The effect of maternal undernutrition on the placental growth trajectory and the uterine insulin-like growth factor axis in the pregnant ewe

J C Osgerby, D C Wathes, D Howard and T S Gadd

Reproduction and Development Group, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Herts AL9 7TA, United Kingdom

(Requests for offprints should be addressed to D C Wathes; Email: dcwathes@rvc.ac.uk)

Abstract

The placenta is a highly efficient multifunctional organ, mediating the exchange of nutrients, gases and waste products between the dam and fetus. This study investigated the effects of chronic maternal undernutrition (70% of estimated requirement) on the placental growth trajectory in the ewe on days 45, 90 and 135 of gestation. The insulin-like growth factor (IGF) system was investigated using *in situ* hybridisation analysis to determine if nutritionally mediated alterations in placental growth were regulated through modifications in placental IGF expression. Placental weight increased between days 45 and 90 ($P < 0.01$), accompanied by a reduction in maternal placentome IGF binding protein (IGFBP)-3, -5 and -6 expression ($P < 0.05$), although IGF-II mRNA levels in maternal villi remained unchanged. Placentome number was unaffected by diet or gestational age. Placental weight remained constant between days 90 and 135 in ewes on 100% maintenance rations but decreased over this period ($P < 0.05$) in ewes on the 70% rations. Gross morphology also altered, so the underfed ewes had more type C and

type D placentomes and fewer type B placentomes than their well-fed counterparts on day 135 ($P < 0.05$). These changes were accompanied by higher IGFBP-6 mRNA expression in the maternal placental villi in undernourished ewes ($P < 0.05$). The change in shape from a type A to a type C placentome was accompanied by flattening of the placentome and a reduction in the ratio of the area of unattached fetal allantochorion to interdigitated maternal and fetal villi. Within the intercotyledonary endometrium, expression of IGFBPs-3 and -5 mRNA in the glandular epithelium increased between days 45 and 90, showing an opposite trend with time to that found in the adjacent placentomes. This indicates tissue-specific control of IGFBP expression. In conclusion, this study has shown clear time-related changes in the uterine IGFBP system during pregnancy, which accompany changes in placental growth. Altered IGFBP expression may play a role in determining placental size in relation to nutritional status, but is unlikely to be the only mediator.

Journal of Endocrinology (2004) **182**, 89–103

Introduction

The placenta is an important determinant of fetal growth, with placental size and birthweight being positively correlated in many species, including sheep (Mellor 1983, Kelly 1992). The ewe has a cotyledonary placenta where fetomaternal exchange takes place at discrete sites known as placentomes. The placentomes reach a maximum weight by approximately day 75 to 80 of gestation (term ~150 days) whilst 80% of fetal growth occurs in the final third of gestation (Ehrhardt & Bell 1995, Wallace *et al.* 1999). In early to mid-pregnancy, insults such as inappropriate maternal nutrition and thermal stress can significantly disrupt placental development and subsequent fetal growth (McCraib *et al.* 1991, 1992, Vatnick *et al.* 1991a, Galan *et al.* 1999). The factors responsible for modifying placental growth are poorly understood.

Evidence suggests that the insulin-like growth factor (IGF) system, a nutritionally sensitive group of proteins, has a fundamental role in placental and fetal development (Reynolds *et al.* 1997, Wathes *et al.* 1998, Han & Carter 2000). These findings are supported by gene deletion studies in mice (Baker *et al.* 1993, Liu *et al.* 1993). The IGF axis consists of two single chain polypeptides, IGF-I and IGF-II, which mediate cellular proliferation, differentiation and metabolism (Jones & Clemmons 1995, Hossner *et al.* 1997). The type 1 IGF-receptor (IGF-1R) primarily mediates the biological activities of IGF-I and IGF-II, whilst the type 2 IGF receptor (IGF-2R) is considered to act as a degradative pathway, removing excess IGF-II from the circulation (Jones & Clemmons 1995, Stewart & Rotwein 1996, Bräulke 1999). A family of high affinity insulin-like growth factor binding proteins (IGFBPs), designated IGFBP-1 to -6, regulate the biological activities of the IGFs (Rechler & Clemmons 1998,

Ferry *et al.* 1999). It is not known whether nutritionally mediated alterations in placental growth occur as a result of the IGF axis becoming modified. The IGFs were thus studied in the placentomes and intercotyledonary region (the endometrial area between the placentomes) using *in situ* hybridisation at three critical time points during pregnancy: day 45 (during the establishment of the placentomes), day 90 (following the development of the placenta) and day 135 (during the period of rapid fetal growth). The associated changes in circulating metabolic hormone concentrations and in fetal growth from animals in this study have been reported previously (Osgerby *et al.* 2002).

Materials and Methods

Experimental design and nutritional treatments

All procedures were performed under the UK Animals (Scientific Procedures) Act 1986 and took place within the normal seasonal breeding cycle. Forty-eight multiparous Welsh Mountain ewes were individually housed under conditions of natural light and ambient temperature on wheat straw bedding with free access to water. Their body condition scores were standardised to 2.5 before the start of the experiment. Ewes were fed a complete diet of pelleted sheep nuts calculated on an individual ewe basis to provide them with 100% of their daily maintenance requirements (based on the criteria of the Meat and Livestock Commission 1988). The complete diet was fed in two equal portions at 0800 h and 1600 h and supplied 10.8 MJ metabolizable energy and 150 g crude protein per kg dry matter.

Following 30 days of acclimatisation, oestrus was synchronised by withdrawing progestagen impregnated sponges (60 mg medoxyprogesterone acetate, Veramix; Upjohn, Crawley, Sussex, UK) 12 days after their insertion. At sponge withdrawal, ewes received an injection of a prostaglandin (PG) $F_{2\alpha}$ analogue, Estrumate (0.5 ml i.m. Schering-Plough Animal Health, Uxbridge, Middlesex, UK) and were presented to the ram 48 h later. Synchronisation was staggered into 3 groups of 16 so that the same ram could be used to fertilise all the ewes and thus minimise genotypic variation. The ram was raddled and the ewes checked four times daily. Day 0 of gestation was taken as the first date at which ewes were observed to have an obvious raddle mark. Pregnancy was confirmed by measuring plasma progesterone concentrations on day 16 of gestation using an enzyme immunoassay kit (Ridgeway Science Ltd, Alvington, Glos, UK). The fetal number was confirmed at day 60 by ultrasonography.

