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## LOWER MATERNAL BODY CONDITION DURING PREGNANCY AFFECTS SKELETAL MUSCLE STRUCTURE AND GLUT-4 PROTEIN LEVELS BUT NOT GLUCOSE TOLERANCE IN MATURE ADULT SHEEP

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### Abstract

Sub-optimal maternal nutrition and body composition are implicated in metabolic disease risk in adult offspring. We hypothesized that modest disruption of glucose homeostasis previously observed in young adult sheep offspring from ewes of a lower body condition score (BCS) would deteriorate with age, due to changes in skeletal muscle structure and insulin signalling mechanisms. Ewes were fed to achieve a lower (L, n=10) or higher (H, n=14) BCS before and during pregnancy. Baseline plasma glucose, glucose tolerance and basal glucose uptake into isolated muscle strips was similar in male offspring at 210±4 weeks. Vastus total myofibre density (HBCS, 343±15; LBCS, 294±14 fibres/mm<sup>2</sup>, p<0.05) and fast myofibre density (HBCS, 226±10; LBCS 194±10 fibres/mm<sup>2</sup>, p<0.05), capillary to myofibre ratio (HBCS, 1.5±0.1; LBCS 1.2±0.1 capillary:myofibre, p<0.05) were lower in LBCS offspring. Vastus protein levels of Akt1 were lower (83±7% of HBCS, p<0.05), and total GLUT-4 was increased (157±6% of HBCS, p<0.001) in LBCS offspring. Despite the reduction in total myofibre density in LBCS offspring, glucose tolerance was normal in mature adult life. However such adaptations may lead to complications in metabolic control in an overabundant postnatal nutrient environment.

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### DISCLOSURES

There are no conflicts of interest.

### AUTHOR CONTRIBUTIONS

LRG, PMC, SEO and MAH contributed to the conception and design of experiments;

LRG, PMC, SEO, MAH, LJH, RLC, NB and HPP, and contributed to the collection, analysis and interpretation of data;

LRG, PMC, SEO, MAH, LJH, AAS and CC contributed to drafting and revision of this article.

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## Keywords

Glucose uptake; myofibres; type 2 diabetes; maternal body condition; skeletal muscle

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## INTRODUCTION

Recent UK guidelines highlight the importance of maternal weight in influencing the health of the offspring, including the risk of adult obesity and metabolic dysfunction (e.g. glucose intolerance and insulin resistance)<sup>1</sup>. Indeed the offspring of low body weight or low body mass index mothers have been shown to be at increased risk of insulin resistance in studies worldwide<sup>2-4</sup>. It is clear that the trajectory of risk of such non-communicable diseases commences early in life and then increases with age<sup>5-7</sup>. We previously reported increased fasting glycaemia, mild glucose intolerance and impaired initial insulin secretion in young male adult offspring of lower body condition score ewes, independent of any change in birthweight<sup>8</sup>. The aim of this study was to determine in the same animals whether this effect worsened with age and if it was mediated by changes in skeletal muscle structure and insulin signalling mechanisms.

In this study we focussed on skeletal muscle as this is the primary tissue for glucose disposal postprandially, and changes in skeletal myofibre composition and capillary density are linked with type 2 diabetes<sup>9</sup> and obesity<sup>10</sup>. We have previously shown in sheep that either early or late gestation under-nutrition reduced total myofibre and capillary density in the late gestation fetus (no effect on body weight), and that the effect of the late gestation maternal under-nutrition was predominantly on the density of the slow-twitch myofibres<sup>11</sup>. In sheep, a 50 % global nutrient restriction in the peri-conceptual period (-18 to 6 dGA) similarly reduced myofibre number in the mid-gestation fetus (no effect on fetal weight)<sup>12</sup> and a 50% maternal under-nutrition from 28 to 70 dGA decreased total myofibre number and increased fast myosin type IIb isoform levels in 8 month old offspring (live weight was increased)<sup>13</sup>. In the present study we investigated the effect of a change in maternal body condition before and throughout gestation to reflect the situation in humans that women who are lean during pregnancy are generally also lean before entering pregnancy. Previously in rodents a 40 % global restriction throughout gestation reduced birth weight and reduced myofibre number in the neonatal guinea pig<sup>14</sup>. Such changes in muscle mass and composition may contribute to later insulin resistance and glucose intolerance, but to our knowledge this has not yet been determined together in one animal model.

Insulin resistance in muscle is one of the earliest identifiable abnormality in pre-diabetic patients<sup>15</sup>. Low birth weight has been associated with reduced expression of protein kinase C zeta (PKC  $\zeta$ ), the p85 and p110 subunits of phosphoinositol-3-kinase and GLUT-4 in skeletal muscle of young Danish men<sup>16</sup>, which could be a precursor to longer term altered glucose handling. Recently we found that GLUT-4 and insulin receptor mRNA levels were increased in fetal sheep skeletal muscle following late gestation maternal under-nutrition and that these effects were accompanied by a decrease in slow-twitch myofibre density<sup>11</sup>. GLUT-4 protein levels were reduced in young adult sheep perirenal fat following late gestation maternal undernutrition and this was associated with impaired glucose tolerance (no effect on birth weight or weight at 1 year)<sup>17</sup>. The content of GLUT-4 was lower in skeletal muscle of 8 month old lambs following a 50% nutrient restriction from 28-70 dGA<sup>13</sup>. A low protein diet during pregnancy and lactation led to insulin resistance in skeletal muscle in 15 month old rat offspring (no effect on weight) and was associated with reduced expression of PKC  $\zeta$  in soleus muscle<sup>18</sup>.

In a range of species including humans, offspring phenotype can be influenced by the developmental environment even within the normal range<sup>19</sup>. We hypothesized that the effect of changes within the normal range maternal body condition on glucose tolerance of their offspring would worsen in mature adulthood. Therefore in sheep we determined the effect of a higher or lower maternal body condition on mature adult offspring response to a glucose load *in vivo*. To investigate mechanisms contributing to glucose tolerance, we measured the uptake of glucose into isolated skeletal muscle strips, skeletal muscle morphology and the expression of insulin signalling proteins in skeletal muscle and fat.

## MATERIALS AND METHODS

All procedures were carried out with local ethics approval and in accordance with the regulations of the UK Home Office Animals (Scientific Procedures) Act, 1986.

### Animals and study design

The body condition score (BCS) is a system used to estimate the amount of muscle and fat coverage in the third lumbar region and is measured on a scale of 1 to 5 (emaciated to obese)<sup>20</sup>. The study design and animal husbandry are described in full in Cripps *et al.*, 2008. Briefly, second-parity welsh Mountain ewes were housed individually on straw and established at a BCS of 2 (lower BCS [LBCS]) or greater than or equal to 3 (higher BCS [HBCS]) by adjusting the daily ration of a complete pelleted diet (Charnwood Milling Co Ltd, Suffolk, UK). Ewes were mated and maintained at the desired BCS by adjusting daily rations and gestational increases in ration were applied according to standard guidelines<sup>21</sup>. To achieve the lower body condition score LBCS animals were fed a daily energy intake of ~57% the HBCS group. The ewes were allowed to deliver spontaneously, and female offspring were removed from the study. From birth, ewes and intact male offspring were housed in 2 groups according to the maternal BCS and the ewes were fed 1.25 times (birth to 2 weeks) and 1.5 times (3 weeks to 12 weeks) their term diet ration in order to maintain their BCS during the lactation period<sup>8</sup>, at 10 weeks: LBCS ewes, 42.3±1.3 kg/1.7±0.0 BCS; vs. HBCS ewes, 55.2±1.8 kg/3.1±0.1 BCS. P<0.001). At 12 weeks the lambs were weaned, housed as one group and allowed creep pelleted diet (As fed providing 10.59 MJ/kg and 18 g crude protein per 100g. Prestige Lamb Pellets + Detox, BOCM Pauls Ltd., UK), hay and water *ad libitum* until 24 weeks. From 24 weeks male offspring were fed a ration of creep pelleted diet (0.75 kg/sheep/day) with hay and water *ad libitum*. From 32 weeks until 1.5 years of age, male offspring were housed as one group and fed standard pelleted diet (0.5 to 1.0 kg/sheep/day. As fed providing 10.38 MJ/kg and 18g crude protein per 100g. Ewbol 18, BOCM Pauls Ltd., UK) with hay and water *ad libitum*.

