Short-term nutritional supplementation of ewes in low body condition affects follicle development due to an increase in glucose and metabolic hormones

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Abstract

This study tested whether the effects of a short period of nutritional supplementation given to ewes during the luteal phase on follicle development and ovulation rate is associated with an increase in circulating concentrations of FSH, glucose or metabolic hormones. Oestrus was synchronised with two prostaglandin injections given 9 days apart and the supplement consisted of corn grain and soybean meal. Corriedale ewes with low body condition were randomly assigned to 2 groups: the control group (C; $n = 10$) received a maintenance diet while the short-term supplemented group (STS; $n = 10$) received double the maintenance diet over days 9 to 14 of the oestrous cycle (day 0 = ovulation). Ovaries were examined daily by ultrasound and blood was sampled three times a day during the inter-ovulatory interval for measuring reproductive and metabolic hormones. On days 9, 11 and 14 of the oestrous cycle, half of the ewes from each group ($n = 5$) were bled intensively to determine the concentrations of glucose, insulin, IGF-I and leptin. Plasma FSH, progesterone, oestradiol and androstenedione concentrations were similar among groups. Dietary supplementation increased plasma insulin concentrations from the first to the sixth day of supplementation and increased glucose concentrations on the third day, compared with control ewes. Plasma leptin concentrations were higher in STS ewes from the second to the fifth day of supplementation. The pattern of IGF-I concentrations was similar among groups. In STS ewes, the nutritional treatment prolonged the lifespan of the last non-ovulatory follicle, so fewer follicular waves developed during the cycle. In STS ewes, increased concentrations of glucose, insulin and leptin one day before ovulatory wave emergence were associated with increased numbers of follicles growing from 2 to 3 mm and with stimulation of the dominant follicle to grow for a longer period. We suggest that the mechanism by which short-term nutritional supplementation affects follicle development does not involve an increase in FSH concentrations, but may involve responses to increased concentrations of glucose, insulin and leptin, acting directly at the ovarian level. This effect is acute, since concentrations of all three substances decrease after reaching peak values on the third day of supplementation. The status of follicle development at the time of maximum concentrations of glucose and metabolic hormones may be one of the factors that determines whether ovulation rate increases or not.

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Introduction

Nutrition is one of the most important factors affecting ovulation rate. For example, supplementation for 4–6 days with lupin grain, a high energy and high protein supplement, is sufficient to increase the ovulation rate in sheep (Gherardi & Lindsay 1982, Oldham & Lindsay 1984, Stewart & Oldham 1986). This effect is not associated with a specific component in the lupin grain but to

an increase in energy-yielding nutrients (Teleni *et al.* 1989b, Downing & Scaramuzzi 1991). To be effective, short-term supplements have to be fed on days 9 to 13 of the oestrous cycle or 6 days before luteolysis (Nottle *et al.* 1985, 1990, Stewart & Oldham 1986, Downing *et al.* 1995). This is the time range when the ovulatory wave emerges (for review see Viñoles 2000). However, the wave-like pattern of follicle development was not clear

when the critical period of supplementation was described, so it was not possible to associate daily follicular growth with the endocrine and metabolic responses to the nutritional treatment.

Data on the effects of nutritional stimuli on follicle-stimulating hormone (FSH) levels during the cycle are equivocal, with reports for (Rhind *et al.* 1985, Rhind & McNeilly 1986) and against (Findlay & Cumming 1976, Rhind *et al.* 1989, Xu *et al.* 1989, Smith & Stewart 1990) the induction of a change. When we used ultrasonography to study the static effect of nutrition on daily follicular development, we found that the increased ovulation rate in ewes with high body condition was associated with increased FSH and decreased oestradiol concentrations during the follicular phase (Viñoles *et al.* 2002). We suggested that lower oestradiol concentrations inhibited FSH less, thus allowing more gonadotrophin responsive follicles to grow and become selected to ovulate.

