Folliculogenesis and ovarian expression of mRNA encoding aromatase in anoestrous sheep after 5 days of glucose or glucosamine infusion or supplementary lupin feeding

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Introduction

Nutrition is a major factor determining the ovulation rate in domestic animals (Lindsay et al., 1991) and the effect of nutrition is thought to be associated with alterations in the metabolic signals that mediate energy availability (Rhind and McNeilly, 1986; Rhind et al., 1989; Downing, 1994). Ovulation rate is determined ultimately by the mechanism that controls selection of the ovulatory follicles from the follicular cohort. This mechanism is characterized by the complex relationship between intrafollicular growth factors and the hypothalamo–pituitary–ovarian feedback system during the final stages of follicular growth and development (Skinner et al., 1987; Lobb and Dorrington, 1992; Scaramuzzi et al., 1993; Monniaux et al., 1994; Monget and Monniaux, 1995; Bao and Garverick, 1998).

An increase in the supply of energy can stimulate follicle development and increase ovulation rate in sheep. Ovulation rate is increased by the infusion of glucose into either the abomasum (Landau et al., 1995) or the jugular vein (Downing et al., 1995a), by the infusion of a mixture of branched chain amino acids into the jugular vein (Downing et al., 1995b) and by dietary supplementation with lupin grain (Nottle et al., 1988, 1990; Downing et al., 1995c). All of these treatments also increase peripheral insulin concentrations (Downing et al., 1995a,b,c), indicating that insulin-mediated glucose uptake, possibly by the ovary, modulates nutritional effects on ovulation rate. In support of this contention, the insulin-dependent glucose transporter

Improved nutrition increases ovulation rate in sheep and there is evidence that intra-ovarian pathways mediate responses to nutrition. An experiment was conducted to examine the effect of dietary energy on folliculogenesis. Anoestrous Merino ewes were fed a diet of wheat straw alone (control, n = 5), or wheat straw supplemented with lupins (300 g day⁻¹, n = 5). Other ewes were fed wheat straw and infused with glucose (50 mmol h⁻¹, n = 5) or with glucosamine (3.5 mmol h⁻¹, n = 5). Intravaginal progestagen sponges were inserted for 12 days, and nutritional treatments were started 5 days before sponge removal. At sponge removal, the ewes were injected with a regimen of GnRH pulses (500 ng every 4 h from 0 to 12 h; 250 ng every 2 h from 14 to 24 h; and 200 ng every 1 h from 25 to 36 h) to simulate normal follicular development. Thirty-six hours after sponge removal, the animals were killed and the ovaries were collected and stored at –80°C. The ovaries were sectioned serially every 10 µm. Every 20th section was stained (to estimate number and diameter of follicles) and every 17–19th section was probed by in situ hybridization for P₄₅₀ aromatase. Data were analysed using ANOVA and chi-squared tests. There was an effect of treatment (P < 0.05) on the number of follicles 2–3, 3–4 and 6–7 mm in diameter. Aromatase-positive follicles (1.6–7.9 mm) were detected in 31 follicles from 15 ewes across all four groups. In ten animals, the largest follicle was aromatase-positive. The diameters of aromatase-positive follicles were larger (P = 0.004) in lupin fed compared with glucose-infused ewes (4.9 ± 0.5, 3.6 ± 0.7, 5.3 ± 0.5 and 4.2 ± 0.5 mm for control, glucose-infused, lupin-fed and glucosamine-infused groups, respectively). Treatment did not affect the plasma concentration of FSH when compared with controls, indicating that the energy supplements were modifying recruited (2–3 mm and 3–4 mm) and selected follicles (> 6 mm) directly. In conclusion, dietary energy can directly stimulate folliculogenesis in recruited and selected follicles, and this effect may be mediated by changes in systemic leptin concentrations and the hexosamine energy-sensing pathway in the follicle.
(GLUT4) is present in both theca and granulosa cells of antral follicles in ewes (Williams et al., 2001). An increase in the supply of energy substrates in this manner is thought to trigger the growth and development of follicles that would normally ovulate or restore the ovulatory capacity of previously compromised follicles.

The mechanism that transduces this energetic input into a reproductive response in the ovary is not clear. The energy demands for folliculogenesis are minimal, so it is not the supply of energy per se that is responsible for this effect. A mechanism based on changes in hypotalamo–pituitary feedback is also unlikely because studies have been unable to establish a link between plasma FSH concentrations and ovulation rate (Findlay and Cumming, 1976; Bindon et al., 1979; Cahill et al., 1981; Driancourt et al., 1988; Downing and Scaramuzzi, 1991). The direct action of a nutrient or metabolite on the follicle is a current theory and two potential mechanisms are leptin signalling in the follicle and nutrient sensing via the hexosamine biosynthetic pathway.

