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

C Viñoles^{1,2}, B L Paganoni^{1,5}, K P McNatty³, D A Heath³, A N Thompson^{4,5,6}, K M M Glover¹, J T B Milton¹ and G B Martin^{1,7}

¹Faculty of Natural and Agricultural Sciences, UWA Institute of Agriculture, University of Western Australia, Crawley, Western Australia 6009, Australia, ²Programa Nacional de Carne y Lana, Instituto Nacional de Investigación Agropecuaria, Ruta 5, km 386, Tacuarembó, Uruguay, ³Victoria University of Wellington, Wellington, New Zealand, ⁴Department of Primary Industries, Hamilton, Victoria 3300, Australia, ⁵Department of Agriculture and Food Western Australia, South Perth, Western Australia 6151, Australia, ⁶School of Veterinary Biology and Biomedical Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia and ⁷Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford OX3 9DU, UK

Correspondence should be addressed to C Viñoles at Programa Nacional de Carne y Lana, Instituto Nacional de Investigación Agropecuaria; Email: cvinoles@adinet.com.uy

Abstract

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 2×2 factorial design was used with nutrition in early life (low or high) and a 6-day supplement (with or without) as factors. All ewes received three prostaglandin (PG) injections 7 days apart, and the supplement (lupin grain) was fed for 6 days from 2 days after the second until the third PG injection. We measured reproductive and metabolic hormones, studied follicle dynamics (ultrasonography), and evaluated granulosa cell numbers, aromatase activity and oestradiol (E₂) 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 vs 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 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 E₂ (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 E₂–FSH balance. *Reproduction* (2014) **147** 101–110

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 (ME) 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 foetal 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 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 3 days after the start of the supplement, in association with an increase in the number of antral follicles selected into the ovulatory wave (Viñoles *et al.* 2005, 2010b, Scaramuzzi *et al.* 2006, 2011). As a consequence, we would expect short-term supplementation to alter the balance of the oestradiol (E_2)–follicle-stimulating hormone (FSH) feedback loop, but this has been difficult to demonstrate experimentally because the waves in FSH concentration, follicle population and E_2 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 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 ovulation rate, in adult life and show a further increase in the number of healthy, potentially ovulatory follicles following a short-term supplementation with lupins, compared with 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 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 4.9-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.

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).

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 allocated to two

groups to apply pre-/post-natal treatments to their foetuses: the 'high' pre-/post-natal nutrition group was run on high-quality pasture (3000 kg dry matter (DM)/ha) from mating through pregnancy (score 3 on day 100 of pregnancy) to weaning at 72 ± 0.2 days; 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 DM/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 in the Merino ewes under paddock conditions (Ferguson *et al.* 2011). So, the foetuses 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).

Only single-born ewes of similar birth weight (5.0 ± 0.1 kg for high, 4.7 ± 0.1 kg for low; mean $\pm$ S.E.M.; $P > 0.05$) 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 (141.7°E $41^\circ 25'\text{S}$, -37.6°S $36'1\text{s}$) following the '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 this study, when the ewes were 4.9 ± 0.1 years old, body 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 (1210 h light and 1150 h darkness). They were acclimatised to these conditions for 3 weeks, during which they were individually fed a nutritionally complete, pelleted diet. The quantity offered to each ewe was calculated to meet their individual 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% DM and supplied 9.0 MJ ME and 110 g of crude protein (CP)/kg (as-fed basis). The ewes were fed daily at 0000 h (0 h) and offered water *ad libitum*. Feed refusals were collected and weighed daily and were $< 10\%$.

