follicular waves on a daily basis. This treatment did not disturb the rhythm of endogenously driven peaks in serum FSH concentrations and follicular wave emergence. Small follicles (1 to 3 mm in diameter) in the ovary appear to be able to respond to physiologic peaks in serum FSH concentrations on a daily basis. The mechanisms for

follicle selection for growth and development into follicular waves and for replenishment of the pool of small ovarian follicles are intriguingly unclear in the ewe.

CHAPTER 6: EFFECTS OF THE RATE AND DURATION OF PHYSIOLOGICAL INCREASES IN SERUM FSH CONCENTRATIONS ON EMERGENCE OF FOLLICULAR WAVES IN CYCLIC EWES

Toosi BM, Seekallu SV and Rawlings NC

6.1. Abstract

There are 3 or 4 follicular waves in the inter-ovulatory interval of cyclic ewes. Emergence of each follicular wave is preceded by a transient peak in serum FSH concentrations which lasts for 3 to 4 days and is essential for emergence of the wave. To study the characteristics of peaks in FSH secretion required for follicular wave emergence, we attempted to investigate the effects of creating a gradual increase in the leading slope of an FSH peak (Experiment 1) and of raising basal serum concentrations of FSH and maintaining them at peak levels for 60 h (Experiment 2). In Experiment 1, 6 cyclic ewes received ovine FSH (0.1 μg/kg, sc) every 6 h for 42 h starting 24 h after ovulation. Control ewes (n = 6) received vehicle. Blood samples were taken every 6 h and ovaries were examined daily by ultrasonography in both experiments. Serum FSH concentrations increased in oFSH treated ewes ($P < 0.05$) resulting in an additional peak between two endogenously driven FSH peaks and therefore, did not give the planned gradual leading slope to an FSH peak. FSH treatment occurred in the early growth phase of wave 1 of the inter-ovulatory interval and increased the growth rate of growing follicles in that wave, compared to control ewes ($P < 0.05$). This apparently induced dominance in follicles in wave 1, causing them suppress wave emergence in response to the injected FSH. In Experiment 2, oFSH was infused constantly (1.98 μg/ewe/h, n = 6) for 60 h starting 3 days after ovulation and at the time of the second endogenously driven FSH peak of the inter-ovulatory interval. Control ewes (n = 5) were infused with

vehicle. Infusion of oFSH resulted in a superstimulatory effect with a peak in the mean number of large follicles on day 2 after the start of FSH infusion (13 ± 1.2 large follicles per ewe; 1.8 ± 0.2 in control ewes; $P < 0.001$). Within oFSH treated ewes there was a decrease in number of small follicles from 16.3 ± 2.1 before the start of treatment to 4.4 ± 1.3 on day 2 ($P < 0.05$). In conclusion, creating an exogenous FSH peak by giving frequent low doses of ovine FSH failed to induce emergence of a new follicular wave due probably to induced dominance exerted by the largest follicles growing concurrent to oFSH treatment. Infusion of a low dose of oFSH constantly for 60 h stimulated the growth of small follicles in the ovary and induced a superovulatory response in cyclic ewes.

6.2. Introduction

In the ewe, ovarian antral follicular growth occurs in a wave-like pattern (Noel et al. 1993, Ravindra et al. 1994, Ginther et al. 1995, Souza et al. 1997, Bartlewski et al. 1999a, Bartlewski et al. 2000b, Evans et al. 2000, Driancourt 2001) which is defined as the emergence and growth of 1 to 3 follicles from a pool of small follicles (1 to 3 mm in diameter) and their growth to ≥5 mm in diameter (Bartlewski et al. 1998, Bartlewski et al. 1999a, Duggavathi et al. 2003a). These follicles may remain in a static phase before regression (anovulatory waves) or ovulation (ovulatory waves) (Bartlewski et al. 1998, Bartlewski et al. 1999a, Bartlewski et al. 2000b). Emergence of each follicular wave is preceded by a transient peak in serum concentrations of follicle stimulating hormone (FSH), which lasts for 3 to 4 days, and is considered the signal for emergence of the wave (Ginther et al. 1995, Bartlewski et al. 1998, Bartlewski et al. 1999a, Driancourt 2001). Truncation of endogenous FSH peaks by treatment with estradiol resulted in

disappearance of follicular waves in cyclic ewes (Barrett et al. 2006). Moreover, treatment of ewes with a physiologic dose of exogenous oFSH to create an FSH peak during the inter-wave interval, resulted in emergence of a new follicular wave without disruption of the normal pattern of FSH peaks and follicular waves (Duggavathi et al. 2004, Duggavathi et al. 2005a). The induced FSH peak resulted in a wave with normal follicle(s) in terms of growth characteristics and estradiol secretion (Duggavathi et al. 2004). It has been suggested that the peaks in serum FSH concentrations need to reach a specific threshold to stimulate the emergence of a follicular wave (Picton and McNeilly 1991, Driancourt 2001). Although this threshold seems to be close to the zenith of the peaks in serum concentrations of FSH, individual variation in this threshold has also been noted among ewes (Picton and McNeilly 1991).

The FSH peaks that precede the emergence of ovarian follicular waves in the ewe appear to vary in characteristics, such as peak height, duration and the shape of the leading and trailing slopes of the peak. However, peaks in serum FSH concentrations, with different characteristics, can trigger emergence of follicular waves when they reach a required threshold (Picton and McNeilly 1991, Driancourt 2001). The relationships between the characteristics of FSH peaks and the growth patterns of their corresponding follicular waves have not been studied in the ewe. In anestrous ewes, when the amplitude of a peak in serum concentrations of FSH was doubled, by giving exogenous oFSH, the ensuing follicular wave did not differ from that seen in control ewes (Duggavathi et al. 2005a).

In Experiment 1, our objective was to see if a very gradual increase in the leading slope of an FSH peak would be detected by the ovary as a proper signal to stimulate

emergence of a new follicular wave. We hypothesized that a new follicular wave with normal follicular dynamics would emerge after an induced FSH peak with a gradual leading slope. A peak in serum FSH concentrations could be simply defined as a temporary and gradual rise in basal concentrations to the threshold levels required for emergence of a follicular wave, since a peak usually occurs over 3 to 4 days. It is not clear whether a discrete peak is required to signal a follicular wave or merely an increase in basal concentrations of FSH to a threshold value. In Experiment 2, our objective was to see if raising the basal serum concentrations of FSH to the concentrations seen at the zenith of a peak and maintaining it for several days would allow multiple follicle waves to emerge. We hypothesized that maintaining elevated basal serum FSH concentrations would induce continuous emergence of new follicular waves in the sheep ovary.

6.3. Materials and methods

All Animal experimentation was performed according to the guidelines of the Canadian Council on Animal Care and was approved by the University of Saskatchewan animal care committee. The cyclic, nulliparous, Western White Face ewes (4 to 6 years of age), used in the present experiments, received daily maintenance rations of alfalfa pellets with water, and cobalt iodized salt licks available *ad libitum*. Ewes were kept outdoors in pens when not involved with experiments. During both experiments, ewes were housed indoors with lighting set to simulate the natural light/dark cycle (October-December). Estrus was synchronized by a 14-day treatment with MAP-releasing intravaginal sponges (Medroxyprogesterone Acetate, 60 mg; Veramix®, Up-John, Orangeville, ON, Canada). Experiments were conducted in the second cycle after synchronization. Estrus was detected with three vasectomized crayon-harnessed rams.

6.3.1. Experiment 1

6.3.1.1. Animals

Twelve ewes with a mean body weight of 81.8 ± 4.7 kg were divided into treatment (n = 6) and control (n = 6) groups.

6.3.1.2. Hormone Preparation and Experimental Procedures

Six ewes were treated with ovine FSH (0.1 μg/kg, sc; Teri.oFSH/ig.1, Tucker Endocrine Research Institute LLC, Atlanta, GA, USA) every 6 h for 42 h. Treatment started 24 h after ovulation. Six control ewes received injections of vehicle only. The treatment regimen was designed to create a gradual increase in serum FSH concentrations over the 42 h prior to the zenith of the second FSH peak of the inter-ovulatory interval. Each 1 mg of oFSH used had a biological potency of FSH equivalent to 90 x NIH-oFSH-S1 and a biological potency of LH less than 0.1 x NIH-oLH-S1. The oFSH was prepared in saline with 0.05% BSA (w/v; Sigma, St. Louis, MO, USA) and 50% polyvinylpyrrolidone (w/v; Sigma; 100 μg oFSH per 50 mL vehicle).

6.3.1.3. Ultrasonography

Transrectal ultrasonographic examination of ovaries was performed using a high-resolution, real-time B-mode echo camera (Aloka SSD-900; Aloka Co. Ltd., Tokyo, Japan) connected to a 7.5 MHz transducer. The examinations were done daily (starting at 08:00 h), starting 2 days before the expected day of estrus. Ovulation was detected by the disappearance of large antral follicles (≥5 mm in diameter) and confirmed by formation of corpora hemorrhagica and/or corpora lutea (Bartlewski et al. 1999d). The

number, diameter and relative position of all follicles ≥1 mm in diameter and copora lutea (CL), were sketched onto ovarian charts, and all ovarian images were recorded on Digital Versatile Discs (DVDs; Verbatim DVD+R 16X, Verbatim Corp. Charlotte, NC, USA), using a compatible DVD recorder (LG, LRA 750, LG Electronics Inc. Canada, Mississauga, ON, Canada), for retrospective analysis of ovarian data. Ultrasongraphic examination of the ovaries was continued until the identification of the emergence of the third follicular wave of the inter-ovulatory interval studied.

6.3.1.4. *Blood Sampling, Hormone Assays and Data Analysis*

Blood samples (10 ml) were collected in vacutainers (Becton Dickinson, Rutherford, NJ, USA) prior to each ultrasound examination and at 08:00, 14:00, 20:00 and 02:00 hr from 24 h before the first, to 48 h after the last treatment, followed by sampling at 08:00 and 20:00 for another 48 h. Serum was harvested from samples and stored at -20 ºC until assayed. Serum samples were analyzed for circulating concentrations of FSH (samples taken daily and every 6 h; (Currie and Rawlings 1987)) and estradiol (samples taken daily; (Joseph et al. 1992)) by validated radioimmunoassays. The sensitivity of the assays (defined as the lowest concentration of hormone capable of significantly displacing labeled hormone from the antibody; unpaired t-test, $P < 0.05$) were as follows: FSH, 0.1ng/mL and estradiol, 1pg/mL. For reference sera with mean FSH concentrations of 0.48 or 3.65 ng/mL, the intra- and inter-assay CVs were 8.3 or 3.4% and 10.5 or 4.8% respectively. For estradiol, the intra- and inter-assay CVs for reference sera with mean concentrations of 8.66 or 25.54 pg/mL were 9.9 or 6.7% and 12.2 or 8.8%, respectively. Peaks in serum concentrations of FSH, in samples taken twice daily, were determined using a cycle-detection computer program modified for Windows® XP

(Clifton and Steiner 1983). Within each peak of FSH secretion, data for all ewes in a group were aligned temporally to the mean day of the zenith of the peak for that group of ewes. To graph the data for each group of ewes, the time of the zenith of each FSH peak was normalized to the mean day of the occurrence of the peak relative to the start of the treatment (Day 0). Peak serum concentrations of estradiol were calculated for each follicular wave.

6.3.1.5. *Analysis of Follicular Data*

A follicular wave was regarded as a follicle or group of follicles that emerged or grew from 3 mm in diameter to an ovulatory size of ≥5 mm in diameter, with emergence restricted to a period of 24 h (Bartlewski et al. 1999a). Data for the first and second follicular waves of the inter-ovulatory interval studied are presented and compared. The mean day of follicular wave emergence for waves 1 and 2 were determined relative to the start of treatment (Day 0). The number of follicles in a wave represents the number of all follicles growing from 1 to 3 mm to ≥5 mm in diameter in each follicular wave. The lengths of the growth, static, and regression phases, maximum diameter, growth rate and the lifespan of the largest follicle in waves 1 and 2 are presented (Bartlewski et al. 1999a). The inter-wave interval is defined as the interval between the time of wave emergence for two consecutive follicular waves.

