The effect of maternal undernutrition on ovine fetal growth

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Abstract

Modifications in maternal nutrition during pregnancy can significantly disrupt fetal growth and subsequent post-natal health and survival. This study investigated the effects of undernutrition on fetal growth and the potential mechanisms involved. Tissue from pregnant ewes (n = 27) was investigated on days 45, 90 and 135 of gestation (term = ~ 150 days). The thoracic girth (P<0.05) was greater in fetuses from nutrient restricted ewes on day 45 and there was also a trend towards an increased gut weight (P<0.08). By day 90, the fetal brain and thymus weight were lighter in underfed than in well-fed animals whilst the weight of the fetal ovaries was heavier (P<0.05). On day 135 the fetal heart, pancreas, thymus, gut and kidney weights were lighter in undernourished ewes (P<0.05). When expressed as a percentage of fetal body weight, significance was retained in the heart, pancreas and thymus (P<0.05). Bone growth was also affected. At day 90 the fetal femur and metatarsal were longer in underfed mothers (P<0.05). In contrast, the fetal humerus and scapula were shorter in underfed than in well-fed animals, on day 135 (P<0.05) when the weight of the semitendinosus muscle (P<0.05) was also reduced. The fall in fetal glucose (P<0.1), insulin (P<0.01) and IGF-I (P<0.01) levels in underfed ewes on day 135 may have compromised fetal growth. Fetal plasma IGF binding protein-2 also increased between days 90 and 135 in underfed ewes (P<0.03), whilst levels were unaltered in well-fed animals. Although maternal and fetal plasma IGF-I levels increased with gestation (P<0.01) and the placentome morphology altered in all ewes (P<0.05), the fall in placental mass (P<0.05), amniotic and allantoic glucose concentrations (P<0.05) and maternal plasma glucose and insulin levels (P<0.05) in underfed ewes in late gestation may have compromised fetal substrate delivery. These perturbations in fetal development may have significant implications on adult health and carcass conformation, raising important health and economic issues in medical and agricultural sectors.

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Introduction

Perturbations in maternal nutrition during pregnancy may ‘programme’ permanent structural and physiological modifications in fetal development (review: Barker 1995, Robinson et al. 1999, Symonds et al. 2001). Whilst these adaptations may occur to sustain fetal development in utero, in extraterine life they are believed to have important pathological implications (review: Barker & Clark 1997, McMillen et al. 2001). At birth, these include reduced neonatal viability (Alexander 1974), whilst in adult life an increased predisposition to cardiovascular, metabolic and endocrine disease has been observed in humans (review: Barker & Clark 1997, Godfrey 1998), with poor wool and carcass quality arising in sheep (review: Black 1983, Bell 1992, Kelly et al. 1996). Despite these significant effects on clinical and agricultural domains, the mechanism by which maternal undernutrition affects fetal growth remains unknown.

Fetal growth depends upon the acquisition of substrate from diverse sites including: (i) the maternal compartment, via the placenta, as products of digestion or mobilised body reserves (McCrabb et al. 1992); (ii) the placental compartment (Chung et al. 1998); (iii) amniotic fluid (Ross & Nijland 1998) or (iv) from endogenous reserves within the fetus itself (Fowden 1997). Substrates such as glucose can promote fetal growth directly by providing energy and/or building blocks required for tissue growth (Fowden 1997). Other substrates act indirectly, regulating factors such as insulin to promote tissue accretion or insulin-like growth factor (IGF-I) to exert metabolic, mitogenic and differentiation activities (Fowden 1995, Harding & Johnston 1995).

Instances where fetal substrate delivery is disturbed may result in fetal development becoming compromised. This is reflected by the positive correlation that exists between placental and fetal weight at term (Kelly 1992). Factors in the maternal circulation including glucose, insulin and IGF-I may also influence fetal substrate availability by regulating nutrient partitioning between the maternal, placental and fetal compartments (Wallace et al. 1997, 2001). Glucose is transported across the placenta by
facilitated diffusion by two closely related glucose transporters, GLUT-1 and GLUT-3, which are regulated by glucose in a time- and concentration-dependent manner (Das et al. 1998, 2000). Insulin, in contrast, is believed to promote lipogenesis within the maternal compartment (Vernon et al. 1981), whilst maternal IGF-I influences glucose and amino acid transfer across the placenta (Kniss et al. 1994, Liu et al. 1994). The actions of the IGFs, IGF-I and IGF-II, are mediated via the type 1 receptor (IGF-1R) (Jones & Clemmons 1995). A family of high affinity insulin–like growth factor binding proteins (IGFBPs), designated IGFBP-1 to –6, regulate the biological activities of the IGFs (Ferry et al. 1999).