At day 22 of gestation ewes were randomly allocated to one of two nutritional treatments: ewes allocated to the 100% ration (well fed) continued to receive 100% of their maintenance requirements throughout gestation whereas ewes allocated to the 70% ration (undernourished) had

their rations gradually reduced over 4 days so that by day 26 of gestation they received only 70% of their daily maintenance requirements. The rations were reviewed weekly and adjusted on an individual basis according to live weight changes, the stage of pregnancy and the number of fetuses carried. There were initially 8 ewes per group at each time point but ewes found to be carrying twins were subsequently excluded from the study (see results section for further details).

Measurements

The gravid uterus was removed *post-mortem* through a midline incision at a consistent point along the length of the cervix at three time points during gestation – day 45 ($n=16$), day 90 ($n=16$) and day 135 ($n=16$). From the body of the uterus four placentomes and six pieces of intercotyledonary endometrium (with fetal membranes attached) were dissected. All tissue samples were wrapped in aluminium foil, rapidly frozen in liquid nitrogen-tempered isopentane and stored at -80°C until required for sectioning. The remaining placentomes were dissected from the uterine wall, counted and individually weighed. Following a procedure developed by Vatnick *et al.* (1991b), placentomes from the day 135 pregnant ewes were typed according to their gross morphology. According to (i) the overall shape of the placentalome i.e. rounded or flat and (ii) the position of the fetal tissue (cotyledon) in relation to the maternal portion of the placentalome (caruncle), the placentalome was classified as type A, B, C or D (see Fig. 1). Type A placentalomes are rounded or concave in shape, with the fetal cotyledon level with the top of the caruncle. In type B placentalomes the fetal cotyledon is raised, protruding from the centre of the rounded cotyledon. Type C placentalomes are more flat when viewed from the top, with the fetal cotyledon covering the top of the caruncle. The lower part of the caruncle is still visible when viewed from the side. Type D placentalomes are flat, with the fetal cotyledon completely covering the caruncle such that it is no longer visible when viewed from the side. These changes in shape are only apparent in late gestation, so only placentalomes from day 135 were classified in this way.

In a follow-up experiment to examine placentalome type in more detail, one further ewe taken from the university flock and carrying a singleton pregnancy was killed on day 135 of gestation. All the placentalomes were removed and sorted according to type. Twelve placentalomes of each of types A, B and C were then selected at random, weighed and fixed in 10% formalin. As only three type D placentalomes were present, these were excluded from the analysis. All the fixed placentalomes were subsequently cut through the midline, and a section was stained with haematoxylin and eosin for histological analysis. Using an image analysis system (SeescanPLC, Cambridge, Cambs, UK), measurements were made on each section of the

Type A **Type B** **Type C** **Type D**

Figure 1 Photomicrographs of four placentomes stained with haematoxylin and eosin to illustrate differences in dimensions according to placentome type. Details of the classification system are given in the text. Measurements taken for analysis included: (a) width of placentome, (b) distance between tips and (c) width of interdigitation of fetal and maternal villi. AC, fetal allantochorion; V, area of interdigitating maternal and fetal villi; PC, placentome capsule. Scale bar represents 1 cm.

following parameters: total width of the placentome, distance between the tips of the placentome, width of the area of interdigitation, width of the capsule, area of interdigitated maternal and fetal villi and area of central core of allantochorion (Fig. 1).

Oligonucleotide probes

All probes used for *in situ* hybridisation were single stranded oligonucleotides (Babraham Institute, Cambridge, Cambs, UK). Sense probes were always included as negative controls as any signal produced on applying this probe could be regarded as non-specific. In the placentomes IGF-II, IGFBP-2, IGFBP-3, IGFBP-5 and IGFBP-6 mRNA were investigated. In the intercotyledonary tissue

(the areas between the placentomes) IGFBP-3, IGFBP-5 and IGFBP-6 mRNA were studied. These probes were chosen based on our previous studies on the localisation of the placental IGF system during ovine pregnancy (Reynolds *et al.* 1997, Gadd *et al.* 2000*a,b*). All probes used in the study are described in Table 1.

Localisation of mRNA by *in situ* hybridisation

This procedure was performed as described previously by Reynolds *et al.* (1997). All chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, UK) or BDH (Poole, Dorset, UK) unless otherwise stated. In summary, frozen placentome and intercotyledonary tissue sections were cut (10 µm thick) and thaw mounted onto 1 mg/ml

Table 1 The sequence of the antisense probes and exposure time for *in situ* hybridisation, X-ray films and emulsions

Probe	Antisense sequence	Exposure time (days)	
		X-ray film	Emulsion
IGF-II	554–598 of ovine IGF-II gene (O'Mahoney & Adams 1989) 5'-AAC-TGG-AGG-GTG-TCC-ACC-AGC-TCC-CCG-CCG-CAC-AGA-GTC-TCG-CTG-3'	21	28
IGFBP-2	452–496 of ovine IGFBP-2 gene (Delhanty & Han 1992) 5'-CAG-AGT-GCT-CCT-CGC-CAT-TGT-CTG-CAA-CCT-GCT-CGG-GGC-TGG-CGC-3'	30	37
IGFBP-3	546–578 of bovine IGFBP-3 gene (Spratt <i>et al.</i> 1991) 5'-AGC-CTG-GTT-CTC-TGT-GCT-CCC-CAT-GCT-GTG-GTC-3'	30	37
IGFBP-5	291–335 of bovine IGFBP-5 gene (Moser <i>et al.</i> 1992) 5'-TCG-GAG-ATG-CGG-GTG-TGC-TTG-GGC-CGG-AAG-ATC-TTG-GGC-GAG-TAG-3'	21	28
IGFBP-6	292–336 of bovine IGFBP-6 gene (Moser <i>et al.</i> 1992) 5'-CCG-CTT-CCG-GTA-GAA-GCC-CCT-ATG-GTC-ACA-ATT-AGG-CAC-GTA-GAG-3'	30	37

poly-L-lysine ($M_r > 300\,000$)-coated glass slides. Sections were fixed in 4% (wt/vol) paraformaldehyde in 0.01 M PBS at pH 7.0 for 5 min at room temperature, prior to three washes in 0.01 M PBS and sequential dehydration in 70% and 95% ethanol.