An alternative mechanism of the immediate nutritional effect on follicular growth might involve direct actions at the ovarian level of glucose and metabolic hormones, such as insulin-like growth factor I (IGF-I) and leptin, since glucose transporter proteins and specific receptors for these hormones are present in the follicles (Williams *et al.* 2001, Muñoz-Gutiérrez *et al.* 2004). Since the final stages of follicle growth are the most sensitive to low FSH levels, but increased FSH concentrations are not a consistent finding, it has been proposed that nutrition changes the ability of gonadotrophin-dependent follicles to use the small amounts of FSH that are available (Scaramuzzi & Radford 1983, Souza *et al.* 1997). Insulin and IGF-I, the concentrations of which increase after a short-term supplementation, could fulfill this role because they increase responsiveness to gonadotrophins and suppress apoptosis in follicles (Monget & Martin 1997, Poretsky *et al.* 1999, Scaramuzzi *et al.* 1999, Williams *et al.* 2001). Leptin influences whole-body glucose homeostasis and the action of insulin, and its concentrations are sensitive to short-term alterations in food intake (Cunningham *et al.* 1999, Marie *et al.* 2001). Insulin stimulates the secretion of leptin by adipocytes and, by promoting lipogenesis, it may indirectly increase leptin production (Poretsky *et al.* 1999). Metabolic hormones also regulate steroidogenesis: glucose and insulin infused together decrease it, while IGF-I infusion is stimulatory (Scaramuzzi *et al.* 1999). Leptin antagonizes the stimulatory effect of insulin on theca cell steroidogenesis, ultimately leading to a decrease in oestradiol secretion (Spicer & Francisco 1997).

The aim of the present study was to test whether a 6-day nutritional supplement, applied over days 9 to 14 of the cycle, affected follicle development and ovulation rate through an increase in circulating FSH concentrations, and whether these responses were associated with increases in circulating concentrations of glucose, insulin or leptin.

Materials and Methods

Animals

The experiment was conducted at the experimental farm of the Agronomy Faculty in Cerro Largo, Uruguay (latitude 31.5° S) during the period February to April 2002, using 20 Corriedale ewes aged 4.6 ± 0.2 years (mean $\pm$ S.E.M.) that had had one lamb the previous year. They had a body weight of 36.4 ± 0.6 kg and a body condition score of 1.8 ± 0.1 units (1 = emaciated, 5 = obese; Suiter 1994). The body condition of the animals was selected based on the experimental model applied to male sheep to study the effects of nutrition on reproduction (Blache *et al.* 2002). Body condition and body weight were measured weekly. Ewes were randomly assigned to 2 groups: the control group (C; $n = 10$) received a maintenance diet, while the short-term supplemented group (STS; $n = 10$) received double the maintenance diet for 6 days, from days 9 to 14 of the cycle (day 0 = ovulation). During the experimental period, all ewes received the diet in one meal at 1200 h, except during the 6-day supplementation period when STS ewes received two meals of equal amounts at 1200 h and 1300 h.

During the experiment, the animals were maintained under 13 h light per day. The mean temperature inside the barn was $20.1 \pm 0.5^\circ\text{C}$, $26.4 \pm 0.5^\circ\text{C}$ and $24.8 \pm 0.5^\circ\text{C}$ at 0730 h, 1400 h and 1930 h respectively. Four weeks before the beginning of the experiment, ewes were placed in individual pens (dimensions: 1.21 m $\times$ 1.51 m) where they were kept. The sequential order of the animals was considered for all procedures.

Diets

Ewes were fed a maintenance diet (AFRC 1993) formulated for restricted intake (46 g dry matter (DM) per kg of metabolic body weight; $\text{BW}^{0.75}$) to avoid refusals. The basal diet consisted of 70% hay (*Trifolium alexandrinum*) and 30% concentrate (80% corn grain and 20% soybean meal). This allocation supplied 6.4 MJ metabolisable energy (ME) and 94 g crude protein (CP) per ewe daily. The ME of the diet was calculated by the equation elaborated by MAAF (1975) and dry matter digestibility (DMD) was calculated by applying the equation described by Oddy *et al.* (1993). Hay was chopped into a particle size of 2 to 5 cm. The chemical composition of the components of the diet is presented in Table 1.

Table 1 Chemical composition of the different components of the diet: dry matter (DM), ash, acid detergent fibre (ADF), crude protein (CP) and fat (values on a dry matter basis).

	DM (g/kg)	Ash (g/kg)	ADF (g/kg)	CP (g/kg)	Fat (g/kg)
Hay	843	79	408	119	25
Corn grain	882	15	46	93	36
Soybean meal	889	65	95	476	17

Water was available to ewes *ad libitum*. Mineral salt (8 g crystalline salt, El Valor, Canelones, Las Brujes Uruguay) and bicarbonate (2% of total diet) were added to the diet. Refusals were removed at 0700 h of the following day and were low throughout the experimental period (less than 10% for hay and concentrates).

Experimental design

A schematic representation of the experimental design is shown in Fig. 1. Oestrus was synchronised with two intramuscular injections of prostaglandin (PG; 30 µg Cloprostenol, Dalmaprost-D, Fatro Laboratory, Montevideo, Uruguay) given 9 days apart. Oestrous behaviour was detected by testing the ewes every 12 h with vasectomised rams from the day of the second PG injection and from day 15 of the next oestrous cycle. Daily ultrasound examinations began on the day of the second PG injection and continued until 7 days after the second ovulation (Fig. 1). Ovulation was detected by the collapse of a large follicle and was considered to be day 0 of the oestrous cycle. During the second period of oestrous detection, ultrasound examinations were increased to every 12 h from oestrous behaviour until the detection of the second ovulation.