This study aimed to determine the effects of maternal nutrition on fetal growth and to elucidate the mechanisms involved. The effects of undernutrition on factors regulating fetal substrate delivery were investigated including the placenta, maternal plasma glucose, insulin and IGF-I levels and amniotic and allantoic protein and glucose concentrations. Tissues from pregnant ewes were studied at three critical time points during gestation: day 45 (when placentomes establish), day 90 (following placental growth) and day 135 (a period of rapid fetal growth). As fetal weight can be an insensitive marker of fetal growth (Hoet & Hanson 1999), individual organ development was studied to determine the effects of maternal undernutrition on fetal growth. Muscle and skeletal development were investigated due to the implications that maternal undernutrition may have on future carcass conformation, a factor of economical significance in agriculture.

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 standardized 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 100% of their daily maintenance requirements (based on the criteria of the Meat and Livestock Commission (MLC) 1988). The complete diet was fed in two equal rations at 0800 and 1600 h and supplied 10·8 MJ metabolizable energy and 150 g crude protein per kg dry matter.

Following 30 days acclimatisation, oestrus was synchronised by withdrawing progesterone impregnated sponges (60 mg medroxyprogesterone acetate, Veramix; Upjohn, Crawley, Sussex, UK) 12 days after their insertion. At sponge withdrawal ewes received an injection of a prostaglandin (PG) F_2α analogue, Estrumate (0·5 ml intra-muscular (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. Day 0 of gestation was taken as the first date at which ewes were observed to have an obvious raddle mark on their rump, indicative of mating by the ram. Pregnancy was confirmed by measuring plasma progesterone concentrations on day 16 of gestation using an enzyme immunoassay kit (Ridgeway Science Ltd, Rodmore Mill Farm, 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% diet (well fed) continued to receive 100% of their maintenance requirements throughout gestation whereas ewes allocated to the 70% diet (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 diets were reviewed weekly and adjusted on an individual basis following the MLC guidelines according to live weight changes, the stage of pregnancy and the number of fetuses carried.

Measurements

The gravid uterus was removed post-mortem at three time points during gestation (day 45 (n = 16), day 90 (n = 16) and day 135 (n = 16)) through a midline incision at a consistent point along the length of the cervix; it was then weighed. Samples of allantoic and amniotic fluid were collected by inserting a needle attached to a syringe into the allantoic and amniotic sac respectively. Fetal blood was collected at days 90 and 135 by cardiac puncture immediately prior to intra-cardiac administration of sodium pentobarbitone to the fetus. The blood and uterine fluids were placed on ice for no more than 20 min before being centrifuged at 1500 g for 15 min at 4 °C and the plasma stored at −20 °C until analysed. The fetus was blotted dry, weighed and sexed. The crown–rump length (CRL) and fetal girth at the fore leg (thoracic girth) and umbilicus was measured. The internal organs were dissected out and weighed with the fluid contents of the fetal gut and heart being removed before weighing. The fore and hind limbs from the left side of the fetus were removed on days 90 and 135 of gestation and X-rayed to determine bone length. The semitendinosus muscle was also removed from the left hind limb on day 135 and weighed. The total volume of uterine fluid was measured. 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...
Blood sampling and assays

Weekly blood samples were taken by jugular venepuncture 5 h after the morning feed from all ewes throughout gestation, and analysed for insulin, glucose and systemic IGF-I concentrations. Fetal blood samples were analysed for insulin, glucose, IGF-I, total protein, IGFBP-2 and IGFBP-3. Allantoic and amniotic fluids were analysed for glucose and total protein content.

Insulin-like growth factor-I

IGF-I in maternal plasma was analysed by radioimmunoassay after ethanol–acetone–acetic acid extraction according to the method of Enright et al. (1989) using recombinant human (rh) IGF-I (Bachem Ltd, Saffron Waldon, Essex, UK) as standard, iodinated rhIGF-I (iodogen method) as tracer and antibody raised against rhIGF-I (Biogenesis Ltd, Poole, Dorset, UK). The detection limit was 8·6 ng/ml and the inter- and intra-assay coefficients of variation were 7·1% and 3·2% respectively. Uterine fluid and fetal plasma glucose levels were measured by an enzymatic colorimetric method according to the method of Werner et al. (1991), placentomes typed according to the overall shape of the placentome i.e. rounded or flat and the position of the fetal tissue (cotyledon) in relation to the maternal portion of the placentome (caruncle), the placentome was classified as type A, B, C or D where the proportion of fetal tissue increased from type A to type D.

