

The Effect of Short-Term Nutritional Supplementation and Body Condition on the Pituitary and Ovarian Responses of Anoestrous Ewes to the “Ram Effect”

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Abstract

In sheep production, the “ram effect” is a technique for inducing fertility in seasonal anoestrus and “flushing”, another technique to increase litter size. Often used individually, we wanted to know if they could be used together to improve reproductive performance of ewes bred during the anoestrus season. Two experiments were conducted; the first with Île-de-France ewes (N=30) comprised a control and a group fed a nutritional supplement and the second with Romane ewes (N=60) replicated these treatments at two levels of body condition. The ewes were stimulated with the “ram effect” and the following responses measured (i) blood concentrations of LH, FSH, oestradiol, progesterone, glucose and insulin (ii) oestrus. Supplementation increased blood glucose and insulin in experiment 1 but not in experiment 2 but it had no effect on FSH; it reduced oestradiol in experiment 2 but not in experiment 1. Higher body condition was associated with higher blood glucose and insulin but not FSH or oestradiol. In addition, higher body condition was associated with a greater proportion of ewes responding to the “ram effect” and greater short-term responses for LH and oestradiol; supplementation had no effect on these responses. In experiment 1 but not experiment 2, supplementation was associated with a higher proportion of ewes in oestrus. The results demonstrate that there are close relationships among the concentrations of LH and oestradiol, the LH surge and the ovarian cyclicity in response to the “ram effect”. These data show an effect of body condition on the “ram effect” that can modify cyclicity and suggest an effect of short-term nutritional supplementation on oestrus. Furthermore these data also suggest that the functional capacity of follicles at the time of the “ram effect” is an important determinant of outcome.

Keywords: Ram effect; Oestradiol; LH; Nutrition; Body condition; Glucose; Insulin

Introduction

The “male effect” is a well known technique that is often incorporated into breeding systems for small ruminants. It is a simple technique for inducing fertile mating outside of the normal breeding season in sheep and goats and thus can be used to effectively extend their natural period of reproduction [1,2]. Equally well known and also often incorporated into breeding systems for small ruminants is the technique of “flushing”, or providing the animals with extra nutrition for a short period prior to mating; that can be used to increase litter size [3,4]. Ordinarily, these two techniques are used individually and although occasionally used together there is little data evaluating their combined effectiveness. Consequently we set out to learn if they could be used together to improve the reproductive performance of sheep mated during seasonal anoestrus, as has been observed for goats [5,6].

These techniques for the management of reproduction in flocks of farm animals have an added important advantage since they meet the criteria for Clean Green and Ethical production systems [7,8]. The “ram effect”, although of great practical value to the hormone free management of reproduction, is subject to a number of shortcomings that limit its efficacy. In particular the response to the “ram effect” is variable both among breeds and within breeds at different times during anoestrus [9]. This variability is expressed in a number of ways. There may be no detectable response to the introduction of rams or there may be a short-term response to the introduction of rams that does not induce ovulation. Additionally, there may be a response to the introduction of rams that does induce ovulation but the resulting corpus

luteum is defective and a so-called short cycle results. The difficulties using the “ram effect” can be reduced to two general problems, first, the failure of the “ram effect” stimulus to evoke a response and second the failure of the follicle to respond adequately to a hormonal stimulus. Unlike the problems related to the strength of the male stimulus the failure of the follicle to respond is an exclusively female problem and possibly exclusively ovarian or perhaps utero-ovarian in origin.

The ovarian response to the “ram effect” consists of a sequence of events commencing with gonadotrophin-stimulated secretion of follicular oestradiol that induces positive feedback, leading to an LH surge, ovulation and the formation of a corpus luteum. Although the later part of this sequence (the LH surge, ovulation and the formation of a corpus luteum) is well established there is very little published data describing the pattern of oestradiol secretion in response to the “ram effect”. It is possible that some of the variability in response to the

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“ram effect” is caused by variability in the follicular population at the time of the “ram effect” and their ability to respond to LH by secreting sufficient oestradiol to induce a positive feedback signal.

Short-term nutritional supplementation of ewes in the breeding season stimulates folliculogenesis by increasing the number of small and medium sized follicles [10,11] and ovulation rate [12,13] an effect which is most likely caused by direct nutrient effects particularly glucose, on the follicle [14,15]. This experiment was designed to determine if a short-term nutritional supplement (flushing) or body condition affected the response of seasonally anoestrous ewes to the “ram effect” and furthermore to determine if nutritional manipulation when used in combination with the “ram effect” could enhance the fertility of ewes mated during seasonal anoestrus.

Materials and Methods

Two experiments were carried out; the first (experiment 1) at the INRA laboratory at Nouzilly used 30 mature Île-de-France ewes and the second (experiment 2) at La Sapinière the INRA field station at Bourges, used 60 young, primiparous Romane ewes. The experiments were carried out in May (experiment 1) and June (experiment 2) during the non-breeding season for these breeds in these locations. The experiments were carried out in accordance with French and European regulations on the care and welfare of animals in research.

Before being used in an experiment the ewes were first confirmed as anoestrus. Blood samples were collected once a week for three weeks before the start of the experiments from a larger group of ewes than were required for the experiments. The plasma samples were then assayed for progesterone and from the pattern of progesterone over this period, anoestrous ewes were identified by a pattern of persistently low (<1ng/mL for three consecutive weeks) concentrations of progesterone indicating the absence of corpora lutea. Only anoestrous ewes were retained for use in the experiments.

Diets and nutritional management

The ewes were weighed (LW) and their body condition score (BCS) recorded regularly; BCS was determined as described [16] using a scale of 0 (emaciated) to 5 (grossly obese). Body condition scores for each experiment, were determined by the same experienced person.

