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

[1] 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.

[2] INRA and University of Nantes, UMR-1280 Physiologie des Adaptations Nutritionnelles, CHU Hôtel Dieu, 44093 Nantes cedex 1, France.

[3] School of Veterinary Medicine and Science, Sutton Bonington Campus, The University of Nottingham, Nottingham LE12 5RD United Kingdom.

[4] Institute of Health Sciences, Centre For Life-Course Epidemiology, and Biocentre Oulu, University of Oulu, Aapistie 5B, 90014 Oulu, Finland.

[5] LUNAM Université, Oniris, Laboratoire d'Etude des Résidus et Contaminants dans les Aliments, USC INRA 1329, Nantes, France.

[6] Department of Animal Science, University of Missouri, Columbia, Missouri 65211, USA.

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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

79 *et al.* 2003). In sheep, the relative importance of enhanced postnatal growth on long term
80 outcomes has not been widely examined, although, when combined with IUGR, accelerated
81 postnatal growth differentially affects energy sensing within the stomach and hypothalamus
82 (Sebert *et al.* 2011). The post-weaning environment is an additional factor which appears to
83 determine the magnitude of the phenotypic response to alterations in maternal diet in
84 pregnancy in rodents (Desai *et al.* 2007). In these studies, the metabolic and related effects in
85 young adult offspring who were nutritionally manipulated *in utero* are minimal unless
86 adiposity has been promoted. The extent to which similar changes in body composition also
87 apply to large mammals has not been investigated.
88
89 Each organ has a set developmental trajectory and therefore they are not all similarly affected
90 by IUGR. The hypothalamus is particularly sensitive to environmental stresses in early life
91 and plays a central role in the regulation of energy homeostasis (Adam *et al.* 2008). Cortisol
92 can influence blood pressure as a consequence of regulating gene expression of arginine
93 vasopressin (*AVP*) and corticotrophin releasing hormone (*CRH*), which when suppressed,
94 acts through negative feedback, to reduce cortisol secretion from the adrenals through
95 decreased adrenocorticotropic hormone action (Lightman 2008). Food intake is regulated by
96 changes in the plasma concentration of markers of energy status, including insulin, leptin, and
97 glucose (Schwartz *et al.* 2000). These factors determine the action of neurotransmitters in the
98 arcuate nucleus of the hypothalamus, especially neuropeptide Y (*NPY*) and pro-opio
99 melanocortin (*POMC*), which have antagonistic actions in signaling peripheral energy status
100 to other hypothalamic nuclei, the cortico-limbic system and the brain stem, which ultimately
101 determine food intake and physical activity (Schwartz *et al.* 2000). Critically, the fetal
102 hypothalamus shows an orexigenic response by increased *NPY* signaling to maternal nutrient
103 restriction in late gestation (Warnes *et al.* 1998) and an anorexigenic response by increased

104 *POMC* signaling to maternal and early postnatal overnutrition (Muhlhausler *et al.* 2006).
105 However, whether these adaptations persist into adulthood is not known. In addition, whilst
106 organogenesis and the developmental maturation of the hypothalamus in altricial species is
107 particularly sensitive to the late gestational nutritional environment (Adam *et al.* 2008),
108 hypothalamic maturation continues after birth when it is particularly responsive to the
109 postnatal energetic environment (Paus 2010). Whether specific changes in the early postnatal
110 environment during key windows, *i.e.* immediately after birth and at weaning, modulate the
111 long-term molecular adaptation of the hypothalamus to IUGR has received no attention. The
112 present study, therefore, not only examines the effects of maternal caloric restriction during
113 late pregnancy on maternal homeostasis but also tests the hypothesis that the adverse effects
114 of IUGR are dependent on the postnatal energetic environment and concomitant differences
115 in peri-partum or post-weaning growth. We investigated the effects of: i) IUGR followed by
116 an accelerated postnatal growth combined with a low activity obesogenic environment after
117 weaning and compared to those offspring born to mothers fed to requirements throughout
118 pregnancy and with the same postnatal treatment, ii) differing postnatal growth rates on adult
119 IUGR offspring submitted to obesogenic conditions, and finally, iii) a differing energetic
120 environment on IUGR offspring submitted to regular postnatal growth rate.
121

