REPRODUCTION
RESEARCH

Effects of pre-mating nutrition on mRNA levels of developmentally relevant genes in sheep oocytes and granulosa cells

Laura F Pisani, Stefania Antonini, Paola Pocar, Stefania Ferrari, Tiziana A L Brevini, Stewart M Rhind[1] and Fulvio Gandolfi

Laboratory of Biomedical Embryology, Department of Animal Science, University of Milan, Via Celoria 10, 20133 Milano, Italy and [1]The Macaulay Institute, Craigiebuckler, Aberdeen, AB15 8QH, UK

Correspondence should be addressed to F Gandolfi; Email: fulvio.gandolfi@unimi.it

L F Pisani and S Antonini contributed equally to this work
L F Pisani is now at Livestock Genomics 2, CERSA, Parco Tecnologico Padano, 26900 Lodi, Italy

Abstract

The present study was designed to investigate the relationship between pre-mating nutrition and the relative amounts of a panel of developmentally relevant genes in ovine oocytes and granulosa cells. Cast age ewes were fed a ration providing 0.5× (0.5 M) or 1.5× (1.5 M) live weight maintenance requirements for 2 weeks before slaughter. The ewes were synchronized and superovulated with FSH and pregnant mares serum gonadotropin. At slaughter, oocytes and granulosa cells were aspirated from follicles >2 mm in diameter and the relative abundance of 8 and 17 transcripts in oocytes and granulosa cells respectively were analyzed by semi-quantitative RT-PCR. In the oocytes, no differences between groups were observed for five transcripts (*GDF9*, *BMP15*, c-kit, glucose transporter 1 (*SLC2A1*), and hexokinase 1), but a lower amount of glucose transporter 3 (*SLC2A3*), sodium/glucose cotransporter 1 (*SLC5A1*), and Na^+/K^+ ATPase mRNAs was detected in the 0.5 M group. Increased expression of *PTGS2*, *HAS2*, and the leptin receptor long form was observed in granulosa cells from the 0.5 M group. No differences between groups were observed for the other transcripts (early growth response factor-1, estrogen receptor-α, LH and FSH receptors, gremlin 1, pentraxin 3, KIT ligand, glucose transporters 1, 3, and 8, *IGF1*, IGF1 receptor, leptin receptor, and tumor necrosis factor-stimulated gene 6). Expression of leptin and sodium/glucose cotransporter 1 was not detected in both groups. The present data indicate that pre-mating nutrition is associated with alteration in the mRNA content in oocytes and surrounding follicle cells in ewes, which may account for the reduced reproductive performance typical of ewes that are fed a restricted ration for a short period of time before mating.

Reproduction (2008) **136** 303–312

Introduction

Nutrition has a significant impact on numerous reproductive functions including hormone production, fertilization, and early embryonic development, both *in vivo* and *in vitro* (Boland *et al.* 2001, Armstrong *et al.* 2003).

Ovulation and lambing rates in sheep are positively influenced by both the level of pre-mating food intake (Gunn *et al.* 1979) and the separate, but related, factor, body condition at mating (Gunn & Doney 1979). While the effects of higher levels of body condition have been shown to be expressed, at least in part, through an increased population of large, potentially ovulatory, ovarian follicles around the time of mating (McNeilly *et al.* 1987), increased levels of food intake before mating do not result in any difference in numbers of large follicles (Rhind & McNeilly 1998). Thus, the differences in ovulation rate associated with the level of food intake must be a function of changes in ovarian activity and in the final processes of oocyte maturation.

It has been shown that reduced intake is associated with a reduced incidence of luteinizing hormone (LH) pulses during the days before ovulation (Rhind *et al.* 1985) and, in recent studies, reduced cleavage rates of oocytes were observed in ewes fed a low energy (0.5×energy requirements) diet in comparison with a high energy (1.5×energy requirements) diet (Papadopoulos *et al.* 2001, Lozano *et al.* 2003, Borowczyk *et al.* 2006). Low cleavage rate was also observed in overfed (*ad libitum* intake) ewes (Lozano *et al.* 2003, Borowczyk *et al.* 2006). Furthermore, Borowczyk *et al.* (2006) observed that the number of blastocysts and the rate of blastocyst formation were lower for underfed ewes compared with control ewes. Taken together, these data indicate that acute changes in sheep nutrition (underfeeding or feeding

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-07-0394
Online version via www.reproduction-online.org

ad libitum) during pre-mating period also result in reduced oocyte quality and embryonic development.

The capacity of an oocyte to develop into a normal embryo is acquired within the ovary during the developmental stages that precede ovulation through a process referred to as 'oocyte capacitation' (Hyttel *et al.* 1997). Although the precise mechanisms have not been fully elucidated, during capacitation, oocytes become equipped for future embryonic development, storing the information acquired during their growth and maturation for use at the appropriate stage of development (Brevini Gandolfi & Gandolfi 2001).

During the interval between fertilization and so-called maternal-embryonic transition, when transcriptional activity is switched on, embryo function is supported by maternal RNAs and proteins synthesized during oogenesis. This implies that RNA and protein molecules can be synthesized as much as several weeks before they are used. A number of strategies to optimize their storage in a quiescent form and to allow their use at the appropriate time during oocyte maturation and early embryonic development have evolved. In particular, it has been shown that oocyte quality and, ultimately, its capacity to lead to sustainable embryonic development depends on the efficiency of such storage process (Gandolfi *et al.* 2005) which, in turn, depends upon an intimate association between the somatic follicular cells and the developing germ cells (Downs & Hunzicker-Dunn 1995). Furthermore, oocyte–granulosa cell communication is bidirectional and essential for both oocyte and follicular somatic cell function and development (Eppig *et al.* 1997).

The aim of this study was to determine whether or not nutrition affects the amount of mRNA molecules stored in the ooplasm and the level of gene expression of granulosa cells. To address this, we determined the relative abundance of a panel of genes in oocytes and granulosa cells derived from underfed versus normally fed ewes. The genes analyzed in the oocytes are involved in important aspects of maturation, such as quality (growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), and c-kit) and metabolism (glucose transporters 1 and 3 (*SLC2A1* and *SLC2A3*), sodium/glucose cotransporter 1 (*SLC5A1*), Na^+/K^+ ATPase, and hexokinase 1 (*HK1*)). The genes selected for the analysis of granulosa cells are related to the endocrine status (estrogen receptor-α (*ESR1*) and LH and follicle-stimulating hormone (FSH) receptors), cell-specific activity (prostaglandin G/H synthase and cyclooxygenase (*PTGS2*), early growth response factor 1 (*EGR1*), hyaluronic acid synthase 2 (*HAS2*), gremlin 1 (*GREM1*), KIT ligand, pentraxin 3 (*PTX3*), and tumor necrosis factor-stimulated gene 6 (*TNFAIP6*)), and metabolism (insulin-like growth factor-1, IGF1 receptor, glucose transporters 1, 3, 8 (*SLC2A8*), *SLC5A1*, leptin, and leptin receptors).

