

Effect of pre and postnatal growth and post-weaning activity on glucose metabolism in the offspring

Neele S. Dellschaft<sup>1,2</sup>, Marie-Cecile Alexandre-Gouabau<sup>2</sup>, David S. Gardner<sup>3</sup>, Jean-Philippe Antignac<sup>5</sup>, Duane H. Keisler<sup>6</sup>, Helen Budge<sup>1</sup>, Michael E. Symonds<sup>1</sup> and Sylvain P. Sebert<sup>1,4</sup>,

<sup>1</sup> Early Life Research Unit, Academic Division of Child Health, Obstetrics & Gynaecology, School of Medicine, Queen's Medical Centre, The University of Nottingham, Nottingham NG7 2UH, United Kingdom.

<sup>2</sup> INRA and University of Nantes, UMR-1280 Physiologie des Adaptations Nutritionnelles, CHU Hôtel Dieu, 44093 Nantes cedex 1, France.

<sup>3</sup> School of Veterinary Medicine and Science, Sutton Bonington Campus, The University of Nottingham, Nottingham LE12 5RD United Kingdom.

<sup>4</sup> Institute of Health Sciences, Centre For Life-Course Epidemiology, and Biocentre, University of Oulu, Aapistie 5B, 90014 Oulu, Finland.

<sup>5</sup> LUNAM Université, Oniris, Laboratoire d'Etude des Résidus et Contaminants dans les Aliments, USC INRA 1329, Nantes, France.

<sup>6</sup> Department of Animal Science, University of Missouri, Columbia, Missouri 65211, USA.

**Running title:** Perinatal growth and insulin resistance

**Key terms:** IUGR, Perinatal growth, Obesity, Appetite regulation, Insulin, Leptin, Cortisol, Metabolomics, Gene expression

**Word count:** 4684

**Number of figures and tables:** 9

Corresponding author and person to whom reprint requests should be addressed:

Professor Michael E. Symonds; Academic Division of Child Health Obstetrics & Gynaecology, School of Medicine, Queen's Medical Centre, The University of Nottingham, Nottingham NG7 2UH, United Kingdom

Phone: +44 1158230625 ; Fax: +44 1158230626.

Email: michael.symonds@nottingham.ac.uk

## **Abstract**

Maternal caloric restriction during late gestation reduces birth weight but whether long-term adverse metabolic outcomes of intra-uterine growth retardation (IUGR) are dependent on either accelerated postnatal growth, or exposure to an obesogenic environment after weaning, is not established. We induced IUGR in twin pregnant sheep using a 40% maternal caloric restriction commencing from 110 days gestation until term (~147 days), compared to mothers fed to 100% of requirements. Offspring were reared either as singletons to accelerate postnatal growth or as twins to achieve standard growth. To promote an adverse phenotype in young adulthood, after weaning, offspring were reared under a low activity obesogenic environment with the exception of a sub-group of IUGR offspring, reared as twins, maintained in a standard activity environment. We assessed glucose tolerance together with leptin and cortisol responses to feeding in young adulthood when the hypothalamus was sampled for assessment of genes regulating appetite control, energy and endocrine sensitivity. Caloric restriction reduced maternal plasma glucose, raised NEFA, and changed the metabolomic profile, but had no effect on insulin, leptin, or cortisol. IUGR offspring whose postnatal growth was enhanced and were obese showed insulin and leptin resistance plus raised cortisol. This was accompanied by increased hypothalamic gene expression for energy and glucocorticoid sensitivity. These long-term adaptations were reduced but not normalised in IUGR offspring whose postnatal growth was not accelerated and remained lean in a standard post-weaning environment. IUGR results in an adverse metabolic phenotype, especially when postnatal growth is enhanced and offspring progress to juvenile-onset obesity.

## **Introduction**

There is increasing evidence to support the early life programming of adult obesity, type 2 diabetes, and hypertension. The prenatal environment's influence depends on organ-specific windows of susceptibility with some, but not all, outcomes linked to mechanisms affecting size at birth (Barker 1997; Roseboom *et al.* 2000). In large mammals, including sheep, pigs, and humans, chronic caloric restriction throughout late gestation results in intra-uterine growth retardation (IUGR) (Roseboom *et al.* 2000; Symonds *et al.* 2009), which contrasts with suboptimal maternal nutrition in earlier gestation, which does not influence birth weight (Roseboom *et al.* 2000; Bispham *et al.* 2003; Sharkey *et al.* 2009).

During diet-induced IUGR maternal homeostasis is altered, affecting the metabolic environment in which the fetus develops (Tygesen *et al.* 2008). It is possible that these metabolic adaptations influence fetal growth independently of changes in the feto-maternal endocrine environment. Nutrients not only fulfill energetic requirements but a range of lipids, non-esterified fatty acids (NEFA), and amino acids act as signaling molecules and could, therefore influence epigenetic processes linked to the long-term regulation of metabolic function (McMillen & Robinson 2005). In the present study, we examined whether a reduction in maternal food intake in late pregnancy, leading to changes in maternal metabolic homeostasis and birth weight, are essential in programming adult predisposition to the following characteristics: i) altered body composition, ii) central and peripheral insulin resistance, iii) the regulation of food intake, and iv) postprandial and post-absorptive endocrine responses to feeding. Furthermore, the extent to which the impact of IUGR on each of these adaptations is dependent on postnatal growth patterns is not known. This is important as many human studies indicate that the long term impact of reduced birth size, in both term and pre-term infants, can be dependent on early postnatal growth (Singhal *et al.* 2003; Stettler

*et al.* 2003). In sheep, the relative importance of enhanced postnatal growth on long term outcomes has not been widely examined, although, when combined with IUGR, accelerated postnatal growth differentially affects energy sensing within the stomach and hypothalamus (Sebert *et al.* 2011). The post-weaning environment is an additional factor which appears to determine the magnitude of the phenotypic response to alterations in maternal diet in pregnancy in rodents (Desai *et al.* 2007). In these studies, the metabolic and related effects in young adult offspring who were nutritionally manipulated *in utero* are minimal unless adiposity has been promoted. The extent to which similar changes in body composition also apply to large mammals has not been investigated.