Leptin is a protein produced by adipose tissue and is involved in the regulation of appetite, energy expenditure and whole-body energy balance (Ingvarsten and Boisclair, 2001). Leptin also affects the reproductive system, acting either at the hypotalamus–pituitary axis, where it appears to help maintain adequate gonadotrophin secretion or by a direct action on the follicular granulosa cells (Spicer, 2001). Thus, in cultured bovine granulosa cells, leptin inhibits oestradiol production (Spicer and Francisco, 1997; B. K. Campbell, unpublished).

The hexosamine biosynthetic pathway can quantitatively assess metabolic status and act as a signalling pathway in some tissues (Wang et al., 1998). Thus, tissues such as skeletal muscle and adipose tissue assess nutritional status so that they can respond to short-term variation in nutritional flux. Intracellular glucose enters the pathway as fructose 1,6-diphosphate and glucosamine is an intermediate in this pathway. One consequence of increased glucose flux through the hexosamine pathway in muscle is the increased expression of genes for leptin (Wang et al., 1998) and two growth factors, fibroblast growth factor 2 (FGF2) (McClain et al., 1992) and transforming growth factor α (TGF-α) (Daniels et al., 1993; Roos et al., 1996), which are potent regulators of granulosa cell proliferation and steroidogenesis (Gospodarowicz et al., 1977; Gospodarowicz and Bialecki, 1979; Adashi et al., 1987, 1988; Skinner and Coffey, 1988).

These potential roles of leptin as a nutritional mediator of follicular function and the hexosamine pathway as a follicular energy sensing mechanism have not been demonstrated. Therefore, the present study tested the hypotheses that: (i) short-term nutritional treatments that stimulate folliculogenesis will increase plasma leptin concentrations; and (ii) increased concentrations of glucose or glucosamine acting through the hexosamine biosynthetic pathway increase the number of follicles positive for aromatase gene expression.

### Materials and Methods

#### Animals and treatments

Twenty-one adult anoestrous Merino ewes were treated for 12 days with medroxyprogesterone sponges (Repronat; Upjohn, Rydalmare). At the same time, the animals were allocated randomly to one of four treatment groups: (i) basal straw diet plus jugular i.v. saline infusion (control group, n = 5); (ii) basal straw diet plus 50 mmol glucose h⁻¹ by jugular intravenous infusion (n = 5); (iii) basal straw diet plus 500 g lupin grain day⁻¹ (n = 6); and (iv) basal straw diet plus 3.5 mmol glucosamine h⁻¹ by jugular intravenous infusion (n = 5). The nutritional treatments lasted for 120 h and ended at the time of sponge removal. The day before the experiment started, bilateral jugular venous canulae were inserted under xylocaine-induced local anaesthesia. One catheter was used exclusively for infusions and the other for collecting blood samples.

An artificial follicular phase to simulate normal follicular development was induced, using a regimen of treatment with GnRH. Starting at sponge removal, each ewe was treated with a regimen of intravenous pulses of GnRH (Sigma, St Louis, MO). The pulses were administered in three stages: 500 ng every 4 h from 0 to 12 h; 250 ng every 2 h from 14 to 24 h; and 200 ng every 1 h from 25 to 36 h. The animals were then killed with an overdose (5–6 g) of intravenous pentobarbital 156 h after the start of nutritional treatments and 36 h after sponge removal. Bilateral samples of ovarian venous blood (2–5 ml) were taken immediately and the ovaries were removed and stored at –80°C.