The oligonucleotide (5 ng) was labelled with ^{35}S dATP (Amersham International, Aylesbury, Bucks, UK) at the 3'-end using deoxynucleotidyl transferase (Pharmacia Biotech, St Albans, Herts, UK) at 34 °C for 60 min. The labelled probe was subsequently diluted to a final concentration of 1 100 000 c.p.m./ml in hybridisation buffer and 100 μl were added to each section. The sections were incubated in a humidified box overnight at 42.5 °C. Following incubation, slides were washed in 1 $\times$ saline sodium citrate (1 $\times$ SSC) 0.2% (wt/vol) sodium thiosulphate pentahydrate solution at room temperature for 30 min, then at a higher stringency in 1 $\times$ SSC 0.2% (wt/vol) sodium thiosulphate pentahydrate solution for 60 min at 57.5 °C. Sections were finally rinsed in 1 $\times$ SSC, 0.1 $\times$ SSC, 70% ethanol, and 95% ethanol, for 1 min each. Slides were then air dried for at least 2 h and exposed to β -max hyperfilm (Amersham International) for the time indicated in Table 1. Uterine samples shown to be positive for each of the probes (Reynolds *et al.* 1997, Wathes *et al.* 1998) were included in each experiment as a positive control.

Photographic emulsions

Slides previously exposed to X-ray film were coated with a photographic emulsion (LM1, Amersham International) according to the manufacturer's instructions and stored at 4 °C for the time indicated in Table 1. Slides were then developed in 20% phenisol, fixed in 1.9 M sodium thiosulphate-pentahydrate and counterstained with haematoxylin and eosin to confirm microscopically the cellular localisation of the radioactive signal.

Optical density (OD) quantification

An image analysis system (Seescan PLC) was used to quantify the level of radioactive signal as OD units using a linear grey scale of 0–2.1 as described previously (Reynolds *et al.* 1997). To determine a background reading of the autoradiograph under analysis, a blank section of the film was placed under the image analyser lens and measured. Measurements were made of the antisense (AS) and sense (S) images obtained from four sections per sample. For each region where localisation had been confirmed, at least six readings per section were taken, giving a minimum of 24 readings per region per sample. The S values were subtracted from the AS values to produce a mean value of specific hybridisation for a particular region for that sample. The detection limit was taken as an OD greater than 0.01. The coefficients of variation for duplicate measurements of the pairs of slides were as follows: IGF-II, 6.5%; IGFBP-2, 11.3%; IGFBP-3, 8.9%; IGFBP-5, 11.4%; IGFBP-6, 6.3%. All samples for each probe were processed in the same batch to avoid any possible inter-batch variation. While this system provides accurate comparisons between animals using the same probe, note that it is not valid to make comparisons of OD units between different probes.

Statistical analysis

All values are given as means $\pm$ S.E.M. The statistical analysis was performed using Statistical Package for the Social Sciences version 7.0 (SPSS, Chicago, IL, USA). All data were tested for homogeneity of variance and log transformed if necessary. The IGF expression data in a given uterine region were analysed by univariate analysis of variance with ration (100% or 70%) and day of gestation (45, 90 or 135) as factors. *Post hoc* tests were by Tukey honestly significantly different (HSD). Data on the histological analysis of placental shape (A, B or C) were

Table 2 The effect of ration and gestational age on the placental growth trajectory in the ewe

	Maternal nutrition	
	100% ration (n=4/5)	70% ration (n=4/5)
Day 45		
Total uterine weight (g)	425 ± 39.2	466 ± 64.9
Total placentome number	75 ± 5	68.0 ± 2.1
Total placentome weight (g)	58 ± 10.8 ^h	52 ± 3.7 ^h
Mean placentome weight (g)	0.79 ± 0.15 ^j	0.77 ± 0.06 ^j
Day 90		
Total uterine weight (g)	2295 ± 39.7	2437 ± 180.2
Total placentome number	80 ± 3.1	87 ± 5.6
Total placentome weight (g)	531 ± 55 ^g	559 ± 33.7 ^{gk}
Mean placentome weight (g)	6.5 ± 0.43 ⁱ	6.5 ± 0.46 ⁱ
Day 135		
Total uterine weight (g)	7748 ± 268.5 ^a	6634 ± 283.0 ^b
Total placentome number	77 ± 8.5	75 ± 9.1
Total placentome weight (g)	540 ± 24.8 ^a	438 ± 44.4 ^{bl}
Mean placentome weight (g)	7.4 ± 1.14	5.9 ± 0.54
Day 135 Distribution of placentome types (%)		
Type A	0	0
Type B	90 ± 3.1 ^c	61 ± 10.6 ^d
Type C	1.9 ± 1.95 ^f	18.8 ± 6.05 ^e
Type D	1.5 ± 0.94 ^f	12.7 ± 3.96 ^e

Within rows (effect of ration): a>b, $P<0.08$; c>d, $P<0.05$; e>f, $P<0.05$.

Within columns (effect of time): g>h, $P<0.01$; i>j, $P<0.01$; k>l, $P<0.05$.

analysed by one-way ANOVA with *post hoc* least significant difference tests. Data were considered statistically significant when P values were equal to or less than 0.05.

Results

Maternal data

Of the 48 ewes mated, 27 had singleton pregnancies and 21 had twins. To avoid any nutritional effects of fetus number, the data presented in this study will concern only those ewes that had singleton pregnancies on day 45 (100%, $n=5$; 70%, $n=4$), day 90 (100%, $n=4$; 70%, $n=5$) and day 135 (100%, $n=4$; 70%, $n=5$). The well-fed ewes (on the 100% ration) maintained their body condition score of 2.5 at mating until the end of the study whereas the undernourished ewes (on the 70% ration) dropped approximately half a body condition score by day 135 of gestation.

Uterine and placental data

The total number of placentomes and the mean placentome weight were unaltered by dietary treatment at each time point investigated (Table 2). However, on day 135 the total uterine weight and the total placentome weight tended to be lower in undernourished ewes (Table 2; a>b, $P<0.08$).

Over time the placental growth trajectory differed between the two dietary treatments. Initially both groups of ewes experienced a significant increase in their total and mean placentome weights between days 45 and 90 (Table 2; g>h, i>j, $P<0.01$). The well-fed ewes maintained these weights to day 135 whilst the placentae from the undernourished ewes significantly decreased from their day 90 weights (Table 2; k>l, $P<0.05$). The placentome number remained unchanged by gestational age. The placentae from the undernourished mothers had significantly more type D and type C placentomes and fewer type B placentomes than their well-fed counterparts (Table 2; c>d, e>f, $P<0.05$).