Each organ has a set developmental trajectory and therefore they are not all similarly affected by IUGR. The hypothalamus is particularly sensitive to environmental stresses in early life and plays a central role in the regulation of energy homeostasis (Adam *et al.* 2008). Cortisol can influence blood pressure as a consequence of regulating gene expression of arginine vasopressin (*AVP*) and corticotrophin releasing hormone (*CRH*), which when suppressed, acts through negative feedback, to reduce cortisol secretion from the adrenals through decreased adrenocorticotrophic hormone action (Lightman 2008). Food intake is regulated by changes in the plasma concentration of markers of energy status, including insulin, leptin, and glucose (Schwartz *et al.* 2000). These factors determine the action of neurotransmitters in the arcuate nucleus of the hypothalamus, especially neuropeptide Y (*NPY*) and pro-opio melanocortin (*POMC*), which have antagonistic actions in signaling peripheral energy status to other hypothalamic nuclei, the cortico-limbic system and the brain stem, which ultimately determine food intake and physical activity (Schwartz *et al.* 2000). Critically, the fetal hypothalamus shows an orexigenic response by increased *NPY* signaling to maternal nutrient restriction in late gestation (Warnes *et al.* 1998) and an anorexigenic response by increased

*POMC* signaling to maternal and early postnatal overnutrition (Muhlhausler *et al.* 2006). However, whether these adaptations persist into adulthood is not known. In addition, whilst organogenesis and the developmental maturation of the hypothalamus in altricial species is particularly sensitive to the late gestational nutritional environment (Adam *et al.* 2008), hypothalamic maturation continues after birth when it is particularly responsive to the postnatal energetic environment (Paus 2010). Whether specific changes in the early postnatal environment during key windows, *i.e.* immediately after birth and at weaning, modulate the long-term molecular adaptation of the hypothalamus to IUGR has received no attention. The present study, therefore, not only examines the effects of maternal caloric restriction during late pregnancy on maternal homeostasis but also tests the hypothesis that the adverse effects of IUGR are dependent on the postnatal energetic environment and concomitant differences in peri-partum or post-weaning growth. We investigated the effects of: i) IUGR followed by an accelerated postnatal growth combined with a low activity obesogenic environment after weaning and compared to those offspring born to mothers fed to requirements throughout pregnancy and with the same postnatal treatment, ii) differing postnatal growth rates on adult IUGR offspring submitted to obesogenic conditions, and finally, iii) a differing energetic environment on IUGR offspring submitted to regular postnatal growth rate.

## Materials and Methods

### Animals and experimental design

All animal procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 with approval from the Local Ethics Committee of the University of Nottingham. The experimental nutritional intervention has previously been described in detail (Sebert *et al.* 2011). In brief, 28 Bluefaced Leicester cross Swaledale twin bearing sheep (*ovis*) were individually housed at 100 days of gestation (dGA) and, at day 110 dGA, randomly allocated to the experimental groups (Figure 1). All pregnancies continued normally until term ( $\sim 145 \pm 1$  days) and produced heterozygous twins. They included a control group (C, n=9) that were fed to requirements through pregnancy (i.e. from  $0.46 \text{ MJ/kg.BW}^{0.75}$  at 110 days gestation, increasing to  $0.72 \text{ MJ/kg.BW}^{0.75}$  at dGA 130), whilst the remaining 19 mothers were caloric restricted (R) and were pair-fed to 60% of control intake, based on their body weight. All mothers were individually weighed once a week prior to feeding in order that their total food requirements could be adjusted. From birth, the offspring born to C mothers were then reared to promote accelerated (A) early postnatal growth (CA, n=8, 4 males and 4 females), accomplished by only one twin being reared by its mother. The offspring born to R mothers were reared to promote an accelerated (RA n=9, 2 males and 7 females) or a regular (RR, n=17) early postnatal growth rate, accomplished by being reared together as twins. After weaning, all offspring were kept in a low activity environment until 17 months of age in order to promote obesity (O, 6 animals on  $19 \text{ m}^2$ , fed *ad libitum* on straw nuts and a micronutrient supplement) with the exception of 9 RR offspring that were kept in a normal physical activity environment, in order to remain lean (RRL, n=9, 5 males and 4 females, 6 animals on  $1125 \text{ m}^2$ , *ad libitum* access to grass and a micronutrient supplement; RRO n=8; 2 males and 6 females; Figure 1). Discrepancies between the total number (n) of mothers and offspring are due to additional offspring intervention groups for the twins that

were removed from their mother on the first day of birth, which included formula-reared twins within the CAO and RAO offspring, which were not included in the present study. The numbers of twin bearing mothers entered into the study for each nutritional group were predicted to be sufficient to produce enough number of male and female offspring for each of the postnatal intervention groups. However due to the uneven distribution of male and females born to R mothers there were fewer male offspring available than anticipated. The resulting groups permit us to draw comparisons between animals with and without IUGR (RAO vs. CAO) and, within those with IUGR, to investigate the effects of early postnatal growth (RAO vs. RRO) and of post-weaning environment (RRO vs. RRL).

### **Timing of samplings and *in vivo* challenges**

**Maternal blood sampling:** At 130 dGA, jugular venous blood samples (5 ml) were collected from the ewes in the morning, prior to, and two hours after, feeding. Venous blood was collected into heparinized or K<sup>+</sup>EDTA coated tubes and the plasma was immediately separated by centrifugation (2500 g x 10 min at 4°C) and stored at -80°C until analysis.

**Offspring blood sampling:** Venous blood samples (prepared and stored under identical conditions as described above) were collected after an overnight fast ( $\geq 18$ h) at both 7 and 16 months of age. Jugular catheters were inserted by percutaneous puncture 1-2 days before sampling. Additional blood samples were collected at 16 months of age following the presentation of a mix of high and low energy-dense feed (3 kg straw nuts, 8.5 MJ/kg and 800 g concentrate pellets, 12.5 MJ/kg) to study the post absorptive and postprandial response at 2, 4, 8, and 24 h after feeding.