#### Collection of blood samples and hormone analysis

Concentrations of FSH, leptin, glucose and insulin were determined every 8 h starting 24 h before the commencement of nutritional treatments and continuing to the end of the experiment. Glucose was measured in whole blood using a Medisense® 2 glucose meter (Medisense Inc., Bedford, MA). The normal reading range was 1.1–33.3 mmol l⁻¹. Coefficients of variation (CV) for fresh human blood for low (2.2 ± 0.2 mmol l⁻¹) medium (4.5 ± 0.2 mmol l⁻¹) high–medium (11.1 ± 0.4 mmol l⁻¹) and high (14.2 ± 0.7 mmol l⁻¹) quality controls were 7.7, 4.1, 3.1 and 4.7%, respectively (Williams et al., 2001). Blood was centrifuged at 1500 g for 10 min and the plasma was recovered and stored at –20°C. Total oestrogen concentrations were measured in ovarian venous blood, and FSH, insulin and leptin concentrations were measured in jugular venous blood using validated radioimmunoassays. Total plasma oestrogen was measured by radioimmunoassay based on the method of Webb et al. (1985). The total oestrogen concentration was measured in plasma after extraction of the samples with 4 ml ethyl acetate:hexane (3:2). The extract was redisolved in 0.10 ml gelatin–phosphate buffer and incubated overnight at 4°C with 0.10 ml antisem (anti-Total Estrogens 1000T; ICN Biomedicals, CA) at a working dilution of 1:15000.
lodinated tracer (0.10 ml containing 10 000 d.p.m. 1,2,6,7-
\(^{1}H\)oestradiol) was added and the mixture incubated
overnight at 4°C. Bound and free tracer were separated by
incubating the assay mixture with 0.5 ml charcoal (0.5%)
coated with dextran (0.05%) at 4°C and centrifuged at
3000 r.p.m. The major crossreactions of the antisera are
oestradiol (100%), oestrone (100%), oestrone-sulphate
(60%), oestradiol-17\(\alpha\) (7%) and oestriol (415%). The limit of
detection was 1.1 pg ml\(^{-1}\). All the samples were measured
in one assay for which the intra-assay CV was 4.7% at
7.4 pg ml\(^{-1}\), 5.0% at 19 pg ml\(^{-1}\) and 4.4% at 32 pg ml\(^{-1}\).
Plasma was assayed for FSH in duplicate by a double-
antibody radioimmunoassay (Atkinson and Adams, 1988)
using NIAMDD-oFSH-RP-1 (bio-potency 75
\(\times\) NIH-FSH-
S1) and NIADDK-anti-oFSH-1 serum. Plasma insulin
concentrations were measured in duplicate by double-anti-
body radioimmunoassay (Tindal et al., 1978). The samples
were assayed as duplicate 200 \(\mu\)l aliquots. A specific
radioimmunoassay based on recombinant bovine leptin
with an antisera raised in an emu (Dromaius novaev-
hollandiae) (Blache et al., 2000) was used to measure leptin.
Briefly, triplicates of standards and duplicates of unknown
samples were incubated overnight at 4°C with diluted
antisera and normal emu serum and then incubated with
labelled leptin for 48 h at 4°C. After incubation, diluted
sheep anti-emu serum was added and the tubes were again
incubated for 48 h at 4°C. Polyethylene glycol 6000 was
added to the tubes before centrifugation. The limit of
detection was 100 pg ml\(^{-1}\) and the intra-assay coefficient of
variation was 6.5% at a bound:free ratio of 65%.

The minimum detectable concentrations of FSH, insulin,
leptin and oestradiol were 0.44 ng ml\(^{-1}\), 4.5 \(\mu\)U ml\(^{-1}\),
0.2 mg ml\(^{-1}\) and 1.0 pg ml\(^{-1}\), respectively. The intra-assay
CVs were 3.4, 7.3, 0.79 and 4.3% for FSH, insulin, leptin
and oestradiol, respectively.

Number of follicles

The frozen ovaries of 21 animals (42 ovaries) were
sectioned serially at 10 \(\mu\)m intervals using a cryostat. The frozen
sections were mounted on to glass slides (2–3
sections per slide) and fixed. The fixed slides were stored in
95% alcohol at 4°C. The number of follicles was deter-
mined by counting all microscopically visible follicles on
every 20th slide stained with Harris’s haematoxylin and
eosin. The follicular diameter was calculated from the mean
of two perpendicular diameters. The follicles were classified
as follows: F < 1: follicles < 1 mm in diameter; F1: follicles
1–2 mm in diameter; F2: follicles > 2–3 mm in diameter; F3:
follicles > 3–4 mm in diameter; F4: follicles > 4–5 mm in
diameter; F5: follicles > 5–6 mm in diameter; F6: follicles
> 6–7 mm in diameter; and F7: > 7 mm in diameter.

In situ hybridization

The expression of cytochrome P\(_{450}\) aromatase was
determined on dehydrated and fixed sections (10 \(\mu\)m) of
frozen ovaries (Wathes et al., 1996; Leung et al., 1999). The
sections were probed with \(\alpha^{35}\)S\(\text{dATP (SJ} 1334; Amersham
Pharmacia Biotech, Bucks) labelled DNA probes specific
for P\(_{450}\) aromatase (antisense; 45mer synthetic oligo-
nucleotide). The probe sequences were based on the
published cDNA sequence for aromatase (Hinshelwood
et al., 1993). The oligonucleotide 45mers was synthesized
using the specific sequence 5’-TCA CCG GGT AGC CAT
CGA TGA CAT CCT CTA AGG TCT TGC GCA-3’
(Leung et al., 1999; Genbank accession number Z69249).

