The effect of short-term nutritional supplementation of ewes with lupin grain (*Lupinus luteus*) on folliculogenesis, the concentrations of hormones and glucose in plasma and follicular fluid and the follicular levels of P₄₅₀ aromatase and IRS-1, -2 and -4

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Abstract

An experiment was conducted on 48 ewes during follicular and luteal phases of the oestrous cycle to determine the effect of a 5-day lupin grain supplementation (500 g/day) on folliculogenesis, plasma concentrations of glucose, insulin, FSH and oestradiol-17 β (E₂), follicular fluid concentrations of glucose, E₂, androstenedione and progesterone and the levels of P₄₅₀ aromatase and insulin receptor substrate 1 (IRS-1), -2 and -4 in theca and granulosa cells. Average weight did not differ between lupin-fed and control groups. The numbers of follicles were increased ($P < 0.05$; χ^2) in the lupin-fed group. The plasma concentrations of glucose ($P < 0.05$; ANOVA) and insulin ($P < 0.001$; ANOVA) were higher in lupin-fed ewes. The plasma concentrations of FSH were not different but those of E₂ were decreased ($P < 0.001$) in the lupin-fed group. Both the follicular fluid concentration of E₂ ($P < 0.05$) and the level of P₄₅₀ aromatase in granulosa cells ($P < 0.05$; ANOVA) were decreased in the lupin-fed group, but only during the follicular phase. The level of P₄₅₀ aromatase in granulosa cells was positively correlated with the concentration of E₂ in follicular fluid ($r = 0.820$; $P < 0.001$; ANOVA). The levels of IRS-1 and -2 in theca and granulosa cell lysates were increased in the lupin-fed group. These data suggest that insulin has a local role in the control of folliculogenesis and is likely to be a mediator of the effects of dietary energy intake on ovulation rate. We suggest that insulin acting through IRS proteins mediates the reproductive actions of insulin in the follicle and that IRS-1 and -2 are nutritionally regulated mediators of the action of insulin in the follicle.

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Introduction

The effects of nutrition on reproduction are well known and widely reported, and of the environmental factors influencing ovulation rate nutrition is one of the most important (Downing & Scaramuzzi 1991, Scaramuzzi *et al.* 2006). This effect of nutrition on ovulation rate in the ewe has major implications for the reproductive performance of sheep and this has been known for some time (Lindsay 1976). Over the years, a considerable body of research has identified three recognized and generally accepted effects of nutrition on ovulation rate; these are i) the static effect, ii) the dynamic effect and iii) the immediate or short-term effect. An example of the short-term effect

of nutrition on ovulation rate is supplementation with lupin grain for 4–6 days before ovulation (Teleni *et al.* 1989). This treatment increases the number of small follicles (Haresign 1981), ovulation rate (Oldham & Lindsay 1984) and prevents atresia of large follicles (Haresign 1981). Most of the investigations describing the effect of nutrition on litter size and more recently ovulation rate and folliculogenesis have been descriptive studies, and although these are detailed and well described, knowledge of their underlying physiological mechanisms is only now beginning to emerge. The current view is that nutrition modulates folliculogenesis through the actions of metabolic hormones such as insulin, insulin-like growth factor 1, GH and leptin. All

these have demonstrated local effects on follicle development and are likely mediators of the effects of nutrition on ovulation rate (Scaramuzzi *et al.* 2011) acting to modulate the action of gonadotrophins, steroids and inhibin in follicles.

Insulin had specific effects on granulosa and theca cell function in the ewe (Campbell *et al.* 1996) and insulin and glucose acted together to influence directly ovarian function *in situ* (Downing *et al.* 1999). The insulin-dependent glucose transporter type 4 (GLUT4) was detected in granulosa and theca cells of ovine follicles implying that changes in insulin-mediated glucose uptake by the follicle modulated its function (Williams *et al.* 2001). Thus, it is apparent that insulin can affect folliculogenesis by regulating the cellular uptake of glucose suggesting a role for insulin in the mechanism of nutritional effects on folliculogenesis in sheep (Scaramuzzi *et al.* 2006).

The insulin receptor that is present in the ovary is a ligand-activated tyrosine kinase. On activation, the insulin receptor phosphorylates a variety of downstream kinases including the insulin receptor substrate (IRS) proteins. The IRS proteins direct and regulate the post-receptor insulin signalling pathways that control glucose uptake, glucose metabolism, protein synthesis, RNA and DNA synthesis and cell survival. Thus, the IRS proteins are potential mediators of dietary effects on folliculogenesis (Brüning *et al.* 2000, Burks *et al.* 2000, Yen *et al.* 2004) possibly by interacting with the gonadotrophin-stimulated cAMP/protein kinase A (PKA) signalling pathways that controls steroid secretion in the follicle. Because the IRSs can specify the action of insulin along diverse signal cascades, it is reasonable to suggest that altered patterns of IRSs expression reflect altered insulin signalling in the follicle. Studies based largely on knockout mice have demonstrated that altered patterns of some IRSs impaired ovarian function (Fantin *et al.* 2000). Thus, the patterns of IRS expressions in theca and granulosa cells in response to diet may be associated with alterations in the physiological function of follicles. The role of the IRSs has not been examined in follicles from ewes fed with a high-energy diet such as lupin grain. We suggest that diet-induced changes in the circulating concentrations of insulin regulate follicular steroidogenesis by modifying follicular responsiveness to gonadotrophin stimulation and that the cellular actions of gonadotrophins are subject to local regulation by insulin acting through the IRSs to mediate the effects of dietary energy intake on folliculogenesis.

The aim of this study was to investigate, in sheep during the follicular and luteal phases of the oestrous cycle, the effects of feeding a high-energy diet on folliculogenesis and on the plasma and follicular fluid concentrations of glucose, insulin, FSH and oestradiol (E_2) and the levels of $P_{450arom}$ and IRS-1, -2 and -4 in theca and granulosa cells.

Results

Three ewes from the lupin-fed groups (one follicular phase and two luteal phases) refused to eat the lupin grain supplement and these three ewes have been excluded from the data set.

Ewe body weight

The average weights (mean $\pm$ S.E.M.) in the lupin-fed groups for both the follicular and the luteal phases ($n=21$) at sponge removal, at the start of lupin feeding and at cull were 45.5 ± 1.18 , 44.2 ± 1.17 and 43.6 ± 1.04 kg respectively, and similarly, for the control groups for both the follicular and the luteal phases ($n=24$), they were 45.5 ± 1.13 , 44.1 ± 0.93 and 43.6 ± 0.88 kg. There were no significant differences within or between groups.

Ovarian morphology

There was at least one corpus luteum (CL) in each ewe. The CLs of ewes in the luteal phase were red or dark pink in colour, with diameters of 7–11 mm. The non-functional CLs of ewes in the follicular phase were pale pink in colour, with diameters of 4–8 mm. The numbers of follicles dissected from the ovaries of lupin-fed and control ewes in the luteal and follicular phases are shown in Table 1. The average number of follicles per ewe in the lupin-fed and control groups were significantly different ($P=0.021$). However, there were no effects of stage of oestrous cycle ($P=0.945$), and the interaction was not significant ($P=0.608$).

