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Follicle development, endocrine profiles and ovulation rate in adult Merino ewes – effects of early nutrition (pre- and post-natal) and supplementation with lupin grain

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Summary

In adult ewes, we tested whether ovarian function, including the response to short-term supplementation, was affected by the nutrition of their mothers during the pre-/post-natal period. A 2x2 factorial was used with nutrition in early life (low or high) and a 6-day supplement (with or without) as factors. All ewes received 3 prostaglandin injections 7 days apart, and the supplement (lupin grain) was fed for 6 days from 2 days after the second until the third prostaglandin injection. We measured reproductive and metabolic hormones, studied follicle dynamics (ultrasonography), and evaluated granulosa cell numbers, aromatase activity and oestradiol concentrations in follicular fluid in healthy follicles at Days 3 and 7 of supplementation. Ovulation rate was increased by 25% by exposure to high pre-/post-natal nutrition (1.5 versus 1.2; $P < 0.05$), in association with a small decrease in FSH concentrations ($P = 0.06$) and a small increase in insulin concentrations ($P = 0.07$). The number of healthy antral

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follicles was not affected. Acute supplementation increased the number of granulosa cells (3.7 ± 0.2 *vs* 3.0 ± 0.2 million; $P < 0.05$) in the largest follicle, and the circulating concentrations of oestradiol (4.6 ± 0.3 *vs* 3.9 ± 0.3 pmol/L; $P < 0.05$) and glucose (3.4 ± 0.03 *vs* 3.3 ± 0.03 mmol/L; $P < 0.01$). Both early life nutrition and acute supplementation appear to affect ovulation rate through changes in glucose-insulin homeostasis that alter follicular responsiveness to FSH and therefore oestradiol-FSH balance.

Other key words: lupin grain, Merino sheep, oestradiol-FSH feedback, metabolic hormones, ovulation rate

Introduction

In sheep genotypes such as the Merino, ovulation rate and fecundity are limited by nutrition and genetic background (Kleemann & Walker 2005). Thus, ovulation rate can be increased by short-term supplementation with lupins, a legume grain with high contents of metabolisable energy and protein that can be safely fed as an acute supplement in large amounts because it contains low concentrations of fermentable starch (White *et al*., 2007). However, responses to short-term lupin supplementation are variable and largely dependent on the ovarian population of antral follicles (Gherardi & Lindsay 1982, Leury *et al.* 1990, Nottle *et al.* 1997; Viñoles *et al.* 2010b). In ewes, the population of antral follicles is affected by several factors, including genotype and body condition (Tassell *et al.* 1983; Viñoles *et al*., 1999), and perhaps the perinatal nutritional history of their mother.

In sheep, low levels of maternal nutrition reduce the ovulation rate and prolificacy of their female offspring (Gunn *et al.* 1995, Rae *et al.* 2002). In cattle, a low level of nutrition during pregnancy decreases the numbers of antral follicles in the offspring, an effect that appears to explain their lifetime reproductive performance (Evans *et al.* 2010). It has long been known that undernutrition from conception to Day 110 of pregnancy delays fetal follicle development in sheep (Rae et al., 2001), but long-term effects on the population of antral follicles in adulthood have not been studied. Importantly, in addressing this issue, it is essential to evaluate the functional status of

the follicles, because only non-atretic follicles can respond to changes in nutrition. Ultrasonography can determine the growth trajectory of follicles but not their functional status, so we need to dissect and biochemically assess follicles at specific
70 stages of the follicular wave (review: Scaramuzzi et al., 2011).

Short-term feed supplements such as corn grain plus soybean meal, glucose infusion and lupin grain, evoke changes in the concentrations of metabolic hormones, with the peak of the response observed three days after the start of the supplement, in
75 association with an increase the number of antral follicles selected into the ovulatory wave (Viñoles *et al.* 2005, Scaramuzzi *et al.* 2006, Viñoles *et al.* 2010b, Scaramuzzi *et al.* 2011). As a consequence, we would expect short-term supplementation to alter the balance of the oestradiol-FSH feed back loop, but this has been difficult to demonstrate experimentally because the waves in FSH concentration, follicle population, and
80 oestradiol concentration are not synchronised among ewes during the oestrous cycle (Viñoles *et al.* 2010b). With respect to the effects of pre- and post-natal nutrition on reproductive performance as adults, the mechanisms are not known, but probably include similar changes in the balance of hormones that are directly responsible for follicle growth and steroid production, perhaps also mediated by changes in the
85 concentrations of the metabolic hormones that affect the responsiveness of follicles to gonadotrophins (Rhind *et al.* 2001, Scaramuzzi *et al.* 2011).

In this experiment, we tested the hypothesis that ewes exposed to high levels of nutrition in pre-/post-natal life would have more antral follicles, and thus a higher
90 ovulation rate, in adult life, and show a further increase in the number of healthy, potentially ovulatory follicles following short-term supplementation with lupins, compared to ewes exposed to lower levels of nutrition in pre-/post-natal life. The presence of extra follicles in ewes that had been well-fed in early life would be associated with higher concentrations of glucose and metabolic hormones, and thus an
95 increase in the steroidogenic capacity of healthy follicles and, therefore, low FSH concentrations. To test this hypothesis, we used the ‘first-wave model’ to synchronise the first follicular wave of the cycles (Viñoles *et al.* 2010b) of 5-year-old Merino ewes that had been born to mothers for which the level of nutrition had been manipulated from mating to weaning, yet were all born as singles and with a similar birth weight.

100

Materials and methods

The experimental procedures were approved by the Eastern Ethics Committee of the Department of Primary Industries, Hamilton, Victoria (Approval Number 2006-15W).

105

Animals and pre-experimental management

This experiment used 40 Merino ewes that were born to mothers that were in body condition score 3 (Russel *et al.* 1969) at mating. Thereafter, the mothers had been
110 allocated to two groups to apply pre-/post-natal treatments to their fetuses: the 'High' pre-/post-natal nutrition group was run on high-quality pasture (3000 kg dry matter/ha) from mating through pregnancy (score 3 on day 100 of pregnancy) to weaning at 72 ± 0.2 d; the 'Low' pre-/post-natal nutrition group was also originally in body condition score 3 at mating, but were then maintained on a low quality pasture (900 kg dry
115 matter/ha) from mating through pregnancy (score 2 by day 100) to weaning (Fig. 1). The 'Low' group was considered to be a control, based on the predicted liveweight changes of Merino ewes under paddock conditions (Ferguson *et al.* 2011). So, the fetuses were exposed to maternal undernutrition around Day 65 of pregnancy, when their ovaries are thought to be most sensitive to metabolic inputs (Rae *et al.* 2001).