6.3.1.6. *Statistical Analysis*

Serum concentrations of FSH and estradiol in samples taken during the experiment were analyzed for effects of time and treatment by two-way repeated measures Analysis of Variance (RM-ANOVA, SigmaStat® Statistical Software for Windows Version 2.03,

1997, SPSS Inc., Chicago, IL, USA). Two-way ANOVA was used to compare characteristics of growing follicles with main effects of treatment and wave (Table 1). Multiple comparisons were made by Fisher's least significant difference (LSD). All values are means ± SEM and statistical significance was set as $P < 0.05$.

6.3.2. Experiment 2

6.3.2.1. Animals

Eleven ewes with a mean body weight of 80.6 ± 3.3 kg were randomly divided into treatment (n = 6) and control (n = 5) groups.

6.3.2.2. Hormone Preparation, Experiment Procedures and Ultrasonography

Ovine FSH (Teri.oFSH/ig.1) was infused intravenously to 6 ewes for 60 h. Infusion started 3 days after ovulation with an infusion rate of 0.5 mL/min/ewe. Peristaltic pumps (Pharmacia P-3, Pharmacia Fine Chemicals, London, UK) were used for infusion of oFSH and were connected to the left jugular vein of the ewes via indwelling jugular catheters (vinyl tubing; 1.00 mm inner diameter x 1.50 mm outer diameter; 530070; Biocorp Australia Propriety Ltd., Huntingdale, Australia). Ovine FSH was prepared in saline (33 µg/500 mL) with 0.05% BSA (w/v; Sigma, St. Louis, MO, USA). All ewes were restrained in individual carts during the infusion with free access to food and water (*ad libitum*). Control animals received the saline-BSA solution. Infusion with oFSH was designed to increase the basal serum concentrations of FSH to the zenith of the FSH peaks that precede follicular waves and to maintain FSH concentrations at this level for 60 hours. The infusion was timed to start prior to the second endogenously driven FSH

peak of the inter-ovulatory interval and to last as long as the endogenous peak. Transrectal ovarian ultrasonographic examination was done daily; starting 2 days before the expected time of estrus and continuing until 10 days after the end of infusion.

6.3.2.3. *Blood Sampling, Hormone Assays and Data Analysis*

Blood samples (10 mL) were taken using vacutainers (Becton Dickinson, Rutherford, NJ, USA) prior to each ultrasound examination. Samples were also taken every 6 h (4 ml) from 6 h before the start of infusion to 24 h after the end of infusion via a second indwelling jugular catheter (vinyl tubing) inserted in the right jugular vein. Cannulae were inserted 24 h before the start of infusion and were filled with heparinized saline between the periods of intensive bleeding (1000 U.S.P. units of heparin sodium per 1 L of saline; Hepalean, Organon Teknika Inc., Toronto, ON, Canada). Serum concentrations of FSH were measured in all blood samples. Serum estradiol and progesterone concentrations were determined in samples taken daily (Rawlings et al. 1984). The sensitivity of the assay for progesterone was 0.02 ng/mL. For reference sera with mean FSH concentrations of 0.91 or 3.91 ng/mL, the intra- and inter-assay CVs were 7.4 or 3.9% and 8.7 or 4.2%, respectively. For estradiol, the intra- and inter-assay CVs for reference sera with mean concentrations of 7.22 or 21.12 pg/mL were 10.8 or 8.7% and 11.9 or 8.8%, respectively. The intra-assay CVs were 4.3, 4.8 and 2.6% for reference sera with mean progesterone concentrations of 2.2, 6.1 and 20.1 ng/mL, respectively. Peaks in serum concentrations of FSH, in samples taken daily, were determined by the cycle detection computer program (Clifton and Steiner 1983). The pattern of serum concentrations of FSH, estradiol and progesterone was normalized to the day of the start of treatment.

6.3.2.4. Analysis of follicular Data

Due to the induced super-stimulatory response in the ovaries of the ewes infused with oFSH, it was not feasible to identify and track individual follicles over the treatment period. Therefore, the length of the growth, static and regression phases of follicles emerging in response to treatment were not determined. The mean number of small (1 to 3 mm in diameter), medium (4mm in diameter) and large (≥ 5 mm in diameter) follicles were calculated and are presented on a daily basis for the period of experiment.

6.3.2.5. Statistical Analysis

Serum concentrations of FSH, estradiol and progesterone in samples taken during the experiment were analyzed for effects of time and treatment by two-way repeated measures Analysis of Variance (RM-ANOVA, SigmaStat® Statistical Software for Windows Version 2.3, 1997, SPSS Inc., Chicago, IL, USA). Two-way ANOVA was also used to assess time and treatment effects for the number of follicles in each size category. Multiple comparisons were made by Fisher's least significant difference (LSD). All values are means ± SEM and statistical significance was set as $P < 0.05$.

6.4. Results

6.4.1. Experiment 1

6.4.1.1. Serum concentrations of FSH

Within the group of ewes treated with a low dose of oFSH every 6 h for 42 h starting 24 h after ovulation, a gradual rise in serum FSH concentrations was observed from 0.7 ±

0.1 ng/mL before the first treatment, reaching a high serum concentration of 2.3 ± 0.4 ng/mL at the end of the treatment period ($P < 0.05$; Fig. 6.1.). This resulted in the occurrence of an additional FSH peak 2.1 ± 0.3 days earlier than the second endogenous peak of the inter-ovulatory interval in oFSH treated ewes ($P < 0.05$; Fig. 6.1.). In oFSH-treated ewes, serum concentrations of FSH were significantly higher from 6 h to 48 h after the start of treatment, compared to vehicle treated ewes (Fig. 6.1.).

Peak estradiol concentrations, corresponding to the first or second follicular waves of the inter-ovulatory interval did not differ between oFSH- and vehicle treated ewes, or within each experimental group (3.9 ± 0.7 or 3.4 ± 0.4 and 3.7 ± 0.5 or 4.5 ± 0.5 pg/mL, respectively; $P > 0.05$).

6.4.1.2. *Development and characteristics of follicular waves*

Injection of a low dose of oFSH every 6 h for 42 h during the growth phase of the first wave of the inter-ovulatory interval did not alter the time of emergence of the second follicular wave in comparison to control ewes ($P > 0.05$; Fig. 6.1.; Table 6.1.). The inter-ovulatory interval and the interval between the first 2 waves of the inter-ovulatory interval did not differ between oFSH- and vehicle-treated ewes ($P > 0.05$; Fig. 6.1.; Table 6.1.). The largest follicle in wave 1 grew faster ($P < 0.05$; Table 6.1.) and had a significantly greater diameter on day 3 after emergence (day 2 after start of treatment; Fig. 6.1.) in ewes that received oFSH compared to vehicle-treated ewes. The growth phase and the lifespan of the largest follicle in wave 1 were shorter in oFSH-treated ewes than in control ewes ($P < 0.05$; Table 6.1.). In both oFSH- and vehicle treated

ewes, there were more follicles emerging in the first wave, compared to the second wave of the inter-ovulatory interval ($P < 0.05$; Table 6.1.).

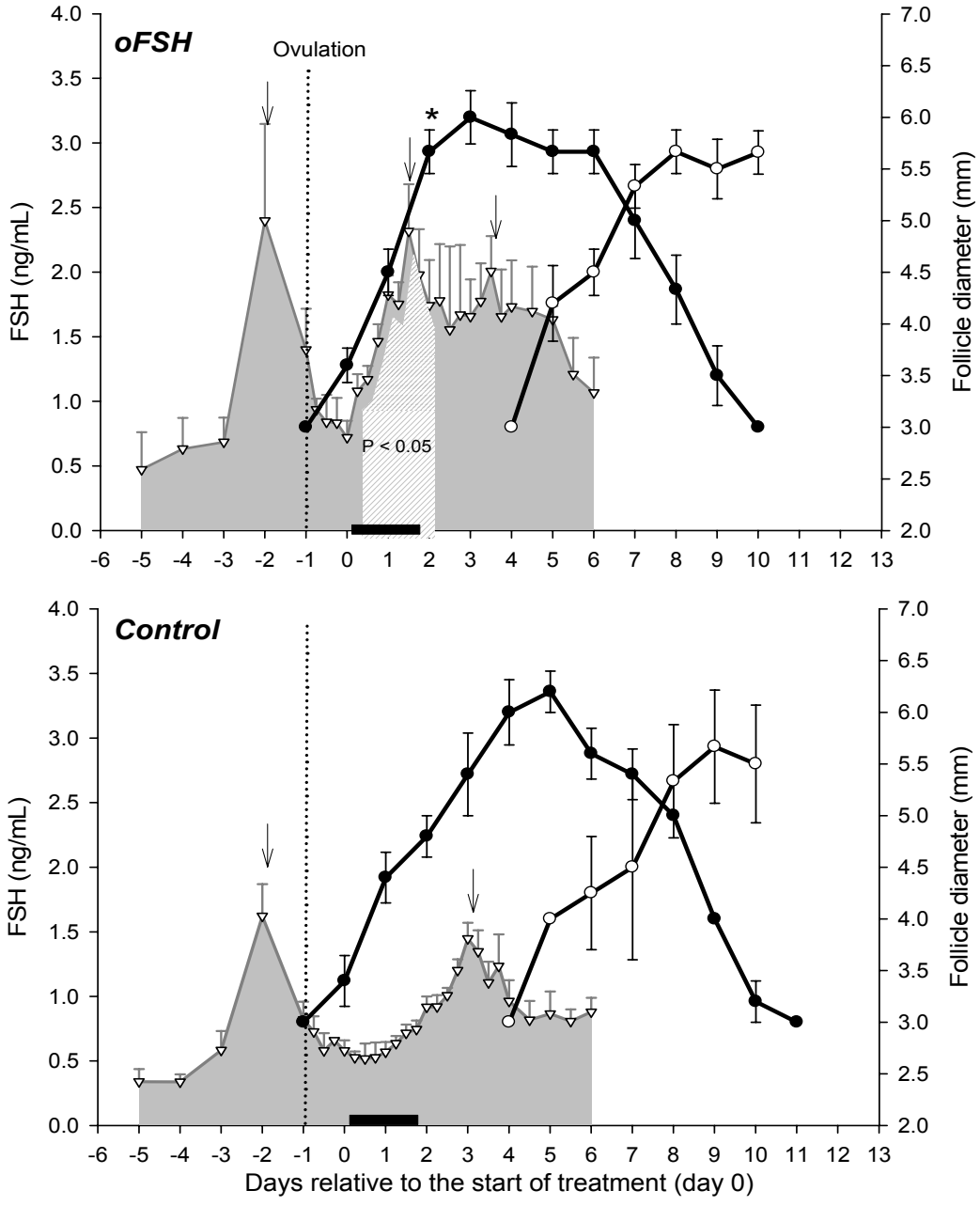

Fig. 6.1. Mean serum concentrations of FSH (outlined with shading) and emerging follicular waves 1 (●) and 2 (○) of the inter-ovulatory interval in cyclic Western White Face ewes treated with oFSH (top panel) and vehicle (bottom panel). Treatment consisted of eight injections of oFSH (0.1 μg/kg) or vehicle 6 h apart, starting 24 h after ovulation. Concentrations of FSH and follicular diameters are expressed as mean ± SEM. Within each peak of FSH secretion, data for all ewes in a group were aligned temporally to the mean day of the zenith of the peak. The time of the zenith of each FSH peak was then normalized to the mean day of the occurrence of the peak relative to the start of the treatment (Day 0). Data for each follicular wave were normalized to the mean day of wave emergence. Asterisk (*) indicates a difference in diameter of follicles between oFSH- and vehicle-treated groups ($P < 0.05$). The area with the diagonal pattern indicates differences in mean serum FSH concentrations between oFSH- and vehicle-treated ewes ($P < 0.05$). Arrows indicate peaks in serum concentrations of FSH detected by the cycle detection computer program. The black bar on the X axis shows the duration of treatment with oFSH or vehicle (42 h).