Insulin

Plasma insulin was measured by ELISA (DRG Diagnostics, Immunodiagnostic Systems Ltd, Tyne and Wear, UK). The detection limit was 0·1 ng/ml and the inter- and intra-assay coefficients of variation were 1·2% and 2·5% respectively.

Glucose

Maternal plasma glucose was measured by an enzymatic colorimetric method according to the method of Werner et al. (1971) using a glucose reagent from Roche Diagnostics (Lewes, East Sussex, UK; catalogue number 2 208 113). Colour development was monitored at 420 nm. The detection limit was 1·5 mmol/l and the inter- and intra-assay coefficients of variation were 2·7% and 2·2% respectively. Uterine fluid and fetal plasma glucose levels were below the limit of detection of this technique and so were measured using an automated glucose analyser (Bayer Opera, Business Group Diagnostics, Tarrytown, NY, USA).

Protein

Protein was measured by an enzymatic colorimetric method using a Bio–Rad diagnostic kit (Bio–Rad Laboratories Ltd, Hemel Hempstead, Herts, UK; catalogue number 500–0116). Colour development was monitored at 690 nm. The detection limit was 0·125 mg/ml and the intra-assay coefficient of variation was 5%.

Western blotting (IGFBP-2, IGFBP-3)

Fetal plasma (4 µl) diluted 1:4 in Laemmli sample buffer (Bio–Rad) was heated to 95 °C for 5 min. Samples and molecular weight markers (Bio–Rad) were loaded onto a 4% stacking gel and electrophoresed through a 16% (w/v) polyacrylamide analysis gel (Bio–Rad). The gel was run at 90 v for 20 min and thereafter at 200 v for 30 min in 1 × Tris–glycine running buffer (Bio–Rad) with 0·1% SDS (pH 8·3) using a Bio–Rad mini–Protein II electrophoresis system. After electrophoresis, the gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 1·3 mM SDS, pH 8·3) containing 20% methanol (v/v) for 30 min before being transferred to 0·2 μm nitrocellulose membranes (Hybond-C pure, Amersham Pharmacia Biotech, Amersham, Bucks, UK) at 15 v for 4 h using a semi–dry electrophoretic blotter (CBS, Scientific Co. Inc., Del Mar, CA, USA). The membranes were blocked in Tris–buffered saline (TBS; 20 mM Tris base, 500 mM NaCl, pH 7·4) containing 3% bovine serum albumin for 2 h and then washed twice for 10 min in TBS alone. The membranes were incubated on a rocking platform overnight at room temperature with 1:1000 dilution of antiserum against bovine IGFBP-2 or –3 in TBS containing 1% gelatine. The antisera were kindly donated by Dr M C Lucy (University of Missouri, Columbia, MO, USA). After incubation, the membranes were rinsed twice in TBS (10 min/wash) and antigen–antibody complexes were detected using a 1:1000 dilution of biotinylated anti–rabbit immunoglobulin (Sigma, Poole, Dorset, UK) in TBS for 1 h. Following two TBS washes (10 min/wash) the membranes were incubated in a TBS solution containing a 1:25 000 dilution of Extravidin AP (Sigma) for 1 h. The membranes were rinsed twice more in TBS (5 min/wash) and placed on a clean flat surface where each was covered with a thin layer of 5–bromo–4–chloro–3–indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate (Sigma). The membrane was observed until bands appeared and was then rinsed in distilled water before finally being left to air dry. IGFBP-2 appeared as a single band of approximately 30 kDa whilst IGFBP-3 was seen as a doublet of approximately 35–40 kDa (Fig. 1). Band intensity was measured using Bio–Rad Molecular

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Results were considered statistically significant when using the MIXED procedures of SAS (version 6.12). Concentrations were analysed using a repeated measures design. Maternal plasma insulin, glucose and IGF-I concentrations did not alter with diet but tended to be lower in undernourished ewes on day 135 (100% diet, n=4, 70% diet, n=5) and day 135 (100% diet, n=4, 70% diet, n=5). The well-fed ewes (on the 100% diet) maintained their body condition score of 2.5 at mating until the end of the study, whereas the undernourished ewes (on the 70% diet) dropped approximately half a body condition score by day 135 of gestation.