Plasma glucose and plasma hormone concentration

Glucose

The plasma glucose concentrations are illustrated in Fig. 1. At the start of lupin feeding (day 0), the glucose concentrations (mean $\pm$ S.E.M.) in lupin-fed ($n=21$) and control groups ($n=24$) were 55.9 ± 1.26 and 56.9 ± 1.09 mg/dl respectively. There was a significant effect of time ($P<0.001$) and a significant interaction between time and supplementation ($P<0.001$). Exploration of this interaction showed that the plasma glucose concentrations in lupin-fed and control groups were significantly different ($P<0.05$) from day 3 to the end of the lupin-feeding period. Following the end of lupin feeding, the plasma concentrations of glucose were not different ($P=0.138$).

Insulin

The plasma insulin concentrations are illustrated in Fig. 1. At the start of lupin feeding (day 0), the insulin concentrations (mean $\pm$ S.E.M.) in lupin-fed and control groups were 0.35 ± 0.05 and 0.35 ± 0.03 ng/ml respectively. By day 2 after the start of lupin feeding, the concentration of plasma insulin increased

Table 1 The mean $\pm$ S.E.M. number of corpora lutea and follicles per ewe classified by diameter (mm) in lupin-fed and control groups at two stages of the oestrous cycle (luteal and follicular).

Treatment	Stage	Corpora lutea	Follicle classes				Total follicles per ewe
			>3.5 mm	2.5– $\leq$ 3.5 mm	2.0–<2.5 mm	<2.0 mm	
Lupin	Luteal ($n=9$)	1.78 ± 0.22^a	2.00 ± 0.24^a	7.22 ± 0.70^a	11.89 ± 1.99^a	$10.56 \pm 1.29^{a,b}$	31.67 ± 3.10^a
	Follicular ($n=8$)	1.63 ± 0.18^a	2.25 ± 0.49^a	$3.38 \pm 0.71^{b,c}$	$10.88 \pm 1.38^{a,b}$	14.75 ± 4.02^a	$31.25 \pm 4.39^{a,d}$
Control	Luteal ($n=9$)	1.44 ± 0.18^a	1.67 ± 0.29^a	4.22 ± 0.76^b	7.89 ± 1.84^b	8.44 ± 0.77^b	$22.22 \pm 2.77^{b,c}$
	Follicular ($n=9$)	1.56 ± 0.18^a	1.89 ± 0.20^a	2.56 ± 0.44^c	$10.78 \pm 1.23^{a,b}$	8.89 ± 1.90^b	$24.11 \pm 2.81^{b,d}$

Within columns, number with different superscripts differ significantly ($P < 0.05$)

significantly in the lupin-fed group and remained higher than that in the controls for the duration of lupin feeding ($P < 0.001$). There was a significant effect of time ($P < 0.001$) and the time by supplementation interaction was significant ($P < 0.001$).

FSH

The plasma FSH concentrations are illustrated in Fig. 2. At the start of lupin feeding (day 0), the plasma FSH concentrations (mean $\pm$ S.E.M.) in lupin-fed and control groups were 1.60 ± 0.12 and 1.68 ± 0.11 ng/ml respectively. After the start of lupin feeding, the concentration of plasma FSH was not different between groups ($P = 0.388$) and the interaction between supplementation and time was not significant ($P = 0.211$).

Oestradiol-17 β

The plasma concentrations of E_2 are illustrated in Fig. 2. At the start of lupin feeding (day 0), the concentrations of E_2 (mean $\pm$ S.E.M.) in lupin-fed and control groups were

6.13 ± 0.61 and 6.42 ± 0.52 pg/ml respectively. After the start of lupin feeding, the concentration of plasma E_2 decreased significantly in the lupin-fed group and remained lower than the controls ($P = 0.001$). There was a significant effect of time ($P < 0.001$) and the time by supplementation interaction was significant ($P = 0.001$). During follicular phase, after the induction of luteolysis with $PGF_{2\alpha}$, plasma concentrations of E_2 in the lupin-fed ($n = 11$) and control groups ($n = 12$) were significantly increased at 14 and 20 h.

Progesterone

The progesterone profiles confirmed that all ewes were undergoing normal oestrous cycles (data not shown).

Follicular fluid glucose and hormone concentrations

Glucose

Follicle diameter. There was a significant effect of follicle size ($P = 0.010$) but not of supplementation ($P = 0.054$) or

Figure 1 The mean ($\pm$ S.E.M.) concentrations of glucose (A) and insulin (B) in jugular venous blood in control ($n = 24$) and lupin-fed ($n = 21$) ewes. Blood samples were taken every second day at 0900 h for 8 days and then from the start of the lupin-feeding period, twice daily at 0900 h (before feeding) and 1400 h, then blood samples were taken at 14, 20 and 40 h after $PGF_{2\alpha}$ injection. Luteolysis was induced in the control–follicular phase ($n = 12$) and lupin–follicular phase ($n = 11$) groups with an injection of $PGF_{2\alpha}$ on day 5 of lupin-feeding period. An asterisk indicates a significant difference ($P < 0.05$) between groups.

Figure 2 The mean ($\pm$ S.E.M.) concentrations of FSH (A) and oestradiol-17 β (B) in jugular venous blood in control ($n=24$) and lupin-fed groups ($n=21$). Blood samples were taken every second day at 0900 h over a period of 8 days, then from the start of lupin feeding, blood samples were taken twice daily at 0900 h (before feeding) and 1400 h, then blood samples were taken at 14, 20 and 40 h after PGF_{2 α} injection. Luteolysis was induced in the control-follicular phase ($n=12$) and lupin-follicular phase ($n=11$) groups with an injection of PGF_{2 α} on day 5 of lupin-feeding period. An asterisk indicates a significant difference ($P<0.05$) between groups.

phase of the oestrous cycle ($P=0.885$) on the concentration of glucose in follicular fluid (Fig. 3).

Oestrogenicity. There were no effects of oestrogenicity ($P=0.460$), supplementation ($P=0.302$) or phase of the oestrous cycle ($P=0.485$) on the follicular fluid concentration of glucose but the supplementation by phase of the oestrous cycle interaction was significant ($P=0.038$). Exploration of this interaction showed that the mean follicular fluid concentration of glucose in oestrogenic and non-oestrogenic follicles in luteal phase was decreased in the lupin-fed groups ($P<0.05$; Fig. 4).

Oestradiol-17 β

Follicle diameter. There were significant effects of follicle size ($P<0.001$), phase of the oestrous cycle ($P<0.001$) and supplementation ($P=0.016$) on the follicular fluid concentration of E₂ (Fig. 3). There were significant interactions between supplementation and follicle size ($P=0.018$), phase of the oestrous cycle and follicle size ($P<0.001$) but not between supplementation and phase of the oestrous cycle ($P=0.956$). The concentration of E₂ in follicular fluid was significantly increased ($P<0.05$) in large follicles (≥ 3.5 mm in diameter; Fig. 3) in all groups. Lupin feeding had effects in follicles ≥ 3.5 mm in diameter where it decreased follicular fluid E₂ in the lupin-fed groups in both luteal phase ($P<0.05$) and follicular phase ($P<0.05$).