120

Only single-born ewes of similar birth weight (5.0 ± 0.1 kg for High, 4.7 ± 0.1 kg for Low; mean ± SEM; P > 0.05) were selected for the experiment. From weaning onwards, they were grazed together on a pasture-based diet on a commercial property near Hamilton in Victoria (141.7ºE 41'25s, -37.6ºS 36'1s) following the
125 'LifetimeWool' guidelines (Young *et al.* 2011). This involved an annual cycle with periods of liveweight loss (2-3 kg) during summer and autumn, periods of liveweight maintenance and periods of liveweight gain.

At the beginning of the present study, when the ewes were 4.9 ± 0.1 years old, body
130 condition score and live weight did not differ (P > 0.05) between the High (2.7 ± 0.1 and 53 ± 1.3 kg) and Low (2.6 ± 0.1 and 51 ± 1.3 kg) groups. For the duration of the experiment, the ewes were randomly allocated to individual pens in an animal house where they were maintained under natural lighting (12:10 h light and 11:50 h

darkness). They were acclimatized to these conditions for 3 weeks during which they
135 were individually fed a nutritionally complete, pelleted diet. The quantity offered to each ewe was calculated to meet their individual metabolisable energy (ME) requirements for maintenance (CSIRO 2007) which was an average of 6.07 MJ ME/day or 674 g of pellets/day. The pellets were 91% dry matter (DM) and supplied 9.0 MJ ME and 110 g of crude protein (CP) per kilogram (as-fed basis). The ewes were
140 fed daily at 00.00 h (Hour 0) and offered water *ad libitum*. Feed refusals were collected and weighed daily and were less than 10%.

Dietary treatments

145 A 2x2 factorial design was used with pre-/post-natal nutrition (Low or High) and lupin supplementation (with or without) as the factors, and 10 animals per group (Fig. 1). During supplementation, whole lupin grain (92% DM, 12.6 MJ ME and 303 g CP per kilogram as-fed basis) was added to the pelleted maintenance diet described above so supplemented animals received double their energy requirements for maintenance
150 (Table 1). Feed refusals remained under 10%.

Cycle synchronisation

All ewes received 3 injections of prostaglandin analogue (each 250 μg cloprostenol;
155 Juramate®, Jurox Pty. Ltd., Australia) 7 days apart in accordance with our 'first-wave' model for synchronising follicle development among ewes (Fig. 1). This allows us to apply the nutritional treatment precisely around the time of emergence of the follicle wave and thus ensure that, for all animals, the peak concentrations of metabolic hormones coincides with follicle selection, when FSH concentrations are low, thus
160 permitting co-dominance (Viñoles *et al.* 2010b). The 6-day period of supplementation began 2 days after the second prostaglandin injection (ie, at the expected time of ovulation and emergence of the first follicular wave of the cycle) and continued until the third prostaglandin injection (Viñoles *et al.* 2010b). To facilitate the processing of tissues, the synchronisation treatment, and thus the beginning of the nutritional
165 supplement, was staged over 3 consecutive days, with two groups of 14 and one group of 12 ewes, with each group containing all treatment combinations.

Ovarian studies

170 The ovaries were examined daily from the day of the second prostaglandin injection until the day of the third prostaglandin injection using transrectal ultrasonography. We used a real-time, B-mode scanner (Aloka SSD 900 Co. Ltd, Insight, Oceania) with a rigid 7.5 MHz transducer modified for external manipulation in the rectum (Viñoles *et al.* 2010a). The ovulation induced by the second prostaglandin injection was detected
175 by observing the collapse of a large (≥ 5 mm) follicle followed by the presence of luteal tissue at the same site 4 days later. For both ovaries on each day, we noted all corpora lutea and the total number, diameter and position of all follicles of diameter ≥ 2 mm. The accuracy and precision of this procedure has been confirmed by analyses of scanned ovaries post-mortem (Viñoles *et al.* 2004). A follicular wave was defined as
180 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 follicular waves were described in relation to measures of the largest follicle: day of emergence, maximum diameter, and day of
185 maximum diameter (Viñoles *et al.* 2001). The day of wave emergence was deemed as the day the largest follicle of the wave (identified retrospectively) reached 2-3 mm in diameter. Lifespan was defined as the number of days between emergence and ovulation.

190 *Dissection of the ovarian follicles*

Ovaries from five animals in each group were recovered 3 days after the start of supplementation (recruitment of follicles into the first wave of the cycle) and from the other five animals 30 h after the third prostaglandin injection (selection of the pre-
195 ovulatory follicles). The numbers of corpora lutea were counted to confirm the ultrasonographic observations. All individual follicles of diameter ≥ 3 mm were dissected free of extraneous tissue in phosphate-buffered saline (PBS) under a stereomicroscope and their diameters recorded to the nearest 0.5 mm. A small slit was made in the follicle wall to allow the antral fluid to escape into a Petri dish, from where

200 it was aspirated through a finely drawn-out Pasteur pipette, taking care not to remove clumps or sheets of granulosa cells. A known volume of the follicular fluid was added to 100µL PBS and frozen at –20°C for the subsequent measurement of oestradiol concentrations, the most reliable indicator of the health status of antral follicles over a wide range of sizes (McNatty *et al.* 1985). The granulosa cells were recovered and
205 counted by haemocytometer. To help classify follicles as non-atretic or atretic, the presence or absence of thecal blood capillaries (at 10 x magnification) and of debris in follicular fluid were noted. In addition, after removal of the granulosa cells, the colour of the theca interna (i.e. red, pink or white) was recorded. For the purpose of this study, a healthy follicle was defined as one with: a) visible thecal blood capillaries, b)
210 follicular fluid devoid of debris, c) a pink- to red-coloured theca interna, d) a normal-looking and intact oocyte-cumulus cell complex and e) more than 25% of the maximum number of granulosa cells for a given follicle size (McNatty *et al.* 1985). Conversely, an atretic follicle was one to which any of these criteria did not apply.

215 *Granulosa cell aromatase assay*

Granulosa cells from individual healthy follicles were collected into PBS and 1% (w/v) BSA. They were washed and resuspended in PBS and 1% BSA so that the final cell concentration was 6 x 10^4 granulosa cells/ml; 0.15 mL aliquants of these cell
220 suspensions were placed in 10 x 75 mm plastic tubes containing 0.15 mL of a PBS and 1% BSA solution with or without 2000 ng testosterone/ml. The cell suspensions were gassed with 5% CO_2 in air, sealed and then incubated for 3 h in a shaking water bath at 37°C. At the end of the incubation, the tubes containing medium plus cells were frozen at –20°C. Subsequently, the contents of the tubes were thawed, centrifuged and the
225 supernatants assayed for oestradiol-17ß.

Blood sampling, glucose and hormone assays

From the day before the initiation of the nutritional treatment until the day of the third
230 prostaglandin injection, jugular blood (5 ml) was sampled daily, 1 h before feeding (Hour 0), into tubes that contained heparin and potassium oxalate. On Days –1, 3 and 6 from the initiation of the nutritional treatment (Day 0), blood was sampled at –1, 3.5 and 7 h relative to feeding. The samples were kept on ice and plasma was separated by

centrifugation within 10 min of sampling and stored at –20°C until assayed.