**Determination of insulin sensitivity:** Glucose tolerance tests (GTT) were undertaken on all offspring at 7 and 16 months of age in which jugular vein catheters had been previously inserted and the area under the curve (AUC) calculated. Animals were fasted overnight

( $\geq 18$ h) and injected intravenously with 0.5 g/kg glucose. Glucose and insulin concentrations were measured in plasma samples before and at 10, 20, 30, 60, 90, and 120 minutes, after the intravenous glucose (Gardner *et al.* 2005). The homeostatic model assessment for insulin resistance (HOMA-IR) index was calculated by multiplication of glucose (mmol/L) and insulin ( $\mu\text{g/L}$ ) concentrations measured in fasted plasma (Wallace *et al.* 2004).

**Determination of body composition, physical activity, and food intake at 16 months of age:**

Total body fat was determined when the animal was sedated (intramuscular injection of 1.5 mg /kg ketamine with 0.1 mg /kg xylazine) and scanned in a transverse position using a Lunar DPX-L (fast-detail whole body smartscan, GE Healthcare, Little Chalfont, UK). The level of spontaneous physical activity in adulthood in their respective environments was determined using uniaxial accelerometers (Actiwatch; Linton Instrumentation, Diss, UK). Average total food intake was measured in 24h intervals over a 10 day period with all animals kept in individual pens and with *ad libitum* access to feed, straw nuts (8.5 MJ/kg) and concentrate pellets (12.5 MJ/kg).

**Post mortem procedures and hypothalamic collection:** At 17 months of age, all offspring were euthanized by electrical stunning and exsanguination after an overnight fast. The entire hypothalamus was dissected according to anatomic landmarks (Sebert *et al.* 2009), snap frozen, and stored at  $-80^{\circ}\text{C}$  until analyzed. The use of entire hypothalamus allows analysis of the entire hypothalamic response but cannot be extrapolated to responses that would require nuclei-specific analyses.

**Laboratory analysis**

**Plasma metabolites and hormones**

Plasma glucose, triglycerides, and NEFA were measured by colorimetric assays (Randox, Crumlin, UK). Insulin was assayed using an ovine specific ELISA assay (Mercodia,



Diagenics Ltd, Milton Keynes, UK). Leptin (Delavaud *et al.* 2000) and cortisol (DPC coat-a-count, Siemens, Camberley, UK) were determined by a radio-immunoassay.

### **Analysis of the plasma metabolome**

Fasted heparin-treated plasma samples taken from mothers at dGA 130 were analyzed for a wide spectrum of metabolites by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). Plasma was defrosted on ice and filtered by centrifugation (Nanosep Omega, Pall, Port Washington, NY) to remove high molecular weight species, proteins in particular (over 10kDa). Metabolomic LC-HRMS profiles were acquired from 15  $\mu$ L of each filtered serum sample using an Agilent 1200<sup>TM</sup> HPLC system equipped with a 150 x 2.1 mm Uptisphere HDO-C<sub>18</sub> column with 3  $\mu$ m particle size (Interchim, Montluçon, France) coupled to a high resolution LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) fitted with an electrospray source operated in the positive ion mode. The detailed conditions applied both for the HPLC separation and mass spectrometric signal acquisition were previously described (Courant *et al.* 2009; Alexandre-Gouabau *et al.* 2011). Quality control standards and samples were randomly included five times into the sequence of injection.

### **Metabolomic data processing**

Open-source *XCMS* software (Smith *et al.* 2006) was used for non-linear alignment of the generated raw data and automatic integration and extraction of the signal intensities measured for each mass-retention time ( $[m/z; rt]$ ) feature constituting these metabolomic fingerprints, which each represent one ion. The *XCMS* parameters were implemented with the algorithm “match-filter” using default settings except for the interval of  $m/z$  value for peak picking which was set to 0.1, the noise threshold set to 6, the group band-width set to 10 and the

minimum fraction set to 0.5 as previously described (21). After *XCMS* processing, the signal abundances observed for identical ions in two groups of samples were statistically analyzed and annotation then subsequent identification of putative metabolites of interest were achieved using an in-house reference databank (34).

### **Gene expression measurements**

Offspring hypothalami were homogenized and RNA isolated, using the RNeasy Plus kit (Qiagen, Hilden, Germany). An aliquot of 4 µg of RNA was reverse transcribed with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was amplified in a real-time thermocycler (Quantica, Techne, Burlington, NJ, USA) using a SYBR green system in Taq polymerase reaction mix (ABsolute blue QPCR SYBR green, Thermo Scientific, Epsom, UK). Specificity of primers was confirmed by sequencing PCR product (Supplementary Information Table 1). Hypothalamic gene expression was assessed for the following pathways: a) orexigenic neurotransmitters: neuropeptide Y (*NPY*); agouti-related peptide (*AGRP*), b) insulin and leptin signaling: protein tyrosine phosphatase non-receptor type 1 (*PTP1B*); suppressor of cytokine signaling 3 (*SOCS3*); insulin receptor (*IR*); and leptin receptor (*OBRB*), c) intracellular energy signaling: AMP-activated kinase (*AMPK2*); mammalian target of rapamycin (*MTOR*); and fat mass and obesity-related gene (*FTO*), d) cortisol regulation: glucocorticoid receptor (*GCR*); corticotropin releasing hormone (*CRH*); and arginine vasopressin (*AVP*). Ribosomal RNA *18S* showed a stable expression and was used as a housekeeping gene. Gene expression was calculated by using the  $2^{-\Delta\Delta Ct}$  method (Livak & Schmittgen 2001).

