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The use of a 'first-wave' model to study the effect of nutrition on ovarian follicular dynamics and ovulation rate in the sheep

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Abstract

We have developed an experimental model in which groups of ewes are simultaneously experiencing the first ovarian follicular wave of their oestrous cycle. We used this 'first-wave model' in a 2×2 factorial experiment (ten ewes per group) to study the effect of body condition (BC) and a short-term supplement on follicular dynamics and ovulation rate. The 'first-wave' was established by giving ewes three injections of prostaglandin (PG), 7 days apart. The 6-day supplement (lupin grain) began 2 days after the second PG injection and continued until the third. Follicles were studied by ultrasound, and blood was sampled to measure glucose and hormones. The supplement increased ($P<0.01$) the concentrations of glucose, insulin and leptin, decreased FSH concentrations ($P<0.01$) and tended to increase oestradiol concentrations ($P=0.06$). The supplement tended to increase the number of 3 mm follicles ($P=0.06$). Compared with low-BC ewes, high-BC ewes had more follicular waves ($P<0.05$), higher concentrations of insulin, leptin and IGF1 ($P<0.05$) and tended to have higher FSH concentrations ($P=0.09$). Leptin and insulin concentrations remained high until the end of supplementation in high-BC ewes, whereas they decreased after the third day of supplementation in low-BC ewes. In conclusion, high concentrations of metabolic hormones in fat ewes are associated with the development of more follicular waves. When a supplement is superimposed on this situation, changes in glucose and metabolic hormones allow more follicles to be selected to ovulate.

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Introduction

The effects of nutrition on ovulation rate in sheep were initially embodied in the concept of 'flushing' in which a period of 3 weeks of high-level nutrition, leading to an increase in body mass, was used to increase litter size (Clark 1934). Later, Coop (1966) expanded upon this 'dynamic effect' by describing a second phenomenon, the 'static effect', reflecting the positive relationship between live weight and ovulation rate. In both the static and dynamic effects, ovulation rate appears to increase because nutrition increases the number of gonadotrophin-dependent follicles (Rhind & McNeilly 1986, Rhind *et al*. 1989, Xu *et al*. 1989, Viñoles *et al*. 2002). Subsequently, a third phenomenon, the 'immediate effect', was defined by Smith & Stewart (1990) to cover increases in ovulation rate following nutritional supplements that are provided for only 4–6 days and do not lead to detectable increases in body mass. Some authors suggested that ewes in low body condition (BC) respond better to the immediate effect of nutrition, while others found a better response in heavier ewes (Morley *et al*. 1978, Leury *et al*. 1990). This issue is yet to be resolved and, to date, there are no reports describing the effect of BC score on the response to a 6-day period of supplementation.

The physiological processes that mediate the effect of nutrition on ovulation rate most probably involve interactions between reproductive and metabolic hormones (Muñoz-Gutierrez *et al*. 2002, 2004, 2005, Scaramuzzi *et al*. 2006). Acute nutritional treatments induce dynamic changes in the metabolic homeostatic systems – for example, over the first 3 days of supplementation, the concentrations of glucose, insulin and leptin rise to peak values and then decline (Teleni *et al*. 1989, Viñoles *et al*. 2005). If these responses allow more follicles to remain viable on reduced FSH concentrations, resulting in an increase in ovulation rate, the maximum concentrations of these factors may need to coincide with the decline in FSH concentrations that makes gonadotrophin-dependent follicles susceptible to atresia. This decline in FSH concentrations occurs 3 days after wave emergence in coincidence with maximum oestradiol (E_2) production by the selected follicle (Viñoles *et al*. 1999). Thus, to be effective, nutritional supplementation may need to begin at the time of the emergence of the ovulatory wave.

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Although FSH regulates follicle growth, links between nutritional stimuli and follicular development via changes in blood concentrations of FSH have been difficult to demonstrate. We contend that the most important uncontrolled variable in most experiments is the relationship between the timing of the supplement and the stage of follicle growth (Viñoles *et al.* 2005). Individual ewes can have 2–4 'waves' of follicular development per cycle (Viñoles 2000); so, when a nutritional supplement is applied from days 9 to 14 of the oestrous cycle, ewes will vary with respect to the phase of development of their follicles. The various phases are associated with specific patterns of FSH and E_2 concentration that may also affect the capacity of the follicles to respond to the metabolic stimulus (Viñoles *et al.* 2005).

The effect of nutrition on FSH and E_2 profiles might be elucidated if a single, synchronised follicular wave could be induced in all ewes during the experimental period. Nutritional treatments could then be imposed during the growing phase of the follicles, a moment when the beneficial effects of glucose and metabolic hormones may have an impact of their steroidogenic capacity, thus on FSH concentrations. One option is to make use of the first wave of the ovulatory cycle – it emerges around the time of ovulation, so its growth pattern is very predictable and similar among ewes, and it avoids the problems introduced as varying numbers of waves develop during the progression of the cycle (Viñoles *et al.* 1999, Viñoles 2000). In this study, we have assessed the value of the 'first-wave' model for testing this hypothesis and also tested whether ewes in high BC will respond better to a 6-day lupin supplement than ewes in low BC.