Table 6.1. Comparison of the characteristics of follicular waves 1 and 2 of the inter-ovulatory interval in cyclic Western White Face ewes treated with oFSH (treatment; n = 6) or vehicle (control; n = 6) every 6 h for 42 h starting 24 h after ovulation.These data represent the characteristics of the largest follicle in a wave.

	Wave 1		Wave 2	
	Treatment	**Control**	**Treatment**	**Control**
Day of wave emergence*	-1.2 ± 0.2[a]	-1.4 ± 0.2[a]	4.0 ± 0.2[b]	3.6 ± 0.2[b]
Day of FSH peak*	-1.5 ± 0.2[a]	-1.5 ± 0.2[a]	3.5 ± 0.3[b]	3.0 ± 0.3[b]
Number of follicles in the wave	2.3 ± 0.3[a]	2.6 ± 0.3[a]	1.7 ± 0.3[b]	1.4 ± 0.3[b]
Length of growth phase (d)	2.8 ± 0.3[a]	4.0 ± 0.3[b]	2.8 ± 0.3[a]	2.0 ± 0.3[a]
Length of static phase (d)	3.2 ± 0.6	2.2 ± 0.6	-	-
Length of regression phase (d)	3.3 ± 0.5	4.4 ± 0.4	-	-
Growth rate (mm/day)	1.2 ± 0.1[a]	0.9 ± 0.1[b]	1.1 ± 0.1[ab]	1.3 ± 0.1[a]
Maximum follicular diameter (mm)	6.2 ± 0.2	6.2 ± 0.3	5.8 ± 0.2	5.6 ± 0.3
Length of follicular lifespan (day)	9.3 ± 0.3[a]	10.6 ± 0.3[b]	-	-
Inter-wave interval (d)	W1 to W2		W2 to W3	
	4.9 ± 0.2	4.8 ± 0.4	4.5 ± 0.3	4.3 ± 0.4

All values are mean ± SEM

[a,b] denotes a significant difference ($P < 0.05$) within and between groups.

* indicates day relative to the start of treatment (Day 0)

W: follicular wave

6.4.2. Experiment 2

6.4.2.1. Serum concentrations of FSH

When oFSH was infused to cyclic ewes for 60 h around the time of the second endogenously driven FSH peak of the inter-ovulatory interval, mean serum FSH concentrations were significantly higher in ewes infused with oFSH (3.9 ± 0.2 ng/mL) compared to control ewes (2.2 ± 0.2 ng/mL). Mean serum concentrations of FSH dropped significantly within 12 h after the end of infusion in oFSH-treated ewes to a nadir of 0.8 ± 0.1 ng/mL; this was lower than the concurrent nadir in serum FSH concentrations in control ewes (1.6 ± 0.2 ng/mL; $P < 0.01$). The duration of the FSH peak (time between the nadirs in oFSH concentrations before and after peaks in FSH serum concentrations) preceding the second follicular wave of the inter-ovulatory interval of control ewes (3.9 ± 0.4 d) did not differ from the duration of the period of elevated serum concentrations of FSH caused by infusion of FSH (4.1 ± 0.3 d; $P < 0.05$; Fig. 6.2.).

6.4.2.2. Emergence of follicular waves and development of antral follicles

The mean day of emergence for follicular waves 1 and 3 of the inter-ovulatory interval did not differ significantly between ewes infused with oFSH or vehicle (days -3.1 ± 0.1 or -2.8 ± 0.3; and days 6.2 ± 0.3 or 6.0 ± 0.1 respectively; Fig. 6.3.)

The mean number of small follicles (≥ 1 mm and ≤ 3 mm in diameter), calculated on a daily basis, decreased significantly on the second day of infusion with oFSH and remained low for 2 days in ewes that received oFSH compared to control ewes ($P <$

0.05; Fig. 6.4.). The mean number of small follicles rebounded in ewes given oFSH and was greater than in control ewes on day 7 after onset of infusion. Compared to control ewes, ewes infused with oFSH had a greater number of medium (4 mm in diameter) and large (≥ 5 mm in diameter) size follicles between days 1 to 7 and 2 to 4 after the start of infusion respectively ($P < 0.05$, Fig. 6.4.). In ewes treated with oFSH, the mean number of large follicles (≥ 5 mm in diameter) reached a maximum on day 2 after the start of treatment (day 0); it then declined rapidly to day 6 ($P < 0.05$; Fig. 6.4.).

6.4.2.3. *Corpus luteum formation and serum concentrations of progesterone and estradiol*

In addition to the corpura lutea (CLs) present in the ovaries at the time of treatment, the presence of newly formed CLs was also noted in the ovaries of ewes treated with oFSH between days 4 and 9 after the start of treatment (Fig. 6.5.). The maximum diameter of the old and newly formed CLs were 14.6 ± 2.4 and 6.4 ± 1.6 mm respectively ($P < 0.05$). Mean serum progesterone concentrations gradually increased from two days prior to the start of treatment (day -2) in both oFSH- and vehicle-treated ewes (Fig. 6.5.). Serum progesterone concentrations were greater in ewes given oFSH compared to control ewes from days 4 to 7 after the start of treatment ($P < 0.05$; Fig. 6.5.). In comparison to control ewes, treatment with oFSH did not result in any significant alteration in the mean serum estradiol concentrations in samples taken daily. Mean serum estradiol concentrations over the period of the experiment were 3.2 ± 0.3 and 3.3 ± 0.3 pg/mL in oFSH and vehicle treated ewes, respectively ($P > 0.05$).

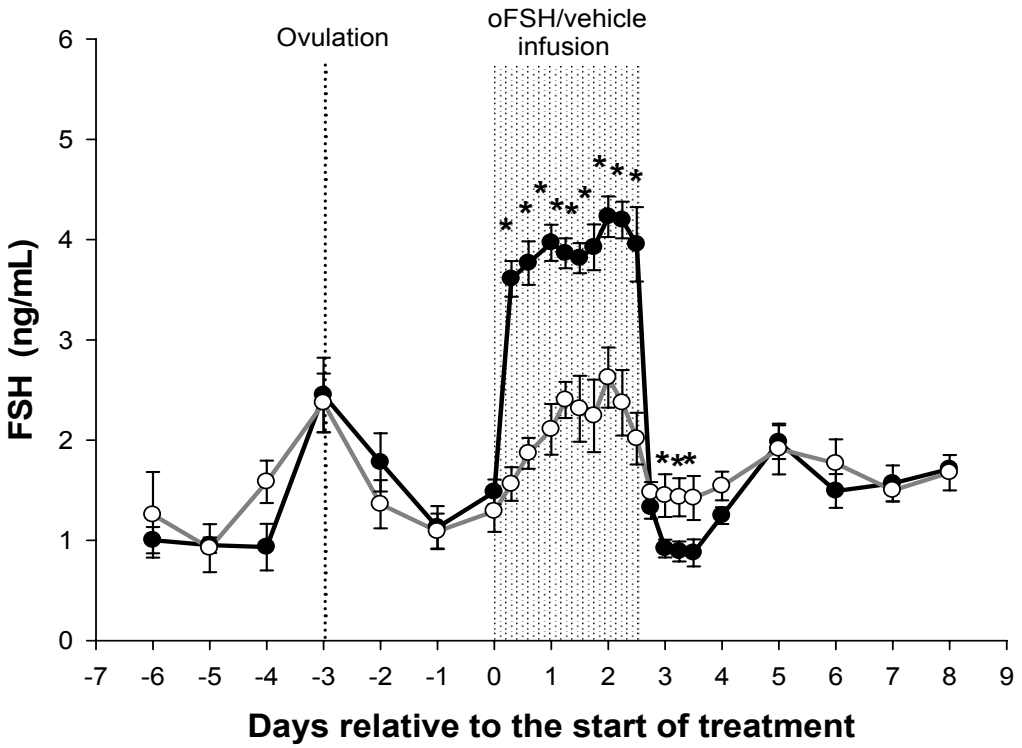

Fig. 6.2. Mean (± SEM) serum FSH concentrations in oFSH-treated (●; n = 6) and control (○; n = 5) cyclic Western White Face ewes from 6 days before to 9 days after the start of treatment (day 0). Treatment consisted of infusion of oFSH (1.98 µg/ewe/h) or vehicle for 60 h, starting 72 h after detection of ovulation by ultrasonographic examination. Asterisk (*) indicates a difference in the mean serum concentrations of FSH between oFSH- and vehicle-treated groups ($P < 0.05$).

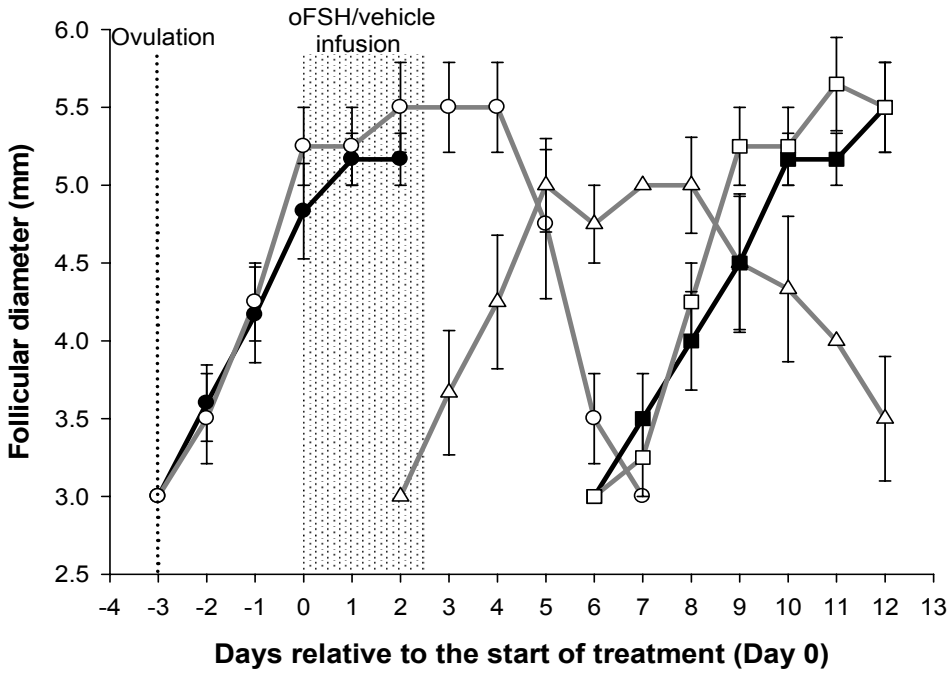

Fig. 6.3. Mean (± SEM) diameter of the largest follicle growing in follicular wave 1 (●/○), wave 2 (Δ) and wave 3 (■/□) of the inter-ovulatory interval in cyclic Western White Face ewes treated with oFSH (closed symbols; n = 6) and vehicle (open symbols; n = 5). Treatment consisted of infusion of oFSH (1.98 μg/ewe/h) or vehicle for 60 h (shaded area), starting 72 h after detection of ovulation by ultrasonographic examination. Within the group of ewes treated with oFSH, there was a super-stimulatory effect on follicle development immediately after onset of treatment; therefore tracking follicles from wave 1 and those growing in response to the treatment was not feasible.