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 (~145 ± 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 MJ/kg.$BW^{0.75}$ at 110 days gestation, increasing to 0.72 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 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 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 for independent intervention groups for

147 the twins that were removed from their mother on the first day of birth. This included
148 formula-reared twins within the CAO and RAO offspring, which were not included in the
149 present study. The numbers of twin bearing mothers entered into the study for each
150 nutritional group were predicted to be sufficient to produce enough numbers of male and
151 female offspring for each of the postnatal intervention groups. However due to the uneven
152 distribution of male and females born to R mothers there were fewer male offspring available
153 than anticipated. The resulting groups permit us to draw comparisons between animals with
154 and without IUGR (RAO *vs.* CAO) and, within those with IUGR, to investigate the effects of
155 early postnatal growth (RAO *vs.* RRO) and of post-weaning environment (RRO *vs.* RRL).
156
157 **Timing of samplings and *in vivo* challenges**
158 **Maternal blood sampling:** At 130 dGA, jugular venous blood samples (5 ml) were collected
159 from the ewes in the morning, prior to, and two hours after, feeding. Venous blood was
160 collected into heparinized or K^+EDTA coated tubes and the plasma was immediately
161 separated by centrifugation (2500 g x 10 min at 4˚C) and stored at -80˚C until analysis.
162 **Offspring blood sampling**: Venous blood samples (prepared and stored under identical
163 conditions as described above) were collected after an overnight fast (≥18h) at both 7 and 16
164 months of age. Jugular catheters were inserted by percutaneous venepuncture 1-2 days before
165 sampling. Additional blood samples were collected at 16 months of age following the
166 presentation of a mix of high and low energy-dense feed (3 kg straw nuts, 8.5 MJ/kg and 800
167 g concentrate pellets, 12.5 MJ/kg) to study the post absorptive and postprandial response at 2,
168 4, 8, and 24 h after feeding.
169 **Determination of insulin sensitivity:** Glucose tolerance tests (GTT) were undertaken on all
170 offspring at 7 and 16 months of age in which jugular vein catheters had been previously
171 inserted and the area under the curve (AUC) calculated. Animals were fasted overnight

(≥18h) 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 (μg/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 euthanazed 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°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 µL of each filtered serum sample using an Agilent 1200^{TM} HPLC system equipped with a 150 x 2.1 mm Uptisphere HDO-C_{18} column with 3 µm 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 (*AMPKA2*); 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).

244

245 **Statistical analysis**

246 **Metabolomic data:** All multivariate data analyses and modeling were performed using
247 *SIMCA-P+*software (v 12, Umetrics Inc., Umeå, Sweden) on log-transformed (van den Berg
248 *et al.* 2006) and Pareto-scaled (Cloarec *et al.* 2005) data as previously described (Alexandre-
249 Gouabau *et al.* 2011). The susceptibility of the metabolic phenotypes of the mothers to
250 caloric restriction in late pregnancy was assessed by using a supervised method, Partial Least
251 Squares Discriminant Analysis (PLS-DA), which was applied to the transformed data set to
252 reveal the potentially existing discrimination between sample groups to be compared within
253 the data set, and to point out the variables more importantly involved in this discrimination.
254 PLS-DA was combined with a multivariate preprocessing filter called Orthogonal Signal
255 Correction (OSC). By removing within-class variability and confounders that may interfere
256 with chemometric analysis, such as LC-MS technical variability, OSC can significantly
257 improve PLS-DA performance, yielding a better discrimination of the clusters (Wagner *et al.*
258 2006). The quality of the generated OSC-PLS-DA model was classically evaluated by several
259 goodness-of-fit parameters and criteria including: R^2 (X), the proportion of the total variance
260 of the dependent variables that is explained by the model; R^2 (Y), defining the proportion of
261 the total variance of the response variable (i.e. the class of the samples) explained by the
262 model; and the predictive ability parameter Q^2(Y), which was calculated by a seven-round
263 internal cross-validation of the data. In addition, a permutation test (n=100) was carried out to
264 validate, and test, the degree of over fitting for OSC-PLS-DA models. The score values from
265 OSC-PLS-DA were subjected to ANOVA to test the model and the validation was considered
266 successful with $P<0.01$. The variables that discriminate the metabolic signatures most
267 significantly were pinpointed by their loadings on PLS-DA.