Established in situ hybridization procedures (Perks,
1994; Wathes et al., 1996; Leung, 1997) were used with
minor modifications. Briefly, the sections were impregnated
with the reaction mixture containing labelled probe in
hybridization buffer (100 000 c.p.m. per 100 \(\mu\)l of
hybridization buffer per slide), covered with a Parafilm
coverslip and incubated overnight at 42°C. After incubation,
the sections were washed at room temperature in citrate
buffer (SSC; 15 mmol sodium chloride l\(^{-1}\), 15 mmol sodium
citrate l\(^{-1}\), pH 7.0, containing 0.2% (w/v) sodium
thiosulphate-5 hydrate) in a shaking bath for 30 min followed
by 1 h at 60°C. The slides were dehydrated in a gradient of
ethanol, air dried and exposed to Kodak Biomax MR-1 film for
15 days. The sense probe was used as the negative control.

Photographic development

The manufacturer’s (LM-1; Amersham Pharmacia
Biotech) instructions were followed. Briefly, dried slides
were dipped vertically into the emulsion for 5 s at 43°C and
allowed to dry horizontally at room temperature and then
on a metal tray pre-cooled with dry ice for 10 min each. The
slides were then placed in a light-tight box with anhydrous
silica gel in the base of the box and incubated for 3 weeks.
After incubation, the slides were dipped into a developer
(Phenisol; Ilford Limited, Ilford) for 5 min and then into a
stop bath of 0.5% acetic acid (w/v) for 1 min. The developed
slides were immersed in a fixative (47% (w/v), sodium
thiosulphate pentahydrate) for 10 min and finally into
distilled water for at least 10 min before counterstaining
with Harris’s haematoxylin and eosin.

Image analysis

After exposure, the photographic images of the ovarian
sections were quantified for specific labelling using an
image analysis system (Seescan, Cambridge) to measure the
absorbance of specific areas (granulosa layer) identified on
adjacent slides. The emulsion-coated slides were also used
to confirm the cellular localization of the aromatase signal.
The results from the autoradiographs were expressed as
arbitrary units of absorbance with a linear range of
0.01–2.10. The non-specific counts (estimated from the
sense probe) were subtracted from the total counts
(estimated from the anti-sense probe) to produce a mean
value for the specific hybridization in each follicle.

Statistical analyses

The concentrations of FSH, leptin, glucose and insulin
were analysed for time and treatment effects using a split-
plot ANOVA. Post-hoc differences between treatment groups were further examined using Tukey’s test (SAS, 1995). Chi-squared tests were used to determine differences in the size distribution of follicles and to identify any differences in the number of follicles positive to P450 aromatase.

Results

One animal in the lupin-fed group had an infected uterus and a persistent corpus luteum. This animal was excluded from the results and analyses.

Number and distribution of ovarian follicles

The mean number of follicles in each class decreased as average follicle size increased (Table 1). There was no effect of treatment on the number of follicles in the < 1 mm, 1–2 mm, 4–5 mm and 5–6 mm size classes. However, there were significant effects of treatment on follicles in the 2–3 mm, 3–4 mm and 6–7 mm classes (P < 0.05; Table 1).

There were effects of the nutritional supplements on both medium-sized (2–3 mm and 3–4 mm) and large (6–7 mm) follicles. Lupin-fed ewes had fewer follicles 2–3 mm in diameter (P < 0.05), whereas glucose-infused ewes had more follicles in this class (P < 0.01) compared with control and glucosamine-infused ewes. However, for follicles of 3–4 mm in diameter, all treated groups had more follicles than the control group. Follicles > 6 mm in diameter were found only in nutritionally supplemented ewes. The lupin-fed group had significantly more follicles of 6–7 mm in diameter than did the control group (P < 0.05).

Ovarian P450 aromatase expression

Aromatase expression was confirmed in the granulosa cell layer (Fig. 1) and the concentration of mRNA in aromatase-positive follicles was not affected by treatment (Table 2). Nutritionally stimulated ewes tended to have more aromatase-positive follicles than did the control ewes but the differences were not significant (Table 2). However, when the treated groups were pooled and the data re-analysed, the difference was significant (control 0.8 ± 0.37 and pooled-treated 1.92 ± 0.35 aromatase-positive follicles per ewe; P < 0.05).