Ultrasound examinations

Transrectal ultrasound examinations were conducted using a real-time, B-mode scanner (Aloka SSD 500 Echo camera, Overseas Monitor Corp. Ltd, Richmond, BC, Canada) and a rigid 7.5 MHz transducer adapted to be manipulated externally in the rectum (Rubianes *et al.* 1997). The total number, the diameter and the position

of all follicles ≥ 2 mm in diameter and all corpora lutea (CL) were assessed in both ovaries each day. A follicular wave was defined as one or more follicles growing to at least 5 mm in diameter. Groups of follicles emerging within 48 h were regarded as a single follicular wave. The subordinate follicle was defined as a follicle that reached 3 mm and could be followed by ultrasonography for at least 3 days. Morphological characteristics of follicular waves were described based on the following parameters of the largest follicle: day of emergence, maximum diameter, lifespan, interwave interval. These variables have been defined previously (Viñoles *et al.* 2001). The day of wave emergence was the day the largest follicle of the wave was retrospectively identified at 3 mm in diameter. Time of deviation was defined as the beginning of the greatest difference in diameter changes between the two largest follicles, at the examination when the second largest follicle reached its maximum diameter (adapted from Ginther *et al.* 1996). The morphological growing phase was defined as the number of days from emergence to maximum diameter of the largest follicle of each wave, and the morphological dominance phase as the number of days from deviation to the emergence of the next wave. Ovulation rate was determined by counting the number of CL 7 days after ovulation. The predictive value and sensitivity of ultrasound for evaluating the presence or absence of CL is 100% and 96% respectively. The predictive value of ultrasound for the number of follicles is 98–100% for 2 mm, 4 mm and ≥ 5 mm diameter follicles except for those of 3 mm diameter (71%). The sensitivity of ultrasonography is high for all sized follicles (90–95%) except those of 2 mm diameter (62%; Viñoles *et al.* 2004).

Figure 1 Schematic representation of the experimental design. PG, injection of a prostaglandin analogue; *, glucose measurements.

Blood sampling for glucose and hormonal measurements

From the day of the second PG injection until the day of the second ovulation, blood was collected in all ewes three times a day into heparinised tubes. Blood was sampled in relation to feeding time (hour 0) at -5 h, 1.5 h and 7 h. To describe acute changes in metabolic hormones and metabolites in relation to feeding, in half of the ewes from each group ($n = 5$) blood was sampled on days 9, 11 and 14 of the cycle. Samples were taken via an indwelling jugular catheter for a period of 24 h – every hour from 2 h before until 10 h after feeding and every second hour thereafter. Frequency was increased to every 30 min in the first 2 h after feeding (Fig. 1). The volume of blood taken each time was 5 ml. Blood was placed in heparinised tubes and maintained on ice until it was centrifuged within 10 min of collection. Plasma was stored at -20°C until assayed.

Progesterone concentrations were estimated in all samples by a direct solid-phase radioimmunoassay (RIA) using DPC kits (Diagnostic Product Co., Los Angeles, CA, USA) as previously described (Meikle *et al.* 1997). The RIA had a sensitivity of 0.2 nmol l^{-1} . The intra-assay coefficients of variation for low (3 nmol l^{-1}), medium (24 nmol l^{-1}) and high (50 nmol l^{-1}) controls were 5%, 6% and 3% respectively. The corresponding inter-assay coefficients of variation were 12%, 10% and 4% respectively.

Oestradiol- 17β concentrations were determined in all samples by RIA using DPC kits (oestradiol double antibody, KE2D, Diagnostic Product Co.) as previously described (Meikle *et al.* 1997). The detection limit of the assay was 4 pmol l^{-1} . The intra-assay coefficient of variation was 25% at 5 pmol l^{-1} and $<10\%$ for concentrations between 11 and 180 pmol l^{-1} . The inter-assay coefficients of variations for three control samples were 30% (7 pmol l^{-1}), 11% (33 pmol l^{-1}) and 16% (63 pmol l^{-1}).