Results

Animal live weights and condition scores

At the time of slaughter, animals of the low intake (L) and high intake (H) groups had mean ($\pm$S.E.M.) live weights of 52.8 ± 1.60 and 56.9 ± 3.25 kg and mean ($\pm$S.E.M.) condition scores of 2.32 ± 0.042 and 2.50 ± 0.060 respectively.

Number of follicles and superovulatory response

The dietary intake, 1.5$\times$maintenance (1.5 M) and 0.5$\times$ maintenance (0.5 M), did not influence either the total number of follicles or the number of follicles in each category we considered (2–4 and >4 mm) as summarized in Table 1.

Oocyte mRNA profiles

Transcripts for all of the eight evaluated genes were detected in oocytes from both 1.5 and 0.5 M animals. The analysis of the three genes related to oocyte quality (e.g. *GDF9*, *BMP15*, c-kit) showed no statistically significant differences between groups (Fig. 1).

Among genes related to oocyte metabolism, *SLC2A3* and *SLC5A1* mRNA levels were found to be lower in ewes fed 0.5 M compared with those fed 1.5 M ($P \leq 0.05$), but only in the oocytes isolated from follicles with a diameter greater than 4 mm. In addition, a lower amount of the Na^+/K^+ ATPase transcript was measured in the 0.5 M group, independently of follicle diameter. No differences between groups were found for the other transcripts investigated (*SLC2A1*, *HK1*, see Fig. 2).

Granulosa cells mRNA profile

No differences in gene expression were observed in relation to follicle diameter for any of the genes analyzed and so data are presented independently of follicle size.

Analysis of the expression levels of genes related to follicle endocrine function (*ESR1*, and FSH and LH receptors) showed no significant differences between groups (Fig. 3).

Table 1 Effect of dietary intake on number of follicles and their distribution by diameter.

Follicle diameter	Dietary intake	
	1.5 M[a]	0.5 M[a]
2–4 mm	9.3 ± 1.3	9.8 ± 0.9
>4 mm	9.2 ± 0.7	8.2 ± 0.6
Total follicles$\times$ovary	18.5 ± 1.2	18 ± 1.5

Data are expressed as mean$\pm$S.E.M.
[a]M, maintenance energy requirement.

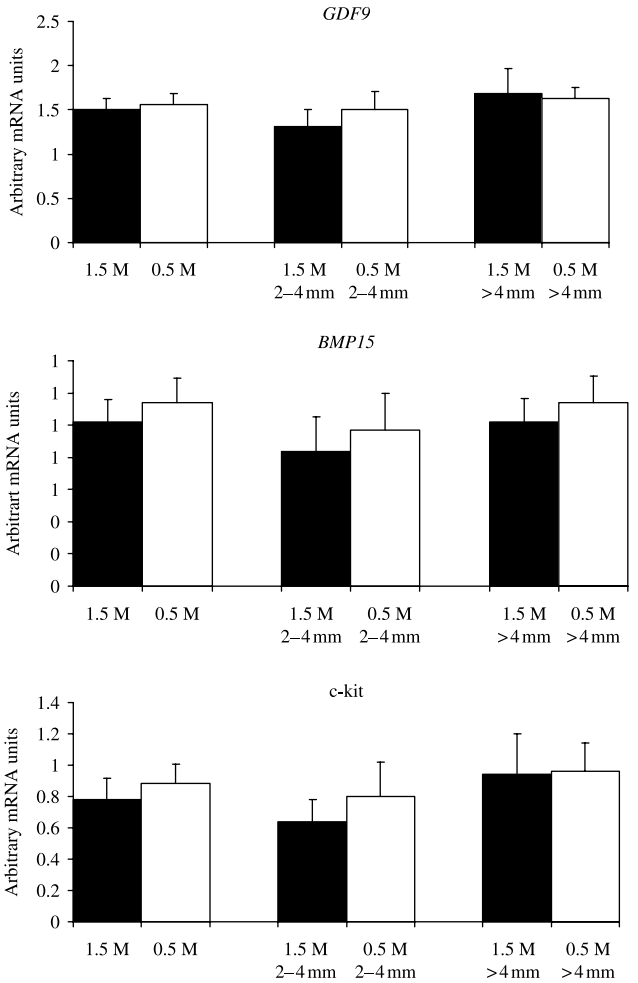

Figure 1 Effect of maternal nutrition on oocyte quality-related gene expression. Values are means ± S.E.M.

Among genes related to follicle development (*HAS2*, *EGR1*, *PTGS2*, KIT ligand, *GREM1*, *PTX3*, and *TNFAIP6*) expression of *PTGS2* in the 1.5 M group was virtually absent, whereas a consistent expression was detected in the 0.5 M group. Furthermore, an up-regulation of *HAS2* mRNA was found in ewes fed with 0.5 M group compared with the 1.5 M one. No differences were observed for any of the other genes analyzed in this group (Fig. 4).

Among genes related to follicle metabolism, an up-regulation of transcript for the leptin receptor long form was observed in the 0.5 M group compared with the 1.5 M. No significant differences were observed for the other transcripts analyzed (*SLC2A1*, *SLC2A3*, *SLC2A8*, *IGF1*, IGF1 receptor, and leptin receptor; Fig. 5). Transcripts for *SLC5A1* and leptin were not found in granulosa cells from the two groups.

Immunoblotting analysis of granulosa cells

PTGS2 protein in granulosa cells was detected as a single strong band of the expected molecular weight only

