Effect of Nutrition on Follicle Development and Ovulation Rate in the Ewe

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Abstract

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The general aim of this thesis was to gain further knowledge on the effect of nutrition on endocrine response and follicular dynamics in the cyclic ewe.

Paper I presents an investigation of the effect of low and high body condition on follicular dynamics and endocrine profiles in ewes. The study showed that ewes in high body condition had a constant pattern of three follicle waves, while ewes in low body condition developed two or three waves during the cycle. Individual follicle tracking by ultrasonography (US) made it possible to study the growing profile of each follicle in relation to its endocrine milieu. Oestradiol and FSH levels were implicated in the regulation of follicle turnover. Ewes in a high body condition had a higher ovulation rate, which was associated with higher FSH and lower oestradiol concentrations during the follicular phase of the cycle.

There was a lack of information regarding the limitations of US to evaluate follicles in different size and ovulation rate, which could replace more invasive techniques. The objective of Paper II was to assess the accuracy of ultrasonography in evaluating the number of follicles and corpora lutea (CL) compared with observations made at post-mortem examination of the ovaries. The predictive value and sensitivity of US was 100% for the presence and 96% for the absence of CL. The sensitivity was 80% for the determination of two CL. The sensitivity was high (90-95%) for follicles ≥ 4 mm. The lower sensitivity (62%) and predictive value (71%) of US for evaluating smaller follicle classes (2 and 3 mm, respectively) may explain inconsistent results found in the literature. Overall, the study demonstrated that US is a good tool for counting the number of CL to evaluate ovulation rate under field conditions.

In Paper III we evaluated if a short-term nutritional supplementation before ovulatory wave emergence could increase the number of follicles recruited into this wave, in ewes in low body condition. The supplement increased the proportion of ewes with a three-wave pattern, but neither FSH concentrations nor follicle recruitment were increased. In the short-term supplemented group the higher feed intake induced a decrease in progesterone concentrations, which prolonged the life span of the largest follicle present at the beginning of the nutritional treatment. A field trail was performed to evaluate the effect of the same nutritional treatment on ovulation rate. Ewes in moderate-high body condition had a higher ovulation rate than ewes in low body condition. In conclusion, short-term supplementation of ewes prolonged the life span of the last non-ovulatory follicle and promoted a 3-wave pattern of follicle development during the inter-ovulatory interval. Supplementation also promoted the development of a more active dominant follicle in ewes with a single ovulation. Short-term supplementation from Day 8 to Day 14 of the oestrous cycle increased ovulation rate in ewes with a moderate to high body condition.

In Paper IV the hypothesis tested was that glucose, insulin, leptin and IGF-I are increased during a short-term supplementation. Acute changes in the peripheral concentrations of glucose and metabolic hormones related to time of feeding were measured. The results showed that glucose, insulin and leptin increased in the supplemented group on the third day after supplementation commenced. IGF-I concentrations were similar among groups. Thus, the effect of a short-term nutritional supplementation on ovulation rate in the ewe may be mediated through an increase in the concentrations of glucose, insulin and leptin.

Key words: sheep, ovary, reproduction, prolificacy, fat stores

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"Todo fue escrito por la misma mano, la mano que despierta el amor, y que hizo un alma gemela para cada persona que trabaja, descansa y busca tesoros bajo el sol. Porque sin esto no tendrían sentido los sueños de los seres humanos".

Paulo Coelho

A Roberto Quadrelli, por haberme dado la energía y el apoyo necesarios para hacer este sueño realidad.

Contents

Appendix

Papers I–IV

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I–IV):

I.   C. Viñoles, M. Forsberg, G. Banchero & E. Rubianes. 2002. Ovarian follicular dynamics and endocrine profiles in Polwarth ewes with high and low body condition. Animal Science 74:539-545.

II.   C. Viñoles, A. Meikle & M. Forsberg. 2003. Accuracy of evaluation of ovarian structures by transrectal ultrasonography in ewes. Animal Reproduction Science, in press.

III.   C. Viñoles, M. Forsberg, G.B. Martin, C. Cajarville & A. Meikle. 2003. Short-term nutritional supplementation of Corriedale ewes: responses in ovulation rate, follicle development and reproductive hormones. Reproduction, submitted.

IV.   C. Viñoles, M. Forsberg, G.B. Martin, J. Repetto & A. Meikle. 2003. Short-term nutritional supplementation of Corriedale ewes: acute changes in glucose and metabolic hormones. Reproduction, submitted.

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General introduction

Reproductive efficiency of sheep flocks is the product of three factors: fertility, prolificacy and the lambs' survival. Prolificacy, which is determined by ovulation rate, is a key factor in reproductive efficiency that can be improved by nutrition (Scaramuzzi, 1988). Since the mechanism by which nutrition promotes an increase in ovulation rate is still obscure, a better understanding of follicular dynamics during the oestrous cycle may help to elucidate the mechanism.

The ovine oestrous cycle

The ewe is a seasonally polyoestrous ruminant with patterns of reproductive function dominated by two distinct cycles. The first is a 16- to 17-day oestrous cycle, which was described by Marshall in 1903; and the second is an annual cycle of ovarian activity determined by the photoperiod (for a review, see Goodman (1994)). Normal ovulatory cycles occur in most sheep breeds in the late summer and autumn (breeding season), but ovulations cease in the winter and spring (non-breeding season). From an adaptive point of view, this restriction of ovarian activity to the late summer and autumn ensures that lambs are born in the late winter and spring, when environmental conditions for their survival are favourable.

The oestrous cycle is a sequence of endocrine events regulated by the hypothalamus (and its secretion of gonadotrophin-releasing hormone (GnRH)), the pituitary gland (with its secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH)), the follicle (which secretes steroids and inhibin), the corpus luteum (CL) (which secretes progesterone and oxytocin) and the uterus (which is responsible for prostaglandin F2α production), with each event in the sequence initiating the subsequent hormonal change. Hypothalamic GnRH stimulates the secretion of LH from the anterior pituitary, which produces ovulation of a large follicle and stimulates luteinization of the follicular remnants. As the CL develops, progesterone concentrations begin to rise and remains elevated during the luteal phase. On days 11–13 of the cycle the minimal luteotropic support allows the increase in prostaglandin to induce luteolysis, and consequently, progesterone concentrations fall (for a review, see Goodman (1994)). Luteolysis marks the beginning of the follicular phase. The decrease in progesterone levels leads to an increase in LH pulse frequency and the stimulation of oestradiol secretion from the ovulatory follicle to elicit both oestrus and the LH surge (Baird & Scaramuzzi, 1976; Karsch *et al.*, 1980). The LH surge, in turn, terminates oestradiol secretion by inducing ovulation and luteinization so that the next cycle can begin. The products secreted by the CL and the ovulatory follicle initiate events leading to the destruction of each structure.

Follicular development and differentiation

Oogonial proliferation is restricted to pre-natal development or shortly after birth, at which time oogonia are transformed into primary oocytes (Hafez, 1993). Nutrition during the foetal life in sheep can influence the number of follicles and subsequent litter size (Robinson *et al.*, 2002). In the post-pubertal ewe the transformation of a given primordial follicle into an ovulatory follicle takes about 6 months (Cahill, 1981). The plane of nutrition of the animal during this 6-month period influences the number of follicles that reach the final stages of growth (Oldham *et al.*, 1990). Once a follicle has entered this phase of growth it has only two alternatives: it will either degenerate through the process called "atresia" or it will ovulate. Since more than 99% of ovarian follicles undergo atresia, it is the norm for a follicle to die rather than to ovulate (Hsueh *et al.,* 1994). The ovulation rate in sheep is therefore determined by the number of follicles that escape atresia.

Scaramuzzi *et al*. (1993) presented a functional model for follicle growth, as given below. The model comprises five functional classes of follicles based on their dependency on and sensitivity to gonadotrophins.

The five functional classes of follicles

(1) Primordial follicles

Primordial follicles constitute the resting stockpile of non-growing follicles, which are progressively depleted during the animal's reproductive life (Greenwald & Shyamal, 1994). Table 1 shows that primordial follicles are small and present in large numbers in the ovaries. These follicles are characterized as having an oocyte devoid of a zona pelucida but surrounded by a flattened layer of pre-granulosa cells. These structures are devoid of a blood capillary network (Scaramuzzi *et al.*, 1993).

(2) Committed follicles

Follicles leave the primordial stage of development in an ordered sequence, and are now irreversibly committed to growth. Before antrum formation follicles grow at a "very slow" rate (Figure 1), taking 130 days to pass through this phase (Cahill & Mauleon, 1980). The primary changes in this stage of development occur in the oocyte, with enlargement and development of the zona pellucida. Once two or three layers of granulosa cells are formed, theca cells differentiate from the surrounding stroma (Scaramuzzi *et al.*, 1993). Insulin-like growth factor II (IGF-II) is probably involved in the differentiation of the stroma cells into the steroid-secreting theca cells (Perks *et al.*, 1995). Receptors for FSH can be identified on granulosa cells of this size class, and theca cells express LH receptors (Table 1). However, the growth of these primary follicles is not dependent on the gonadotrophins, but is influenced instead by autocrine and paracrine factors (McNeilly *et al.*, 1991; Findlay *et al.*, 2000).

(3) Gonadotrophin-responsive follicles

After antrum formation follicles grow at a "slow" rate (Figure 1), with the follicle taking about 30 days to increase from 0.2 mm to 0.7 mm. However, by the end of

Table 1. *Functional classification and characteristics of ovine follicles. LHr= luteinizing hormone receptor; FSHr= follicle-stimulating hormone receptor. The positive (+) and negative (–) symbols represent the expression of receptors and aromatase activity. Adapted from Scaramuzzi et al. (1993).*

Follicle class	Number	Size (mm)	Theca cells LHr	Granulosa cells		
				FSHr	LHr	Aromatase activity
Primordial	40 000–300 000	0.03	–	–	–	–
Committed	4 000	0.03–0.1	+	+	–	–
Gonadotrophin-responsive	25	1–2.5	++	++	–	+
Gonadotrophin-dependent	1–8	2.5	+++	++	+	++
Ovulatory	1–2	2.5–6	+++	++	++	+++

this stage the speed of growth increases, with the follicle taking 5 days to increase from 0.8 mm to 2.5 mm. Figure 1 describes this period as the "fast" phase of growth. The maximum rate of proliferation of granulosa cells is reached when the follicle is 0.85 mm in diameter, whereafter it decreases (Turnbull *et al.*, 1977; Cahill & Mauleon, 1980). The induction of aromatase activity, which is a crucial step in follicle development, occurs in this follicle class (Table 1). However, appreciable amounts of oestradiol are not detected until the follicle reaches approximately 0.5 mm (Scaramuzzi *et al.*, 1993). Aromatase activity per cell increases in parallel with the increasing sensitivity of granulosa cells to FSH (Scaramuzzi *et al.*, 1993). Insulin-like growth factor I (IGF-I) stimulates proliferation of granulosa and synergizes with FSH in granulosa cell differentiation (Monget & Monniaux, 1995; for a review, see Poretsky *et al.* (1999)). Most gonadotrophin-responsive follicles contain androgen in follicular fluid and the more advanced ones have some aromatase activity and consequently have oestradiol in their follicular fluid. There are about 25 gonadotrophin-responsive follicles that make up the pool from which the ovulatory follicles develop (McNatty *et al.*, 1982; Scaramuzzi *et al.*, 1993). There is a clear link between the size at which follicles become gonadotrophin-dependent and the size at which follicles are recruited to ovulate (Driancourt, 2001). The recruitment window in sheep lasts 36–48 hours (Souza *et al.*, 1998; Bartlewski *et al.*, 1999).

(4) Gonadotrophin-dependent follicles

For a follicle to progress from gonadotrophin responsiveness to gonadotrophin dependency, there is an absolute requirement for FSH. With adequate FSH support, there is a further increase in aromatase activity and follicles secrete oestradiol in increasing amounts. Luteinizing hormone receptors (LHrs) appear on the granulosa cells of this follicle class (Table 1). In the rat LHrs are induced by the combined action of oestradiol and FSH (Richards, 1980). Without adequate FSH support aromatase activity is not maintained, oestradiol secretion falls and androgen accumulates within the follicle, leading to atresia (Scaramuzzi & Campbell, 1990).