All diets were designed using the INRA recommendations for the growth and maintenance needs of adult, non-pregnant ewes [17]. In experiment 1, the ewes were fed *ad-libitum* a basal diet of two thirds hay (0.73 Mcal of net energy and 106 g of metabolizable protein per kilogram of Dry Matter) and one third straw (0.75 Mcal of net energy and 35 g of metabolizable protein per kilogram of DM) which corresponded to an average daily intake of 0.91 Mcal of net energy and 108.72 g of metabolizable protein. For experiment 2, the ewes in the low BCS group were fed a basal diet of the same hay which corresponded to an average daily intake of 0.96 Mcal of net energy and 138.86 g of metabolizable protein while the ewes in the medium BCS group were fed a basal diet of hay plus 300g per day of a standard sheep concentrate (1.96 Mcal of net energy and 140 g of metabolizable protein per kilogram DM) which corresponded to an average daily intake of 1.77 Mcal of net energy and 207.28 g of metabolizable protein. These basal diets were designed to maintain a constant body weight and body condition score reflecting a stable metabolic state during the experiment. In both experiments all ewes had *ad-libitum* access to water and mineral licks. The control (maintenance fed, non-supplemented) groups were fed the basal diet for the duration of the experiments and the supplemented groups were fed individually, a daily supplement of 400g crushed

maize (2.13 Mcal of net energy and 104 g of metabolizable protein per kilogram DM) and 100g soya meal (2.11 Mcal of net energy and 402 g of metabolizable protein per kilogram DM) per ewe per day which corresponded to an extra daily intake of 1.21 Mcal of net energy and 93.17 g of metabolizable protein that raised their energy intake to at least twice maintenance. The supplement was fed in equal portions at 1000h and 1600h over two five day periods; the first commencing five days before the introduction of rams and the second 13 days after the introduction of rams. These two times were selected so as to provide short-term supplementation over about five days before the ram-induced and subsequent ovulations.

Experiment 1: In May, thirty anoestrous Île-de-France ewes were allocated to two equal groups randomised by age and BCS; a control group and a supplemented group. The ewes were parous and of mixed ages ranging from 1.5 to 8.5 years. They had all lambed in February-March and their lambs had been weaned at birth.

Experiment 2: In early June, sixty anoestrous Romane ewes were allocated at random to four equal groups in a two by two factorial design. The first factor was body condition score (BCS) at two levels; low BCS and medium BCS; the median BCS of these two groups at the start of the experiment were 1.75 (range – 1.75-2.00) and 2.25 (range - 2.00-2.75). The second factor was diet tested at two levels (supplemented and control [non-supplemented]). The ewes were 18 months old and primiparous, having lambed between mid-February and mid-March. Their lambs had been weaned in mid-April, two months before the beginning of the experiment.

The “ram effect”

The ewes were kept completely isolated from all contact with rams until the time the rams were introduced. The ewes were housed in group pens of 10 (experiment 1) or 15 (experiment 2) ewes and a single sexually active ram was placed in each pen. The time the rams were introduced to the ewes was designated “time 0”. The rams were left in contact with the ewes for the next 15 days they were then replaced with fresh rams fitted with mating harnesses and crayons. To ensure an even stimulus and to avoid variability associated with individual rams during the “ram effect”, the rams were rotated amongst the pens every 30 minutes for the first five hours and then in experiment 1, once a day and in experiment 2, once a week.

A short-term LH response was defined as a concentration of LH more than three standard deviations above the mean concentration of LH before the introduction of rams, in the four hours immediately following the introduction of rams. A short-term oestradiol response was defined as a concentration of oestradiol that was significantly greater than the concentration before the introduction of rams, in the eight hours immediately following the introduction of rams and a preovulatory increase in oestradiol was defined as a concentration of oestradiol >2.0pg/mL, in two consecutive samples between 8 and 52h. A LH surge was defined as a concentration of LH >8.0ng/mL (the upper detection limit of the assay), in two consecutive samples between 8 and 52h [18].

The type of cycle induced by the “ram effect” was determined from the pattern of progesterone after the “ram effect”. The length of the luteal phase was defined as the number of days that the plasma concentration of progesterone was continuously above 0.5 ng/mL and the length of the oestrous cycle was defined as the number of days between the first and second time following the introduction of rams, that the plasma concentration of progesterone rose above 0.5 ng/mL. A normal luteal phase was defined as a one in which the concentration of progesterone

was above 0.5 ng/mL for more than 10 days and a short luteal phase was defined as one in which the concentration of progesterone rose above the mean baseline before the “ram effect” for at least one day in the first seven days after the introduction of rams.

The detection of oestrus

From day 15 rams fitted with harness and crayons were used to detect oestrus. The ewes were examined once a day, from day 15 to the end of the experiment. The presence of a crayon mark on the rump of a ewe was regarded as evidence of oestrus. In experiment 1, oestrus was confirmed by direct observation: a ewe suspected in oestrus was placed in a pen with a ram and the paired watched by an observer to confirm if the ewe was in oestrus.

Blood sampling and plasma preparation

Jugular venous blood was collected at the following times relative to the day rams were introduced to the ewes (designated day 0). From days -6 to -1 blood samples were taken twice daily at 0900h (one hour before feeding) and at 1300h (three hours after feeding). These samples were assayed for glucose, insulin, oestradiol (only the sample at 0900h) and FSH (only the sample at 0900h). Furthermore, samples were taken at -4, 0.5, 1, 2, 3, 4h and then at intervals of four-hours for the next 48 hours. These samples were assayed for LH, and of them, selected samples were assayed for oestradiol (-4, 0.5, 8, 16, 24, 32, 40 and 48h). Finally samples were taken once a day from day 2 to day 11 and then three times a week until the end of the experiment on day 33. These samples were assayed for progesterone. Within an hour of blood collection, the samples were centrifuged at 2500g for 10 min. The plasma was then decanted and stored at -20°C.

Hormone and metabolite analyses

The analysis of glucose used plasma from blood samples collected into fluoride EDTA vacuum tubes. Samples collected into Heparin-lithium vacuum tubes were analysed for insulin, progesterone, LH and oestradiol. All assays were carried out in duplicate.

ELISAs for LH, FSH and progesterone: Plasma samples were analysed by ELISA to determine the concentrations of FSH, LH [19] and progesterone [20]. The limit of detection of LH was 0.01ng/mL, the upper limit of detection was 8.0 ng/mL and the inter-assay coefficient of variation was 13.6%. The limit of detection of FSH was 0.1ng/mL and the inter-assay coefficient of variation was 12.2%. The limit of detection of progesterone was 0.25ng/mL and the inter-assay coefficient of variation was 14.3%.

Radio immunoassays for oestradiol and insulin: The plasma concentrations of oestradiol were determined by radioimmunoassay [21] of solvent extracted plasma using a commercial oestradiol radioimmunoassay kit (Estradiol-2 kit P2210; Diasorin, SA, Antony, France). The limit of detection of oestradiol was 0.15 pg/mL and the intra-assay coefficient of variation was 6% (within experiments all samples were analysed in a single assay). Insulin was measured using a heterologous radioimmunoassay [22]. The sensitivity of the assay was 0.05 ng/mL and the inter-assay and intra-assay coefficients of variation were 10% and 15% respectively. The cross reactivity of the antiserum with ovine insulin was 100% relative to the homologous standard.