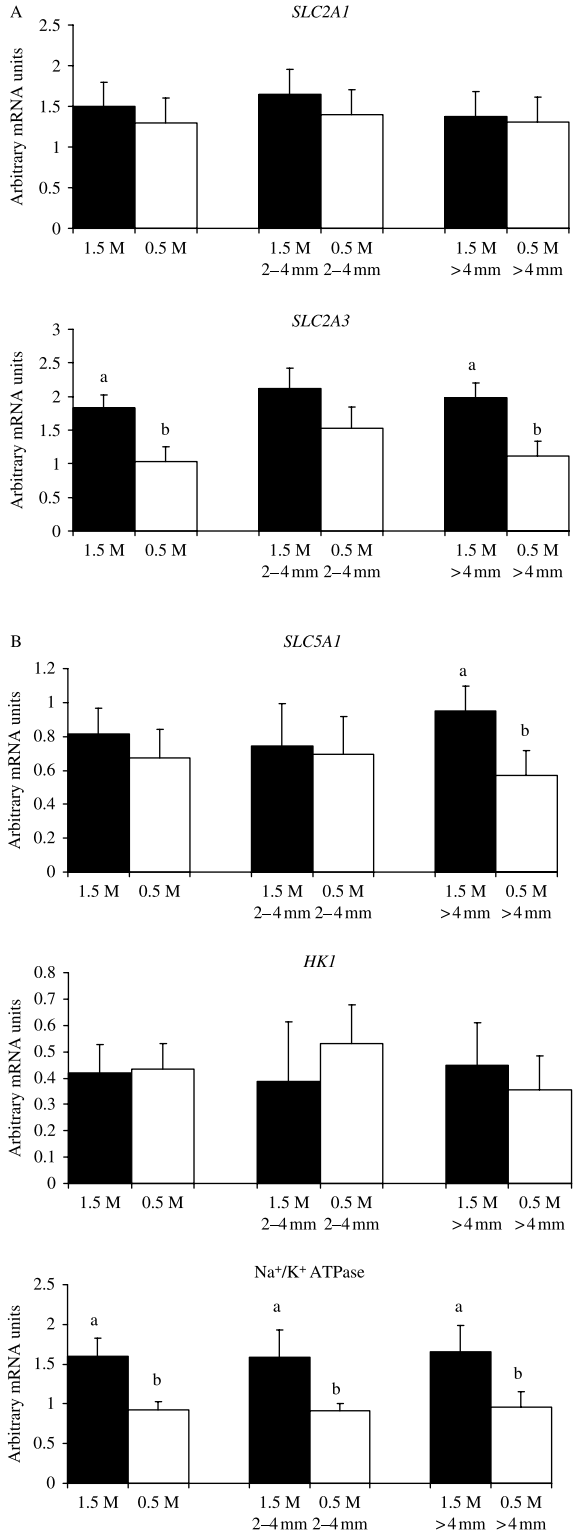

Figure 2 (A and B) Effect of maternal nutrition on oocyte metabolism-related gene expression. Values are means ± S.E.M. Different letters within columns indicate statistical difference ($P<0.05$).

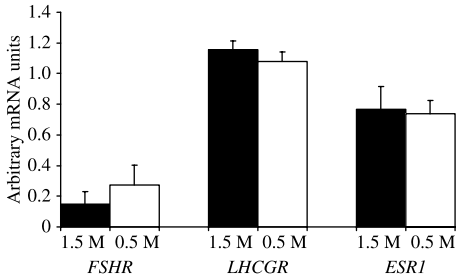

Figure 3 Effect of maternal nutrition on granulosa cells endocrine-related genes expression. Values are means±S.E.M.

among the constitutive proteins of cells isolated from ewes fed 0.5 M, whereas no specific signal was visible in the 1.5 M group (Fig. 6). This reflected exactly the data obtained analyzing *PTGS2* transcript. Unfortunately, it was not possible to perform a similar analysis on the other genes that showed differences between the experimental groups either because suitable antibodies were not commercially available, as in the case of granulosa cells, or not enough proteins were available, as in the case of oocytes.

Discussion

Pre-mating levels of food intake can influence the ovulation rate in sheep without changing the numbers of large, potentially ovulatory follicles present in the ovary (Rhind & McNeilly 1998). However, the underlying differences in gene expression and physiology that result in differential rates of follicle maturation and ovulation during the 48 h before ovulation have not been

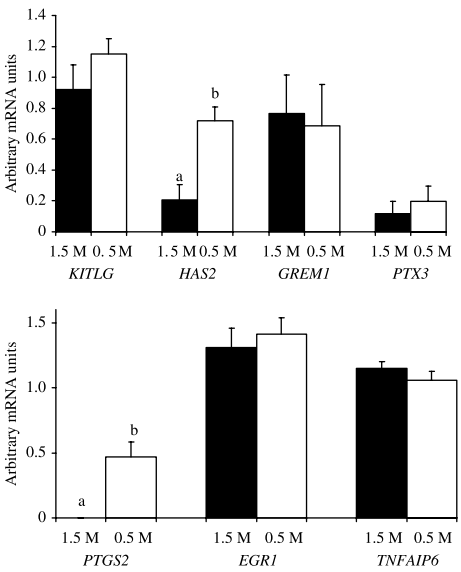

Figure 4 Effect of maternal nutrition on granulosa cells quality-related genes expression. Different letters within columns indicate statistical difference ($P<0.05$).

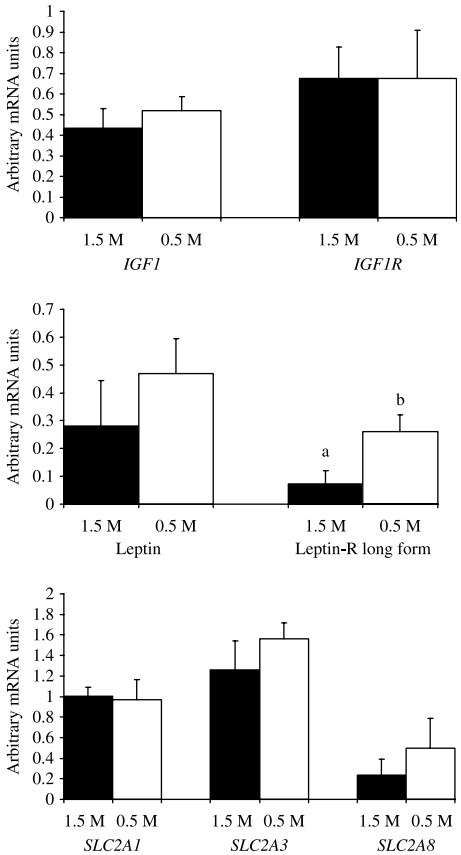

Figure 5 Effect of maternal nutrition on granulosa cells metabolism-related genes expression. Different letters within columns indicate statistical difference ($P<0.05$).

elucidated. To our knowledge, this is the first study investigating the association between the pre-mating nutritional status and the expression level of a panel of developmentally and metabolically relevant genes in the

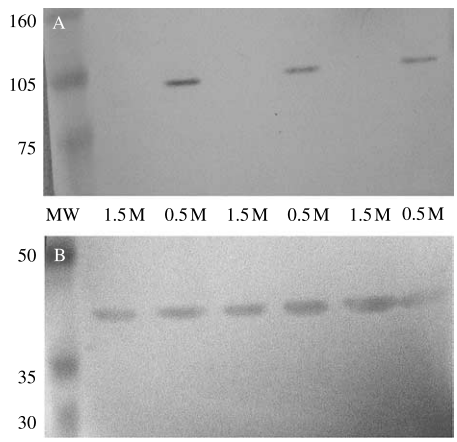

Figure 6 Effect of maternal nutrition on the presence of PTGS2 protein in granulosa cells. (A) Equal amounts of protein were loaded on each lane and probed with an antibody specific for *PTGS2*. PTGS2 protein is detected only in the 0.5 M group confirming the results obtained analyzing its transcript. (B) Loading control stained with an antibody specific for β-actin. MW, molecular weight marker.

oocyte and granulosa cells in ewes. The present data show that an acute reduction of energy intake during the pre-mating period is associated with changes in mRNA levels in both oocyte and surrounding cells.

Analysis of the expression levels of genes related to oocyte quality, such as *GDF9* (a gene that promotes the progression of folliculogenesis and oocyte development and maturation), *BMP15* (which is involved in oocyte and granulosa cell development), and c-kit (which acts in combination with its ligand to determine the growth of the follicle and the oocyte) showed no differences between groups, independently of follicle diameter. By contrast, differences in pre-mating nutritional planes influenced the expression level of some genes related to the metabolic activity of the oocyte. Specifically, our data indicate a statistical down-regulation of *SLC2A3*, *SLC5A1*, and Na^+/K^+ ATPase mRNAs in ewes fed with 0.5×live weight maintenance requirements.