Expression of components of the IGF axis in placentomes

In the placentomes, IGF-II mRNA was localised in the mesoderm of the fetal villi (OD 0.18 ± 0.007 , $n=26$) and at lower levels in the maternal placentome capsule (OD 0.06 ± 0.006) (Fig. 2A). In both regions, expression was unaltered by ration or stage of gestation.

IGFBPs-2, -3, -5 and -6 were all localised in the maternal (but not the fetal) component of the placentomes, in both the villi and the surrounding capsule. IGFBP-2 mRNA expression was higher in the capsule (OD 0.17 ± 0.015 , $n=25$) compared with the maternal villi (OD 0.06 ± 0.006) (Fig. 2C, D, Fig. 3A-C) and expression was unaltered by time or ration in either location. IGFBP-3 mRNA was expressed in the placentome

Figure 2 Autoradiographs showing the localisation of IGF-II (A, B), IGFBP-2 (C, D) and IGFBP-3 (E, F) mRNA in the ovine placenta of a well-fed ewe on day 45 of gestation. Sections were treated with antisense (AS) (A, C, E) or sense (S, control) (B, D, F) oligonucleotide probes. The areas described are placenta capsule (PC), fetal mesoderm (FM) and caruncular stroma (CS). Scale bars represent 1.25 mm.

capsule ($OD\ 0.10 \pm 0.008$, $n=27$) and in the maternal villi (Fig. 2E, F, Fig. 3D, E). IGFBP-3 expression in the maternal villi, IGFBP-5 expression in the maternal villi (Fig. 4A–C, Fig. 5A–C) and IGFBP-6 mRNA expression in the placenta capsule (Fig. 4E, F) all showed time-related changes, with a major reduction in expression between 45 and 90 days of gestation (Fig. 6). IGFBP-5 expression in the capsule also decreased with time but not until after day 90. None of these measurements was affected by ration. IGFBP-6 mRNA expression in the

maternal villi (Fig. 4E) also decreased over time, but in this case there was also an effect of ration, with expression lower on days 90 and 135 in sheep on the 100% compared with the 70% ration (Fig. 7).

Expression of components of the IGF axis in intercotyledonary endometrium

IGF-II mRNA expression was detected in both the luminal epithelium ($OD\ 0.07 \pm 0.007$, $n=25$) and the

Figure 3 Sections were hybridised with antisense (AS) (A, C–G) and sense (S, control) (B, H) oligonucleotide probes, coated with photographic emulsion and counterstained with haematoxylin and eosin. The silver grains confirmed that IGFBP-2 (A–C) and IGFBP-3 (D, E) mRNA is expressed in the placental capsule (PC) and caruncular stroma (CS) in the ovine placentalome of well-fed ewes on days 45 and 90 of gestation respectively. In the intercotyledonary region, IGFBP-3 (F–H) mRNA was localised in the luminal epithelium (LE) and endometrial glands (EG) in well-fed ewes on day 90 of gestation. SC, stratum compactum. Scale bars represent 200 μ m.

Figure 4 Autoradiographs showing the localisation of IGFBP-5 and IGFBP-6 mRNA in the pregnant ovine uterus. Sections were hybridised with antisense (AS) (A, C, D, E) or sense (S, control) (B, F) oligonucleotide probes. Sections A to C show placentome sections taken from well-fed ewes on day 45 (A and B) and day 135 of gestation (C). IGFBP-5 mRNA is expressed in the placental capsule (PC) and caruncular stroma (CS) with levels decreasing between days 45 and 135 of gestation (A–C). Section D demonstrates the expression of IGFBP-5 mRNA in the luminal epithelium (LE) and endometrial glands (EG) in the intercotyledonary region of a well-fed ewe on day 90 of gestation. Sections E and F show the expression of IGFBP-6 mRNA in the placentome of a well-fed ewe on day 45 of gestation where levels are confined to the placental capsule (PC) and caruncular stroma (CS). Scale bars represent 1.25 mm.

underlying stratum compactum ($OD\ 0.12 \pm 0.023$). As in the placentomes, expression was not altered by gestation stage or ration. IGFBPs-3 and -5 mRNA were detected in the luminal epithelium (IGFBP-3, $OD\ 0.08 \pm 0.008$, $n=26$; IGFBP-5 0.37 ± 0.027 , $n=23$) and glandular epithelium (Fig. 3G, Fig. 4D, Fig. 5D). In the glands, expression of IGFBP-3 and IGFBP-5 mRNA both

showed changes related to stage of gestation but in the opposite direction to those observed in the maternal villi, as concentrations increased between days 45 and 90 of gestation (Fig. 6). IGFBP-6 mRNA was also expressed in the glandular epithelium ($OD\ 0.06 \pm 0.004$, $n=26$) and at a higher level in the stratum compactum ($OD\ 0.21 \pm 0.011$) (Fig. 5F). Within the intercotyledonary

Figure 5 Sections were hybridised with antisense (AS) (A, C, D, F) or sense (S, control) (B, E) oligonucleotide probes, coated with photographic emulsion and counterstained with haematoxylin and eosin. IGFBP-5 mRNA was localised in the placental capsule (PC) and caruncular stroma (CS) in the placental region (A–C), and in the luminal epithelium (LE) and endometrial glands (EG) in the intercotyledonary region (D, E) of a well-fed ewe on day 45 of gestation. IGFBP-6 mRNA was detected in the stratum compactum (SC) underlying the luminal epithelium (LE) in the intercotyledonary region of a well-fed ewe on day 90 of gestation (F). Scale bars represent 200 μ m (A–C, F) and 800 μ m (D, E).

endometrium, no significant changes in expression of the IGF system were detected due to ration.

Changes in placental shape

Results of the main experiment indicated that there was a shift to more C and D type placentomes on the restricted diet by day 135 gestation. In order to examine the implication of such a change on placental histology, 12 placentomes of each of types A, B and C were collected from one ewe on day 135. There were insufficient type D placentomes present for statistical analysis. Examination of

cross sections through the centre of each placental type suggested that the main change with type was an opening out and flattening of the placental type from type A to type D (Fig. 1). This was associated with an increase in weight. In this process, the overall width of the placental type and the distance between the tips both increased significantly (Table 3). There were no significant changes in the width of the area of interdigitation between maternal and fetal villi or in the width of the capsule. The total area of interdigitation and of the central core of fetal allantochorion both increased. However, the ratio of interdigitated to fetal only tissue also increased between types A and C.