Follicles in this gonadotrophin-dependent stage have a higher requirement for FSH than do gonadotrophin-responsive and ovulatory follicles, a distinguishing
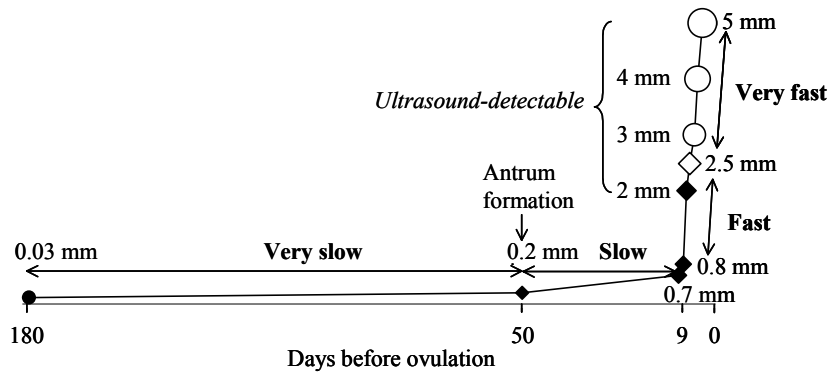
Figure 1. The phase of growth from primordial (●) to antrum formation represents the very slow phase of growth. The slow phase of growth covers antrum formation until the gonadotrophin-responsive follicle (◆) reaches 0.7 mm. From when the follicle is 0.8 mm in size to the gonadotrophin-dependent stage (◇) is the fast phase of growth, after which follows the very fast phase of growth culminating in the ovulatory follicle (○). Modified from Dowing & Scaramuzzi (1991).

characteristic that makes them the most vulnerable to atresia (Scaramuzzi *et al.*, 1993).

(5) Ovulatory follicles

In 1980 Cahill & Mauleon described some of the problems of evaluating the mean growth rate of large follicles by histological means. The technological breakthrough that led to the study on a daily basis of follicle development in ruminants came a little later with transrectal ultrasonic imaging (Pierson & Ginther, 1984). Using this methodology, which permits to visualize ovarian follicles ≥2 mm in size, it was possible to elucidate that the growth rate of a follicle from recruitment until it reaches pre-ovulatory size is approximately 1 mm/day (Bartlewski *et al.*, 1999; Evans *et al.*, 2000). Figure 1 shows this period as a "very fast" growth phase.

In single-ovulating ewes the ovulatory follicle usually reaches a diameter of ≥5 mm. Ewes homozygous for the Booroola gene can have more than 5 ovulations per cycle, with follicles reaching a pre-ovulatory size of 2–4 mm (Scaramuzzi & Radford, 1983; Souza *et al.*, 2001; Montgomery *et al.*, 2001). The transformation of a gonadotrophin-dependent follicle to one capable of ovulating requires a low, but critical concentration of FSH (Campbell *et al.*, 1999). Ovulatory follicles have granulosa cells with a larger number of LHrs and FSH receptors (FSHrs) (Table 1). The increase in size of the ovulatory follicle is due to an increase in granulosa cell number and to the accumulation of follicular fluid in the antrum (Turnbull *et al.*, 1977). Aromatase activity is maximal (Table 1) and for this reason, the ovulatory follicle has the highest intra-follicular levels of oestradiol (for a review, see Hsueh *et al.* (1984)). The ovulatory follicle is responsible for over 90% of the

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circulating concentrations of this hormone (Baird & Scaramuzzi, 1976; Baird *et al.*, 1991).

The crucial factor in the continued development of the ovulatory follicle is its ability to synthesize oestradiol (Baird, 1983). Fortune & Quirk (1988) suggested a model for steroidogenesis in the bovine, which involves the coordinated actions of FSH and LH. The progestins pregnenolone and progesterone are precursors of synthesis of androstenedione in theca cells. Androgens then cross the basal membrane of the follicle. In the granulosa cells androgens are metabolized to oestradiol 17-β. Stimulated by LH, the granulosa cells secrete pregnenolone which can be converted to androgens by the theca cells (Fortune & Quirk, 1988).

The number of follicles that passes from one stage of development to the next decreases with each step, and most of the follicles are lost in the process of atresia (Table 1). In this way the number of follicles destined to ovulate and the ovulatory quota are strictly regulated. During the whole developmental process follicles sequentially become more sensitive to gonadotrophins (Webb *et al.*, 1999). The stages of follicular development that can be modulated by gonadotrophins occur in an organized and cyclic manner, referred to as the "waves of follicular development".

From gonadotrophin-responsive to ovulatory follicle

Wave-like patterns of follicular development in sheep

The role of FSH in timing the events of the oestrous cycle was revealed after the occurrence of waves of follicular development was confirmed. Bister & Paquay (1983), who described a regular 5-day endogenous FSH rhythm, observed three surges within a 17-day cycle in the ewe. The authors proposed that these three 5–6-day waves of FSH secretion were probably related to the waves of follicle growth reported previously (Smeaton & Robertson, 1971; Brand & de Jong, 1973). By means of endoscopy, it was revealed that the growth of follicles ≥5 mm in diameter occurs in an orderly fashion, at approximately 5-day intervals throughout the oestrous cycle (Nöel *et al.*, 1993). The wave-like pattern of follicular growth and a temporal association between a surge in FSH and the emergence of each wave for sheep was confirmed by ultrasonography (Ginther *et al.*, 1995; Bartlewski *et al.*, 1998; Leyva *et al.*, 1998; Souza *et al.*, 1998).

Figure 2 shows that a follicular wave, as characterized by ultrasonography, involves the initial "recruitment" of a group of gonadotrophin-responsive follicles from which one, in monovular breeds, is "selected" to continue its growth and becomes the "dominant" follicle. While growing, the dominant follicle promotes atresia in the other follicles from the same cohort (Driancourt *et al.*, 1985). Depending on whether the CL regresses or not, the dominant follicle either becomes atretic (the anovulatory dominant follicle) or ovulates (Sirois & Fortune, 1988).

In sheep a limiting aspect of studying follicular dynamics by ultrasonography is the size of the ovaries and their structures. For this reason, the wave pattern has
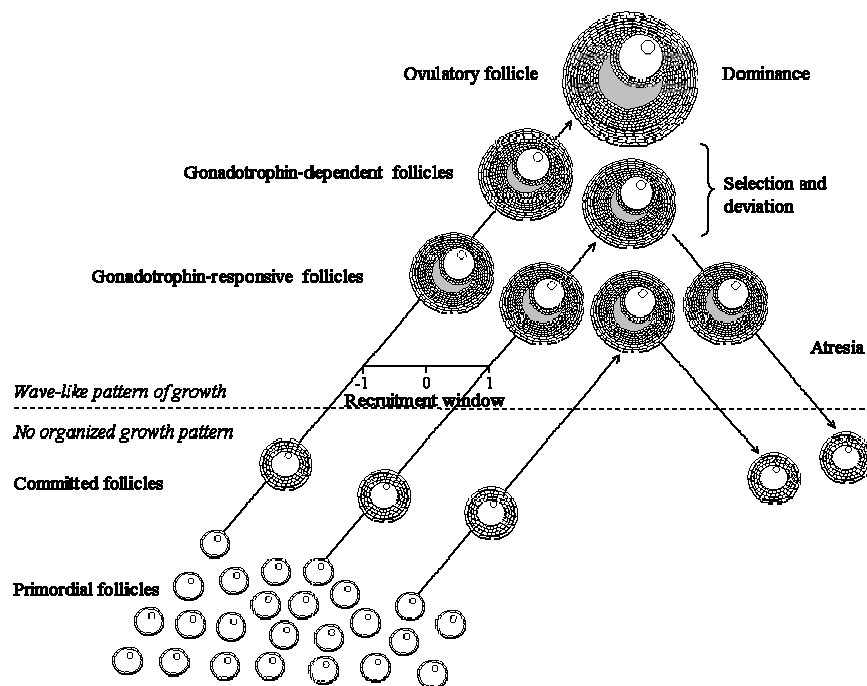
Figure 2. An illustration of follicular development in the ewe, showing a group of primordial follicles from which the committed follicles develop. The gonadotrophin-responsive follicles will be recruited into a wave of development. The gonadotrophin-dependent follicles are the most sensitive to atresia and represent the step during which the selection process regulates the ovulatory quota. The recruitment window marks the time period when a surge of FSH initiates the wave. Modified from Scaramuzzi *et al.* (1993).

been detected only in follicles destined to grow up to 5 mm or more. Consequently, very few follicles (one to three per wave) are detectable for characterizing the wave pattern, complete with follicular selection and dominance (Adams, 1999). Another obstacle to studying the mechanisms of follicular dominance in ewes is that the difference in size between the largest and second largest follicle within a wave is small (1–2 mm). Many studies on follicular dynamics have not examine changes in the number of follicles over the cycle, as per the original definition of waves in cattle (Rajakoski, 1960). Some studies have revealed an increase in the number of small follicles at the time of wave emergence (Schrick *et al.*, 1993; Viñoles *et al.*, 1999b; Evans *et al.*, 2000), while others were unable to describe such a phenomenon (Ginther *et al.*, 1995; Bartlewski *et al.*, 1999). One explanation for the diverging results may be that the accuracy of ultrasonography varies between follicles in different size classes, and that the power of this methodology therefore needs to be tested.

The mechanism of selection and dominance in sheep

Each follicular wave is preceded by a rise in FSH concentration (Souza *et al.*, 1998). This is followed by the stimulation of a group of gonadotrophin-responsive follicles to be transformed into gonadotrophin-dependent follicles (Figure 2)

(Souza *et al.*, 1997a; Viñoles *et al.*, 1999b). At this stage all the growing follicles secrete inhibin, which promotes a reduction in FSH concentrations (Souza *et al.*, 1998). The fall in the concentration of FSH plays a key role in limiting the number of follicles that eventually ovulate (Baird, 1983). It is the ability of a follicle to respond to the switch in gonadotrophic support that is the central mechanism of follicle selection (Campbell *et al.*, 1999). This switch to LH dependence provides the selected follicle with the capacity to survive and continue growing under low FSH concentrations (McNeilly *et al.*, 1991). While the selected follicle increases in size, there is a parallel increase in its production of oestradiol, androstenedione and inhibin until it reaches maximum size (Souza *et al.*, 1997a; 1998). Inhibin sets the overall level of negative feedback while oestradiol is responsible for day-to-day fluctuations in the concentration of FSH which determines the emergence of follicular waves (Baird *et al.*, 1991). Three days after emergence the follicle reaches 5 mm in diameter and stops producing oestradiol, which signifies the end of its functional dominance. Nevertheless, the period of morphological dominance lasts longer, since the follicle can still be identified by ultrasonography for a few days after it has stopped producing oestradiol (Souza *et al.*, 1997a; Viñoles *et al.*, 1999b).

The suppressive effects of high progesterone levels and the stimulatory effects of low progesterone levels on the growth of the largest follicle have been documented in sheep (Johnson *et al.*, 1996; Rubianes *et al.*, 1996; Viñoles *et al.*, 1999b; Flynn *et al.*, 2000; Viñoles *et al.*, 2001). This evidence provides a rationale for the hypothesis that the smaller size attained by follicles in mid-cycle waves is the result of progesterone-induced suppression of LH.

The existence of follicular dominance in the ewe has generated some debate in the past (Driancourt *et al.*, 1985; Schrick *et al.*, 1993; Lopez-Sebastian *et al.*, 1997). One reason for this debate is that the magnitude of dominance is defined by the size difference between the dominant and the largest subordinate follicle, which difference is small in sheep (Driancourt, 2001). However, enough evidence supports the hypothesis of the dominance phenomenon, particularly during the first and last waves of the cycle. Within each wave in mono-ovular sheep one follicle grows larger than the next largest follicle and one follicle per wave contains more oestradiol and has a higher oestradiol:progesterone ratio than other follicles in the same wave do (Evans *et al.*, 2000). Depending upon the number of waves developing during the cycle, 1 or 2 days after wave emergence, when the largest follicle reaches 4 mm diameter, it continues to grow at a higher rate and becomes the dominant follicle, while the next largest follicle regresses, a process that is called "deviation" (Ginther *et al.*, 1996; Evans *et al.*, 2000).