Glucose: The concentration of glucose in plasma was determined by colourimetry using the glucose oxidase method. The reagents were supplied as a kit (Glucose Assay Kit; Sigma Aldrich Inc, Saint-Quentin Fallavier, France) and the assay method followed the instructions provided by the manufacturer. Plasma samples were diluted to obtain

concentrations that fell within the range of the standard curve (20 to 80 mg/dL). The sensitivity of the assay was 20 mg/dL.

Statistical analyses

Proportions were tested using the Chi squared test or Fisher's exact test. The data on hormone concentrations were analysed by a repeated measures analysis of variance (ANOVA) to test the effects of body condition score and dietary supplementation on (i) the plasma concentrations of glucose, insulin, oestradiol and FSH (ii) the short-term responses of LH and oestradiol and (iii) the pre-ovulatory secretion of LH and oestradiol. The effect of the presence of an LH surge on the plasma concentration of oestradiol were also analysed by a repeated measure ANOVA to test the effects of body condition score and/or dietary supplementation on (i) the short-term responses of LH and oestradiol and (ii) the pre-ovulatory secretion of LH and oestradiol. Analysis of variance was followed when appropriate, with post-hoc comparisons using the Bonferroni correction or the least significant difference test. The effect of dietary supplementation on the acute response of glucose and insulin were tested using the paired t-test. Main effects from the ANOVAs are reported as estimated marginal means \pm the pooled standard errors (sem). Other data are reported as means \pm sem except body condition scores which are reported as medians and ranges.

Results

General

In experiment 1, two ewes (one from each group) had ovulated spontaneously before the rams were introduced to the ewes; data from these two ewes were excluded from the experiment. For experiment 1, the ewes had a mean (\pm sem) body weight of 59.6 ± 1.4 kg and a median BCS of 2.25 (range 1.75 to 3.50). They were not significantly different between the experimental groups or over the period of the experiment. For experiment 2, the median BCS for the low BCS group was 1.75 (range 1.75 to 2.00) and for the medium BCS group it was 2.25 (range 2.00 to 2.50) and it did not change over the period of supplementation. The mean (\pm sem) body weight was 50.9 ± 0.68 kg for the low BCS group and 56.8 ± 1.30 kg for the medium BCS group and they were significantly different ($P < 0.001$). Body weight did not change significantly for any of the groups for the duration of the experiment.

Effect of diet and body condition on glucose and insulin

In experiment 1, supplementation with maize and soya meal had no statistically significant overall effect ($P = 0.291$) on the blood concentrations of glucose over the five day period of dietary supplementation (Figure 1). During the period of supplementation, the 0900h, pre-feeding concentrations of insulin tended to be higher from two days after the start of dietary supplementation but in no case did the increase reach statistical significance (Figure 1). The concentrations of glucose three hours after supplementary feeding (Figure 1) were significantly higher only on day 3 ($P = 0.006$). There was, however, a highly significant effect of supplementary feeding three hours after feeding, when the concentration of insulin was significantly increased compared to control ewes (days 2 ($P = 0.03$), 3 ($P = 0.01$), and 4 ($P = 0.01$)) and also when compared to the concentration 1hour before feeding in supplemented ewes [days 2 ($P = 0.027$), 3 ($P = 0.003$) and 4 ($P = 0.009$)]. In the non-supplemented group there were no significant differences in the concentration of insulin or glucose among these times.

In experiment 2, the blood concentration of glucose (data not presented) was not significantly affected by supplementation (control, 68.4 vs. supplemented, 67.5 mg/dL; pooled sem=0.50, $P = 0.085$)

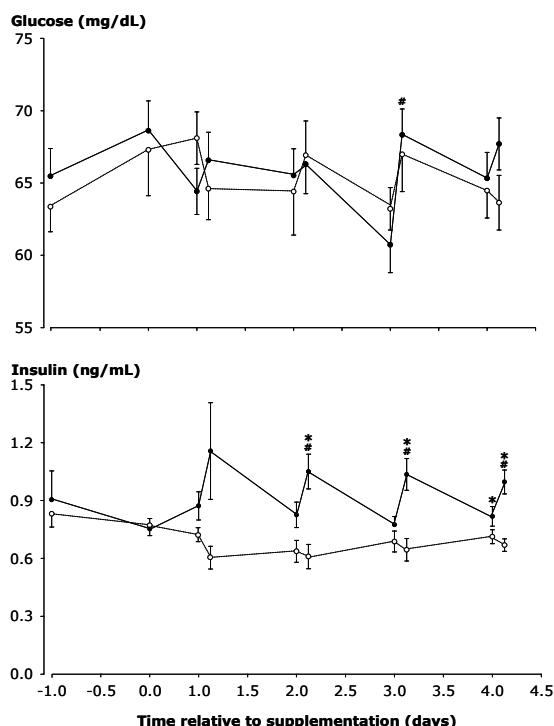


Figure 1: Experiment 1 - The pre- and post-feeding plasma concentrations of glucose and insulin during the first period of nutritional supplementation (days minus five to zero in relation to the "ram effect" in ewes that were fed a basal diet of hay and straw without (○; control: n= 14) or with a daily supplement of 400 g of crushed maize and 100g of soya meal for 5 days (●; supplemented: n = 14). An asterisk (*) indicates a significant difference between treatments within times (<0.05) and a hash (#) indicates a significant difference 1h before and 3h after feeding on the same day (<0.05).

but it was significantly higher in ewes with a higher body condition score (medium BCS, 68.8 vs. low BCS, 67.2 mg/dL; pooled sem=0.30, $P=0.001$). The interaction between nutritional supplementation and BCS was also significant ($P=0.003$) and indicated that nutritional supplementation eliminated the difference in concentrations of blood glucose that were associated with differences in body condition score. In experiment 2, the plasma concentrations of insulin (data not presented) were not significantly affected by supplementation (control, 0.973 vs. supplemented, 1.028 ng/mL; pooled sem=0.021, $P=0.089$) but it was significantly higher in ewes with a higher body condition score (medium BCS, 1.046 vs. low BCS, 0.956 mg/dL; pooled sem=0.023, $P=0.002$); the interaction between the two was not significant ($P=0.578$).

Effect of diet and body condition on oestradiol and FSH

In experiment 1, supplementation with maize and soya meal had no significant effect on the blood concentrations of oestradiol (control, 0.27 vs. supplemented, 0.28 pg/mL; pooled sem=0.03; $P=0.610$) or FSH (control, 0.82 vs. supplemented, 0.87 ng/mL; pooled sem=0.05; $P=0.875$) over the five-day period of supplementation. In experiment 2, dietary supplementation significantly reduced the blood concentrations of oestradiol (control, 0.71 vs. supplemented, 0.59 pg/mL; pooled sem=0.042; $P=0.009$) but had no effect on the concentration of FSH (control, 0.39 vs. supplemented, 0.37 ng/mL; pooled sem=0.024; $P=0.606$). There was no significant effect of BCS on the plasma concentration of either oestradiol (medium BCS, 0.63 vs. low BCS, 0.59 pg/mL; pooled sem=0.042; $P=0.169$) or FSH (medium BCS, 0.32 vs. low BCS, 0.43 ng/mL; pooled sem=0.065; $P=0.089$).