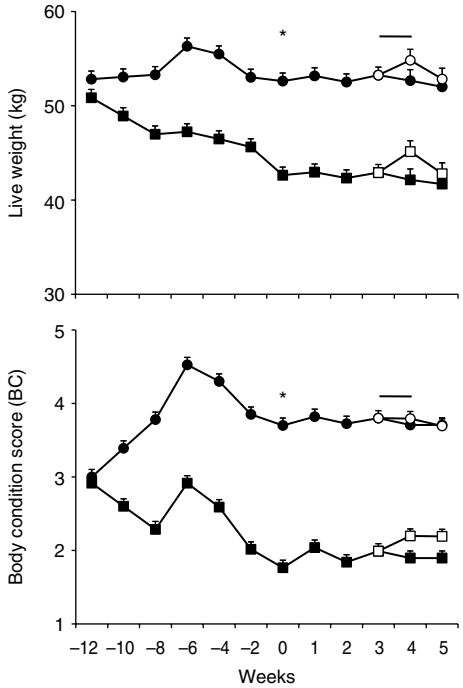

Figure 1 Patterns of change in live weight and BC during the period before (−12 to −2 weeks) and after (0–5 weeks) the time at which high-BC (closed circle) and low-BC ewes (closed square) were moved into the animal house, where they were fed to maintenance diet (weeks 0–3) with half of each group then fed a 6-day supplement with lupin grain (open circle, open square; between weeks 3 and 4). * Ewes were weighed after transport; the black bar indicates the period of supplementation.

Results

Condition score and live weight

The targets for BC (1.8±0.1 vs 3.7±0.1) and live weight (42.6±0.9 vs 52.6±0.9 kg) were reached after 12 weeks of differential feeding (Fig. 1). After they were moved into the animal house (week 0), the low-BC ewes were offered 5.3±0.2 MJ metabolisable energy (ME)/day, and the high-BC ewes were offered 6.1±0.2 MJ ME/day. These dietary regimes maintained live weight and BC at approximately constant values for 3 weeks. During the period of supplementation treatment, the supplemented low-BC ewes were offered 10.8±0.2 MJ ME/day, and the supplemented high-BC ewes were offered 12.4±0.2 MJ ME/day, double their requirements for maintenance. The supplement appeared to induce transient increases in live weight in both groups of ewes, but these effects were not significant ($P>0.05$, Fig. 1). By the end of the supplementation period, BC was higher in low-BC supplemented ewes than in low-BC non-supplemented ewes ($P<0.05$, weeks 4 and 5; Fig. 1), but the high-BC groups did not differ.

The 'first-wave model'

The expected emergence and the actual emergence of the first wave of the cycle were correlated ($r^2=0.96$; $P<0.001$; data not shown), so the model effectively synchronised first-wave emergence among ewes. Thus, for the experiment, the 'first-wave model' was operational, and nutritional treatments effectively began at the time of wave emergence (day 0).

Progesterone

Progesterone (P_4) concentrations increased after ovulation and reached maximum values on the day of the third prostaglandin (PG) injection (Fig. 2). One ewe from the low-condition, supplemented group failed to respond to the third injection of PG, and showed no luteal regression, so her data were omitted from analysis. In all other ewes, the decrease in P_4 concentrations led to initiation of the final stages of follicular growth and

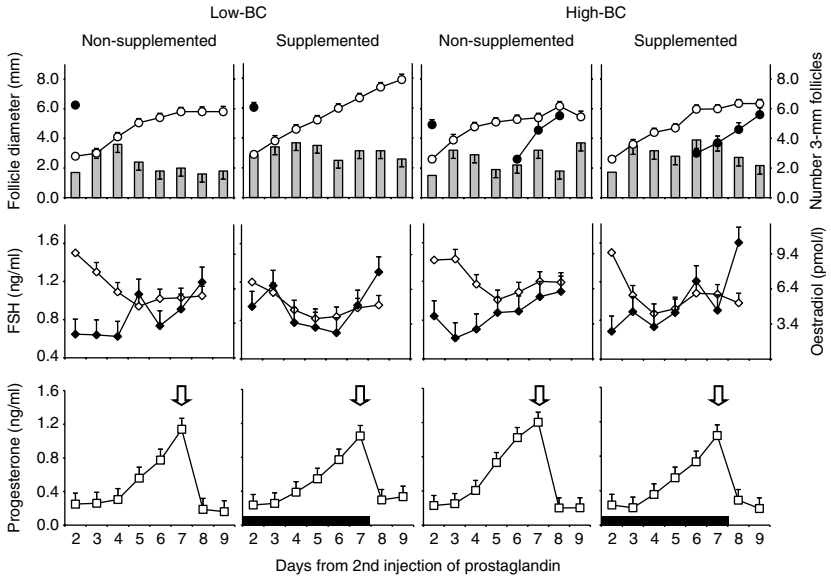

Figure 2 Growth profile of the dominant follicle from the first (open circle) and the second follicular waves (closed circle), in association with the numbers of 3 mm follicles (grey bars, upper panel), and the plasma concentrations of FSH (open diamond) and oestradiol (closed diamond; middle panel) and progesterone (lower panel; open square) in ewes of low and high body condition (BC), supplemented (black bars) or not with lupin grain for 6 days. Arrows indicate the time of the third prostaglandin injection. All values are least squares means (±S.E.M).

ovulation (Fig. 2). BC, supplementation and the interaction between the two factors had no significant effect on P_4 concentrations.