Oestrogenicity. There were significant effects of oestrogenicity ($P<0.001$) and phase of the oestrous cycle

($P=0.007$) but not of supplementation ($P=0.188$) on the follicular fluid concentration of E₂ and the oestrogenicity by phase of the oestrous cycle interaction was significant ($P=0.008$); exploration of this interaction shows that lupins decreased follicular fluid E₂ in the lupin-fed groups in follicular phase ($P<0.05$) but not in the luteal phase (Fig. 4).

Androstenedione

Follicle diameter. The follicular fluid concentrations of androstenedione are shown in Fig. 3. There were significant effects of follicle size ($P=0.017$) and phase of the oestrous cycle ($P=0.031$) but not of supplementation ($P=0.122$) but the supplementation by phase of the oestrous cycle interaction was significant ($P=0.007$). Thus, in the luteal but not in the follicular phase, the concentration of androstenedione in follicular fluid was decreased ($P<0.05$) in the lupin-fed group when compared with controls.

Oestrogenicity. There were significant effects of oestrogenicity ($P=0.025$) and phase of the oestrous cycle ($P=0.026$) but not of supplementation ($P=0.138$) on the follicular fluid concentration of androstenedione and the supplementation by phase of the oestrous cycle interaction was significant ($P<0.001$). The follicular fluid concentration of androstenedione in luteal phase was decreased in the lupin-fed group for both oestrogenic ($P<0.05$) and non-oestrogenic ($P<0.05$) follicles (Fig. 4).

Figure 3 The mean ($\pm$ S.E.M.) concentrations of glucose (A), oestradiol (B), androstenedione (C) and progesterone (D) in follicular fluid from follicles <3.5 and ≥ 3.5 mm in diameter from four groups of Welsh Mountain ewes. The letters a, b and c compare differences within follicle size and the letters x and y compare differences between follicle sizes. Different letters indicate a significant difference ($P < 0.05$).

Progesterone

Follicle diameter. The concentrations of progesterone in follicular fluid are shown in Fig. 3. There were significant effects of follicle size ($P = 0.026$) and phase of the oestrous cycle ($P < 0.001$) but not of supplementation ($P = 0.750$). The concentrations of progesterone in follicular fluid were significantly decreased ($P < 0.05$) in the follicular phase. The follicular fluid progesterone concentrations in the lupin-fed ewes during the luteal phase were significantly decreased ($P < 0.05$) in follicles ≥ 3.5 mm compared with those < 3.5 mm in diameter.

Oestrogenicity. There was a significant effect of phase of the oestrous cycle ($P < 0.001$) but not of oestrogenicity ($P = 0.586$) or supplementation ($P = 0.497$) on the follicular fluid concentration of progesterone. The concentration of progesterone was decreased ($P < 0.05$) in the control-follicular and lupin-follicular groups from both non-oestrogenic and oestrogenic follicles (Fig. 4).

The levels of $P_{450arom}$ in theca and granulosa cells

Follicle diameter

There was no detectable $P_{450arom}$ in the theca cell lysates in any of the samples. For granulosa cells (Table 2), levels of aromatase were not significantly different in small and medium follicles. There was a significant effect of follicle size ($P < 0.001$) and the level of aromatase was significantly increased ($P < 0.05$) in large follicles compared with medium and small follicles. For large follicles, the level of aromatase was decreased in lupin-fed ewes but the decrease was significant only in the follicular phase ($P < 0.05$).

Oestrogenicity

There was a significant effect of oestrogenicity ($P < 0.001$) but not of supplementation ($P = 0.546$) or of phase of the oestrous cycle ($P = 0.986$) on the levels of aromatase in granulosa cells. The supplementation by oestrogenicity interaction was significant ($P = 0.038$). The level of aromatase was significantly increased ($P < 0.05$) in oestrogenic follicles when compared with non-oestrogenic follicles in all groups. The level of $P_{450arom}$ was decreased ($P < 0.05$) in oestrogenic follicles during follicular phase in the lupin-fed group compared with the control group (Table 3).

Correlation between $P_{450arom}$ and follicular fluid E_2

The level of aromatase in granulosa cell lysates was positively correlated with the concentration of E_2 in follicular fluid from the same follicle ($r = 0.820$; $P < 0.001$; Fig. 5).

Insulin receptor substrate 1

Follicle diameter

IRS-1 was detectable in theca and granulosa cells from all follicle classes. In theca cells, the level of IRS-1 from large follicles was increased ($P < 0.05$) in the lupin-follicular group compared with control-follicular group. In granulosa cells, there was a significant effect of follicle size ($P = 0.001$), and the level of IRS-1 in granulosa cells from the lupin-follicular group was increased ($P < 0.05$) in large follicles (Table 2).

Oestrogenicity

The level of IRS-1 in theca cells from oestrogenic follicles was increased ($P < 0.05$) in the lupin-follicular group when compared with control-follicular group. There was a significant effect of oestrogenicity ($P = 0.006$) on the level of IRS-1 in granulosa cells. The levels of IRS-1 in theca and granulosa cells from the lupin-luteal and lupin-follicular groups were significantly increased ($P < 0.05$) in oestrogenic follicles (Table 3).

Insulin receptor substrate 2

Follicle diameter

In theca cells, there was a significant effect of supplementation ($P = 0.004$) and the interaction between the phase of the oestrous cycle and follicle size was significant ($P = 0.005$). The levels of IRS-2 in theca cells were increased ($P < 0.05$) in the lupin-fed group during follicular phase compared with the control group (Table 2). The levels of IRS-2 in granulosa cells from all follicle classes were increased ($P < 0.05$) in the lupin-fed groups compared with the control groups.

Oestrogenicity

The level of IRS-2 in theca and granulosa cells from oestrogenic follicles was increased ($P < 0.05$) in the lupin-fed groups during both the luteal and the follicular phases (Table 3).

Insulin receptor substrate 4

Follicle diameter

In theca cells, there was a significant supplementation by phase of the oestrous cycle interaction ($P = 0.010$) on the level of IRS-4. The level of IRS-4 in theca cells was decreased ($P < 0.05$) in the lupin-fed group

Figure 4 The mean ($\pm$ S.E.M.) concentrations of glucose (A), oestradiol (B), androstenedione (C) and progesterone (D) in follicular fluid from non-oestrogenic (< 100 ng/ml) and oestrogenic (≥ 100 ng/ml) follicles from four groups of Welsh Mountain ewes. The letters a and b compare differences within oestrogenicity class and the letters x and y compare differences between oestrogenicity class. Different letters indicate a significant difference ($P < 0.05$).