Ovarian responses to the "ram effect"

The numbers of ewes that responded to the "ram effect" with a rise in progesterone within 7 days of the introduction of the rams, were 11/28 (39%) in experiment one and 18/60 (30%) in experiment 2. The responses to the "ram effect" are summarised in (Table 1).

Experiment 1: The proportion of ewes with a significant short-term LH response to the introduction of rams was 20/28 (71%) and did not differ between the supplemented and control groups. Of the 20 ewes that responded to the "ram effect" only seven (35%) had a pre-LH surge increase in oestradiol and of these, five (25%) went on to have a LH surge and again there was no significant differences between the groups for these two end points. Of the 20 ewes with short term responses eleven (55%) had a subsequent rise in progesterone including seven in which no LH surge was detected in the 52 hours after the introduction of the rams. There was no difference between groups (Table 1). Of the eleven ewes with a rise in progesterone, six had short luteal phases and five had a normal oestrous cycle and went on to show oestrus 19.2 ± 1.20 (mean \pm sem) days after the introduction of rams. The proportion displaying oestrus was significantly different ($P=0.048$) between supplemented ewes (4/5) and control ewes (1/6).

Because, there was a significant effect of BCS on the responses to the "ram effect" in experiment 2 (see below) the data from the 28 ewes in experiment 1 were pooled and retrospectively re-grouped into two groups based on BCS, as follows; medium BCS (n=16; median, 2.25; range 1.75-2.25) and high BCS (n= 12; median 2.75; range 2.50-3.50) and re-analysed. The proportion of ewes with a rise in progesterone for medium (3/16) and high BCS groups (8/12) was significantly different ($P=0.012$) while the proportion displaying oestrus (1/3 versus 4/8 for medium and high BCS groups) was not ($P=0.776$).

Experiment 2: The responses to the "ram effect" are summarised in (Table 1). The proportion of ewes with a significant short-term increase in LH in response to the introduction of rams was the proportion responding was significantly ($P=0.00001$) in the non-supplemented, low BCS group (Table 1). The proportion of control ewes in the low BCS group showing a short-term increase in the concentration of LH in low BCS non-supplemented ewes (6/14; 43%) was significantly lower than in low BCS supplemented ewes (15/15; 100%; $P<0.001$) and both the medium BCS groups (13/15; 87%; $P=0.013$; 15/15; 100%; $P<0.001$). Of the 49 ewes that responded to the "ram effect", 18 (37%) had a pre-LH surge increase in oestradiol and of these 13 (27%) went on to have an LH surge. The proportion of ewes with a pre-LH surge increase in oestradiol (Table 1) did not differ significantly ($P=0.161$) among groups. Similarly, the proportions of ewes having a LH surge (Table 1) did not differ significantly ($P=0.343$) among groups.

Of the 49 ewes with a short-term increase in LH, 18 (37%) went on to have increased concentrations of progesterone after the introduction of rams including 7 ewes in which no LH surge was detected and of these, 6 had short luteal phases. The proportions of ewes with a rise in progesterone ($P=0.016$) and displaying oestrus ($P=0.001$) were both significantly affected by BCS. Further examination of these effects show that the medium BCS group had an increased proportion of ewes with a rise in progesterone ($P=0.023$) and oestrus ($P=0.001$) and a decreased proportion with a short cycle ($P=0.025$) and that supplementation had no effect on any of these (proportion of ewes with a rise in progesterone [$P=0.576$], a short cycle [$P=0.576$] or oestrus [$P=0.813$]).

Endocrine (LH and oestradiol) responses to the "ram effect"

Experiment 1: The plasma concentrations of LH were significantly elevated ($P=0.002$) by the time of the first blood sample (15 to 30

Experiment	Treatment groups		Short-term increase in LH within 4h	E2 Surge (>2.0 pg/mL between 4 and 52h)	LH Surge (between 4 and 52h)	Increased P4 (>0.5 ng/mL within 7 days)	Short Luteal Phase	Oestrus
One	Control (n=14)		10	3	2	6	3	1 ^a
	Supplemented (n=14)		10	4	3	5	3	4 ^b
Two	Control	Low BCS (n=15)	6 ^a	2 ^a	1 ^a	1 ^a	1 ^{a,b}	0 ^a
		Med BCS (n=15)	13 ^b	7 ^a	5 ^a	6 ^b	1 ^{a,b}	6 ^b
	Supplemented	Low BCS (n=15)	15 ^b	3 ^b	3 ^a	6 ^{a,b}	4 ^a	1 ^a
		Med BCS (n=15)	15 ^b	6 ^a	4 ^a	5 ^b	0 ^b	5 ^b

Table 1: The number of ewes responding to the “ram effect” in control ewes and in ewes supplemented with 500g of maize/soya per day for five days immediately before the introduction of rams. Experiment two involved the additional factor of body condition score (BCS) at two levels; low (median BCS 1.75) and medium (median BCS 2.25). Within columns and within experiments values with different superscripts are significantly different (P<0.05)

