In

the name of

God

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Chapter 1

Introduction

Superovulation can be defined as increased ovulatory response by external hormone therapy. Both eCG (equine chorionic gonadotrophin) and FSH (follicle stimulating hormone) are widely used to induce superovulation in sheep. Premature luteal regression is much more common when superovulation is induced with eCG than when FSH is used as the superovulatory treatment(Espinosa-Ma´rquez et al.,2004, Saharrea et al.,1998,Cervantes et al.,2007). This is one of the main reasons for which the use of PMSG for superovulation in goats has been abandoned in spite of the potential advantage derived from a one-injection regimen, that is simpler than the 6 to 8 injections needed when FSH is used (Armstrong et al., 1983 and Pendleton 1992). Treatment with eCG is economically inexpensive and, because of its long half-life, can be administered in a single injection; however, when it is given at greater doses, eCG has a detrimental effect on hormonal profiles, ovulation and fertilization (Shelton and Moore, 1967; Evans and Robinson, 1980; Bindon and Piper., 1982). When superovulation is induced with eCG, follicular growth often remains stimulated after ovulation and the concentration of estradiol remains elevated throughout the early luteal phase, reaching a peak around four days after the onset of estrus (Armstrong et al., 1983),that induces the synthesis and release of prostaglandin F2a, resulting in early luteal regression (Battye et al., 1988). As a result, progesterone concentrations decline before embryos can be collected (Armstrong et al., 1983), thus compromising both embryo quality and embryo survival. Different treatments have been developed to prevent early luteal regression in animals superovulated with eCG, including the use of prostaglandin F2a inhibitors (Battye et al., 1988) and the administration of human chorionic gonadotropin (hCG) or GnRH to luteinize estradiol-producing follicles (Battye et al., 1988, Saharrea et al., 1998). Some investigators (Rajamahendran and P. C. Sianangama 1992 and Saharrea et al., 1998) have used hCG to luteinize estradiol-producing follicles and prevented premature luteal regression. The administration of hCG can promote ovulation of the first-wave dominant follicle leading to accessory CL formation and increased progesterone concentrations in sheep (Farin et al., 1988; Nephew et al., 1994). Armstrong et al. (1982) administered either hCG or GnRH at the time of the onset of estrus in goats superovulated with eCG, being unable to increase the number of ovulations and to reduce the number of follicles present in the ovaries during the subsequent luteal phase, thus suggesting that the problem was not an inadequate preovulatory LH surge, but rather a lack of response of some follicles to the LH secreted at that time. Thus, the ovulatory failure may be due to asynchronous follicular growth (Pendleton et al., 1992) , which could result in lack of LH receptors in those follicles that are in earlier stages of development at the time of the endogenous preovulatory LH peak (Armstrong et al., 1982; Pendleton et al., 1992). Furthermore, the continued presence of eCG in the circulation, due to its long half-life, could induce the growth of new estradiol-producing follicles after the first wave of superovulation has taken place (Pendleton et al., 1992). Up-to-date, there is little information on a repeated intramuscular injection of hCG on luteal characteristics and serum progesterone concentration in eCG-superovulated sanjabi ewes during the breeding season. The ability of hCG to enhance CL development was assessed by administration of single or multiple injection of hCG at a dose of 500 IU to eCG-superovulated sanjabi ewes after sponge removal, as a fundamental study with the aim of enhance serum progesterone concentration at the time of embryo collection.

Chapter 2

Literature Review

2.1. The ovine estrous cycle

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

There is also an annual cycle of ovarian activity that is superimposed on the normal estrous cycle. In most breeds of sheep (McKinzie and Terrill, 1937; Asdell, 1946; Hafez, 1952), normal estrous cycles occur in the fall andwinter (breeding season), but ovarian cycles cease in the spring and summer (anestrus; Bartlewski *et al* 1998). This ensures that lambs are born in the spring, when environmental conditions are favourable for their survival (Gordon, 1997). There is considerable variation in seasonal reproductive patterns between different breeds (Goodman, 1994). The length of the breeding season seems to depend on the location of the breed of ewe (Hafez, 1952). Breeds with marked anestrous periods (e.g. Scottish Blackface) reproduce under much harsher environmental conditions than those with a limited anestrous season (e.g. Merino) (Marshall, 1937; Hafez, 1952; Robinson, 1959). For breeds of sheep in regions of high latitude (temperate regions) the breeding season begins in late summer and continues until late winter (Legan and Karsch 1979; Karsch *et al* 1979). Whereas breeds located closer to the equator (tropical regions) do not show distinct seasonality and some are even able to continue to reproduce throughout the year (Robinson 1959,). Even though some breeds have marked anestrous periods, antral follicular wave development is still maintained throughout the anestrous period (Smeaton and Robertson 1971; Bartlewski *et al* 1998). The annual variation in day length remains unchanged from year to year (Goodman 1994). This explains why photoperiod is one of many environmental variables capable of influencing seasonal breeding in the ewe (Legan and Karsch, 1980). Sheep are short-day breeders because they become fertile (i.e. estrous cycles commence) as day length decreases in the autumn months (Robinson, 1959).