An initial study was conducted at 1.5 yr<sup>8</sup> (17 LBCS and 19 HBCS intact rams). Between 1.5 and 4 years of age (life expectancy of sheep is approximately 10-12 years) animals were kept on grass (as one group) from May to October or indoors (November to April) with access to standard pelleted diet (0.9 to 1.2 kg/sheep/day, Ewbol 18 following assessment of flock weight and BCS) with hay and water *ad libitum*. There were no differences between the groups at 2 years of age in body weight (HBCS, 82.9±1.4; LBCS, 84.1±2.0 kg P=0.6) and BCS (HBCS, 2.5±0.1; LBCS, 2.5±0.1 P=1.0), or at 3 years of age in body weight (HBCS, 85.7±1.6; LBCS, 87.6±1.1 kg P=0.4) and BCS (HBCS, 2.8±0.1; LBCS, 3.0±0.1 P=0.3). After 1.5 years, 5 rams died due to either infection or fighting accidents (rams were intact). At 210 ± 4 weeks in 10 LBCS and 14 HBCS intact male singleton offspring we measured body weight, BCS by palpitation and fat and muscle depth by ultrasound scanning<sup>22</sup>.

### Glucose tolerance test

At  $210 \pm 4$  weeks the mature intact adult male offspring were acclimatized to metabolic carts for 5 days with daily pelleted ration and *ad libitum* access to hay. Pelleted food was given 27 hr before, and hay was removed 19 hr before, the administration of the glucose bolus. Sheep were given *ad libitum* access to water throughout the protocol. A temporary intravenous catheter (Radiopaque FEP 14G  $\times$  140 mm; Abbott Laboratories Ltd, Maidenhead, UK) was placed in the jugular vein via a small incision in the skin under local anaesthesia (2 ml Lignol, Arnolds Veterinary Products Ltd, Shrewsbury, UK). A 2-hour recovery period was allowed prior to the *i.v.* glucose tolerance test (GTT). Blood samples (7 ml) were collected before ( $-30$ ,  $-15$ , 0 min) and after (5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 210 min) an *i.v.* glucose bolus (0.5 g/kg body weight over 2 min) onto chilled EDTA/fluoride tubes (Teklab Ltd, Durham, UK). These were centrifuged, and the plasma frozen in aliquots and stored at  $-80$  °C. Once the procedure was completed animals were given a standard pelleted ration and hay was returned.

### Post mortem

The last pelleted feed was given 27 hours before, and hay removed 19 hrs before, the post-mortem. Water was provided *ad libitum*. Animals were killed with an overdose of sodium pentobarbitone (*i.v.* 145 mg/kg). Body weight, crown-rump length, abdominal circumference, biparietal diameter, femur length and shoulder height were recorded. Under sterile conditions major organs were weighed. A 1 g sample of pancreas was placed into 10 ml of 180 mmol HCl on ice and the tissue minced with scissors before being placed in a sonication bath for 30 s. The sample was then left overnight at 4 °C, centrifuged at  $1500 \times g$  for 20 min and the supernatant collected and frozen at  $-80$  °C.

Gastrocnemius and soleus muscle weights and mid-belly circumferences were recorded. Mid-belly muscle samples of the vastus lateralis and soleus muscles were removed immediately after death and were frozen by immersion into freezing isopentane (fibres in a vertical orientation, for histology) and liquid nitrogen (for molecular biology). All samples were stored at  $-80$  °C.

### Glucose uptake studies

The uptake of glucose into isolated muscle strips was measured using techniques adapted from<sup>18</sup>. Briefly, small strips (~50 mg) were taken from the vastus, soleus and gastrocnemius muscles and placed in chilled Tyrode's solution (137 mM NaCl, 5 mM glucose, 5 mM KCl, 12 mM NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 10 mM HEPES, 2 mM pyruvate, 38 mM mannitol and 0.1% bovine serum albumin, pH 7.4) until commencement of the uptake experiment. All incubations were then carried out at 38 °C in a shaking water bath. The strips were placed in 5 ml of warmed Tyrode's solution for 10 min and then incubated for a further 20 min in an identical medium in the presence or absence of insulin (16 nM, Novo Nordisk, UK). The strips were blotted on filter paper and incubated for 10 min in 3 ml Tyrodes solution supplemented with 8 mM [<sup>3</sup>H]methyl glucose (437  $\mu$ Ci/mmol) and 32 mM [<sup>14</sup>C]mannitol (8  $\mu$ Ci/mmol; GE Healthcare, UK). After incubation the muscle strips were blotted on filter paper to remove any residual radioactive buffer and then frozen in liquid nitrogen and stored at  $-80$  °C. Frozen muscle strips were homogenized in 400  $\mu$ l of water and centrifuged at  $10,000 \times g$  for 5 min. Supernatant <sup>3</sup>H and <sup>14</sup>C activity was determined simultaneously by liquid scintillation counting (Tricarb 2100TR, PerkinElmer, UK). Briefly, 300  $\mu$ l was put into a scintillation vial along with 6ml of Optiphase 'Hisafe' II scintillation fluid (PerkinElmer, Massachusetts, USA) and each vial run on the counter for 5 min. Protein levels were measured using Coomassie blue protein assay and glucose uptake corrected for the amount of protein. Extracellular space was corrected by subtracting mannitol levels.

## Immunohistochemistry

All chemicals were from Sigma (USA), unless otherwise stated. Primary antibodies were mouse anti-skeletal fast myosin antibody, clone MY32 (to positively identify fast myofibres, cat. no. M4276) and polyclonal rabbit anti-human von Willebrand factor (to positively identify capillary endothelial cells, DakoCytomation, cat no. A0082). In brief, 10  $\mu\text{m}$  transverse cryosections from the mid-belly of the skeletal muscles were fixed in water-free acetone at room temperature for 15 minutes, and endogenous peroxidase activity was inhibited by incubation in 0.5 % hydrogen peroxide in methanol for 30 minutes. Non-specific protein interactions were blocked with DMEM containing 20% fetal calf serum and 1% bovine serum albumin for 30 minutes and then incubated with either anti-skeletal fast myosin antibody (1:100) or anti-human von Willebrand factor antibody (1:300) at 4 °C overnight. All antibodies were diluted in Tris buffered saline (TBS). After rinsing with TBS, sections were incubated for 30 minutes with biotinylated anti-mouse (1:400) or anti-rabbit (1:400) antibody. Sections were washed and treated for 15 minutes with streptavidin biotin-peroxidase complex (1+1:200) and then for 10 minutes in amino ethyl carbazole. Finally, sections were counterstained with Mayers haematoxylin and baked with crystal mount (AbD Serotec, Kidlington, UK) before being mounted in Pertex (Surgipath, Peterborough, UK). A negative control section was processed simultaneously (methodology as above, replacing the primary antibody with TBS).