Dietary treatments

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

Cycle synchronisation

All ewes received three injections of prostaglandin (PG) analogue (each 250 μg cloprostenol; Juramate, Jurox Pty Ltd., Rutherford, NSW, 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

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 animal house (grey area) where they were fed once per day at 0000 h. Their follicle waves were synchronised using three injections of PG analogue (PG1, PG2 and 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, recruitment of follicles into the first wave of the cycle) and five ewes were sacrificed 30 h after PG3 (S2, selection of the pre-ovulatory follicles). BC, body condition; FOO, food on offer (kg DM/ha).

wave and thus ensure that, for all animals, the peak concentrations of metabolic hormones coincide with follicle selection, when FSH concentrations are low, thus permitting co-dominance (Viñoles *et al.* 2010b). 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 (Viñoles *et al.* 2010b). To facilitate the processing of tissues, the synchronisation treatment, and thus the beginning of the nutritional 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

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

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 PG injection (selection of the pre-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 PBS under a stereomicroscope with 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 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 100 μ l PBS and frozen at -20°C for subsequent measurement of E_2 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 counted by using haemocytometer. To help classify follicles as non-atretic or atretic, the presence or absence of thecal blood

Table 1 Average amount 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

capillaries (at 10× 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 i) visible thecal blood capillaries, ii) follicular fluid devoid of debris, iii) a pink- to red-coloured theca interna, iv) a normal-looking and intact oocyte–cumulus cell complex and v) 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.

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 6×10^4 granulosa cells/ml; 0.15 ml aliquants of these cell suspensions were placed in 10×75 mm plastic tubes containing 0.15 ml of PBS and 1% BSA solution with or without 2000 ng testosterone/ml. The cell suspensions were gassed with 5% CO₂ 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 supernatants were assayed for E₂.

Blood sampling, glucose and hormone assays

From the day before the initiation of the nutritional treatment until the day of the third PG injection, jugular blood (5 ml) was sampled daily, 1 h before feeding (0 h), 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.

Plasma progesterone was assayed 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 assayed. The limit of detection was 0.3 ng/ml. The intra-assay coefficients of variation (CV) were 2.8% for low (1.2 ng/ml) and 4.2% for high (9.9 ng/ml) progesterone values in plasma, and inter-assay CV were 2.3 and 17.4% respectively.

E₂ was assayed in a single RIA using an Adaltis MAIA Oestradiol Kit from Diagnostic Technology (Belrose, NSW, Australia). Plasma samples collected at –1, 3.5 and 7 h relative to feeding were pooled to obtain a daily profile. E₂ was extracted from 400 µl plasma and 200 µl quality controls and standards using 2 ml diethyl ether with an extraction efficiency of $88 \pm 2\%$. The limit of detection was 3 pmol/l. The intra-assay CV was 4.9% for concentrations of 11 pmol/l and 16.4% for concentrations of 35 pmol/l. The inter-assay CV was 25 and 18.5% for low and high E₂ concentrations respectively. Concentrations of E₂ in follicular fluid and the medium from granulosa cell cultures were measured using the same kit described earlier, except that samples were not extracted.

The limit of detection of the assay was 3.7 pmol/l. The intra-assay CV was 10% for concentrations of 21 pmol/l and 11% for concentrations of 58 pmol/l. The inter-assay CV was 8 and 2% for the low and high E₂ concentrations respectively.

As samples of follicular fluid were further diluted 1/100 in preparation for the assay, values were multiplied by 1000 to express the concentrations (nmol/l). The E₂ production by granulosa cells was calculated based on the following formula: (E₂ concentrations (pmol/tube)/assay volume (µl) × 600/1000)/(cell number (×10⁶) × 1 000 000), and expressed (nmol E₂/million granulosa cells).

Plasma concentrations of FSH were measured in a single RIA using reagents kindly supplied by Dr A F Parlow of the National Institute of Diabetes, Digestive and Kidney Disease (Baltimore, MD, USA), 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 (six replicates) containing 1.5, 2.4 and 3.2 ng/ml were used to determine intra-assay CV: 2.2, 3.0 and 3.2%. The inter-assay CV were 1.6, 1.9 and 2.2% for the low, medium and high 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) with an Infinity Glucose Hexokinase Kit (cat no. TR15421, Thermo Electron Co., Melbourne, VIC, Australia). The intra and inter-assay CV 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.* 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 six replicates of control samples containing 2.7, 4.1 and 9.3 mU/ml, for which the intra-assay CV were 9.1, 5.5 and 7.6%.