The mean diameter of aromatase-positive follicles was affected by lupin and glucose treatments (P < 0.01; Table 2). The average diameter of aromatase-positive follicles was greatest in lupin-fed ewes and smallest in glucose-infused ewes (Table 2), although differences from average diameters of aromatase-positive follicles in control or glucosamine-infused groups were not significant.

Total oestrogen concentration in ovarian venous blood

The mean concentration of oestrogen was significantly greater (P < 0.05) in the ovarian venous blood from ovaries containing aromatase-positive follicles (46.8 ± 12.8 pg ml⁻¹) compared with that in ovarian venous blood from ovaries containing aromatase-negative follicles (19.6 ± 3.3 pg ml⁻¹). The mean concentrations of oestrogen in ovarian venous blood were not affected by treatment (Table 2).

Glucose concentration in jugular venous blood

The mean blood concentrations of glucose were increased by glucose infusion but were not affected by the other treatments (Table 3). The mean concentration of glucose in blood increased rapidly once the glucose infusion started and remained high for the duration of the infusion. At the end of infusion, the glucose concentrations in blood fell rapidly to pre-infusion values (Fig. 2).

Hormone concentration in jugular venous blood

The mean plasma concentrations of insulin were significantly increased by glucose infusion and feeding lupins (Table 3). However, the patterns of these increases differed (Fig. 3). In the glucose-infused group, the insulin concentration was increased significantly (P < 0.05) by 8 h after the beginning of infusion and remained high (P < 0.05) until the end of infusion at 120 h. In the lupin-fed group, the concen-

<table>
<thead>
<tr>
<th>Follicle class (diameter, mm)</th>
<th>Control</th>
<th>Glucose</th>
<th>Lupin grain</th>
<th>Glucosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>F0 (&lt; 1)</td>
<td>36.7 ± 2.8</td>
<td>46.6 ± 9.1</td>
<td>42.7 ± 6.62</td>
<td>38.8 ± 10.3</td>
</tr>
<tr>
<td>F1 (1–2)</td>
<td>15.5 ± 2.2</td>
<td>24.4 ± 5.4</td>
<td>22.0 ± 5.9</td>
<td>15.8 ± 3.7</td>
</tr>
<tr>
<td>F2 (2–3)</td>
<td>4.1 ± 1.7a</td>
<td>8.2 ± 3.4b</td>
<td>2.7 ± 0.4c</td>
<td>5.6 ± 1.9a</td>
</tr>
<tr>
<td>F3 (3–4)</td>
<td>0.6 ± 0.3a</td>
<td>2.6 ± 1.6b</td>
<td>1.7 ± 0.2b</td>
<td>2.0 ± 0.6c</td>
</tr>
<tr>
<td>F4 (4–5)</td>
<td>1.3 ± 0.5</td>
<td>1.2 ± 0.2</td>
<td>0.5 ± 0.3</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>F5 (5–6)</td>
<td>1.1 ± 0.3</td>
<td>0.6 ± 2.4</td>
<td>1.2 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>F6 (6–7)</td>
<td>0a</td>
<td>0.2 ± 0.2ab</td>
<td>0.7 ± 0.2b</td>
<td>0.2 ± 0.2ab</td>
</tr>
<tr>
<td>F7 (&gt; 7)</td>
<td>0</td>
<td>0.2 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values with different superscripts within rows differ significantly (P < 0.05).
tration of insulin was increased at 32 h, 80 h and from 96 to 120 h ($P < 0.05$) after the start of lupin feeding. Concentrations of insulin in the control and glucosamine-infused groups were not different from each other (Table 3; Fig. 3).

The mean plasma concentrations of leptin were affected by treatment with significantly higher concentrations in the glucose-infused and lupin-fed groups compared with the control and glucosamine-infused groups ($P < 0.05$; Table 3). The pattern of response differed in the two affected groups (Fig. 4). In glucose-infused ewes, leptin concentrations increased to a maximum at 16 h after the start of infusion ($P < 0.05$) and remained high for a further 32 h ($P < 0.05$), after which time they returned to control concentrations (Fig. 4). In the lupin-fed group, leptin concentrations increased more slowly to a maximum at 24 h after the start of feeding ($P < 0.05$) and remained high for another 72 h ($P < 0.05$) after which they returned to control concentrations (Fig. 4).

The mean plasma concentrations of FSH were not affected by any treatment when compared with the control ewes (Table 3). However, the mean plasma concentration of FSH in glucosamine-infused ewes was significantly lower.