235

Plasma progesterone was assayed in duplicate using a standard radioimmunoassay (RIA) kit (Diagnostic Systems Laboratories Inc, Webster, TX) as described elsewhere (Gray *et al.* 2000). Only samples collected at –1 h relative to feeding were assayed. The limit of detection was 0.3 ng/mL. The intra-assay coefficients of variation were
240 2.8% for low (1.2 ng/mL) and 4.2% for high (9.9 ng/mL) progesterone values in plasma, and inter-assay coefficients of variation were 2.3% and 17.4%, respectively.

Oestradiol-17β was assayed in a single RIA using an Adaltis MAIA Oestradiol Kit from Diagnostic Technology (Suite 45, 7 Narabang Way, Belrose, NSW 2085,
245 Australia). Plasma samples collected at –1, 3.5 and 7 h relative to feeding were pooled to obtain a daily profile. Oestradiol was extracted from 400 µl plasma and 200 µl quality controls and standards using 2 ml diethyl ether with an extraction efficiency of 88 ± 2%. The limit of detection was 3 pmol/L. The intra-assay coefficient of variation was 4.9% for concentrations of 11 pmol/L and 16.4% for concentrations of 35 pmol/L.
250 The inter-assay coefficient of variation was 25% and 18.5 % for low and high oestradiol concentrations, respectively. Concentrations of oestradiol in follicular fluid and the medium from granulosa cell cultures were measured using the same kit described above, except that samples were not extracted. The limit of detection of the assay was 3.7 pmol/L. The intra-assay coefficient of variation was 10% for
255 concentrations of 21 pmol/L and 11% for concentrations of 58 pmol/L. The inter-assay coefficient of variation was 8% and 2% for the low and high oestradiol concentrations. Since samples of follicular fluid were further diluted 1/100 in preparation for the assay, values were multiplied by 1000 to express the concentrations in nmol/L. The oestradiol production by granulosa cells was calculated base on the following formula= estradiol
260 concentrations (pmol/tube)/assay volume (µl) x 600/1000)/(cell number (x10^6) x 1000000, and expressed as nmoL oestradiol/million granulosa cells.

Plasma concentrations of FSH were measured in a single RIA using reagents kindly supplied by Dr AF Parlow of the National Institute of Diabetes, Digestive and Kidney
265 Disease (Baltimore, MD), as 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 (6 replicates) containing 1.5, 2.4 and 3.2 ng/mL were used to determine intra-assay coefficients of variation: 2.2%, 3.0% and 3.2%. The inter-assay coefficients of variation were 1.6%, 1.9% and 2.2% for the low, medium and high
270 quality controls.

Glucose concentrations were determined spectrophotometrically in samples collected with fluoride-oxalate as the anticoagulant. Concentrations were measured in a Cobas Mira Autoanalyser (Hoffman-La Roche Diagnostica, Basal, Switzerland) with an
275 Infinity™ Glucose hexokinase kit (Cat. No. TR15421, Thermo Electron Co., Melbourne, Australia). The intra and inter-assay coefficients of variation for the quality control (5.3 mmol/L) were 3.2% and 4.9%.

Plasma insulin was assayed in duplicate in a single double-antibody RIA (Tindal *et al.*
280 1978) that had been validated for sheep plasma in our laboratory (Miller *et al.* 1995). The limit of detection was 1.2 mU/mL. The assay included 6 replicates of control samples containing 2.7, 4.1 and 9.3 mU/mL, for which the intra-assay coefficients of variation were 9.1%, 5.5% and 7.6%.

285 Leptin was assayed in all samples in a single double-antibody RIA using an antibody raised against recombinant bovine leptin in an emu, as described in detail by Blache *et al.* (2000). 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 0.1, 0.4 and 1.04 ng/mL included in the assay to estimate the intra-assay coefficients of
290 variation, which were 20.4%, 6.8% and 7.2%.

Plasma concentrations of IGF-I were measured in one double-antibody RIA (Gluckman *et al.* 1983), after interference by binding proteins had been minimized using an acid-ethanol cryoprecipitation, as validated for ruminant samples (Breier *et al.*
295 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 0.44 and 2.42 ng/mL IGF-I, were included in the assay and were used to estimate the intra-assay (11.1% and 10.3%) coefficients of variation.

300

Statistical analyses

Categorical data, such as the proportions of animals that developed a second follicular wave during the experimental period was analysed by the *genmod* procedure in the
305 Statistical Analysis System (SAS, 9.1.3, SAS Institute Inc., Cary, NC, USA). The effects of pre-/post-natal nutrition, supplementation, and the interaction of these two factors, on ovulation rate were compared using a generalized lineal model with a binomial distribution (0 = single ovulation and 1 = double ovulation) after log transformation of the data. A logistic regression was used to test whether the presence
310 or absence of follicles was affected by pre-/post-natal nutrition, supplementation, and day (3 or 7) from the start of feeding. A second set of data was created using the animals that had follicles, to analyse the impact of pre-post-natal nutrition, supplementation, and day (3 or 7) on the status (healthy or atretic) and size class (3, 4 and 5 mm) of follicles. Data that involved repeated measurements (eg, plasma
315 hormone concentrations, follicular development) were analysed by the mixed-model procedure of SAS, including the fixed effects of time, day of sacrifice, pre-/post-natal nutrition, and supplement, and their interactions. The normality of the data and the presence of outliers were checked using the univariate procedure available in SAS. The covariance structure was modelled using the random effect of ewe-within-group plus
320 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 $P < 0.05$ and tendencies for $0.05 < P < 0.1$. Data are presented as least square means ± pooled standard errors.

325

Results

Follicular dynamics

330 The growth profile and maximum diameter of the follicles that were induced to ovulate by the second prostaglandin injection (before the beginning of the supplementation period) did not differ among groups ($P > 0.05$). Fig. 2 shows that the ovulatory follicle

grew in parallel with a decrease in the concentrations of progesterone and an increase in the concentrations of oestradiol. The characteristics of the dominant follicle of the
335 first wave (day of emergence, lifespan, maximum diameter, growth rate) also did not differ among groups ($P > 0.05$). FSH concentrations increased in all groups prior to the emergence of the first follicular wave, with associated changes in the number of follicles ≥ 3 mm in diameter (Fig. 2). Progesterone concentrations increased from Day 3 after the start of the nutritional treatment and peaked on Day 6 in all groups (Fig. 2).
340 The proportion of ewes developing a second follicular wave (high early nutrition, non-supplemented = 2/5; high early nutrition, supplemented =1/5; low early nutrition, non-supplemented = 3/5; low early nutrition, supplemented = 2/5) and the growth profile of the largest follicle of the second wave did not differ among groups ($P > 0.05$). In the supplemented groups, FSH concentrations increased significantly with the emergence
345 of the second follicular wave. The final growth of the pre-ovulatory follicles induced by the third PG injection occurred in parallel with a decrease in progesterone and an increase in oestradiol concentrations (Fig. 2).