The estrous cycle can be divided into two distinct phases; the follicular phase and the luteal phase (Senger, 2003). These two phases can then be further sub-divided. The follicular phase of the estrous cycle includes pro-estrus and estrus (Arthur *et al* 1989). Pro-estrus is characterised by declining serum concentrations of progesterone as a consequence of luteal regression (Arthur *et al* 1989; Senger 2003). There is also an increase in serum estradiol concentrations due to the emergence and growth of the ovulatory follicle (Goodman 1994; Senger 2003). The estrus period immediately follows pro-estrus (Goodman 1994). Estradiol is the dominant hormone during this period and is the cause of major behavioural changes and the period of sexual receptivity and mating, in the ewe (Robertson 1977). Estrus lasts between 24 to 48

hours, depending on the breed (Land 1973). Ovulation in sheep occurs 24 to 30 hours after the onset of estrus behaviour (McKinzie and Terrill 1937; Robertson 1977). The luteal phase of the cycle includes metestrus and diestrus. The first period is metestrus, during which ovulation and the formation of a corpus luteum (CL) occur (Keyes *et al* 1983). A structure called the corpus hemorrhagicum forms prior to the CL and is due to the rupture of blood vessels in the follicle wall (Senger 2003). Once the CL is fully functional and secretes high levels of progesterone, this period is referred to as diestrus and is the longest stage of the estrous cycle (Senger 2003). Cyclic activity in the ewe is mainly regulated by the hypothalamic-pituitary-ovarian axis (Goodman 1994).

2.2. Hormonal profiles during the ovine estrous cycle

Within the brain, the hypothalamus and pituitary are involved in the secretion of gonadotropin releasing hormone (GnRH), whereas the pituitary gland has the role of releasing follicle stimulating hormone (FSH), luteinising hormone (LH), prolactin and oxytocin. Ovarian follicles secrete estrogens and inhibins and post-ovulation, the corpus luteum (CL) releases progesterone. Finally, the uterine endometrium releases prostaglandin $F_{2\alpha}$.

2.2.1. Secretion of gonadotropins

There are two functionally distinct modes of LH secretion in the ewe (Dyer 1985; Arthur 1989), and each control different aspects of ovarian function. The preovulatory LH surge (Fig. 2.1) which reaches a peak of 39.28 ± 4.21 ng/ml (Rawlings and Cook 1993) 14 hours before ovulation (Arthur *et al* 1989) induces ovulation and formation of the corpus luteum (Goodman 1994). This gonadotropin surge is primarily induced and sustained by decreased progesterone and increased estradiol secretion during the final stage of the estrous cycle (Moenter *et al* 1990). Tonic or pulsatile LH secretion (Fig. 2.1) occurs throughout the ewes' cycle (Rawlings and Cook 1993) and is important for ovarian steroidogenesis (Goodman 1994). Rhythmic LH pulses are generated in response to GnRH release from the hypothalamus and reach a peak amplitude of 0.33 ng/ml (Bartlewski *et al* 2000). GnRH controls both the synthesis and release of pituitary gonadotropins through binding to specific receptors in the plasma membrane of the gonadotrophs (Stojilkovic *et al* 1994). Intensive blood sampling has revealed low-amplitude pulses of LH occurring 1 to 6 times an hour (Goodman *et al* 1981). Investigations by Baird (1978) demonstrated an increase in tonic LH secretion during the pro-estrus period resulting from an increased LH pulse frequency, from one pulse every 3 to 4 hours during the mid-luteal phase to a maximum of one pulse every 20 to 30 minutes just before the LH surge. During the preovulatory surge release of LH pulse frequency and amplitude of LH increases (Baird 1978; Goodman *et al* 1981) as does basal serum concentrations of LH (Rawlings and Cook 1993).The preovulatory surge release of LH is accompanied by an FSH surge with a peak magnitude of 4.36 ± 0.39 ng/ml (Rawlings and Cook 1993) (Fig. 2.1; Wheaton *et al* 1984; Baird *et al* 1991). A second FSH surge occurs within 20 to 36 hours after the preovulatory gonadotropin surge and has lower amplitude (3.00 ± 0.53 ng/ml; Bartlewski *et al* 1999) but is longer in duration (20 to 24 hours) as compared to the preovulatory surge (11 to 12 hours) (Fig. 2.1; Pant *et al* 1977; Bister and Paquay 1983; Wheaton *et al* 1984; Findlay *et al* 1990). FSH secretion during the ovine estrous cycle is non-pulsatile, when measured from the jugular vein (Bister and Paquay 1983; Wheaton *et al* 1984), however there is a day-to-day variation in serum FSH concentrations (Baird *et al* 1981). The combination of ultrasound examination