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**Fig. 1.** (a,c,d) Antisense and (b,e,f) sense autoradiographic localization of mRNA for cytochrome P$_{450}$ aromatase in ovaries from GnRH-stimulated anoestrous Merino ewes. Note the intense localization of aromatase mRNA in the granulosa cells and its absence in theca cells. ar: aromatase; fa: follicular antrum; g: granulosa cells; t: theca cells. Scale bars represent (a,b) 2.5 μm, (c–e) 10 μm, and (f) 5 μm.
than it was in the lupin-fed ewes ($P < 0.05$; Table 3). There was a significant effect of time ($P < 0.05$) on plasma FSH concentrations (Fig. 5) and FSH declined significantly in all groups after the withdrawal of progestagen sponges at the end of the infusions.

### Discussion

The regimen of GnRH injections used in the present study could simulate normal development of follicles in anoestrous ewes, and aromatase-positive follicles were

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**Table 2.** Number and diameter of aromatase-positive follicles, aromatase (arbitrary units of absorbance) and the ovarian venous concentration of oestradiol of GnRH-treated anoestrous Merino ewes infused with glucose or glucosamine, or fed a supplement of lupin grain for 5 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of aromatase-positive follicles per ewe</th>
<th>Diameter of aromatase-positive follicles (mm)</th>
<th>Aromatase mRNA concentration (units of absorbance)</th>
<th>Total oestrogen concentration in ovarian venous blood (pg ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8 ± 0.3</td>
<td>4.9 ± 0.5$^{a,b}$</td>
<td>0.41 ± 0.1</td>
<td>18.0 ± 3.9</td>
</tr>
<tr>
<td>Glucose infusion</td>
<td>2.0 ± 0.8</td>
<td>3.6 ± 0.7$^{a}$</td>
<td>0.32 ± 0.07</td>
<td>39.9 ± 18.9</td>
</tr>
<tr>
<td>Lupin fed</td>
<td>2.0 ± 0.0</td>
<td>5.3 ± 0.5$^{b}$</td>
<td>0.48 ± 0.04</td>
<td>37.2 ± 9.3</td>
</tr>
<tr>
<td>Glucosamine infusion</td>
<td>1.8 ± 0.6</td>
<td>4.2 ± 0.5$^{a,b}$</td>
<td>0.47 ± 0.07</td>
<td>40.0 ± 29.4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Values with different superscripts within columns differ significantly ($P < 0.05$).

**Table 3.** Blood concentration of glucose (mean ± SEM) and the plasma concentrations of insulin, FSH and leptin (mean ± SEM) of GnRH-treated anoestrous Merino ewes infused with glucose or glucosamine, or fed a supplement of lupin grain for 5 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Glucose infused</th>
<th>Lupin fed</th>
<th>Glucosamine infused</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol l$^{-1}$)</td>
<td>3.4 ± 0.04$^{a}$</td>
<td>4.3 ± 0.08$^{b}$</td>
<td>3.5 ± 0.04$^{a}$</td>
<td>3.3 ± 0.04$^{a}$</td>
</tr>
<tr>
<td>Insulin (μU ml$^{-1}$)</td>
<td>12.7 ± 0.8$^{a}$</td>
<td>33.1 ± 2.1$^{b}$</td>
<td>20.9 ± 2.3$^{c}$</td>
<td>12.7 ± 1.2$^{a}$</td>
</tr>
<tr>
<td>Leptin (ng ml$^{-1}$)</td>
<td>0.5 ± 0.01$^{a}$</td>
<td>0.7 ± 0.03$^{b}$</td>
<td>0.7 ± 0.03$^{b}$</td>
<td>0.5 ± 0.02$^{a}$</td>
</tr>
<tr>
<td>FSH (ng ml$^{-1}$)</td>
<td>1.39 ± 0.05$^{ab}$</td>
<td>1.42 ± 0.04$^{ab}$</td>
<td>1.46 ± 0.06$^{a}$</td>
<td>1.29 ± 0.04$^{b}$</td>
</tr>
</tbody>
</table>

Values with different superscripts within rows differ significantly ($P < 0.05$). Glucose, FSH and leptin showed an effect of day of treatment ($P < 0.05$).