Capillaries were hard to identify in the frozen sections and therefore these were re-embedded in paraffin to allow thinner sections to be cut. In brief, muscle cryosections (4  $\mu\text{m}$ ) were re-embedded in paraffin and then deparaffinised in Clearene (2  $\times$  5 min) and rehydrated through graded alcohols (5 min in each) to 70 %. Endogenous peroxidase activity was inhibited by incubation with 0.5 % hydrogen peroxide in methanol for 10 min. Slides were covered with working pronase solution (DakoCytomation, Denmark) and incubated at room temperature for 10 min. Sections were then washed in tris-buffered saline (TBS; 3 washes  $\times$  2 min), immersed in avidin solution for 20 min, rinsed in TBS, immersed in biotin solution for 20 min, and rinsed in TBS. Sections were incubated in DMEM containing 20 % calf serum and 1 % BSA culture medium for 20 min to block non-specific protein interactions. Following this, anti-human von Willebrand factor antibody (1:300) was applied and the sections were incubated at 4 °C overnight. After rinsing with TBS, sections were incubated with biotinylated anti-rabbit (1:400) antibody for 30 min. Sections were washed and treated with streptavidin biotin-peroxidase complex (1 + 1:200) for 30 min and then with Diaminobenzidine (DAB) for 5 min. Sections were rinsed in TBS and washed in running tap water (5 min). Finally, sections were counterstained with Mayers haematoxylin and dehydrated through graded alcohols, cleared in Clearene and mounted in Pertex (Surgipath, Peterborough, UK).

## Image analysis

The myofibre density and size in the sections was assessed using a photomicroscope (Zeiss, Axioskop II) and the KS-400 image analysing system (Image Associates, Bicester, UK). Five microscopic images (validated as good representation of overall myofibre density, with < 2 % error), with  $\times 40$  objective, were selected randomly from each section and imported into the KS-400 image analysing program. In each of these fields of view the total fascicular area was calculated, and using a non-biased counting frame, all myofibres, fast-twitch myofibres (identified as the red positively stained fibres) and slow myofibres (negatively stained white fibres, Fig. 1A) were counted, and their density expressed as the number of fibres per  $\text{mm}^2$  fascicle. The average cross sectional area of the myofibres was defined by manually drawing around 41 individual fast-twitch and 24 slow-twitch fibres, with the cursor. Obvious deviations from a true cross section were excluded from the analysis.



Capillaries were counted within the fascicular area of 6 random fields (validated as good representation of overall capillary density, with < 2.4 % error) with  $\times 40$  objective (Fig. 1B) and capillary density was expressed as capillary number per  $\text{mm}^2$  fascicle. The numbers of myofibres from the same fields were counted and capillary/fibre ratio calculated.

All measurements were made by one observer and the intra-observer variability tested by reproducing the counts from the same section, at different times. The intra-observer variability was less than 5.6 % for all variables.

### Western blotting

These were performed as previously stated<sup>8</sup>. In brief, total protein was extracted from vastus muscle and abdominal fat. The cleared protein lysates from each animal were standardised to a final concentration of 1 mg/ml in Laemmli's sample buffer and equal amounts of protein for each animal (20  $\mu\text{g}$ ) were loaded onto 10 % SDS polyacamide gels for separation by electrophoresis. The separated proteins were transferred onto polyvinylidene difluoride membrane in singleton and Western blotting was carried out. The primary antibodies used in this study were to insulin receptor  $\alpha$  subunit (InsR, 1:200), PI3-kinase p85 regulatory subunit (p85, 1:1000, Upstate Biotech, NY, USA), PKC  $\alpha$  (1:200), insulin-like growth factor-I receptor  $\beta$ -subunit (IGF-IR  $\beta$ , 1:200), glucose transporter-4 (GLUT-4, 1:5000, Abcam, Cambridge, UK) and Akt1 (1:2000). All antibodies were rabbit polyclonal, except Akt1 (monoclonal mouse) and were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) unless stated otherwise. Autoradiographs of Western blots were imaged and the optical densities of the immunoreactive protein bands were measured. Linearity of signal was confirmed by the inclusion of 20 and 10  $\mu\text{g}$  of a pooled sample on each gel. Positive controls (where available) were also included on each gel.

### RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from liver using TRI Reagent (Sigma, USA). Quality and quantity of the RNA was assessed by spectrophotometry (A260nm/A280nm), and the integrity of the RNA was checked by formaldehyde gel electrophoresis, staining with ethidium bromide and visualization of intact 18S and 28S ribosomal RNA bands under ultraviolet light. Total RNA from each sample was reverse transcribed using standard protocols with random primers, RNase inhibitors and reverse transcriptase (Promega, UK). The three most stable housekeeping genes were determined using an ovine gene normalising (geNorm<sub>TM</sub>) kit developed from liver samples from our laboratory (Primer Design Ltd., UK) to be  $\beta$ -Actin, RPL-19 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Housekeeping gene expression was measured using SYBR Green, and primers/probes were supplied in kit form (Primer Design, UK [www.primerdesign.co.uk](http://www.primerdesign.co.uk)). Then real-time PCR (7700 Sequence Detection System, USA) was used to evaluate mRNA levels of InsR, glucocorticoid receptor (GR), Phosphoenolpyruvate carboxykinase (PEPCK), Glucose 6-phosphatase (G6Pase) using primers and probes (Eurogentec, UK) designed (Primer Express Software, Applied Biosystems, USA) with reference to published sequences (Table 1). InsR, GR, PEPCK and G6Pase were expressed relative to the geometric mean of the three housekeeping genes.

### Biochemical analysis

Plasma was analysed by autoanalyser using commercial kits as follows: glucose (hexokinase-6-phosphate dehydrogenase method [Dade-Behring]), high-density lipoprotein (HDL; Dade-Behring), low-density lipoprotein (LDL; calculated using the Friedwald formula) total cholesterol (Dade-Behring), total serum protein (Biuret reaction [Dade-Behring]), urea (urease enzyme assay [Dade-Behring]) and non-esterified fatty acid (NEFA; Alpha Laboratories Ltd, Eastleigh, UK). These analyses were measured as part of routine assays carried out at the National Health Services, Clinical Biochemistry Department,

Addenbrookes Hospital, Cambridge (Dade-Behring Dimension RXL analyser) and at the Institute of Human Nutrition, University of Southampton, Southampton (Konelab 20 autoanalyser).

Plasma triacylglyceride (TAG) was measured as described previously in full<sup>23</sup>. In brief total lipids were extracted and separated by solid phase extraction. TAG was converted to fatty acid methyl esters (FAME) and were separated and analysed by gas chromatography (HP6890 Hewlett Packard GC system with a BPX-70 column, Agilent Technologies, Cheshire, UK).

Insulin concentrations in plasma (25 µl, collected on EDTA tubes) and extracted pancreatic tissue were measured in duplicate by Enzyme-Linked ImmunoSorbent Assay (ELISA; DRG Sheep Insulin ELISA; ImmunoDiagnostic Systems, Tyne and Wear, UK). The range of the assay was 0.1 to 2.5 µg/l. The inter-assay and intra-assay coefficients of variance were 7.0 % and 4.4 %, respectively.