Placental data

The effect of diet on placental development will be reported in a companion paper (J C Osgerby, D C Wathes, D Howard & T S Gadd, work in progress) and is only summarised briefly here. Over time the growth of the placenta differed between the two dietary treatments. Initially, both groups of ewes experienced a significant increase in their total and average placentome weights between days 45 and 90 (P<0.05). The well-fed ewes maintained these weights to day 135 whilst the placenta from the undernourished ewes decreased significantly from their day 90 weights (day 90, n=5, 559 ± 33·7 g vs day 135, n=5, 438 ± 44·4 g, P<0.05). The total placental weight, therefore, tended to be lower in undernourished than in well-fed mothers on day 135 (100% diet, n=4, 540 ± 24·8 g vs 70% diet, n=5, 438 ± 44·4 g, P<0.08). Placental morphology was also altered as placentae from the undernourished mothers had significantly more D type (70% diet, n=5, 12·7 ± 3·9% vs 100% diet, n=4, 1·5 ± 0·9%) and C type (70% diet, n=5, 18·8 ± 6·0% vs 100% diet, 1·95 ± 1·95%) placentomes and fewer B type (70% diet, n=5, 61·1 ± 10·6% vs 100% diet, n=4, 89·7 ± 3·1%) placentomes than their well-fed counterparts at this stage (P<0.05). There were no A type placentomes in either group. The total placenta number was unaffected by diet and gestational age (77·7 ± 2·72, n=27).

Fetal data

In undernourished fetuses the thoracic girth was increased at day 45 of gestation (P<0.05), unchanged by diet at day 90 but decreased significantly by day 135 (P<0.01) (Table 1). The umbilical girth was not different at days 45 or 90 but was lower in undernourished ewes on day 135 (P<0.01) (Table 1). The fetal body weight, in contrast, did not alter with diet but tended to be lower in undernourished ewes on day 135 (Table 1; P<0.01). At day 45 of gestation the gut weight tended to be greater in underfed ewes (P<0.08) and this change was
### Table 1
Effect of maternal undernutrition on weights and measurements of fetuses at days 45, 90 and 135 of gestation. Values are expressed as the mean ± S.E.M.