Ovulation rate, as determined by the number of corpora lutea detected by
350 ultrasonography after the second prostaglandin injection and confirmed by direct observation post-mortem, was higher with exposure to High (1.5 ± 0.1) than to Low pre-/post-natal nutrition (1.2 ± 0.3; $P < 0.05$).

Pre-post-natal nutrition, supplementation and day had no effect on the presence or
355 absence of follicles ($P > 0.05$), or on the status (atretic or healthy) of follicles. However, the number of healthy follicles in the different size classes was affected by day after feeding commencement (Table 2) – there were more 3 and 4 mm healthy follicles on Day 3, but more 5 mm healthy follicles on Day 7 ($P < 0.001$).

360 *Granulosa cells and oestradiol concentrations*

The size of the largest healthy follicle (6.1 ± 0.2 mm) and its number of granulosa cells (3.7 ± 0.2 million) were greater in supplemented than in non-supplemented ewes (5.4 ± 0.2 mm and 3.0 ± 0.2 million; $P < 0.05$) irrespective of day of supplementation or of
365 pre-/post-natal nutrition treatment. The antral fluid concentrations of oestradiol in

healthy follicles were affected by the supplement ($P < 0.05$) and by the interaction between supplement, pre-/post-natal nutrition and day ($P < 0.01$). The supplement decreased the oestradiol concentrations in follicular fluid in ewes from both the Low and High pre-/post-natal nutrition groups on Day 7 (Table 3).

370

The capacity of the granulosa cells from healthy follicles to produce oestradiol from testosterone was not affected by pre-/post-natal nutrition, supplementation, or the interaction between these factors. The concentration of oestradiol was higher on Day 7 (85.2 ± 5.5 nmoL oestradiol/million granulosa cells) than on Day 3 (50.7 ± 5.9 nmoL

375 oestradiol/million granulosa cells; $P < 0.001$).

Circulating concentrations of hormones and glucose

380 Ewes that had been exposed to High pre-/post-natal nutrition tended ($P = 0.055$) to have lower FSH concentrations during the experimental period (1.0 ± 0.03 ng/ml) than ewes that had been exposed to Low pre-/post-natal nutrition (1.1 ± 0.03 ng/ml). Supplemented ewes had lower FSH concentrations (1.0 ± 0.03 ng/ml) than non-supplemented ewes (1.1 ± 0.03 ng/ml; $P < 0.01$). Plasma oestradiol concentrations

385 were not affected by pre-/post-natal nutrition but, overall, values were higher in supplemented (4.6 ± 0.3 pmol/L) than in non-supplemented ewes (3.9 ± 0.3 pmol/L; $P<0.05$). Maximum oestradiol concentrations tended to be higher in supplemented (11.2 ± 0.8 pmol/L) than in non-supplemented ewes (9.2 ± 0.8 pmol/L; $P=0.09$).

390 Concentrations of glucose were affected by supplementation, day and the interaction between these two factors (Fig. 3, 4), but not by pre-/post-natal or time after feeding ($P > 0.05$). In non-supplemented groups, concentrations decreased from Day 2 to 7 (Fig. 4) while, in supplemented groups, concentrations reached maximum values on Day 4 and decreased thereafter. Overall, supplemented ewes had slightly higher glucose

395 concentrations (3.4 ± 0.03 mmol/L) than non-supplemented ewes (3.3 ± 0.03 mmol/L; $P < 0.01$), but concentrations were similar in ewes from the High (3.3 ± 0.03 mmol/L) and Low (3.3 ± 0.03 mmol/L) pre-/post-natal nutrition treatments (Fig. 4). However, the effect of supplementation on pre-feeding glucose concentration was greater in ewes

that had received Low nutrition than those had received High nutrition during pre-
400 /post-natal life (Days 3 and 6 of feeding; Fig. 4).

Concentrations of insulin were affected by pre-/post-natal nutrition, supplementation, day, hour and the interaction among these factors. Ewes that had received High pre-/post-natal nutrition tended ($P = 0.07$) to have higher insulin concentrations (7.7 ± 0.28
405 μU/ml) compared to those that had received Low pre-/post-natal nutrition (7.0 ± 0.28 μU/ml). On the day before the initiation of the nutritional treatment, peak insulin values were observed at 7 h after feeding in all groups, and the peaks were generally advanced to 3.5 h on Days 3 and 6 ($P < 0.001$; Fig. 3). In non-supplemented groups, concentrations remained unchanged during the treatment period (Fig. 3, 4). In
410 supplemented groups, on the other hand, insulin dynamics differed between ewes that had been exposed to High or Low pre-/post-natal nutrition. Concentrations increased from Day 1 to reach maximum values on Day 3 in the High group, whereas maximum values were delayed until Day 5 in the Low group, with a subsequent decrease only observed in the Low group.
415

Concentrations of leptin were affected by supplementation, day and the interaction between the two factors, but they were not affected by pre-/post-natal nutrition. Within each day, values remained relatively stable with time after feeding (Fig. 3). They also did not change over Days -1 to 7 of the supplementation period in the non-
420 supplemented groups (Fig. 4) but, in the supplemented groups, they increased on Day 1 and remained elevated until Day 7. Overall, concentrations were higher in supplemented (1.2 ± 0.03 ng/ml) than in non-supplemented ewes (0.9 ± 0.03 ng/ml), the differences being significant from Days 2 to 7 only in High pre-/post-natal nutrition ewes ($P < 0.01$; Fig. 4).
425

Plasma IGF-I concentrations were also affected by supplementation, day and the interaction between the 2 factors, but not by pre-/post-natal nutrition or time after feeding (Fig. 3). Supplemented ewes (33.0 ± 1.28 ng/ml) had higher IGF-I concentrations than non-supplemented ewes (22.0 ± 1.28 ng/ml; $P < 0.001$; Fig. 4).
430 Overall, concentrations decreased from Day -1 to Day 6 in non-supplemented ewes while, in supplemented ewes, they remained similar or increased (Fig. 4). A steep

decay was observed from Day 2 to Day 4 in supplemented ewes that had received High pre-/post-natal nutrition, but the values recovered thereafter (Fig. 4).