For non-metabolomic outcomes: Statistical analysis of the data was performed using PASW® 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 = $9.9x10^{-19}$), and of the 2629 detected features constituting these metabolomic profiles, 133 differed significantly ($P<0.01$). Of these, 95 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 were downregulated with a fold change of <0.71. Due to species-specific technical constraints, only five of these compounds could be precisely identified (Table 1B) as phenylalanine, tryptophan, and three forms of o-acetyl-carnitine which were all upregulated in R mothers.

Offspring

The primary characteristics of each offspring group over the study are summarized 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 within 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.

346 **Discussion**

347 We have established that the long-term adverse outcomes of IUGR on insulin sensitivity can
348 be dependent on exposure to accelerated early postnatal growth together with an obesogenic
349 post-weaning environment. Accelerated early postnatal growth and post-weaning obesity
350 following IUGR resulted in central resistance to insulin and leptin and was accompanied by
351 an upregulation of gene expression for markers primarily recruited in energy sensing. In an
352 absence of adult obesity, the detrimental effects of IUGR appeared to be much less
353 pronounced. We have, therefore, indicated the important association between raised plasma
354 insulin and *in utero* programmed changes of hypothalamic sensitivity previously observed
355 following juvenile onset obesity (Sebert *et al.* 2011).
356
357 Both acute and chronic reductions in maternal food intake in late gestation stimulate maternal
358 catabolism resulting in hypoglycemia, ketoacidosis (Herrera & Amusquivar 2000; Tygesen *et*
359 *al.* 2008) and an increased lipolysis (Symonds *et al.* 1989). In the present study, caloric
360 restriction over the same period not only induced fasting hypoglycemia but was accompanied
361 by a more pronounced rise in plasma glucose immediately after feeding. These substantial
362 fluctuations in maternal plasma glucose are likely to be paralleled within the fetus, and thus
363 possibly resetting metabolic homeostasis. We, therefore, propose that the metabolic stimuli
364 following maternal nutrient restriction in late gestation not only promotes NEFA oxidation
365 (Symonds *et al.* 1989) but stimulates protein catabolism as indicated by raised plasma
366 acetylcarnitine identified in the metabolomic analysis.
367
368 This is the first study to analyze the maternal metabolomic response to caloric restriction in
369 any species. Given the substantial dichotomy in the maternal metabolic profiles with maternal
370 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 *PTP1B* or *SOCS3*, suggesting further mechanistic studies are required.

Lean IUGR offspring (RRL *vs* RRO) were characterised as exhibiting reduced hypothalamic gene expression for *PTP1B* 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

421 differences in birth weight and post weaning growth (Hancock *et al.* 2012). Only twin
422 bearing mothers were selected for the present study, so it is not possible to ascertain whether
423 similar interventions designed to impact on postnatal growth rates would lead to identical
424 outcomes in singleton offspring. Our study demonstrates, however, that both the postnatal
425 and post weaning environments are important determinants of long-term outcomes following
426 IUGR. To date, there are no large animal studies which have looked at the developmentally
427 exacerbated effects of adult onset obesity together with the extent to which all symptoms of
428 the metabolic syndrome become manifest. This is due to a number of practical considerations
429 which include the extended time period required, well beyond the three year time frame of
430 most project grant awards and the very high cost of such studies. In addition, the sex of the
431 offspring is not predictable in naturally conceived pregnancies. A study designed to analyze
432 the biological interaction between the sex of the offspring and the outcomes of fetal
433 programming would clearly require a much larger number of mothers to reach the appropriate
434 number of male and female offspring. Given the current limitations and knowledge, our
435 present data support the evidence that some long term impacts of fetal programming are
436 common to both sexes. However, future studies that are able to include sufficient numbers of
437 males and females are warranted to analyze further the effect of the sex of the offspring and
438 its interaction with the fetal and postnatal environments.
439
440 In conclusion, in sheep, manipulation of the maternal metabolic status alone, without
441 significant changes in maternal plasma insulin, leptin and cortisol, is sufficient to have long
442 term consequences for the offspring's health. The adverse phenotype of IUGR is enhanced by
443 accelerated postnatal growth and exposure to an obesogenic environment in juvenile life.
444

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

446

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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 intra-venous glucose injection at **(A)** 7 (i.e. puberty) and **(B)** 16 months of age (i.e. young adulthood).