## Statistical analysis

**Metabolomic data:** All multivariate data analyses and modeling were performed using *SIMCA-P* software (v 12, Umetrics Inc., Umeå, Sweden) on log-transformed (van den Berg *et al.* 2006) and Pareto-scaled (Cloarec *et al.* 2005) data as previously described (Alexandre-Gouabau *et al.* 2011). The susceptibility of the metabolic phenotypes of the mothers to caloric restriction in late pregnancy was assessed by using a supervised method, Partial Least Squares Discriminant Analysis (PLS-DA), which was applied to the transformed data set to reveal the potentially existing discrimination between sample groups to be compared within the data set, and to point out the variables more importantly involved in this discrimination. PLS-DA was combined with a multivariate preprocessing filter called Orthogonal Signal Correction (OSC). By removing within-class variability and confounders that may interfere with chemometric analysis, such as LC-MS technical variability, OSC can significantly improve PLS-DA performance, yielding a better discrimination of the clusters (Wagner *et al.* 2006). The quality of the generated OSC-PLS-DA model was classically evaluated by several goodness-of-fit parameters and criteria including:  $R^2(X)$ , the proportion of the total variance of the dependent variables that is explained by the model;  $R^2(Y)$ , defining the proportion of the total variance of the response variable (i.e. the class of the samples) explained by the model; and the predictive ability parameter  $Q^2(Y)$ , which was calculated by a seven-round internal cross-validation of the data. In addition, a permutation test ( $n=100$ ) was carried out to validate, and test, the degree of over fitting for OSC-PLS-DA models. The score values from OSC-PLS-DA were subjected to ANOVA to test the model and the validation was considered successful with  $P<0.01$ . The variables that discriminate the metabolic signatures most significantly were pinpointed by their loadings on PLS-DA.

**For non-metabolomic outcomes:** Statistical analysis of the data was performed using PASW<sup>®</sup> statistics software (v 17.02, IBM, Chicago, USA). Kolmogorov-Smirnoff tests were realized on every parameter analyzed to determine the Gaussian distributions of the variables. The influence of maternal nutrition (CAO vs. RAO), early postnatal growth (RAO vs. RRO), and obesogenic environment (RRL vs. RRO) were determined, according to parametric distribution, using ANOVA with a pairwise a priori test or Mann-Whitney U tests. Of the metabolites and hormones measured over 24 hours, changes over time were tested with the use of a paired t-test. Data is expressed as mean values with their standard errors. To address the limitations of multiple testing, statistical trend was accepted with a 95% interval of confidence ( $P < 0.05$ ) and significance was accepted with a confidence interval of 99% ( $P < 0.01$ ). Correlations were tested with the non-parametric Spearman's test and slope of the correlation was reported on the linear fit. Each variable was tested for sex. Body weight and fat mass are known to differ, in absolute scale, between male and female sheep (Bloor *et al.* 2013) thus sex-specific Z-score transformation was used prior to analyses. Specifically, we saw no indication of a difference in male and female offspring in glucose homeostasis, which is consistent with earlier studies (Gardner *et al.* 2005). Moreover, comparison for each variable between groups for females only demonstrated similar outcomes, although without reaching statistical significance, hence data for each sex were combined for further analyses and greater statistical power.

## Results

### Mothers

Relative to their weight at the beginning of the caloric restriction, R mothers gained less weight up to term compared with C mothers (Figure 2A). At 130 dGA, plasma glucose was reduced in fasted R mothers but there was a greater increase after feeding (Table 1A). Plasma NEFA concentrations were higher in fasted R mothers but did not differ between groups after feeding, whilst plasma triglycerides, cortisol, insulin, and leptin were unaffected by maternal diet (Table 1A). Metabolomic analysis showed a specific biological signature associated with the caloric restricted mothers, with a strong overall difference between the groups (OPLS-DA model of all the 2629 [m/z, rt] features detected, using 2 latent factors for maternal plasma metabolomic profiles (describing 43% of variable information); C mothers n=7 and R mothers n=17; Validation parameters:  $R^2X$  (cum) = 0.434,  $R^2Y$  (cum) = 0.999,  $Q^2$  (cum) = 0.994, permutation test (n = 100) with  $R^2$  intercept = 0.331 and  $Q^2$  intercept=-0.333, ANOVA  $P$ -value =  $9.9 \times 10^{-19}$ ), and of the 2629 detected features constituting these metabolomic profiles, 133 differed significantly ( $P < 0.01$ ). Of these, 95 which were upregulated with a fold change (expressed as a ratio of the mean abundance in R group compared to the mean abundance in the C group) of  $>1.4$  and only 7 which were downregulated with a fold change of  $<0.71$ . Due to technical constraints, only five of these compounds could be precisely identified (Table 1B), i.e. phenylalanine, tryptophan, and three forms of o-acetyl-carnitine were upregulated in R mothers.

### Offspring

The primary characteristics of each offspring group over the study are summarised in Table 2. Offspring of R mothers were smaller at birth and, when subjected to an intervention of accelerated early postnatal growth (RA), gained weight faster before weaning than either CA

or RR groups, suggesting that nutrient restriction during late pregnancy did not diminish milk production. By 7 months of age, body weight was similar between groups. At 16 months of age, as expected, physical activity was higher in those offspring kept in an unrestricted environment (RRL vs RRO) but did not differ between those maintained within an obesogenic environment. RRL animals were further smaller and consumed less feed each day.

**Insulin sensitivity:** At 7 months of age, the glucose AUC during the GTT was higher in obese compared to lean animals (RRO vs RRL, Figure 3 and Table 3). RAO offspring showed twice the insulin response to a standard glucose challenge in comparison to RRO. By 16 months of age, glucose AUC did not differ between groups but the insulin response during the GTT was significantly higher in RAO compared to CAO ( $P<0.05$ ), as was the HOMA-IR, an index of insulin resistance, which was also higher in RAO than in the RRO offspring ( $P<0.01$ ).

**Effect of feeding on plasma profiles of leptin and cortisol:** Prior to the feeding challenge, plasma leptin was higher in RAO than in RRO (Figure 4A). In the RAO group, plasma leptin initially declined on feeding ( $P<0.05$ ), to gradually increase between 8 and 24 h after feeding ( $P<0.05$ ). This effect was not observed in any other group as plasma leptin remained unchanged. Plasma glucose and insulin differed between RRO and RRL animals during the 24h of measurements ( $P<0.05$ ) but not between groups raised in an obesogenic environment (Figure 4B and 4C). Plasma cortisol both peaked 4 hours after feeding and was highest in RAO compared with CAO offspring, a difference that persisted until at least 8 h after feeding (Figure 4D).