and blood sampling has confirmed that peaks in serum FSH concentrations every 5 days are associated with follicular wave emergence (Ginther *et al* 1995; Bartlewski *et al* 1998; Souza *et al* 1997; Evans *et al* 2000).

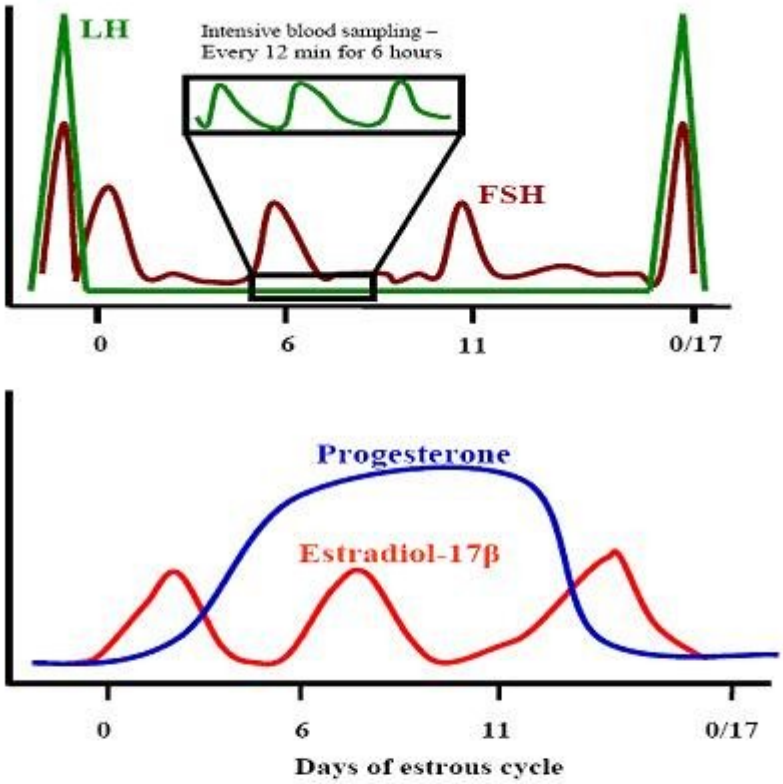

Figure 2 1. Schematic representation of serum profiles of LH and FSH (top panel), and estradiol-17β and progesterone (bottom panel) throughout an estrous cycle in the ewe (x-axis: d0 = day of ovulation, y-axis: relative concentrations of hormones). Except for high concentrations during preovulatory surge, serum LH concentrations remain basal throughout the luteal phase of the cycle. Pulses of LH secretion are detectable in frequently collected blood samples. FSH secretion remains almost non-pulsatile and periodic peaks in FSH secretion occur once every 4-5 days throughout the estrous cycle. Periodic peaks in estradiol secretion also occur, but they tend to coincide with nadirs in serum FSH concentrations. Serum progesterone concentrations increase from day 0 to day 11 and then reach a nadir by day 15 after ovulation. Based on data from Pant *et al* 1977; Rawlings and Cook 1993; Bartlewski *et al* 1999a; Evans 2003b and reproduced by permission of Duggavathi 2004.

2.3. Regulation of LH secretion

2.3.1. Hypothalamic regulation

GnRH regulates the synthesis and release of pituitary gonadotropins through its specific membrane-bound receptors on the gonadotrophs (Stojilkovic *et al* 1994;). LH secretion has been used as an indirect measurement of GnRH release (Goodman 1994); it was assumed that the pulsatile nature of LH release monitored in the peripheral circulation reflected the existence of pulsed GnRH secretion. Techniques developed by Levine *et al* (1982), Clarke and Cummins (1982) and Moenter *et al* (1991) allowed the direct measurement of GnRH concentrations by either perfusion of the median eminence or by sampling portal blood in ewes. These techniques provided

evidence for the episodic release of GnRH and the close temporal relationship between GnRH and LH pulses. Although each pulse of GnRH is followed by an LH pulse, there are some small elevations in GnRH concentrations that fail to induce LH pulses (Clarke and Cummins 1982; Levine *et al* 1982). It has been suggested that these small GnRH pulses maintain LH synthesis, leading to accumulation of releasable LH in the pituitary (Clarke and Cummins 1982). The relationship between GnRH and LH is maintained during both the follicular and luteal phase in the breeding season (Baird 1978; Moenter *et al* 1991) as well as throughout the non-breeding season (Barrell *et al* 1992). However, during anestrous the pulse frequency and amplitude of GnRH/LH pulses are significantly lower compared to the breeding season.