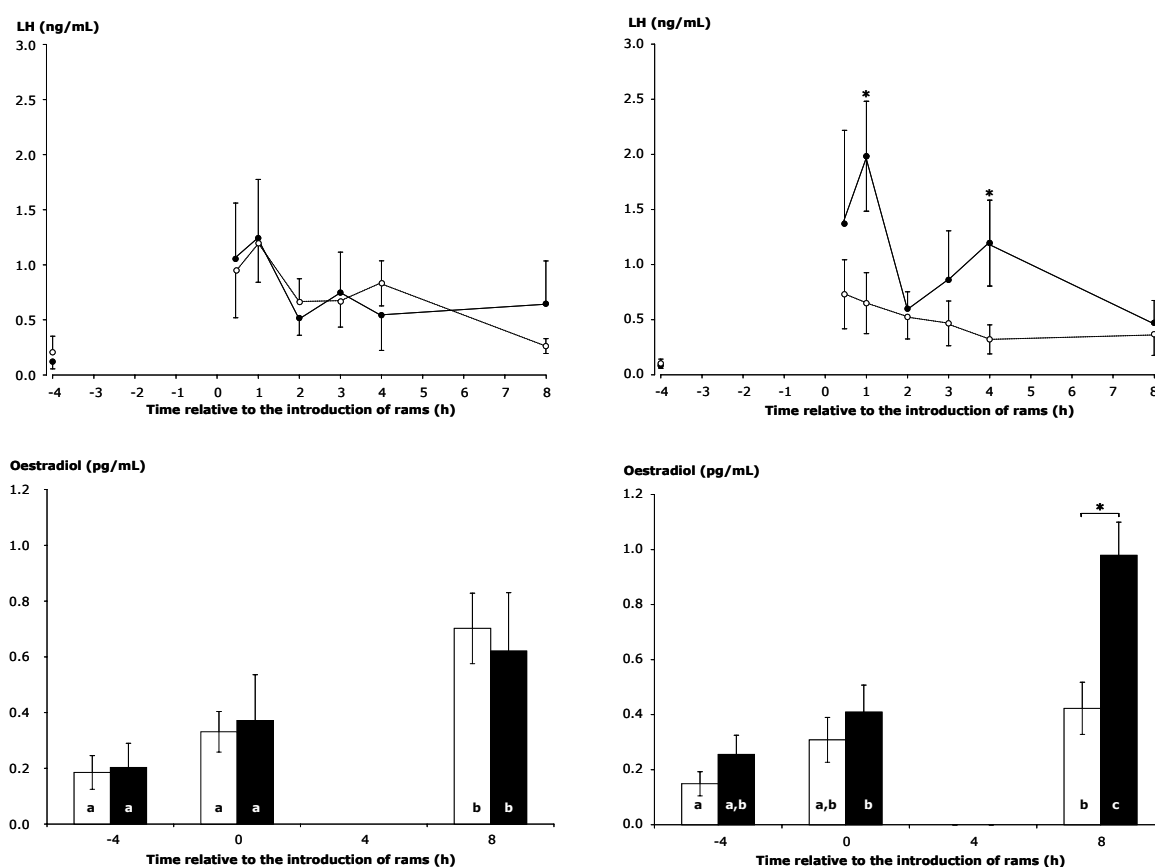
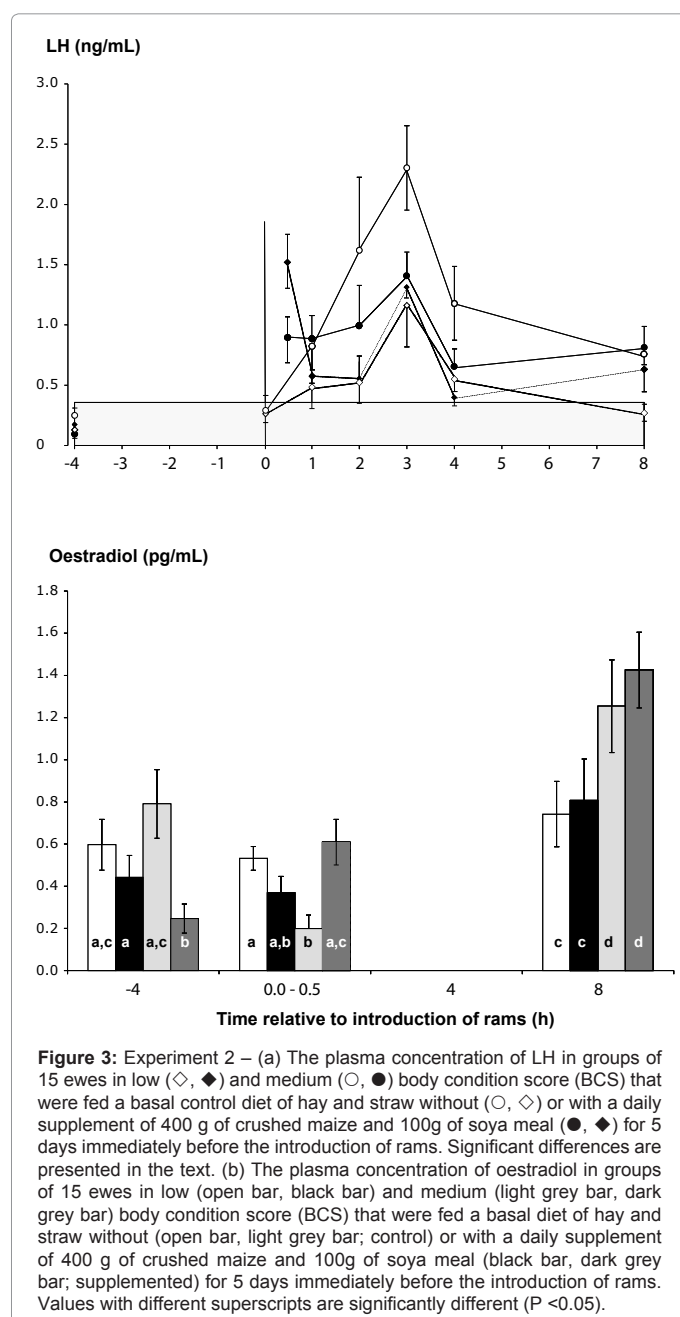


Figure 2: Experiment 1 – The left hand graphs show the plasma concentrations of LH and oestradiol in ewes before and after the introduction of rams, in control ewes (○ and open bars; n=14) and ewes that were fed a daily supplement of 400 g of crushed maize and 100g of soya meal (● and hatched bars; n=14) for 5 days immediately before the introduction of rams. The right hand graphs show the plasma concentrations of LH and oestradiol in the same ewes before and after the introduction of rams, but categorised by body condition score into medium (○ and open bars; n=16) and high BCS (● and black bars; n=12) groups. There was no overlap of BCS between the groups. Time points with different superscripts are significantly different and an asterisk (*) indicates a significant difference between treatments within times (P<0.05).

minutes) after the introduction of rams and remained significantly above the concentration measured before the introduction of rams (all <0.002; (Figure 2), top). The concentration of oestradiol was significantly increased (P<0.001) 8h hours after the introduction of rams but not at 0h (P=0.067) and the concentration at 8h was significantly greater than at 0h (P=0.008; (Figure 2), bottom). Dietary supplementation had no significant effect on the magnitudes of these short-term responses to the “ram effect” for both LH (P=0.785; (Figure 2), top left)) and oestradiol (P=0.824; (Figure 2), bottom left). However,

when the data were re-analysed using body condition score to define categories significant effects of BCS were seen on the concentrations of both LH (P<0.001, top right) and oestradiol (P<0.001; (Figure 2), bottom right). Ewes in the high BCS class had higher concentrations of LH at all times after the introduction of rams and the differences were statistically significant at 1h (P=0.002), and 4h (P=0.057) compared to the medium BCS group at the same times. The concentration of oestradiol was significantly higher in the high BCS group compared to the medium BCS group at 8h after the introduction of rams (P=0.002).