Figure 5 Positive correlation between the level of $P_{450arom}$ in granulosa cell lysates and the concentration of oestradiol-17 β in follicular fluid from the same follicle (A: C/L, control-luteal; L/L, lupin-luteal; C/F, control-follicular; L/F, lupin-follicular group; FF E_2 , the concentration of oestradiol-17 β in follicular fluid (ng/ml)). The individual $P_{450arom}$ levels in granulosa cells $\blacklozenge$ and the concentration of oestradiol-17 β in follicular fluid from the same follicle (B).

during follicular phase compared with the control group for all follicle classes, but in large follicles it was increased ($P < 0.05$) in the lupin-fed group during luteal phase compared with the control group. The levels of IRS-4 in granulosa cells were increased in the lupin-fed group during the luteal phase (medium follicles) and the follicular phase (large follicles) compared with control follicles (both $P < 0.05$) (Table 2).

Oestrogenicity

In theca cells, there was a significant supplementation by phase of the oestrous cycle interaction ($P = 0.006$) on the level of IRS-4. Lupins decreased ($P < 0.05$) the level of IRS-4 in theca cells during follicular phase for both oestrogenic and non-oestrogenic follicles. In granulosa cells there was a significant effect of phase of the oestrous cycle ($P = 0.039$) but not of oestrogenicity ($P = 0.092$) or supplementation ($P = 0.096$) on the level of IRS-4. In non-oestrogenic follicles the level of IRS-4 in granulosa cells from the follicular phase was increased ($P < 0.05$) in the lupin-fed group compared with control. The level of IRS-4 in granulosa cells from the lupin-luteal group was increased ($P < 0.05$) in oestrogenic compared with non-oestrogenic follicles (Table 3).

Discussion

The results of this study confirm that short-term dietary supplementation with lupin grain increased the number of follicles in ewes (Table 1). These data agree with several other studies (Downing & Scaramuzzi 1991, Williams *et al.* 2001, Muñoz-Gutiérrez *et al.* 2002,

Viñoles *et al.* 2005, Letelier *et al.* 2009) showing that short-term nutritional supplementation of ewes increased the number of follicles. This short-term effect was independent of body weight again confirming the findings of several similar studies (Oldham & Lindsay 1984, Nottle *et al.* 1985, King *et al.* 1991, Pearce *et al.* 1994, Somchit *et al.* 2007). These results suggest that rapid follicular responses to nutrition are associated with specific metabolic or nutritional signals rather than with body weight or body condition *per se* (Downing & Scaramuzzi 1991).

It is clear that short-term feeding of lupin grain increased plasma concentrations of both glucose and insulin. The administration of glucose (Downing *et al.* 1995a, Rubio *et al.* 1997), feeding high-energy diets (Downing *et al.* 1995b) or infusing gluconeogenic amino acids (Downing *et al.* 1995c) to ewes increased circulating plasma insulin concentrations and they also increased follicle number and/or ovulation rate. Many papers have reported that short-term nutritional supplementation increased the plasma concentrations of insulin in ewes (Downing & Scaramuzzi 1991, Downing *et al.* 1995b, Williams *et al.* 2001, Viñoles *et al.* 2005, Somchit *et al.* 2007), cows (Gutiérrez *et al.* 1997, Landau *et al.* 2000, Gong *et al.* 2002) and gilts (Ferguson *et al.* 2003). All these data suggest that the concentration of plasma insulin is key variable associated with the nutritional modulation of ovulatory responses. We suggest that increased plasma insulin leads to an increase in insulin-mediated glucose uptake by the ovary during the late luteal phase of the oestrous cycle that stimulates the growth of small follicles and/or prevents atresia in medium to large follicles, thereby increasing the pool of ovulatory follicles.

Table 2 The mean $\pm$ s.e.m. levels of aromatase, IRS-1, -2 and -4 in theca cells and granulosa cells from four groups, classified by follicle diameter.

Stage	Suppl	Follicle diameter (mm)	P _{450arom} GC	IRS-1		IRS-2		IRS-4	
				TC	GC	TC	GC	TC	GC
Luteal	Control	2.0–<2.5	0.038 $\pm$ 0.027 ^{a,x}	0.408 $\pm$ 0.093 ^x	0.568 $\pm$ 0.082 ^a	0.695 $\pm$ 0.107 ^{a,x,y}	0.264 $\pm$ 0.055 ^x	1.387 $\pm$ 0.504 ^{x,y}	0.124 $\pm$ 0.058 ^y
Luteal	Control	2.5–<3.5	0.113 $\pm$ 0.053 ^{a,x}	0.477 $\pm$ 0.106 ^x	0.402 $\pm$ 0.104 ^a	0.636 $\pm$ 0.098 ^{a,x,y}	0.217 $\pm$ 0.060 ^x	0.939 $\pm$ 0.209 ^y	0.087 $\pm$ 0.030 ^y
Luteal	Control	≥ 3.5	0.522 $\pm$ 0.143 ^{b,x,y}	0.565 $\pm$ 0.117 ^x	0.600 $\pm$ 0.095 ^a	0.477 $\pm$ 0.084 ^{a,x,y}	0.432 $\pm$ 0.085 ^x	0.949 $\pm$ 0.379 ^y	0.188 $\pm$ 0.052 ^y
Luteal	Lupins	2.0–<2.5	0.087 $\pm$ 0.020 ^{a,x}	0.398 $\pm$ 0.051 ^x	0.524 $\pm$ 0.091 ^a	0.989 $\pm$ 0.113 ^{a,x}	0.663 $\pm$ 0.076 ^y	1.171 $\pm$ 0.086 ^y	0.354 $\pm$ 0.165 ^{x,y}
Luteal	Lupins	2.5–<3.5	0.068 $\pm$ 0.027 ^{a,x}	0.429 $\pm$ 0.117 ^x	0.316 $\pm$ 0.090 ^a	0.860 $\pm$ 0.134 ^{a,b,x}	0.645 $\pm$ 0.090 ^y	1.451 $\pm$ 0.248 ^{x,y}	0.494 $\pm$ 0.083 ^x
Luteal	Lupins	≥ 3.5	0.481 $\pm$ 0.087 ^{b,y}	0.469 $\pm$ 0.079 ^x	0.465 $\pm$ 0.077 ^a	0.635 $\pm$ 0.083 ^{b,x,y}	0.690 $\pm$ 0.072 ^y	1.576 $\pm$ 0.206 ^x	0.258 $\pm$ 0.089 ^y
Follicular	Control	2.0–<2.5	0.031 $\pm$ 0.014 ^{a,x}	0.279 $\pm$ 0.064 ^x	0.421 $\pm$ 0.079 ^a	0.382 $\pm$ 0.061 ^{a,y}	0.164 $\pm$ 0.055 ^x	1.894 $\pm$ 0.254 ^x	0.289 $\pm$ 0.090 ^{x,y}
Follicular	Control	2.5–<3.5	0.009 $\pm$ 0.004 ^{a,x}	0.207 $\pm$ 0.093 ^x	0.290 $\pm$ 0.092 ^a	0.324 $\pm$ 0.132 ^{a,y}	0.412 $\pm$ 0.152 ^{x,y}	1.839 $\pm$ 0.543 ^x	0.219 $\pm$ 0.097 ^{x,y}
Follicular	Control	≥ 3.5	0.772 $\pm$ 0.135 ^{b,x}	0.257 $\pm$ 0.044 ^y	0.545 $\pm$ 0.069 ^a	0.429 $\pm$ 0.066 ^{a,y}	0.311 $\pm$ 0.062 ^x	1.960 $\pm$ 0.131 ^x	0.372 $\pm$ 0.091 ^y
Follicular	Lupins	2.0–<2.5	0.103 $\pm$ 0.046 ^{a,x}	0.458 $\pm$ 0.096 ^x	0.378 $\pm$ 0.074 ^{a,b}	0.705 $\pm$ 0.115 ^{a,x}	0.575 $\pm$ 0.059 ^y	1.074 $\pm$ 0.126 ^y	0.504 $\pm$ 0.085 ^x
Follicular	Lupins	2.5–<3.5	0.082 $\pm$ 0.033 ^{a,x}	0.335 $\pm$ 0.078 ^x	0.211 $\pm$ 0.073 ^a	0.628 $\pm$ 0.132 ^{a,x,y}	0.637 $\pm$ 0.083 ^y	0.885 $\pm$ 0.170 ^y	0.346 $\pm$ 0.091 ^{x,y}
Follicular	Lupins	≥ 3.5	0.530 $\pm$ 0.101 ^{b,y}	0.514 $\pm$ 0.064 ^x	0.450 $\pm$ 0.056 ^b	0.704 $\pm$ 0.096 ^{a,x}	0.754 $\pm$ 0.085 ^y	0.929 $\pm$ 0.090 ^y	0.651 $\pm$ 0.083 ^x