2.3.2. Gonadal regulation

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

During the greater portion of the luteal phase and throughout anestrus, FSH secretion, unlike that of LH, is non-pulsatile (Wallace and McNeilly 1986) suggesting that FSH and LH secretion are differentially regulated by GnRH (Clarke *et al* 1986). Van Cleeff *et al* (1995) reported that during the luteal phase in the ewe, FSH release was episodic, with each FSH pulse produced by a GnRH pulse, but the release pattern of FSH monitored in peripheral circulation was uncoupled from GnRH stimulus. More recent studies (Padmanabhan and McNeilly 2001; Padmanabhan *et al* 2002; Padmanabhan *et al* 2003) provided evidence of a hypothalamic-independent regulation of FSH secretion.

2.4. Regulation of FSH secretion

2.4.1. Hypothalamic regulation

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2.4.2. Gonadal regulation

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

2.5. Secretion and regulation of secretion of estradiol

The main source of estradiol are the largest (≥5 mm in diameter), non-atretic follicles (Evans *et al* 2000). Studies involving the ultrasonographic monitoring of ovarian follicular development and blood sampling have shown 3 to 4peaks (peak amplitude of 4.6 ± 0.6 pg/ml; Bartlewski *et al* 1999) in serum estradiol concentrations per cycle and those peaks coincide with the attainment of the largest diameter of a follicle in each follicular wave (Fig. 2.1.; Souza *et al* 1998; Bister *et al* 1999; Bartlewski *et al* 1999). Increased estradiol secretion during the follicular phase of the estrous cycle is a reflection of increased maturation of the preovulatory follicles and is associated with an increase in LH receptor content in both granulosa and theca cells (Carson *et al* 1979; Armstrong *et al* 1981). The secretion of estradiol results from LH binding to its receptor on the follicular theca cells which stimulates androgen synthesis, and from FSH inducing aromatization of this substrate to estradiol in the granulosa cells (Carson *et al* 1979; Armstrong *et al* 1981). There is an increase in estradiol secretion within 5 minutes of a pulse of LH, and concentrations remain elevated for around 2 hours (Martin 1984). In both cyclic (Baird *et al* 1976) and anestrous ewes (Scaramuzzi and Baird 1977), each pulse of LH is followed by a rise in the secretion of estradiol-17β. Progesterone concentrations in the follicular fluid increase at the time of the preovulatory LH surge, while estradiol concentrations decline to a minimal value, within 16 to 24 hours of the LH surge (Baird 1978; Campbell *et al* 1990). Once serum concentrations of LH exceed 5 ng/ml, the largest ovarian follicles are no longer able to respond to LH by producing estradiol (Baird 1978). In further studies a decline in estradiol concentrations on the day of ovulation was seen coinciding with the secondary peak of FSH secretion (Bister and Paquay 1983; Findlay *et al* 1990; Baird *et al* 1991).

In sheep, estradiol plays an important role in regulating the secretory activity of the hypothalamus (Clarke 1987). Estradiol can exert both a positive and a negative feedback effect on the secretory activity of the hypothalamus and pituitary gland. During the follicular phase of the estrous cycle in ewes, the hypothalamus is the main site for the positive feedback effects of estradiol (Herman and Adams 1990; Moenter *et al* 1990). Estradiol also enhances the response of the anterior pituitary to GnRH (Clarke and Cummins 1984; Phillips *et al* 1990). Physical disconnection of the pituitary from hypothalamic GnRH (Clarke *et al* 1983; Clarke and Cummins 1984; Girmus and Wise 1992) completely blocks pituitary response to estradiol. Therefore, full expression of the estradiol-dependent positive feedback effects on gonadotropin secretion requires continued input from the hypothalamus (Clarke *et al* 1989). However, estradiol enhances the negative feedback effects of progesterone on

pulsatile LH secretion, during the luteal phase of the estrous cycle of the ewe, and acts primarily at the level of the hypothalamus (Goodman *et al* 1981a,b; Martin *et al* 1983). In ovariectomized ewes, Kasa-Vubu *et al* (1992) found that progesterone blocks the estradiol-induced LH surge by preventing the increase in GnRH pulse frequency and amplitude, but Koligian and Stormshak (1977) suggest that perhaps this occurs by decreasing the sensitivity of pituitary gonadotrophs to estradiol. The inhibitory effect of progesterone is all the more pronounced in seasonally anestrous ewes (Karsch *et al* 1987).