Experiment 2: The results of experiment 2 closely match those of experiment 1. The short-term responses to the “ram effect” were significantly increased in medium BCS ewes compared to low BCS ewes (Figure 3) for both LH ($P<0.001$) and oestradiol ($P=0.033$). The increases for LH tending towards significance at 0.5h ($P=0.064$) and 1h ($P=0.083$) and they were significant at 2h ($P=0.047$), 3h ($P=0.050$) and 4h ($P=0.027$). Similarly, the concentrations of oestradiol were significantly higher 8h after the introduction of rams in medium BCS ewes ($P=0.004$) compared to the low BCS group. However, there was no effect of dietary supplementation on the short-term responses of either LH ($P=0.859$) or oestradiol ($P=0.658$; (Figure 3). The concentrations of oestradiol were not significantly higher 8h after the introduction of rams in supplemented ewes compared to control ewes ($P=0.535$; (Figure 3).



Relationships among the “ram effect”, oestradiol and the LH Surge

For this analysis, the data for LH and oestradiol from both experiments were categorized according to the presence of an LH surge, into three groups: (i) non-responders defined as ewes without an LH surge and with progesterone levels below 0.5ng/mL for the duration of the experiment (ii) responders defined as ewes with an LH surge within 52h of the introduction of the rams and an increase in progesterone following the LH surge and (iii) atypical responders defined as ewes in which the LH surge was not detected over the 52 hour period of 4-hourly blood sampling but in which there was an increase in progesterone following the introduction of rams suggesting that an LH surge occurred after the end of the period of 4-hourly blood sampling.

In both experiments, there was clear relationship between the concentration of oestradiol and the detection of an LH surge (Figure 4). Ewes with LH surges had significantly higher concentrations of oestradiol compared to both “no LH surge” ($P<0.001$) and “atypical” groups in both experiments (both $P<0.001$; (Figure 4). In experiment 2 the “atypical” group had its highest concentration of oestradiol 48 hours after the rams were introduced and at this time it was significantly higher than both the “LH surge” ($P=0.001$) and “no LH surge” ($P<0.001$) groups.

Similarly, in both experiments, the presence of a LH surge was also strongly related to the magnitude of the short-term responses of LH and oestradiol (Figure 5). Those ewes with a LH surge had significantly greater short-term responses for LH compared to the “no LH surge” (both $P<0.001$) and “atypical” groups (both $P<0.001$) and in experiment 1, the “no LH surge” group was significantly ($P=0.01$) lower than the “atypical” group. These effects were also reflected in the oestradiol concentrations and in both experiments they were significantly lower at 8 hours after the “ram effect” in the “no LH surge” (both $P<0.001$) and “atypical” groups (both $P<0.01$). In experiment 1, the “atypical” responders tended ($P=0.067$) to have a significantly higher concentrations of oestradiol 8 hours after the introduction of rams, compared to the “no LH surge” group.

Discussion

The proportion of ewes responding to the “ram effect” were unexpectedly low (Table 1) in both experiments and typifies the difficulties often encountered with “ram effect” experiments. Nevertheless we were able to collect valuable data and to draw some tentative conclusions from the results of the two experiments.

The results of these experiments suggest that the interaction of nutrition and the “ram effect” is affected by at least two components of nutrition. Both the body condition score (fatness) and the level of dietary supplementation influenced the response to the “ram effect” but in different ways and in this respect the two independent experiments in this study produced some consistent results. Short-term dietary supplementation had no effect in either experiment, on the proportion of ewes that responded to the “ram effect” whereas the body condition score of the ewes at the time of the “ram effect” had a highly significant effect. In both experiments, a greater proportion of ewes responded to the “ram effect” in the higher BCS group compared to lower BCS group. By contrast, in experiment 1, short-term nutritional supplementation increased the proportion of ewes showing oestrus whereas there was no effect of body condition score on this response but, exactly the opposite effects were seen in experiment 2. These contradictory findings are

obviously problematic, but they may be explained by any one of several differences between experiments 1 and 2. In experiment 2, the ram-induced ovulation tended to be later (Figure 4) and thus the timing of the second period of supplementation was early in relation to the second ovulation. Other differences include the breed and age of the ewes and the BCS categories that were compared (medium vs. high in experiment 1 and low vs. medium in experiment 2) all of which may have also affected the expression of oestrus.

The expression of oestrus in the ewe is an oestrogen-dependent phenomenon that is dependent on a period of prior exposure to progesterone [23,24] thus in ewes, the ram-induced ovulation during anoestrus when progesterone levels are low for a prolonged period of time, is not generally accompanied by oestrus. For this reason a second period of nutritional supplementation between days 13 and 18 after the "ram effect", was introduced into the experimental design and in experiment 1, this was associated with an enhanced expression of oestrus at the following ovulation because of exposure to progesterone following the ram-induced ovulation. However this effect was not seen in experiment 2 (discussed above). The findings from experiment 1 are consistent with recent findings in goats [5,6,25] where it was reported that short-term nutritional supplementation improved oestrus and ovulation rate of anoestrous female goats in semi-arid north-central Mexico but not the proportion responding to the "buck effect".

Despite the fact that the supplementation was similar, it produced different effects on the blood concentrations of glucose and insulin between the two experiments; surprisingly, supplementation had little effect on these parameters in experiment 2. In hindsight, the basal diet of the medium BCS groups in experiment 2 provided more energy than intended and this could have reduced the impact of the supplementation. The inconsistent nature of these observations may originate from the differences in the basal diets or they may reflect underlying differences between the Île-de-France and Romane breeds. Both the glucose and insulin data were more variable in Romane ewes and could have masked any effect of supplementation. The Romane breed (formerly known as INRA 401) is a synthetic breed developed by INRA and contains a high proportion (50%) of Romanov genes and as a breed they are nervous and easily stressed [26,27] which could alter carbohydrate metabolism as reported in rats [28,29] potentially explaining the differences in the two sets of data.