Figure 6 Histograms showing changes in IGFBP expression with stage of gestation in different regions of the ovine placenta. (a) IGFBP-6 and (b) IGFBP-5 mRNA expression in the placentome capsule (CAP). (c) IGFBP-3 and (d) IGFBP-5 mRNA expression in the maternal villi (MV). (e) IGFBP-3 and (f) IGFBP-5 mRNA in the glandular epithelium (GE). Values are measured as OD units from autoradiographs on days 45, 90 and 135 ($n=9$ ewes at each stage of gestation) and are shown as means $\pm$ S.E.M. The effect of stage of gestation on mRNA expression is indicated: $b > a$, $P < 0.001$; $d > c$, $P < 0.06$. None of the IGFbps illustrated was affected by ration. Note that with the *in situ* hybridisation system used, it is not valid to make comparisons of OD units between different probes.

Figure 7 Histograms showing changes in IGFBP-6 mRNA expression with stage of gestation and ration in maternal villi. Values are measured as OD units from autoradiographs on days 45, 90 and 135. Ewes were fed either 100% (open bars) or 70% (solid bars) of their estimated nutrient requirements for the stage of gestation, with 4 or 5 ewes per group. Values are means $\pm$ S.E.M. Differences due to stage of gestation are indicated by the superscript letters a > b ($P < 0.01$). Differences due to ration at a particular stage are indicated by * $P < 0.05$.

This implied that there was proportionately less uninterdigitated fetal allantochorion present in the type C placentomes.

Discussion

During pregnancy, inappropriate maternal nutrition can significantly disrupt the placental growth trajectory (Kelly

1992, Wallace *et al.* 1999) but the factors that instigate these changes remain unknown. The IGF axis, localised in the reproductive tract and placentae of many species, may play a fundamental role in placental and fetal growth (sheep: Reynolds *et al.* 1997, cow: Robinson *et al.* 2000, rodent, primate, human: Han & Carter 2000). Recent studies have demonstrated the nutritional sensitivity of the IGFs, both systemically (Thissen *et al.* 1994, Bauer *et al.* 1995, Sohlström *et al.* 1998) and locally within the uterine tract (Gadd *et al.* 2000a). In this study we have investigated the utero-placental IGF system using *in situ* hybridisation to enable tissue-specific localization of mRNA. We did not attempt to co-localise protein expression in the different uterine regions as locally synthesised protein cannot be distinguished from that which has arrived at the region via the circulation. However, by focusing upon gene expression we realise that the post-transcriptional regulation of the protein cannot be addressed.

The total and average placentome weights increased between days 45 and 90 of gestation. Earlier studies have demonstrated that placentome proliferation reaches a maximum rate between days 50 and 60 of gestation, with placentomes attaining a maximum weight by days 75 to 80 (Ehrhardt & Bell 1995). Nutritional insults during this period have thus been associated with changes in placental growth (McCrabb *et al.* 1992), with maternal factors including weight, age and body condition influencing this interaction (DeBarro *et al.* 1992, Wallace *et al.* 1997, Osgerby *et al.* 2003a,b). In this study, placentome weight was not, at this early stage, influenced by nutrition although we have previously reported that the 70% reduction in ration did result in altered fetal growth by day 90, with more obvious signs of fetal growth retardation apparent by day 135 of gestation (Osgerby *et al.* 2002). Ration did, however, significantly affect the placental growth trajectory between days 90 and 135 of gestation, with the total placentome weight decreasing in underfed ewes whilst remaining unchanged in their well-fed counterparts.

The factors responsible for regulating placentome size remain to be elucidated. IGF-I mRNA was not

Table 3 Comparison of measurements of placentomes of different types from the same ewe

	Type A (n=12)	Type B (n=12)	Type C (n=12)	Significance
Measurement				
Width of placentome (mm)	18.7 $\pm$ 0.01 ^a	19.5 $\pm$ 1.10 ^a	24.3 $\pm$ 1.11 ^b	0.001
Distance between tips (mm)	4.9 $\pm$ 0.33 ^a	5.2 $\pm$ 0.68 ^a	9.3 $\pm$ 0.66 ^b	0.000
Weight (g)	4.9 $\pm$ 0.46 ^a	5.8 $\pm$ 0.79 ^a	8.3 $\pm$ 0.73 ^b	0.003
Width of interdigitation (mm)	5.0 $\pm$ 0.19	4.6 $\pm$ 0.23 ^b	5.5 $\pm$ 0.23 ^a	0.016
Width of capsule (mm)	0.67 $\pm$ 0.032	0.60 $\pm$ 0.018	0.63 $\pm$ 0.038	NS
Area of interdigitated villi (mm ²) (V)	118 $\pm$ 9.1 ^a	123 $\pm$ 13.2 ^a	178 $\pm$ 12.4 ^b	0.001
Area of central allantochorion (mm ²) (AC)	33.5 $\pm$ 3.33 ^a	44.2 $\pm$ 5.26 ^a	48.9 $\pm$ 6.54 ^b	0.003#
Ratio V:AC	3.7 $\pm$ 0.30	2.9 $\pm$ 0.20 ^a	4.3 $\pm$ 0.53 ^b	0.045#

Within rows, values with different superscript letters were significantly different. For rows marked with #, analysis was on log transformed data.

NS, not significant.

investigated as previous studies had demonstrated that levels were below the limit of detection after implantation (Reynolds *et al.* 1997). However, IGF-II mRNA was localised in the placental capsule and mesoderm of the fetal villi as previously described (Reynolds *et al.* 1997, Gadd *et al.* 2000a). Mouse embryos carrying null mutations for IGF-II exhibit placental growth retardation, emphasising the significant role which this growth factor has in placental development (Baker *et al.* 1993). However, in this study maternal ration had no significant effect on placental IGF-II mRNA and this lack of effect due to nutrition is consistent with two previous studies (Gadd *et al.* 2000a, Osgerby *et al.* 2003b). IGF-II mRNA levels were also unaltered by gestational age.

The IGFBPs localised in the placental capsule and stroma of the maternal villi as previously described (Reynolds *et al.* 1997, Gadd *et al.* 2000a,b) may, however, regulate the mitogenic activity of IGF-II in the placental capsule. IGFBP-3 and IGFBP-5 expression in the caruncular stroma of the maternal villi and IGFBP-6 mRNA in the placental capsule all decreased significantly between days 45 and 90 of gestation. These reductions in IGFBP expression may result in lower levels of protein being translated. Consequently, the availability and thus the mitogenic capacity of the IGFs may rise, contributing to the increase in placental mass between days 45 and 90 of gestation.