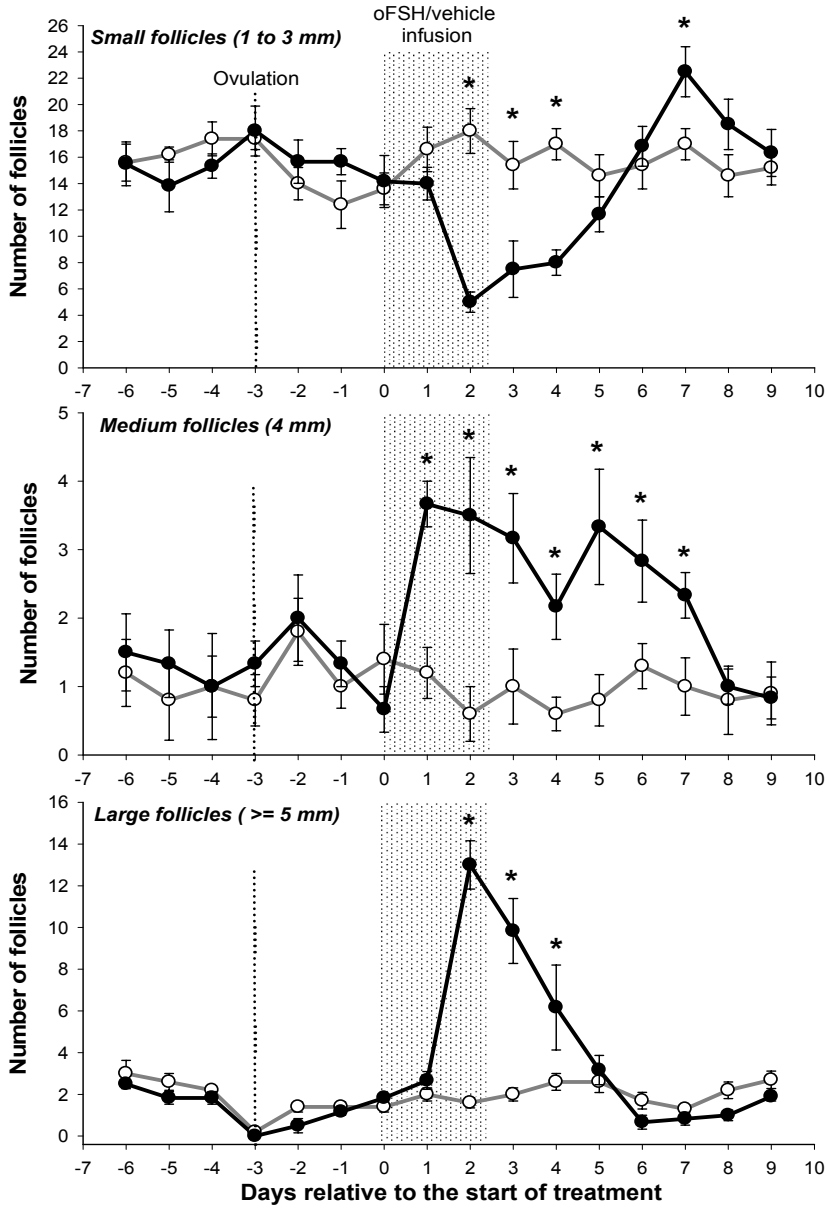
oFSH/vehicle infusion
Small follicles (1 to 3 mm)
Ovulation
Medium follicles (4 mm)
Large follicles (>= 5 mm)
Number of follicles
Days relative to the start of treatment

Fig. 6.4. Mean (± SEM) number of small (1-3 mm in diameter; top panel), medium (4 mm in diameter; middle panel) and large (≥ 5 mm in diameter; bottom panel) size follicles calculated on a daily basis from 6 days before to 9 days after the start of treatment (Day 0) of cyclic Western White Face ewes given oFSH (●; n = 6) or vehicle (○; n = 5). Treatment consisted of infusion of oFSH (1.98 μg/ewe/h) or vehicle for 60 h (shaded area), starting 3 days after detection of ovulation by ultrasonographic examination. Asterisk (*) indicates a difference in number of follicles between oFSH- and vehicle-treated ewes ($P < 0.05$).

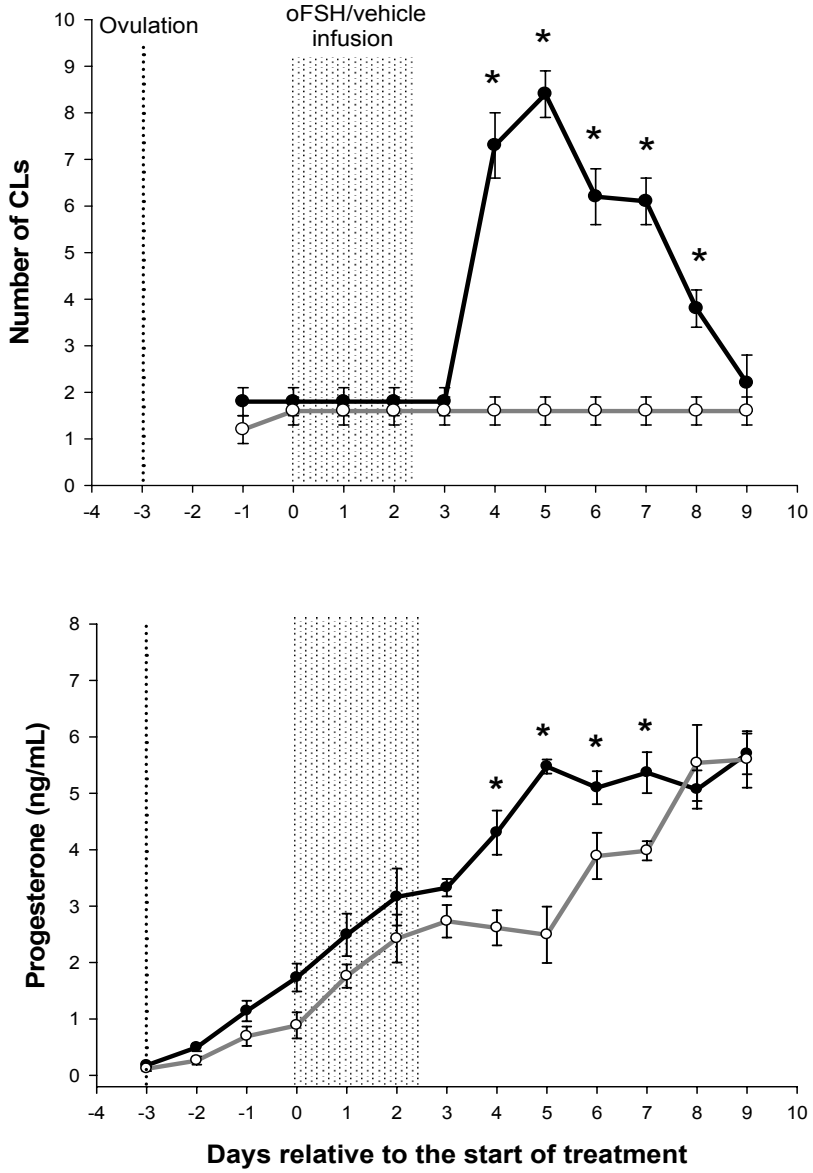
Ovulation
oFSH/vehicle infusion
Number of CLs
Progesterone (ng/mL)
Days relative to the start of treatment

Fig. 6.5. Mean (± SEM) number of corpora lutea (CLs, top panel) and serum concentrations of progesterone (bottom panel) in the ovaries of cyclic Western White Face ewes given oFSH (●; n = 6) or vehicle (○; n = 5). Treatment consisted of infusion of oFSH (1.98 μg/ewe/h) or vehicle for 60 h (shaded area), starting 72 h after detection of ovulation by ultrasonographic examination. The mean number of CLs was calculated on a daily basis from 1 day before to 9 days after the start of treatment (Day 0), Asterisk (*) indicates a difference in the number of CLs, or a difference in mean serum progesterone concentration, between oFSH- and vehicle-treated ewes ($P < 0.05$).

6.5. Discussion

In previous studies from our laboratory The treatment of Western White Face ewes with two injections of oFSH (0.5 μg/kg, sc), 8-h apart, resulted in a peak in circulating FSH concentrations with a similar peak amplitude to endogenously driven peaks (Duggavathi et al. 2004, Duggavathi et al. 2005a). Creation of such FSH peaks in between two consecutive, endogenously driven FSH peaks, induced emergence of additional follicular waves without interrupting the rhythmic occurrence of endogenously driven FSH peaks and follicular wave emergence (Duggavathi et al. 2004, Duggavathi et al. 2005a). Waves were induced in the growth phase of endogenously driven waves and subsequent endogenously driven waves emerged during the growth phase of waves caused by injection of oFSH. These observations bring follicular dominance into question in the ewe. In Experiment 1 of the present study, we gave approximately the same total dose of oFSH used previously, but in 8 injections (0.1 μg/kg in each injection) 6-h apart. We had intended for this treatment to create a gradual leading slope to the second endogenously driven FSH peak of the inter-ovulatory interval. However, treatment increased serum FSH concentrations gradually to a physiological peak 2.1 days before the second endogenously driven peak of the inter-ovulatory interval. The induced peak occurred 3.5 days after the first endogenously driven peak of the inter-ovulatory interval. The inter-peak interval in the ewe is generally within the range of 3 to 5 days (Ginther et al. 1995, Bartlewski et al. 1999a, Duggavathi et al. 2004). In this set of ewes, the second endogenously driven peak of the inter-ovulatory interval occurred at an interval of 5.5 days from the first endogenously driven peak. This second peak was later than we expected; therefore, we did not achieve our experimental goal for

FSH treatment. However, this discrepancy in timing of FSH peaks was fortuitous and led to an interesting observation. The ovary did not respond to the early gradual increase in serum FSH concentrations that we created with a new wave, but rather the second endogenously driven peak appeared to initiate the second wave of the inter-ovulatory interval. It could be argued that the presence of a direct suppressive effect of the growing follicles in the first follicular wave of the inter-ovulatory interval might explain the lack of additional follicular wave emergence in response to the treatment with oFSH. However, the existence of ovarian follicular dominance is much less clear in sheep than cattle; there may be some weak dominance in the follicular phase and early luteal phase (Baird 1983, Lopez-Sebastian et al. 1997, Driancourt 2001, Evans et al. 2002). Recently, it has been suggested that a direct inhibitory effect from a large follicle on the emergence of a new follicular wave probably does not exist in the ewe (Davies 2005, Duggavathi et al. 2005a). Studies demonstrating this were discussed above in the opening of our discussion. However, in the present study, the large growing follicles of wave 1 were stimulated by treatment with oFSH resulting in a greater growth rate compared to control ewes. This rapidly growing follicle, stimulated by greater than normal serum concentrations of FSH early in its growth phase, could have produced inhibitory secretory products that prevented the injected FSH from inducing a fresh follicular wave (induced dominance). The later occurring, second endogenously driven FSH peak of the inter-ovulatory interval, may have caused emergence of a new wave when the first wave was in its static phase. In cattle, follicular dominance is lost during the static phase of a wave (Adams 1999). Interestingly, these observations suggest that enhanced FSH stimulation of a growing follicle in a wave, in the cyclic ewe, can induce dominance and a delay of the emergence of the next expected follicular wave. This

could also explain why we did not achieve our experimental goal as discussed above. Therefore, although dominance is weak or absent in the ewe it would appear that the mechanism to exert dominance is conserved in large antral follicles and can be induced by injection of oFSH. The present data do not reveal how dominance was achieved; it would not appear to have involved estradiol secretion as this did not differ between experimental groups in Experiment 1. In sheep, the larger growing antral follicles in each follicular wave have been shown to be the major source of estradiol production with maximum estradiol production seen at the end of the growth phase of those follicles (Souza et al. 1996, Souza et al. 1997, Bartlewski et al. 1999a).

The eventual emergence of the second follicular wave of the inter-ovulatory interval in ewes given oFSH in Experiment 1 was delayed but occurred following what appeared to be the second endogenously driven FSH peak. This peak occurred at a similar time to the second peak of the inter-ovulatory interval of the control ewes. It is not clear whether the second wave in the ewes given oFSH was simply delayed by treatment or could have been induced by the rather minimal endogenously driven peak in serum FSH concentrations that preceded it. This FSH peak could have occurred as the first follicular wave of the inter-ovulatory interval lost its induced dominance.