Follicles

The supplement tended to increase the number of 3 mm follicles (2.9±0.2 vs 2.4±0.2; $P=0.06$). The characteristics of the dominant follicle of the first wave were similar for all ewes (Fig. 2). The emergence of a second follicular wave 6.0±0.2 days after the second PG injection was observed more frequently in high-BC than in low-BC ewes (7/20 vs 1/19; $P<0.05$). The second follicular wave emerged during the growing phase of the dominant follicle of the first wave, and in three of seven supplemented high-BC ewes, it yielded ovulatory follicles (Fig. 2).

Ovulation rate

Ovulation rate was affected by BC ($P<0.05$) and tended to be affected by the interaction between BC and supplementation ($P=0.07$). High-BC ewes had a higher ovulation rate than low-BC ewes ($P<0.05$), and the supplement tended ($P=0.07$) to increase ovulation rate in high-BC but not in low-BC ewes (Table 1).

Plasma FSH concentrations

In all groups, FSH concentrations decreased as the dominant follicle of the first wave developed and remained unchanged thereafter (Fig. 2). The effect of the interaction between BC and supplementation was not significant ($P>0.05$). BC tended to increase FSH concentrations ($P=0.09$), but supplemented ewes had lower values than non-supplemented ewes (Table 2; $P<0.01$). The characteristics of the FSH wave that stimulated the emergence of the first follicular wave are presented in Table 3.

Plasma concentrations of E_2

The concentrations of E_2 were affected by day ($P<0.001$), being higher after the third PG injection than before it (Fig. 2). There was no significant effect of BC, but overall values tended to be higher in supplemented than in non-supplemented ewes (Table 2; $P=0.06$).

Blood concentrations of glucose

In all groups, glucose concentrations increased from −1 to +3.5 h relative to feeding (1.9±0.0 vs 2.3 ±0.0 mmol/l; $P<0.001$; Fig. 3), remained high at the 7-h sampling and then usually decreased by 15-h sampling (2.2±0.0 mmol/l). During the period of supplementation, there was a transitory decrease in glucose

Table 1 Ovulation rate in ewes of low and high body condition (BC), supplemented or not with lupin grain for 6 days.

Treatment		Ovulation rate		
Body condition	Supplement	Single	Twin	Mean
Low BC	−	8	2	1.2^{a}
Low BC	+	7	1	1.1^{a}
High BC	−	6	4	1.3^{ax}
High BC	+	3	7	1.7^{by}

$^{ab}P<0.05$; $^{xy}P=0.07$.

Table 2 Blood concentrations of glucose and plasma concentrations of FSH, oestradiol (E_2), progesterone (P_4), and metabolic hormones in ewes of low body condition (BC) and high BC that had received or not (±) a 6-day supplement of lupin grain. All values are pooled least square means ± S.E.M. for the 7-day sampling period.

	Low BC	High BC	−Supplement	+Supplement
FSH (ng/ml)	1.1 ± 0.04^{x}	1.2 ± 0.04^{y}	1.2 ± 0.04^{a}	1.0 ± 0.04^{b}
E_2 (pmol/l)	4.5 ± 0.4	4.8 ± 0.4	4.1 ± 0.4^{x}	5.2 ± 0.4^{y}
P_4 (ng/ml)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Glucose (mmol/l)	2.3 ± 0.04^{a}	2.1 ± 0.04^{b}	2.1 ± 0.04^{b}	2.3 ± 0.04^{a}
Insulin (μU/ml)	5.2 ± 0.4^{a}	6.6 ± 0.4^{b}	4.4 ± 0.4^{a}	7.4 ± 0.4^{b}
Leptin (ng/ml)	1.6 ± 0.1^{a}	2.4 ± 0.1^{b}	1.7 ± 0.1^{a}	2.3 ± 0.1^{b}
IGF1 (ng/ml)	57 ± 2.4^{a}	73.1 ± 2.4^{b}	63 ± 2.4	66 ± 2.4

Within rows and effects (BC and supplement): $^{ab}P<0.05$; $^{xy}P<0.09$.

concentrations from the second to the third day after second PG injection, in all groups except the high-BC supplemented group (Fig. 4). Overall, supplemented ewes had higher glucose concentrations than non-supplemented ewes ($P<0.01$; Table 2), and high-BC ewes had lower concentrations than low-BC ewes ($P<0.05$; Table 2).

Plasma concentrations of insulin

During the day, insulin concentrations were maximal 3.5 h after feeding and then decreased slowly to 15 h after feeding (7.5 ± 0.3 vs 4.9 ± 0.3 μU/ml, $P<0.001$; Fig. 3). High-BC ewes had higher values than low-BC ewes ($P<0.001$; Table 2), and supplemented ewes had higher values than non-supplemented ewes ($P<0.05$; Table 2, Fig. 4). Over the 7-day sampling period, both supplementation and BC affected the patterns of insulin concentration, and the response to supplementation depended on BC. In supplemented high-BC ewes, insulin values were elevated from day 3 until the last day of feeding, whereas in supplemented low-BC ewes, values decreased significantly on day 5 after the second PG injection, although they remained higher than those in low-BC non-supplemented ewes ($P<0.001$, Table 2, Fig. 4).