<table>
<thead>
<tr>
<th>Maternal nutrition</th>
<th>100% diet (n=4/5)</th>
<th>70% diet (n=4/5)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean weight</td>
<td>% Body weight</td>
</tr>
<tr>
<td></td>
<td>or length</td>
<td></td>
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<tr>
<td><strong>Day 45</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal body weight  (g)</td>
<td>8.6 ± 0.56</td>
<td></td>
</tr>
<tr>
<td>Thoracic girth (cm)</td>
<td>4.3 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Umbilical girth (cm)</td>
<td>5.0 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Gut (g)</td>
<td>0.2 ± 0.02</td>
<td>2.5 ± 0.16</td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>0.08 ± 0.006</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>0.71 ± 0.06</td>
<td>8.3 ± 0.46</td>
</tr>
<tr>
<td>Adrenals (g)</td>
<td>0.03 ± 0.006</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0.1 ± 0.004</td>
<td>1.26 ± 0.05</td>
</tr>
<tr>
<td>Lungs (g)</td>
<td>0.31 ± 0.04</td>
<td>3.6 ± 0.46</td>
</tr>
<tr>
<td><strong>Day 90</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal body weight  (g)</td>
<td>574 ± 31.0</td>
<td></td>
</tr>
<tr>
<td>Thoracic girth (cm)</td>
<td>17.7 ± 0.46</td>
<td></td>
</tr>
<tr>
<td>Umbilical girth (cm)</td>
<td>19.8 ± 0.67</td>
<td></td>
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<tr>
<td>Gut (g)</td>
<td>19.5 ± 1.71</td>
<td>3.37 ± 0.14</td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>5.2 ± 0.3</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>41.9 ± 2.77</td>
<td>7.36 ± 0.55</td>
</tr>
<tr>
<td>Adrenals (g)</td>
<td>0.14 ± 0.02</td>
<td>0.02 ± 0.002</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>3.7 ± 0.31</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td>Lungs (g)</td>
<td>31.0 ± 2.35</td>
<td>5.46 ± 0.51</td>
</tr>
<tr>
<td>Brain (g)</td>
<td>15.4 ± 0.61</td>
<td>2.70 ± 0.09</td>
</tr>
<tr>
<td>Thymus (g)</td>
<td>2.3 ± 0.24</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>0.76 ± 0.04</td>
<td>0.13 ± 0.009</td>
</tr>
<tr>
<td>Pancreas (g)</td>
<td>0.63 ± 0.01</td>
<td>0.11 ± 0.005</td>
</tr>
<tr>
<td>Thyroid (g)</td>
<td>0.23 ± 0.02</td>
<td>0.04 ± 0.003</td>
</tr>
<tr>
<td>Ovaries (mg)</td>
<td>340 ± 1.53</td>
<td>0.006 ± 0.0001</td>
</tr>
<tr>
<td>Testes (g)</td>
<td>0.27 (n=1)</td>
<td>0.05</td>
</tr>
<tr>
<td>Femur (cm)</td>
<td>2.6 ± 0.06</td>
<td></td>
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<tr>
<td>Metatarsal (cm)</td>
<td>2.4 ± 0.06</td>
<td></td>
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<tr>
<td><strong>Day 135</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal body weight  (g)</td>
<td>4395 ± 99.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Thoracic girth (cm)</td>
<td>37.3 ± 0.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Umbilical girth (cm)</td>
<td>37.8 ± 0.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Gut (g)</td>
<td>196 ± 5.9&lt;sup&gt;x&lt;/sup&gt;</td>
<td>4.45 ± 0.03&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>210 ± 1.27&lt;sup&gt;xc&lt;/sup&gt;</td>
<td>0.40 ± 0.03&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>133 ± 8.1</td>
<td>3.01 ± 0.13</td>
</tr>
<tr>
<td>Adrenals (g)</td>
<td>0.45 ± 0.05</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>38.2 ± 1.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87 ± 0.03&lt;sup&gt;3c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lungs (g)</td>
<td>130 ± 14.0</td>
<td>2.98 ± 0.35</td>
</tr>
<tr>
<td>Brain (g)</td>
<td>53.5 ± 1.11</td>
<td>1.20 ± 0.05</td>
</tr>
<tr>
<td>Thymus (g)</td>
<td>280 ± 1.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64 ± 0.05&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>80 ± 0.96</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>Pancreas (g)</td>
<td>48 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11 ± 0.007&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thyroid (g)</td>
<td>0.89 ± 0.05</td>
<td>0.02 ± 0.001</td>
</tr>
<tr>
<td>Ovaries (mg)</td>
<td>67.7 ± 1.57&lt;sup&gt;n&lt;/sup&gt;(n=3)</td>
<td>0.002 ± 0.0003</td>
</tr>
<tr>
<td>Testes (g)</td>
<td>1.7 (n=1)</td>
<td>0.04</td>
</tr>
<tr>
<td>Semitendinosus muscle (g)</td>
<td>84 ± 0.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Humerus (cm)</td>
<td>7.7 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Scapula (cm)</td>
<td>6.5 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Femur (cm)</td>
<td>8.9 ± 0.06</td>
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Within rows: a>b, P<0.01; c>d, P<0.05; g>h, P<0.05; e>f, P<0.01.
significant when expressed as a percentage of body weight (Table 1; $P<0.05$). The other organs measured on day 45 of gestation were not altered by diet when expressed either as weight or as percentage body weight. These included the kidneys, liver, adrenals, heart and lungs. By day 90, however, significant organ weight differences could be detected for the ovaries (which were heavier) and the thymus and brain (which were lighter) in the undernourished ewes (Table 1; $P<0.05$). When expressed as a percentage of body weight the thymus and ovaries were significantly different between treatment groups (Table 1). The following organs were unaffected by diet on day 90 of gestation when expressed as weight or percentage body weight: gut, kidneys, liver, adrenals, heart, lungs, spleen, testes, pancreas and thyroid. At day 135 a wide range of organs were growth retarded by the 70% diet (Table 1). These included the gut, kidneys, heart, thymus and pancreas ($P<0.05$). The heart, thymus, pancreas ($P<0.05$) and gut ($P<0.1$) were lower in underfed ewes when expressed as a percentage of body weight (Table 1). The following organs were not growth retarded on day 135 when expressed as weight or percentage body weight: liver, adrenals, lungs, brain, spleen, thyroid, ovaries and testes.