The emergence and growth of more follicles to ≥5 mm in diameter in the first wave, compared to the second wave of the inter-ovulatory interval, in the ewes of Experiment 1, has not been previously reported in Western White Face ewes (Bartlewski et al. 1999a). In the Western White Face ewe, there is a transient increase in the number of small antral follicles in the ovary around the time of ovulation (Duggavathi et al. 2003a). In Polypay ewes more follicles have been shown to grow to >4 mm in diameter in the

first follicular wave of the inter-ovulatory interval; however, this effect did not seem to be induced by variation in gonadotropin secretion (Ginther et al. 1995).

In Experiment 2 of the present study, serum FSH concentrations were increased and maintained at about 75% higher than the corresponding peak in serum FSH concentrations in control ewes. However, the increased serum concentrations of FSH during oFSH infusion were within the physiologic range for the peak FSH concentrations in cyclic Western White Face ewes (1.7 to 5.9 ng/mL) (Bartlewski et al. 1999a). Infusion of a physiologic dose of oFSH for 60 h around the expected time of the second FSH peak of the inter-ovulatory interval resulted in a superstimulatory response in the ovaries of all treated ewes. Superstimulatory/superovulatory doses of FSH have been shown to stimulate the growth of multiple ovulatory sized follicles in sheep; however, the effects of these doses are not physiological (Riesenberg et al. 2001a, Riesenberg et al. 2001b, Gonzalez-Bulnes et al. 2002a, Gonzalez-Bulnes et al. 2002b, Gonzalez-Bulnes et al. 2002c). The results of Experiment 2 were unexpected and quite exciting. Ovarian follicular superstimulation/superovulation resulted from a dose of oFSH of 119 μg (equal to 10.7 NIH-FSH-S1 units) infused over 60 h, compared to currently used superstimulatory regimens using high concentrations of FSH given in several injections over a few days (oFSH: 176 (Simonetti et al. 2008) or 180 (Gonzalez-Bulnes et al. 2002a, Gonzalez-Bulnes et al. 2002b, Mitchell et al. 2002, Veiga-Lopez et al. 2005) NIH-FSH-S1 units over 4 days; pFSH: 17 mg over 1.5 days (Riesenberg et al. 2001a) or 18 mg over 3 days (Gonzalez-Bulnes et al. 2000)). This indicates that a very low dose of oFSH given constantly over only two to three days could produce an effective superstimulatory tool.

The observed superstimulatory response in Experiment 2 resulted in a significant drop in the number of small follicles concurrent with an increase in the number of medium and large size follicles, indicating that most members of the ovarian pool of small follicles are capable of growing when they are continuously exposed to a physiological concentration of FSH equivalent to the peaks that precede follicular waves. It is interesting that raising serum FSH concentrations in the ewe for 60 h, to a level equivalent to the zenith of the FSH peaks that precede follicular waves, caused a large number of small follicles to advance in growth and development. This suggests that the major attribute of the FSH peak that precedes emergence of ovarian follicular waves is to reach a critical threshold value. Interestingly, doubling the amplitude of a peak does not increase the number of follicles in a wave or affect any characteristics of the wave (Duggavathi et al. 2005a). Differing degrees of responsiveness of small follicles present in the ovary may determine the number of growing follicles in each follicular wave in response to a physiological peak in serum FSH concentrations when circulating peak concentrations are available for only a few hours (Bartlewski et al. 1998, Bartlewski et al. 1999a). However, discrete peaks in serum FSH concentrations or discrete and limited doses of FSH could be critical in releasing a limited number of responsive small follicles into a new follicular wave, if the whole pool is responsive. The shape and height of an FSH peak is quite variable (Bartlewski et al. 1999a); again, emphasizing the existence of a critical threshold to induce a wave.

Treatment of ewes with physiological concentrations of oFSH in Experiment 2 did not cause an increase in serum concentrations of estradiol, even though a superstimulatory effect was seen on emergence and growth of antral follicles in that experiment.

Stimulatory and inhibitory effects of FSH have been noted on granulosa cell production of estradiol in the ewe (Monniaux 1987, McNatty et al. 2007). Peak estradiol production in a follicular wave is seen when the largest follicle of a wave reaches its maximum size and at that stage estradiol production is probably more LH dependent (Souza et al. 1997, Bartlewski et al. 1999a). Estradiol production from follicular waves is lower in anestrous ewes compared to cyclic ewes probably because the frequency of secretion of LH pulses is much lower in anestrus than cyclic ewes (Bartlewski et al. 2000c). Serum concentrations of estradiol can increase during super stimulatory treatments in the ewe, but these treatments utilize very high doses of FSH and the preparations of FSH often have some contamination with LH (Gonzalez-Bulnes et al. 2002a, Simonetti et al. 2008).

It was also intriguing that in ewes given FSH in Experiment 2, the occurrence of the next endogenously driven FSH peak and emergence of the corresponding follicular wave happened simultaneously with the third FSH peak and follicular wave of the inter-ovulatory interval in control ewes. There was no interruption in the periodicity of peaks in serum FSH concentrations or ovarian follicular waves, when a period of ovarian superstimulation was induced by infusion of oFSH at the expected time of a follicular wave. In cattle, ablation of the dominant follicle of the first follicular wave of the estrous cycle advances the emergence of the second wave of the inter-ovulatory interval (Ginther et al. 2002). However, Evans et al. (2002) found that ablation of the largest antral follicle, in the ewe, did not significantly advance the following peak in FSH concentrations and subsequent follicular wave emergence. This indicates that, unlike cattle, the lifespan of the largest follicle in a wave does not influence the time of occurrence of the next endogenous FSH peak. The occurrence of rhythmic peaks in

serum FSH concentrations has been also reported in ovariectomized ewes, with those peaks having a similar inter-peak interval and amplitude as those seen in ovary-intact ewes (Duggavathi et al. 2005a). In another study, a 10-day application of estradiol-releasing implants in cyclic ewes resulted in truncation of peaks in serum FSH concentrations and interruption of follicular wave emergence; however, those truncated peaks showed a rhythmic occurrence during the treatment period (Barrett et al. 2006). There is perhaps, an endogenous rhythm that drives the peaks in serum FSH concentrations that precede follicular waves in the ewe that is not influenced by ovarian follicular secretory products.

Ovulation of growing follicles in response to oFSH treatment was observed in Experiment 2, in the presence of at least one functional CL. All newly formed CLs were characterized by compromised morphology and function. This suggests a lack of LH support for ovulation and formation of the new CLs. Variation in the responsiveness of large follicles (≥5 mm in diameter) for ovulation and/or formation of proper CLs has been previously reported in the presence of a full-lifespan CL, in the ewe (Bartlewski et al. 1999b, Bartlewski et al. 2000a). Based on the results of other studies it was also suggested that this variation could be due to differing degrees of follicular maturation (Haresign and Lamming 1978, McNatty et al. 1981a, Carriere et al. 1995, Duggavathi et al. 2003b) or differing responsiveness of follicles with the same size and age to gonadotropins (Bartlewski et al. 2001a, Bartlewski et al. 2004, Liu et al. 2007b).

In conclusion, injections of oFSH every 6 h for 42 h in the early to mid growth phase of a follicular wave in the ewe appeared to cause follicles in this wave to block the emergence of a follicular wave. In contrast to cattle, this marked dominance of a

follicular wave is not usually seen in the ewe (Driancourt et al. 1991, Ginther et al. 1995, Evans et al. 2002, Duggavathi et al. 2004, Duggavathi et al. 2005a). In Experiment 2, infusion of oFSH, at physiologic dose, increased and maintained basal serum FSH concentrations at a slightly higher level than peak concentrations in control ewes. This resulted in a superstimulatory/superovulatory response at a dose of about 17-fold lower than commercially employed superstimulatory regimens in the ewe. Based on these results, we suggest that most of the population of small antral follicles in the ovine ovary become responsive to physiologic peak concentrations of FSH and are cable of advancing into a follicular wave.

CHAPTER 7: SUSCEPTIBILITY OF FOLLICLES TO APOPTOSIS IN AN EXTENDED OVARIAN FOLLICULAR LIFESPAN MODEL IN THE EWE

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7.1. Abstract

In prolific breeds of sheep, such as the Finnish Landrace, the extended lifespan of follicles growing in the penultimate wave of the cycle enables them to ovulate with follicles growing in the final wave. The extended lifespan of those follicles was associated with lower serum progesterone concentrations, compared to non-prolific breeds. Treatment of ewes with prostaglandin and intravaginal sponges containing medroxyprogesterone acetate (MAP) creates an endocrine milieu similar to low serum concentrations of progesterone. Such treatment of non-prolific Western White Face ewes increased ovulation rate by extending the lifespan of the follicles in the penultimate wave of the cycle; however, fertility was not enhanced. In the present experiment, the incidence of apoptosis in large antral follicles (≥5 mm in diameter) with an extended lifespan to apoptosis was investigated. Six cyclic, non-prolific, Western White Face ewes were treated with a single dose of PGF2α on day 8 of the estrous cycle, followed immediately by a 6-day intravaginal treatment with MAP-sponges. Ultrasonographic examination of ovaries was done daily. Ovariectomy was performed one day before the expected day of ovulation and large follicles (≥5 mm in diameter) originating from both the final and penultimate waves of the cycle were fixed in paraformaldehyde and processed for TUNEL staining. The lifespan of the penultimate and final waves of the cycle was 7.1 ± 0.2 and 3.0 ± 0.2 d, respectively ($P < 0.05$). The percentage of TUNEL-positive cells in follicles of the penultimate wave of the cycle was greater than in

follicles developed in the final wave (25.8 ± 4.0 and 7.3 ± 3.3 percent, respectively; $P < 0.05$). In conclusion, creation of an endocrine environment similar to low serum progesterone concentrations delayed follicular atresia in large antral follicles in the penultimate wave of an estrous cycle; but this delay was accompanied by a greater degree of apoptosis in the follicular somatic cells compared to follicles in the final wave of the cycle at about one day before expected ovulation.

7.2. Introduction

In the ewe, an estrous cycle is characterized by emergence of 3 or 4 antral follicular waves. In each follicular wave, one to three antral follicles grow from 2 or 3 mm to ≥5 mm in diameter before regression or ovulation (Ginther et al. 1995, Bartlewski et al. 1998, Bartlewski et al. 1999a, Evans et al. 2000, Driancourt 2001, Duggavathi et al. 2005a). In non-prolific breeds such as the Western White Face ewe, follicles that ovulate, such as the Finnish Landrace, originate from the final wave of the cycle. However, in prolific breeds of ewe, it has been suggested that the increased ovulation rate occurs due to ovulation of follicles recruited from both final and penultimate waves of the estrous cycle (Bartlewski et al. 1999a, Bartlewski et al. 1999d). The lifespan of follicles growing in the penultimate wave of the cycle is prolonged assuring their presence at the time of ovulation.

In normal cyclic ewes, emergence and growth of follicles in the penultimate wave of the cycle usually occurs at around 8 to10 days after ovulation (Bartlewski et al. 1999a, Toosi et al. 2009), which is concurrent with the maximum secretion of progesterone from the corpus luteum (Edgar and Ronaldson 1958, Duggavathi et al. 2003b). It has

been suggested that lower serum progesterone concentrations in prolific ewes, compared to non-prolific ewes, is associated with the longer lifespan of follicles in the penultimate wave of the cycle (Bartlewski et al. 2003). Treatment of ewes with intravaginal sponges containing medroxyprogesterone acetate (MAP), after removal of the corpus luteum with prostaglandin treatment, appeared to mimic the condition of low serum progesterone concentrations (Flynn et al. 2000). The ovulation rate in a non-prolific breed of sheep was increased significantly when ewes were given a 6-day treatment with MAP sponges after prostaglandin $F_{2\alpha}$ induced luteolysis at midcycle (Bartlewski et al. 2003). This treatment resulted in the extended lifespan of follicles growing in the penultimate wave of the cycle and ovulation of about 50% of those follicles with follicles emerging in the final wave of the cycle (Bartlewski et al. 2003). However, when a group of ewes were bred after treatment to increase the ovulation rate, fertility was not enhanced (Davies 2005). Clearly, in this treatment regime for enhanced ovulation rate, the follicles ovulating from the penultimate wave of the cycle would be older compared to follicles in the final wave of the cycle. In cattle, oocytes from aged follicles have a lower fertility (Austin et al. 1999) but this does not appear to be the case in sheep (Evans et al. 2001b).