TC, theca cell; GC, granulosa cell. Within columns, the letters a and b compare follicle sizes and the letters x and y compare groups. Different letters indicate a significant difference ($P < 0.05$).

Table 3 The mean $\pm$ s.e.m. levels of aromatase, IRS-1, -2 and -4 in theca cells and granulosa cells from four groups, classified by their oestrogenicity.

Stage	Suppl	Oestrogenicity	P _{450arom} GC	IRS-1		IRS-2		IRS-4	
				TC	GC	TC	GC	TC	GC
Luteal	Control	Non-oestrogenic	0.067 $\pm$ 0.044 ^{a,x}	0.514 $\pm$ 0.182 ^{a,x}	0.512 $\pm$ 0.293 ^{a,x}	0.505 $\pm$ 0.174 ^{a,x}	0.395 $\pm$ 0.135 ^{a,x}	0.754 $\pm$ 0.293 ^{a,x}	0.167 $\pm$ 0.166 ^{a,x}
Luteal	Control	Oestrogenic	0.901 $\pm$ 0.103 ^{b,x,y}	0.600 $\pm$ 0.166 ^{a,x}	0.643 $\pm$ 0.047 ^{a,x}	0.459 $\pm$ 0.095 ^{a,x}	0.446 $\pm$ 0.110 ^{a,x}	1.060 $\pm$ 0.588 ^{a,x}	0.194 $\pm$ 0.058 ^{a,x}
Luteal	Lupins	Non-oestrogenic	0.114 $\pm$ 0.045 ^{a,x}	0.328 $\pm$ 0.077 ^{a,x}	0.294 $\pm$ 0.099 ^{a,x}	0.474 $\pm$ 0.106 ^{a,x}	0.501 $\pm$ 0.050 ^{a,x}	1.698 $\pm$ 0.312 ^{a,x,y}	0.065 $\pm$ 0.074 ^{a,x}
Luteal	Lupins	Oestrogenic	0.876 $\pm$ 0.079 ^{b,x,y}	0.621 $\pm$ 0.132 ^{b,x}	0.649 $\pm$ 0.098 ^{b,x}	0.808 $\pm$ 0.113 ^{b,y}	0.894 $\pm$ 0.116 ^{b,y}	1.465 $\pm$ 0.281 ^{a,x,y}	0.451 $\pm$ 0.147 ^{b,x,y}
Follicular	Control	Non-oestrogenic	0.008 $\pm$ 0.003 ^{a,x}	0.352 $\pm$ 0.089 ^{a,x}	0.427 $\pm$ 0.105 ^{a,x}	0.490 $\pm$ 0.151 ^{a,x}	0.219 $\pm$ 0.084 ^{a,x}	1.997 $\pm$ 0.117 ^{a,y}	0.264 $\pm$ 0.149 ^{a,x}
Follicular	Control	Oestrogenic	1.077 $\pm$ 0.023 ^{b,x}	0.219 $\pm$ 0.047 ^{a,y}	0.592 $\pm$ 0.085 ^{a,x}	0.405 $\pm$ 0.074 ^{a,x}	0.347 $\pm$ 0.079 ^{a,x}	1.945 $\pm$ 0.180 ^{a,y}	0.416 $\pm$ 0.114 ^{a,x,y}
Follicular	Lupins	Non-oestrogenic	0.166 $\pm$ 0.061 ^{a,x}	0.378 $\pm$ 0.107 ^{a,x}	0.266 $\pm$ 0.054 ^{a,x}	0.768 $\pm$ 0.138 ^{a,x}	0.778 $\pm$ 0.112 ^{a,y}	1.050 $\pm$ 0.170 ^{a,x}	0.730 $\pm$ 0.080 ^{a,y}
Follicular	Lupins	Oestrogenic	0.699 $\pm$ 0.123 ^{b,y}	0.578 $\pm$ 0.076 ^{b,x}	0.536 $\pm$ 0.068 ^{b,x}	0.675 $\pm$ 0.127 ^{a,y}	0.743 $\pm$ 0.115 ^{a,y}	0.865 $\pm$ 0.106 ^{a,x}	0.618 $\pm$ 0.113 ^{a,y}

TC, theca cell; GC, granulosa cell. Within columns, the letters a and b compare oestrogenicity and the letters x and y compare groups. Different letters indicate a significant difference ($P < 0.05$).

We have shown that the plasma concentrations of E_2 during the period of lupin feeding and in the subsequent early follicular phase of the oestrous cycle were decreased in the lupin-fed group; this result agrees with other studies that reported decreased concentrations of plasma E_2 in supplemented ewes (Adams *et al.* 1994, Letelier *et al.* 2008a, 2008b) and gilts (Ferguson *et al.* 2003). At the same time, the concentrations of FSH were not elevated by these treatments and this leads us to suggest that short-term nutritional supplementation may be acting locally in the follicle to perturb the negative feedback control systems that regulate folliculogenesis. It is not clear why the decreased secretion of E_2 did not result in a compensatory increase in FSH, perhaps this was inhibited by an increased inhibin secretion from the increased number of small- and medium-sized follicles, known sources of inhibin (Campbell *et al.* 1990) as has been suggested (Scaramuzzi *et al.* 2011). This hypothesis remains to be tested. The concentrations of E_2 in follicular fluid reported in this study show unsurprisingly that it was higher in follicles ≥ 3.5 mm than in follicles < 3.5 mm (Chang *et al.* 1976, Carson *et al.* 1981, Henderson & Franchimont 1981, Evans & Martin 2000). We have also shown that the short-term feeding of lupin grain decreased follicular fluid E_2 in large follicles during both the luteal and the follicular phases; other studies have reported similar results (Peluso *et al.* 1991, Muñoz-Gutiérrez *et al.* 2005) and they are consistent with our other observations from this study, that is decreased concentration of plasma E_2 and decreased levels of granulosa cell aromatase in follicles from ewes supplemented with lupin grain.