Plasma concentrations of leptin

In non-supplemented ewes, leptin concentrations peaked 7 h after feeding (2.1 ± 0.05 ng/ml) to decrease 8 h later (1.9 ± 0.05 ng/ml; $P<0.001$; Fig. 3). Overall, concentrations were higher in high-BC than in low-BC ewes ($P<0.001$), and higher in supplemented than in non-supplemented ewes throughout the 7-day sampling period ($P<0.001$; Table 2, Fig. 1). During the final 2 days of the supplementation treatment (days 5–7), in ewes receiving the lupin supplement, leptin values decreased in low-BC ewes but remained elevated in high-BC ewes (Fig. 4).

Plasma concentrations of insulin-like growth factor 1

Overall, the plasma concentrations of insulin-like growth factor 1 (IGF1) were higher in high-BC than in low-BC ewes ($P<0.001$) but were not affected by supplementation (Table 2 and Fig. 4). Concentrations increased in all the groups in the final sample, taken 1 day after the last PG injection and during the early follicular phase (Fig. 4).

Discussion

The series of three PG injections led to an alignment of wave emergence among ewes and allowed us to synchronise the growing phase of the follicles with the beginning of a supplementation period that promoted increases in ovulation rate. With this 'first-wave model', we were able to reveal interrelationships among follicular development, short-term supplementation, FSH concentrations and follicular recruitment. Our observations suggest that there is a better response to the immediate effect of nutrition in high-BC ewes than in low-BC ewes. This outcome did not appear to be linked, in ewes with high BC, to the presence of larger numbers of small follicles (3 mm) but did appear to be related to higher concentrations of insulin and leptin. The secretion of insulin and leptin was sustained until after the end of the supplement and stimulated the emergence of a second follicular wave. The E_2 results suggest that supplementation increased the steroidogenic capacity of follicles, perhaps in response to the higher insulin concentrations. This study cannot determine cause and effect, but our observations are consistent with the hypothesis that high concentrations of the metabolic hormones help the follicles to cope with the reduction in FSH concentrations that accompanies follicular maturation, thereby helping them to 'escape' atresia and allowing for an increase in ovulation rate.

The effect of nutrition on FSH concentrations has been difficult to demonstrate – in some studies, an increase, rather than a decrease, in FSH concentrations has

Table 3 Characteristics of the FSH 'wave' associated with the development of the largest follicle, in ewes with low and high body condition (BC), supplemented or not with lupin grain for 6 days (all values are least-squares means ± S.E.M).

Group	Supplement	Maximum FSH concentration (ng/ml)	Duration of FSH increase (h)	Number of FSH decreases
Low BC	−	2.2 ± 0.2^{abc}	42.4 ± 0.6^{b}	0.6 ± 0.3^{a}
Low BC	+	1.9 ± 0.2^{ac}	23.2 ± 0.6^{ac}	1.6 ± 0.3^{b}
High BC	−	2.7 ± 0.2^{b}	29.6 ± 0.6^{c}	0.8 ± 0.3^{ab}
High BC	+	2.1 ± 0.2^{ac}	28.8 ± 0.6^{c}	0.9 ± 0.3^{ab}

Within columns: $^{ab}P<0.05$; $^{bc}P<0.1$.

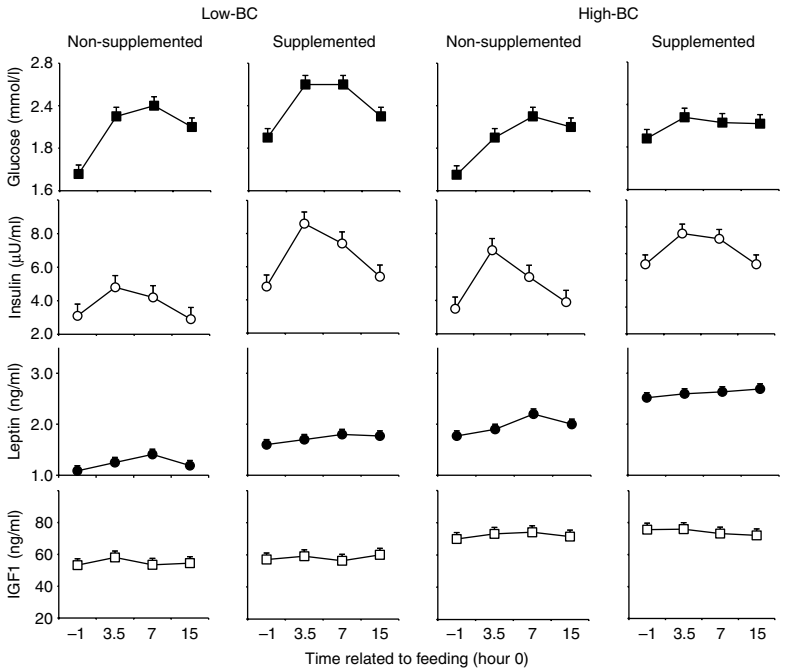

Figure 3 Changes in the concentrations of glucose (closed square), insulin (open circle), leptin (closed circle) and IGF1 (open square) related to the time of feeding in ewes of low and high body condition (BC), supplemented or not with lupin grain for 6 days. All values are least squares means (±S.E.M).