High concentrations of blood glucose are taken up primarily in muscle and adipose tissue by insulin-dependent, GLUT4-mediated mechanisms. However, the ovarian follicle also contains GLUT4 [30,31] and therefore it is likely that the follicle also increases its insulin-dependent uptake of glucose when the blood concentrations of glucose are elevated but, the effect that this has on follicular function is not known. Short-term dietary supplementation had no effect on

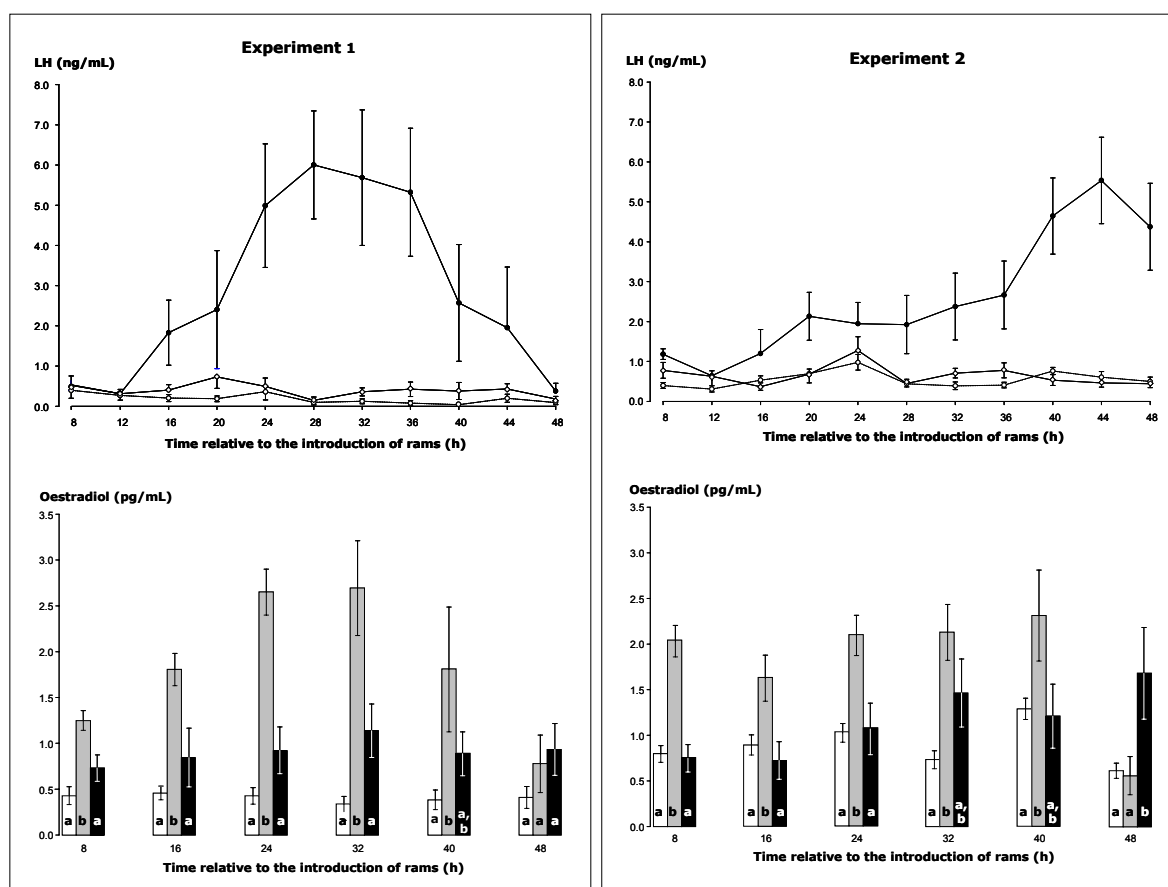


Figure 4: Experiments 1 and 2 –The plasma concentrations of LH and oestradiol in ewes of ewes between 8 and 48 hours after the introduction of rams with (i) a LH surge (● and grey bars), (ii) ewes without a LH surge and no increase in the concentration of progesterone following the introduction of rams (○ and open bars) and (iii) ewes without a LH surge but with an increase in the concentration of progesterone following the introduction of rams (◇ and black bars). Experiment 1 on the left; LH surge (n=5), no LH surge (n=14) and Atypical (n=9) and experiment 2 on the right LH surge (n=13), no LH surge (n=39) and Atypical (n=8). For the LH data, P values are presented in the text and for the oestradiol data values with different superscripts are significantly different (P < 0.05).