SLC2A3 is one of the passive transporters expressed in pre-implantation embryos and it is involved in glucose uptake (Pantaleon *et al*. 1997, Augustin *et al*. 2001). Glucose is the main energy substrate for nuclear maturation in cattle, mice, and pigs (Downs *et al*. 1998, Downs & Utecht 1999, Hashimoto *et al*. 2000, Wongsrikeao *et al*. 2007) and a deficiency can compromise the ability of the oocyte to reach the second metaphase and to extrude the first polar body (Dominko & First 1997). The down-regulation of *SLC2A3* observed in the present study is in accordance with the reduced level of glucose transporters reported in low quality sheep oocytes (Leoni *et al*. 2007). In addition, low numbers of glucose transporters have been shown to be associated with low rates of blastocyst production in bovines (Oropeza *et al*. 2004) and have been postulated to affect embryo compaction and blastulation (Thompson 2000, Block *et al*. 2007). It is also noteworthy that, in contrast to what has been observed in mice and in humans, where the expression of this glucose transporter begins only at the activation of the embryo genome (Dan-Goor *et al*. 1997, Pantaleon *et al*. 1997), in cattle (Augustin *et al*. 2001) and the present study, *SLC2A3* is already expressed in immature oocytes. This suggests that SLC2A3 has an important role in early glucose metabolism in these species.

Na^+/K^+ ATPase is involved in the regulation of cell volume and the transport of glucides and amino acids into cells. Reduced production of Na^+/K^+ ATPase could impact adversely on oocyte and embryo metabolism. Our study demonstrates that an acute reduction in food intake induces a down-regulation of Na^+/K^+ ATPase transcript in oocytes; this could account for a reduced level of developmental competence. In fact, this enzyme has been reported to be more abundant in oocytes selected for *in vitro* maturation compared with discarded ones (De Sousa *et al*. 1998), and it is also known that antisense oligonucleotide specific for the Na^+/K^+ ATPase inhibits blastocysts formation in cattle (Watson *et al*. 1999). In addition, levels of Na^+/K^+ ATPase transcript in low competence sheep oocytes are lower than that in high competence ones (Leoni *et al*. 2007).

The detection, in the 0.5 M group ewes, of lower levels of mRNAs coding for three out of five genes related to oocyte metabolic function suggests that under-nutrition alters oocyte metabolism. It is possible that such changes in transcript levels at this early stage of development could contribute not only to short-term effects on reproductive function but also to long-term reductions in adult reproductive performance (Gunn *et al*. 1995, Rhind *et al*. 1998).

In the present study, the expression level of genes related to granulosa cell endocrine functionality (FSH and LH receptors, and estrogen receptor-α) was not affected by the energy intake levels. This is partially in contrast to the findings of Lozano *et al*. (2003) who observed that a hypocaloric diet did not affect pituitary FSH levels, but could reduce LH levels. However, this discrepancy could be attributable to the superovulation treatment used in our experiments. Since exogenous gonadotropins cause an increased expression of FSH and LH receptors (LaPolt *et al*. 1990, 1992), it is possible that superovulation overrode any underlying differences in hormone receptor gene expression induced by the dietary intake. The possibility of effects on oocyte quality of administration of exogenous hormones has been postulated, frequently, but at present it remains conjecture. Although changes in the methylation status of several imprinted genes have been reported in oocytes from stimulated cycles in both the mouse and the human in the latter, the effects of superovulation could not be distinguished from those of donor age and fertility (Sato *et al*. 2007), indicating that additional, unquantified effects may be involved. While the biological significance of these findings remains unclear, it is possible that the endocrine milieu around the time of conception may affect the outcome of pregnancy (Sinclair 2008). However, within our experimental design, ovarian stimulation was essential in order to obtain enough biological material without incurring the high costs associated with using very large numbers of individual animals. Two genes involved in follicle growth and cumulus expansion, *PTGS2* and *HAS2*, showed a significant increase in transcript levels in the 0.5 M group, relative to the 1.5 M group animals. This suggests that the effect of nutrition on these genes is specific since all the other genes involved in follicular development were not influenced by the diet. Moreover, we could confirm that changes of *PTGS2* mRNA levels were reflected in the parallel changes of its protein.

PTGS2 is an inducible enzyme essential for follicle prostaglandin production, which is expressed according to a precise time scale during follicular development. In physiological conditions, PTGS2 is expressed by secondary and pre-antral follicles, but it is absent in primary and antral follicles (Tokuyama *et al*. 2003). Diet restriction

causes a reduction in availability of lipids (Ye *et al*. 2005) for prostaglandins synthesis and so the maintenance of PTGS2 expression in antral follicles isolated from sheep subjected to diet restriction may represent a compensatory mechanism for suboptimal amounts of intracellular lipids. At present, it is unclear how diet regulates *PTGS2* expression since the expression levels of *EGR1* and *TNFAIP6* were unaffected even if both genes can up-regulate *PTGS2* expression (Richards 2005, Sayasith *et al*. 2006). *HAS2* is normally expressed in the early follicle stages and expression decreases thereafter, followed by a rapid increase some hours before ovulation (Fulop *et al*. 1997, Salustri *et al*. 2004). It regulates the formation of a composite extracellular matrix by the cumulus–oocyte complex (Chen *et al*. 1990, Camaioni *et al*. 1993), a process critical to ovulation and cumulus expansion. The higher level of *HAS2* transcripts measured in the 0.5 M group suggests an anomaly in extracellular matrix deposition, possibly owing to an alteration in the timing of follicular gene expression, which may impair the dynamics of oocyte maturation and the subsequent ovulation process.

Both the leptin receptor and its long form were detected in the granulosa cells of all follicles but only the latter showed a significant up-regulation in the 0.5 M group. Leptin is a protein hormone synthesized by the adipose tissue and transported to the ovaries where it regulates glucose uptake and induces LH secretion, thereby linking nutritional state and reproductive function (Bucholtz *et al*. 1996, Nagatani *et al*. 1996). In the present study, no leptin expression was observed in any of the groups analyzed, a finding in broad agreement with the study of Munoz-Gutierrez *et al*. (2005) who described a weak expression of leptin only in a small proportion of antral follicles in the sheep, confirming that leptin is mainly produced by adipose tissue and delivered to the ovary through the blood stream.

The level of leptin expression in the hypothalamus increases in well-fed ewes (Dyer *et al*. 1997), and a high concentration of circulating leptin causes a reduction in the expression of its receptors (Ohtani *et al*. 2001). On the other hand, nutritional restrictions reduce the blood levels of leptin, which induces an increase of appetite and stimulates the search of food (Chilliard *et al*. 2005). Taken together, these data suggest that the increase in receptor expression in the underfed ewes may reflect a decrease in circulating leptin with possible consequences for follicle maturation and ovulation. In fact, the leptin/leptin receptor complex represents the main connection between nutrition and hormonal secretion by the hypothalamus–hypophysis–gonad axis (Elmquist *et al*. 1998, Sawchenko 1998), since a reduction in leptin secretion causes a reduction in LH and a consequent delay in estrus cycle (Stanley *et al*. 2000). Furthermore, the leptin receptor long form is specifically involved in this process, influencing GNRH secretion and the following LH outflow (Barb *et al*. 2005, Kaminski *et al*. 2006).