been reported, but most reports show no statistically significant change (Rhind *et al*. 1985, Rhind & McNeilly 1986, Scaramuzzi *et al*. 2006). By using the 'first-wave model' to promoted a high degree of synchrony among waves of FSH, follicles and E_2, we were able to reveal a decrease in FSH concentrations during short-term supplementation, associated with extra negative feedback by E_2, and perhaps inhibin, from the follicles. The supplement not only decreased mean FSH concentrations but also affected the characteristics of the FSH 'wave': in low-BC supplemented ewes, the wave was shorter and often associated with significant decreases in FSH concentrations. This probably limited the ovulatory quota of follicles, in agreement with previous results (Viñoles *et al*. 2002).

It is feasible that the endocrine environment created by the first-wave model, that is low P_4 concentrations that we would expect to lead to high LH pulse frequencies, may have increased the steroidogenic capacity of the follicles (Evans *et al*. 2001). The hormonal

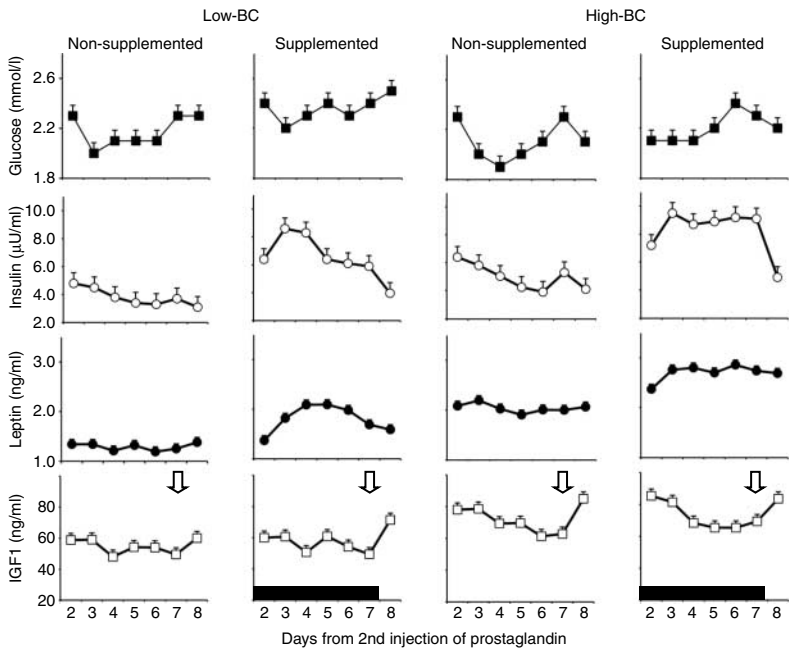

Figure 4 Changes in the blood concentrations of glucose (closed square) and plasma concentrations of insulin (open circle), leptin (filled circle) and IGF1 (open square) in ewes of low and high body condition (BC), supplemented (black bars) or not with lupin grain for 6 days. Arrows indicate the time of the third prostaglandin injection. All values are least squares means (±S.E.M).

outcome may be different if the nutritional treatment is applied under the higher P_4 concentrations of the mid-luteal phase. For this reason, we suggest that the FSH–E_2 pattern observed in this study occurs if ewes are supplemented during the early-luteal phase of their oestrous cycle.

In a previous study (Viñoles *et al*. 2005), we suggested that the effect of short-term supplementation on ovulation rate depends on four factors: i) the status of follicular development at the beginning of the treatment; ii) the plasma concentrations of glucose and metabolic hormones; iii) the changing patterns in hormone concentrations and iv) the pool of follicles available for the action of these hormones at the time the supplement is introduced. We also suggested that there was a need to standardise the model to study the effect of nutrition on follicle growth (Viñoles *et al*. 2005). The 'first-wave model' satisfies these requirements – it allows us to begin feeding the supplement at the expected time of wave emergence so that the peak concentrations of the metabolic hormones coincide with the time of maximum requirement for FSH in the growing follicles. In low-BC supplemented ewes, insulin and leptin reached peak concentrations on day 3 of supplementation and then decreased, as observed previously (Teleni *et al*. 1989, Viñoles *et al*. 2005). Although the supplement increased the number of small follicles, ovulation rate was not affected in low-BC ewes in this study. In contrast, in high-BC ewes, the concentrations of insulin and leptin increased and remained elevated until supplementation ended. This permitted more follicle(s) to be selected into the ovulatory wave. This reinforces the view that the impact of nutrition on ovulation rate depends, at least partly, on the persistence of high concentrations of the metabolic hormones.