Plasma FSH concentrations were determined in samples taken at -5 h and 7 h with respect to feeding by RIA (Viñoles *et al.* 1999). The sensitivity of the assay was $0.2\text{ }\mu\text{g l}^{-1}$. The intra-assay coefficient of variation was 9% at $3\text{ }\mu\text{g l}^{-1}$, 10% at $5\text{ }\mu\text{g l}^{-1}$ and 9% at $7\text{ }\mu\text{g l}^{-1}$. The inter-assay coefficients of variations for three control samples were 13% ($3\text{ }\mu\text{g l}^{-1}$), 13% ($5\text{ }\mu\text{g l}^{-1}$) and 14% ($7\text{ }\mu\text{g l}^{-1}$).

Androstenedione concentrations were determined twice a day by RIA (DSL-4200, Diagnostic Systems Laboratories, Inc., Texas, TX, USA) in samples taken at -5 h and 7 h daily, from the day of emergence of the last non-ovulatory wave to ovulation. To increase the sensitivity of the assay, $150\text{ }\mu\text{l}$ tracer and $25\text{ }\mu\text{l}$ antibody were used (50% binding for the 0 standard). On the first day, samples ($250\text{ }\mu\text{l}$) were extracted with 2.5 ml ether and $500\text{ }\mu\text{l}$ of the standard (1725 pmol l^{-1}) was extracted in 4.5 ml ether. A pool of plasma from ewes known to have low (110 pmol l^{-1}), medium (225 pmol l^{-1}) and high (385 pmol l^{-1}) androstenedione concentrations from previous studies were used as quality controls. After extraction, $25\text{ }\mu\text{l}$ PBS were added

to each tube and $1000\text{ }\mu\text{l}$ PBS were added to the standards. The samples and standards were incubated overnight at 4°C . On the second day, the standard curve was made by serial dilutions of the standard (1:2). The standard curve ranged from 7 pmol l^{-1} to 432 pmol l^{-1} . An aliquot of $25\text{ }\mu\text{l}$ from each dilution was used in the standard curve. The antibody and tracer were added to the samples and the standard curve and incubated overnight at room temperature. On the third day, $500\text{ }\mu\text{l}$ precipitating reagent were added to the tubes and, after incubation for 30 min at room temperature, the tubes were centrifuged for 30 min at 4°C . The detection limit of the assay was 16 pmol l^{-1} . The intra-assay coefficients of variation were 7% for the low, 3% for the medium and 9% for the high control. The inter-assay coefficients of variations for the low, medium and high controls were 14%, 6% and 9% respectively.

IGF-I concentrations in plasma were measured in all samples by double-antibody RIA (Gluckman *et al.* 1983). Interference by binding proteins was minimised by acid-ethanol cryoprecipitation, as validated for ruminant samples (Breier *et al.* 1991). The samples were assayed as duplicate $100\text{ }\mu\text{l}$ aliquots and the limit of detection was 3.0 ng/ml . Six replicates of two control samples containing 7.7 and 27.7 ng/ml were included in the assay and were used to estimate the intra-assay variation (10.5% and 6.9%).

Leptin was analysed in all samples by RIA using antibodies raised in an emu (Blache *et al.* 2000). The samples were assayed as duplicate $100\text{ }\mu\text{l}$ aliquots and the limit of detection was 0.1 ng/ml . Six replicates of three control samples containing 1.3 , 1.6 and 2.4 ng/ml were included in the assay. The intra-assay coefficients of variation were 6.9%, 7.9% and 10.2%. All the samples were analysed in one assay.

Insulin concentrations were measured in all samples by a direct solid-phase radioimmunoassay (RIA) using DPC kits (Diagnostic Product Co.). According to the manufacturer, the sensitivity of the assay is 8.6 pmol l^{-1} . For low (14 pmol l^{-1}), medium (251 pmol l^{-1}) and high (667 pmol l^{-1}) controls, the intra-assay coefficients of variation were 11%, 5% and 2% and the inter-assay coefficients of variation were 38%, 7% and 4% respectively.

Glucose concentrations were measured in fresh blood from one hour before until six hours after feeding (10 samples each day, Fig. 1) using an Elite glucose meter (Bayer, Montevideo, Uruguay). The normal reading was in the range of 20 to 600 mg/dl l^{-1} .