The demise of functional dominance indicates the beginning of follicle death. Apoptotic cell death is the mechanism behind follicle atresia. Gonadotrophins and oestrogens have a role as follicle survival factors, while androgens have a role as atretogenic factors in rodents. Atresia is correlated with a decline in oestrogen synthesis concomitant with increased progesterone production (for a review, see Hsueh *et al.* (1984)).

Upon cessation of oestradiol secretion by the dominant follicle, FSH is again allowed to surge, and stimulates the emergence of a new wave. Studies on sheep

disagreed on the number of follicular waves that develop during the oestrous cycle. Investigations report between two and four waves per oestrous cycle (Ginther *et al.*, 1995; Leyva *et al.*, 1998; Souza *et al.*, 1998; Bartlewski *et al.*, 1999; Evans *et al.*, 2000). Owing to the variable number of waves that develop during the oestrous cycle, the ovulatory wave emergence occurs between day 9 and day 14 (for a review, see Viñoles (2000)).

Although it is not clear why there is variation in the number of waves that develop during the cycle, body condition of ewes may have an effect on hormonal profiles and therefore the speed of follicle turnover (Viñoles *et al.*, 1999a). Deviation occurs more quickly in ewes with three waves than in ewes with two waves, suggesting that the duration of dominance is shorter in ewes with three-wave than in ewes with two-wave cycles. Early atresia of the dominant follicle, which allows early increase in FSH, will promote earlier emergence of the next wave (Evans *et al.*, 2000).

In cattle the number of follicular waves appears to be related to ovulation rate. The occurrence of twin ovulation is associated with a three-wave rather than a two-wave pattern of follicle development (Bleach *et al.*, 1998). In sheep a three-wave pattern of follicle development is associated with an increased number and speed of turnover of follicles (Evans, 2003). If there is a positive association between number of follicular waves and ovulation rate, then it may be that more follicles are available for being recruited into the ovulatory wave. However, information is lacking on how different nutritional treatments, applied to increase ovulation rate, affect follicle recruitment and selection.

Co-dominance: the development of more than one dominant follicle

Two mechanisms have been proposed by Scaramuzzi *et al.* (1993) to account for multiple-ovulation in ewes: an increase in the number of gonadotrophin-responsive follicles available for further development, and a wider window of opportunity for FSH to act on these follicles. Ewes carrying the Booroola gene have been reported to select a larger number of follicles into the first follicular wave of the cycle than ewes not carrying this gene (Souza *et al.*, 1997b). Normally, the last follicular wave of the oestrous cycle is the one containing the ovulatory follicles. However, in one study in which twin ovulation occurred, the penultimate wave contained ovulatory follicles in 10% of the Western white-faced ewes studied and in 57% of Finn ewes (Bartlewski *et al.*, 1999). The increased ovulation rate appeared to have been due primarily to an extended period of recruitment of follicles to ovulate (Bartlewski *et al.*, 1999).

Nutritional manipulation

Feeding energy/protein supplements to increase ovulation rate

Ovulation rate is influenced by several factors, one of the most important of which is nutrition. That nutrition influences lambing was reported by Marshall (1905) who wrote "that differences in food and environment exercise an influence over fertility in the sheep as in other animals has long ago been recognised". He

classified the information obtained from various flock-masters in eastern Scotland in six groups, depending on the amount of extra food that the flocks received. Marshall's remarkable conclusions were that "…the percentage of lambs was, as a rule, larger among flocks which underwent a process of artificial stimulation during the sexual season" and that "the percentage of barren ewes was generally relatively less in such flocks".

Over the years several definitions of the effect of nutrition have been formulated. These include the so-called "static effect", the "dynamic effect" and the "immediate effect". The term "static effect" refers to the higher ovulation rate observed in heavy compared with light ewes, while the "dynamic effect" refers to increases in ovulation rate due to increases in live weight and body condition during short periods (e.g. 3 weeks) before mating (Smith & Stewart, 1990). Furthermore, it has been shown that 4-6 days' supplementation with lupin grain (a high-energy and high-protein supplement) increases ovulation rate with no changes in live weight or body condition (Knight *et al.*, 1975; Lindsay, 1976; Oldham & Lindsay, 1984; Stewart & Oldham, 1986; Downing *et al.*, 1995b). This effect has been called the "immediate nutrient effect".

The different effects of nutrition on ovulation rate forms part of one nutritional continuum, measured at different times relative to the commencement of feeding (Smith & Stewart, 1990). The effect of nutrition on the reproductive processes may depend upon the "net nutritional status", a term which encompasses endogenous and exogenous sources of nutrients available to the ewe (Downing & Scaramuzzi, 1991).

Effect of dietary components

When Knight *et al*. (1975) first reported an increase in ovulation rate following supplementation with lupin, it was unclear whether the response to lupin supplementation was a response to energy, to protein or to some specific compound in the feed.

Most attempts to determine the relative importance of protein and energy contents of the diet have indicated that energy is the more important (Teleni *et al*., 1989a; b). In ruminants up to 35% of glucose requirements can be met by amino acids. An increase in protein will therefore result in an increase in glucose. Teleni *et al*. (1989b) have shown that intravenous infusions of energy substrates, including glucose, will increase ovulation rate in ewes. They concluded that energy provides important regulatory signals for ovulation.

The immediate effect of nutrition can be achieved with supplements and a mixture of grains whose protein and energy content is equivalent to that in the lupin supplement (Davis *et al.*, 1981; Teleni *et al.*, 1989b; Pearse *et al.*, 1994). A short-term infusion of glucose and a mixture of branched chain amino acids have also been reported to result in increases in ovulation rate (Downing *et al.*, 1995b; c; 1997).

Influence of nutrition on the reproductive axis

Gonadotrophin-releasing hormone-dependent pathways

Since FSH and LH are necessary for follicle growth and development, the mechanisms by which the level of feed intake influences ovulation rate may be mediated through changes in the concentrations of these hormones. In general, efforts to find correlations between gonadotrophins and ovulation rate have produced negative results.

In studying the "static effect" of nutrition it was found that LH pulse frequency and amplitude was higher in ewes in high body condition during the follicular phase than in ewes in lower body condition (Rhind *et al.*, 1985; Rhind & McNeilly, 1986). The same authors found no effect of condition score on mean LH pulse frequency or amplitude during the cycle (Rhind *et al.*, 1989). In studying the "immediate effect" Stewart (1990) found that short-term supplementation with lupins had no significant effect on LH pulse frequency but that it did increase LH pulse amplitude from day 8 to day 5 before ovulation in supplemented ewes. Luteinizing hormone pulse frequency was reported to be higher during the luteal phase in ewes that had twin ovulations than in ewes with single ovulations (Thomas *et al.*, 1984; Rhind & McNeilly, 1986). However, since increasing the frequency of GnRH pulses artificially from day 11 to day 13 of the cycle did not affect ovulation rate, it was concluded that GnRH pulse frequency does not control ovulation rate (Martin & Thomas, 1990).

Effects of nutrition on FSH levels during the cycle are equivocal, with reports for and against the induction of a change. In some studies greater dietary intake was reported to increase FSH concentrations in the late luteal phase and the follicular phase of the cycle and was associated with an increase in ovulation rate (Rhind *et al.*, 1985; Smith & Stewart, 1990). However, ewes about to have twin ovulations had higher concentrations of FSH in the luteal phase, but lower FSH concentrations in the follicular phase, than ewes about to have single ovulations (Scaramuzzi & Radford, 1983; Thomas *et al.*, 1984; Lindsay *et al.*, 1993). It would be expected that an increase in ovarian follicular growth leads to the secretion of extra oestrogens and inhibin, which would decrease gonadotrophin secretion. Thus, the normal levels of gonadotrophins observed when ovulation rate is changed may acutely reflect a shift in the equilibrium of the feedback system (Monget & Martin, 1997).

There is evidence that hepatic metabolism changes in relation to levels of feeding (Farningham & Whyte, 1993). Smith & Stewart (1990) reported that increased nutrient intake can produce an increase in liver size and the concentrations of hepatic enzymes. This results in an increased level of metabolism of steroid hormones, which in turn may decrease negative feedback effects on gonadotrophins. It has been suggested that enhanced nutrition may increase oestradiol clearance by the liver. In one study oestradiol in the peripheral circulation was 10% lower in ewes on a high than in ewes on a low plane of nutritional intake. This decrease in oestradiol was associated with an increase in FSH (Adams *et al.*, 1997).

Metabolic clearance rate of progesterone changes with feeding, and is directly related to the level of feed offered to the ewes (Parr *et al.*, 1993). A decrease in progesterone concentrations associated with higher intake is a consistent finding, but its relationship with the pattern of LH secretion remains to be elucidated (Parr *et al.*, 1992; Lozano *et al.*, 1998).

Gonadotrophin-releasing hormone-independent pathways

Nutritional treatments that promote an increase in ovulation rate seem to affect particularly the last two stages of follicle development (i.e. the gonadotrophin-dependent and ovulatory follicles). Follicles at these stages are dependent on FSH for their growth and development; however, in several studies nutritional supplementation did not modify FSH concentrations (Findlay & Cumming, 1976; Knight, 1980; Radford *et al.*, 1980; Scaramuzzi & Radford, 1983; Downing *et al.*, 1995a). For this reason, it is unlikely that the mechanism that transduces the energetic inputs into an ovarian response is based on changes in the hypothalamo-pituitary feedback system. It is likely therefore that nutrition affects the sensitivity of these follicles to the action of FSH at the ovarian level (Scaramuzzi & Campbell, 1990).

In studying the "static effect" of nutrition Rhind *et al*. (1986, 1989) found that ewes in high body condition had a higher ovulation rate and a higher number of large follicles (defined as ≥4 mm and as >2.5 mm by the same authors in different studies) than did ewes in low body condition but with a similar number of small follicles. Ewes with a high live weight had more follicles ≥2 mm. Also in these ewes a greater number of follicles were recruited to ovulate and the rate of atresia was lower than in ewes with low live weight (Xu *et al.*, 1989). The "dynamic effect" of nutrition promoted similar changes in the follicle population. A nutritional treatment applied during one oestrous cycle in ewes did not change the number of follicles 1–2 mm in size but increased the number of follicles 2–3 mm large in supplemented ewes. It was concluded that the effect of flushing was exerted through the prevention of atresia of large follicles in the last 30 hours before ovulation (Haresign, 1981).

The "immediate effect" of nutrition also promotes changes in the follicle population. In ewes supplemented with lupins for 7 days, there was a significant reduction in the number of follicles that became atretic (Nottle *et al.*, 1985). Similarly, ewes supplemented with lupins for 4 days had more 2-mm follicles visible on their ovaries than did ewes that were fed oats (Stewart, 1990).

According to Gherardi & Lindsay (1982), it is not necessary to feed lupins for 3 weeks, and 9 days of supplementation before ovulation are adequate for promoting an increase in ovulation rate. The minimum period of supplementation was later reduced to 6 days (Oldham & Lindsay, 1984). Stewart & Oldham (1986) found that the intake of lupins promoted a positive effect if fed between day 8 and day 5 before ovulation, but the effect was either negative or neutral if fed between day 4 and day 1 before ovulation. After these studies the effective period for nutritional treatments was dramatically reduced for the ewe. This major breakthrough in research leads to a reduction in the cost of supplementary feeding, which is
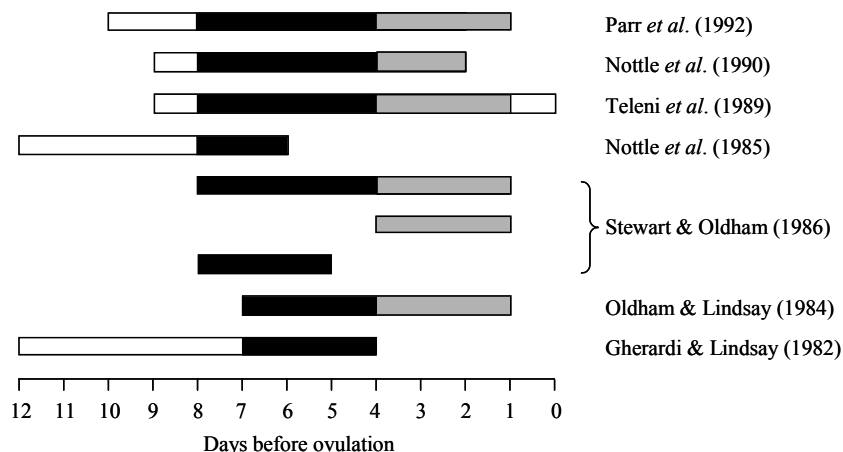
Figure 3. Schematic representation of the duration of short-term nutritional treatments applied in different experiments. The length of the bar represents the entire treatment, which was sub-divided based on the findings by Stewart & Oldham (1986). Black indicates the period during which short-term nutritional supplementation has a positive effect, while grey indicates a negative/neutral effect of supplementation.

important in terms of farm economics (Young *et al.*, 1990). However, since there is a critical time during the oestrous cycle, when short-term supplementation increases ovulation rate, the oestrus of ewes should be synchronized (Gherardi & Lindsay, 1982).