Alternatively, high-BC ewes may have responded better to the supplement than low-BC ewes, due to the changes in live weight and BC that had been induced during the previous 12 weeks (i.e. carry-over effects). Although the metabolic hormones were not measured during pre-experimental period, we would expect glucose and metabolic hormone concentrations to have been affected by the level of nutrition (Sosa *et al*. 2006). Moreover, long-term effects of the metabolic status have been documented, with individual animals showing different responses to the same nutritional stimulus, a phenomenon that we have termed 'metabolic memory' (Blache *et al*. 2007). This is reinforced by observations showing that undernutrition 6 months prior to ovulation has a negative influence on ovulation rate (Nottle *et al*. 1997). Although low- and high-BC ewes had pools of gonadotrophin-responsive follicles of similar sizes, the previous 12 weeks of exposure to low concentrations of the metabolic hormones may have rendered the follicles in low-BC ewes less responsive to the combined effect of FSH, glucose, insulin and leptin.

In our study, maximum concentrations of glucose, insulin and leptin were temporally associated with feeding, as previously reported for ewes (Marie *et al*. 2001, Viñoles *et al*. 2005). Insulin and leptin concentrations increased with supplementation and with BC, and IGF1 concentrations increased only with BC. These hormones have direct and opposite actions at ovarian level: insulin and IGF1 potentiate the steroidogenic effects of the gonadotrophins, whereas leptin antagonises the stimulatory effect of insulin and IGF1 on theca cell steroidogenesis (Poretsky *et al*. 1999, Kendall *et al*. 2004, Scaramuzzi *et al*. 2006). In this study, we observed an increase in E_2 concentrations in supplemented animals, despite the increase in leptin concentrations, suggesting that leptin was not able to antagonise the effects of insulin and IGF1. Some of the disagreement may be due to the different experimental models ('first-wave model' versus cycle synchronisation), since the supplement is applied at different stages of the oestrus cycle (early- versus mid–late-luteal phase respectively). It has been shown that, during the normal cycle, insulin and IGF1 normally reach peak concentrations around oestrus, while leptin increases in the mid-luteal phase (Spicer *et al*. 1993, Sosa *et al*. 2004, Viñoles *et al*. 2004*a*). The impact of these hormone dynamics on follicular steroidogenesis needs to be explored.

Lupin-supplemented ewes had higher glucose concentrations that non-supplemented ewes, as observed in previous studies with lupin grain and other supplements (Viñoles *et al*. 2005, Letelier *et al*. 2008*a*, 2008*b*). The disagreement with previous reports (Blache *et al*. 2000*a*, Muñoz-Gutierrez *et al*. 2002) is probably related to the time the sample was taken related to feeding, the method used to measure glucose concentrations, or the feeding strategy (i.e. once versus twice daily feeding). The glucose values observed in this study were variable; suggesting that it may have been influenced by a stress induced by, for example, the daily ultrasonography. However, the fact that ewes maintained live weight and BC after being moved into the animal house suggests that they were not greatly affected by stress. High-BC ewes had lower glucose than low-BC ewes, as observed by Sosa *et al*. (2006) in their 'well-nourished' and 'undernourished' ewes, but this is consistent with their higher concentrations of insulin, a factor that would drive glucose uptake. The tissues of high-BC ewes, including their ovarian follicles, would have been using more glucose perhaps growing faster, thus helping to explain the increased frequency of twin ovulations.

In conclusion, we suggest that the 'first-wave model' offers a useful approach for studying the effect of nutrition on ovulation rate because it permits control over the timing of nutritional supplementation and the physiological responses that it induces, with respect to the stages of follicle development, reducing between-animal variation and permitting more precise measures of the physiological and ovulatory responses. From this

study, we have thus been able to conclude that BC affects follicle development and the response to supplementation, and that both BC and the immediate effect of nutrition act to promote an efficient use of FSH by follicles, counteracting the inhibitory effect of increased steroidogenesis on FSH secretion. This allows extra follicles to develop and ovulate.

Materials and Methods

The experimental procedures were approved by the Animal Ethics Committee of the University of Western Australia (Approval RA/3/100/534), according to the recommendations of the Australian National Health and Medical Research Council.

Animals and pre-experimental management

We selected 150 Merino ewes 3 months before the beginning of the experiment, aged 4.5±0.1 years (mean±S.E.M.), with a BC score of 3.0±0.03 (0=emaciated, 5=obese; Russel *et al.* 1969) and live weight 51.3±0.9 kg. All animals grazed the same type of dry summer pasture. To modify live weight and BC, the feed on offer was restricted in the 'low-BC' group (target BC=2, n=75) and increased to *ad libitum* in the 'high-BC' group (target BC=4, n=75) by adjusting the stocking rate and by feeding different amounts of lupin grain (6.5 ewes/ha and 100 g/head daily to low-BC ewes to provide 4.2 MJ ME/day; 5 ewes/ha and 300 g/head daily to high-BC ewes to provide >6 MJ ME/day; Fig. 5).