Statistical analyses

The number of follicular waves and the number of ovulatory follicles in three size classes ($\leq 5\text{ mm}$, 6 mm and $>6\text{ mm}$) were compared using Fisher's exact probability test. The morphological characteristics of the largest follicle of the last non-ovulatory wave and the ovulatory wave were compared by analysis of variance using

the mixed procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). The model included the fixed effect of treatment and the random effect of ewe within group. For the analysis of repeated measurements, the mixed procedure of the SAS was used. The model included the fixed effects of treatment and day, and their interaction. The covariance structure was modelled using the random effect of ewe within group plus autoregressive order 1, to account for the correlation between sequential measurements within the same animal (Littell *et al.* 2000). Data with repeated measurements included: (1) body weight, (2) body condition, (3) plasma concentrations of progesterone, FSH, oestradiol, IGF-I and leptin, all of which were analysed considering the period before treatment (day 0 to day 8) as a covariate in the model, and (4) plasma concentrations of glucose, insulin, leptin and IGF-I included the fixed effect of time after feeding in the model. Mean values were compared by the method of Least Square Means. The relationships between increases in FSH concentrations and follicular wave emergence were analysed using a skewness method (Viñoles *et al.* 2002). Profiles of oestradiol and androstenedione associated with the growing profile of the largest follicle of the last two waves of the interovulatory interval were studied individually. For each ewe and each hormone, the baseline and standard deviation of the baseline value were calculated as the average of the lowest 25% of all values measured. The time at which the concentration of a hormone began to increase was defined as the time when at least two consecutive values increased above the baseline by threefold the standard deviation of the baseline. Factors affecting plasma leptin concentrations were evaluated by multiple regression analysis using a backwards elimination procedure in SAS, using the data obtained during the frequent bleeding. The mean values for concentrations of leptin, glucose, insulin, and IGF-I from -2 to 6 h after feeding (period 1; $n = 30$), and for leptin, insulin and IGF-I from 7 and 24 h after feeding (period 2; $n = 30$), were calculated. The dependent variable was plasma leptin concentrations and the independent variables included group, day, period and plasma concentrations of glucose, insulin and IGF-I. Since the effects of period and glucose concentrations were not significant in the model, a mean for the 24-h period was calculated for leptin, insulin and IGF-I and included in the final model ($n = 30$). Data are presented as least square means $\pm$ S.E.M. Differences were considered significant if $P < 0.05$.

Results

Body weight and body condition score

Body condition score and body weight were similar in groups C (1.8 ± 0.1 and 36.3 ± 0.7 kg respectively) and STS (1.9 ± 0.1 and 36.0 ± 0.7 kg respectively) during the period before treatment, but body weight was higher in the STS group (38.3 ± 0.7 kg) than in the C group

(36.2 ± 0.7 kg; $P < 0.01$) during the period after nutritional treatment, perhaps due to the increased ruminal and intestinal content in the supplemented animals.

Follicular measurements

Ewes from the STS group developed fewer follicular waves than ewes from the C group (2.9 ± 0.1 vs 3.4 ± 0.2 ; $P < 0.05$). In the STS group, 9 of the 10 ewes developed three follicular waves while the other ewe developed two waves during the cycle. Ewes in the control group developed three ($n = 6$) and four ($n = 4$) follicular waves. The mean time of emergence of the last non-ovulatory wave and the ovulatory wave was on days 5.5 ± 0.7 and 11 ± 0.7 for the STS group and on days 5.8 ± 0.7 and 12 ± 0.7 for the C group respectively.

Table 2 shows the morphological characteristics of the last non-ovulatory follicle and the ovulatory follicle for each group. The last non-ovulatory follicle had a longer lifespan in the STS group (Fig. 2 and Table 2). The ovulatory follicle had a longer morphological growing phase and its morphological dominance phase ($P = 0.06$) and lifespan ($P = 0.07$) tended to be longer in STS ewes (Fig. 2).

Ovulation rate was similar in the STS (1.1 ± 0.3) and C groups (1.1 ± 0.3). The number of follicles recruited into the ovulatory wave was similar among groups. However, in the STS group, more follicles that had been recruited into the ovulatory wave grew from 2 to 3 mm (2.0 ± 0.4 vs 0.9 ± 0.4) and less follicles grew from 2 to 4 mm (2.0 ± 0.3 vs 3.0 ± 0.3). More ewes had ovulatory follicles of 6 mm (7/11) and > 6 mm (3/11) in the STS group than in the C group (5/11 and 1/11 respectively; $P < 0.05$).

Progesterone, FSH, oestradiol and androstenedione concentrations

Figure 3 shows that the concentrations of progesterone, oestradiol and FSH were similar among groups from day 9

Table 2 Morphological characteristics of the last non-ovulatory follicle and the ovulatory follicle (emergence = day 0) in ewes that received a short-term nutritional supplement (STS, $n = 10$) and in control ewes ($n = 10$). Results are least squares means $\pm$ S.E.M.