Several authors have confirmed the effectiveness of the short-term nutritional supplementation in increasing ovulation rate. Figure 3 shows that the critical period during which short-term nutritional supplementation stimulates ovulation rate is from day 8 to day 4 before ovulation, in other words, during days 10–14 of the oestrous cycle (Gherardi & Lindsay, 1982; Oldham & Lindsay, 1984; Nottle *et al.*, 1985; Stewart & Oldham, 1986; Teleni *et al.*, 1989b; Nottle *et al.*, 1990; Parr *et al.*, 1992). This is the time range when the ovulatory wave emergences. However, since the wave-like pattern of follicle development was not yet clear when the above investigations were performed, the authors were unable to associate the critical period with the emergence of the ovulatory wave.

Glucose and metabolic hormones

The changes in circulating levels of metabolic hormones are important signals of the metabolic state of the animal (Lindsay *et al.*, 1993). Metabolic hormones such as insulin, growth hormone, IGF-I and leptin have important roles in the control of follicle development and are likely to be mediators of the effects of dietary intake on ovulation rate (Muñoz-Gutierrez *et al.*, 2002).

The insulin related ovarian regulatory system plays an important role in regulating ovarian responsiveness to gonadotrophins. This system is composed of insulin and IGF-I and IGF-II, whose receptors are present in granulosa and theca cells (for a review, see Poretsky *et al.* (1999)). Insulin binding to its receptor results in a number of metabolic effects, the most important of which is the

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stimulation of glucose transport into the cells. Glucose is the major source of energy for the ovary (Rabiee *et al.*, 1997).

In one study plasma glucose and insulin increased in ewes fed lupin supplementation for 8 days (Teleni *et al.*, 1989a). The insulin peak occurred on the third day of supplementation and decreased thereafter, reaching basal levels 5–6 days after supplementation commenced. It was hypothesized that the dramatic and transient increase in insulin may provide the signal for the initiation of anabolic processes with subsequent increased fat and protein deposition (Teleni *et al.*, 1989a).

The positive relationship between nutrition and ovulation rate has been investigated in sheep infused intravenously with glucose for 5 days from day 8 to day 12 of the oestrous cycle. This treatment increased glucose and insulin levels in serum and increased ovulation rate. The authors concluded that insulin was involved in mediating ovulation responses to nutritional stimuli, either directly or by stimulation of insulin-mediated glucose uptake (Downing *et al.*, 1995a). Similarly, the increase in ovulation rate promoted by intravenous infusion of a mixture of leucine, isoleucine and valine was associated with an increase in insulin concentrations (Downing *et al.*, 1995c).

However, data arguing for the direct effect of insulin on ovulation rate are contradictory. Hinch & Roelofs (1986) found an increase in ovulation rate when ewes were infused daily with insulin during the luteal phase of the oestrous cycle. Leury *et al*. (1990) found no effect on ovulation rate in response to injections of insulin in the absence of lupin supplementation. There was no relationship between insulin concentration and presence of one or two CL.

Insulin-like growth factor I, the concentrations of which increase after short-term supplementation, affects the responsiveness of follicles to gonadotrophins (Blanche *et al*., 1996; Monget & Martin, 1997; for a review, see Poretsky *et al.* (1999)). Insulin-like growth factor I suppresses apoptosis in ovarian follicles, thus reducing their rates of atresia and increasing the number of follicles that develop to the ovulatory stage (for a review, see Poretsky *et al.* (1999); Scaramuzzi *et al.*, 1999). Insulin-like growth factor I is not produced locally but is produced by the liver after GH stimulation. It has been suggested that IGF-I can reach the ovine ovary through a local pathway, since follicular oestradiol increases IGF-I synthesis in the uterus, which could then act in an endocrine manner to affect follicular cell development (Perks *et al.*, 1995).

Another metabolic signal that affects reproductive function is leptin, a protein hormone produced in adipose cells (Zhang *et al.*, 1994; Dyer *et al.*, 1997). Leptin acts directly in the hypothalamus to regulate food intake and the whole-body energy balance, and has a direct action at the level of the ovary (Spicer, 2003). This hormone is thought to be the metabolic signal that regulates nutritional status effects on reproductive function (Houseknecht & Portocarrero, 1998). Plasma concentrations of leptin vary with acute changes in nutrition and are correlated with body fatness in sheep (Chilliard *et al.*, 1998; Blache *et al.*, 2000a).

There is a dual control of leptin in ruminants: background concentrations in the fed animal reflect adiposity, together with an acute sensitivity to short-term

alterations in food intake (Marie *et al.*, 2001). Insulin stimulates the secretion of leptin by adipocytes. By promoting lipogenesis, insulin may indirectly increase leptin production (for a review, see Poretsky *et al.* (1999)). Moreover, leptin concentrations increase 2–8 hours after a meal, further supporting the association between insulin and leptin (Marie *et al.*, 2001). In one study, an increase in plasma insulin and leptin concentrations occurred when ewes were fed lupin grains. Plasma leptin concentrations increased 24 hours after the start of supplementation and remained high for another 72 hours, after which they returned to basal concentrations (Muñoz-Gutierrez *et al.*, 2002).

Metabolic hormones also regulate steroidogenesis. Acting at the ovarian level, the insulin-related ovarian regulatory system appears to potentiate the steroidogenic effect of gonadotrophins (for a review, see Poretsky *et al.* (1999)). In one study, the infusion of IGF-I via the ovarian artery increased the secretion of oestradiol during the follicular phase, showing that IGF-I is a potent stimulator of steroidogenesis in sheep (Scaramuzzi *et al.*, 1999). By contrast, in another study insulin and glucose infused together decreased the secretion of androstenedione and to a lesser extent oestradiol, suggesting that nutritional surplus may modulate the steroidogenic capacity of the follicle (Downing *et al.*, 1999). Leptin antagonizes the stimulatory effect of insulin on theca cell steroidogenesis. Leptin may exert a direct inhibitory effect on bovine ovarian function by inhibiting both granulosa and theca cell steroidogenesis, ultimately leading to a decrease in oestradiol secretion (Spicer & Francisco, 1997). However, the precise role and mechanisms of action of leptin in regulating hormone-induced ovarian steroidogenesis require further elucidation (Spicer, 2003). In a low-leptin environment (i.e. in animals in low body condition/with a low feed intake) ovarian function is dictated primarily by gonadotrophins and insulin/IGF-I. In a moderate-to high-leptin environment (i.e. animals in high body condition/with a high feed intake) the ovary is kept from "over producing" oestradiol via leptin inhibition of insulin-induced androstenedione production by the theca cells and aromatase activity by granulosa cells (Spicer, 2001).

The role of gonadotrophins in determining ovulation rate in the ewe remains confusing because consistent relationships between nutrition, secretion of gonadotrophins and ovulation rate are difficult to demonstrate. One reason for this confusion may be the difficulty in studying two discontinuous variables (ovulation rate and pulse frequency) as end-points of nutritional stimulation (Lindsay *et al.*, 1993). Moreover, the experimental designs suitable for studying these two variables are not compatible, since a large number of ewes are needed to evaluate ovulation rate, while a small number of ewes are needed to perform frequent blood sampling to measure LH pulses. Although there is consistency in the decrease in progesterone concentrations in ewes fed at a higher intake level, there are certain difficulties in evaluating LH pulse frequency. Serial blood sampling to measure GnRH/LH pulse frequency seems to increase or decrease ovulation rate depending on the time of the cycle at which the samples are taken (Adams *et al.*, 1993).

Although an increase in insulin concentrations is a common endocrine response to a variety of nutritional treatments (Downing & Scaramuzzi, 1991) insulin administration in ewes has been reported to have no effect on ovulation rate,

probably owing to the consequent reduction in glucose concentrations (Leury *et al.*, 1990; Downing & Scaramuzzi, 1997). It has been claimed that ewes in body condition 2, on a scale from 1 to 5, respond better to nutritional supplementation, but few authors describe the body condition of the animals used in their experiments (e.g. Parr *et al*. (1992)). Moreover, a better ewe response to nutritional supplementation has been described in animals in high condition than in those in low body condition (Leury *et al.*, 1990). If leptin concentrations are positively correlated with fat stores, and leptin indirectly inhibits oestradiol production by the follicles, then leptin may be the variable that best describes the equilibrium of the feedback system (by "equilibrium" is meant the lack of changes in oestradiol and FSH concentrations).

Despite considerable research efforts the mechanisms dictating the nutrition-dependent response are still not fully understood. The effect of nutrition on ovulation rate is not promoted by any specific nutrient, hormone or metabolite, since a cause and effect relationship has not been demonstrated. Therefore, the control of ovulation rate provides a good example of the need for a holistic approach to the nutritional management of reproduction in livestock (Robinson *et al.*, 2002). Since responses to nutrition at the gonadal level are complex, Blache *et al*. (2000a) have put forward the concept of a central "sensor integrator", which may process the information on nutritional status and translate it into the appropriate reproductive response.

The present study

Outline and aims

From the aforegoing it is clear that there is a large amount of information on the effect of nutrition on ovulation rate; however, the mechanism behind the static and immediate effects of nutrition on follicle development is still not clear. The largest follicle is the main source of steroids, which has a negative feedback on gonadotrophins. Fluctuations in the concentration of gonadotrophins affect the recruitment and selection of follicles and consequently the number of follicles destined to ovulate. Studying follicular dynamics may therefore help to clarify the mechanism by which nutrition increases ovulation rate. The general aim of this thesis was to gain further knowledge on the effect of nutrition on endocrine response and follicular dynamics in the cyclic ewe.

As has been mentioned, the length of supplementation given to increase ovulation rate and the cost of such supplementation can be reduced by synchronizing oestrus in ewes. Most synchronization treatments in sheep utilize intra-vaginal medroxyprogesterone acetate (MAP) sponges. Although helpful for oestrus synchronization, MAP leaves residues in the environment, which may be regarded as potential contaminants and could possibly act as endocrine disrupters in the ecological system. We have found that a single dose of prostaglandin 14 days before mating is as effective for oestrus synchronization as a 12-day MAP treatment, with no risk of environmental contamination and, moreover, a reduction in the cost of the treatment (Viñoles *et al*., 2002). These results encouraged us to apply a routine prostaglandin treatment in our experimental designs.

In the ewe the number of follicular waves that develop during the oestrous cycle is variable. This variability can be attributed to several factors, among which nutritional status of the animals may play an important role. In cattle poor nutrition is associated with a reduction in the size of the dominant follicle of all waves and a reduced persistence of the dominant follicle of the first wave (Murphy *et al.*, 1991). Follicle turnover is regulated by the concentrations of progesterone, FSH and oestradiol in cattle (Adams *et al.*, 1992; Ginther *et al.*, 1996; Evans *et al.*, 1997). In **Paper I** we tested the hypothesis that static body condition of the ewe affects progesterone, FSH and oestradiol, with implications for subsequent follicular growth and turnover and therefore the number of follicular waves.