In sheep, apoptosis or programmed cell death has been shown to be a major component of ovarian follicular atresia (Jablonka-Shariff et al. 1996, Jolly et al. 1997a, Jolly et al. 1997b). The evolutionarily conserved biochemical process of apoptosis includes specific DNA fragmentation at internucleosomal sites (Gavrieli et al. 1992, Hussein 2005). DNA fragmentation can be detected in histological sections using terminal dideoxynucleotidyl transferase dUTP nick end labelling (TUNEL) (Gavrieli et al. 1992, Tilly and Perez

1997). In the present experiment, we were interested to investigate the viability of follicles in the penultimate and final waves of the estrous cycle in non-prolific ewes given the MAP and prostaglandin treatment above, by looking at the levels of apoptosis just before the expected time of ovulation. We hypothesized that the aged antral follicles of the penultimate wave of the cycle in ewes given prostaglandin and MAP would show a greater degree of follicular apoptosis compared to follicles in the final wave of the cycle.

7.3. Materials and methods

7.3.1. Animals and experiment procedures

All animal experimentation was performed according to the guidelines of the Canadian Council on Animal Care and was approved by the local animal care committee. Six cyclic, non-prolific, Western White Face ewes (5 to 6 years of age) with an average body weight of 77.5 ± 3.2 kg were used in this study (October-December). Ewes were kept indoors with lighting set to simulate the natural light/dark cycle and they received daily maintenance rations of alfalfa pellets with water, and cobalt iodized salt licks available *ad libitum*. Estrus was synchronized by a 14-day treatment with MAP-releasing intravaginal sponges (Medroxyprogesterone Acetate, 60 mg; Veramix®, Up-John, Orangeville, ON, Canada). Experiments were conducted in the second cycle after synchronization. Estrus was detected with three vasectomized crayon-harnessed rams.

All ewes were treated with an intravaginal MAP sponge (60 mg; Veramix, Upjohn, ON, Canada) for 6 days starting on Day 8 of the estrous cycle (Day 0: Day of ovulation). A single dose of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 15 mg, im; Lutalyse, Upjohn, Orangeville, ON,

Canada) was given to all ewes immediately after sponge insertion. During the treatment period and after sponge removal we were able to clearly detect and monitor the emergence and development of follicles in the penultimate and final waves of the cycle. Two days after sponge removal (Day 16) or one day before the expected day of subsequent ovulation, the ovaries which had large follicles (≥5 mm in diameter) from both the penultimate and final waves of the cycle were removed.

7.3.2. Ultrasonography

Transrectal ovarian ultrasonography was performed with a B-mode, real-time echo camera (Aloka SSD 900; Aloka Co. Ltd., Tokyo, Japan) equipped with a stiffened 7.5 MHz linear array transducer. All ewes underwent daily ultrasonographic examination, starting two days before the expected day of estrus. The day of ovulation was defined as the day on which a large, previously identified ovarian follicle of ≥5 mm in diameter was no longer seen. After detection of ovulation, ultrasonography was continued from 8 days after ovulation or when treatment commenced, to the day of ovariectomy to monitor ovarian antral follicular growth. The size and relative position of all follicles ≥1 to 2 mm in diameter were sketched on ovarian charts.

7.3.3. Tissue collection and preparation

Ovariectomy was performed by mid-ventral laparotomy within 5 min after euthanasia by Euthanyl Forte® (IV; 1 mL/5 kg of body weight; Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). Collected ovaries were cut into separate small pieces which contained either the preovulatory follicle originating from penultimate or final wave of the cycle. The follicles were then fixed in 4% paraformaldehyde for 24 h at room

temperature. The tissues were embedded in paraffin and 5-µm sections were cut at the point of the largest diameter of the follicle and mounted on poly-L-Lysine coated slides. Each of four sequential sections of a follicle was separately mounted on a slide and used for TUNEL, hematoxylin and eosin (H & E) or positive and negative control staining.

7.3.4. TUNEL assay

A terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL; *in situ* cell death detection kit AP; catalogue no.: 1168409910, Roche Diagnostics Co., Indianapolis, IN, USA) was used to identify the extent of cell apoptosis in granulosa and theca cells of the follicles collected. The procedure of labelling DNA fragments used in this study was a modification of the protocol described by (Weber et al. 2002). For permeabilisation, deparafinized and rehydrated tissue sections were irradiated with microwaves at 800 W for 90 sec (Kenmore Microwave Oven 1000 W, Model no: 87103, Sears Canada Inc., Toronto, ON, Canada) with 0.1 M Citrate buffer (Sigma, St. Louis, MO, USA). After 3'-end labelling of DNA fragments and blocking the slides with 3% bovine serum albumin in tris buffered saline (TBS), samples were analyzed in a drop of PBS under a fluorescence microscope (Zeiss® Axioskop 40, Carl Zeiss Canada Ltd., Toronto, Canada) using a standard filter (520 nm, green). Every TUNEL assay included a separate positive control slide that was incubated with 1 µg/mL DNase (DNase I recombinant, catalogue no.: 04716728001, Roche Diognostics Co., Indianapolis, IN, USA) for 10 min at 22 °C. Negative control slides were incubated with 50 µl of label solution without terminal transferase for 60 min at 37 °C.

7.3.5. TUNEL quantitation and data analysis

The sections which were stained with TUNEL or H&E were subjected to blinded evaluation. The number of TUNEL positive and the total number of follicular somatic cells were counted in ten different fields at 2, 4, 6, 8, 10 and 12 o'clock (1000X). TUNEL positive cells were expressed as a percentage of the total number of follicular somatic cells. The percentage of apoptotic cells in follicles originating from the penultimate or final wave of the estrous cycle were compared by the Wilcoxon Signed Rank test (Statistix® Statistical Software for Windows, Version 8, Analytical Software, Tallahassee, FL, USA. The lifespan of follicles from the penultimate and final waves of the cycle were compared by student's t-test (SigmaStat® Statistical Software for Windows, Version 2.03, 1997, SPSS Inc.).

7.4. Results

At the time of ovariectomy, the lifespan of follicles originating from the penultimate and final waves of the cycle were 7.1 ± 0.2 and 3.0 ± 0.2 d, respectively ($P < 0.05$). There was no difference in maximum follicle diameter at the time of follicle collection between the preovulatory follicles from the penultimate (5.4 ± 0.2 mm) and final (5.5 ± 0.2 mm) wave of the estrous cycle ($P < 0.05$). The percentage of apoptotic (TUNEL-positive) cells in follicles of the penultimate wave of the cycle was greater than in follicles in the final wave (25.8 ± 4.0 and 7.3 ± 3.3%, respectively; $P < 0.05$). The percentage of TUNEL-positive cells in follicles growing in the final and penultimate waves of the cycle are shown in Table 7.1.

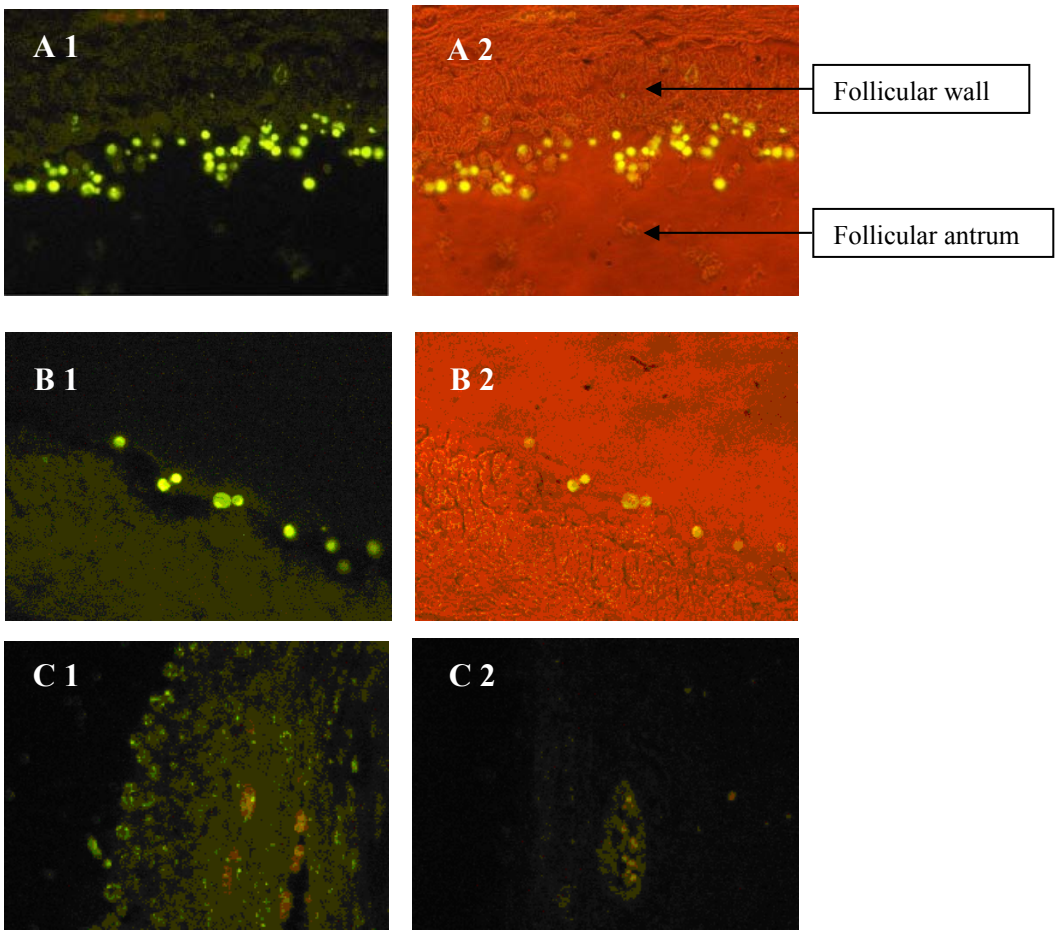

Fig. 7.1. Representative images of apoptosis in preovulatory follicles from penultimate (A 1 & A 2) and final (B 1 & B 2) waves obtained from seven cyclic Western White Face ewes.

A1 and B1 : TUNEL-positive cells in the follicular wall are identifiable with green fluorescence. These cells were mainly located towards the follicular antrum. A2 and B2: A combination of florescent and phase contrast microscopy was used to confirm the location of the apoptotic cells. C1: Positive DNase treated control sample. C2: Negative control sample.

Table 7.1. Percentage of apoptotic (TUNEL-positive) cells in large antral follicles (≥5 mm in diameter) originating from the final and penultimate waves of the estrous cycle and collected one day before expected ovulation. All ewes were treated with a single dose of $PGF_{2\alpha}$ on day 8 of the cycle followed by a 6-d treatment with a MAP sponge.