In conclusion, our results show that short-term food restriction alters the levels of a few specific transcripts in both oocyte and granulosa cells. Our data indicate that genes involved in oocyte metabolic activity are affected, specifically, and that this phenomenon is accompanied by the deregulation of the correct dynamic of gene expression in granulosa cells. The present study describes the effect of total dietary intake but the future investigations that address specific dietary components should provide further insight into the underlying mechanisms associated with changes in reproductive performance in ewes.

Materials and Methods

Animals and treatments

The study was conducted using 16 cast age ewes of proven fertility. They were allocated randomly to two groups with initial mean (±S.E.M.) live weights of 56.1±1.89 kg (low intake, $n=10$) and 56.0±2.80 kg (high intake, $n=6$) and mean body condition scores (Russel *et al*. 1969) of 2.37±0.034 and 2.33±0.064 respectively, at the start of the study. During the 2-week period before slaughter, low intake animals were fed pelleted rations (Ewe pellets; Harbro Ltd, Turriff, UK) and chopped straw at rates that provided 0.5×live weight maintenance requirements (0.5 M) and high intake animals were offered the same feeds in amounts that provided 1.5×live weight maintenance requirements (1.5 M), a level of intake consistent with normal, pre-mating levels of nutrition.

In order to obtain enough material to perform the study with the available number of animals, it was necessary to superovulate them. The estrous cycles of all ewes were synchronized, to control the time of ovulation, by the insertion of intravaginal pessaries containing 30 mg progestagen (Intervet, Cambridge, UK) for 14 days before slaughter. During the days before slaughter, the following protocol was applied: day 3, Ovagen (oFSH, Synergy Products, Melksham, UK), i.m. 1.05 mg at 0800 h+1.05 mg at 2000 h; day 2, Ovagen, 1.05 mg at 0800 h+1.05 mg at 2000 h; day 1, Ovagen, 1.05 mg at 0800 h+1.05 mg Ovagen +400 i.u. pregnant mares serum gonadotropin (PMSG; 2 ml, Intervet) i.m. at 2000 h; and day 0 remove sponge.

Ovary handling and oocyte and granulosa cell recovery

Reproductive tracts were collected at slaughter and stored in PBS at ~37 °C during transport to the laboratory. Ovaries were removed and washed once with PBS. The number and diameter of visible follicles were determined. The follicles were individually punctured and follicular fluid was released in a Petri dish. Cumulus oocyte complexes (COCs) were isolated and divided into two groups split according to follicle diameter (2–4 or >4 mm). Under a stereomicroscope, COCs were recovered from each dish and transferred to a Petri dish containing washing mediums TCM 199. For every COC, oocytes were denuded with 2.9% citric acid and collected in two pools from each sheep, based on follicle diameter. The

oocytes were washed in washing medium TCM 199 and stored in RNAlater, RNA Stabilization Reagent (Qiagen SpA) until use. Granulosa cells obtained at oocyte retrieval were collected by centrifugation and stored in RNAlater as above.

RNA isolation

Oocytes of each follicle category of each sheep were pooled for poly(A)+RNA extraction using the Dynabeads mRNA DIRECT Micro-kit (Invitrogen S.r.l) according to the manufacturer's instructions. Briefly, pools were lysed for 10 min at room temperature in 200 µl lysis buffer (100 mmol Tris–HCl (pH 8.0), 500 mmol LiCl, 10 mmol EDTA, 1% (w/v) SDS, and 5 mmol dithiothreitol). After lysis, 10 µl pre-washed Dynabeads oligo$(dT)_{25}$ were pipetted into the tube and binding of poly$(A)^+$RNAs to oligo(dT) was allowed for 5 min at room temperature. The beads were then separated with a magnetic separator, washed twice with 30 µl washing buffer (10 mmol Tris–HCl (pH 8.0), 0.15 mmol LiCl, 1 mmol EDTA, and 0.1% (w/v) SDS) and thrice with 30 µl washing buffer (10 mmol Tris–HCl (pH 8.0), 0.15 mmol LiCl, and 1 mmol EDTA). Poly$(A)^+$RNAs were then eluted from the beads by incubation in 11 µl DEPC-treated sterile water at 65 °C for 2 min. Aliquots were immediately used for RT. Prior to RNA isolation, 1 µl exogenous mRNA of rabbit globin (6.25 pg/µl) RNA was added to each oocyte sample as a standard for semi-quantitative analysis of gene expression.

Total RNA was extracted from granulosa cells using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was dissolved in 20 µl DEPC–H_2O, and the concentration and purity were judged by measuring the absorbance at 260 nm and calculating the ratio of absorbance at 260–280 nm using a u.v. spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories S.r.l).

RT

RNA from each oocytes pool and granulosa cells replicate (1 µg total RNA) were reverse transcribed into cDNA in a total volume of 20 µl reaction mixture containing 8.5 µl sterile water, 1 µl of 10 mM dNTP mix, and 1 µl oligo$(dT)_{12–18}$ (500 ng/µl). RNA was denatured at 65 °C for 5 min, and then 4 µl of 5× first-strand buffer (250 mM Tris–HCl (pH 8.3), 375 mM KCl, and 15 mM $MgCl_2$), 1.5 µl of 50 mM $MgCl_2$, 2 µl of 0.1 M dithiothreitol, and 1 µl of RNaseOUT recombinant RNase inhibitor (Invitrogen, S.r.l, 40 U/µl) were added. RT was performed with 200 U Superscript II reverse transcriptase (Invitrogen, S.r.l) for 1 h at 42 °C. Enzymes were inactivated at 70 °C for 15 min.

Semi-quantitative PCR

An aliquot of each RT product was subjected to gene-specific PCR (PCR, Table 2) in an automated thermal cycler (iCycler, Bio-Rad Laboratories S.r.l). PCR was performed with cDNA equivalents corresponding to 0.25 oocyte or 100 ng total RNA for granulosa cells. The reaction mix consisted of 0.8 µl of 50 mM $MgCl_2$, 0.3 µl Taq DNA polymerase (5 U/µl), 2 µl of 10× PCR buffer (200 mM Tris–HCl (pH 8.4), 500 mM KCl),

Table 2 Primer pairs used for PCR amplifications of cDNA.