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 were included in the assay to estimate the intra-assay CV, which were 20.4, 6.8 and 7.2%.

Plasma concentrations of insulin-like growth factor 1 (IGF1) were measured in a single double-antibody RIA (Gluckman *et al.* 1983) after interference by binding proteins had been 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 0.44 and 2.42 ng/ml IGF1, were included in the assay and were used to estimate the intra-assay CV (11.1 and 10.3%).

Statistical analyses

Categorical data, such as the proportions of animals that developed a second follicular wave during the experimental period, were analysed by the GENMOD procedure in the 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 generalised 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 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 (e.g. plasma hormone concentrations and 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 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 $\pm$ pooled standard errors.

Results

Follicular dynamics

The growth profile and maximum diameter of the follicles that were induced to ovulate by the second PG injection (before the beginning of the supplementation period) did not differ between groups ($P > 0.05$). Figure 2 shows that the ovulatory follicle grew in parallel with a decrease in the concentrations of progesterone and an increase in the concentrations of E_2 . The characteristics of the dominant follicle of the first wave (day of emergence, lifespan, maximum diameter and growth rate) also did not differ between groups ($P > 0.05$). FSH concentrations increased in all groups before 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

Figure 2 Growth profile of the follicle induced to ovulate after the second PG 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, 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 PG injections. The broken lines on day 3 indicate sacrifice of five ewes, reducing the number of ewes by half in each treatment group. All values are least squares means ($\pm$ S.E.M.). a vs b vs c, indicates significant changes in the profile of follicle growth and hormonal 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.

Table 2 Numbers of atretic and healthy follicles in 3, 4 and 5 mm size classes found in the groups of five ewes sacrificed on days 3 or 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	
	3	7	3	7
Days of sacrifice				
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.

peaked on day 6 in all groups (Fig. 2). 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 and low early nutrition, supplemented = 2/5) and the growth profile of the largest follicle of the second wave did not differ between groups ($P > 0.05$). In the supplemented groups, FSH concentrations increased significantly with the emergence 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 E_2 concentrations (Fig. 2).

Ovulation rate, as determined by the number of corpora lutea detected by ultrasonography after the second PG 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 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 commencement of feeding (Table 2) – there were more 3 and 4 mm healthy follicles on day 3, but more 5 mm healthy follicles on day 7 ($P < 0.001$).

Granulosa cells and E_2 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 pre-/post-natal nutrition treatment. The antral fluid concentrations of E_2 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 E_2 concentrations in follicular fluid in ewes from both the low and high pre-/post-natal nutrition groups on day 7 (Table 3).

The capacity of the granulosa cells from healthy follicles to produce E_2 from testosterone was not affected by pre-/post-natal nutrition, supplementation, or the interaction between these factors. The concentration of E_2 was higher on day 7 (85.2 ± 5.5 nmol E_2 /million granulosa cells) than on day 3 (50.7 ± 5.9 nmol E_2 /million granulosa cells) ($P < 0.001$).

Circulating concentrations of hormones and glucose

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 E_2 concentrations 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 E_2 concentrations tended to be higher in supplemented (11.2 ± 0.8 pmol/l) than in non-supplemented ewes (9.2 ± 0.8 pmol/l; $P = 0.09$).

Concentrations of glucose were affected by supplementation, day and the interaction between these two factors (Figs 3 and 4), but not by pre-/post-natal or time after feeding ($P > 0.05$). In non-supplemented groups, concentrations decreased from days 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 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 that had received high nutrition during pre-/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 between these factors. Ewes that had received high pre-/post-natal nutrition tended ($P = 0.07$) to have higher insulin concentrations (7.7 ± 0.28 μ U/ml)

Table 3 Oestradiol (nmol/l) concentrations in follicular fluid in ewes that had been exposed to low and high pre-/post-natal nutrition and then had received or not ($\pm$) a 6-day supplement of lupin grain.