Plasma cortisol (µg/dl) was measured in duplicate using an Immulite analyser (DPC, UK) in 10 µl of plasma (collected by EDTA tubes) by a solid-phase, competitive chemiluminescent enzyme immunoassay, with an incubation cycle of 30 min. The range of the assay was 0.42 – 1.70 ng/ml and the intra-assay coefficient of variance was 5.7 %.

## Data analysis

Non-parametric data were log transformed prior to testing and are expressed as the geometric mean (95 % confidence intervals). Parametric data are expressed as the mean ± S.E.M. Area-under-the-curve (AUC) measurements for glucose, insulin, TAGs and NEFA were calculated (GraphPad Prism, version 3, GraphPad Software Inc., San Diego, USA) between 5 and 210 min (i.e. following glucose administration) from baseline (pre-glucose bolus) (i.e. 'incremental' AUC, Fig. 1 and Table 3). For the purpose of comparison between these data and our previous study of these animals at 1.5 years<sup>8</sup>, we also report the AUC measurements from 0 mM glucose to represent the total metabolic state. Dietary groups were compared with an unpaired Student's t-test (SPSS version 12, SPSS Inc., Chicago, USA). To test the effect of age (i.e. data at year 1.5 [Cripps *et al.* 2008] versus year 4) and its interaction with dietary group, a repeated measures ANOVA was used. Where a significant interaction was found between age and nutritional group a paired t-test was used to compare age within dietary group. For all comparisons, statistical significance was accepted when  $P < 0.05$ . Trends are discussed when  $0.05 < P < 0.1$ .

## RESULTS

### Weight and body composition

There was no difference between dietary groups in birth weight (HBCS,  $4.02 \pm 0.14$ ; LBCS,  $3.92 \pm 0.23$  kg), postnatal growth during suckling (0-12 weeks: HBCS,  $29.6 \pm 0.8$ ; LBCS,  $29.3 \pm 0.7$  kg), weaning to young adulthood (12 weeks – 1.5 years: HBCS,  $35.0 \pm 1.3$ ; LBCS,  $34.0 \pm 1.7$  kg) and young adulthood to mature adulthood (1.5 years – 2.5 years: HBCS,  $14.2 \pm 1.6$ ; LBCS,  $16.9 \pm 1.7$  kg). There was no difference between dietary groups in weight, BCS or back fat depth at 4 yrs of age (Table 2), but back muscle depth was greater in LBCS than in HBCS offspring ( $p < 0.05$ ). There was a significant increase between 1.5 and 4 yrs in body weight (1.5 yr,  $68.0 \pm 1.1$ ; 4yr,  $81.4 \pm 1.2$  kg.  $P < 0.001$ ), BCS (1.5 yr,  $2.1 \pm 0.1$ ; 4yr,  $2.6 \pm 0.1$ .  $P < 0.001$ ) and back fat depth (1.5 yr,  $2.0 \pm 0.2$ ; 4yr,  $3.3 \pm 0.2$  mm.  $P < 0.001$ ) that was not different between dietary groups (data shown are the combined dietary groups). For muscle depth there was a significant interaction between age and dietary group whereby back muscle depth increased between 1.5 and 4 yrs in LBCS offspring only (1.5 yr,  $29.4 \pm 0.9$ ; 4 yr,  $32.3 \pm 0.7$  mm.  $P < 0.05$ ).

There were no differences between the dietary groups in the weight of soleus and gastrocnemius muscles (Table 2), in gastrocnemius:femur length ratio (HBCS,  $1.88 \pm 0.08$ ; LBCS,  $2.04 \pm 0.07$ ), nor in gastrocnemius (HBCS,  $13.13 \pm 0.20$ ; LBCS,  $12.68 \pm 0.24$  cm) and soleus (HBCS,  $2.73 \pm 0.13$ ; LBCS,  $2.58 \pm 0.18$  cm) muscle circumference. There were no differences between the dietary groups in the major organ weights recorded (Table 2) nor in offspring body proportions (including crown-rump length and abdominal circumference. Data are not shown).

### Basal hormone and nutrient levels

There was no difference between the dietary groups in basal plasma hormone and nutrient levels (Table 3), nor in pancreatic insulin levels at post-mortem (HBCS, 11.2 (5.4-12.9); LBCS, 6.1 (3.3-8.2) nmol/g  $P=0.15$ ).

There was a small negative correlation between weight gain during suckling and basal glucose at 4 yrs ( $R^2=0.197$ ,  $P<0.05$ ). Basal glucose (1.5 yr,  $3.7 \pm 0.1$ ; 4 yr,  $4.0 \pm 0.1$  mmol/L,  $P<0.01$ ), insulin (1.5 yr,  $39.3 \pm 2.5$ ; 4 yr,  $106.8 \pm 10.2$  pmol/L,  $P<0.001$ ) and basal insulin:glucose ratio (1.5 yr,  $10.7 \pm 0.7$ ; 4 yr,  $26.2 \pm 2.5$ ,  $P<0.001$ ) increased with age, regardless of dietary group (data shown are the combined dietary groups).

### Response to a glucose load

**Intravenous Glucose Tolerance Test**—There was no difference between the dietary groups in peak glucose, peak insulin, or AUC analysis (from baseline; Table 3). For AUC calculations from 0 units (as index of total metabolic state), the lack of difference between the dietary groups persisted in glucose AUC (HBCS,  $2030.07 \pm 67.22$ ; LBCS,  $2123.19 \pm 62.41$  mmol.min.L<sup>-1</sup>), initial insulin AUC 0-5 min (HBCS,  $0.51 \pm 0.06$ ; LBCS,  $0.64 \pm 0.19$  nmol.min.L<sup>-1</sup>), insulin AUC (HBCS,  $52.68 \pm 5.77$ ; LBCS,  $66.07 \pm 6.42$  nmol.min.L<sup>-1</sup>) and insulin:glucose AUC (HBCS,  $25.80 \pm 2.63$ ; LBCS,  $31.09 \pm 2.96$ ).

Weight gain between weaning and young adulthood was positively correlated with peak glucose ( $R^2=0.175$ ,  $P<0.05$ ) and insulin (trend only,  $R^2=0.119$ ,  $P<0.1$ ). There was a decrease with age in glucose AUC (from baseline) (1.5 yr,  $1425 \pm 48$ ; 4 yr,  $1226 \pm 58$  mmol.min.L<sup>-1</sup>,  $P<0.01$ ) and initial insulin AUC 0-5min (1.5 yr,  $0.51 \pm 0.24$ ; 4 yr,  $0.24 \pm 0.08$  nmol.min.L<sup>-1</sup>.0-5 min,  $P<0.01$ ). There was a trend for an increase with age in insulin:glucose AUC (from baseline) (1.5 yr  $25.6 \pm 1.6$ , 4 yr  $31.5 \pm 2.7$  insulin:glucose,  $P<0.1$ ). These age effects were not affected by dietary group.

There was no difference between dietary groups in the overall TAG or NEFA response to a glucose load (Fig. 1, AUC analysis). For NEFA, but not TAG, the minimum level was reached more quickly in HBCS than in LBCS group ( $P<0.05$ ; see Table 4).