435

Discussion

The effect of nutrition in early life on ovulation rate in adult life agrees with
440 observations by Rae *et al.* (2002), who studied 20-month old ewes. However, the level of nutrition in early life did not affect the size of the population of healthy antral follicles or the numbers of granulosa cells in the largest follicles so, as would be expected it did not affect oestradiol production. On the other hand, FSH concentrations were lower in ewes that received high pre-/post-natal nutrition than in those that
445 received low pre-/post-natal nutrition. Although we did not detect an increase in the number of follicles recruited into the ovulatory wave, ovulation rate was higher in ewes fed well in early life than those fed poorly in early life. Acute supplementation did not affect the size of the population of healthy follicles, but it did increase the size of the largest follicle and its number of granulosa cells, and thus the concentrations of
450 oestradiol, probably explaining the lower concentrations of FSH in supplemented than non-supplemented ewes. Thus, as with acute supplementation (Viñoles *et al*., 2010), the increase in ovulation rate caused by good early nutrition is associated with a reduction in FSH concentrations. This suggests that, in both situations, increases in ovulation rate must be accompanied by increases in the responsiveness of follicles to
455 FSH, perhaps due to the actions of metabolic factors within the ovary, including those involved in glucose homeostasis (Scaramuzzi *et al*., 2011). The level of nutrition in early life had no major effect on the circulating concentrations of glucose or metabolic hormones, but did elicit a small change in the concentrations of insulin. Short-term supplementation markedly increased the circulating concentrations of glucose and
460 metabolic hormones. We therefore suggest that the level of nutrition in early life leads to long-term effects on the dynamics of the metabolic and reproductive hormones that affect the final stages of follicular selection, and thus ovulation rate. However, early nutrition does not seem to affect the metabolic or ovarian responses to acute supplementation.

465

Nutrition in early life did not influence the effect of short-term supplementation on the number of healthy pre-ovulatory follicles, but the pooled data from the two early-nutrition groups leads to new insights into the mechanisms involved in the ovarian response to short-term supplementation. Supplemented ewes had a larger healthy
470 follicle with 20% more granulosa cells and produced 20% more circulating oestradiol. Granulosa cells are the richest source of aromatase activity and thus oestradiol (McNatty *et al.* 1984), but the increased steroidogenic capacity of the follicles in lupin-fed ewes was associated with the increases in granulosa cell numbers but not aromatase activity. The increased production of oestradiol was associated with a
475 decrease in FSH concentrations in supplemented ewes. These observations are coherent with our previous studies of 6-day supplementation with the 'first-wave model' (Viñoles *et al.* 2010b). Using an alternative to the 'first-wave' experimental model, we have also shown that supplementation with corn plus soya bean meal for 6-days during the mid-luteal phase leads to the development of larger preovulatory
480 follicles (Viñoles *et al.* 2005) and, under field conditions, induces a 15% increase in ovulation rate (Viñoles *et al*., 2009). Therefore, across a range of experimental protocols, the common theme is that acute supplementation induces a larger and more oestrogenic pre-ovulatory follicle that reduces FSH concentrations yet allows the selection of extra follicles, thus leading to an increase in ovulation rate.

485

As mentioned above, decreased FSH concentrations are compatible with increased selection of pre-ovulatory follicles if the response to FSH is amplified by, for example, increased concentrations of glucose and metabolic hormones (Scaramuzzi *et al.* 2011). In this experiment, the 6-day lupin supplement increased the circulating concentrations
490 of glucose, insulin, leptin and IGF-I, as seen previously (Viñoles *et al.* 2005, Scaramuzzi *et al.* 2006, Viñoles *et al.* 2010b, Scaramuzzi *et al.* 2011). In the present study, the changes associated with feed intake were evident for insulin, but not for glucose or leptin concentrations, in contrast to previous observations (Marie *et al*., 2001; Viñoles *et al.* 2005). The peak concentrations of glucose and the metabolic
495 hormones occurred between Days 2 and 5 after the start of feeding, a degree of variation that contrasts with the consistent increase on Day 3 observed previously (Teleni *et al*., 1989, Viñoles *et al.* 2005), and that appears to be explained by the level of pre-/post-natal nutrition. These metabolic factors can change the internal endocrine milieu of the follicle – for example, leptin decreases the stimulatory action of IGF-I on

500 steroidogenesis – decreasing the concentrations of oestradiol in the antral fluid and increasing the likelihood that growing follicles will survive in the presence of low FSH concentrations (Spicer *et al.*, 2002; Scaramuzzi *et al.* 2006).

This study is based on our 'first-wave' experimental model in which waves of FSH,
505 follicular growth and oestradiol are tightly synchronised among ewes so as to increase experimental power (Viñoles *et al.* 2010b). The first-wave model is effective in that regard, but the low progesterone concentrations produced by a newly formed and short-life corpus luteum may create an abnormal endocrine milieu (e.g. increased LH pulse frequency), with negative consequences for the development of the pre-ovulatory
510 follicle, the competence of the oocyte and fertility (Sirois & Fortune 1990, Viñoles *et al.* 2001, Fierro *et al.*, 2011, Viñoles *et al.* 2012). The development of an alternative approach for synchronising the emergence of waves of follicles during the luteal phase might be useful; therefore, for testing whether changes in the oestradiol-FSH feedback loop are indeed an effect of nutrition or a consequence of the 'first-wave' model.
515

Conclusions

Exposure to high levels of nutrition in early life increases ovulation in adult ewes, 5 years later, in association with changes in insulin-glucose homeostasis and oestradiol-FSH balance. These responses are similar those following acute nutritional
520 supplementation. It appears that, in both situations, metabolic factors amplify the effect of FSH on follicle development, thus overcoming any effects of a decrease in FSH concentrations caused by the development of a larger steroidogenic follicle, allowing co-dominance and an increase in ovulation rate. The responses to early nutrition and acute supplementation do not appear to interact.
525

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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535

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Legends to Figures

Figure 1. A schematic representation of the experimental design, beginning with the nutritional treatments to which the experimental ewes were exposed during pre-/post-natal life. All ewes were run together until 4.9 years of age, they were moved into an
710 animal house (grey area) where they were fed once per day at 00.00 h. Their follicle waves were synchronized using 3 injections of prostaglandin analogue (PG1, PG2, PG3). On Day 1 (2 days after PG2 on Day –1) half of the ewes in each group were fed daily (F) with a supplement supplying twice their requirements for maintenance for 6 days (black bar). Five ewes from each treatment were sacrificed on Day 3 (S1:
715 recruitment of follicles into the first wave of the cycle) and 5 ewes were sacrificed 30 h after PG3 (S2: selection of the pre-ovulatory follicles). BC = body condition; FOO = food on offer in kg DM/ha.

Figure 2. Growth profile of the follicle induced to ovulate after the second
720 prostaglandin injection (black circle), the dominant follicle of the first (white circle) and the second follicular waves (black square) of the cycle, in association with the numbers of 3-mm follicles (grey bars, upper panel) and the plasma concentrations of FSH (white diamond), oestradiol (black diamond; middle panel) and progesterone (lower panel; white square) in ewes exposed to low and high pre-/post-natal nutrition,
725 that were not supplemented or fed a nutritional supplement for 6 days (from Day 1 shaded area). Arrows indicate the time of the second and third prostaglandin injections. The broken lines on Day 3 indicate sacrifice of 5 ewes, reducing the number of ewes by half in each treatment group. All values are least squares means (± SEM). ^{a} vs ^{b} vs ^{c}, indicates significant changes in the profile of follicle growth and hormonal
730 concentrations within the same group of ewes. ^{x} vs ^{y} vs ^{z}, indicates significant changes in the number of > 3 mm follicles between days within the same group of ewes.