The reduced total placental weight in underfed ewes between days 90 and 135 was associated with higher IGFBP-6 mRNA expression in maternal villi. However, IGFBP-2, IGFBP-3 and IGFBP-5 expression in the caruncular stroma of the maternal villi and IGFBPs-2, -5 and -6 mRNA in the placental capsule remained unaltered by ration. IGFBP-3 has previously been shown to decrease in the plasma of undernourished animals whilst circulating IGFBP-2 is inversely regulated by insulin, increasing in response to dietary restriction (Lee *et al.* 1997, Maxwell *et al.* 1998, Oldham *et al.* 1999). In our study, the insulin concentration in late gestation was reduced on the restricted ration (Osgerby *et al.* 2002). Although circulating concentrations of IGFBPs were not measured, it is thus possible that raised IGFBP-2 levels in the plasma of undernourished ewes coupled with higher IGFBP-6 expression in the maternal villi may have been responsible for reducing placental weight by inhibiting the IGF-II mediated proliferation. This is supported by findings in the guinea pig, where placental weight and maternal plasma IGFBP-2 concentrations were negatively correlated (Roberts *et al.* 2001). A reduction in placental weight between mid and late pregnancy has previously been described (Vatnick & Bell 1992, Ehrhardt & Bell 1995, Schneider 1996, Jenkinson *et al.* 1999). This may have significant implications on subsequent milk production as placental lactogen and progesterone, important mediators of mammary gland development, are produced from the placenta at levels that are proportional to the placental mass (Mellor 1987).

The shapes of the placentomes on day 135 of gestation were also modified by diet. Undernourished ewes had significantly fewer type B placentomes and more types C and D placentomes than well-fed ewes. Similar structural adaptations have previously been reported in ewes that have been undernourished (Crowe *et al.* 1996), undergone a unilateral fetectomy (Vatnick *et al.* 1991b) or been exposed to hypoxic conditions (Penninga & Longo 1998). Women living at high altitude also have an increased incidence of irregular shaped placentas (reviewed by Penninga & Longo 1998). It has been suggested that this change in shape is due to a compensatory increase in fetal vascularisation in response to nutrient restriction (Hoet & Hanson 1999). Histological analysis in this study showed that the structural change was due to an opening of the placental capsule which was not accompanied by a proportionate increase in the area of fetal allantochoion. The trend was rather in the reverse direction. This result corresponds to a study by Heasman *et al.* (1999) who manually separated fetal and maternal components of the placentomes and found that dietary restriction to 50–60% of maintenance energy requirements between 30 and 80 days of gestation caused a significant decrease in the weight of the fetal component, whereas the weight of the maternal component remained the same. The shift from the type A to the type C placental capsule in the single ewe examined in the follow-up study described here was accompanied by an increase in placental weight. We have, however, also observed small type D placentomes (J C Osgerby, T S Gadd and D C Wathes, unpublished observations) and in the main study the shift from type B to type D placentomes was accompanied by an overall decrease in placental weight. It is therefore unlikely that the flattening process is caused just by an increase in size and as we did not assess the expression of the IGF axis in relation to placental type it remains uncertain what does cause the flattening.

The number of placentomes that formed was unaltered by diet and gestational age. Holst *et al.* (1992) similarly found that a moderate nutritional restriction implemented for four weeks from days 79, 87 and 95 of gestation exerted a negligible effect on placental number, which is thought to be fixed by day 56 of gestation (Wallace 1948). There are, however, exceptions with infusions of bovine somatotrophin from days 97 to 124 of gestation increasing the placental number (Stelwagen *et al.* 1994) while Heasman *et al.* (1999) found that feeding ewes 50–60% maintenance energy on days 30 to 80 of gestation caused a significant increase in placental number in the nutrient-restricted group. The factors that prevent further placentomes from developing in late gestation are unclear. Neither body condition score at mating nor ration (100 or 70% of maintenance requirements) affected placental number at day 65 of gestation (Osgerby *et al.* 2003a,b). Placental number at this time was, however, positively related to the circulating maternal IGF-I concentration

and negatively related to IGFBP-3 expression in the luminal epithelium (Osgerby *et al.* 2003b). It is thus possible that a high local concentration of IGFBP-3 can inhibit placentome formation.

In the intercotyledonary region, the expression of IGFBPs-3 and -5 mRNA increased between days 90 and 135 of pregnancy. In contrast, expression of both these binding proteins in the placentomes decreased as pregnancy progressed. Therefore, there were opposite trends in closely adjacent maternal uterine tissues, which were presumably exposed to similar circulating hormone concentrations. In the Dorset ewe in mid gestation we have previously reported reduced expression of both IGFBPs-3 and -5 mRNA in the glandular epithelium of ewes of fat compared with thin body condition (Osgerby *et al.* 2003a). Surprisingly little is known about local factors regulating IGFBP expression in different tissues. In liver, growth hormone (GH), IGF-I and insulin concentrations all contribute to the control of IGFBP-3 synthesis (Rajaram *et al.* 1997, Phillips *et al.* 1998). Type 1 IGF receptors are localised to the epithelium but not the placentome in the pregnant ovine uterus (Reynolds *et al.* 1997), and GH receptor expression is also highest in this location (Klemp *et al.* 1993) providing a possible differential control mechanism. Uterine IGFBP-5 mRNA is up-regulated by an embryonic factor in early pregnancy (Gadd *et al.* 2000b) with evidence from other systems suggesting that this could be IGF-II (e.g. Matsumoto *et al.* 1996). However, local expression of IGF-II in this study did not change with time. In the quail, Fu *et al.* (2001) reported that retinoic acid could cause a rapid up-regulation in IGFBP-5 mRNA expression in a tissue-specific manner. Further work is therefore required to determine both the control mechanisms involved and the functional significance of changing IGFBP expression in the uterus during pregnancy.