Crown–rump length was not affected by maternal diet (day 45, $6.78 \pm 0.05$ cm, day 90, $27.2 \pm 0.32$ cm and day 135, $45.9 \pm 0.73$ cm), but X-rays of the fetal hind and forelegs at days 90 and 135 showed that skeletal development was significantly altered (Table 1). At day 90 there was a trend for the leg bones of fetuses from undernourished mothers to be longer; however only the femur and metatarsal reached levels of significance ($P<0.05$). In contrast, at day 135 the trend was for bones from the undernourished fetuses to be shorter. The humerus and scapula were significantly shorter ($P<0.05$) with the femur not quite reaching significance. In addition, at day 135 the weight of the semitendinosus muscle was significantly reduced in fetuses from undernourished mothers (Table 1; $P<0.05$).

Allantoic and amniotic fluid

The total volume of uterine fluid was unaltered by diet but increased significantly with time between days 90 and 135 of gestation (day 90, $n=9$, $742 \pm 35.9$ ml vs day 135, $n=9$, $1259 \pm 104$ ml, $P<0.05$). Protein levels differed between the allantoic and amniotic fluid and increased with gestational age (Fig. 2A,B; $P<0.001$). Glucose concentrations altered in allantoic fluid with time and diet, decreasing with gestation and with a 70% diet on day 135 (Fig. 2C; $P<0.02$). A similar trend was observed in the amniotic fluid with glucose levels decreasing between days 45, 90 and 135 of gestation (day 45, $n=9$, $1.5 \pm 0.08$ mmol/l; day 90, $n=9$, $0.79 \pm 0.02$ mmol/l; day 135, $n=8$, $0.23 \pm 0.04$ mmol/l; day 45>day 90>day 135, $P<0.01$) and with a 70% diet on day 135 (100% diet, $n=4$, $0.32 \pm 0.01$ mmol/l vs 70% diet, $n=4$, $0.14 \pm 0.03$ mmol/l; 100% diet>70% diet, $P<0.05$).

Figure 2 The concentration of (A) protein in allantoic fluid, (B) protein in amniotic fluid and (C) glucose in allantoic fluid in well-fed (100% diet; solid bars) and underfed (70% diet; open bars) ewes on days 45, 90 and 135 of gestation. Values are means ± S.E.M. Bars with different letters are significantly different ($P<0.05$).
Maternal metabolic profiles

**Glucose** Undernourished ewes had lower plasma glucose concentrations than their well-fed counterparts between days 27 and 132 of gestation (Fig. 3A; *P* < 0.02). There was no significant effect of time on glucose concentrations.

**Insulin** Plasma insulin concentrations tended to be higher in underfed ewes between days 0 and 83 of gestation although this did not reach significance. Between days 97 and 132 this trend was reversed with underfed ewes exhibiting lower levels of insulin than well-fed animals (Fig. 3B; *P* < 0.01). Insulin levels altered with time in well-fed ewes, increasing between days 55 and 111. This slight rise was not significant in underfed animals.

**IGF-I** Plasma IGF-I concentrations increased between days 0 and 97 and days 0 and 104 of gestation in underfed and well-fed ewes respectively (Fig. 3C; *P* < 0.01). There were no significant effects of diet although levels tended to be higher in undernourished than in well-fed ewes between days 48 and 97 (Fig. 3C; *P* < 0.08) with this trend reversing from day 104.

**Fetal plasma IGF-I, insulin, glucose and protein**

Fetal IGF-I concentrations mirrored maternal levels with fetuses from undernourished mothers tending to be higher at day 90 but significantly lower than their well-fed counterparts at day 135 (Table 2; *P* < 0.01). Fetal IGF-I concentrations in both treatment groups also increased with time, being significantly higher at day 135 than at day 90 of gestation (Table 2; *P* < 0.001). Insulin concentrations were not affected by maternal diet at day 90 but by day 135 concentrations in the undernourished fetuses were significantly lower (Table 2; *P* < 0.01). At day 90 and day 135 there was a trend for glucose levels in undernourished fetuses to be lower but levels did not reach significance (Table 2; *P* < 0.1). The concentration of total protein in fetal plasma increased significantly between days 90 and 135 in both groups (Table 2; *P* < 0.003) but was not affected by maternal diet.

**Fetal IGFBP-2 and IGFBP-3**

IGFBP-2 levels increased significantly between days 90 and 135 in fetuses from undernourished but not well-fed ewes (Table 2; *P* < 0.03). Maternal diet did not significantly affect IGFBP-3 levels, although in both the lower and upper bands of the IGFBP-3 doublet there was a trend for levels in fetuses from the undernourished mothers to be lower (*P* < 0.2) (Table 2). The upper band in both treatment groups increased significantly with the stage of gestation (Table 2; *P* < 0.01).