In a previous investigation (Somchit *et al.* 2007), we reported that nutritional supplementation of ewes with lupin grain increased the follicular fluid concentrations of glucose during the luteal phase of the oestrous cycle. We were unable to confirm this finding in this study and in fact we found the opposite effect, that is, glucose concentrations in follicular fluid were reduced in the luteal phase of the oestrous cycle in ewes fed lupin grain.

The reasons for this discrepancy are not apparent, but in both studies, there were wide variations in the concentration of glucose in follicular fluid both among animals and among follicles within animals and perhaps our contradictory observations are a reflection of the inherent variability of glucose concentrations in follicular fluid.

The concentrations of androstenedione in follicular fluid were decreased in the lupin-fed ewes during the luteal phase and this decrease was associated with a reduction in follicular fluid E_2 . In addition, we found that androstenedione in follicular fluid tended to be higher in small follicles < 3.5 mm compared with follicles ≥ 3.5 mm in diameter. Androstenedione is increased in small follicles (Chang *et al.* 1976, Wise 1987), probably because it is a substrate for E_2 synthesis, and in large follicles, there is a high rate of utilization for E_2 synthesis.

The results of western immunoblotting from granulosa and theca cell lysates in this study show that $P_{450arom}$, with a molecular weight of ~ 50 kDa (Albrecht *et al.* 2001, Gen *et al.* 2001, Turner *et al.* 2002), was detected in granulosa cells, but not at all in theca cells (Fig. 6). Aromatase otherwise known as $P_{450arom}$ is an enzyme responsible for converting androgens to oestrogens in granulosa cells (Hillier *et al.* 1994, Johnson & Everitt 1995, Brodie *et al.* 1999). Aromatase is a microsomal member of the cytochrome P_{450} superfamily, namely aromatase cytochrome P_{450} , the product of the *CYP19A1* (*CYP19*) gene (Simpson *et al.* 2002). Studies by Manikkam *et al.* (2001) and Garverick *et al.* (2002) reported that the expression of mRNA for $P_{450arom}$ in the follicle was localized to granulosa cells. We have shown that aromatase levels were significantly increased in large follicles compared with small and medium follicles. More importantly and for the first time, to our knowledge, the results show that the level of aromatase was significantly decreased in large follicles in the follicular phase of lupin-fed ewes compared with control ewes. This suggests that lupin feeding may affect aromatase activity by decreasing its level in granulosa cells during the follicular phase of the oestrous cycle.

Figure 6 The example of immunoblotting of $P_{450arom}$ (A), IRS-1 (B), IRS-2 (C) and IRS-4 (D). Lane M, molecular weight marker (kDa); lanes 1–9, lysate samples. The $P_{450arom}$, IRS-1, -2 and -4 had approximate molecular masses of 50, 165, 200 and 155 kDa respectively.

In addition, the level of aromatase in granulosa cells was positively correlated with the concentration of E_2 in follicular fluid.

We determined the level of IRS-1, -2 and -4 in theca and granulosa cells, but we did not measure IRS-3 because recent research attempting to clone the IRS-3 gene revealed the absence of a functional IRS-3 gene in humans and none of the molecular approaches have provided evidence for a functional IRS-3 gene in human tissue (Giovannone *et al.* 2000, Björnholm *et al.* 2002, Kokk *et al.* 2005). Furthermore, the absence of IRS-3 does not appear to influence fertility (Liu *et al.* 1999) and mice lacking IRS-3 have normal growth patterns and glucose homeostasis (Liu *et al.* 1999).

There is little published information about the IRS proteins in ovarian tissue from normal animals, IRS-1 is expressed in the luteinized rat ovary (Talavera *et al.* 1996), in follicular cells from the human (Wu *et al.* 2000, Dunaif *et al.* 2001) and in ovaries from the female rat (Richards 1994, Richards *et al.* 1995) while IRS-2 expression has been reported in theca and granulosa cells from female mice (Neganova *et al.* 2007) and studies using gene knockout mice suggest that IRS-2 and -4 are likely to be involved in mediating follicular responses to insulin (Fantin *et al.* 2000). At present, there are no published reports of IRSs in the sheep follicle, and our data report for the first time the presence of IRS-1, -2 and -4 in theca and granulosa cell lysates from sheep.

The IRSs are important components of insulin signalling pathways. This study shows that both theca and granulosa cells contain IRS-1, -2 and -4 at approximate molecular weights of 165, 200 and 155 kDa respectively (Sun *et al.* 1992, Fantin *et al.* 1998, Ross *et al.* 1998, Wu *et al.* 2000, Tsuruzoe *et al.* 2001). IRS-1 studies in human (Wu *et al.* 2000) and in mouse (Neganova *et al.* 2007) found that the level of IRS-1 in theca and granulosa cells increased with follicular development but our own data do not agree with this finding. Studies from knockout mice suggest that IRS-1 mainly mediates mitogenic pathways of insulin signalling (Tamemoto *et al.* 1994, Withers *et al.* 1998) and increased levels of phosphorylated IRS-1 amplify insulin-stimulated granulosa cell proliferation (Yen *et al.* 2004). Our study used follicles of 2 mm diameter and greater; in ewes, follicles of this size are not highly proliferative (Scaramuzzi *et al.* 2011). Our results show that IRS-1 was present at both stages of the oestrous cycle. We also found that the level of IRS-1 in theca cells from large follicles was higher in the lupin-fed ewes during follicular phase. Concerning IRS-2, lupins increased its level in theca cells during the follicular phase and in granulosa cells during both the luteal and the follicular phases. This study provides some evidence to suggest that both IRS-1 and -2 are nutritionally regulated in sheep and that they are implicated in the nutritional regulation of folliculogenesis.

In this study, the general patterns of expression of IRS-1 and -2 in theca and granulosa cells were higher in response to nutrition in both luteal and follicular phases whereas IRS-4 in the theca was higher during the luteal phase but lower in the follicular phase. This pattern of expression of IRS-1, -2 and -4 in ovine follicles therefore supports our hypothesis that the insulin–glucose system is associated with nutritionally stimulated folliculogenesis. However, additional research is needed to precisely define the roles of the IRSs in modulating gonadotrophin-stimulated folliculogenesis.