In **Paper I** we realized the difficulty in predicting ovulation rate of the ewe at the time of disappearance of the largest follicle, since ewes with twin ovulations may ovulate a 4 mm follicle recruited a few days before ovulation. In these cases ovulation of two follicles was evident 5 days after ovulation when two luteal structures appeared on the ovarian surface. There was a lack of information regarding the limitations of ultrasonography, which could replace more invasive techniques such as laparotomy and laparoscopy. Information regarding the increase in number of small follicles (≥ 2 mm) at the time of wave emergence is inconsistent. This inconsistency may in part be explained by the inaccuracy of ultrasonography to distinguish follicle size. In **Paper II** the objective was

therefore to assess the accuracy of estimating the size and number of corpora lutea and ovarian follicles by comparing results of ultrasound scanning of the ewe with results of post-mortem examination of the ovaries.

In cattle an increase in ovulation rate is associated with a three-wave rather than a two-wave pattern of follicle development (Bleach *et al.*, 1998). Similarly, we found that ewes in high body condition had a three-wave pattern and also, had higher ovulation rates than did ewes in low body condition (**Paper I**). In **Paper III** our hypothesis was that short-term nutritional supplementation around the time of ovulatory wave emergence will increase the proportion of ewes in low body condition developing a three-wave pattern. The increase in the number of waves will be promoted by changes in the concentrations of FSH and oestradiol, which will increase the number of follicles recruited to ovulate. To test the effect of the nutritional treatment on ovulation rate, a field trial was performed in parallel with a controlled design.

Giving ewes high-energy/high-protein supplementation did not increase FSH concentrations, nor did it decrease oestradiol concentrations; rather, it induced an increase in ovulation rate (**Paper III**). It is therefore unlikely that the mechanism that transduces the energetic inputs into a reproductive response in the ovary is based on changes in the hypothalamus-pituitary feedback system (Muñoz-Gutierrez *et al.*, 2002). Nutrition changes the level of metabolites and metabolic hormones, which acting at the ovarian level may promote an increase in ovulation rate. The aim of **Paper IV** was to test the hypothesis that short-term supplementation induces an increase in plasma concentrations of glucose, insulin, leptin and IGF-I, which may rescue follicles from atresia.

Methodological considerations

Location of the studies

All the experiments were carried out in Uruguay (30-35°SL) during the breeding season (March to June) with an average natural light:dark ratio of 13:11 hours. The first study (**Paper I**) was carried out at the Sheep Unit of the National Institute of Agricultural Research (INIA) in Colonia (35°SL), Uruguay. The second study (**Paper II**) was performed at the slaughterhouse "La Tablada" in Melo, Cerro Largo (31.5°SL). The third study (**Papers III and IV**) was performed at the Experimental Station "Bañado de Medina" at the Agronomy Faculty in Cerro Largo. The field trial (**Paper III**) was performed at the stud farm "La Carolina" in Cerro Largo.

General procedures

Oestrus was synchronized with two prostaglandin (PG) injections given 9 days apart (150 µg Glandinex®, Universal Laboratory, Montevideo, Uruguay (**Paper I**), and 30 µg Cloprostenol, Dalmaprost-D®, Fatro Laboratory, Montevideo, Uruguay (**Papers III and IV**). The ewes remained with marked vasectomized rams from day 10 of the oestrous cycle (**Paper I**) or else, the vasectomized ram was presented to the ewes every 12 hours (**Papers III and IV**) for the observation

of oestrous behaviour. Body weight and body condition (BC) were measured weekly. Body condition was estimated using a subjective system based on a five-point scale from 1 (emaciated) to 5 (obese), as described by Suiter (1994) (**Papers I**, **III** and **IV**).

Experimental designs

In **Paper I** Polwarth ewes were selected from a flock according to their BC score. High-BC (HBC) ewes (n = 8) had a score of 4.1 ± 0.1 (mean ± SEM) and low-BC (LBC) ewes (n = 8) had a score of 1.9 ± 0.1. Controlled time of grazing (5 hours) was used to maintain BC in ewes with LBC throughout the experiment, while ewes with HBC grazed ad libitum except during ultrasound measurement (5 hours). Daily ultrasound examinations began 1 week before the expected onset of the first spontaneous oestrus and continued until the second ovulation was observed. Daily blood sampling started 1 week before the expected onset of the first spontaneous oestrus until the occurrence of the second ovulation.

In **Paper II** 50 Corriedale ewes of unknown reproductive history and at random stages of the oestrous cycle were used for the study. In all ewes the number and size of the CL were evaluated. In 17 out of 50 ewes the diameter and position of all follicles 2–6 mm large were recorded by ultrasonography. One ultrasound examination was performed to each animal. The ewes were slaughtered 12 hours after the ultrasound examination. The ovaries were collected and dissected and the number and size of the CL and follicles evaluated.

In **Paper III** two experiments were carried out in parallel. In a controlled experiment 20 Corriedale ewes aged 4.6 ± 0.2 years and with a body condition score of 1.8 ± 0.1 and a body weight of 36.4 ± 0.6 kg were used. Ewes were randomly assigned to two groups: (1) the control group (n = 10) received the maintenance diet, while (2) the short-term supplemented group (n = 10) received twice the maintenance diet from day 9 to day 14 after ovulation. In **Papers III** and **IV** ewes were fed a maintenance diet (AFRC, 1993) formulated for restricted intake (46 g dry matter (DM) per kg of metabolic body weight; BW $^{0.75}$) to avoid refusals. The basal diet consisted of 70% hay (Trifolium alexandrinum) and 30% concentrate (80% corn grain and 20% soybean meal). The food supplied 7 MJ metabolizable energy and 95 g crude protein per kg of dry matter per day. Four weeks before the beginning of the experiment ewes were handled in individual pens. Ultrasonographic examinations started the day of the second PG injection to establish ovulation and monitor follicle development, and continued until 7 days after the second ovulation. From the day of the second PG injection until occurrence of the second ovulation blood was collected three times a day. Thereafter samples were taken daily until day 7. Blood was sampled with respect to time of feeding (hour 0) at -5 hours, 1.5 hours and 7 hours.

In the field trial (**Paper III**) 282 Corriedale ewes aged 6.5 ± 0.1 years, weighing 58.8 ± 0.7 kg and with a body condition score of 2.9 ± 0.1 units were used. Ewes were randomly assigned to a control group (n = 141) and a short-term supplemented group (n = 141). All ewes grazed native pasture for 5 hours per day. Eight days after the peak in oestrus the ovaries of ewes (n = 65 per group) that

showed oestrous behaviour from day 16 to day 19 of the cycle and had eaten the supplement for at least 4 days were scanned by ultrasonography to count the number of CL. An equal number of ewes from the control group that showed oestrus in the same period were likewise scanned.

In **Paper IV** we used half of the animals from each group of the controlled experiment described in **Paper III**. In the short-term supplemented group all the ewes (n = 5) ovulated the same day. To determine acute changes in glucose and metabolic hormones blood was sampled frequently before and after feeding on days 9, 11 and 14 after ovulation. The samples were obtained for a period of 24 hours as follows: every hour starting from 2 hours before until the start of feeding, every 30 minutes in the first 2 hours after feeding, every hour from 2 hours until 10 hours after feeding, and every second hour thereafter.

Ultrasonographic examinations of the ovaries

The same operator performed all the ultrasonographic examinations. The day of ovulation was defined as the day when the largest identified follicle was no longer seen and was substituted by luteal tissue in the same location. Transrectal ultrasonographic examinations were performed using a real-time B-mode machine (Aloka SSD 500, Aloka Co., Ltd., Tokyo, Japan) equipped with a 7.5 MHz linear array transducer (**Papers I**, **II** and **III**). Either a transducer designed for human prostate examination (**Paper I**) or a transducer adapted to be manipulated externally in the rectum was used (**Papers II** and **III**). Examinations were performed with the ewe in standing position by external intra-rectal manipulation of the transducer (Figure 4). The number, diameter and relative position of all ovarian follicles with a diameter of ≥2 mm and corpora lutea in both ovaries were mapped daily. Figure 5 shows an example of the ovarian sketches obtained during ultrasonographic examinations.

Sampling procedure, glucose and hormone determinations

Blood samples (10 ml) were taken by jugular venipuncture (**Papers I** and **III**). In **Paper IV** the samples (5 ml) were taken from an indwelling jugular catheter. Samples were chilled at 4°C and centrifuged within 3 hours of collection (**Paper I**). In **Paper III** samples were extracted by jugular venipuncture and maintained on ice until they were centrifuged within 10 minutes of collection. The samples of serum (**Paper I**) and plasma (**Papers III** and **IV**) were stored at –20°C until assayed for hormone content.

In **Papers I** and **III** progesterone was assayed daily during the inter-ovulatory period with a direct solid-phase radioimmunoassay (RIA) (Meikle *et al.*, 1997). Oestradiol 17β (Meikle *et al.*, 1997) and FSH concentrations (Viñoles *et al.*, 1999b) were determined by liquid-phase RIA every 24 h (**Paper I**) or every 12 h (**Paper III**) during the inter-ovulatory period. In **Papers III** and **IV**, since oestradiol concentrations are low in the peripheral circulation of sheep and since androstenedione concentration which is closely related to oestradiol concentration, circulates in higher concentrations, we measured both steroids to gain a more
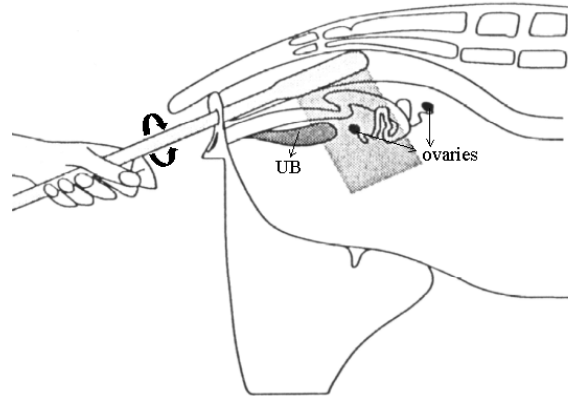
Figure 4. Transrectal ultrasonographic examination of the reproductive tract with the ewe in standing position. In cranial position to the urinary bladder (UB) the transducer is moved clockwise and anti-clockwise to locate the ovaries. Modified from Kähn (1994).

accurate picture of steroidogenesis (Baird & Scaramuzzi, 1976; Souza *et al.*, 1997b). Androstenedione concentrations were measured by liquid-phase RIA (DSL-4200, Diagnostic Systems Laboratories, Inc., TX, USA). Parallelism of the standard curve with serial dilution of ovine plasma samples was demonstrated for androstenedione.

In **Paper IV** glucose concentrations were measured in fresh blood using an Elite® glucose meter (Bayer Laboratory, Montevideo, Uruguay). Insulin concentrations were measured by a direct solid-phase RIA using DPC kits (Diagnostic Product Co., Los Angeles, CA, USA). Parallelism of the standard curve with serial dilution of ovine plasma samples was demonstrated for insulin. Insulin-like growth factor I concentrations in plasma were measured by double-antibody RIA (Gluckman *et al.*, 1983). Leptin was analysed by RIA (Blache *et al.*, 2000b). Cortisol was measured by a direct solid-phase RIA (Diagnostic Product Co., Los Angeles, CA, USA) (van Lier *et al.*, 1998).

Statistical analyses

The numbers of follicular waves and the number of ovulatory follicles in different size classes were compared using Fisher's exact probability test (**Papers I** and **III**). Ovulation rate and the effect of body condition on ovulation rate were analysed using Fisher's exact probability test or Chi-square tests (**Papers I** and **III**).