In summary, the 70% ration used in this study only had a relatively small effect on placental size although we have shown previously that it did result in fetal growth retardation (Osgerby *et al.* 2002). The increase in placental weight between days 45 and 90 of gestation was accompanied by a reduction in placentome IGFBPs-3, -5 and -6 expression whilst IGF-II mRNA levels remained unchanged. The placental weight decreased in underfed ewes between days 90 and 135 of gestation and the gross placentome morphology on day 135 was altered with fewer type B and more type C and D placentomes expressed than in well-fed animals. This was accompanied by a higher expression of IGFBP-6 mRNA in the maternal villi over this period but other aspects of the placental IGF system were not altered by the reduced ration. These data suggest that changing placental expression of IGF binding proteins may affect placental growth but additional factors probably contribute to the fine-tuning in size associated with diet.

Acknowledgements

We would like to thank Mr D Manners and Mr J Thompson for their care of the animals, Dr Z Cheng for assistance with the statistical analysis, Ms J Montgomery for technical assistance and Mrs B Wilshire for the preparation of the photographs.

Funding

The authors wish to thank the Scottish Executive Environment and Rural Affairs Department (SEERAD), the Wellcome Trust and the Biotechnology and Biological Sciences Research Council (BBSRC) for their financial support.

References

- Baker J, Lui J-P, Robertson EJ & Efstratiadis A 1993 Role of insulin-like growth factors in embryonic and post-natal growth. *Cell* **75** 73–82.
- Bauer MK, Breier BH, Harding JE, Veldhuis JD & Gluckman PD 1995 The fetal somatotrophic axis during long term maternal undernutrition in sheep: evidence for nutritional regulation *in utero*. *Endocrinology* **136** 1250–1257.
- Braulke T 1999 Type-2 IGF receptor: a multi-ligand binding protein. *Hormone Metabolism and Research* **31** 242–246.
- Crowe CP, Hawkins P, Saito T, Stratford L, Noakes DE & Hanson MA 1996 Nutritional plane in early pregnancy: effect on fetal and placental size in late gestation. *Programme and Abstracts, Society for the Study of Fetal and Newborn Physiology*, Arica, Chile, Abstract 11.
- DeBarro TM, Owens JA, Early CR & Robinson JS 1992 Nutrition during early/mid pregnancy interacts with mating weight to affect placental weight in sheep. *Proceedings of the 24th Annual Conference of the Australian Society for Reproductive Biology* **36** (abstract).
- Delhanty PJD & Han VKM 1992 The characterization and expression of ovine insulin-like growth factor binding protein-2. *Journal of Molecular Endocrinology* **9** 31–38.
- Ehrhardt RA & Bell AW 1995 Growth and metabolism of the ovine placenta during mid-gestation. *Placenta* **16** 727–741.
- Ferry RJ, Katz LEL, Grimberg A, Cohen P & Weinzimer SA 1999 Cellular actions of insulin-like growth factor binding proteins. *Hormone and Metabolic Research* **31** 192–202.
- Fu Z, Noguchi T & Kato H 2001 Differential regulation of IGFBP-2 and IGFBP-5 gene expression by vitamin A status in Japanese quail. *American Journal of Physiology, Endocrinology and Metabolism* **281** E138–E146.
- Gadd TS, Aitken RP, Wallace JM & Wathes DC 2000a Effect of a high maternal dietary intake during mid-gestation on components of the utero-placental insulin-like growth factor (IGF) system in adolescent sheep with retarded placental development. *Journal of Reproduction and Fertility* **118** 407–416.
- Gadd TS, Osgerby JC & Wathes DC 2000b Regulation and localisation of insulin-like growth factor binding protein-5 gene expression in the uterus and placenta of the cyclic and early pregnant ewe. *Biology of Reproduction* **62** 1415–1421.
- Galan HL, Hussey MJ, Barbera A, Ferrazzi E, Chung M, Hobbins JC & Battaglia FC 1999 Relationship of fetal growth to the duration of heat stress in an ovine model of placental insufficiency. *American Journal of Obstetrics and Gynecology* **180** 1278–1282.
- Han VKM & Carter AM 2000 Spatial and temporal patterns of expression of messenger RNA for insulin like growth factors and their binding proteins in the placenta of man and laboratory animals. *Placenta* **21** 289–305.