Figure 3. Changes in the plasma concentrations of glucose (square), insulin (circle), leptin (diamond) and IGF-I (triangle) in ewes exposed to low and high pre-/post-natal
735 nutrition, non-supplemented (black symbols) or supplemented with lupins (white symbols) for 6 days (shaded area). Samples were taken at -1, 3.5 and 7 h (relative to the time of feeding at Hour 0) on Day -1 (two days before the start of supplementation)

and the third and the final day of supplementation. The broken line indicates the day of sacrifice of 5 ewes, reducing the number of ewes by half in each treatment group. *=
740 Significant differences between supplemented and non-supplemented ewes. All values are least squares means (± SEM). Note that on Day 6, the number of ewes was reduced to 5 in each treatment.

Figure 4. Changes in the plasma concentrations of glucose (square), insulin (circle),
745 leptin (diamond) and IGF-I (triangle) in ewes exposed to low and high pre-/post-natal nutrition, and either non-supplemented (black symbols) or supplemented with lupin grain (white symbols) for 6 days (shaded area). Samples were taken daily, from two days before supplementation started until the day after the end of the supplementation. Arrows indicate the time of the second and third prostaglandin injections. The broken
750 line indicates the day of sacrifice of 5 ewes, reducing the number of ewes by half in each treatment group. *= Significant differences between supplemented and non-supplemented ewes. All values are least squares means (± SEM).

Table 1. Average amounts of metabolisable energy and crude protein offered to control and supplemented ewes that been previously exposed to low or high pre-/post-natal nutrition.

Pre-/post-natal nutrition	Acute supplementation	Metabolisable energy (MJ/day)	Crude protein (g/day)
low	+	11.9	215.4
low	–	6.0	73.4
high	+	12.2	220.7
high	–	6.2	76.4

Table 2. Numbers of atretic and healthy follicles in 3, 4 and 5 mm size classes found in the groups of 5 ewes sacrificed on Day 3 or Day 7 of the period of supplementation. Data are combined across all treatments (exposure to low and high pre-/post-natal nutrition; maintenance-fed or supplemented with lupin grain).

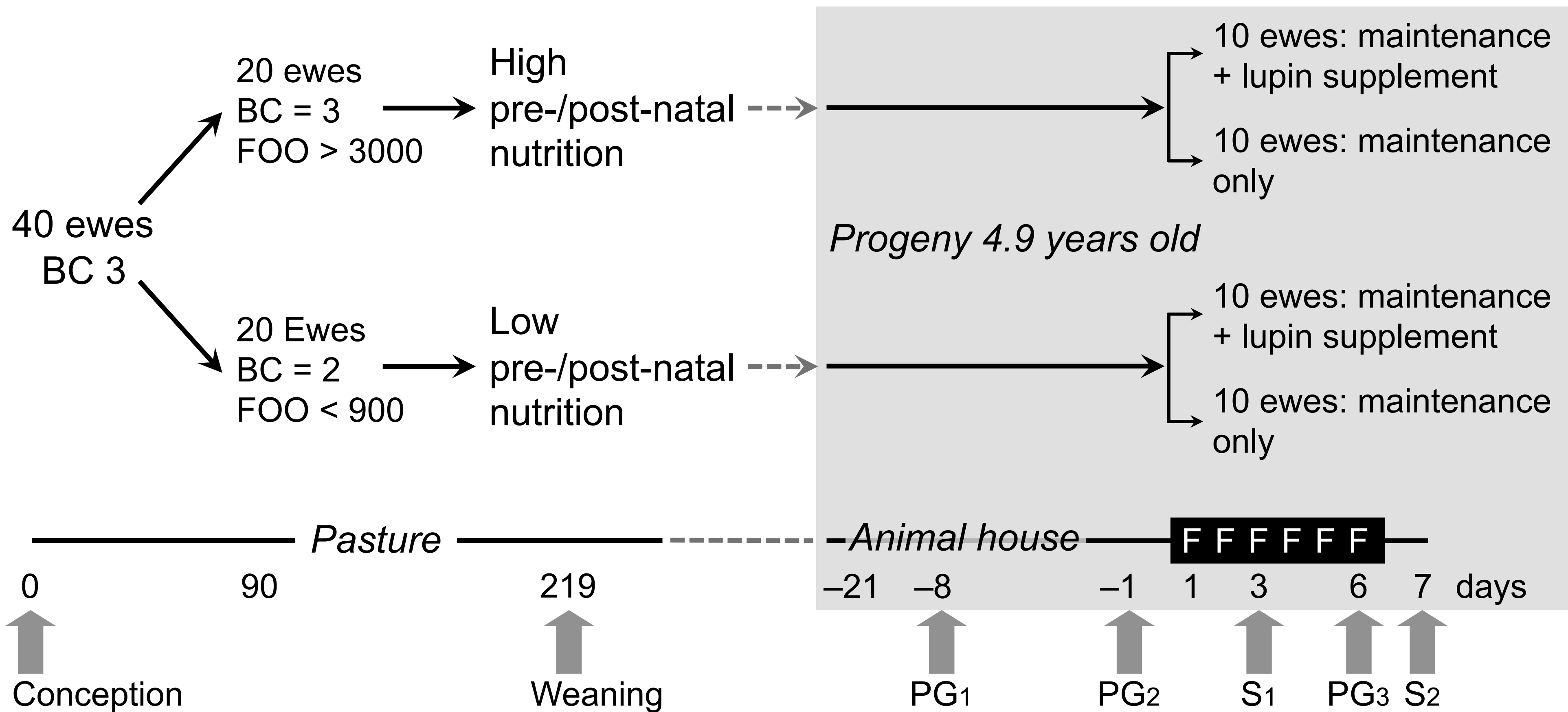
Status	**Atretic**		**Healthy**	
Day of sacrifice	**3**	**7**	**3**	**7**
3 mm	6	2	15[a]	7[b]
4 mm	8	4	11[a]	3[b]
5 mm	4	5	6[a]	20[b]
Total	18	11	32[a]	30[a]