**Isolated muscle strips**—As expected, basal ( $P<0.001$ ) and insulin-stimulated ( $P<0.01$ ) glucose uptake was greater in soleus than in vastus or gastrocnemius muscles. In all muscles, there was no difference in basal glucose uptake between the dietary groups (Table 5). In the soleus muscle, insulin-stimulated glucose uptake tended to be lower in the LBCS as compared to HBCS offspring ( $P<0.1$ , Table 5), but there was no difference in uptake between the dietary groups in the vastus and gastrocnemius muscles.

### Re-evaluation of 1.5 year data

Five rams were removed from the cohort in the period of time following the study at 1.5 years<sup>8</sup>. Glucose handling data at 1.5 years was reanalyzed using only the animals which were studied at 4 years and showed that there were no differences between the groups in basal glucose (HBCS,  $3.66 \pm 0.06$ ; LBCS,  $3.80 \pm 0.08$  mmol/L  $P=0.18$ ) or initial insulin AUC



0-5 min (HBCS,  $0.69 \pm 0.08$ ; LBCS,  $0.60 \pm 0.06$  nmol.min.L<sup>-1</sup> P=0.41) but that glucose AUC remained significantly increased in the LBCS group (HBCS,  $2183 \pm 31.0$ ; LBCS,  $2283 \pm 25.2$  mmol.min.L<sup>-1</sup> P<0.05).

### Muscle morphology

In the vastus muscle there was a reduction in total myofibre density, fast myofibre density (P<0.05), and a trend for a reduction in slow myofibre density (P<0.1) in the LBCS compared to HBCS offspring (Fig. 2). Fast (HBCS,  $2671 \pm 110$ ; LBCS,  $2617 \pm 360$  μm<sup>2</sup>) and slow (HBCS,  $2969 \pm 202$ ; LBCS,  $3319 \pm 164$  μm<sup>2</sup>) myofibre cross-sectional area was similar between dietary groups. In the soleus muscle, slow myofibre density (Fig. 2) and CSA (HBCS,  $3793 \pm 242$ ; LBCS,  $3665 \pm 280$  μm<sup>2</sup>) was similar between dietary groups.

In the vastus muscle capillary density tended to be reduced in the LBCS compared to HBCS offspring (Fig. 2, P<0.1). The capillary to myofibre ratio was significantly reduced in LBCS compared to HBCS offspring (Fig. 2, P<0.05). In the soleus muscle, capillary density and capillary to myofibre ratio was similar between dietary groups (Fig. 2).

### Gene expression

**Muscle and fat**—Protein levels of Akt1 were lower (P<0.05) and IGF-IR levels tended to be lower ( $p < 0.1$ ) in the vastus muscle of LBCS offspring (Fig. 3). GLUT-4 protein levels were increased in the vastus muscle of LBCS offspring (P<0.001). There were no differences in InsR, PI3-kinase p85 subunit and PKC protein in the vastus muscle between dietary groups. Protein levels of Akt1 tended to be reduced in the abdominal fat of LBCS offspring (P<0.1, Fig. 3). There were no differences in GLUT-4, InsR, IGF-IR, PI3-kinase p85 subunit and PKC protein in the abdominal fat between dietary groups.

**Liver**—InsR mRNA levels were increased in liver of LBCS offspring (HBCS, 0.51 (0.37 – 0.65); LBCS, (0.72 (0.55 – 0.89) arbitrary units normalized to housekeeping gene, P<0.05). There was no significant difference between dietary groups in liver GR, PEPCK or G6Pase mRNA levels (data not shown).

## DISCUSSION

Epidemiological and animal studies implicate the fetal nutrient environment as a determinant of metabolic disease in later life. Maternal body composition is linked to maternal diet and food intake and is important since the mother provides nutrition for the fetus from both her dietary intake and her own body reserves. The offspring of low body mass index mothers have been shown to be at increased risk of insulin resistance<sup>2-4</sup>. Our previous observations showed that lowering the BCS of the ewe during pregnancy reduced glucose tolerance of young adult offspring<sup>8</sup>. In this study we had the rare opportunity to study the same animals again in mature adulthood and found that rather than worsening, the LBCS animals displayed no signs of altered glucose metabolism, despite reduced myofibre, fast-fibre density and capillary to myofibre ratio in the vastus lateralis muscle. Our findings suggest that this may be due in part to adaptations in the muscle insulin-signalling pathway.

An important and novel finding of this study is that the altered glucose handling at 1.5 yrs in offspring of lower body condition score mothers<sup>8</sup> did not persist into mature adult life, and indeed no other sheep study of glucose metabolism has followed up animals postnatally for so long following *in utero* under-nutrition. The difference between dietary groups at 1.5 years in the response to a glucose load was still apparent when the data set was restricted to only the animals that were also studied at 4 years. Fatty acid metabolism (indicated by plasma NEFA and TAG levels) was similar between offspring groups, although the slightly

longer time to minimum levels of NEFA during the glucose tolerance test in LBCS animals may be a weak marker of altered insulin sensitivity in other organs such as adipose and liver. There was no difference between dietary group in insulin signalling pathway in adipose tissue but we observed that hepatic InsR expression at the mRNA level was increased in LBCS animals. This was not associated with any change in fasting blood glucose and there was no effect on mRNA levels of genes involved with gluconeogenesis in the liver (i.e. GR, PEPCK, G6Pase). Increased liver insulin sensitivity could impact on the LCBS offspring response to dietary excess or deficiency through fatty acid synthesis, gluconeogenic or glycogen storage pathways; however this speculation was not tested in the current study design. In sheep offspring, exposure to a late gestation 50% under-nutrition challenge increased insulin and glucose AUC at 1 year of age<sup>17</sup> and exposure to a 50 % reduction from 28-78 dGA increased glucose AUC in 63 and 250 day old offspring<sup>24</sup>. Glucose tolerance was reduced among men and women exposed to famine during late gestation and this was more pronounced in those exposed to famine who then became obese as adults<sup>25</sup>. In rats, glucose tolerance of offspring of mothers fed a low protein diet was improved at 6-9 weeks<sup>26;27</sup> unaltered at 44 weeks<sup>27</sup> and significantly worse by 17 months and exacerbated by an over-abundant postnatal diet<sup>28;29</sup>. Sheep are ruminants but have advantages as a model in that singleton offspring can be studied and also they develop key organ systems fully prenatally. In the present study, back fat and muscle and BCS increased modestly with age but at the 4 year study the animals still were of average BCS. It remains possible that if they had become obese differences between the lower and higher condition score groups in glucose handling may have persisted or worsened with age. Thus, overall these findings provide additional insight into our understanding of the influence of nutrition in early life on the risk of disorders of metabolism in adult life such as obesity and type 2 diabetes<sup>19</sup>.

Our data show that basal glucose, basal insulin, and basal insulin to glucose ratio increased with age which is consistent with signs of altered glucose homeostasis and insulin resistance. The initial insulin response to glucose (insulin 0-5min AUC, a measure of the first phase insulin response) decreased from 1.5 to 4 yr and this is consistent with several human studies that have reported a significant age-dependent decrease in glucose-stimulated insulin secretion<sup>30-32</sup>. However, glucose AUC (from baseline) was lower at 4 yr of age than at 1.5 yrs (independent of dietary group) suggesting that glucose tolerance had in fact improved with age. In humans glucose tolerance is generally thought to decrease with age<sup>33</sup>, not as a consequence of age *per se* but possibly due to lower activity and weight gain. In the current study activity levels were not assessed, but it is of note that weight gain was modest (~10 Kg) and the increase in BCS and adiposity was small.