	STS group	Control group
Last non-ovulatory follicle		
Deviation (day)	1.9 ± 0.4	1.5 ± 0.4
Morphological growing phase (day)	2.8 ± 0.5	3.4 ± 0.5
Morphological dominance phase (day)	4.8 ± 0.5	3.8 ± 0.5
Lifespan (days)	10.2 ± 0.6^a	7.8 ± 0.6^b
Ovulatory follicle		
Deviation (day)	1.2 ± 0.4	1.8 ± 0.4
Morphological growing phase (day)	4.5 ± 0.4^a	3.4 ± 0.4^b
Morphological dominance phase (day)	5.2 ± 0.5^x	3.7 ± 0.5^y
Lifespan (days)	6.0 ± 0.3^x	5.1 ± 0.3^y

^{a,b}Means within rows with different superscripts are significantly different ($P < 0.05$); ^{x,y}means within rows with different superscripts tended to be different ($P > 0.05$).

Figure 2 Growth profile of the last non-ovulatory follicle (LNOF) and the ovulatory follicle (OF) in ewes that received a short-term nutritional supplement (STS, $n = 10$, ●) and in ewes fed at maintenance level ($n = 10$, ○). Results are least squares means $\pm$ S.E.M.

to day 17 of the oestrous cycle (Fig. 3). Concentrations of FSH reached a maximum ($2.3 \pm 0.2 \mu\text{g l}^{-1}$) 1.3 $\pm$ 0.2 days before the emergence of the ovulatory wave, remained high for 1.6 ± 0.2 days and fell to minimum values 2.5 $\pm$ 0.2 days after wave emergence. Oestradiol concentrations reached a maximum ($19.5 \pm 0.9 \text{ pmol l}^{-1}$) 4.1 $\pm$ 0.3 days after emergence and remained high for 2.9 ± 0.2 days. Androstenedione concentrations reached a maximum ($356 \pm 30 \text{ pmol l}^{-1}$) 3.9 $\pm$ 0.4 days after emergence and remained high for 1.0 ± 0.1 days.

Leptin and IGF-I concentrations

Figure 3 shows that leptin concentrations were higher in STS than in C ewes from day 10 to day 13 of the oestrous cycle ($P < 0.05$). In both groups, leptin concentrations started to increase on day 9 of the oestrous cycle and then decreased from day 11 to the end of the oestrous cycle.

Plasma leptin concentrations fluctuated with time relative to feeding but there was no clear post-prandial surge (Fig. 4). In the STS group, leptin concentrations were higher on day 11 than on days 9 and 14 while, in the C group, leptin concentrations were lower on day 14 than on days 9 and 11 ($P < 0.05$), in agreement with the observed decrease in leptin concentrations from day 11 of the cycle (Fig. 3). Factors affecting plasma leptin concentrations were group, day and plasma insulin concentrations ($P < 0.001$; $r^2 = 0.5$; $n = 30$). The effects of period (-2 to 6 h and 7 to 24 h after feeding) and glucose concentrations were not significant.

The IGF-I pattern was similar in STS and control ewes (Fig. 3). IGF-I concentrations decreased from days 9 to 11–12 ($P < 0.05$) and increased from day 14 to day 17 of the oestrous cycle ($P < 0.001$). The effects of time after feeding, day and their interaction were all significant ($P < 0.001$). Figure 4 shows that IGF-I concentrations were higher on day 9 than on days 11 and 14 in both STS and control ewes ($P < 0.001$), in agreement with the pattern observed during the oestrous cycle (Fig. 3).

Glucose and insulin concentrations

Glucose concentrations decreased in both groups from -1 h to 1.5 h relative to feeding ($P < 0.05$) and increased gradually from 2 to 5 h after feeding ($P < 0.001$) on days 9, 11 and 14 (Fig. 4). In the control group, glucose concentrations were similar on days 9 and 11 but increased on day 14 ($P < 0.001$). In STS ewes, glucose concentrations on days 11 and 14 were higher than on day 9 ($P < 0.001$). Glucose concentrations on day 11 were consistently higher in STS than in control ewes (Fig. 4).

Plasma insulin concentrations also changed after feeding and the changes were more pronounced in STS ewes. In the control ewes, insulin concentrations were similar on days 9 and 11 but increased on day 14 ($P < 0.001$; Fig. 4). In STS ewes, insulin concentrations increased from day 9 to day 11 and decreased on day 14, although the values on day 14 were still higher than those on day 9 ($P < 0.001$). Plasma insulin concentrations were higher in STS than in C ewes on days 9, 11 and 14 ($P < 0.001$; Fig. 4), with the greatest difference between groups being observed on day 11.

Discussion

Short-term supplementation with corn grain and soybean meal from days 9 to 14 of the oestrous cycle prolonged the lifespan of the last non-ovulatory follicle and thus decreased the number of follicle waves. This effect was not associated with changes in FSH concentrations, but it was correlated with an increase in the circulating concentrations of glucose, insulin and leptin. The short-term supplementation did not affect ovulation rate.