[a] vs [b] compare numbers of healthy follicles at different days of sacrifice

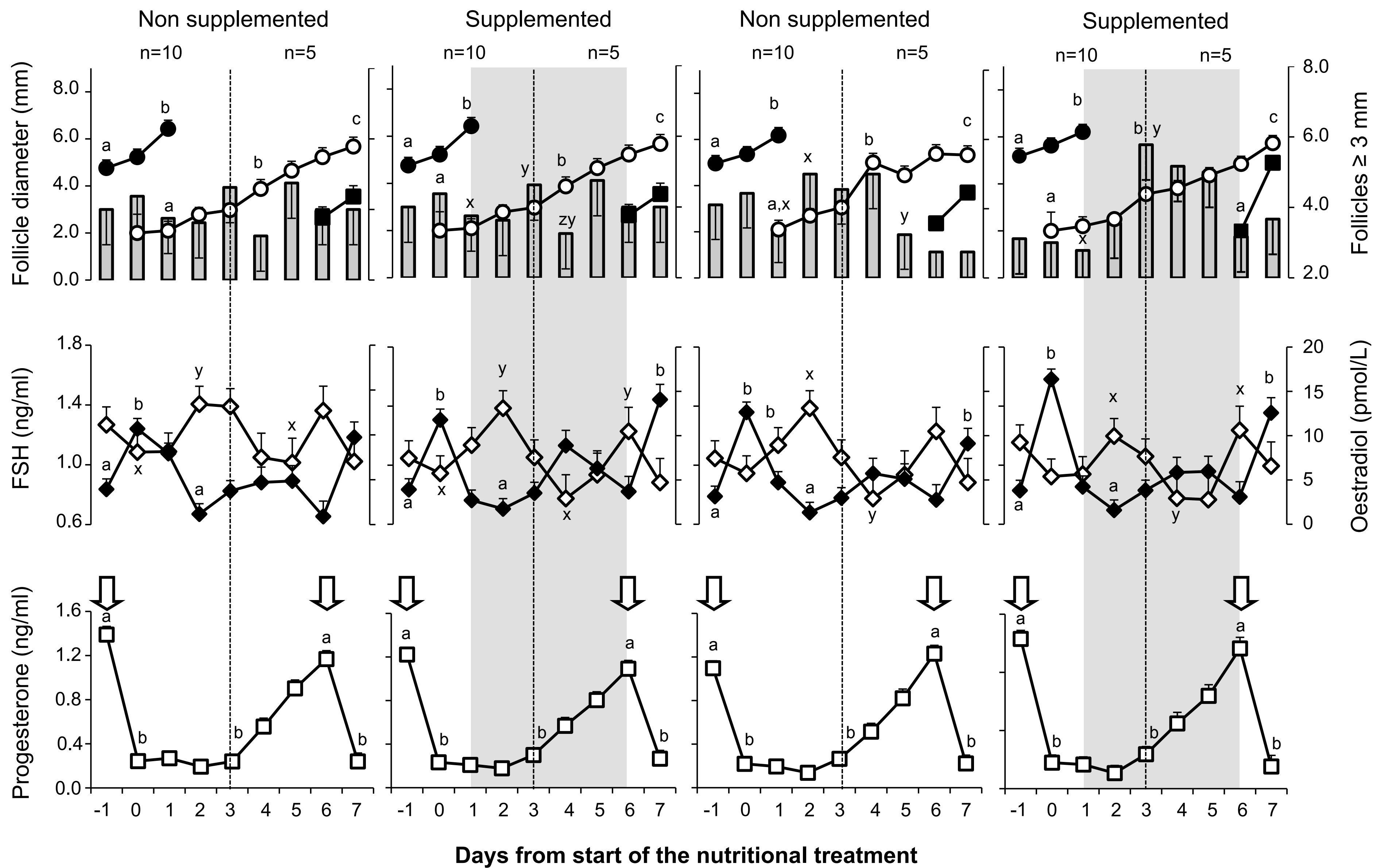
Table 3. Oestradiol **(nmol/L) c**oncentrations in follicular fluid in ewes that had been exposed to low and high pre-/post-natal nutrition and then had received or not (±) a 6-day supplement of lupin grain.

	-Supplement		**+Supplement**	
Day	**3**	**7**	**3**	**7**
Low	61.7 ± 20.9[a]	259.2± 54.0[b]	88.8 ± 31.2[a]	70.8± 46.6[a]
High	47.0 ± 20.9[a]	242.3± 47.0[b]	77.5 ± 35.2[a]	121.9 ± 35.2[a]

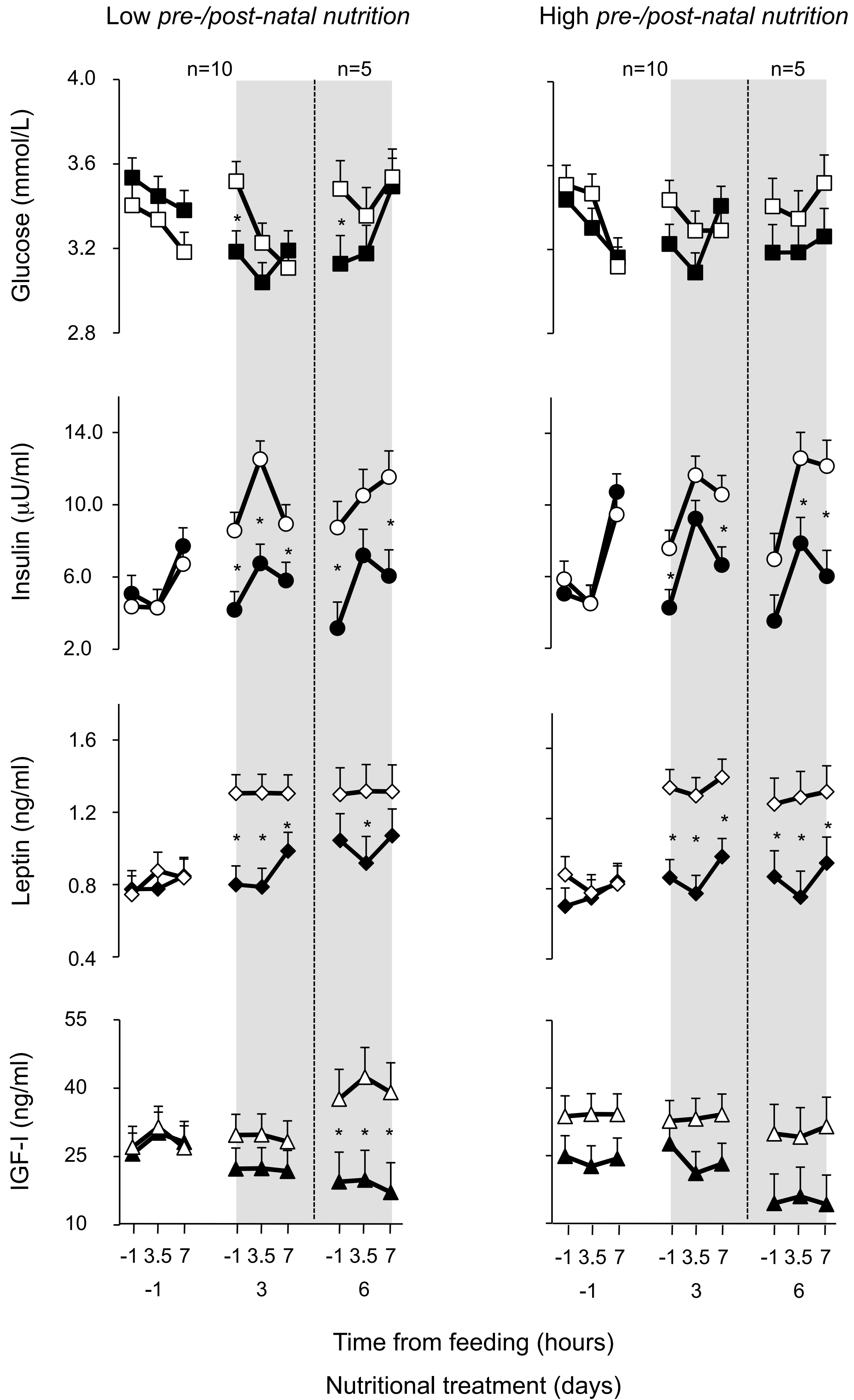
Within rows and between columns [a] vs [b], $P < 0.01$.