599 Offspring of C and R mothers were subjected to an accelerated (CA, n=8; RA, n=8) or
600 regular growth (RR, n=15) during lactation. After weaning, offspring were then exposed to
601 an obesogenic environment (O) with the exception for a subset of RR, which remained lean
602 (RRL, n=8; RRO, n=7). Open square, CAO; filled square, RAO; open circle, RRO; filled
603 circle, RRL. Values are mean ± SEM. Significant differences between groups $P<0.05$; *
604 between CAO and RAO; # between RAO and RRO; ‡ between RRO and RRL.
605
606 **Figure 4: Influence of fetal intrauterine growth restriction, accelerated postnatal**
607 **growth, and obesity on A) leptin, B) glucose, C) insulin, and D) cortisol response to**
608 **feeding in the young adult offspring at 16 months of age.**
609 Insert in D depicts the relative change in plasma cortisol concentrations between 2h and 4h
610 after feeding in the intervention groups.
611 Offspring of C and R mothers were subjected to an accelerated (CA, n=7; RA, n=8) or
612 regular growth (RR, n=14) during lactation. After weaning, offspring were then exposed to
613 an obesogenic environment (O) with the exception for a subset of RR, which remained lean
614 (RRL, n=7; RRO, n=7). Open square, CAO; filled square, RAO; open circle, RRO; filled
615 circle, RRL. Values are mean ± SEM. Significant difference between time points $P<0.05$; a
616 within RAO. Significant differences between groups $P<0.05$; * between CAO and RAO; #
617 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 ± 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.

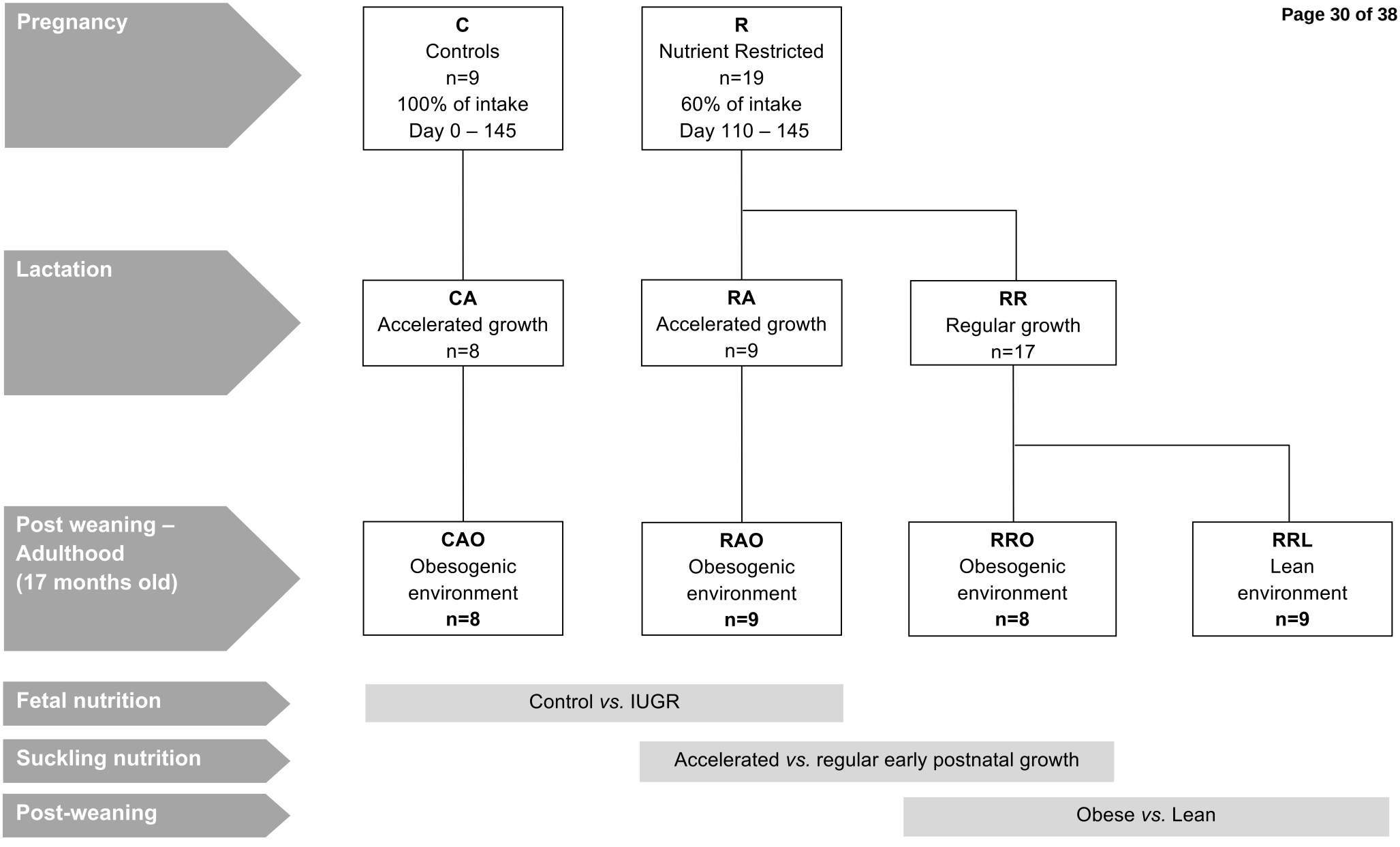
Offspring of C and R mothers were subjected to an accelerated (CA, n=8; RA, n=9) or regular growth (RR, n=17) during lactation. After weaning, offspring were then exposed to an obesogenic environment (O) with the exception for a subset of RR, which remained lean (RRL, n=9; RRO, n=8). Values are mean ± 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.