2.6. Secretion and regulation of secretion of progesterone

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

The mechanisms involved in the synthesis and secretion of progesterone are complex in nature. Niswender and Nett (1988) reviewed, in detail, the steriodogenic pathways involved in progesterone synthesis and secretion. In brief, cholesterol bound to low density lipoprotein (LDL) produced by the liver is the primary substrate for progesterone synthesis. The steroidogenic luteal cells contain LDL receptors that are involved in thetransport of lipoprotein from outside to inside the cell, where cholesterol is liberated. For the biosynthesis of progesterone, cholesterol is transported to the mitochondria. LH is the single most important endocrine factor involved in the regulation of synthesis and secretion of progesterone in the corpus luteum (Schomberg *et al* 1967). Several authors have shown that, LH administration consistently increases progesterone secretion (McCracken *et al* 1971; Baird and Collett 1973) and maintains luteal function in hypophysectomised ewes (Kattenbach *et al* 1968). Elevations in LH also prolong the luteal life span in normal ewes (Karsch *et al* 1970), whereas injections of LH antisera cause premature luteal regression (Fuller and Hansel 1970). However, other reviewers (Goodman 1994) have suggested that this may well be a pharmacological effect. Endogenous LH pulses have no obvious effect on progesterone secretion in late luteal phase ewes (Baird 1978; Campbell *et al* 1990). These *in vivo* observations are consistent with *in vitro* data demonstrating that most progesterone secretion derives from large luteal cells that are unresponsive to LH (Goodman 1994). However, it has been suggested that these luteal cells normally function at maximal capacity so that they cannot respond further to an LH stimulus (Niswender *et al* 1985; Wiltbank *et al* 1991).

2.7. Follicular growth and development

2.7.1. Folliculogenesis

Populations of primordial (resting pool; primary oocytes surrounded by a squamous layer of pre-granulosa cells; Greenwald and Terranova 1988) and primary (growing pool; single layer of granulosa cells surrounding the oocyte) ovarian follicles constitute thereserve pool of follicles formed just before or soon after birth (40,000 to 3000,000 primordial follicles in ewe lambs; Driancourt *et al* 1991). There is a continual migration of (3 to 4 ovarian follicles per day) primordial follicles from the non-growing pool of follicles into the growing pool of primary follicles (van Wezel and Rodgers 1996; Turnbull *et al* 1977). When follicles leave the resting pool, they become secondary or preantral follicles with two or three layers of granulosa cells (Driancourt *et al* 1991). At this stage the granulosa cells become cuboidal and begin to express markers of cell proliferation (Wandji *et al* 1997; Fortune 2003). The next stage is early antral or tertiary follicular development followed by the formation of a complete antrum (i.e. the Graafian follicle; Driancourt *et al* 1991). The period of follicular growth from the primordial to the preovulatory stage in ewes exceeds 6 months (Cahill and Mauleon 1980). Growth from the primordial to the early preantral stage (0.2 mm in diameter) takes an average of 130 days (Cahill and Mauleon 1980). It takes an additional 24 to 35 days to reach 0.5 mm in diameter, 5 days to reach 2.2 mm in size (Turnbull *et al* 1977) and about 4 days to reach a preovulatory size of 4.5 to 5 mm in diameter (Turnbull *et al* 1977; McNeilly 1984).

2.7.2. The early stage of follicular development

The growth of follicles from the primordial to the preantral stage is termed early follicular development (Cahill and Mauleon 1980). The control of early follicular development is not fully understood, but is thought to be independent of gonadotropic hormones (McNatty *et al* 1981). Tisdall *et al* (1995) provided evidence, in sheep, suggesting FSH receptors are present on granulosa cells as early as the primary follicularstage, although several other authors (reviewed by Fortune 2003) have demonstrated a varying ability of FSH to stimulate preantral follicle development. Wu *et al* (2000), from i*n vitro* experiments in mice, indicated that LH is needed for development of smaller preantral follicles to the antral follicle stage; however, in general, there is a poor understanding of the potential effects of LH on the growth of preantral follicles (Fortune 2003). Nevertheless, follicles become responsive to gonadotropins towards the end of this early stage of folliculogenesis, and this is a prerequisite to subsequent antral follicular growth and maturation (Campbell *et al* 1995).