The static effect of nutrition is consistently associated with an increase in ovulation rate (Rhind & McNeilly 1986, Rhind *et al.* 1989, Xu *et al.* 1989). We have previously found that ewes in high body condition have a high ovulation rate which is accompanied by high FSH and low oestradiol concentrations during the follicular

Figure 3 Changes in the concentrations of progesterone (diamonds) and oestradiol (triangles; top panel), FSH (triangles, middle panel), and leptin (circles) and IGF-I (squares; bottom panel) from days 9 to 17 of the oestrous cycle in ewes that received a short-term nutritional supplement (STS, $n = 10$) from day 9 to 14 of the oestrous cycle (solid symbols) and in ewes fed at maintenance level ($n = 10$, open symbols). Results are least squares means $\pm$ S.E.M.

phase, compared with ewes in low body condition (Viñoles *et al.* 2002). However, the effectiveness of the immediate effect of nutrition in increasing ovulation rate is not consistent (Stewart 1990). In the present study, a 6-day nutritional treatment, fed from days 9 to 14 of the oestrous cycle, did not affect ovulation rate. However, a 7-day supplement fed from days 8 to 14 of the oestrous cycle in the same breed and during the same season, increased ovulation rate by 14% (Viñoles 2003). The inconsistent effect of short-term supplementation on ovulation rate suggests that an increase in ovulation rate may

depend on follicular status at the beginning of the nutritional treatment, among other factors.

At the beginning of the treatment, the last non-ovulatory follicle was at the end of its growing phase and the supplementation prolonged its lifespan (Fig. 2). This delayed atresia may have been induced by changes in the circulating concentrations of glucose and metabolic hormones. Insulin concentrations increased from the first day of supplementation, with maximum values for glucose and insulin observed on the third day after supplementation commenced, in agreement with Teleni *et al.* (1989a). Insulin has a direct effect on adipocytes to stimulate leptin secretion (Poretsky *et al.* 1999, Marie *et al.* 2001). In our study, the supplement increased leptin concentrations from the second to the fifth day of supplementation, with higher values on the third day after the start of feeding. We suggest that increasing concentrations of glucose, insulin and leptin, from the first to the third day of feeding, prolonged the lifespan of the last non-ovulatory follicle and thus delayed the occurrence of atresia, slowing down follicle turnover (fewer follicular waves in supplemented ewes).

The impact of a short-term supplementation at the ovarian level may depend upon hormone dynamics. In supplemented ewes, peak glucose, insulin and leptin concentrations occurred one day before ovulatory wave emergence. Although we are aware of the limitations of ultrasonography for determining follicles in smaller size classes (Viñoles *et al.* 2004), the supplement increased the number of follicles growing from 2 to 3 mm. However, fewer follicles continued to grow up to 4 mm one day after ovulatory wave emergence compared with the untreated controls. If increased concentrations of glucose, insulin and leptin are important signals for the initial stimulation of gonadotrophin-responsive follicles, their concentrations may need to remain high in order to promote the selection of more than one follicle into the ovulatory wave. Still, high but decreasing concentrations of insulin and leptin on the fifth day of supplementation stimulated the ovulatory follicle to grow for a longer period, thus increasing the proportion of ewes with follicles larger than 6 mm. Similarly, Muñoz-Gutiérrez *et al.* (2002) found that the diameter of aromatase-positive follicles was increased by a nutritional supplement (lupins), and follicles >6 mm in diameter were seen only in supplemented ewes. Alternatively, increased concentrations of metabolic hormones favoured the selected follicle which exerted its dominant effect and reduced the number of follicles reaching 4 mm in supplemented ewes.

The changes in follicle development observed in the present study were independent of FSH action. The results of this study and a previous one (Viñoles *et al.* 2002) suggest that the static and the immediate effects of nutrition on follicle development are not mediated through common mechanisms, since, unlike the static effect, the immediate effect of nutrition is not mediated by changes in the FSH–oestradiol feedback system, but by an increase

Figure 4 Concentrations of glucose in entire blood, and plasma concentrations of insulin, leptin and IGF-I, during the first (day 9), third (day 11) and sixth (day 14) days after the start of a short-term supplement in supplemented ewes (●; $n = 5$) and in control ewes fed a maintenance diet (○; $n = 5$). Horizontal dotted line, time of feeding. Results are least square means $\pm$ S.E.M.

in glucose, insulin and leptin concentrations (Fig. 5). Glucose and metabolic hormones act directly at the ovarian level to regulate steroidogenesis (Poretsky *et al.* 1999, Williams *et al.* 2001, Muñoz-Gutiérrez *et al.* 2004). Insulin and glucose infused together decrease the secretion of androstenedione and, to a lesser extent, oestradiol, while leptin can directly attenuate insulin-induced steroidogenesis in theca and granulosa cells (Spicer & Francisco 1997, Downing *et al.* 1999). In this study, androstenedione and oestradiol concentrations were similar among supplemented and control ewes, suggesting that the immediate effect of nutrition is not acting via the regulation of steroidogenesis, but most probably by stimulating

glucose uptake by the follicles (Fig. 5, Muñoz-Gutiérrez *et al.* 2004).