641 **Table 3: Influence of fetal intrauterine growth restriction, accelerated postnatal growth,**
642 **and obesity on the onset of insulin resistance in the offspring.** Plasma glucose and insulin
643 responses to an intravenous glucose injection at 7 (i.e. puberty) and 16 months (i.e. young
644 adulthood) of age.
645 Offspring of C and R mothers were subjected to an accelerated (CA, n=8; RA, n=8) or
646 regular growth (RR, n=15) during lactation. After weaning, offspring were then exposed to
647 an obesogenic environment (O) with the exception for a subset of RR, which remained lean
648 (RRL, n=8; RRO, n=7). Values are mean ± SEM. Significant differences between groups
649 represented by different superscripts, a vs b $P<0.05$; c vs d $P<0.01$.
650
651 **Table 4: Effect of maternal caloric restriction, accelerated postnatal growth, and**
652 **juvenile-onset obesity on the regulation of energy balance and endocrine sensitivity in**
653 **the hypothalamus of young adults.**
654 Offspring of C and R mothers were subjected to an accelerated (CA, n=5; RA, n=8) or
655 regular growth (RR, n=15) during lactation. After weaning, offspring were then exposed to
656 an obesogenic environment (O) with the exception for a subset of RR, which remained lean
657 (RRL, n=8; RRO, n=7). Values are mean ± SEM and n=5-8 per time point. Statistical
658 significance for the effect of maternal diet (i.e. CAO vs RAO), accelerated postnatal growth
659 (i.e. RAO vs RRO) and obesity (i.e. RRO vs RRL). NS, not significant.
660 *NPY*, neuropeptide Y; *AGRP*, agouti-related peptide; *PTP1B*, protein tyrosine phosphatase,
661 non-receptor type 1; *SOCS3*, suppressor of cytokine signalling 3; *IR*, insulin receptor; *OBRB*,
662 leptin receptor, long form; *AMPKA2*, AMP-activated protein kinase α2; *MTOR*, mammalian
663 target of rapamycin; *FTO*, fat mass and obesity associated gene; *GCR*, glucocorticoid
664 receptor; *CRH*, corticotropin releasing hormone; *AVP*, arginine vasopressin.
665

Table 5: Correlations between plasma insulin and leptin concentrations and hypothalamic gene expression for *NPY* and *AGRP* (2^-ΔΔCt) 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 [a)] $*10^{-5}$ and [b)] $*10^{-6}$. *AGRP*, agouti-related peptide; *NPY*, neuropeptide Y.