The offspring of LBCS ewes had reduced total myofibre and fast-twitch density, and a tendency for reduced slow-twitch myofibre density, in the vastus muscle that does not appear to be due to changes in CSA and therefore could involve changes to the extracellular space, although this was not assessed. This is consistent with our previous findings that early and late gestation under-nutrition periods reduced fibre density in the late gestation fetus<sup>11</sup>. A reduction in fast fibre number has been seen in the vastus lateralis muscle of 14 day old lambs following a 50 % nutrient reduction 30-70 dGA<sup>34</sup>. A reduction in fast fibres is more likely to have a greater effect on muscle strength than a reduction in slow fibres, due to their faster speed of contraction and reduced resistance to fatigue. Studies suggest that individuals of lower birth weight have reduced muscle strength<sup>35</sup> and a lower myofibre score<sup>36</sup> in adult life, with a consequent increase in disability and frailty with age. Decreased strength with aging has been associated with a reduction in type II fibres<sup>37</sup>.

Our observation of a decrease in capillary to myofibre ratio and a trend for reduced overall capillary density in the vastus muscle, but not in the soleus, suggests that blood flow and muscle growth were related. Indeed, the reduction in capillary to myofibre ratio in the vastus

muscle indicates that each fibre was being supplied by fewer blood vessels. In mice, impaired insulin signalling in endothelial cells led to attenuated insulin-induced capillary recruitment and insulin delivery, and reduced glucose uptake in skeletal muscle<sup>38</sup>. Our observation of decreased Akt1 (significant) and IGF-IR (trend only) levels in LBCS offspring vastus muscle are consistent with decreased muscle growth. There was a trend for only Akt1 to be lower in LBCS offspring abdominal fat. Myofibre hypertrophy and hypertrophy-associated vascular endothelial growth factor (VEGF) expression is inhibited by a dominant-negative mutant of Akt1 and conversely, transduction of a constitutively active form of Akt1 increase myofibre size and VEGF production<sup>39</sup>. Hypertrophy of myotubes, in response to IGF-I or insulin (both ligands of IGF-IR), increases secretion of VEGF<sup>39</sup>. VEGF is implicated in capillary remodelling/angiogenesis<sup>40</sup>. Interestingly, Akt1 has also been implicated in angiogenesis<sup>41</sup>. Thus, Akt1 is a mechanism through which blood vessel recruitment and muscle growth may be coupled, although in the present study reduced myofibre and capillary density in LBCS offspring vastus muscle was not accompanied by a change in myofibre CSA.

Our observed changes in skeletal muscle morphology, in particular the reduced total myofibre density in hindlimb muscle, in LBCS offspring were not associated with reduced glucose tolerance at 4 yrs (only a small non-significant decrease in insulin-sensitive glucose uptake in the soleus muscle strips of LBCS offspring was observed). The small increase in back muscle depth in the LBCS group may have contributed positively to glucose tolerance in these animals, similar to that associated with hypertrophy of muscle following resistance training<sup>42:43</sup>. In theory such improvement in glucose tolerance could partially attenuate any reduction in whole body glucose tolerance that might have resulted from reduced myofibre density in limb muscle.

In the vastus muscle, there were no differences in InsR, PI3-kinase p85 subunit and PKC protein between dietary groups but GLUT-4 (the major insulin-responsive glucose transporter in skeletal muscle and the last stage in the insulin pathway signalling to glucose uptake) was increased in LBCS offspring. In contrast, there was no effect on these key genes in abdominal fat. In contrast previous studies observed reduced skeletal muscle GLUT-4 in much younger (8 month) lambs following early gestation maternal undernutrition<sup>13</sup>. So we speculate that the effects of altered skeletal muscle composition that were apparent at 1.5 yrs in terms of glucose tolerance<sup>8</sup> may be offset at least in part by compensatory increases in GLUT-4 resulting in normal glucose tolerance by 4 yrs. While not assessed directly in the present study, changes to the GLUT-4 system might involve altered GLUT-4 translocation (from intracellular vesicular storage to the plasma membrane). Indeed, GLUT-4 protein content was unchanged but translocation was increased in the muscle of 10 day old pups from protein restricted dams and this was associated with normal glucose tolerance<sup>44</sup>.

Thus we have shown that the mild glucose intolerance in young adulthood caused by gestational lower maternal diet and body composition<sup>8</sup> was no longer detectable by mature adulthood in terms of total body glucose handling. Our observation of decreased myofibre and capillary density is likely to have originated *in utero* and at the young adult stage may have contributed to altered glucose handling – but by mature adulthood we speculate that the observed up-regulation of GLUT-4 and increase in back muscle depth may form part of a compensatory mechanism to regulate glucose homeostasis. However, these adaptations may have limitations in an over-abundant postnatal environment, where there would be increased propensity for weight gain and obesity. These findings have implications for dietary choices of mothers, even pre-pregnancy.