- Heasman L, Clarke L, Stephenson TJ & Symonds ME 1999 The influence of maternal nutrient restriction in early to mid pregnancy on placental and fetal development in sheep. *Proceedings of the Nutrition Society* **58** 283–288.
- Hoet JJ & Hanson MA 1999 Intrauterine nutrition: its importance during critical periods for cardiovascular and endocrine development. *Journal of Physiology* **514** 617–627.
- Holst PJ, Allan CJ & Gilmour AR 1992 Effects of a restricted diet during mid pregnancy of ewes on uterine and fetal growth and lamb birth weight. *Australian Journal of Agricultural Research* **43** 315–324.
- Hossner KL, McCusker RH & Dodson MV 1997 Insulin-like growth factors and their binding proteins in domestic animals. *Animal Science* **64** 1–15.
- Jenkinson CMC, Min SH, Mackenzie DDS, McCutcheon SN, Breier BH & Gluckman PD 1999 Placental development and fetal growth in growth-hormone treated ewes. *Growth Hormone and IGF Research* **9** 11–17.
- Jones JI & Clemmons DR 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews* **16** 3–34.
- Kelly RW 1992 Nutrition and placental development. *Proceedings of the Nutrition Society of Australia* **17** 203–211.
- Klemp M, Bingham B, Breier BH, Baumbach WR & Gluckman PD 1993 Tissue distribution and ontogeny of growth hormone receptor messenger ribonucleic acid and ligand binding to hepatic tissue in midgestation sheep fetus. *Endocrinology* **132** 1071–1077.
- Lee WH, Gaylord TD, Bowsher RR, Hlaing M, Moorehead H & Liechty EA 1997 Nutritional regulation of circulating insulin-like growth factors (IGFs) and their binding protein in the ovine fetus. *Endocrine Journal* **44** 163–173.
- Liu JP, Baker J, Perkins AS, Robertson EJ & Efstratiadis A 1993 Mice carrying null mutations of the genes encoding insulin-like growth factor I (IGF-I) and type 1 IGF receptor (IGF1R). *Cell* **75** 59–72.
- McCraib GJ, Egan AR & Hosking BJ 1991 Maternal undernutrition during mid-pregnancy in sheep. Placental size and its relationship to calcium transfer during late pregnancy. *British Journal of Nutrition* **65** 157–168.
- McCraib GJ, Hosking BJ & Egan AR 1992 Changes in the maternal body and feto-placental growth following various lengths of feed restriction during mid-pregnancy in sheep. *Australian Journal of Agricultural Research* **43** 1429–1440.
- Matsumoto T, Gargosby SE, Oh Y & Rosenfeld RG 1996 Transcriptional and post-translational regulation of insulin-like growth factor-binding protein-5 in rat articular chondrocytes. *Journal of Endocrinology* **148** 355–369.
- Maxwell A, Butterwick R, Yateman M, Batt RM, Cotterill A & Camacho-Hübner C 1998 Nutritional modulation of canine insulin-like growth factors and their binding proteins. *Journal of Endocrinology* **158** 77–85.
- Meat and Livestock Commission 1988 Sheep Improvement Services. Feeding the ewe. 2nd Edn, Meat and Livestock Commission, UK.
- Mellor DJ 1983 Nutritional and placental determinants of foetal growth rate in sheep and consequences for the newborn lamb. *British Veterinary Journal* **139** 307–324.
- Mellor DJ 1987 Nutritional effects on the fetus and mammary gland during pregnancy. *Proceedings of the Nutrition Society* **46** 249–257.
- Moser DR, Lowe WL, Dake BL, Booth BA, Boes M, Clemmons DR & Bar RS 1992 Endothelial cells express insulin-like growth factor binding proteins 2 to 6. *Molecular Endocrinology* **6** 1805–1814.
- Oldham JM, Martyn JAK, Hua KM, MacDonald NA, Hodgkinson SC & Bass JJ 1999 Nutritional regulation of IGF-II, but not IGF-I, is age dependent in sheep. *Journal of Endocrinology* **163** 395–402.
- O'Mahoney JV & Adams TE 1989 Nucleotide sequence of an insulin-like growth factor II cDNA. *Nucleic Acids Research* **17** 5392.
- Osgerby JC, Wathes DC, Howard D & Gadd TS 2002 The effect of maternal undernutrition on ovine fetal growth. *Journal of Endocrinology* **173** 131–141.
- Osgerby JC, Gadd TS & Wathes DC 2003a Effect of maternal body condition on placental and fetal growth and the insulin-like growth factor axis in Dorset ewes. *Reproduction* **125** 717–731.
- Osgerby JC, Gadd TS & Wathes DC 2003b The effects of maternal nutrition and body condition on placental and foetal growth in the ewe. *Placenta* **24** 236–247.
- Penninga L & Longo LD 1998 Ovine placentome morphology: effect of high altitude, long term hypoxia. *Placenta* **19** 187–193.
- Phillips LS, Pao CI & Villafuerte BC 1998 Molecular regulation of insulin-like growth factor-I and its principle binding protein, IGFBP-3. *Progress in Nucleic Acid Research and Molecular Biology* **60** 195–265.
- Rajaram S, Baylink DJ & Mohan S 1997 Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocrine Reviews* **18** 801–831.
- Rechler MM & Clemmons DR 1998 Regulatory actions of insulin-like growth factor binding proteins. *Trends in Endocrinology and Metabolism* **9** 176–183.
- Reynolds TS, Stevenson KR & Wathes DC 1997 Pregnancy-specific alterations in the expression of the insulin-like growth factor system during early placental development in the ewe. *Endocrinology* **138** 886–897.
- Roberts CT, Sohlström A, Kind AL, Grant PA, Earl RA, Robinson JS, Khong TY, Owens PC & Owens JA 2001 Altered placental structure induced by maternal food restriction in guinea-pigs: a role for circulating IGF-II and IGFBP-2 in the mother? *Placenta* **22** S77–S82.
- Robinson RS, Mann GE, Gadd TS, Lamming GE & Wathes DC 2000 The expression of the IGF system in the bovine uterus throughout the oestrous cycle and early pregnancy. *Journal of Endocrinology* **165** 231–243.
- Schneider H 1996 Ontogenic changes in the nutritive function of the placenta. *Placenta* **17** 15–26.
- Sohlström A, Katsman A, Kind KL, Roberts CT, Owens PC, Robinson JS & Owens JA 1998 Food restriction alters pregnancy-associated changes in IGF and IGFBP in the guinea pig. *American Journal of Physiology - Endocrinology and Metabolism* **274** E410–E416.
- Spratt SK, Tatsuna GP & Sommer A 1991 Cloning and characterisation of bovine insulin-like growth factor binding protein-3 (bIGFBP-3). *Biochemical and Biophysical Research Communications* **177** 1025–1033.
- Stelwagen K, Grieve DG, Walton JS, Ball JL & McBride BW 1994 Effect of bovine somatotropin administration during the last trimester of gestation on maternal growth, and fetal and placental development in primigravid ewes. *Animal Production* **58** 87–94.
- Stewart CEH & Rotwein P 1996 Growth differentiation and survival: multiple physiological functions for insulin-like growth factors. *Physiological Reviews* **76** 1005–1026.
- Thissen J, Ketelslegers J & Underwood LE 1994 Nutritional regulation of the insulin-like growth factors. *Endocrine Reviews* **15** 80–95.
- Vatnick I & Bell AW 1992 Ontogeny of fetal hepatic and placental growth and metabolism in sheep. *American Physiological Society* **263** R619–R623.
- Vatnick I, Ignatz G, McBride BW & Bell AW 1991a Effect of heat stress on ovine placental growth in early pregnancy. *Journal of Developmental Physiology* **16** 163–166.
- Vatnick I, Schoknecht PA, Darrigrand R & Bell AW 1991b Growth and metabolism of the placenta after unilateral fetectomy in twin pregnant ewes. *Journal of Developmental Physiology* **15** 351–356.
- Wallace JM, Da Silva P, Aitken & Cruickshank MA 1997 Maternal endocrine status in relation to pregnancy outcome in rapidly growing adolescent sheep. *Journal of Endocrinology* **155** 359–368.

- Wallace JM, Bourke DA & Aitken RP 1999 Nutrition and fetal growth: paradoxical effects in the overnourished adolescent sheep. *Journal of Reproduction and Fertility Supplement* **54** 385–399.
- Wallace LR 1948 The growth of lambs before and after birth in relation to the level of nutrition. *Journal of Agricultural Science* **38** 93–153.
- Wathes DC, Reynolds TS, Robinson RS & Stevenson KR 1998 Role of insulin-like growth factor system in uterine function and placental development in ruminants. *Journal of Dairy Science* **81** 1778–1789.

Received 26 February 2004
 Accepted 30 March 2004
 Made available online as an
 Accepted Preprint 8 April 2004