Pregnancy
C
Controls
n=9
100% of intake
Day 0 – 145
R
Nutrient Restricted
n=19
60% of intake
Day 110 – 145
Lactation
CA
Accelerated growth
n=8
RA
Accelerated growth
n=9
RR
Regular growth
n=17
Post weaning – Adulthood (17 months old)
CAO
Obesogenic environment
n=8
RAO
Obesogenic environment
n=9
RRO
Obesogenic environment
n=8
RRL
Lean environment
n=9
Fetal nutrition
Control vs. IUGR
Suckling nutrition
Accelerated vs. regular early postnatal growth
Post-weaning
Obese vs. Lean

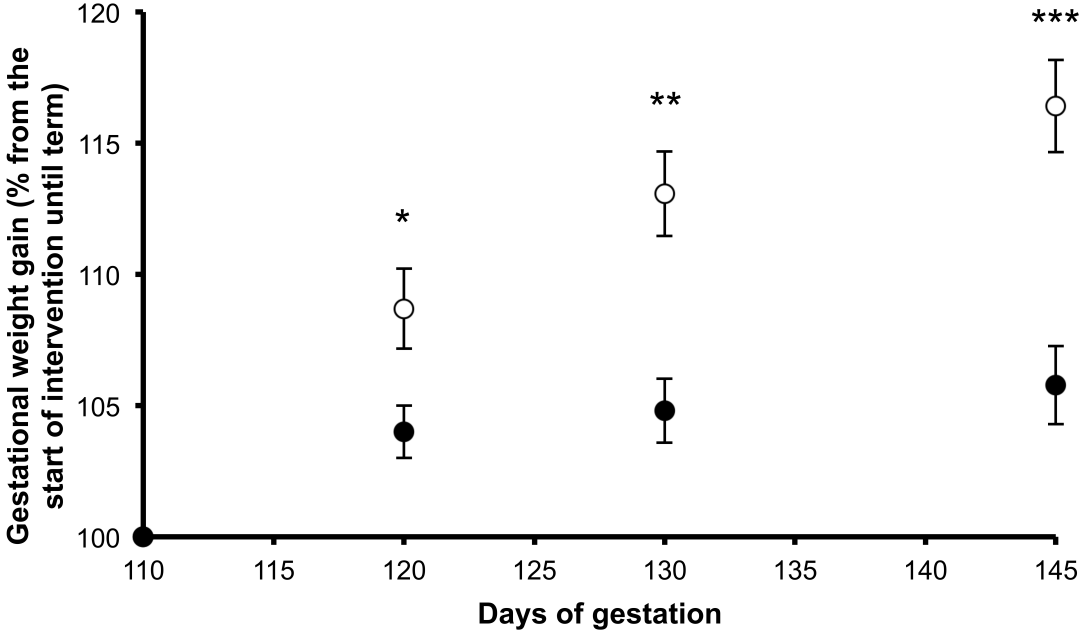
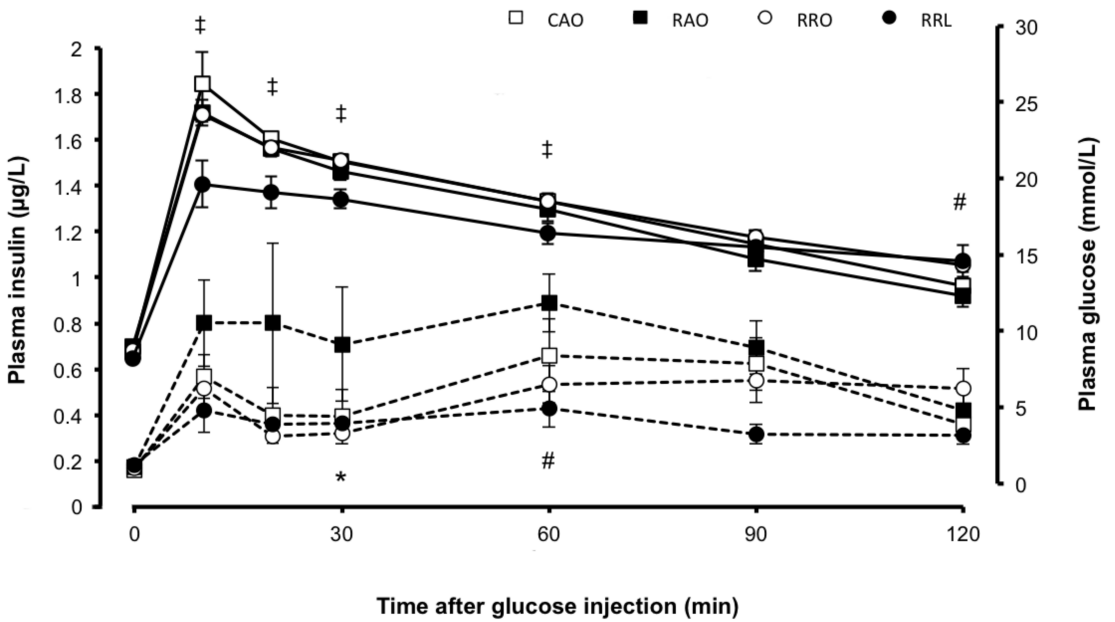