The pool of follicles available for the action of glucose and metabolic hormones may play a key role in stimulating an increase in ovulation rate. Ewes in high body condition had a higher number of gonadotrophin-dependent follicles than did ewes in low body condition (Rhind & McNeilly 1986, Rhind *et al.* 1989, Xu *et al.* 1989, Viñoles *et al.* 2002). Moreover, a better response to nutritional supplementation has been described in ewes that are in high rather than low body condition (Leury *et al.* 1990). We suggest that the lack of an increase in ovulation rate observed in this study may be due to the low body

Figure 5 Schematic representation of the static effect (left) and the immediate effect (right) of nutrition on ovarian activity. The static effect of nutrition promotes: (1) an increase in FSH concentrations, (2) the development of more follicles, and (3) a decrease in oestradiol (E2) concentrations. Acting at the ovarian level, FSH stimulates the development of more follicles and this promotes an increase in ovulation rate (OR). Lower oestradiol production by the follicles is most probably associated with the higher leptin levels inhibiting steroidogenesis in ewes in high body condition. The lower oestradiol concentrations reduce negative feedback at the hypothalamus and pituitary gland, leading to higher circulating FSH concentrations. By contrast, the immediate effect of nutrition promotes: (1) an increase in glucose concentrations, (2) an increase in insulin concentrations, (3) an increase in leptin concentrations, and (4) stimulation of follicular growth. Insulin stimulates glucose uptake by the follicles and an increase in leptin concentrations that together stimulate follicle growth. Insulin-like growth factor I (IGF-I) is probably acting to stimulate follicle growth, due to a local decrease in the concentrations of binding proteins (BP). Increased follicular growth is not associated with changes in steroidogenesis so the feedback mechanism remains unchanged. The scheme presented here is meant to emphasize the possibility that the static and immediate effects of nutrition can be additive. LH, luteinising hormone; GnRH, gonadotrophin releasing hormone.

condition of the animals, since metabolic signals acting on a larger pool of follicles may have a better chance of rescuing follicles from atresia, thus increasing ovulation rate.

IGF-I plays a critical role in the selection of the dominant follicle and stimulates glucose metabolism by acting through the type I receptor (Fortune *et al.* 2004, Muñoz-Gutiérrez *et al.* 2004). In the present experiment, plasma IGF-I concentrations were similar between supplemented and control ewes, as shown in other studies (Downing *et al.* 1995). However, circulating concentrations of IGF-I may not be a good indicator of the amount available to the follicle, since IGF-I availability is regulated by proteases and binding proteins produced at the level of the follicle (Roche 1996, Mihm & Austin 2002, Fortune *et al.* 2004). Short-term nutritional supplementation decreases the percentage of IGF binding protein-2 positive follicles and changes the expression of mRNA IGF-I receptor (Muñoz-Gutiérrez *et al.* 2004) so, if IGF-I is involved in the response of the follicles to improved nutrition, then studies of the follicular microenvironment are necessary to clarify its role.

Our findings suggest that the effect of a short-term nutritional supplement on follicle development may be

mediated by glucose and metabolic hormones acting directly at the ovarian level (Fig. 5). However, the impact of short-term supplementation on ovulation rate may depend on the factors mentioned above, such as follicular status, circulating concentrations of glucose and metabolic hormones, hormone dynamics, and the pool of follicles available for the action of these hormones at the time the supplement is fed. Considering all these factors together, it is not surprising that the outcome of the immediate effect of nutrition on ovulation rate is not consistent between and within laboratories, since the model for studying the effect has not yet been standardised for the female sheep (for review see Viñoles 2003).

We conclude that the effect of short-term nutritional supplementation on follicle development is not mediated by an increase in FSH concentrations, but by increased concentrations of glucose, insulin and leptin acting directly at the ovarian level. This effect is acute, since concentrations decrease after reaching peak values on the third day of supplementation. The status of follicle development at the time of maximum concentrations of glucose and metabolic hormones may be one of the factors that determine whether ovulation rate increases or not in response to nutritional treatment.

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