**Discussion**

The fetal origins hypothesis suggests that in humans poor nutrition *in utero* is associated with an increased predisposition to major illnesses in later life, in particular cardiovascular disease and non-insulin dependent diabetes (review: Barker & Clark 1997). In this study, nutritionally mediated alterations in ovine fetal growth were evident from day 45 of gestation. As pregnancy progressed, the
Table 2 Factors measured in fetal plasma on days 90 and 135 from well-fed (100% diet) and undernourished (70% diet) ewes

<table>
<thead>
<tr>
<th></th>
<th>Day 90</th>
<th>Day 135</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100% (n=4)</td>
<td>70% (n=5)</td>
</tr>
<tr>
<td></td>
<td>100% (n=4)</td>
<td>70% (n=5)</td>
</tr>
<tr>
<td>IGF-I (ng/ml)</td>
<td>46·6 ± 6·71e</td>
<td>57·2 ± 6·00f</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0·33 ± 0·02a</td>
<td>0·33 ± 0·02a</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>1·43 ± 0·27c</td>
<td>0·99 ± 0·27b</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>6·36 ± 0·58b</td>
<td>6·42 ± 0·52b</td>
</tr>
<tr>
<td>IGFBP-2 (OD × mm²)</td>
<td>2·03 ± 0·30e</td>
<td>1·53 ± 0·30e</td>
</tr>
<tr>
<td>IGFBP-3 lower band (OD × mm²)</td>
<td>1·27 ± 0·14a</td>
<td>1·07 ± 0·14a</td>
</tr>
<tr>
<td>IGFBP-3 upper band (OD × mm²)</td>
<td>0·19 ± 0·06b</td>
<td>0·13 ± 0·06b</td>
</tr>
</tbody>
</table>

Within rows: a>b>c, P<0·01; d>e>f, P<0·05; g>h, P<0·1.
OD, optical density.

The effects of chronic maternal undernutrition became widespread, with the heart and pancreas, organs central to the fetal origins hypothesis, becoming severely growth retarded. Bone and muscle development were also affected, parameters which are critical to subsequent carcass conformation and meat quality in sheep. Despite these alterations in fetal growth, the fetal weight did not alter significantly, confirming the insensitivity of fetal weight as an indicator of fetal growth as proposed by Harding & Johnston (1995).

On day 45 of gestation, the thoracic girth was greater in fetuses from underfed than well-fed ewes, with the weight of the gut showing a similar trend. When expressed as a percentage of fetal body weight, the gut was greater in undernourished ewes suggesting fetal growth had been altered asymmetrically. By day 90, the brain and thymus were lighter in fetuses from underfed mothers, whilst the weight of the ovaries increased. When expressed as a percentage of fetal body weight, the thymus and ovaries were significantly different in undernourished ewes. Maternal dietary restriction in sheep has previously been shown to reduce the fetal brain weight (Harding & Johnston 1995, Clarke et al. 1998), disrupt ovarian development (Borwick et al. 1998) and compromise lifetime reproductive performance in subsequent female offspring (Gunn et al. 1995, review: Rhind et al. 2001). Isocaloric low protein maternal diets have also altered brain development in the offspring of rats (review: Hoet & Hanson 1999). A relationship between malnutrition and thymic atrophy has also been observed in humans (Beisel 1992) with chronic maternal undernutrition compromising fetal thymus growth in sheep (Bauer et al. 1995). Alterations in fetal thymic development may contribute to the detrimental effects maternal malnutrition can have on the immunocompetence of offspring (Moore 1998).

Fetal development may have altered in early to mid-gestation due to the changes in maternal metabolic status. Maternal plasma glucose concentrations were lower in underfed ewes between days 27 and 90 of gestation whilst maternal insulin and maternal and fetal IGF-I concentrations all tended to be higher in these animals on or prior to day 90. The placental mass and amniotic and allantoic glucose and protein levels were unaltered between groups during this period.

On day 135 of gestation, the effects of maternal undernutrition on fetal growth were diverse. The weight of the fetal gut, heart, pancreas, kidneys and thymus were all significantly lighter in undernourished ewes, although when expressed as a percentage of fetal body weight only the heart, pancreas and thymus were different. Contrary to observations on day 90 of gestation, the brain and ovaries were no longer different between groups. This may be attributed to a brain-sparing reflex whereby nutrients are diverted to spare brain metabolism at the expense of the trunk, limbs and abdominal viscera (Rudolph 1984, Godfrey & Robinson 1998).