		Animal #					
		1	***8***	***77***	***79***	***91***	***94***
TUNEL-positive cells (%)	**Final Wave**	**6**	**7**	**4**	**9**	**13**	**5**
	Penultimate Wave	**23**	**20**	**17**	**40**	**36**	**19**

7.5. Discussion

In the present experiment, in ewes given prostaglandin and a short term treatment with MAP, regression of follicles emerging in the penultimate wave of the cycle was delayed and therefore, follicles originating from both penultimate and final waves of the cycle were collected at the same time before ovulation. The extended lifespan of follicles in this model is due to prolongation of the static phase of those follicles (Bartlewski et al. 2003). In this experiment, the proportion of apoptotic cells in the follicular somatic cells was greater in follicles originating from the penultimate wave of the cycle compared with those growing in the final wave. At the time of follicle collection, follicles originating from the penultimate wave of the cycle had a longer lifespan and were mainly in their static phase compared to follicles from the final wave. Follicles from the final wave of the cycle were at the end of the growth or in the early static phase of the follicular wave. Apoptosis is a marker of atresia in ovarian follicles (Hussein 2005, Krysko et al. 2008). Advanced apoptosis was observed in atretic follicles in sheep ovaries (Jablonka-Shariff et al. 1996). Therefore, high rates of apoptosis indicate that follicles from the penultimate wave were becoming atretic. Thus, these data demonstrate that in follicles from the penultimate wave with an extended static phase, there is a decrease in granulosa and theca cell proliferation and enhanced apoptosis leading to follicle atresia and likely poor oocyte quality. Although these follicles can ovulate with follicles from the final wave of the cycle, results of our previous studies with prostaglandin and MAP treatment, led us to conclude that they do not appear to be able to contribute to increasing fertility when ewes are bred. It is interesting that the prolific Finish Landrace ewe achieves an enhanced ovulation rate and fertility by ovulating

follicles from both the final and penultimate follicular waves of a cycle (Bartlewski et al. 1999a).

Although aged follicles from the penultimate wave of the cycle showed a greater degree of atresia/apoptosis, their sizes were not different from those emerging in the final wave. It is interesting that PGF2α and MAP treatment delays follicular regression, but the mechanism involved is not clear. Gonadotropins have been shown to support follicular development and decrease the incidence of atresia (Jablonka-Shariff et al. 1996, Matsuda-Minehata et al. 2006). However, no differences in parameters of secretion of FSH and LH were reported before in Western White Face ewes given the same treatment as in the present study (Bartlewski et al. 2003). Perhaps, the progestogen treatment had some direct effects on the ovary (Bartlewski et al. 2003); however, this needs further elucidation.

Apoptosis is a primary degenerative process that occurs during follicular atresia in different species (Hughes and Gorospe 1991, Tilly et al. 1991, Hurwitz and Adashi 1992, Tilly et al. 1992, Billig et al. 1994, Jolly et al. 1994). In the ewe, it has been shown that apoptosis occurs before other changes in morphological or biochemical indices of follicular status become apparent (Jolly et al. 1997a). Although follicles from the penultimate wave of the cycle showed a greater degree of apoptosis in this study, previous studies using this extended follicular lifespan model, showed an increased ovulation rate in non-prolific ewes (Bartlewski et al. 2003, Davies 2005). These results suggest that although follicles from the penultimate wave of the cycle were not as

healthy as follicles from the final wave at the time of ovulation, they were sill capable of responding to an LH surge with ovulation.

On the other hand, based on results from the present study we suggest that development of atresia in aged follicles affects some aspects of their viability, leading to decreased fertility of the oocyte they contain. It would seem that mechanisms leading to atresia of a follicle in the ewe are triggered during the static phase of a follicle before any noticeable decline in follicular diameter is detectable by ultrasound examination. In sheep, estradiol synthesis by a follicle occurs concurrent with the growth of that follicle (Souza et al. 1997, Bartlewski et al. 1999a, Duggavathi et al. 2006) and it is minimal during the static phase (Souza et al. 1997, Bartlewski et al. 1999a). Initiation of follicular atresia during the static phase of the follicular lifespan is likely involved in declining estradiol secretion by the follicle, since follicular atresia has been shown to be associated with a decreased aromatase activity of granulosa cells (Jolly et al. 1997a).

Most of the apoptotic cells observed in the present study were located on the antral side of the zona granulosa. This is in agreement with other reports indicating that cell layers closer to the follicular antrum are the predominant site of detection of apoptosis in atretic ovine follicles (Jablonka-Shariff et al. 1996, Jolly et al. 1997b). Perhaps, this is due to the extrusion of apoptotic debris toward the follicular antrum, since they accumulate in the follicular antrum (Jolly et al. 1997b). In sheep, apoptotic cells/nuclei were also observed in the antrum, suggesting that apoptotic bodies are released into the follicular fluid in atretic follicles (Jablonka-Shariff et al. 1996).

In the present study, the prostaglandin and MAP treatment created an endocrine milieu similar to low serum concentrations of progesterone (Bartlewski et al. 2003). This milieu delayed follicular regression of the penultimate follicular wave of a cycle as reported by others (Evans et al. 2001b, Bartlewski et al. 2003, Davies 2005). However, the delayed follicular atresia was accompanied by a greater degree of apoptosis in follicular somatic cells. We concluded that an increase in the incidence of apoptosis, as a marker of follicular atresia, occurs in aged antral follicles in sheep prior to any morphological changes detectable by ultrasonography and that likely leads to a decreased follicular viability and lowered fertility of the oocytes the follicles contain.

CHAPTER 8: GENERAL DISCUSSION AND FUTURE DIRECTIONS

8.1. General summary discussion and conclusions

In the present thesis, regulation of ovarian antral follicular development was investigated in sheep. The results of the experiments presented in this study, enhanced our understanding of the mechanisms controlling ovarian antral follicular waves in the ewe, providing the potential to develop methods to improve reproductive management in sheep and other species.

Follicular development occurs in a wave-like pattern in the ewe (Noel et al. 1993, Ravindra et al. 1994, Ginther et al. 1995, Souza et al. 1997, Bartlewski et al. 1999a, Evans et al. 2000). Emergence of each follicular wave is preceded by a transient peak in serum FSH concentrations. These FSH peaks are required for development of antral follicular waves (Ginther et al. 1995, Bartlewski et al. 1998, Bartlewski et al. 1999a, Driancourt 2001, Barrett et al. 2007). *However, it is not clear what facets of an FSH peak cause follicular wave emergence and what aspects of development of a follicular wave are regulated by its preceding FSH peak.* Experiments presented in this thesis were designed to address these subjects.

We applied two different approaches to our investigations. Peaks in serum FSH concentrations appear to vary in their characteristics, such as duration, peak concentration and amplitude. Our first approach was to look for patterns of these variations across the inter-ovulatory interval and see if these were associated with any alteration in the characteristics of follicular waves. Our second approach was to

manipulate different characteristics of FSH peaks within a physiologic range and to investigate the resulting changes in follicular wave dynamics.

8.1.1. First Approach

Some variation in the characteristics of peaks in serum FSH concentrations have been noted across the inter-ovulatory interval in sheep (Ginther et al. 1995, Bartlewski et al. 1999a). However, no consistent pattern has been previously reported. In the present study (Chapter 3), using nineteen Western White Face ewes, we showed that the amplitude of FSH peaks declined across the inter-ovulatory interval while basal serum FSH concentrations increased. Intriguingly, FSH peaks declined in amplitude by up to 50% across the inter-ovulatory interval and yet still induced follicular waves. However, no associated changes in characteristics of follicular waves were observed in that experiment. Based on previous studies we concluded that *there is a threshold for serum concentrations of FSH to induce the emergence of a follicular wave (Duggavathi et al. 2005a). In addition, based on chapter 3 of the present studies, we concluded that variation in the peak amplitude and duration and basal serum concentrations of FSH, across the inter-ovulatory interval, do not appear to have a marked influence on the characteristics of follicles in those waves.*

Ultrasonographic imaging has enhanced our understanding of ovarian function by allowing repeated, non-invasive examination of the ovaries (Schrick et al. 1993, Ravindra et al. 1994, Ginther et al. 1995, Souza et al. 1997, Bartlewski et al. 2000b). Quantitative evaluation of the changes in the echotexture of a tissue is not feasible by the human eye (Baxes 1994, Singh et al. 2003) and requires custom designed computer

algorithms (Pierson and Adams 1995, Singh et al. 2003). Since no consistent pattern for changes in antral follicular dynamics were noted across the inter-ovulatory interval in Chapter 3, we investigated the potential changes in the image attributes of follicles emerging at different stages of the inter-ovulatory interval (Chapter 4). Image attributes of antral follicles reflect the cellular and vascular composition of the follicular wall and consistency of the follicular fluid (Singh et al. 1998, Tom et al. 1998b, Singh et al. 2003, Vassena et al. 2003a). In chapter 4, we showed that the NPV of the wall, in follicles emerging in follicular wave 3 of the inter-ovulatory interval, was greater at emergence (3 mm in diameter) and then dropped as follicles in that wave grew to ≥5 mm in diameter. A tendency for a similar pattern in the changes in NPV of the wall was also observed for ovulatory follicles in the final wave of the inter-ovulatory interval. This observation suggested a higher degree of proliferation and/or vascularization in the wall of those follicles at emergence. We concluded that *there are potential morphological and functional differences amongst antral follicles emerging at different stages of the inter-ovulatory interval in cyclic ewes. Interestingly, those trends were more evident for follicles emerging after mid-cycle (Wave 3 and the last or Ovulatory wave).*

In chapter 3, we noted that changes in the characteristics of peaks in serum FSH concentrations across the inter-ovulatory interval were not associated with differences in the characteristics of the follicular waves that followed each peak. However, in Chapter 4, trends in echotextural variation of the follicular wall among follicles emerging at different stages of the inter-ovulatory interval appeared to be more marked towards the end of the inter-ovulatory interval as FSH peak amplitude declined but basal serum FSH concentrations increased. It has been shown that FSH controls the proliferation of

granulosa cells (Rao et al. 1978, Monniaux 1987, McNatty et al. 1993). Granulosa cells play a central role in supporting the development of follicles that are destined for ovulation and formation of a corpus luteum. Perhaps, the increase in basal serum concentrations of FSH seen in Chapter 3, towards the end of the inter-ovulatory interval, caused a greater proliferation and density of cells in the follicular wall as reflected by the greater NPV in the follicular wall at the onset of those waves emerging late compared to early in the inter-ovulatory interval. Although ovulation occurs mainly from the last wave of the inter-ovulatory interval in non-prolific breeds of ewe, it can also occur from the penultimate wave (Bartlewski et al. 1999a, Evans et al. 2000, Driancourt 2001). Based on the ultrasonographic data discussed above, it is appealing to speculate that follicles emerging after the middle of the inter-ovulatory interval are more adapted for ovulation and transformation into corpora lutea.

8.1.2. Second Approach

We also designed experiments to manipulate characteristics of peaks in serum FSH concentrations and to investigate their potential effects on follicular wave dynamics. We studied the influence of a supraphysiologic amplitude, enhanced frequency of peaks and creating a gradual leading slope to the FSH peaks on emergence and characteristics of the follicular waves that followed. The effects of basal serum FSH concentrations on follicular wave dynamics were also studied.

In sheep, usually 1 to 3 follicles grow in each follicular wave (Ginther et al. 1995, Bartlewski et al. 1999a, Evans et al. 2000). It has been shown that in most species, recruitment of follicles into waves is regulated by FSH (Driancourt 2001). However, the

mechanisms involved in recruitment of follicles into each follicular wave are not clear in the ewe (Baird and Campbell 1998, Driancourt 2001, Fabre et al. 2006). We think that initiation of a follicular wave in the ewe requires that the preceding FSH peak reaches a threshold of serum FSH concentrations; however, this threshold varies among ewes (Picton and McNeilly 1991, Driancourt 2001, Duggavathi et al. 2005a). It is not clear if the number of follicles emerging in each follicular wave is associated with the absolute concentration or amplitude of the peak that precedes it.

In this thesis, we showed that the number of follicles emerging in each follicular wave did not change among different waves of the inter-ovulatory interval (Chapter 3). Although the amplitude of FSH peaks and basal serum concentrations of FSH varied across the inter-ovulatory interval, the peak concentrations of FSH remained unchanged (Chapter 3). Therefore, emergence of a similar number of follicles in different follicular waves across the inter-ovulatory interval might reflect the absolute concentrations of FSH at the zenith of each FSH peak.