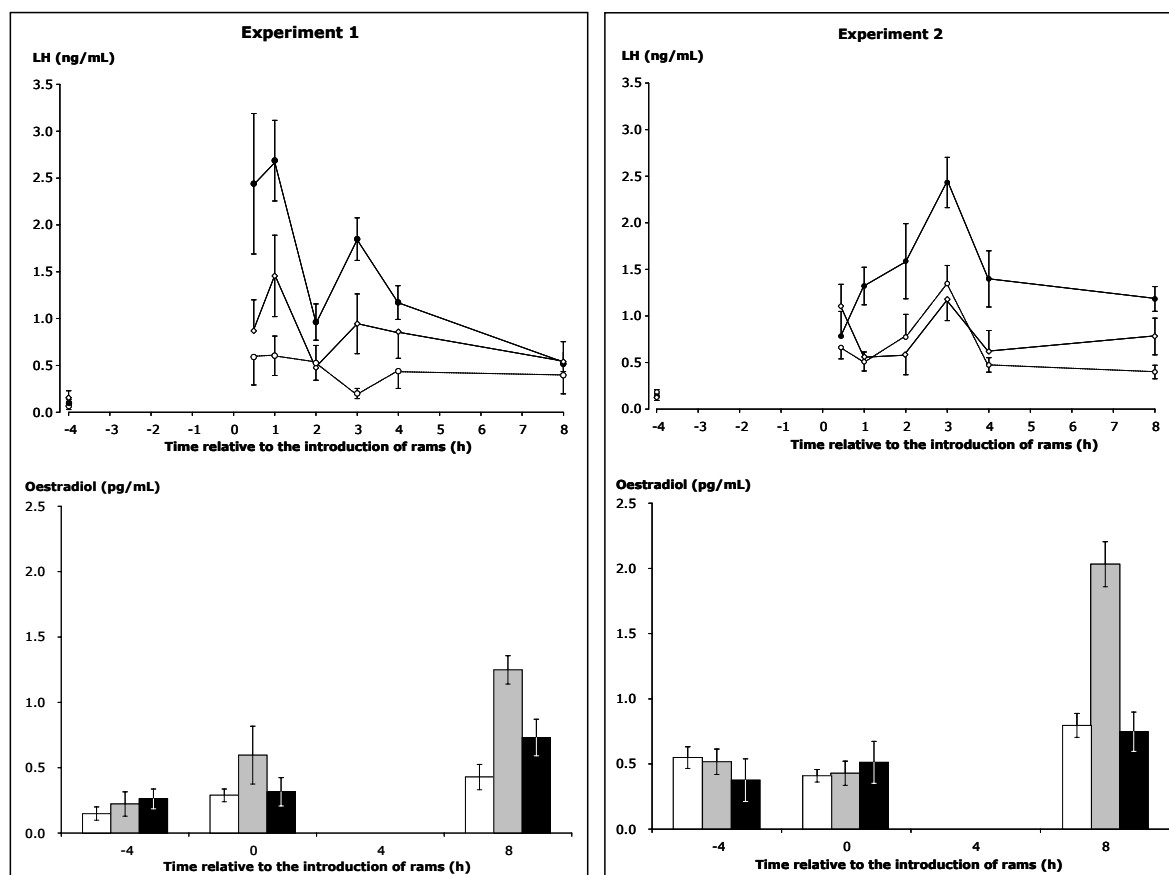


Figure 5: Experiments 1 and 2 – The short term changes in the plasma concentrations of LH and oestradiol in ewes of ewes between -4 and 8 hours after the introduction of rams with (i) a LH surge (● and grey bars), (ii) ewes without a LH surge and no increase in the concentration of progesterone following the introduction of rams (○ and open bars) and (iii) ewes without a LH surge but with an increase in the concentration of progesterone following the introduction of rams (◇ and black bars). Experiment 1 on the left; LH surge (n=5), no LH surge (n=14) and Atypical (n=9) and experiment 2 on the right LH surge (n=13), no LH surge (n=39) and Atypical (n=8). For the LH data, P values are presented in the text and for the oestradiol data values with different superscripts are significantly different (P < 0.05).

the blood concentration of FSH in either experiment, while the blood concentrations of oestradiol were unchanged by dietary supplementation in experiment 1 but they were suppressed in experiment 2. This apparent contradiction arises most likely because the average concentrations of oestradiol in experiment 1 were very low in control ewes and very close to the limit of detection of the RIA that was used. Experiment 1 was carried out in May at the deepest part of anoestrus and using a more seasonal breed (Île-de-France) than experiment 2 which was carried out in June with the less seasonal Romane breed and thus it is not surprising that endogenous concentrations of oestradiol were so low in experiment 1. Short-term nutritional supplementation with glucogenic diets [32,33] and the short-term infusion of glucose [15] into sheep during the breeding season reduced the concentration of oestradiol and the level of aromatase in oestrogenic follicles while at the same time increasing the number of small and medium sized follicles and the results of experiment 2 are consistent with these observations at least with respect to oestradiol.

The effects of body condition score are particularly interesting because the differences were relatively modest at about half a unit of body condition score (approximately 5 kg). We have recently established that each unit of body condition score equates to a difference in back fat thickness of approximately 10 mm for Île-de-France ewes (Jean-Baptiste Menassol, Benoît Malpau and Rex Scaramuzzi; unpublished data). Our experiments show that quite small differences in body

condition score can have major effects on the ability of ewes to respond to the "ram effect". Ewes in the high body condition score groups had a better short-term response to the "ram effect". The mean concentration of LH during the short-term response was greater and peaked earlier in ewes in the high body condition score groups and this resulted in higher concentrations of pre-ovulatory oestradiol and a greater overall response that is, the LH surge, ovulation and the formation of a corpus luteum in ewes with a higher body condition score.

From the results of these studies examining the effects of body condition score and nutritional supplementation on the "ram effect", we suggest tentatively that the effect of body condition score is mediated primarily at a hypothalamic level so that ewes in good body condition have an enhanced response to "ram effect"; they secrete more LH presumably because they release more GnRH in response to the pheromonal stimulus of the "ram effect". We also suggest that the effect of short-term nutritional supplementation under some conditions, is probably mediated at an ovarian level to enhance the expression of oestrus and based on the results of other investigations [32,33], possibly also ovulation rate.

The "ram effect" involves a co-ordinated sequence of hypothalamic, pituitary and follicular events starting with increased secretion of GnRH followed by increased secretion of LH and oestradiol culminating in an LH surge and ovulation. Within this sequence of endocrine events

mainly because of technical difficulties, only one hormone has been examined in detail, defining for LH both its short-term LH response and the pre-ovulatory surge. There is very little information about the secretion of GnRH and oestradiol in response to the “ram effect”. In this study we were able to determine the pattern of secretion of oestradiol following the “ram effect”. On one hand, ewes that did not have any type of oestrous cycle after the “ram effect” consistently had a very poor short-term LH and oestradiol responses to the “ram effect” and this was not followed by a pre-ovulatory increase in the concentration of oestradiol, consequently these ewes did not have a LH surge and did not ovulate. On the other hand ewes that had an oestrous cycle of some sort, after the “ram effect” consistently had a better short-term LH and oestradiol responses to the “ram effect” and these were followed by a pre-ovulatory increase in the concentration of oestradiol and an LH surge within 48h of the “ram effect”. All these ewes ovulated. A third pattern of response was observed and these were classed as “atypical” responders. These ewes all had oestrous cycles of some sort, after the “ram effect” despite the fact that no LH surge was detected over the first 48h after the “ram effect”. The short-term LH and oestradiol responses to the “ram effect” in this group was intermediate between the “LH surge” and “no LH surge groups” and we suggest that in these ewes an LH surge did take place but later than 48h and so it was not detected. Indeed in the “atypical group” the blood concentration of oestradiol was either not decreasing (experiment 1) or still increasing (experiment 2) 48h after the “ram effect” suggesting that its preovulatory increase was delayed (Figure 4). The later rise in LH and oestradiol following the “ram effect” in the Romane breed (Figure 4), (experiment 2) is consistent with the observation that positive feedback in the Romanov breed (the Romane is a Romanov cross) is less sensitive to oestradiol than the Île-de-France breed [21]. These observations are as far as we are aware the first detailed descriptions of the pattern of secretion of oestradiol following the “ram effect” and show that the sequence of reproductive endocrine events in an ovulation induced in anoestrus using the “ram effect” are qualitatively similar to those following a spontaneous ovulation during the breeding season.

In conclusion, the results of the two experiments reported in this paper suggest that there is a dual effect of nutrition on the response to the “ram effect”. First is an effect of body condition score on the proportion of ewes ovulating in response to the “ram effect” that is associated with greater short-term responses of LH and oestradiol induced by the “ram effect” and this effect is most likely hypothalamic in origin. Second in experiment 1, there was an effect of short-term nutritional supplementation that was associated with a higher proportion of oestrus ewes in response to the “ram effect” and because this effect was not associated with differences in the short-term LH response, it probably has an ovarian origin. Finally, the results of the two experiments also show that there is a very close relationship between a follicles ability to secrete oestradiol and the likelihood of a LH surge in response to the “ram effect”, suggesting that the functional capacity of the follicle at the time of the “ram effect” is an important determinant of outcome and point to the need for further investigations of follicular physiology in anoestrous ewes.

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