Gestational weight gain (% from the start of intervention until term)
120
115
110
105
100
110
115
120
125
130
135
140
145
Days of gestation
*
**
***

Figure 2


A. 7 months of age
CAO
RAO
RRO
RRL
Plasma insulin (µg/L)
Plasma glucose (mmol/L)
Time after glucose injection (min)
0
30
60
90
120

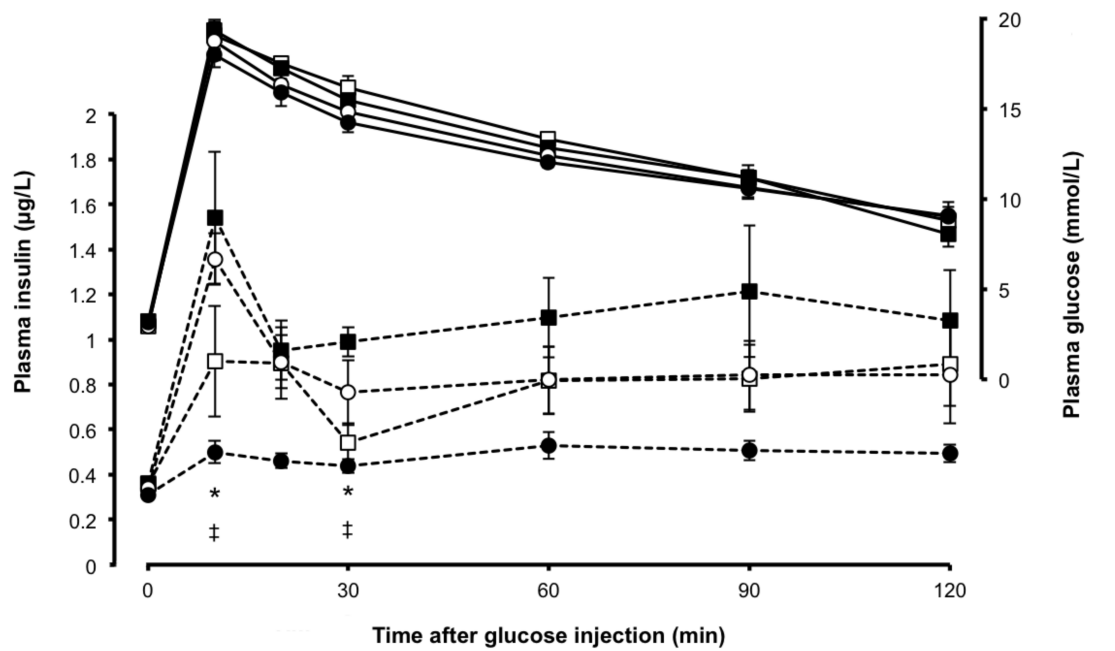

B. 16 months of age