2.7.3. Antral follicular waves in sheep

There are two stages of ovarian antral follicular development in both sheep and cattle (Mihm and Bleach 2003). The first is a ‘slow growth phase’ which, as discussed earlier, is believed to be independent of gonadotropins (Lussier *et al* 1987). The second is a ‘fast growth phase’ that requires gonadotropin support, and is usually described as a follicular wave (Sunderland *et al* 1994). In sheep, a follicular wave is defined as a follicle or group of follicles that grows from 2 or 3 mm in diameter to an ostensibly ovulatory size of ≥5 mm in diameter, with emergence restricted to a 24 hour period (Duggavathi *et al* 2003) (Figure 2.2). Of the mammalian species studied, ovarian follicular dynamics has been most closely studied in cattle (Adams et al 1995), therefore follicular dynamics in sheep will be compared to that of cattle in the following section. It is the antral follicular wave stage that will be focused on next;

however, in order to aid in the interpretation of this information a few terms must first be defined. In domestic ruminants, the *growth phase* is defined as the time taken by the individual antral follicle to grow from emergence (e.g. 2 or 3 mm in diameter in sheep as recorded by transrectal ultrasonography), to its maximum size. The time taken by this follicle to regress to the minimal recordable size is termed the *regression phase*, and the time period between the end of the growing phase and the onset of regression is defined as the *static phase* (Schrick *et al* 1993; Ravindra *et al* 1994). Follicle *recruitment* refers to the synchronised growth of a group of ovarian antral follicles that eventually gain the ability to fully respond to endocrine (gonadotropic) stimuli. *Selection* is the process by which only limited numbers of these follicles are rescued from atresia and continue to grow to an ovulatory size. *Dominance* is a characteristic of a large selected ovarian antral follicle (dominant follicle) of a wave or cohort of follicles, that permits its survival and further development in an endocrine environment suppressive to other co-existing follicles (subordinate follicles). *Follicle emergence or follicular wave emergence* is the beginning of the growth of a group of follicles from the minimum recordable size, which subsequently ovulate or undergo atresia (Ginther *et al* 1996).

The development and application of transrectal ultrasonography brought about a large increase in the understanding of follicular waves in both cyclic and anestrous ewes (Schrick *et al* 1993; Ravindra *et al* 1994; Ginther *et al* 1995; Souza *et al* 1998; Bartlewski *et al* 1999a; Gibbons *et al* 1999; Vinoles *et al* 1999; Evans *et al* 2000). The wave-like pattern of antral follicular emergence and growth occurs more frequently in sheep (every 4 to 5 days; Ginther *et al* 1995; Bartlewski *et al* 1998; Evans *et al* 2000) than in cattle (every 7 to 10 days; Savio *et al* 1988; Sirois and Fortune 1988), and each wave is preceded by a transient increase in serum FSH concentrations (Adams *et al* 1992; Ginther *et al* 1995; Bartlewski *et al* 1998; Souza *et al* 1998; Bister *et al* 1999; Bartlewski *et al* 1999a; Evans *et al* 2000; Evans *et al* 2001; Duggavathi *et al* 2003a; Duggavathi *et al* 2004). In the ewe, a follicular wave consists of 1 to 4 follicles growing from 2 to 3 mm in diameter to a maximum size of 4 to 12 mm in diameter before regression or ovulation (Noel *et al* 1993; Ravindra *et al* 1994; Ginther *et al* 1995; Bartlewski *et al* 1999a; Evans *et al* 2000; Vinoles *et al* 2001). The number of follicular waves per cycle can vary between breeds of sheep, but range from 2 to 4 waves per cycle (Noel *et al* 1993; Ravindra *et al* 1994; Ginther *et al* 1995). In cattle, there is a significant increase in the number of small antral follicles at the time of follicular wave emergence (6 to 9 follicles in the 4 to 6 mm diameter range; Gong *et al* 1993; Ginther *et al* 1996), and then a gradual reduction in the number of small follicles during the growth of the dominant follicle (Ginther *et al* 1996). In a recent study in sheep, Duggavathi *et al* (2003) surmised that, unlike in cattle, there is no increase in the numbers of small antral follicles (2-3 mm in diameter) at follicular wave emergence. In cattle, one of the follicles of the wave becomes dominant, and the others become atretic (subordinate; Ginther *et al* 1989b); this stage has been given the term deviation (reviewed by Ginther *et al* 1996). In sheep, ovulatory follicles originate from the final follicular wave of the cycle, like in cattle (Ginther *et al* 1995; Bartlewski *et al* 1999a). However, in some prolific breeds of sheep (Finnish Landrace and Rambouille x Booroola ewes) about 50% of all ovulatory sized follicles from the penultimate wave ovulate along with the ovulatory follicles from the final wave of the cycle (Bartlewski *et al* 1999a; Gibbons *et al* 1999). The ovulatoryfollicles (8 to 20 mm in diameter) in cattle originate only from the last follicular wave of the interovulatory interval (Ginther *et al* 1996). Therefore, selection, deviation and dominance are not obvious in sheep.