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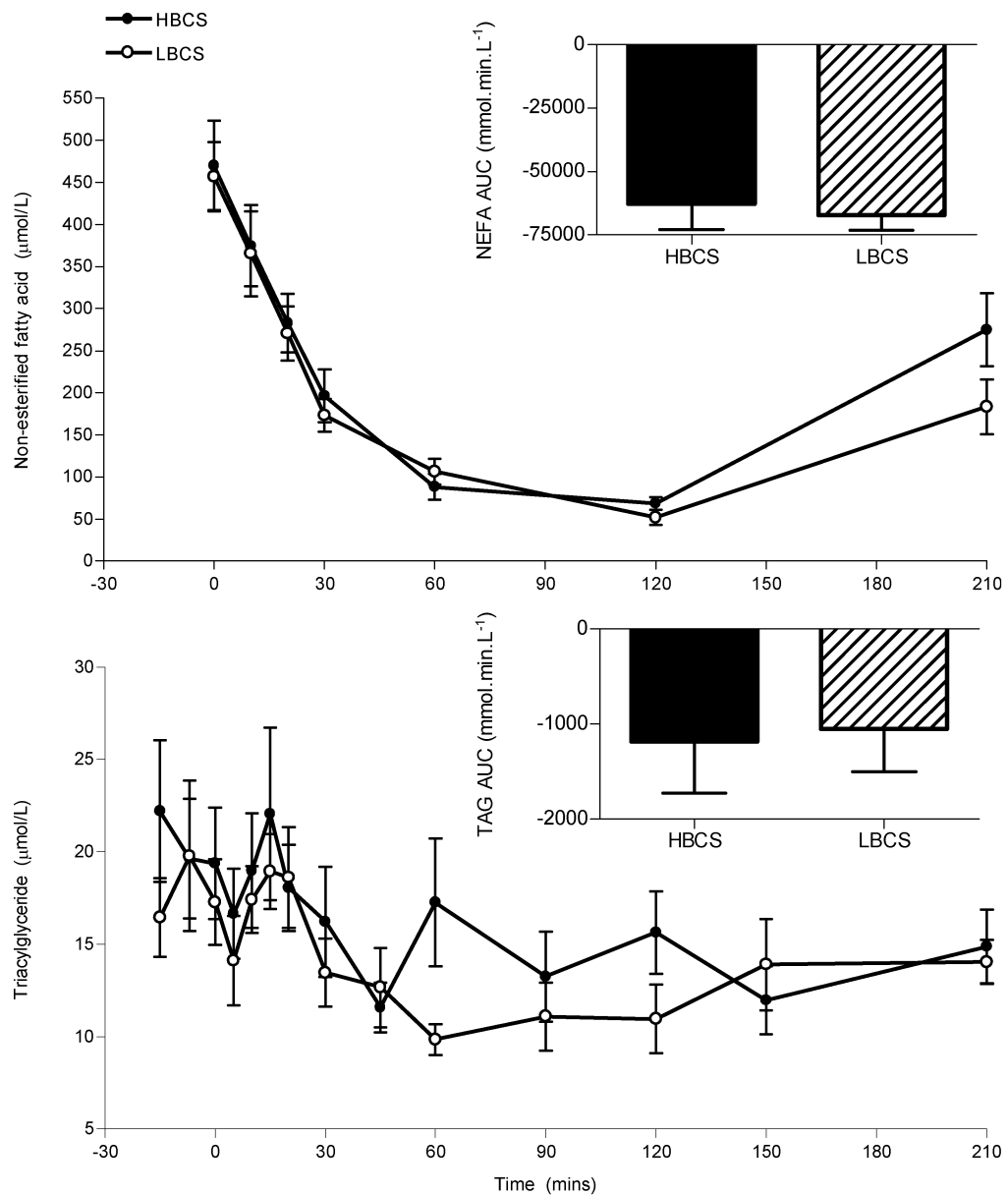
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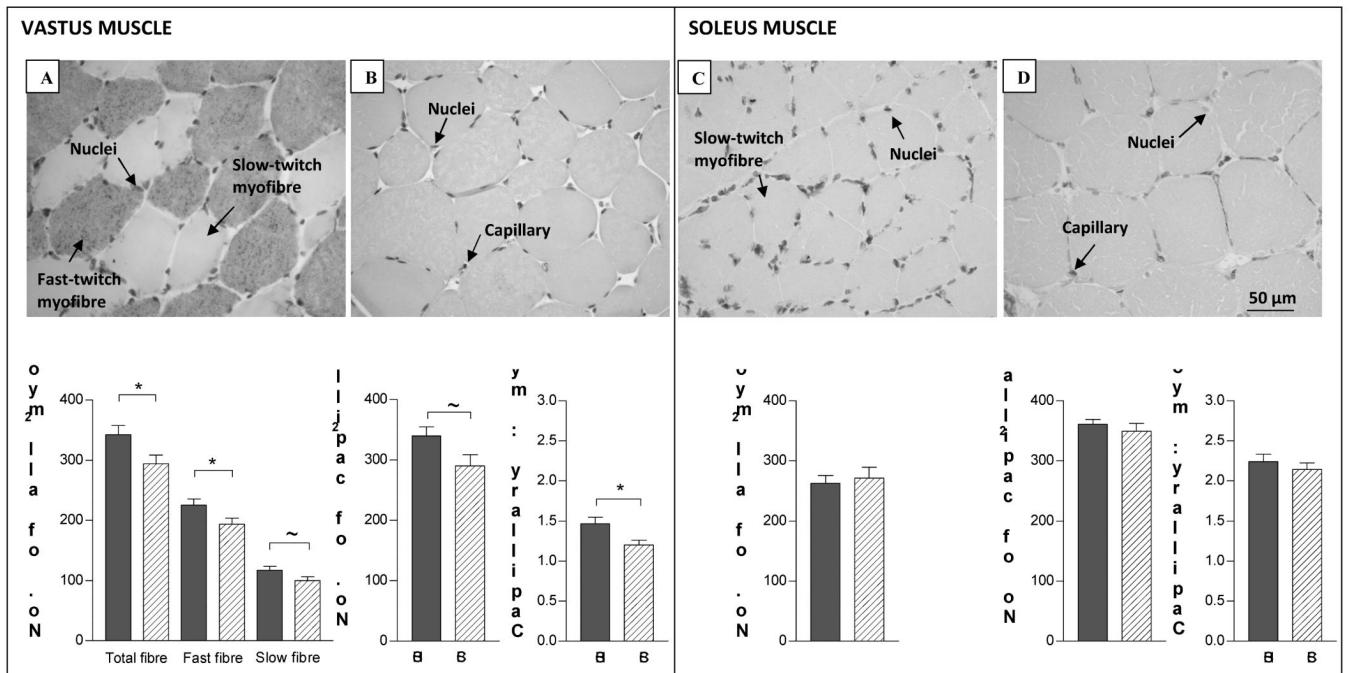
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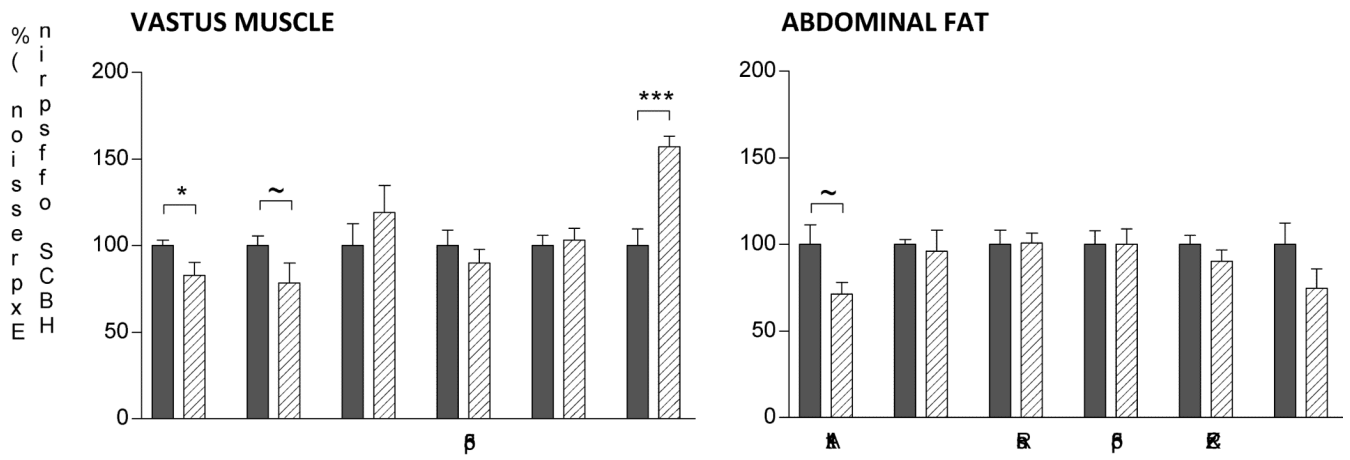
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**Figure 1.** Plasma non-esterified fatty acid and triacylglyceride during the intravenous glucose tolerance test of offspring of higher body condition score (HBCS, filled bars/symbols) and lower body condition score (LBCS, hatched bars/open symbols) ewes. Data are shown as the mean  $\pm$  SEM. Area under the curve (AUC) were calculated from baseline plasma levels prior to the glucose bolus.



**Figure 2.** Myofibre and capillary density in the vastus (A and B) and soleus (C and D) muscles of offspring of high body condition score HBCS, filled bars) and low body condition score (LBCS, hatched bars) ewes. Data are shown as the mean  $\pm$  SEM, \*P<0.05 and ~P<0.1.



**Figure 3.** Insulin signalling pathway protein expression in the vastus muscle and abdominal fat of offspring of high body condition score (HBCS, filled bars) and low body condition score (LBCS, hatched bars) ewes. Data are shown as the mean  $\pm$  SEM. ~P<0.1, \*P<0.05, \*\*\*P<0.001.