**Fig. 2.** Mean blood concentration of glucose in anoestrous Merino ewes in which follicular growth was induced with a regimen of GnRH. Ewes were infused with saline (control, ○; $n = 5$), glucose (■, 50 mmol h$^{-1}$; $n = 5$) or glucosamine (○, 3.5 mmol h$^{-1}$; $n = 5$) or fed a supplement of lupin grain (▲, 500 g day$^{-1}$; $n = 4$) for 5 days until the time of progesterone pessary withdrawal (arrow). Animals were killed 156 h after the start of infusion or feeding.
found in 15 of 20 ewes; in ten of these ewes, the largest follicle was also aromatase-positive. The results of the present study also show that the concentration of oestrogen in the ovarian vein draining an ovary containing an aromatase-positive follicle was significantly higher than the concentration of oestrogen in the ovarian vein draining an ovary containing only aromatase-negative follicles. These data are in agreement with other reports demonstrating that: (i) the largest follicle is the principal source of ovarian venous oestradiol (Baird and Scaramuzzi, 1976); (ii) aromatase activity of granulosa cells and oestradiol concentration in follicular fluid are positively correlated in follicles > 3.5 mm in diameter (Monniaux, 1987); and (iii) aromatase activity is found principally in the granulosa cells of peri-ovulatory follicles in superovulated sheep (Lautinick et al., 1994). The results of the present study show that the

Fig. 3. The mean ± SEM plasma concentration of insulin in anoestrous Merino ewes in which follicular growth was induced with GnRH. The ewes were infused with saline (control, ◇; n = 5), glucose (■, 50 mmol h⁻¹; n = 5) or glucosamine (○, 3.5 mmol h⁻¹; n = 5) or fed a supplement of lupin grain (▲, 500 g day⁻¹; n = 4) for 5 days until the time of progesterone pessary withdrawal (arrow). Animals were killed 156 h after the start of infusion or feeding.

Fig. 4. The mean ± SEM plasma concentration of leptin in anoestrous Merino ewes in which follicular growth was induced with GnRH. The ewes were infused with saline (control, ◇; n = 5), glucose (■, 50 mmol h⁻¹; n = 5) or glucosamine (○, 3.5 mmol h⁻¹; n = 5) or fed a supplement of lupin grain (▲, 500 g day⁻¹; n = 4) for 5 days until the time of progesterone pessary withdrawal (arrow). Animals were killed 156 h after the start of infusion or feeding.
number of large, potentially ovulatory follicles was always greater than the number of aromatase-positive follicles. Therefore, the number of large follicles is not necessarily a reliable indicator of potential ovulation rate because large follicles must also be oestrogenic if they are going to ovulate (Webb et al., 1989).

The following hypotheses were tested: (i) that short-term nutritional treatments that stimulate folliculogenesis will alter circulating plasma leptin concentrations; and (ii) that increased glucose or glucosamine acting through the hexosamine biosynthetic pathway will alter aromatase gene expression in the granulosa cells.

The results of the present study show that short-term nutritional supplementation can stimulate folliculogenesis and that the numbers of follicles 2–4 mm in diameter and > 6 mm were increased by nutritional supplementation. Follicles > 2.5 mm in diameter are normally considered to be capable of ovulating (Rhind and McNeilly, 1998). The data from the present study are in agreement with earlier results (Allen and Lamming, 1961), showing that ewes fed a high energy diet had increased numbers of follicles 2–3 mm in diameter. However, this is not a consistent observation and, in one study (Rhind and McNeilly, 1998), a high food intake increased the number of follicles 1.0–2.5 mm in diameter but not follicles > 2.5 mm in diameter. When ewes were fed lupin grain, the increase in ovulation rate was correlated with a greater number of follicles > 2 mm in diameter. Nottle et al. (1985) and Downing (1994) suggested that lupin grain supplementation decreased the number of follicles undergoing atresia. Increased energy supply did not affect the early stages of follicular development, but did increase ovulation rate by preventing atresia in follicles 2–3 mm in diameter without affecting the number of follicles > 3 mm in diameter (Haresign, 1981). Follicles > 6 mm in diameter were seen only in ewes given the nutritional supplement, and the diameter of the aromatase-positive follicles was increased by lupin feeding. These findings indicate that nutritional supplementation increases ovulation rate by preventing atresia in large dominant follicles.

The number of aromatase-positive follicles was not affected by the treatments. However, closer inspection of the data showed that the number of aromatase-positive follicles in the energy-supplemented animals was about twice the number in the control group. There have been consistent reports that nutritional supplementation increases the number of large follicles. For example, 600 or 1200 g of a mixture of 10% lupin grain in hammer milled oaten hay for 14 weeks increased the number of oestrogenic follicles and the total ovarian content of oestradiol in growing follicles (Boukhliq et al., 1996), and the increased ovulation rate observed when feeding grain supplements has been associated with a larger number of follicles > 4 mm in diameter (Howland et al., 1966).