Gene	Forward 5′–3′	Reverse 5′–3′	Annealing temperature (°C)	PCR cycles no.	Product length (bp)	GenBank accession no.
Globin	GCAGCCACGGTGGCGAGTAT	GTGGGCAGGAGCTTGAAAT	60	35	259	V00875
β-actin	CCAAGGCCAACCGTGAGAAG	CCATCTCCTGCTTCGAAGTCC	57	26	350	U39357
BMP15	TCTATTGCCCACCTGCCTGAG	TGAAGCTGATGGCGGTAAACC	59	30	322	AY885263
GDF9	TAGTCAGCTGAAGTGGGACA	AGCCATCAGGCTCGATGGCC	55	30	400	DQ304681
c-kit	AGGCATATCCCAAACCTGAACACC	CAACAGGAACAGAACACCTCTGCT	60	32	351	D45168
SLC2A1	TCGTCGTCGGCATCCTCATC	TCCACCACAAACAGCGAAACG	60	32	496	U89029
SLC2A3	AGGAGGAGGAGAAGGCAAAGG	AAGGCCACAAAGACCAGGTG	58	33	478	NM001009770
SLC2A8	ATGGCTGCCATGCAGTTC	TTTGGTCTCAGGGACACAG	56	34	702	AF495799
SLC5A1	TCGCAGGACGGTTGTTCATG	CGCTCCTCTTTGCTGTTACGC	58	33	439	X82411
Na^+/K^+ATPase	ACCTGTTGGGCATCCGAGAGA	AGGGGAAGGCACAGAACCACCA	60	35	335	NM012504
HK1	TGCGGCTCTCTGATGAAACT	TCCAGGGCGATGAAATCTCC	57	31	166	AF542053
KIT ligand	CGTGTGACTGATGATGTGAAAG	GCTACTGCTGTCATTCCTAAGG	53	34	507	Z50743
PTGS2	AGGTGTATGTATGAGTGTAGGA	GTGCTGGGCAAAGAATGCAA	54	32	483	U68486
HAS2	TTTACAATACTCCTGGGTGGTG	ACAATGCCGTACAGTCCCTAG	54	32	274	DY517053
GREM1	AAGGCCCAGCACAATGACTC	AACGACACTGCTTCACACGC	59	33	421	AY942576
PTX3	GAGAACTCCGAGTGGACAAGC	CTGCACAGATGGGTCCATGTTC	58	32	442	EE829599
IGF1	CCTCGCATCTCTTCTATCTGGC	CACTCCCTCTGCTTGTGTTCTTC	56	30	351	NM001009774
IGF1R	AGCGCCTCTAACTTTGTCTTTG	CCCATTCCCAGAGAGAGAGG	56	30	297	AY162434
Leptin	GAACTGTTCCTGGGCACAAG	TTTCAAGGTCCGGAGTTGTC	55	36	215	AF102856
Leptin R	AGGTGAGGTGCAAGAGATTGGA	CGGGGTTTGGTTGCATTCAAG	55	33	286	NM001009763
Leptin R long form	TCCAAACCCCAAGAACTGTTCC	TCCTGCTCTCATCCTCACAGGTTA	55	35	315	AB199589
FSHR	AATGTCCTGGCCTTTGTGGTC	GGTCTGGGCTTGCACTTCATAG	57	30	348	L07302
LHCGR	GGGAATGGGTGTAGTGTTGCTG	GCAGCTGAGATGGCAAAGAAAG	57	32	473	L36329
ESR1	CCATGGAGCATCCAGGGAAG	AGAGGCACCACGTTCTTGCAC	60	28	423	AY033393
EGR1	CCGACTATCTGTTTCCACAAC	TGAGCTCATCTGAGCGAGAG	58.5	30	309	AY924307
TNFAIP6	TACAAGCAGCTAGAGGCAGCC	CTTCAAGGTCATGACATTTCCTG	58	26	483	AY919871

1 μl of 10 mM dNTPs, 1 μl (25 pmol) of each sequence-specific primer, and sterile water up to 20 μl.

For each set of primers, the optimal cycle number at which the transcript was amplified exponentially was established running a linear cycle series, and the number of PCR cycles was kept within this range (Table 2).

An aliquot of each cDNA sample was amplified by PCR with rabbit globin and β-actin gene-specific primers, for oocytes and granulosa cells respectively.

RT-PCR products were subjected to electrophoresis on a 2% agarose gel in 1× TAE buffer (40 mM Tris–acetate and 1 mM EDTA) containing 0.5 μg/ml ethidium bromide. After electrophoresis at 80 V for 45 min, the fragments were visualized on a 312 nm u.v. transilluminator. The image of each gel was recorded using a Kodak digital camera (DC290, Sigma–Aldrich). The intensity of each band was assessed by densitometric analysis performed with the Labimage software (Kapelan GmbH, Halle, Germany).

The quantification procedure based on direct digitalization of the PCR product after separation on agarose gel provides a well-established and sensitive method to detect even small differences in amounts of mRNA from different biological samples (Grover *et al.* 2001, Ringhoffer *et al.* 2001). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each gene of interest by the intensity of the globin and β-actin band for oocyte and granulosa cells respectively, as described previously (Wrenzycki *et al.* 2002, Brevini *et al.* 2004).

To account for gel to gel variations, each band was normalized against the 350 bp band of a 50 bp ladder marker (Invitrogen cat n. 10416-014, Stenman *et al.* 1999).

Protein extraction, gel electrophoresis, Western blotting, and immunostaining

Cells were homogenized, lysed, and constitutive proteins were extracted. Protein concentration was assessed by the Coomassie Blu-G Dye-binding methods (Read & Northcote 1981). Aliquots of 100 μg were prepared and resuspended in sample buffer consisting of 10% (w/v) glycerol, 2.3%(w/v) SDS, and 6.25 M Tris–HCl (pH 6.8) and electrophoresed on a 10% SDS-polyacrylamide slab gel (Laemmli 1970). Proteins were then transferred onto nitrocellulose filters according to (Towbin *et al.* 1979), using 0.5 A/cm^2. Equal sample loading and transfer efficiency were confirmed by staining of the membrane with Ponceau Red. The membrane was probed with a specific rabbit polyclonal antibody (diluted in the ratio of 1:500) raised against PTGS2 (ab15839; Abcam, Cambridge, UK) and with a MAB specific for β-actin (Sigma, A5441) as a loading control. The specific IgG biotin conjugates (Calbiochem, San Diego, CA, USA) were used as secondary antibodies, and the presence of immunoreactivity for the molecules of interest was visualized using Auroprobe BL plus streptavidin/Silver enhancement Reagent IntenSE BL (Amersham, GE Health Care).

Statistical analysis

Statistical analysis was performed using the unpaired *t*-test or the Mann–Whitney rank sum test, as appropriate, using the Sigma Stat statistical package (Systat Software Inc., San Jose, CA, USA). Data are presented as mean percentages (±S.E.M.) of a minimum of three independent animals. In all cases, differences of $P \leq 0.05$ were considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

This work was supported by the University of Milan FIRST 2006 and the Scottish Executive Environment and Rural Affairs Department.

Acknowledgements

The authors are grateful to Valentina Tosetti for her help with the collection of the experimental material.

References

Armstrong DG, Gong JG & Webb R 2003 Interactions between nutrition and ovarian activity in cattle: physiological, cellular and molecular mechanisms. *Reproduction Supplement* **61** 403–414.