**Table 1**  
**Primer and probe sequences (5' to 3') used in the measurement of mRNA levels by real-time polymerase chain reaction**

Target Gene	Primer/probe	Sequence	Accession no.
GR	Forward Primer	ACTGCCCCAAGTGAAAACAGA	X70407
	Reverse Primer	GCCCAGTTTCTCCTGCTTAATTAC	
	TaqMan Probe	AGAAGATTTTATCGAACTCTGCACCCCTGG	
G6Pase	Forward Primer	TGGAGTCTTTTCAGGCATTGC	EF062861
	Reverse Primer	CTTGAGACTGGCATTGTAGATGCT	
	TaqMan Probe	TTGCTGAGACTTTCCGCCACATCCA	
PEPCK	Forward Primer	GATTGGCATCGAGCTGACAGA	EF062862
	Reverse Primer	CGCCCATCCTCGTCATG	
	TaqMan Probe	TCGCCCTACGTGGTGACCAGCA	
InsR	Forward Primer	ACC-GCC-AAG-GGC-AAG-AC	AY162434
	Reverse Primer	AGC-ACC-GCT-CCA-CAA-ACT-G	
	TaqMan Probe	AAC-TGC-CCT-GCC-ACT-GTC-ATC-AAC-G	



**Table 2**  
**Body biometry and post-mortem organ weights in 4 year old offspring**

	HBCS (n=14)	LBCS (n=10)	P value
<b>Body biometry</b>			
Age, wk	210.2 ± 1.1	210.4 ± 1.0	0.90
Weight, kg	80.54 ± 1.78	82.50 ± 1.45	0.43
BCS (1-5 units)	2.61 (2.41-2.88)	2.64 (2.50-2.80)	0.96
Fat depth (mm)	3.4 ± 0.4	3.3 ± 0.3	0.90
Muscle depth (mm)	29.2 ± 0.9	32.3 ± 0.7	0.02 *
<b>Organ weights (as % of body weight)</b>			
Left kidney	0.116 ± 0.003	0.110 ± 0.003	0.57
Right kidney	0.113 ± 0.003	0.112 ± 0.002	0.85
Liver	1.11 ± 0.03	1.08 ± 0.03	0.89
Heart	0.44 ± 0.02	0.49 ± 0.03	0.15
Lung	1.10 ± 0.09 (11)1	1.17 ± 0.12 (9)	0.62
Gastrocnemius muscle	0.077 ± 0.004	0.081 ± 0.003	0.47
Soleus muscle	0.0037 ± 0.0002	0.0038 ± 0.0004	0.87

Measurements on offspring of high body condition score (HBCS) and low body condition score (LBCS) ewes.

\* P<0.05. Bracketed numbers indicate where group size is different from maximum. Data are shown as the mean ± SEM, except for body condition score (BCS) which is shown as the geometric mean (95 % CI).

**Table 3**  
**Plasma hormones and nutrients under baseline (fasted) conditions and during the glucose tolerance test**

	HBCS (n=14)	LBCS (n=10)	P value
<b>Basal plasma levels</b>			
Glucose (mmol/L)	4.06 ± 0.12	4.14 ± 0.14	0.66
Insulin (pmol/L)	102 (40-144)	91 (48-130)	0.53
Insulin : glucose	28.02 ± 3.73	23.72 ± 3.05	0.41
Cortisol (ng/ml)	0.95 ± 0.11	0.93 ± 0.10	0.13
TAG (umol/L)	20.49 ± 2.97 (13)	17.83 ± 2.23	0.51
NEFAs (umol/L)	470.17 ± 53.07	457.08 ± 41.22	0.86
Total protein (g/L)	71.50 ± 0.99	73.60 ± 0.86	0.14
Cholesterol (mmol/L)	1.39 ± 0.09	1.46 ± 0.07	0.53
HDL (mmol/L)	0.84 ± 0.05	0.88 ± 0.04	0.61
LDL (mmol/L)	2.01 ± 0.08 (13)	2.11 ± 0.13	0.51
Urea (mmol/L)	5.61 ± 0.37	5.64 ± 0.35	0.69
<b>Glucose tolerance test</b>			
Peak glucose (mmol/L)	23.41 ± 0.43	24.04 ± 0.39	0.31
Glucose AUC (mmol.min.L <sup>-1</sup> )	1193.00 ± 50.70	1259.00 ± 64.53	0.42
Peak insulin (pmol/L)	487.94 ± 49.81	598.72 ± 59.36	0.16
Initial insulin AUC 0-5 min (nmol.min.L <sup>-1</sup> )	0.24 ± 0.07	0.24 ± 0.23	0.96
Insulin AUC (nmol.min.L <sup>-1</sup> )	33.87 ± 4.50	43.98 ± 3.87	0.12
Insulin AUC : glucose AUC	28.38 ± 3.56	35.98 ± 3.79	0.16

Measurements on offspring of high body condition score (HBCS) and low body condition score (LBCS) ewes. AUC calculations are from baseline. Data are shown as the mean ± SEM except for basal insulin which is shown as the geometric mean (95 % CI). Bracketed number indicates where n differs from column heading.

**Table 4**  
**Minimum plasma non-esterified fatty acid (NEFA) and triacylglyceride (TAG) reached during the intravenous glucose tolerance test**

	<b>HBCS (n=13)</b>	<b>LBCS (n=10)</b>	<b>P value</b>
Minimum NEFA ( $\mu\text{mol/L}$ )	63.36 $\pm$ 8.38	51.74 $\pm$ 9.21	0.37
Delta minimum NEFA ( $\mu\text{mol/L}$ ) (from baseline)	-406.82 $\pm$ 52.28	-405.34 $\pm$ 36.55	0.98
Time to minimum NEFA (min)	98.57 $\pm$ 7.97	120.00 $\pm$ 0.00	0.03 *
Minimum TAG ( $\mu\text{mol/L}$ )	7.79 $\pm$ 0.85	6.59 $\pm$ 0.93	0.36
Delta minimum TAG ( $\mu\text{mol/L}$ ) (from baseline)	-12.70 $\pm$ 2.87	-11.24 $\pm$ 2.06	0.84
Time to minimum TAG (min)	63.85 $\pm$ 9.56	80.50 $\pm$ 19.09	0.41

Measurements on offspring of higher body condition score (HBCS) and lower body condition score (LBCS) ewes. Data are shown as the mean  $\pm$  SEM.

\*  $P < 0.05$ , significant difference between dietary groups.

**Table 5**  
**Glucose uptake into isolated muscle strips**

	HBCS (n=10)	LBCS (n=7)	P value
<b>Basal glucose uptake (pmol/min/mg)</b>			
Soleus muscle	0.55 ± 0.07	0.43 ± 0.04	0.20
Vastus muscle	0.24 ± 0.03	0.22 ± 0.03	0.69
Gastrocnemius muscle	0.29 ± 0.07	0.25 ± 0.02	0.57
<b>Insulin stimulated glucose uptake (pmol/min/mg)</b>			
Soleus muscle	1.01 ± 0.06	0.84 ± 0.07	0.08 <sup>~</sup>
Vastus muscle	0.61 ± 0.07	0.54 ± 0.08	0.46
Gastrocnemius muscle	0.56 ± 0.05	0.44 ± 0.04	0.11

Measurements on offspring of high body condition score (HBCS) and low body condition score (LBCS) ewes. Data are shown as the mean ± SEM,

<sup>~</sup>P<0.1.