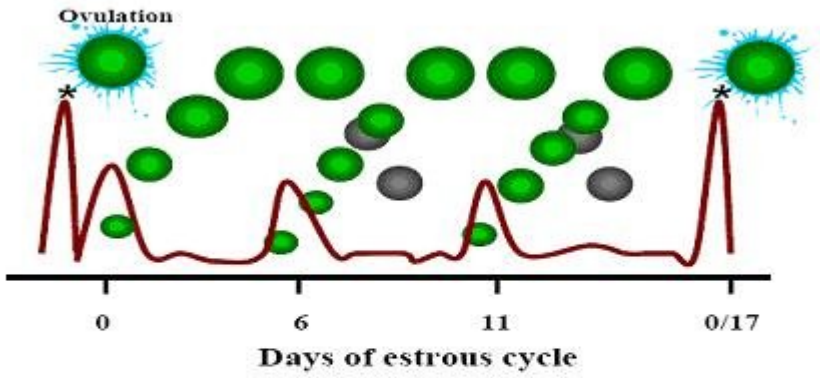

Figure 2. A schematic representation of ovarian antral follicular waves in sheep during an estrous cycle. Three follicular waves (defined as 1 or more 2-3 mm follicles emerging and growing together to reach an ovulatory diameter of ≥5 mm, before regression (dark colored spheres) or ovulation) are shown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

2.8. Regulation of antral follicular growth and development

2.8.1. Gonadotropic hormones

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

2.8.2. Gonadal steroids as regulators of follicular growth

Richards (1994) suggested that estrogens,acting endocrinologically, may enhance the response of ovarian follicles to gonadotropins in hypophysectomised rates.From the results of other studies in rodents (Richards 2001; Britt and Findlay 2003) it was concluded that estradiol is required for early folliculogenesis. It is also thought that the combination of FSH and estradiol enhances the formation of LH receptors in granulosa cells of mature ovarian follicles (Richards *et al* 2002). There is contradictory evidence for the role of the CL in follicle growth. Dailey *et al* (1982)

deduced that the CL acts locally to increase the numbers of all follicles visible on the ovarian surface. However, more recently, Bartlewski *et al* (2001) suggested that the presence of the CL locally inhibits the numbers of antral follicles not growing beyond 3 mm in diameter in ewes. Bartlewski *et al* (2001) also concluded that there was no inhibitory effect of the CL on the numbers of follicles growing beyond 3 mm in diameter.

2.9. Ovulation in the ewe

There are notable variations in ovulation rate among different breeds of sheep (Lahlou Kassi and Mariana 1984; Campbell et al 1995) and among different strains of sheep within breeds (Scaramuzzi and Radford 1983; Driancourt *et al* 1988). The mean ovulation rate of non-prolific breeds of sheep is 1 to 3 follicles whereas the mean ovulation rate of prolific sheep is roughly 3 (Bartlewski *et al* 1999).

Ovulation, in the ewe, is a distinct biological phenomenon that requires the rupture of healthy tissue at the surface of the ovary. This is achieved through finely orchestrated biochemical changes regulated by multiple pathways and modulated by an even larger number of factors and processes (Tsafriri and Chun 1996).

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

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

1988). Once the egg-bearing cumulus mass is expelled from the ovary, ovulation is complete (Espey 1999).

2.10. Corpus luteum formation and development

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

In ewes, the greatest number of active LH receptors is located on the small luteal cells (Harrison *et al* 1987). Luteal cells require LH receptors in order to respond to gonadotropic stimuli. However, the large luteal cells are unresponsive to LH stimulation (Hoyer and Niswender 1986), suggesting that large luteal cells are not dependent on LH for the production of progesterone (Alila and Dowd 1991). The increase in total luteal mass during the early and mid-luteal phase of the cycle is due to both small and large luteal cells. Between days 4 and 12 there is an increase in size of large luteal cells; however, the number of cells remains constant until the onset of luteolysis (Farin *et al* 1989). On the other hand, there is an increase in the number of small luteal cells from days 4 to 8 but no change in the actual size of the small luteal cells (Farin *et al* 1989). The capillary endothelial cells and luteal fibroblasts increase in number between days 4 and 12, and between days 8 and 16 of the cycle (Farin *et al* 1989). Many of the proliferating cells contribute to the extensive capillary network of the corpus luteum (Juengel *et al* 1999). Once established, the capillary network supports blood flow to the corpus luteum at a rate that exceeds that in other tissues (Juengel *et al* 1999).