To investigate whether variation in peak FSH concentrations over a wider but still physiologic range, could affect the characteristics of follicular waves in the ewe, treatment with ovine FSH was used to increase the amplitude of an FSH peak by 5 to 6 fold (Chapter 3). We concluded that *enhancing FSH peak amplitude/peak concentration to the upper end of the physiological range had only a small effect on the characteristics of the subsequent follicular wave and did not increase the number of follicles recruited or emerging into that wave.*

An interesting and important question is what mechanisms are involved in the regulation of the periodicity of peaks in serum FSH concentrations and the resulting recurrent emergence of antral follicular waves every 3 to 5 days in the ewe. The ability of a growing follicle in a wave to exert direct and indirect dominance on the emergence of new follicular waves has been investigated in the ewe. Induction of dominance by injecting LH, to create pulses in serum concentrations of LH, decreased serum FSH concentrations and suppressed the growth of follicles beyond 4 mm in diameter (Gonzalez-Bulnes et al. 2004). In the same study, the ovary ipsilateral to the largest follicle had the greatest decrease in number of growing small follicles. However, the presence of a large follicle may (Gonzalez-Bulnes et al. 2003) or may not (Driancourt et al. 1991, Gonzalez-Bulnes et al. 2000) decrease the ovarian response to superovulatory treatments in sheep. Furthermore, ablation of the largest follicle of a follicular wave did not affect the time of emergence of the next follicular wave in the ewe (Evans et al. 2002). In more recent studies in sheep, injections of oFSH to create a physiological FSH peak during the growth phase of a follicular wave, induced emergence of a new follicular wave (Duggavathi et al. 2004, Davies 2005, Duggavathi et al. 2005a). Moreover, an endogenously driven peak in serum FSH concentrations and emergence of its corresponding follicular wave occurred during the growth phase of a follicular wave induced by injection of oFSH (Davies 2005). These findings caused us to question the existence of both direct and indirect follicular dominance in the ewe. Another potential mechanism for the regulation of the periodicity of FSH peaks and follicular wave emergence could be the existence of an intrinsic rhythm that drives the occurrence of peaks in serum FSH concentrations independent of any effect of follicular dominance. This notion was supported by the occurrence of repeated peaks in serum FSH

concentrations in ovariectomized ewes (Duggavathi et al. 2005a). Also, there was no interruption in the normal train of FSH peaks after creation of an exogenously driven follicular wave in middle of an inter-wave interval (Duggavathi et al. 2004, Davies 2005). Our results from Chapter 5 of the present thesis also led us to suggest the existence of such an endogenous rhythm that could drive FSH peaks.

In chapter 5 of the present thesis, the ovine ovary responded to discrete peaks in serum FSH concentrations, created on a daily basis, by emergence of new follicular waves. Emergence of recurrent follicular waves on a daily basis indicated that direct dominance is not as evident in sheep as it is in cattle. We concluded that *in the ewe, the periodicity of antral follicular wave emergence can follow the daily occurrence of discrete peaks in serum FSH concentrations; in addition, small FSH-sensitive follicles are available on a daily basis to enter a wave in response to a physiological FSH stimulus.*

It appears that as long as discrete peaks in serum FSH concentrations occur and reach a specific threshold, they can drive the emergence of follicular waves in the ewe, even when the discrete FSH peaks occur on a daily basis (Chapter 5). We were interested to see if a very gradual increase in the leading slope of an FSH peak would be detected by the ovary as a proper signal to stimulate emergence of a new follicular wave. We treated ewes with low physiological doses of FSH every 6 h for 42 h (Chapter 6). This treatment resulted in the gradual increase in serum concentrations of FSH to a peak; however, this peak was not followed immediately by emergence of a new follicular wave and did not delay the occurrence of the next endogenously driven FSH peak. Interestingly, our treatment started early in the growth phase of the existing follicular wave in the ovary and we observed an increased growth rate of follicles growing in that wave (Chapter 6).

We concluded that *stimulation of those growing follicles by injected oFSH could have produced inhibitory secretory products that prevented emergence of a new follicular wave in response to our treatment (induced dominance)*. Some evidences for local suppression of the final development of antral follicles by a large pre-existing follicle in the ovary have been reported in the ewe (Gonzalez-Bulnes et al. 2004). It was suggested that follicle growth inhibitory factors (FGIFs) could be produced by dominant follicles to directly and locally inhibit development of subordinate follicles (Armstrong and Webb 1997). However, several lines of evidence support the argument that the mechanisms used to explain dominance in cattle are not as active in sheep (Driancourt et al. 1991, Ginther et al. 1995, Adams 1999, Bartlewski et al. 1999a, Driancourt 2001, Evans et al. 2001a, Evans et al. 2002, Duggavathi et al. 2004, Davies 2005, Duggavathi et al. 2005a). An earlier study in this thesis (Chapter 5) and other studies from our laboratory (Duggavathi et al. 2004, Davies 2005, Duggavathi et al. 2005a) provided strong evidence for the absence of direct dominance in Western White Face ewes as described above. It is not clear how the exposure of a growing follicle(s) in a follicular wave to injected FSH, as explained in Chapter 6, could be involved in the induction of direct dominance and this needs to be further elucidated. In general, the presence or absence of dominance is controversial in sheep. However, it should be noted that these contradictory evidences may reflect differences in mechanisms controlling follicular wave dynamics in different breeds of sheep, from the strictly mono-ovular to poly-ovular breeds (Baird and Campbell 1998, Bartlewski et al. 1999a, Gonzalez-Bulnes et al. 2001). *It is very interesting to speculate and conclude that potential mechanisms of dominance are conserved in ovine growing antral follicles; however, they do not show dominance at normal serum FSH concentrations and follicle sizes.*

A peak in serum FSH concentrations usually last for 3 to 4 days (duration between nadirs before and after a peak). Therefore, *a peak in serum FSH concentrations could be simply defined as a temporary rise in basal concentrations to the threshold levels required for emergence of a follicular wave*. But, again the question is why only 1 to 3 follicles respond to that increase in serum FSH concentrations and grow further in a follicular wave? Is there any difference in the maturity or differentiation of follicles in the pool of small follicles that respond to FSH compared to other small follicles? Our data from Chapter 5 showed that small follicles that are responsive to FSH peaks are present in the ovary on a daily basis. Furthermore, based on the presence of a relatively constant number of small antral follicles in the ovine ovary across the inter-ovulatory interval we suggest that this population of follicles could be fairly homogenous (Duggavathi et al. 2003a). In this thesis we also showed that peak concentrations of FSH, or FSH peak amplitude, had no effect on the number of follicles emerging in each follicular wave (Chapter 3). We further tested the critical attributes of the FSH peak and the availability of FSH sensitive follicles in the pool of small follicles (Chapter 6). This was done by infusing a physiological dose of ovine FSH to increase basal serum concentrations of FSH to a level equivalent to the zenith of a peak; this concentration was maintained for 60 h (Chapter 6). Amazingly, this treatment resulted in emergence and growth of the largest proportion of the small follicles present in the ovary to ovulatory diameters (super-stimulatory effect). These results led us to conclude that *the bulk of follicles in the pool of small follicles can respond to oFSH at any given time. The transient nature of discrete peaks in serum concentrations of FSH may provide a short threshold stimulation that only initiates onward growth in a limited number of the most*

sensitive follicles in the pool of small follicles in the ewe. However, this would not seem to adequately explain the precise limitation of the number of follicles in a wave in sheep.

Our findings presented in Chapter 5 are also in agreement with the conclusions above. However, in the experiment described in Chapter 5, frequent treatment of ewes with oFSH, to create discrete peaks in serum FSH concentrations on a daily basis, resulted in an elevation in basal serum FSH concentrations during the treatment period. We have to conclude that each treatment with oFSH created a discrete peak in serum FSH concentrations above the elevated basal serum concentrations, and activated the emergence of a new follicular wave. Basal serum FSH concentrations rose above the concentrations reached by many FSH peaks seen in normal untreated ewes (Bartlewski et al. 1999a). However, basal serum FSH concentrations were not maintained for 60 h as in the study described above in Chapter 6. If discrete FSH peaks above increased baseline can elicit follicular waves then the amplitude of a peak above baseline must be critical to initiate such waves, not just the absolute FSH concentration at the zenith of the peak. Again, it is intriguing that the follicular waves induced by FSH peaks on an increasing baseline of serum FSH concentrations contained the expected number of follicles. There was no perturbation of the numbers of small follicles entering each wave.

Finally, we investigated the viability of aged ovulatory follicles originating from the penultimate wave of the estrous cycle in non-prolific ewes, when their lifespan was extended by giving prostaglandin and MAP. We looked at the level of apoptosis just before the expected time of ovulation. This treatment delayed regression of the penultimate follicular wave of a cycle; however, those follicles with an extended

lifespan had a greater degree of apoptosis in follicular somatic cells. *We concluded that an increase in the incidence of apoptosis, as a marker of follicular atresia, occurs in aged antral follicles in sheep prior to any morphological changes detectable by ultrasonography and that likely leads to decreased follicular viability and lowered fertility of the oocytes the follicles contain.*

8. 2. Future directions

1. We showed variation in the image attributes of antral follicles among different follicular waves across the inter-ovulatory interval. Variation in the image attributes of the follicular wall reflect morphological changes and perhaps, changes in physiological function of follicles. This needs to be clarified and confirmed in the ewe by histomorphological investigations.

2. By using ultrasonography at a higher resolution, it would be interesting to study the image attributes of small and medium size follicles around time of follicular wave emergence to find out if follicles destined for emergence into a follicular wave are distinguishable from other follicles in a similar size range.

3. Based on the experimental model developed in our laboratory to create physiological peaks in serum FSH concentrations and to induce new follicular waves, it would be interesting to investigate the molecular characteristics of the oocyte at different stages of follicular development.

4. In this thesis, we showed that giving multiple injections of a low physiologic dose of oFSH, early after recruitment of a follicle in a follicular wave, may induce dominance in

that follicle. It would be interesting to test the degree of induced dominance in growing follicles of a follicular wave in response to different doses of oFSH and to investigate the differences in dominant and non-dominant follicles by molecular and ultrasonographic approaches.

5. According to the results of this thesis, infusion of a low physiological dose of oFSH will result in a superovulatory response in the ewe. It would be interesting to address the potential applications of this finding in practice. Development of FSH implants or the use of osmotic pumps, to release a constant physiological dose of oFSH, in order to superovulate the ewe, would be very appealing. Moreover, oocytes could be recovered after superovulation, using this model, to compare their viability with other superovulatory regimens commercially used in this species.

6. The possible presence of an endogenous rhythm that could drive the peaks in serum FSH concentrations needs to be further investigated in the ewe. An interesting approach to address the existence of an endogenous rhythm for FSH peaks would be to ablate all medium and large size follicles in the ovary, one day after the expected day of an endogenously driven peak in serum FSH concentrations, and look to see if that hastens emergence of the next FSH peak.

7. In each follicular wave, only 1 to 3 follicles grow from the pool of small follicles in the ovary to ovulatory diameters. It is not possible to differentiate between the emerging and resting follicles when they are 1-3 mm in diameter. If we knew which small follicles within the ovarian pool were more prepared for further growth and development, then we could investigate potential mechanisms involved in initiation of further development

of those small antral follicles. Since infusion of FSH stimulated most of the small follicles in the ovary to grow to an ovulatory diameter, we could speculate that those mechanisms were activated in most of the small follicles in the ovary rather than only in 1 to 3 of them. Therefore, it would be interesting to collect ovaries after 24-48 h of oFSH infusion, when most of those stimulated follicles are still in the small and medium size range (≤4 mm in diameter), and investigate the expression of molecular factors mediating follicular development, such as members of the IGF system and BMPs in those follicles. When we infused FSH to ewes (Chapter 6), follicles did not emerge and grow markedly from the pool of small follicles until close to the second day of treatment. Small follicles at a similar stage of development and collected from untreated ewes, could be used as controls.

8. In this thesis, we showed that follicles ovulating from the penultimate wave of the inter-ovulatory interval, after MAP sponge treatment, had a greater degree of apoptosis. FSH has been shown to increase follicular resistance to apoptosis; therefore, it would be intriguing to see if supporting those follicles in the penultimate wave with oFSH would delay aging of those follicles and improve follicle viability.

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