**Hypothalamic gene expression:** Expression of appetite regulatory genes was unchanged, whilst *AMPKA2*, *MTOR*, and *FTO* were all higher in RAO compared to CAO groups (Table 4). A statistically significant negative correlation between anorexigenic circulating hormones insulin and leptin and the expression of orexigenic genes *NPY* and *AGRP* was observed in RAO and RRO, but not CAO or RRL groups. Taken together, these different relationships suggest a potential change in insulin and leptin sensitivity with the hypothalamus after IUGR (Table 5). Gene expression of both *AVP* and *CRH* was higher in RAO offspring as compared to CAO. Expression of *NPY* was three times higher and of *PTP1B*, *AMPKA2*, *MTOR*, and *GCR* was lower in the RRL offspring as compared to RRO. Importantly, postnatal growth rate (RAO vs RRO) did not have any effect on hypothalamic gene expression in any of the pathways investigated.

## Discussion

We have established that the long-term adverse outcomes of IUGR on insulin sensitivity can be dependent on exposure to accelerated early postnatal growth together with an obesogenic post-weaning environment. Accelerated early postnatal growth and post-weaning obesity following IUGR resulted in central resistance to insulin and leptin and was accompanied by an upregulation of gene expression for markers primarily recruited in energy sensing. In an absence of adult obesity, the detrimental effects of IUGR appeared to be much less pronounced. We have, therefore, indicated the important association between raised plasma insulin and *in utero* programmed changes of hypothalamic sensitivity previously observed following juvenile onset obesity (Sebert *et al.* 2011).

Both acute and chronic reductions in maternal food intake in late gestation stimulate maternal catabolism resulting in hypoglycemia, ketoacidosis (Herrera & Amusquivar 2000; Tygesen *et al.* 2008) and an increased lipolysis (Symonds *et al.* 1989). In the present study, caloric restriction over the same period not only induced fasting hypoglycemia but was accompanied by a more pronounced rise in plasma glucose immediately after feeding. These substantial fluctuations in maternal plasma glucose are likely to be paralleled within the fetus, and thus possibly resetting metabolic homeostasis. We, therefore, propose that the metabolic stimuli following maternal nutrient restriction in late gestation not only promotes NEFA oxidation (Symonds *et al.* 1989) but stimulates protein catabolism as indicated by raised plasma acetylcarnitine identified in the metabolomic analysis.

This is the first study to analyze the maternal metabolomic response to caloric restriction in any species. Given the substantial dichotomy in the maternal metabolic profiles with maternal nutrient restriction or free access to food, the present study suggests that the source of energy



available to the fetus may be a primary determinant of long term energy homeostasis in the offspring, especially when subsequently exposed to an obesogenic environment. The brain is dependent on the availability of glucose and ketone bodies (Robinson & Williamson 1980) and this switch in energy source may be essential to hypothalamic plasticity. Although insulin resistance following maternal caloric restriction can be exacerbated further with age (Kongsted *et al.* 2014), at 16 months of age we observed an effect of both postnatal growth rate and a clear influence of exposure to an obesogenic environment.

Despite higher insulin and leptin concentrations, IUGR offspring raised in an obesogenic environment (RAO *vs* CAO) did not exhibit alterations in gene expression for orexigenic neurotransmitters such as *NPY* and *AGRP* and correlations between plasma leptin with *NPY* and *AGRP* suggest a blunted response in RAO as compared to CAO, i.e. early-onset hypothalamic resistance to leptin (Schwartz & Baskin 2013). No reduction in gene expression for insulin and leptin receptors was found, which could have suggested a potential mechanism. Whether these effects are mediated through changes in downstream signaling has yet to be confirmed. We were unable to detect any significant changes in expression of *PTPIB* or *SOCS3*, suggesting further mechanistic studies are required.

Lean IUGR offspring (RRL *vs* RRO) were characterised as exhibiting reduced hypothalamic gene expression for *PTPIB* but the abundance of the orexigenic neurotransmitter *NPY* was raised, reflecting a high central sensitivity to insulin and leptin, as expected in animals of normal body weight (Ahmad *et al.* 1997). Glucose homeostasis and the hormonal response to feeding in RRL were similar to CAO offspring. Taken together, these findings indicate a degree of maladaptation as lean IUGR individuals would be expected to exhibit lower plasma concentrations of fasted metabolites and hormones and show a smaller response to those

challenges than obese animals, at least in terms of NEFA, insulin, and leptin (Sebert *et al.* 2009). One hypothalamic outcome of IUGR was increased expression of genes involved in energy sensing, which were also higher in the offspring reared within an obesogenic, compared with a lean environment (RRO vs RRL). In the lean IUGR group, the expression for those genes was reduced to values very similar to obese controls, even though *FTO* is known to be more highly expressed in obese than lean sheep (Sebert *et al.* 2010). This further suggests that IUGR has a long-term effect which is not fully corrected with exposure to a high activity environment. However, these assumptions will need to be tested further with a more appropriate control group and in both male and female offspring.

IUGR also resulted in raised gene expression for hypothalamic genes involved in cortisol regulation, i.e. *CRH* and *AVP* which, when combined with the higher plasma cortisol response to feeding seen in the obese IUGR group subject to an accelerated postnatal growth rate, may be indicative of reduced negative feedback control (Lightman 2008). The same higher expression of *AVP* and *CRH* was observed in the obese IUGR animals subjected to a slower postnatal growth rate and lean IUGR animals, which both had a lower cortisol response to feeding. Therefore, we did not see a similar loss of negative feedback in these latter offspring. This difference in cortisol regulation is novel and requires further investigation. It has recently been described that female sheep with juvenile-onset obesity have elevated plasma cortisol concentrations (Bloor *et al.* 2013), a difference not found in the present study.