In late gestation, maternal undernutrition may have retarded fetal growth by disrupting a series of placental and systemic factors. As the ovine fetus undergoes a rapid phase of growth between days 90 and 135 of gestation (Symonds et al. 2001), the reduction in placental weight and alteration in placentome morphology in undernourished ewes during this period may perturb fetal growth by altering nutrient delivery. Similar nutritionally mediated changes in placental weight (Holst et al. 1992, review: Mellor 1983) and shape (Wallace et al. 2001) have previously been reported in the ewe. The trend for maternal plasma glucose and IGF-I to decrease with undernutrition in late gestation may also alter fetal glucose and amino acid delivery (Kniss et al. 1994, Liu et al. 1994, Hay 1995). Reductions in maternal glucose and IGF-I concentrations have previously been reported in underfed pregnant ewes (Bauer et al. 1995). Plasma insulin levels also decreased in undernourished animals, potentially depicting a change in maternal adipose tissue metabolism in favour of lipid mobilisation. This may explain the fall in maternal body condition in underfed ewes in late gestation (Vernon et al. 1981). Amniotic and allantoic glucose levels were also reduced in underfed ewes on day 135, although the biological significance of this remains to be determined.

The reduction in placental weight and maternal systemic factors in underfed ewes in late gestation may
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Contribute to the decline in fetal plasma glucose, insulin and IGF-I levels observed in undernourished animals on day 135. Reductions in ovine fetal plasma glucose, insulin and IGF-I have been reported earlier following periods of maternal dietary restriction (Bauer et al. 1995, Gallaher et al. 1998). Glucose provides the fetus with 30% of its daily carbon and energy requirements in the fed state (Fowden 2001), whilst fetal insulin promotes tissue accretion and cell proliferation in bone and soft tissue (Fowden 1989, 1995). As a primary regulator of fetal IGF-I, the fall in fetal insulin levels may explain the reduction in plasma IGF-I in undernourished fetuses on day 135 (Fowden 1995). IGF-I is also critical to fetal growth and anabolism, influencing gut (Kimble et al. 1995), renal (Hammerman & Miller 1993) and pancreatic β cell development (Hugl et al. 1998) as well as cardiac growth and function (Fsgaard et al. 1999). The decline of fetal glucose, insulin and IGF-I may thus contribute to nutritionally mediated changes in fetal growth.

In vivo, the biological actions of the IGFs are regulated by the IGFBPs. Fetal plasma IGFBP-2 levels increased between days 90 and 135 of gestation in undernourished ewes whilst levels remained unchanged in their well-fed counterparts. IGFBP-2 has previously been shown to increase following dietary restriction (sheep: Lee et al. 1998). The fall in fetal insulin in underfed fetuses may have mediated this change due to IGFBP-2 being inversely regulated by this factor (review: Kelley et al. 1996). In contrast, fetal plasma IGFBP-3 levels tended to decrease in fetuses from underfed mothers (P<0.2) as previously reported in sheep (Lee et al. 1997, Gallaher et al. 1998). Earlier studies have reported reduced plasma IGFBP-3 and IGF-I levels and IGF-I mRNA expression in the muscle, lung and kidney of fetal sheep when placental growth was restricted (Kind et al. 1995).

Although the umbilical and thoracic girths were suppressed in underfed ewes on day 135 of gestation, the CRL was unaltered. From day 90, however, the length of the fetal femur and metatarsal were increased by maternal undernutrition. The trend for IGF-I to be higher in underfed mothers in mid-gestation, and in their fetuses on day 135, the fetal somatotropic axis during long term maternal undernutrition in sheep: evidence for nutritional regulation in utero. Endocrinology 136 1250–1257.


In summary, chronic maternal undernutrition altered fetal growth from early in gestation. As pregnancy pro-gressed, the detrimental effects of undernutrition became widespread and severe. Maternal undernutrition may alter fetal growth by moderating fetal substrate delivery. This, in turn, may disrupt the fetal glucose–insulin–IGF-I axis, a system that is pivotal to fetal development. These perturbations in fetal growth may have significant implications on adult health and carcass conformation, raising significant health and economic issues in both the medical and agricultural sectors.

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