The characteristics of the largest follicle of each wave (day of emergence, day of maximum diameter, maximum diameter, and life span), inter-wave intervals and inter-ovulatory interval were studied by analysis of variance in the GLM procedure of the Minitab computer program (Minitab Inc., 1994, State College, PA, USA; **Paper I**) or by the mixed procedure in the Statistical Analysis System (SAS Institute Inc., 1999-2000, Cary, Nc, USA; **Paper III**). The difference in

Day	OVARIO IZQUIERDO	– OVARIO DERECHO	Left ovary	Right ovary
11				
12				
13				
14				
15				
16				
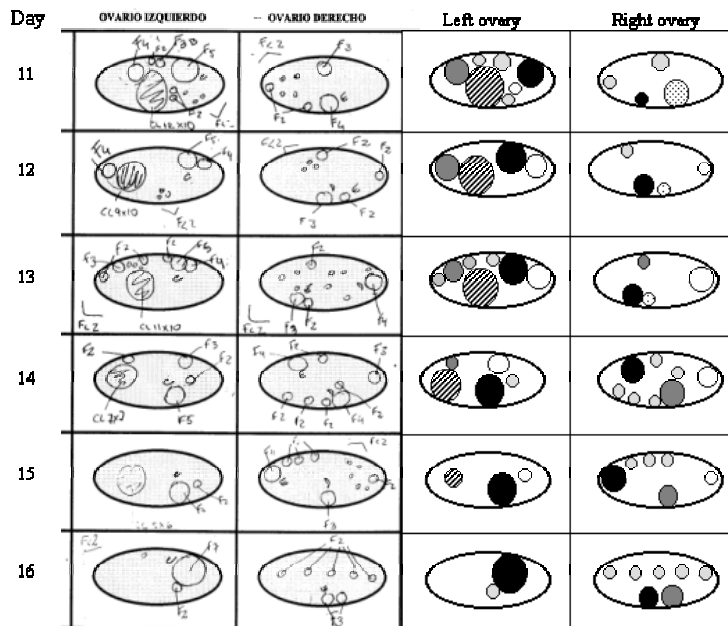
Figure 5. Daily maps obtained by ultrasound examinations of ovarian follicles and the CL, presented as raw data (left) and analysed data (right) for pairs of ovaries. Circles with the same colours in the same ovary represent the evolution of the same structure.

maximum diameter attained by the ovulatory follicle in ewes that had a single ovulation was compared using Student's *t*-test (**Paper I**).

In **Papers I** and **III** the relationship between changes in the concentrations of FSH, oestradiol and androstenedione, and the growth profile of the dominant follicles was analysed using a skewness method (Zarco *et al.*, 1984). For each ewe and each hormone, the baseline and standard deviation (SD) of the baseline value were calculated. A significant increase was defined as a threefold increase in the concentration of a hormone above the SD of the baseline. A significant FSH decrease was defined as a threefold decrease in the concentration of FSH above the SD of the baseline.

For the analysis of repeated measurements, the mixed procedure of the SAS was used. The model included the fixed effects of day, group (**Paper I**) and time (**Papers III** and **IV**) and their interactions. The covariance structure was modelled using the random statement ewe within group (**Paper I**) plus autoregressive order 1 (**Paper III**) (Littell *et al.*, 2000). Data with repeated measurements included: the total number of follicles ≥2 mm in diameter; diameters of the largest and second largest follicles; concentrations of progesterone, oestradiol and FSH; body weight and body condition (**Papers I** and **III**); and concentrations of glucose, insulin, leptin, IGF-I, androstenedione and cortisol (**Paper IV**).

Correlations were analysed using the procedure available in SAS. The variables studied were: the peak and nadir concentrations of FSH and oestradiol for individual animals (**Paper I**); and the number of follicles in different size classes

observed by ultrasonography and at post-mortem evaluation of the ovaries (**Paper II**).

Linear regression analyses were performed using the procedure available in SAS. The variables studied by simple regression were the diameters of CL and follicles observed by ultrasonography and post-mortem, with ultrasonography as the dependent variable (**Paper II**). The variable studied by multiple regression was plasma leptin concentration. The dependent variable was plasma leptin concentrations and the independent variables included group, day, period and plasma concentrations of glucose, insulin and IGF-I (**Paper IV**).

In **Paper II**, the positive predictive value (100 a/(a+b)) and the sensitivity (100 a/(a+c)) of ultrasonography were calculated from the number of correct positive diagnoses (a), the number of false positive diagnoses (b) and the number of false negative diagnoses (c).

In all the papers the data are presented as means ± standard error of the mean (SEM) and differences were considered significant if P≤0.05.

Results

Ovarian follicular dynamics and endocrine profiles in Polwarth ewes with high and low body condition (Paper I)

All HBC ewes developed three follicular waves, while four LBC ewes had two waves and four had three waves of follicle development during the inter-ovulatory interval. High-BC ewes had higher progesterone concentrations from day 5 to day 12 after ovulation than did LBC ewes. The higher progesterone concentrations in HBC ewes were due to the presence of ewes with two corpora lutea. Progesterone concentrations were similar among LBC ewes that developed two waves and LBC ewes with three waves.

The emergence of 75% of the follicular waves was preceded by significant increases in FSH concentration, which occurred 1 day before wave emergence. Oestradiol concentrations increased significantly during the growth phase in 86% of the large follicles. In HBC ewes we observed a negative correlation between the maximum and minimum concentrations of oestradiol and FSH (P≤0.001; r = -0.5; n = 48). A similar inhibitory effect of oestradiol on FSH was observed in LBC ewes irrespective of whether they developed two or three follicular waves. Figure 6 shows that FSH concentrations increased during a longer period before the emergence of the second wave in ewes that developed two waves compared with ewes that developed three waves. In ewes with a two-wave pattern FSH concentration was higher and oestradiol was lower than in ewes with a three-wave pattern. In HBC ewes FSH concentration was similar but oestradiol concentration was lower than in LBC ewes that developed a three-wave pattern (unpublished data).
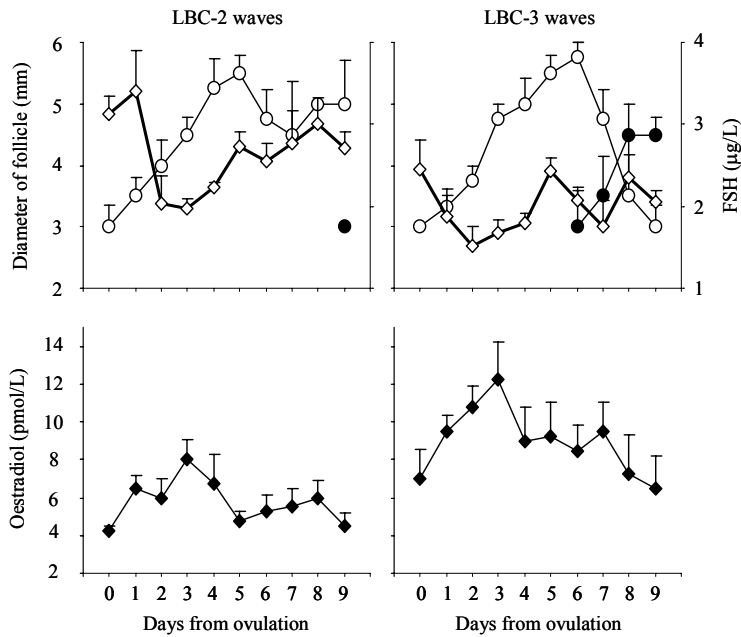
Figure 6. Concentrations of FSH ($\Diamond$) and growth profiles of the dominant follicle of wave 1 ($\bigcirc$) and wave 2 ($\bullet$; upper panel) and oestradiol ($\blacklozenge$) concentrations (lower panel) in ewes in low body condition that developed two or three waves during the cycle (unpublished data from **Paper I**).
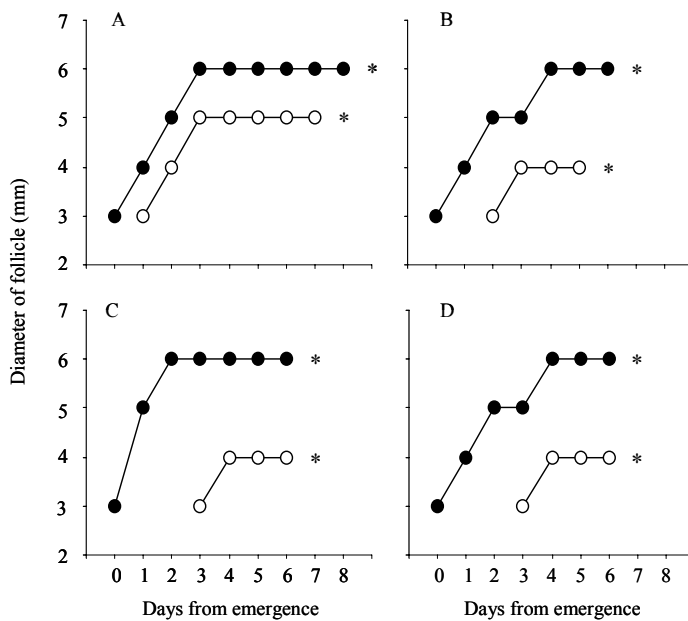

Figure 7. Growth profile of the two ovulatory follicles ($\bullet$ and $\bigcirc$) in four ewes in high body condition. A: both follicles originated from the same wave. B–D: recruitment of a smaller follicle 48–72 hours after the emergence of the largest follicle (unpublished data from **Paper I**).

Twin ovulations were observed in four HBC ewes but in no ewes in the LBC group. In one of the four cases in which a twin ovulation occurred, two follicles ≥5 mm that originated from the last follicular wave became the ovulatory follicles. In the other three cases, in addition to the largest follicle originating from the last wave, another follicle emerged 48–72 hours later, reaching a smaller diameter (4 mm) and then ovulating. Figure 7 shows the growth profiles of the two ovulatory follicles (unpublished data). In HBC ewes the follicular phase was characterized by lower oestradiol but higher mean FSH concentrations than in LBC ewes.

In HBC ewes we found that the increase in FSH concentration that preceded ovulatory wave emergence was less frequently followed by a significant inhibition in FSH concentrations (5/16) than in ewes with LBC (11/16).

In the HBC ewes that had a single ovulation (n = 11), the ovulatory follicle attained a larger diameter (6.2 ± 0.2 mm) than did that of ewes in the LBC group (n = 16; 5.6 ± 0.2 mm).

*Accuracy of evaluation of ovarian structures by transrectal ultrasonography in ewes (**Paper II**)*

The predictive value and sensitivity of ultrasonography was 100% for the presence and 96% for the absence of a CL. In four out of five ewes with double ovulations, the presence of the two corpora lutea was correctly diagnosed by ultrasonography.

Figure 8 shows two situations which may lead to incorrect estimation of corpora lutea number: (A) overestimation due to the presence of CL with large papillae; (B) underestimation due to the presence of a CL with a big central cavity, which can be mistaken for a big follicle.

For functional corpora lutea, the sensitivity of ultrasonography was 100%. The regression coefficient for CL diameter was significant (P≤0.001; r^2 = 0.4; n = 35). The size of 85 out of 117 follicles was accurately determined. The correlation between numbers of follicles counted by ultrasonography and follicles counted post-mortem increased with size of follicle from r = 0.44 (P≤0.01) for 2 mm to r = 0.85 (P≤0.001) for ≥5 mm diameter follicles. The regression between the diameters of follicles determined by the two methods was significant (P≤0.001; r^2 = 0.8; n = 117). The predictive value of ultrasonography for the number of follicles was high (98–100%) for all follicle sizes except for 3 mm diameter follicles (predictive value 71%). Similarly, the sensitivity was high for all sizes of follicles (90–95%) except for those of 2 mm diameter (62%).

*Short-term nutritional supplementation of Corriedale ewes: responses in ovulation rate, follicle development and reproductive hormones (**Paper III**)*

In Experiment 1 the number of follicles recruited into the ovulatory wave was similar between groups and as a consequence ovulation rate did not differ between the short-term supplemented and the control group. From day 9 to day 12 progesterone concentrations were lower in the short-term supplemented than in the
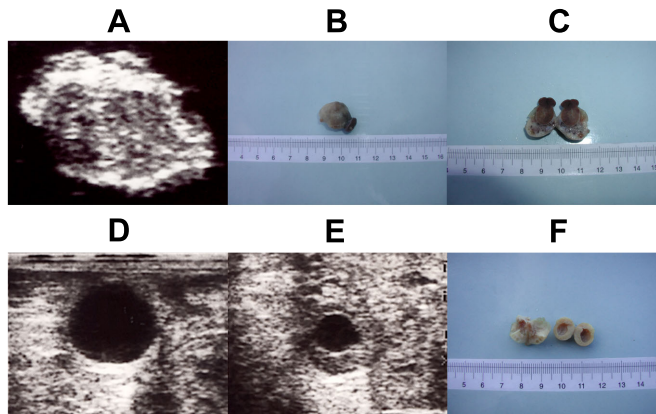
Figure 8. A CL with large papillae presented as (A) a printout of the ultrasound image in vitro after ovariectomy; and photographs of the ovary before (B) and after dissection (C; upper panel). Evolution of a CL with a large cavity shown in (D) a printout of the ultrasound image in vivo 3 days after ovulation; (E) a printout of the same ovary 6 days after ovulation; and (F) a photograph of the CL after ovariectomy.

control group. Plasma FSH and oestradiol concentrations were similar among the groups. Ewes from the short-term supplemented group developed fewer follicular waves than ewes from the control group did and the last non-ovulatory follicle had a longer life span in the short-term supplemented than in the control group. Half of the ewes in the short-term supplemented group had a second androstenedione increase during the last non-ovulatory wave, which occurred on day 12 of the cycle. In ewes from the control group the single androstenedione increase lasted longer than it did in short-term supplemented ewes.