Days	– Supplement		+ Supplement	
	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, $P < 0.01$.

compared with those that had received low pre-/post-natal nutrition ($7.0 \pm 0.28 \mu\text{U/ml}$). 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 (Figs 3 and 4). In 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.

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-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 ($1.2 \pm 0.03 \text{ ng/ml}$) than in non-supplemented ewes ($0.9 \pm 0.03 \text{ ng/ml}$), the differences being significant from days 2 to 7 only in high pre-/post-natal nutrition ewes ($P < 0.01$; Fig. 4).

Plasma IGF1 concentrations were also affected by supplementation, day and the interaction between the two factors, but not by pre-/post-natal nutrition or time after feeding (Fig. 3). Supplemented ewes ($33.0 \pm 1.28 \text{ ng/ml}$) had higher IGF1 concentrations than non-supplemented ewes ($22.0 \pm 1.28 \text{ ng/ml}$; $P < 0.001$; Fig. 4). Overall, concentrations decreased from days -1 to 6 in non-supplemented ewes while, in supplemented ewes, they remained similar or increased (Fig. 4). A steep decay was observed from days 2 to 4 in supplemented ewes that had received high pre-/post-natal nutrition, but the values recovered thereafter (Fig. 4).

Discussion

The effect of nutrition in early life on ovulation rate in adult life agrees with 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 E_2 production. On the other hand, FSH concentrations were lower in ewes that received high pre-/post-natal nutrition than in those that 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 in 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 E_2 , probably explaining the lower concentrations of FSH in supplemented than non-supplemented ewes. Thus, as with acute supplementation (Viñoles *et al.* 2010a, 2010b), the increase in ovulation rate caused by good early nutrition is associated with a reduction in FSH concentrations.

Figure 3 Changes in the plasma concentrations of glucose (square), insulin (circle), leptin (diamond) and IGF1 (triangle) in ewes exposed to low and high pre-/post-natal 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 0 h) on day -1 (2 days before the start of supplementation) and the third and the final day of supplementation. The broken line indicates the day of sacrifice of five 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 ($\pm$ S.E.M.). Note that on day 6, the number of ewes was reduced to five in each treatment.

Figure 4 Changes in the plasma concentrations of glucose (square), insulin (circle), leptin (diamond) and IGF1 (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 2 days before supplementation started until the day after the end of the supplementation. Arrows indicate the time of the second and third PG injections. The broken line indicates the day of sacrifice of five 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 ($\pm$ S.E.M.).

This suggests that, in both situations, increases in ovulation rate must be accompanied by increases in the responsiveness of follicles to 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 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.

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 lead to new insights into the mechanisms involved in the ovarian response to short-term supplementation. Supplemented ewes had a larger healthy follicle with 20% more granulosa cells and produced 20% more circulating E_2 . Granulosa cells are the richest source of aromatase activity and thus E_2 (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 E_2 was associated with a 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 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.

As mentioned earlier, 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 of glucose, insulin, leptin and IGF1, as seen previously (Viñoles *et al.* 2005, 2010b, Scaramuzzi *et al.* 2006, 2011). In this 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 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 IGF1 on steroidogenesis – decreasing the concentrations of E_2 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, follicular growth and E_2 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 follicle, the competence of the oocyte and fertility (Sirois & Fortune 1990, Viñoles *et al.* 2001, 2012, Fierro *et al.* 2011). 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 E_2 –FSH feedback loop is indeed an effect of nutrition or a consequence of the 'first-wave' model.

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 E_2 –FSH balance. These responses are similar to those following acute nutritional 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.

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.

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