From each group, 20 ewes were relocated from pasture to individual pens in an animal house, under a natural photoperiod (for 32°S, 115°E) provided by indoor lighting. Only ewes that reached the target BC and had a single ovulation in two consecutive ultrasonographic evaluations were selected for the experiment. Selected ewes were allocated to the supplementation treatment according to their live weight and BC and randomly distributed in different pens in the animal house and acclimatised to these conditions (3 weeks; Fig. 5). Ewes were numbered in a sequential order according to their location in the animal house, and the sequence was kept for all procedures. The animals stayed in the same pen during the whole experimental period. BC and live weight were measured weekly in all the animals.

Experimental design and dietary management

Once moved into the animal house, the ewes were individually fed an 80:20 mix of oat chaff and lupin grain and a macro and trace mineral mix to meet their requirements for maintenance (CSIRO 2007). This mix supplied 8.9 MJ ME and 101 g crude protein (CP) per kilogram as fed basis (90% dry matter). The dry matter digestibility (DMD) and CP of the oat chaff and lupin grain were measured by near-infrared reflectance spectroscopy with appropriate calibrations (Independent Lab Services, Perth, Western Australia, Australia). The ME values were then calculated from DMD according to the equations in CSIRO (2007). The ewes were offered water *ad libitum* and fed daily at 0700 h (hour 0). Refusals were collected and weighed daily and were low throughout the experimental period (<10% for chaff and lupin grain).

A 2×2 factorial design was used with BC (low or high), with or without 6 days of supplementation with lupin grain as factors and with ten animals per group (Fig. 5). The supplemented ewes were fed additional lupin grain which, along with the maintenance diet, provided the ewes with twice their ME requirement for maintenance (CSIRO 2007). Further information on the nutritional attributes of lupin grain is given by Boukhliq & Martin (1997).

Study of follicular development with the 'first-wave model'

The first follicular wave was synchronised in all ewes with two injections of 250 μg cloprostenol, a PG analogue (Juramate, Jurox Pty Ltd, Rutherford, NSW, Australia) administered at 7-day intervals. The 6-day period of supplementation began 2 days after the second PG injection (i.e. at the expected time of ovulation and emergence of the first follicular wave of the cycle) and continued until the third PG injection, used to induce the ovulation of the follicular wave that was the focus of the experiment (Fig. 5). The ovulation rate was measured 8 days after the third PG injection.

Using transrectal ultrasonography, the ovaries were examined daily, from the day of the second PG injection until the ovulation induced by the third PG injection. We used a real-time, B-mode scanner (Aloka SSD 900 Co. Ltd, Tokyo, Japan) with a rigid 7.5 MHz transducer modified for external manipulation in the rectum (Viñoles *et al.* 2010). Ovulation

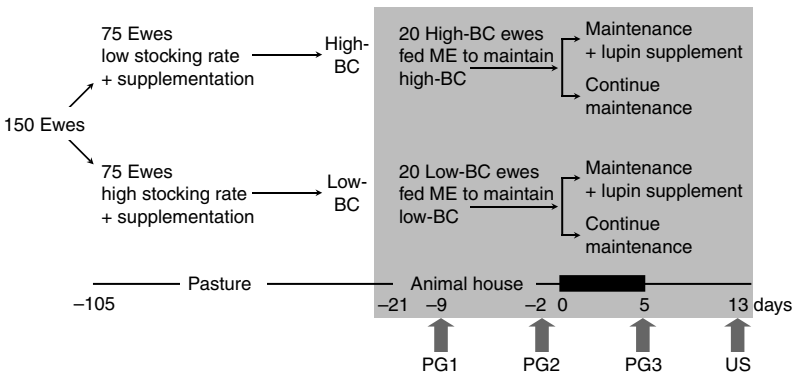

Figure 5 A schematic representation of the design of the experiment, showing nutritional pre-treatment in the field followed by confinement in an animal house where, after acclimatisation, the 6-day lupin supplement (or control) period was imposed. The 'first-wave model' is indicated by the block arrows indicating the three prostaglandin (PG) injections, with the lupin supplement imposed 2 days after the second injection. Day 0, first day that ewes received double their requirements for maintenance; US, ultrasound for measurement of ovulation rate.

was detected by observing the collapse of a large follicle (≥5 mm) followed by the presence of luteal tissue at the same site 4 days later. For both ovaries on each day, we observed all corpora lutea and the total number, diameter and position of all follicles ≥2 mm in diameter, and recorded their images on videotape. The accuracy and precision of this procedure have been confirmed by analyses of scanned ovaries postmortem (Viñoles *et al.* 2004*b*). 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. A subordinate follicle was defined as a follicle that reached 3 mm and could be followed by ultrasonography for at least 3 days. The characteristics of the follicular waves were described in relation to the measures of the largest follicle: day of emergence, maximum diameter and day of maximum diameter (Viñoles *et al.* 2001). The day of wave emergence was the day that the largest follicle of the wave was retrospectively identified as being 2–3 mm in diameter. Life-span was defined as the number of days between emergence and ovulation.

Blood sampling, glucose and hormone assays

From the first day of feeding until the day after the third PG injection, jugular blood (5 ml) was collected into heparinised tubes at −1, 3.5, 7 and 15 h relative to feeding (hour 0). Immediately after the extraction of the blood sample (i.e. within a minute) from each ewe, glucose concentration was measured in all samples using an Accu-Chek Advantage glucose meter (Roche Diagnostics Australia Pty Ltd). The normal reading range was 0.6–33.3 mmol/l. The samples were kept in ice, and plasma was separated from the blood by centrifugation within 10 min of sampling and stored at −20 °C until assayed.