Augustin R, Pocar P, Navarrete-Santos A, Wrenzycki C, Gandolfi F, Niemann H & Fischer B 2001 Glucose transporter expression is developmentally regulated in *in vitro* derived bovine preimplantation embryos. *Molecular Reproduction and Development* **60** 370–376.

Barb CR, Hausman GJ & Czaja K 2005 Leptin: a metabolic signal affecting central regulation of reproduction in the pig. *Domestic Animal Endocrinology* **29** 186–192.

Block J, Fischer-Brown AE, Rodina TM, Ealy AD & Hansen PJ 2007 The effect of *in vitro* treatment of bovine embryos with IGF1 on subsequent development *in utero* to day 14 of gestation. *Theriogenology* **68** 153–161.

Boland MP, Lonergan P & O'Callaghan D 2001 Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. *Theriogenology* **55** 1323–1340.

Borowczyk E, Caton JS, Redmer DA, Bilski JJ, Weigl RM, Vonnahme KA, Borowicz PP, Kirsch JD, Kraft KC, Reynolds LP *et al.* 2006 Effects of plane of nutrition on *in vitro* fertilization and early embryonic development in sheep. *Theriogenology* **84** 1593–1599.

Brevini TA, Cillo F, Colleoni S, Lazzari G, Galli C & Gandolfi F 2004 Expression pattern of the maternal factor zygote arrest 1 (Zar1) in bovine tissues, oocytes, and embryos. *Molecular Reproduction and Development* **69** 375–380.

Brevini Gandolfi TAL & Gandolfi F 2001 The maternal legacy to the embryo: cytoplasmic components and their effects on early development. *Theriogenology* **55** 1255–1276.

Bucholtz DC, Vidwans NM, Herbosa CG, Schillo KK & Foster DL 1996 Metabolic interfaces between growth and reproduction. V. Pulsatile luteinizing hormone secretion is dependent on glucose availability. *Endocrinology* **137** 601–607.

Camaioni A, Hascall VC, Yanagishita M & Salustri A 1993 Effects of exogenous hyaluronic acid and serum on matrix organization and stability in the mouse cumulus cell–oocyte complex. *Journal of Biological Chemistry* **268** 20473–20481.

Chen L, Wert SE, Hendrix EM, Russell PT, Cannon M & Larsen WJ 1990 Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. *Molecular Reproduction and Development* **26** 236–247.

Chilliard Y, Delavaud C & Bonnet M 2005 Leptin expression in ruminants: nutritional and physiological regulations in relation with energy metabolism. *Domestic Animal Endocrinology* **29** 3–22.

Dan-Goor M, Sasson S, Davarashvili A & Almagor M 1997 Expression of glucose transporter and glucose uptake in human oocytes and preimplantation embryos. *Human Reproduction* **12** 2508–2510.

Dominko T & First NL 1997 Timing of meiotic progression in bovine oocytes and its effect on early embryo development. *Molecular Reproduction and Development* **47** 456–467.

Downs SM & Hunzicker-Dunn M 1995 Differential regulation of oocyte maturation and cumulus expansion in the mouse oocyte–cumulus cell complex by site-selective analogs of cyclic adenosine monophosphate. *Developmental Biology* **172** 72–85.

Downs SM & Utecht AM 1999 Metabolism of radiolabeled glucose by mouse oocytes and oocyte–cumulus cell complexes. *Biology of Reproduction* **60** 1446–1452.

Downs SM, Humpherson PG & Leese HJ 1998 Meiotic induction in cumulus cell-enclosed mouse oocytes: involvement of the pentose phosphate pathway. *Biology of Reproduction* **58** 1084–1094.

Dyer CJ, Simmons JM, Matteri RL & Keisler DH 1997 Leptin receptor mRNA is expressed in ewe anterior pituitary and adipose tissues and is differentially expressed in hypothalamic regions of well-fed and feed-restricted ewes. *Domestic Animal Endocrinology* **14** 119–128.

Elmquist JK, Bjorbaek C, Ahima RS, Flier JS & Saper CB 1998 Distributions of leptin receptor mRNA isoforms in the rat brain. *Journal of Comparative Neurology* **395** 535–547.

Eppig JJ, Chesnel F, Hirao Y, O'Brien MJ, Pendola FL, Watanabe S & Wigglesworth K 1997 Oocyte control of granulosa cell development: how and why. *Human Reproduction* **12** 127–132.

Fulop C, Salustri A & Hascall VC 1997 Coding sequence of a hyaluronan synthase homologue expressed during expansion of the mouse cumulus–oocyte complex. *Archives of Biochemistry and Biophysics* **337** 261–266.

Gandolfi F, Brevini TA, Cillo F & Antonini S 2005 Cellular and molecular mechanisms regulating oocyte quality and the relevance for farm animal reproductive efficiency. *Revue Scientifique et Technique* **24** 413–423.

Grover PK, Stapleton AM, Miyazawa K & Ryall RL 2001 Simple, sensitive and accurate method for the quantification of prothrombin mRNA by using competitive PCR. *Biochemical Journal* **356** 111–120.

Gunn RG & Doney JM 1979 Fertility in cheviot ewes. 1. The effect of body condition at mating on ovulation rate and early embryo mortality in North Country and South Country Cheviot ewes. *Animal Production* **29** 11–16.

Gunn RG, Doney JM & Smith WF 1979 Fertility in cheviot ewes. 2. The effect of level of premating nutrition on ovulation rate and early embryo mortality in North Country and South Country Cheviot ewes in moderately-good condition at mating. *Animal Production* **29** 17–23.

Gunn RG, Sim D & Hunter EA 1995 Effects of nutrition *in utero* and in early life on the subsequent lifetime reproductive performance of Scottish Blackface ewes in two management systems. *Animal Science* **60** 223–230.

Hashimoto S, Minami N, Yamada M & Imai H 2000 Excessive concentration of glucose during *in vitro* maturation impairs the developmental competence of bovine oocytes after *in vitro* fertilization: relevance to intracellular reactive oxygen species and glutathione contents. *Molecular Reproduction and Development* **56** 520–526.

Hyttel P, Fair T, Callesen H & Greve T 1997 Oocyte growth, capacitation and final maturation in cattle. *Theriogenology* **47** 23–32.

Kaminski T, Smolinska N, Gajewska A, Siawrys G, Okrasa S, Kochman K & Przala J 2006 Leptin and long form of leptin receptor genes expression in the hypothalamus and pituitary during the luteal phase and early pregnancy in pigs. *Journal of Physiology and Pharmacology* **57** 95–108.

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227** 680–685.

LaPolt PS, Oikawa M, Jia XC, Dargan C & Hsueh AJ 1990 Gonadotropin-induced up- and down-regulation of rat ovarian LH receptor message levels during follicular growth, ovulation and luteinization. *Endocrinology* **126** 3277–3279.

LaPolt PS, Tilly JL, Aihara T, Nishimori K & Hsueh AJ 1992 Gonadotropin-induced up- and down-regulation of ovarian follicle-stimulating hormone (FSH) receptor gene expression in immature rats: effects of pregnant mare's serum gonadotropin, human chorionic gonadotropin, and recombinant FSH. *Endocrinology* **130** 1289–1295.