In Experiment 2 mean ovulation rate was 15% higher in the short-term supplemented group than in the control group. In the short-term supplemented group, ewes with a body condition score ≥ 3 tended to have a higher ovulation rate than did ewes with a body condition score ≤ 2.5 (P = 0.07).

Short-term nutritional supplementation of Corriedale ewes: acute changes in glucose and metabolic hormones (*Paper IV*)

Dietary supplementation increased glucose concentrations on day 11, and increased plasma insulin concentrations on days 9, 11 and 14 compared with control ewes. The greatest difference in insulin concentrations between groups was observed on day 11. Plasma leptin concentrations on day 11 were higher in short-term supplemented ewes than in control ewes. No differences between groups were observed in plasma IGF-I concentrations. Plasma leptin concentrations were related to group, day and plasma insulin concentrations (P$\leq$0.001; r^2 = 0.5; N = 30). Plasma androstenedione concentrations on day 11 were similar between short-term supplemented and control ewes.

General discussion

In **Paper I** the hypothesis that FSH and oestradiol concentrations are implicated in the regulation of follicle turnover was confirmed.

All ewes in high body condition developed three follicular waves during the inter-ovulatory interval, while ewes in low body condition developed fewer follicular waves. This variation in the number of waves in ewes in different body condition may in part explain the large variation in the number of waves reported in the literature (Ginther *et al.*, 1995; Leyva *et al.*, 1998; Bartlewski *et al.*, 1999; Evans *et al.*, 2000). The negative feedback effects of steroids secreted by the dominant follicle are critical to the regulation of the follicular waves in cattle (Evans *et al.*, 1997). In our study we found a negative correlation between concentrations of oestradiol and FSH in ewes in high body condition. Similarly, oestradiol and FSH were negatively associated in ewes in LBC, independently of whether they developed two or three follicular waves. However, ewes that developed a two-wave pattern had a longer period with higher FSH concentrations prior to the emergence of the second follicular wave. If a decrease in FSH concentrations is part of the mechanism of selection of the dominant follicle, then the higher FSH levels delayed the process of selection, which resulted in the development of fewer waves during the cycle (Baird, 1983). Alternatively, since gonadotrophin-dependent follicles show large variability in their FSH requirements, the higher the individual threshold, the fewer waves develop during the cycle (Brown, 1978; Fry & Driancourt, 1996; McNatty *et al.*, 1999).

The ovulatory follicle becomes less dependent on FSH 48 hours before ovulation and less favoured follicles become atretic when FSH falls below the threshold level necessary to keep them alive (Baird *et al.*, 1991). If a higher nutritional plane reduces the secretion of negative feedback hormones from dominant follicles, then more follicles may be able to reach the pre-ovulatory stage (Scaramuzzi *et al.*, 1993). This may have been the case in our study, since ewes in HBC had a higher ovulation rate associated with lower oestradiol and higher FSH concentrations before ovulation than did ewes in the LBC group. There are two possible explanations for the lower oestradiol concentrations observed in ewes in high body condition. One explanation may be an increase in the clearance rate of oestradiol by the liver (Adams *et al.*, 1997). Another explanation may be a reduction of steroidogenesis via leptin inhibition of insulin-induced androstenedione production by theca cells and aromatase activity in granulosa cells (Spicer, 2001). The first alternative is less likely, since a similar reduction in progesterone concentrations would be expected if steroids were metabolized faster. The second explanation is more probable, since a positive correlation between body condition and leptin concentrations is well established in sheep (Chilliard *et al.*, 1998; Blache *et al.*, 2000a). Aside from the mechanism, our results suggest that in our study a reduction in the negative feedback exerted by oestradiol promoted an increase in FSH concentration 5 days before ovulation. Higher FSH availability increased the number of follicles selected to ovulate from the larger pool of gonadotrophin-dependent follicles present in the ovaries of HBC ewes.

In contrast to our previous studies (Viñoles *et al.*, 1999b) we did not observe significant changes in the number of small or total follicles before the emergence of each wave in this study. If an increase in the number of small follicles is indicative of wave emergence, then it may be that the accuracy of the technique is low for measuring small follicles, which may explain the inconsistent results between studies.

In **Paper II** we found that transrectal ultrasound scanning of the ewe in standing position with a 7.5 MHz transducer is an accurate method for estimating the size and number of corpora lutea and ovarian follicles. However, the accuracy of ultrasonography may decrease with an increase in the number of corpora lutea (Dickie *et al.*, 1999). In our study the sensitivity of ultrasonography was lower for the presence of double than for the presence of single corpora lutea. However, this may have been a function of the low incidence of double corpora lutea, where one error has a proportionately large effect on the calculated sensitivity. If ultrasound scanning for estimating the number of corpora lutea is performed from day 0 to day 4 after oestrus, and a big central cavity is mistaken for a big follicle, then the number of corpora lutea may be underestimated. Moreover, the presence of a non-functional CL from the previous cycle may lead to overestimation of the number of corpora lutea. The limitations of ultrasonography for determining the number of corpora lutea can be overcome by analysing the ovulation rate at a time when a well-developed CL can be clearly identified, for example on day 10 after oestrus (Dickie *et al.*, 1999). The study demonstrates that ultrasonography is a good tool for counting the number of corpora lutea to evaluate ovulation rate under field conditions.

The sensitivity of ultrasonography was lower for 2 mm follicles and the predictive value was lower for 3 mm follicles. The majority of the follicles in the range of 2–6 mm were 2 mm follicles. This is an important finding considering that the fluctuation in the number of 2 mm and 3 mm follicles is used to describe the recruitment of follicles at wave emergence and their inhibition by the dominant follicle. This finding may in part explain the inconsistency in the description of this phenomenon between investigators, since it is difficult to accurately determine these follicles by ultrasonography. However, ultrasonography may be a better tool for studying other aspects of follicle dynamics, such as the growing profile of the largest and second largest follicles, since follicles ≥4 mm are determined with high accuracy when utilizing this technique.

Based on our findings in **Paper I**, our hypothesis in **Paper III** was that a short-term nutritional supplementation would increase the proportion of ewes in low body condition developing three follicular waves. The proportion of supplemented ewes developing three waves increased as compared with the untreated ewes, but some of the untreated ewes developed four waves, which did not occur in the supplemented ewes. The supplement did not increase the number of follicles recruited into the ovulatory wave, and the treatment did not evoke an increase in FSH concentrations or a decrease in oestradiol concentrations during the follicular phase. There was no effect of the supplementation on ovulation rate in the small groups of ewes used for the controlled experiment; however, when supplementation was tested with larger groups of ewes in the field, it increased

ovulation rate by 15%. It must be added that these ewes had a moderately high body condition and that the supplement was fed over days 8–14 of the cycle.

The increased feed intake in supplemented ewes promoted a decrease in progesterone levels, which was accompanied by fewer follicular waves during the inter-ovulatory period. The lower progesterone levels in the short-term supplemented group may have changed the pattern of LH pulse frequency and thus lengthened the life span of the largest follicle. If the life span of the largest follicle present at the beginning of the treatment is lengthened by this process, then we have an explanation for the development of fewer follicular waves in supplemented ewes. Progesterone concentration is one of the factors that affect the duration of the dominant effect of a follicle. The positive effect of low and the negative effect of high progesterone concentrations on follicle growth and the maintenance of the dominance of the follicle have been documented in sheep (Dutt & Casida, 1948; Johnson *et al.*, 1996; Rubianes *et al.*, 1996; Viñoles *et al.*, 1999b; Flynn *et al.*, 2000; Viñoles *et al.*, 2001). However, in **Paper I** and **III** we found no association between endogenous progesterone concentrations and the number of follicular waves, which is in agreement with results reported in cattle (Ahmad *et al.*, 1997). We therefore suggest that it is not the concentration of progesterone as such but changes in the concentrations of progesterone during the luteal phase of the cycle that affect follicle turnover.

An increase in ovulation rate may be the consequence of an increase in the number of gonadotrophin-responsive follicles available to be recruited, and/or a longer period under high FSH levels that prolongs the recruitment window (Scaramuzzi *et al.*, 1993). In **Paper I** ewes with HBC had a larger pool of gonadotrophin-responsive follicles and higher FSH concentrations during the follicular phase. Since this endocrine environment promoted the selection of a second ovulatory follicle a combination of the two mechanisms proposed by Scaramuzzi *et al*. (1993) may explain the increase in ovulation rate. However, in **Paper III** our results suggest that the increase in the number of gonadotrophin-responsive follicles is the less likely mechanism, since supplemented ewes recruited similar numbers of follicles as recruited by control ewes into the ovulatory wave.

In **Paper III** ewes that received the supplement developed a more active dominant follicle, as reflected by the earlier divergence in the growth profile between the dominant and the first subordinate follicle. These results are in agreement with results reported in cows with a better energy status (Lucy *et al.*, 1992). In **Paper I** we found that in HBC ewes with a single ovulation the ovulatory follicle attained a larger diameter compared with the ovulatory follicle in LBC ewes. It has been proposed that gradual rather than acute reductions in FSH concentrations are associated with the presence of a larger antral follicle (Campbell, 1999). The larger diameter attained by the ovulatory follicle in HBC ewes may be associated with the less frequent inhibition of FSH concentrations below the threshold level compared with LBC ewes. In **Paper III** we found an increased frequency of follicles >6 mm in diameter in supplemented compared with control ewes. Similarly, Muñoz-Gutierrez *et al*. (2002) found that the diameter of aromatase-positive follicles was increased by nutritional supplement

(lupin), and follicles >6 mm in diameter were seen only in supplemented ewes. In our study, an increase in the availability of FSH (**Paper I**) or a more efficient use of FSH by the dominant follicle (**Paper III**) may have resulted in an increase in follicle diameter.

In **Paper I** twin ovulations occurred in four out of 16 cases, while in **Paper III** twin ovulations occurred in one out of ten cases. Owing to limitation in numbers it was not possible to study the phenomenon of selection of more than one follicle into the ovulatory wave. However, there seems to be consistency in the selection of a follicle that reaches its ovulatory capacity at a smaller size, which is similar to the situation found in ewes carrying the Booroola gene (Scaramuzzi & Radford, 1983; Souza *et al.*, 2001).

In the field trial (**Paper III**) performed with the same breed and during the same season, short-term supplementation increased ovulation rate. Moreover, supplemented ewes in higher body condition had a higher ovulation rate than did supplemented ewes in lower body condition, suggesting that a certain level of fat stores may be required for increased ovulation rates (Leury *et al.*, 1990; Williams *et al.*, 2001). One possible explanation for this dependence of response to body condition is that ewes both in good body condition and with high feed intake may have higher leptin concentrations, which in turn, may reduce steroid concentrations and thus allow an increase in ovulation rate (Spicer, 2001). Moreover, short-term supplementation may have a greater impact on a larger population of gonadotrophin-responsive follicles, as we found in the ovaries of HBC ewes in **Paper I**.