These results show at least in the ewe that nutrition can directly alter the physiological function of ovarian follicles without any change in the circulating concentration of FSH. There was a significant increase in the number of follicles in the lupin-fed ewes, as well as increased granulosa and thecal IRSs and decreased levels of aromatase and these changes were associated with an increased number of follicles and lower concentrations of E_2 in follicular fluid and in peripheral blood.

In summary, a short-term supplement with lupin grain increased the number of follicles with no detectable change in live weight. However, there were increased plasma concentrations of insulin and glucose and decreased concentrations of E_2 in blood and follicular fluid; there were no effects on either FSH or progesterone. Thus, the gonadal responses to short-term nutritional supplementation appear to be associated with metabolic or nutritional signals to the follicle and specifically insulin rather than with weight. The IRSs are intracellular intermediates of insulin signalling and our data show that their levels are nutritionally regulated in the follicle suggesting a functional role in the nutritional stimulation of folliculogenesis in sheep. Beyond this, further interpretation is speculation, but it is tempting to suggest that the intra-follicular actions of insulin in the follicle may involve crosstalk with FSH-stimulated aromatase (Scaramuzzi *et al.* 2010).

Materials and Methods

Animals

Forty-eight clinically healthy, Welsh Mountain ewes 2–3 years old were obtained from approved commercial sources. The animals were acclimatized for a week before the onset of the experiment. They were kept on straw bedding in group pens, fed with 500 g/ewe per day of a commercial concentrate diet (Ewe Feed, BOCM PAULS Ltd., Ipswich, Suffolk, UK; 18% protein; Metabolizable energy (ME)=15.7 MJ/kg dry matter (DM) and given free access to good quality meadow hay (10.8% crude protein and ME=8.3 MJ/kg DM); water was available *ad libitum*. The ewes were divided into four equal treatment groups: control–luteal phase (C–L group), lupin-fed–luteal phase (L–L group), control–follicular phase (C–F group) and lupin-fed–follicular phase (L–F group). The experiment was carried out in October, during the breeding season. Animals in the lupin-fed groups were moved into individual

pens (1.5×2.5 m) a day before start of the lupin-feeding period. Oestrus was synchronized in all ewes with intra-vaginal progestagen sponges containing 30 mg flugestone acetate (Chronogest, Intervet Ltd., Milton Keynes, Buckinghamshire, UK), for 12 days. During the period of sponge insertion, the ewes were trained to eat lupin grain (*Lupinus luteus*, variety Wodjil (spring sown), 40–44% protein, ME=14.5 MJ/kg DM, and 6.5% oil, Innoseeds Ltd., Downham Market, Norfolk, UK). Ewes in the two lupin-fed groups were fed with a commercial concentrate diet until the day of sponge removal; they were then switched to a diet of meadow hay (10.8% crude protein and ME=8.3 MJ/kg DM) and 8 days later the ewes in these groups were fed a supplement of 500 g/ewe per day of lupin grain for 5 days (days 6–11 of the oestrous cycle): 250 g in the morning (after blood collection at 0900 h) and 250 g at 1700 h. Animals in the two control groups were fed a commercial concentrate diet until sponge removal; then they were switched to a diet of meadow hay until the end of the experiment. Animals in the follicular phase groups received i.m. injections of 125 µg of an analogue of PGF_{2α} (Estrumate, Schering-Plough Animal Health Ltd., Harefield, Middlesex, UK) on day 11 of the synchronized oestrous cycle (i.e. day 5 of the lupin-feeding period). The ewes were weighed at sponge removal, at the start of lupin feeding and at cull. All procedures involving live animals were conducted with the authorization of the Home Office and in compliance with the Animal (Scientific Procedures) Act, 1986.

Blood collection

Blood samples were taken at 0900 h every second day starting at the time of sponge removal until the start of the lupin-feeding period. At the start of lupin-feeding period, blood sampling regime was altered to twice daily at 0900 h (immediately before feeding) and 1400 h (5 h after feeding), this regime continued to the end of the lupin-feeding period. After PGF_{2α} injection, blood samples were taken at 14, 20 and 40 h after luteolysis induction in follicular phase group. All animals were culled at 40 h after PGF_{2α} injection. The blood was placed immediately in heparinized (200 IU) glass tubes. Plasma was separated by centrifugation at 4 °C and stored at –20 °C. Blood samples for glucose analysis were collected separately using 2.7 ml glucose syringes (S-Monovette, containing 1.2 mg EDTA/ml blood and 1.0 mg fluoride/ml blood, SARSTEDT, Aktiengesellschaft & Co., Nümbrecht, Germany). The plasma was separated by centrifugation and stored as described earlier. The plasma containing heparin was analysed for insulin, FSH, E₂ and progesterone and the plasma containing EDTA and fluoride was analysed for glucose.

Collection and dissection of ovaries

At the end of the experiment, the animals were killed with a captive bolt pistol followed by exsanguination. The ovaries were collected within 5 min of death and placed in ice-cold sterile saline. Both ovaries from nine ewes per group were dissected. The remaining ovaries were fixed in buffered formal saline. All visible follicles >1.0 mm in diameter were dissected free from the ovarian stroma, using fine scissors and fine-toothed

dissecting forceps, and placed in PBS in a sterile plastic Petri dish. Follicle diameter was measured to the nearest millimeter, using a graph paper grid placed under the dish. Each follicle was then hemisected in 1 ml sterile PBS in a sterile plastic mini-Petri dish (NUNCCLON, Nunc A/S Ltd., Roskilde, Denmark). The granulosa cell layer was gently scraped into the PBS using a sterile, fine plastic loop. The theca shell was placed in a sterile 1.5 ml microtube (STAR Labs GmbH, Ahrensburg, Germany). The PBS containing granulosa cells and follicular fluid was then placed in another 1.5 ml microtube and centrifuged at 4 °C for 10 min at 13 000 r.p.m. (approximately 18 894 g). Following centrifugation, the supernatant containing diluted follicular fluid was transferred into a 1.5 ml microtube and stored at –20 °C. The separated theca shell and the granulosa cell pellet were stored at –80 °C. Follicular fluid was assayed for glucose, androstenedione, E₂ and progesterone. Theca and granulosa cell lysates were prepared and the lysates were analysed for $P_{450arom}$, IRS-1, -2 and -4 by western immunoblotting.

Follicle classification

Follicles were classified and processed according to their diameter as follows: i) follicles >2.5 mm: individual follicles were separated into theca, granulosa and follicular fluid for individual analysis; ii) follicles 2.0–2.5 mm: follicles were pooled within ovaries (in batches of two to seven follicles), the pooled follicles were then separated into theca, granulosa and follicular fluid; and iii) follicles <2.0 mm: all follicles were pooled within ovaries, the pooled follicles were then separated into theca, granulosa and follicular fluid. The theoretical volume of follicular fluid volume (V) was calculated as described previously (Carson *et al.* 1981) using the formula: $V=0.52(D)^{2.7}$, where V is the volume of fluid (mm³) and D is the follicle diameter (mm).

For the analysis of follicle number, follicles were grouped into four classes based on their diameter: i) <2.0 mm, ii) 2.0–<2.5 mm, iii) 2.5–≤3.5 mm and iv) >3.5 mm.