All offspring raised in an obesogenic environment became equally obese irrespective of their *in utero* diet, and this may reflect the more physiological, long term exposure we adopted to induce this condition. Both twin and singleton pregnancies are common in sheep, leading to

differences in birth weight and post weaning growth (Hancock *et al.* 2012). Only twin bearing mothers were entered into the present study, so it is not possible to ascertain whether similar interventions designed to impact on postnatal growth rates would lead to identical similar outcomes in singleton offspring. Our study demonstrates, however, that both the postnatal and post weaning environments are important determinants of longterm outcomes following IUGR. To date, there are no large animal studies which have looked at the developmentally exacerbated effects of adult onset obesity together with the extent to which all symptoms of the metabolic syndrome become manifest. This is due to a number of practical considerations which include the extended time period required, well beyond the three year time frame of most project grant awards and the very high cost of such studies. In addition, the sex of the offspring is not predictable in naturally conceived pregnancies. A study designed to analyze the biological interaction between the sex of the offspring and the outcomes of fetal programming would clearly require a much larger number of mothers to reach the appropriate number of male and female offspring. Given the current limitations and knowledge, our present data support the evidence that some long term impacts of fetal programming are common to both sexes. However, future studies that are able to include sufficient numbers of males and females are warranted to analyze further the effect of the sex of the offspring and its interaction with the fetal and postnatal environments.

In conclusion, in sheep, manipulation of the maternal metabolic status alone, without significant changes in maternal plasma insulin, leptin and cortisol, is sufficient to have long term consequences for the offspring's health. The adverse phenotype of IUGR is enhanced by accelerated postnatal growth and exposure to an obesogenic environment in juvenile life.

**Declaration of Interest:** The authors have nothing to disclose.

### **Acknowledgements**

This work was supported by the European Union Sixth Framework Program for Research and Technical Development of the European Community, The Early Nutrition Programming Project FOOD-CT-2005-007036, the Nutricia Research Foundation, and the Nottingham Respiratory Biomedical Research Unit. S.P.S. was also supported by a Wellcome Trust Value-in-People award.

## References

- Adam CL, Findlay PA, Chanet A, Aitken RP, Milne JS & Wallace JM 2008 Expression of energy balance regulatory genes in the developing ovine fetal hypothalamus at midgestation and the influence of hyperglycemia. *AJP: Regulatory, Integrative and Comparative Physiology* **294** R1895–R1900. (doi:10.1152/ajpregu.00163.2008)
- Ahmad F, Azevedo JL, Cortright R, Dohm GL & Goldstein BJ 1997 Alterations in skeletal muscle protein-tyrosine phosphatase activity and expression in insulin-resistant human obesity and diabetes. *Journal of Clinical Investigation* **100** 449.
- Alexandre-Gouabau M-C, Courant F, Le Gall G, Moyon T, Darmaun D, Parnet P, Coupé B & Antignac J-P 2011 Offspring metabolomic response to maternal protein restriction in a rat model of intrauterine growth restriction (IUGR). *Journal of Proteome Research* **10** 3292–3302. (doi:10.1021/pr2003193)
- Barker DJ 1997 Fetal nutrition and cardiovascular disease in later life. *British Medical Bulletin* **53** 96–108.
- Van den Berg RA, Hoefsloot HCJ, Westerhuis JA, Smilde AK & van der Werf MJ 2006 Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics* **7** 142. (doi:10.1186/1471-2164-7-142)
- Bispham J, Gopalakrishnan GS, Dandrea J, Wilson V, Budge H, Keisler DH, Broughton Pipkin F, Stephenson T & Symonds ME 2003 Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. *Endocrinology* **144** 3575–3585. (doi:10.1210/en.2003-0320)
- Bloor ID, Sébert SP, Saroha V, Gardner DS, Keisler DH, Budge H, Symonds ME & Mahajan RP 2013 Sex differences in metabolic and adipose tissue responses to juvenile-onset obesity in sheep. *Endocrinology* **154** 3622–3631. (doi:10.1210/en.2013-1207)
- Cloarec O, Dumas M-E, Craig A, Barton RH, Trygg J, Hudson J, Blancher C, Gauguier D, Lindon JC, Holmes E *et al.* 2005 Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic <sup>1</sup>H NMR data sets. *Analytical Chemistry* **77** 1282–1289. (doi:10.1021/ac048630x)
- Courant F, Pinel G, Bichon E, Monteau F, Antignac J-P & Le Bizec B 2009 Development of a metabolomic approach based on liquid chromatography-high resolution mass spectrometry to screen for clenbuterol abuse in calves. *The Analyst* **134** 1637. (doi:10.1039/b901813a)
- Delavaud C, Bocquier F, Chilliard Y, Keisler DH, Gertler A & Kann G 2000 Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA in sheep. *Journal of Endocrinology* **165** 519–526.
- Desai M, Babu J & Ross MG 2007 Programmed metabolic syndrome: prenatal undernutrition and postweaning overnutrition. *American Journal of Physiology. Regulatory,*

- Integrative and Comparative Physiology* **293** R2306–R2314.  
(doi:10.1152/ajpregu.00783.2006)
- Gardner DS, Tingey K, Van Bon B, Dandrea J, Keisler DH, Stephenson T & Symonds ME 2005 Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **289** R947–R954. (doi:10.1152/ajpregu.00120.2005)
- Hancock SN, Oliver MH, McLean C, Jaquiere AL & Bloomfield FH 2012 Size at birth and adult fat mass in twin sheep are determined in early gestation. *The Journal of Physiology* **590** 1273–1285.
- Herrera E & Amusquivar E 2000 Lipid metabolism in the fetus and the newborn. *Diabetes/metabolism Research and Reviews* **16** 202–210.
- Kongsted AH, Tygesen MP, Husted SV, Oliver MH, Tolver A, Christensen VG, Nielsen JH & Nielsen MO 2014 Programming of glucose-insulin homeostasis: long-term consequences of pre-natal versus early post-natal nutrition insults. Evidence from a sheep model. *Acta Physiologica* **210** 84–98. (doi:10.1111/apha.12080)
- Lightman SL 2008 The neuroendocrinology of stress: a never ending story. *Journal of Neuroendocrinology* **20** 880–884. (doi:10.1111/j.1365-2826.2008.01711.x)
- Livak KJ & Schmittgen TD 2001 Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods* **25** 402–408.  
(doi:10.1006/meth.2001.1262)
- McMillen IC & Robinson JS 2005 Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiological Reviews* **85** 571–633.  
(doi:10.1152/physrev.00053.2003)
- Muhlhausler BS, Adam CL, Findlay PA, Duffield JA & McMillen IC 2006 Increased maternal nutrition alters development of the appetite-regulating network in the brain. *The FASEB Journal* **20** 1257–1259. (doi:10.1096/fj.05-5241fje)
- Paus T 2010 A primer for brain imaging: a tool for evidence-based studies of nutrition? *Nutrition Reviews*, Vol. 68, No. S1. *Nutrition Reviews* **68** S29–S37.  
(doi:10.1111/j.1753-4887.2010.00327.x)
- Robinson AM & Williamson DH 1980 Physiological roles of ketone bodies as substrates and signals in mammalian tissues. *Physiological Reviews* **60** 143–187.
- Roseboom TJ, van der Meulen JH, Osmond C, Barker DJ, Ravelli AC, Schroeder-Tanka JM, van Montfrans GA, Michels RP & Bleker OP 2000 Coronary heart disease after prenatal exposure to the Dutch famine, 1944–45. *Heart (British Cardiac Society)* **84** 595–598.
- Schwartz MW & Baskin DG 2013 Leptin and the brain: then and now. *Journal of Clinical Investigation* **123** 2344–2345. (doi:10.1172/JCI69346)
- Schwartz MW, Woods SC, Porte D, Seeley RJ & Baskin DG 2000 Central nervous system control of food intake. *Nature* **404** 661–671.