In **Paper III** we found that the effect of short-term supplementation was not mediated by an increase in FSH levels, in contrast to our findings in **Paper I**. That nutritional stimuli do not exclusively act through FSH concentrations has been reported previously (Findlay & Cumming, 1976; Knight, 1980; Radford *et al.*, 1980; Scaramuzzi & Radford, 1983; Downing *et al.*, 1995a). For this reason, a direct effect of metabolic hormones on the follicles has been proposed as the likely mechanism of action (Downing & Scaramuzzi, 1991; Downing *et al.*, 1999). However, since oestradiol and FSH concentrations are critical for follicle growth and turnover, changes in the number of follicular waves are expected to promote changes in the patterns of both hormones. In **Paper I** LBC ewes that developed a three-wave pattern had a dominant follicle that produced higher oestradiol concentrations, and this follicle had a shorter life span than did that in ewes with a two-wave pattern. In **Paper III** ewes that developed more follicular waves had shorter intervals between their increases in FSH and oestradiol concentrations. In supplemented ewes the follicle with a prolonged life span promoted a delay in the FSH surge that promoted the emergence of the ovulatory wave (Viñoles *et al.*, 1999b; Viñoles *et al.*, 2001). If nutrition does affect the number of follicular waves, it does so by changing the time of occurrence of the FSH surge rather than by changing FSH concentrations.

In **Paper IV** we confirmed that short-term nutritional supplementation in the animals studied induced an increase in glucose, insulin and leptin on the third day after commencement of supplementation but was not associated with an increase in IGF-I concentration.

Glucose and insulin increased in supplemented ewes and maximum concentrations occurred on the third day after supplementation commenced, as was found in ewes supplemented with lupin (Teleni *et al.*, 1989a). It has been suggested that the dramatic and transient increase in insulin may provide the signal for the initiation of anabolic processes and that the rapidity of the ovulatory response in lupin-fed ewes is consistent with the rapid insulin response (Teleni *et al.*, 1989a; Downing *et al.*, 1995a). The increase in glucose and insulin induced by short-term supplementation found in **Paper IV** may explain the higher ovulation rate observed when the same treatment was applied under field conditions in **Paper III**. If insulin-stimulated glucose uptake by the follicle determines the increase in ovulation rate, then follicles are rescued from atresia, since we found that the number of follicles recruited into the ovulatory wave was not increased by short-term supplementation.

In **Paper IV** plasma leptin concentrations reached a maximum on the third day after the start of supplementation, as has been reported previously in ewes supplemented with lupin (Muñoz-Gutierrez *et al.*, 2002). We found a daily association between insulin and leptin concentrations in ewes that received short-term nutritional supplementation, as reported previously (for a review, see Poretsky *et al.* (1999); Marie *et al.*, 2001). If the increased concentrations of leptin and insulin are important signals for follicle development, then this effect is acute since both hormones decreased before the end of the supplementation period, probably due to the equilibrium of a new energy balance.

Follicles efficiently aromatize androstenedione to oestradiol until they reach maximum diameter, after which the aromatase activity decreases (Souza *et al.*, 1997a; Bartlewski *et al.*, 1999; Viñoles *et al.*, 1999b). In **Paper III** maximum concentrations of androstenedione and oestradiol occurred concomitantly. However, in supplemented ewes a second androstenedione increase was observed, probably as a consequence of the prolonged life span of the last non-ovulatory follicle that had decreased aromatase activity. We speculate that the nutritional treatment changed the concentrations of metabolites and/or metabolic hormones, thus promoting a short-term decrease in androstenedione production in the middle of the treatment period (Downing *et al.*, 1999). However, in **Paper IV** we found that increased leptin concentrations on the third day after the start of supplementary feeding were not associated with lower androstenedione concentrations. The decrease in progesterone concentrations in supplemented ewes (**Paper III**) may be associated with an increase in LH pulse frequency. Since androstenedione production by the follicle depends upon LH stimulation, it may be that the frequency of blood sampling was not adequate to visualize such an increase.

An increase in ovulation rate promoted by supplementation with lupin was not associated with changes in plasma IGF-I concentrations (Downing *et al.*, 1995a). In **Paper IV** plasma IGF-I concentrations were similar between supplemented and control ewes. However, circulating concentrations of IGF-I may not be a good indicator of the amount available to the follicle. In cattle it has been shown that IGF-I availability is regulated by proteases produced by granulosa and theca cells, which induce proteolytic activity on the binding proteins (BP) (Roche, 1996;

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Mihm & Austin, 2002). In sheep follicular growth is accompanied by a decrease in IGFBP-2 and IGFBP-4 (Monget & Monniaux, 1995). If IGF-I is involved in the response of the follicles to improved nutrition, then studies at the follicular level are necessary to clarify its role. However, the increased frequency of follicles >6 mm in diameter in supplemented compared with control ewes (**Paper III**) may be indirect evidence of the stimulatory effect of IGF-I on granulosa cells and may explain the presence of a more active dominant follicle in supplemented ewes.

Integration of the major findings of this work

The ovary forms the core of the scheme presented in Figure 9, where the static and the immediate effects of nutrition influence the follicular dynamics, and the changes that occur on follicle steroidogenesis can be translated at the central level by the feedback mechanisms.

In the present work the increase in ovulation rate promoted by the static effect of nutrition was associated with a decrease in oestradiol production by the follicle that reduced the negative feedback, promoting an increase in FSH, which in turn stimulated the development of more gonadotrophin-responsive follicles and the selection of extra follicles to ovulate. The immediate effect of nutrition was not associated with changes in the steroidogenic capacity of the follicle and as a consequence the feedback mechanisms remained unchanged. The increase in ovulation rate promoted by the immediate effect of nutrition is most probably due to an increase in glucose, insulin and leptin. Acting at the ovarian level they may increase the capacity of the follicles to utilize FSH, which is when follicles are rescued from atresia resulting in an increase in ovulation rate.

The conceptualization presented in Figure 9 is meant to emphasize that the static and immediate effects of nutrition can be additive. The common endocrine signal is probably leptin. At the ovarian level leptin modulates insulin actions on steroidogenesis. In ewes in high body condition receiving short-term supplementation, glucose, insulin and leptin acting on a larger population of gonadotrophin-responsive follicles may increase the number of follicles selected to ovulate.

The development of a more active dominant follicle may be part of the same mechanism, reflecting the ability of nutrition to enhance the utilization of low FSH concentrations by the follicle. The variability between gonadotrophin-dependent follicles in requirements of FSH, both within and between animals is large. For this reason, although short-term supplementation may provide all the follicles with the same opportunity to increase their utilization of FSH, the response may depend upon the threshold levels of individual follicles. The improved endocrine environment may be effective in increasing ovulation rate in a proportion of the ewes, while in the majority of the animals it may promote the development of a more active dominant follicle.

If the two effects of nutrition are the same response measured at different times, then an increase in the number of follicular waves and an increase in the number of gonadotrophin-responsive follicles are expected to occur if feed is supplemented for a sufficient time to promote an increase in body condition.
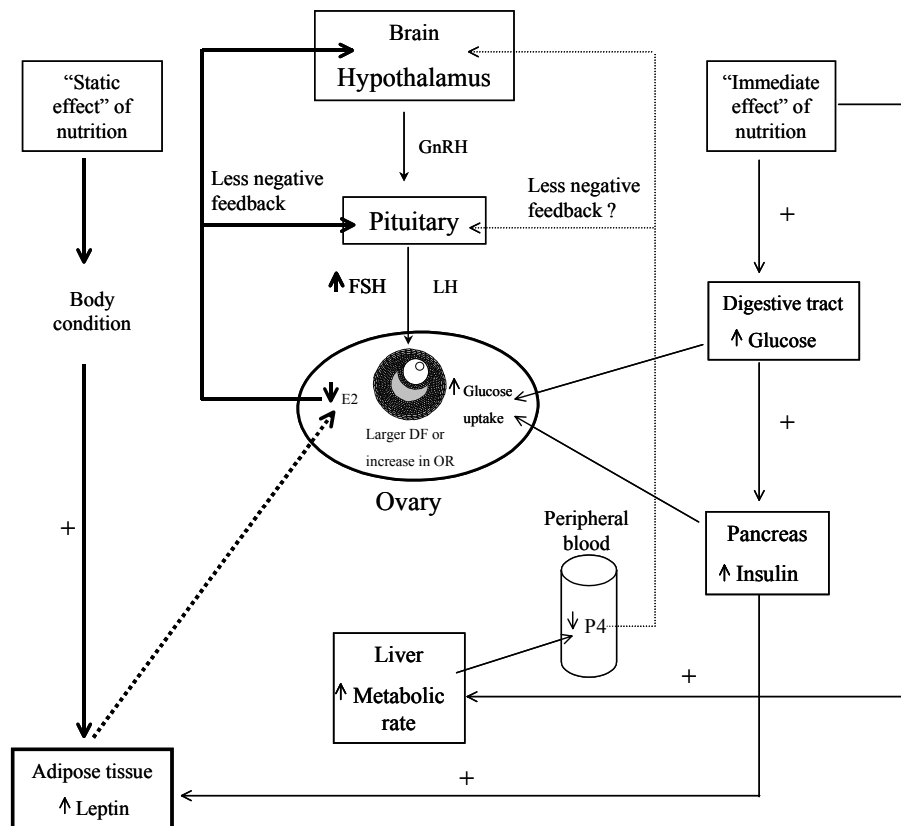
Figure 9. Schematic representation of the "static effect" (left) and the "immediate effect" (right) of nutrition on ovarian activity. E2 = oestradiol; P4 = progesterone; DF = dominant follicle; OR = ovulation rate.

Conclusions

- Body condition influences the pattern of follicular dynamics through changes in the endocrine milieu

- Ewes in high body condition had significantly larger pool of small antral follicles in the ovaries than did ewes with low body condition

- Ewes in high body condition had higher FSH and lower oestradiol concentrations during the follicular phase, which allowed an extended period of follicular recruitment and an increase in ovulation rate

- Ultrasound scanning of ewes in standing position provides a practical and accurate method for determination of the number of corpora lutea and also, for studying the growth profile of large follicles

- The predictive value and sensitivity of ultrasonography are lower for follicles of small diameter

- Short-term nutritional supplementation reduced the number of waves that developed during the inter-ovulatory interval owing to the increase in the life span of the last non-ovulatory follicle

- Nutritionally supplemented ewes with a single ovulation developed a more active dominant follicle than did control ewes

- Short-term nutritional supplementation from day 8 to day 14 of the oestrous cycle increases ovulation rate in ewes in moderate to high body condition

- The effect of short-term supplementation on ovulation rate may be mediated through an increase in the concentrations of glucose, insulin and leptin

Future perspectives

The increase in ovulation rate obtained with the diet used in our experiment is promising. However, more research involving larger numbers of animals may be needed to prove the efficacy of this technique under field conditions. There is a need to stimulate the interest of the animals in the supplement, to increase the proportion of animals eating the extra food. Our data from the field trial suggest that short-term nutritional supplementation based on corn grain and soybean meal may be more effective in ewes with a moderate body condition score. However, since this had not been an a priori hypothesis the experimental design was not adequate to evaluate this phenomenon. It was not our objective to evaluate the impact of this treatment on the number of lambs born. However, this is the key factor that will increase the productivity of the flock, and needs to be tested in the future. Any attempt to increase reproductive performance by increasing prolificacy should be accompanied by identification of ewes bearing multiple foetuses (e.g. by ultrasonography). Good nutrition in late pregnancy of ewes carrying twins increases birth weight of lambs and the production of colostrum and milk by the ewe, being a key factor in ensuring a vigorous ewe-lamb unit and consequently, lamb survival (Lindsay *et al.*, 1990).

The more likely mechanism by which the immediate effect of nutrition influences ovulation rate involves an interplay between glucose and metabolic hormones acting at the ovarian level. The metabolic hormones play an important role in regulation of steroidogenesis by the follicle. Androstenedione appears to play an important role as an atretogenic factor, since immunization against this steroid consistently increases ovulation rate in sheep (Scaramuzzi *et al.*, 1977; Scaramuzzi, 1979; Scaramuzzi & Martin, 1984). However, our bleeding protocol was not frequent enough to evaluate the effects of the decreased progesterone concentration on LH pulse frequency and its effects on steroid production. This is a hypothesis than needs to be further explored.

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"Llegar al objetivo es importante si hemos asimilado las lecciones del recorrido. La conquista, además, se transforma en un nuevo punto de partida para iniciar otra búsqueda y continuar aprendiendo..."

Paulo Coelho

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