Plasma P_4 was measured once a day from the first day of feeding until ovulation, in samples collected at −1 h relative to feeding. Samples were analysed in duplicate using a standard RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX, USA) as described elsewhere (Gray *et al.* 2000). Only samples collected at −1 h relative to feeding were analysed. The limit of detection was 0.4 ng/ml. The intra-assay coefficients of variation (CV) were 2.6% for low (0.9 ng/ml) and 4.1% for high (10.4 ng/ml) P_4 values in plasma, and the inter-assay CV were 5.7 and 11.1% respectively.

E_2 concentrations were determined in all plasma samples in a single RIA using an Adaltis MAIA E_2 kit from Diagnostic Technology (Suite 45, 7 Narabang Way, Belrose, NSW 2085, Australia). Samples collected at −1, 3.5, 7 and 15 h relative to feeding were pooled to obtain a daily profile. The limit of detection was 1.3 pmol/l. The intra-assay CV was 8.4% for E_2 concentrations of 5 pmol/l, 2.4% for E_2 concentrations of 22 pmol/l and 23% for E_2 concentrations of 51 pmol/l.

FSH, insulin, leptin and IGF1 were measured in the samples collected at −1, 3.5, 7 and 15 h relative to feeding during the 7-day sampling period. Plasma concentrations of FSH were measured in a single RIA using the reagents kindly supplied by Dr A F Parlow of the National Institute of Diabetes, Digestive and Kidney Disease (Baltimore, MD, USA) using a method described previously (Martin *et al.* 1994). The samples were assayed as duplicate 100 μl aliquants, and the limit of detection was 0.5 ng/ml. Pooled plasma samples (six replicates) containing 1.4, 2.3 and 3.4 ng/ml were used to determine the intra-assay CV: 7.2, 4.5 and 1.9%.

Plasma insulin was assayed in duplicate in a single double-antibody RIA (Tindal *et al.* 1978) that had been validated for sheep plasma in our laboratory (Miller *et al.* 1995). The limit of detection was 1.6 μU/ml. The assay included six replicates of control samples containing 3.0, 4.3 and 10.5 μU/ml, for which the intra-assay CV were 2.6, 3.9 and 5.4%.

Leptin was analysed in all samples in a single double-antibody RIA using an antibody raised against recombinant bovine leptin in an emu (Blache *et al.* 2000*b*). The samples were assayed as duplicate 100 μl aliquants, and the limit of detection was 0.3 ng/ml. Six replicates of three control samples containing 0.5, 0.9 and 1.9 ng/ml included in the assay to estimate the intra-assay CV: 5.7, 2.9 and 4.0%.

Plasma concentrations of IGF1 were measured in all samples in one double-antibody RIA (Gluckman *et al.* 1983). Interference by binding proteins was minimised using an acid–ethanol cryoprecipitation, as validated for ruminant samples (Breier *et al.* 1991). The samples were assayed as duplicate 100 μl aliquants, and the limit of detection was 0.4 ng/ml. Six replicates of two control samples, containing 1.6 and 0.24 ng/ml IGF1, were included in the assay and were used to estimate the intra-assay CV (6.1 and 22.2%).

Statistical analyses

The frequencies of animals developing a second follicular wave were analysed by Fisher's exact test. The effects of supplementation, BC and their interaction on ovulation rate (ovulations per ewe ovulating) were compared using a generalised lineal model with a binomial distribution (0=single ovulation and 1=double ovulation) and a log transformation of the data. Two supplemented low-BC ewes that failed to ovulate after the second or the third PG injections were not used to analyse ovulation rate (Table 1). The morphological characteristics of the ovulatory follicles were compared by ANOVA using the mixed-model procedure in the Statistical Analysis System (SAS), including the fixed effects of BC and supplement and the random effect of ewe-within-group. The relationships between the FSH wave that stimulated the growth of the first follicular wave and the growth profile of the largest follicle of that wave were analysed using a skewness method (Viñoles *et al.* 2002). For each individual ewe, the mean and S.D. were calculated in an excel worksheet. Significant increases and decreases in FSH concentrations were defined as the mean±3 S.D.s. The significant increases and decreases were then eliminated to calculate the baseline concentration. The maximum FSH concentration was defined as the maximum significant increase, the duration of the FSH increase as the number of hours FSH remained significantly elevated and the number of decreases as the number of times FSH concentrations decreased significantly below the baseline concentration. After all the individual values were generated, the effect of BC and supplement was analysed using the general linear model (GLM) procedure in SAS. The normality of the data and the presence of outliers were checked using the

univariate procedure available in SAS. Data that involved repeated measurements (e.g. live weight, BC, plasma hormone concentrations) were also analysed by the mixed-model procedure of SAS, including the fixed effects of time, BC and supplement and their interactions. 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). Mean values were compared using least squares means and considered significant if $P<0.05$. Data are presented as least square means ± pooled S.E.M.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported in this manuscript.

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