For the analysis of follicular fluid, follicles were grouped into two classes based on their diameter: i) <3.5 mm and ii) ≥3.5 mm; and oestrogenicity: i) non-oestrogenic (E₂<100 ng/ml) and ii) oestrogenic follicles (E₂≥100 ng/ml).

For the western blot analysis, granulosa and theca lysates from follicles ≥3.5 mm class were analysed individually, while follicles in the 2.0–<2.5 and 2.5–≤3.5 mm classes were pooled within ovaries and analysed as a pool. This was necessary because there was insufficient protein from single follicles <3.5 mm in diameter. Follicles in the <2.0 mm class were not analysed.

Glucose and hormone assays

Plasma analyses

Glucose was analysed in 2 µl plasma, by colorimetry using the glucose oxidase phenol 4-aminoantipyrine peroxidase method (Randox test kits; Randox Laboratories Ltd., Crumlin Co., Antrim, UK) and an IMOLA automated analyzer (Randox Laboratories Ltd., Crumlin Co.). All samples were analysed in a single assay. The intra-assay coefficient of variation (CV) was

5.1%. The limit of sensitivity for the glucose assay was 10.8 mg/dl.

Insulin was analysed by RIA (Williams *et al.* 2001). The intra-assay CV was 4.7%. The inter-assay CV was 6.0% and the limit of sensitivity for the insulin assay was 0.05 ng/ml.

FSH was analysed by RIA (Campbell *et al.* 1994). The intra-assay CV was 11.3%. The inter-assay CV was 8.8% and the limit of sensitivity for the FSH assay was 0.78 ng/ml.

E₂ was analysed by RIA (Mann & Lamming 1995). The intra-assay CV was 9.6%. The inter-assay CV was 5.2% and the limit of sensitivity for the E₂ assay was 0.62 pg/ml.

Progesterone was determined by RIA (Campbell *et al.* 1990). The intra-assay CV for low- (0.5 ng/ml), medium- (1.0 ng/ml) and high- (4.0 ng/ml) quality control plasmas were 17.8, 10.8 and 6.0% respectively. The inter-assay CV was 2.8% and the limit of sensitivity for the progesterone assay was 0.04 ng/ml.

Follicular fluid analyses

Follicular fluid concentrations were corrected for dilution in 1 ml PBS and are reported as concentrations of undiluted follicular fluid. Glucose was determined in individual follicles ≥ 2.5 mm in diameter, by colorimetry using the GOD/PAP method as described earlier but with 35 μ l of diluted follicular fluid. All the samples were analysed in a single assay. The intra-assay CV was 3.6%. The limit of sensitivity for the glucose assay was 0.018 mg/dl.

Androstenedione was analysed by RIA (Campbell *et al.* 1998). The intra-assay CV for low- (50 ng/ml), medium- (250 ng/ml), high-1- (400 ng/ml) and high-2- (800 ng/ml) quality controls were 11.2, 10.0, 6.4 and 16.8% respectively. The inter-assay CV was 9.2% and the limit of sensitivity for the androstenedione assay was 4.4 ng/l.

E₂ was analysed by RIA (Campbell *et al.* 1996). The intra-assay CV for low- (50 ng/ml), medium- (250 ng/ml), high-1- (400 ng/ml) and high-2- (800 ng/ml) quality controls were 9.0, 19.1, 4.9 and 5.6% respectively. The inter-assay CV was 12.5% and the limit of sensitivity for the E₂ assay was 0.04 ng/ml.

Progesterone was analysed by RIA (Campbell *et al.* 1998). The intra-assay CV for low- (50 ng/ml), medium- (250 ng/ml), high-1- (400 ng/ml) and high-2- (800 ng/ml) quality controls were 15.7, 8.5, 11.8 and 3.1% respectively. The inter-assay CV was 13.9% and the limit of sensitivity for the progesterone assay was 3.9 ng/ml.

Western blot analysis

Theca and granulosa cell lysates were analysed for P_{450arom}, IRS-1, -2 and -4 by western immunoblotting (Somchit 2008). Briefly, SDS-PAGE was used to separate the proteins in the theca and granulosa cell lysates. Electrophoresis was carried out at 150 V and 400 mA for 75–100 min. Following electrophoresis, the gel was transferred to a PVDF membrane (Immobilon-P Transfer membranes, pore size 0.45 μ m, Millipore Corporation, Billerica, MA, USA) and incubated in a blocker solution (10% BSA or 10% skimmed milk). The PVDF membrane was then probed with a primary antibody against mouse anti-human cytochrome P_{450arom} (Serotec Ltd., Kidlington, Oxfordshire, UK), rabbit polyclonal anti-IRS-1 (C-20, sc-559, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-IRS-2 (H-205,

sc-8299, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-IRS-4 (Upstate Biotechnology, Inc., Lake Placid, NY, USA) or mouse monoclonal β -actin (AC-15, ab6276, abcam, Abcam plc, Cambridge Science Park, Cambridgeshire, UK) at working dilutions of 1:500, 1:250, 1:500, 1:1000 and 1:4000 respectively. After overnight incubation, the PVDF membrane was washed with TBST for 3 $\times$ 20 min. Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin specific polyclonal antibody (BD Biosciences Pharmingen, Oxford Science Park, Oxfordshire, UK) or ImmunoPure goat anti-rabbit IgG peroxidase conjugated (Pierce Biotechnology, Inc., Cramlington, Northumberland, UK) were used as the secondary antibodies at a working dilution of between 1:1000 and 1:10 000. The membrane was then washed in TBST, developed using an ECL detection method (ECL western blotting detection reagents, Amersham Biosciences) and exposed to film (Hyperfilm ECL high performance chemiluminescence film, Amersham Biosciences) for 5–10 min. The optical density of P_{450arom}, IRS-1, -2 and -4 bands were measured using the Quantity One Software Program version 4.4.0 (Bio-Rad Laboratories). The specific density of the P_{450arom}, IRS-1, -2 and -4 for each sample was calculated by subtraction of the background density and divided by the specific density of corresponding β -actin band or quality control band.

Statistical analysis

The statistical analyses were performed using the SPSS Statistical Software programme version 15.0 (SPSS, Inc.). All data are presented as the means $\pm$ S.E.M. Where data were not normally distributed, they were transformed to logarithms. Data for hormone concentrations in plasma and body weight were analysed using a repeated measures, mixed model ANOVA with time as the within subject (within sheep) repeated measure and treatments (diet and stage of the oestrous cycle) as between subject (between sheep) factors. Data on hormone concentrations in follicular fluid and the levels of P_{450arom}, IRS-1, -2 and -4 were analysed using a repeated measures, mixed model ANOVA with follicles within sheep as the within subject factor and treatments (diet and stage of the oestrous cycle) as between subject (between sheep) factors. For both methods, interactions were explored using 95% CIs. Follicle numbers and distributions were tested by the χ^2 test and correlation (either Pearson's or Spearman's) was used to examine the relationships among constituents of follicular fluid.

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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