- Sebert SP, Hyatt MA, Chan LLY, Patel N, Bell RC, Keisler D, Stephenson T, Budge H, Symonds ME & Gardner DS 2009 Maternal nutrient restriction between early and midgestation and its impact upon appetite regulation after juvenile obesity. *Endocrinology* **150** 634–641. (doi:10.1210/en.2008-0542)
- Sebert SP, Hyatt MA, Chan LLY, Yiallourides M, Fainberg HP, Patel N, Sharkey D, Stephenson T, Rhind SM, Bell RC *et al.* 2010 Influence of prenatal nutrition and obesity on tissue specific fat mass and obesity-associated (FTO) gene expression. *Reproduction* **139** 265–274. (doi:10.1530/REP-09-0173)
- Sebert SP, Dellschaft NS, Chan LLY, Street H, Henry M, Francois C, Sharma V, Fainberg HP, Patel N, Roda J *et al.* 2011 Maternal nutrient restriction during late gestation and early postnatal growth in sheep differentially reset the control of energy metabolism in the gastric mucosa. *Endocrinology* **152** 2816–2826. (doi:10.1210/en.2011-0169)
- Sharkey D, Gardner DS, Fainberg HP, Sebert S, Bos P, Wilson V, Bell R, Symonds ME & Budge H 2009 Maternal nutrient restriction during pregnancy differentially alters the unfolded protein response in adipose and renal tissue of obese juvenile offspring. *The FASEB Journal* **23** 1314–1324. (doi:10.1096/fj.08-114330)
- Singhal A, Fewtrell M, Cole TJ & Lucas A 2003 Low nutrient intake and early growth for later insulin resistance in adolescents born preterm. *The Lancet* **361** 1089–1097.
- Smith CA, Want EJ, O'Maille G, Abagyan R & Siuzdak G 2006 XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Analytical Chemistry* **78** 779–787. (doi:10.1021/ac051437y)
- Stettler N, Kumanyika SK, Katz SH, Zemel BS & Stallings VA 2003 Rapid weight gain during infancy and obesity in young adulthood in a cohort of African Americans. *The American Journal of Clinical Nutrition* **77** 1374–1378.
- Symonds ME, Bryant MJ & Lomax MA 1989 Lipid metabolism in shorn and unshorn pregnant sheep. *British Journal of Nutrition* **62** 35–49.
- Symonds ME, Sebert SP, Hyatt MA & Budge H 2009 Nutritional programming of the metabolic syndrome. *Nature Reviews. Endocrinology* **5** 604–610. (doi:10.1038/nrendo.2009.195)
- Tygesen MP, Nielsen MO, Nørgaard P, Ranvig H, Harrison AP & Tauson A-H 2008 Late gestational nutrient restriction: effects on ewes' metabolic and homeorhetic adaptation, consequences for lamb birth weight and lactation performance. *Archives of Animal Nutrition* **62** 44–59. (doi:10.1080/17450390701780276)
- Wagner S, Scholz K, Donegan M, Burton L, Wingate J & Völkel W 2006 Metabonomics and biomarker discovery: LC–MS metabolic profiling and constant neutral loss scanning combined with multivariate data analysis for mercapturic acid analysis. *Analytical Chemistry* **78** 1296–1305. (doi:10.1021/ac051705s)
- Wallace TM, Levy JC & Matthews DR 2004 Use and abuse of HOMA modeling. *Diabetes Care* **27** 1487–1495.

Warnes KE, Morris MJ, Symonds ME, Phillips ID, Clarke IJ, Owens JA & McMillen IC  
1998 Effects of increasing gestation, cortisol and maternal undernutrition on  
hypothalamic neuropeptide Y expression in the sheep fetus. *Journal of  
Neuroendocrinology* **10** 51–57.



## Figure Titles

**Figure 1: Schematic depiction of the study design.** Twin pregnant ewes were randomly assigned to one of two diets for late gestation (110-145 days): C diet meeting requirements or macronutrient-restricted diet meeting 60% of caloric requirements (R). After birth twins were either both reared by their mother (regular early postnatal growth rate, R) or separated with only one twin being reared by the mother (accelerated early postnatal growth rate, A). After weaning a majority of animals were kept in restricted space, representing a mildly obesogenic (O) environment but a proportion of the RR group were kept within an unrestricted space, leading to lean (L) animals. Discrepancies between numbers (n) of mothers and offspring are due to additional offspring intervention groups which were not included in the present study.

## **Figure 2: Maternal characteristics of diet-induced intrauterine growth restriction.**

Effect of a 40% reduction in maternal food intake from 110 days gestation on maternal weight gain throughout the remainder of pregnancy (relative to their weight at dGA 110, the start of intervention).

Twin-pregnant sheep were either fed to requirements (C, n=9) or pair-fed to 60% of that amount from 110 days gestation (R, n=19). In (A) Values are mean and SEM with 10 animals per group. Significant differences between groups: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

## **Figure 3: Influence of fetal intrauterine growth restriction, accelerated postnatal growth, and obesity on the onset of insulin resistance in the offspring.**

Time course of changes in plasma glucose (continuous line) and insulin (dashed line) following an intravenous glucose injection at (A) 7 (i.e. puberty) and (B) 16 months of age (i.e. young adulthood).