Leoni GG, Bebbere D, Succu S, Berlinguer F, Mossa F, Galioto M, Bogliolo L, Ledda S & Naitana S 2007 Relations between relative mRNA abundance and developmental competence of ovine oocytes. *Molecular Reproduction and Development* **74** 249–257.

Lozano JM, Lonergan P, Boland MP & O'Callaghan D 2003 Influence of nutrition on the effectiveness of superovulation programmes in ewes: effect on oocyte quality and post-fertilization development. *Reproduction* **125** 543–553.

McNeilly AS, Jonassen JA & Rhind SM 1987 Reduced ovarian follicular development as a consequence of poor body condition in ewes. *Acta Endocrinologica* **115** 75–83.

Munoz-Gutierrez M, Findlay PA, Adam CL, Wax G, Campbell BK, Kendall NR, Khalid M, Forsberg M & Scaramuzzi RJ 2005 The ovarian expression of mRNAs for aromatase, IGF-I receptor, IGF-binding protein-2, -4 and -5, leptin and leptin receptor in cycling ewes after three days of leptin infusion. *Reproduction* **130** 869–881.

Nagatani S, Bucholtz DC, Murahashi K, Estacio MA, Tsukamura H, Foster DL & Maeda KI 1996 Reduction of glucose availability suppresses pulsatile luteinizing hormone release in female and male rats. *Endocrinology* **137** 1166–1170.

Ohtani K, Sakamoto H, Kikuchi A, Nakayama Y, Idei T, Igarashi N, Matukawa T & Satoh K 2001 Follicle-stimulating hormone promotes the growth of human epithelial ovarian cancer cells through the protein kinase C-mediated system. *Cancer Letters* **166** 207–213.

Oropeza A, Wrenzycki C, Herrmann D, Hadeler KG & Niemann H 2004 Improvement of the developmental capacity of oocytes from prepubertal cattle by intraovarian insulin-like growth factor-I application. *Biology of Reproduction* **70** 1634–1643.

Pantaleon M, Harvey MB, Pascoe WS, James DE & Kaye PL 1997 Glucose transporter GLUT3: ontogeny, targeting, and role in the mouse blastocyst. *PNAS* **94** 3795–3800.

Papadopoulos S, Lonergan P, Gath V, Quinn KM, Evans AC, O'Callaghan D & Bolan MP 2001 Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. *Theriogenology* **55** 1059–1069.

Read SM & Northcote DH 1981 Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Analytical Biochemistry* **116** 53–64.

Rhind SM & McNeilly AS 1998 Effects of level of food intake on ovarian follicle number, size and steroidogenic capacity in the ewe. *Animal Reproduction Science* **52** 131–138.

Rhind SM, Leslie ID, Gunn RG & Doney JM 1985 Plasma FSH, LH, prolactin and progesterone profiles of Cheviot ewes with different levels of intake before and after mating and associated effects on reproductive performance. *Animal Reproduction Science* **8** 301–313.

Rhind SM, Elston DA, Jones JR, Rees JR, McMillen SR & Gunn RG 1998 Effects of restriction of growth and development of Brecon Cheviot ewelambs on subsequent lifetime reproductive performance. *Small Ruminant Research* **30** 121–126.

Richards JS 2005 Ovulation: new factors that prepare the oocyte for fertilization. *Molecular and Cellular Endocrinology* **234** 75.

Ringhoffer M, Schmitt M, Karbach J, Jager E, Oesch F & Arand M 2001 Quantitative assessment of the expression of melanoma-associated antigens by non-competitive reverse transcription polymerase chain reaction. *International Journal of Oncology* **19** 983–989.

Russel AJF, Doney JM & Gunn RG 1969 Subjective assessment of body fat in live sheep. *Journal of Agricultural Science* **72** 451–454.

Salustri A, Garlanda C, Hirsch E, De Acetis M, Maccagno A, Bottazzi B, Doni A, Bastone A, Mantovani G, Beck Peccoz P *et al.* 2004 PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in *in vivo* fertilization. *Development* **131** 1577–1586.

Sato A, Otsu E, Negishi H, Utsunomiya T & Arima T 2007 Aberrant DNA methylation of imprinted loci in superovulated oocytes. *Human Reproduction* **22** 26–35.

Sawchenko PE 1998 Toward a new neurobiology of energy balance, appetite, and obesity: the anatomists weigh in. *Journal of Comparative Neurology* **402** 435–441.

Sayasith K, Brown KA, Lussier JG, Dore M & Sirois J 2006 Characterization of bovine early growth response factor-1 and its gonadotropin-dependent regulation in ovarian follicles prior to ovulation. *Journal of Molecular Endocrinology* **37** 239–250.

Sinclair KD 2008 Assisted reproductive technologies and pregnancy outcomes: mechanistic insights from animal studies. *Seminars in Reproductive Medicine* **26** 153–161.

De Sousa PA, Westhusin ME & Watson AJ 1998 Analysis of variation in relative mRNA abundance for specific gene transcripts in single bovine oocytes and early embryos. *Molecular Reproduction and Development* **49** 119–130.

Stanley SA, Todd JF, Small CJ, Kim MS, Heath MM, Anand P, Ghatei MA & Bloom SR 2000 The effects of ciliary neurotrophic factor on the hypothalamo–pituitary gonadal axis *in vitro* in female rats. *Journal of Neuroendocrinology* **12** 1009–1013.

Stenman J, Finne P, Stahls A, Grenman R, Stenman UH, Palotie A & Orpana A 1999 Accurate determination of relative messenger RNA levels by RT-PCR. *Nature Biotechnology* **17** 720–722.

Thompson JG 2000 *In vitro* culture and embryo metabolism of cattle and sheep embryos – a decade of achievement. *Animal Reproduction Science* **60–61** 263–275.

Tokuyama O, Nakamura Y, Musoh A, Honda K, Ozaki K & Ishiko O 2003 Expression and distribution of cyclooxygenase-2 in human ovary during follicular development. *Osaka City Medical Journal* **49** 39–47.

Towbin H, Staehelin T & Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *PNAS* **76** 4350–4354.

Watson AJ, Westhusin ME, De Sousa PA, Betts DH & Barcroft LC 1999 Gene expression regulating blastocyst formation. *Theriogenology* **51** 117–133.

Wongsrikeao P, Nagai T, Agung B, Taniguchi M, Kunishi M, Suto S & Otoi T 2007 Improvement of transgenic cloning efficiencies by culturing recipient oocytes and donor cells with antioxidant vitamins in cattle. *Molecular Reproduction and Development* **74** 694–702.

Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K & Niemann H 2002 *In vitro* production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. *Biology of Reproduction* **66** 127–134.

Ye X, Hama K, Contos JJ, Anliker B, Inoue A, Skinner MK, Suzuki H, Amano T, Kennedy G, Arai H *et al.* 2005 LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature* **435** 104–108.

Received 31 August 2007
First decision 10 October 2007
Revised manuscript received 16 May 2008
Accepted 28 May 2008