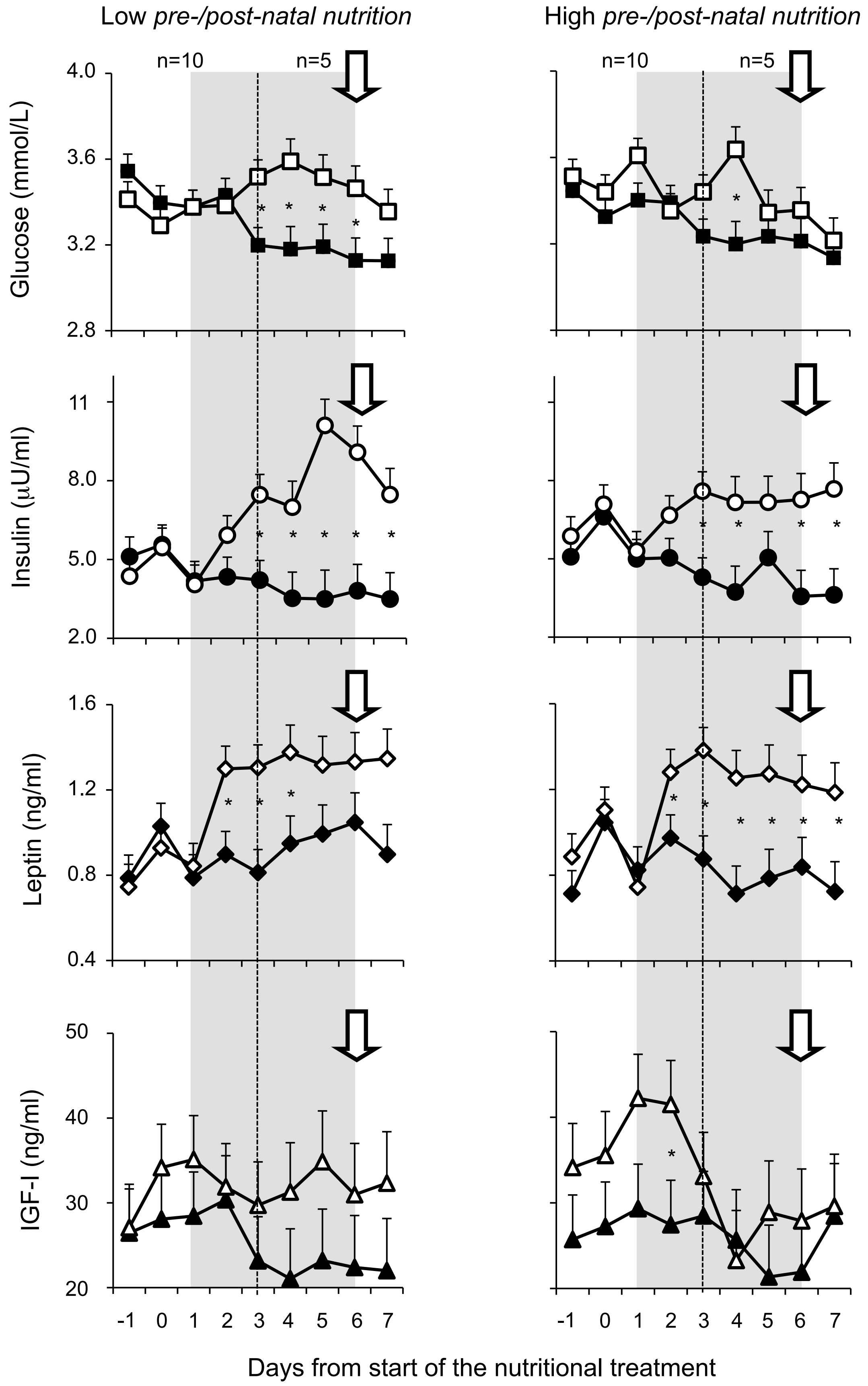
40 ewes
BC 3
20 ewes
BC = 3
FOO > 3000
High
pre-/post-natal
nutrition
20 Ewes
BC = 2
FOO < 900
Low
pre-/post-natal
nutrition
10 ewes: maintenance
+ lupin supplement
10 ewes: maintenance
only
Progeny 4.9 years old
10 ewes: maintenance
+ lupin supplement
10 ewes: maintenance
only
Pasture
Animal house
F F F F F F
0
90
219
–21
–8
–1
1
3
6
7
days
Conception
Weaning
PG1
PG2
S1
PG3
S2



Low pre-/post-natal nutrition
High pre-/post-natal nutrition
Non supplemented
Supplemented
Non supplemented
Supplemented
n=10
n=5
Follicle diameter (mm)
Follicles ≥ 3 mm
FSH (ng/ml)
Oestradiol (pmol/L)
Progesterone (ng/ml)
Days from start of the nutritional treatment



Low pre-/post-natal nutrition
High pre-/post-natal nutrition
n=10
n=5
Glucose (mmol/L)
Insulin (μU/ml)
Leptin (ng/ml)
IGF-I (ng/ml)
4.0
3.6
3.2
2.8
14.0
10.0
6.0
2.0
1.6
1.2
0.8
0.4
55
40
25
10
-1 3.5 7
-1
3
6
Time from feeding (hours)
Nutritional treatment (days)



Low pre-/post-natal nutrition
High pre-/post-natal nutrition
n=10
n=5
Glucose (mmol/L)
4.0
3.6
3.2
2.8
Insulin (μU/ml)
11
8.0
5.0
2.0
Leptin (ng/ml)
1.6
1.2
0.8
0.4
IGF-I (ng/ml)
50
40
30
20
-1 0 1 2 3 4 5 6 7
Days from start of the nutritional treatment