2.11. Endocrine regulation of luteolysis

The luteolytic factor in ruminants is prostaglandin F2α (PGF2α) and is released from the endometrial glands of the uterus (reviewed by Knickerbocker *et al* 1988). PGF2α travels to the ovary by way of the uterine venous and lymphatic vessels and ovarian artery (Koziorowski *et al* 1989). In the ewe, small luteal cells are insensitive to PGF2α, while large luteal cells contain PGF2α receptors (Fitz *et al* 1982). Functional luteolysis (a decline in the capacity to release progesterone) can be induced by PGF2α through interference with the transfer of cholesterol through membranes of the mitochondria and by way of large luteal cell receptors (Spencer 1998). However, the CL of the ewe is only responsive to PGF2α between days 4 and 14 of the estrous cycle (Day 0 = oestrous; Chamley *et al* 1972).

Ovarian estradiol, progesterone, and oxytocin are regulators of PGF2α secretion, in the ewe. Exposing the uterus to high levels of progesterone for a specific period of time prepares the endometrium for PGF2α synthesis (Silvia *et al* 1991). Zelinski *et al* (1982) reported high concentrations of endometrial receptors for progesterone at estrus but then a gradual decline during the luteal phase of the cycle. The exposure to luteal phase progesterone allows the build up of prostaglandin endoperoxidase and arachidonic acid, which are required for PGF2α production (Knickerbocker *et al* 1988; Silvia *et al* 1991). Towards the end of the luteal phase, the formation of endometrial receptors for oxytocin and estradiol increases and is stimulated by follicular estradiol (Roberts *et al* 1975, 1976; Koligan and Stormshak 1977; Spencer

1988; Juengel and Niswender 1999). Early exposure to progesterone greatly amplifies the effect of estradiol on the recruitment of oxytocin receptors and estradiol amplifies the secretion of (Fogwell *et al* 1985; Homanics and Silvia 1988; Vallet *et al* 1990). It is interesting to note that an increase in pulsatile PGF2α secretion and an elevation in the number of oxytocin receptors are related to the decrease in circulating progesterone concentrations (Sheldrick and Flint 1985). Leyva *et al* (1998) found that the increase in endometrial oxytocin receptors can be detected as early as 6 hours after the withdrawal of progesterone in the ewe.

2.12. Synchronization

manipulation of either the Estrus synchronization (**ES**) in livestock focuses on the the estrous cycle. In does and ewes, the opportunity luteal or the follicular phase of duration and more control is greater during the luteal phase, which is of longer for can be employed to extend the luteal phase by responsive to manipulation. Strategies regressing exogenous progesterone or to shorten this phase by prematurely supplying techniques must not only establish tight existing corpora lutea (**CL**). Successful provide an acceptable level of fertility upon artificial insemination synchrony, but also accomplished through co-treatments using or natural mating. The latter is commonly gonadotropin.

Successful AI and After these conditions are met, ES becomes the basis for embryo transfer programs. In small ruminants, ES is affected by the seasonal breeding patterns in most temperate breeds of goats and sheep. In avovular does and ewes, estrus may not only have to be synchronized, but also initiated. Systems requiring the regression of an active CL will not be effective under these conditions. However, after cyclic activity can be induced in anovular goats and sheep, seasonal breeding can be manipulated and the production cycle can be shortened. A second opportunity in small ruminants is the propensity of many breeds to carry and raise multiple offspring, which can often be controlled by adjustments in dosage levels and nutritional manipulations as part of the ES regimen.

2.12.1.Intravaginal Sponges

Intravaginal sponges have been the traditional treatment of choice for ES in small ruminants, during the breeding and anestrus seasons. They are impregnated with progestagens that are effective at lower dose levels than natural progesterone. Two types of sponges are currently commercially available, based on either flurogestone acetate (**FGA**), marketed as Chronogest (Intervet, Angers, France), or medroxyprogesterone acetate (**MAP**), marketed as Veramix (Pharmacia & Upjohn, Orangeville, Canada).

Intravaginal sponges are usually inserted over periods of 9 to 19 d and used in conjunction with PMSG, particularly for out-of-season breeding, injected at time of sponge removal or 48 h prior to sponge removal. Intravaginal sponges have high retention rates (> 90%), and females usually exhibited estrus within 24 to 48 h after sponge removal.

2.12.2. Efficacy of Intravaginal Sponges

Estrus response and fertility vary greatly when intravaginal sponges are applied, dependent on species, breed, co-treatment, management, and mating system. A comparison of intravaginal sponges containing 15, 30, 45, or 60 mg of MAP in seasonally anovular Corriedale ewes showed no differences between doses in the