Offspring of C and R mothers were subjected to an accelerated (CA, n=8; RA, n=8) or regular growth (RR, n=15) during lactation. After weaning, offspring were then exposed to an obesogenic environment (O) with the exception for a subset of RR, which remained lean (RRL, n=8; RRO, n=7). Open square, CAO; filled square, RAO; open circle, RRO; filled circle, RRL. Values are mean  $\pm$  SEM. Significant differences between groups  $P<0.05$ ; \* between CAO and RAO; # between RAO and RRO; ‡ between RRO and RRL.

**Figure 4: Influence of fetal intrauterine growth restriction, accelerated postnatal growth, and obesity on A) leptin, B) glucose, C) insulin, and D) cortisol response to feeding in the young adult offspring at 16 months of age.**

Insert in D depicts the relative change in plasma cortisol concentrations between 2h and 4h after feeding in the intervention groups.

Offspring of C and R mothers were subjected to an accelerated (CA, n=7; RA, n=8) or regular growth (RR, n=14) during lactation. After weaning, offspring were then exposed to an obesogenic environment (O) with the exception for a subset of RR, which remained lean (RRL, n=7; RRO, n=7). Open square, CAO; filled square, RAO; open circle, RRO; filled circle, RRL. Values are mean  $\pm$  SEM. Significant difference between time points  $P<0.05$ ; a within RAO. Significant differences between groups  $P<0.05$ ; \* between CAO and RAO; # between RAO and RRO; ‡ between RRO and RRL.

## Table Titles

**Table 1: Effect of maternal diet in late gestation commencing on 110 days gestation on plasma endocrine and metabolic characteristics.** Plasma was sampled from mothers at 130 days gestation and **(A)** concentrations of metabolites and hormones, determined immediately prior to and 2 hours after feeding and **(B)** metabolites in pre-feeding samples that were identified by metabolomic fingerprinting to have significantly changed with maternal diet (Mann-Whitney test,  $p < 0.01$ ).

Twin-pregnant sheep were either fed to requirements (C,  $n=9$ ) or pair-fed to 60% of that amount from 110 days gestation (R,  $n=19$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . M, monoisotopic mass; all compounds were identified using authentic standards. Fold change of each feature is reported as mean  $\pm$  SEM of the abundance for caloric restricted mothers relative to controls.

**Table 2: Influence of fetal intrauterine growth restriction, accelerated postnatal growth, and obesity on offspring body weight and on adult body composition, physical activity, and food intake as measured at 16 months of age.**

Offspring of C and R mothers were subjected to an accelerated (CA,  $n=8$ ; RA,  $n=9$ ) or regular growth (RR,  $n=17$ ) during lactation. After weaning, offspring were then exposed to an obesogenic environment (O) with the exception for a subset of RR, which remained lean (RRL,  $n=9$ ; RRO,  $n=8$ ). Values are mean  $\pm$  SEM. Significant differences between groups represented by different superscripts, a vs b  $P < 0.05$ ; c vs d  $P < 0.01$ .

NEFA, non-esterified fatty acids; TG, triglycerides.

**Table 3: Influence of fetal intrauterine growth restriction, accelerated postnatal growth, and obesity on the onset of insulin resistance in the offspring.** Plasma glucose and insulin responses to an intravenous glucose injection at 7 (i.e. puberty) and 16 months (i.e. young adulthood) of age.

Offspring of C and R mothers were subjected to an accelerated (CA, n=8; RA, n=8) or regular growth (RR, n=15) during lactation. After weaning, offspring were then exposed to an obesogenic environment (O) with the exception for a subset of RR, which remained lean (RRL, n=8; RRO, n=7). Values are mean  $\pm$  SEM. Significant differences between groups represented by different superscripts, a vs b  $P < 0.05$ ; c vs d  $P < 0.01$ .

**Table 4: Effect of maternal caloric restriction, accelerated postnatal growth, and juvenile-onset obesity on the regulation of energy balance and endocrine sensitivity in the hypothalamus of young adults.**

Offspring of C and R mothers were subjected to an accelerated (CA, n=5; RA, n=8) or regular growth (RR, n=15) during lactation. After weaning, offspring were then exposed to an obesogenic environment (O) with the exception for a subset of RR, which remained lean (RRL, n=8; RRO, n=7). Values are mean  $\pm$  SEM and n=5-8 per time point. Statistical significance for the effect of maternal diet (i.e. CAO vs RAO), accelerated postnatal growth (i.e. RAO vs RRO) and obesity (i.e. RRO vs RRL). NS, not significant.

*NPY*, neuropeptide Y; *AGRP*, agouti-related peptide; *PTP1B*, protein tyrosine phosphatase, non-receptor type 1; *SOCS3*, suppressor of cytokine signalling 3; *IR*, insulin receptor; *OBRB*, leptin receptor, long form; *AMPKA2*, AMP-activated protein kinase  $\alpha 2$ ; *MTOR*, mammalian target of rapamycin; *FTO*, fat mass and obesity associated gene; *GCR*, glucocorticoid receptor; *CRH*, corticotropin releasing hormone; *AVP*, arginine vasopressin.

**Table 5: Correlations between plasma insulin and leptin concentrations and hypothalamic gene expression for *NPY* and *AGRP* ( $2^{-\Delta\Delta Ct}$ ) at 16 months of age.**

Offspring of C and R mothers were subjected to an accelerated (CA, n=5; RA, n=8) or regular growth (RR, n=15) during lactation. After weaning, offspring were then exposed to an obesogenic environment (O) with the exception for a subset of RR, which remained lean (RRL, n=8; RRO, n=7). \* $P < 0.05$ ; \*\* $P < 0.01$ . Slope is expressed as <sup>a)</sup>  $\times 10^{-5}$  and <sup>b)</sup>  $\times 10^{-6}$ . *AGRP*, agouti-related peptide; *NPY*, neuropeptide Y.