One of the objectives of the present study was to examine the potential role of the hexosamine pathway as a nutrient-signalling pathway in the follicle. There is considerable data to show that the hexosamine pathway is a nutrient-signalling pathway in muscle and adipose tissue. Glucosamine is present in the follicular fluid of small and medium-sized follicles, but not in that of large follicles of buffalo (Boushehri et al., 1996). In the ovary, eCG stimulates the rapid uptake of glucosamine by the zona pellucida and increases its concentration in follicular fluid from large follicles, whereas hCG enhances glucosamine uptake by the theca and granulosa of preovulatory follicles (Fowler et al., 1989).
and Guttridge, 1987). Although these data show that glucosamine is present and active in the follicle, it is not directly concerned with its potential nutrient signalling role. The results of the present study indicate that glucosamine infusion stimulates folliculogenesis, as it increased the number of follicles of between 3 and 4 mm in diameter and, in this respect, mimicked the effect of glucose. Glucosamine infusion also tended to increase the number of aromatase-positive follicles and the number of follicles > 6 mm in diameter. Thus, glucosamine may be a nutrient-signalling pathway in the follicle, although further studies are required to confirm this tentative finding.

In addition to stimulating folliculogenesis, the nutritional supplements had a range of effects on circulating hormones. Lupin supplementation and glucose infusion both increased the plasma concentrations of insulin, indicating increased cellular uptake of glucose by follicles using the insulin-mediated GLUT4 pathway (Williams et al., 2001). The effects of insulin on follicular cells include: (i) enhancement of glucose and amino acid metabolism; (ii) stimulation of cell proliferation and growth; (iii) stimulation and inhibition of follicular steroid secretion; and (iv) modulation of gonadotrophin receptor function (Allen et al., 1981; Savio et al., 1981; Rein and Schomberg, 1982; Poretsky et al., 1985; Veldhius et al., 1985; Davoren et al., 1986; Downing et al., 1999).

There is evidence that leptin provides a signal of nutritional status to the reproductive system, and is the long sought-after mediator of nutritional status. Leptin is produced in adipose tissue, which actively responds to nutritional and metabolic changes. Leptin can act as a metabolic signal to the reproductive system by direct actions on the follicle (Spicer and Francisco, 1997). There are leptin receptors in the human ovary (Greisen et al., 2000) and leptin can attenuate insulin-induced steroidogenesis of granulosa cells directly without affecting cell proliferation (Spicer and Francisco, 1997).

Leptin secretion is subject to both long-term and short-term regulation. Glucose, pyruvate and insulin all act on rat adipocytes in vitro to stimulate release and to increase synthesis of leptin (Levy and Stevens, 2001). Leptin concentrations in sheep are not responsive to short-term (< 2 h) changes in blood glucose or insulin (Kauter et al., 2000). The present results confirm that, in sheep, a long-term glucose infusion increased plasma leptin concentrations by 8 h. The lupin supplement also increased plasma leptin concentrations but this effect was not observed until 24 h after the start of feeding. The results of the present study reveal some interesting temporal relationships among leptin, insulin and glucosamine. The leptin concentration was increased after either glucose infusion (increased by 8 h) or lupin feeding (increased by 24 h), but the increased concentration of leptin was not maintained despite the continued high concentration of glucose or insulin. Rats on high-fat diets may become resistant to leptin (Walder et al., 1995; Souza et al., 1996, 1997). The results of the present study in conjunction with other published information (Spicer, 2001) are consistent with the contention that leptin is a mediator of nutritional response on follicular function.

Glucosamine treatment did not alter the circulating concentrations of glucose, insulin or leptin. Nevertheless, glucosamine treatment did have effects on folliculogenesis and aromatase expression, indicating that its actions in follicular cells are intracellular. These findings are consistent with a proposed nutrient signalling role for glucosamine and the hexosamine pathway in the follicle, as occurs in skeletal muscle (Wang et al., 1998; Ukropec et al., 2001).

In conclusion, nutritional treatments have a direct stimulating action on folliculogenesis, which is independent of changes in circulating FSH. The effects were not the same across all three treatments and, furthermore, were observed at two different stages of folliculogenesis. The effect of lupin supplementation was mainly in large follicles > 6 mm in diameter, and this effect was not mimicked by infusion of either glucose or glucosamine. The effect of lupin grain supplementation is not completely mediated by increased insulin-mediated glucose uptake by the ovary. However, insulin-mediated glucose uptake did stimulate folliculogenesis, especially in medium-sized follicles 2–4 mm in diameter, an effect that was also observed in the glucosamine-infused ewes.

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