Plasma insulin (µg/L)
Plasma glucose (mmol/L)
Time after glucose injection (min)
0
30
60
90
120

Figure 3

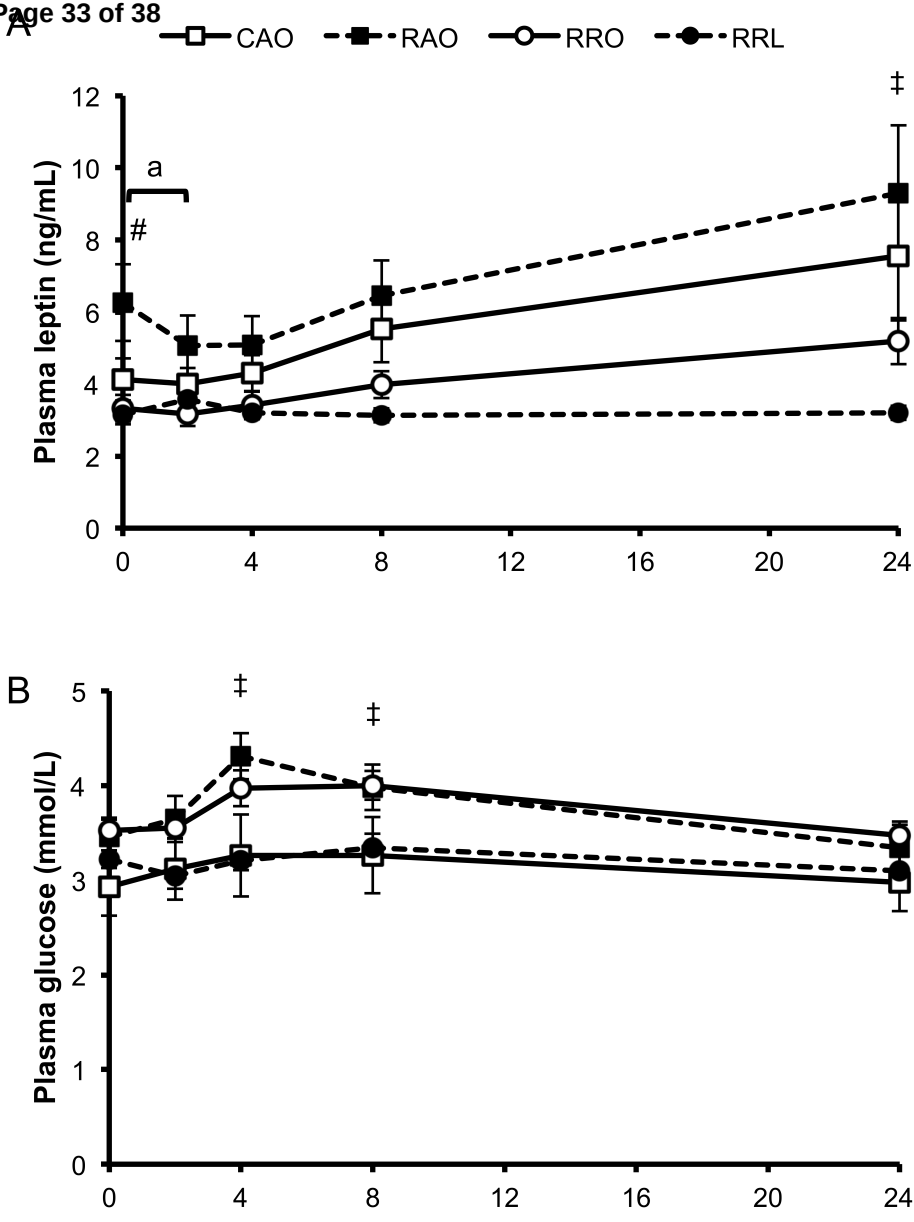

A
CAO
RAO
RRO
RRL
Plasma leptin (ng/mL)
0
2
4
6
8
10
12
a
#
‡
0
4
8
12
16
20
24
B
Plasma glucose (mmol/L)
0
1
2
3
4
5
‡
‡
0
4
8
12
16
20
24

Table 1

A

Variables	Control	Restricted
Glucose (mmol/L)		
Fasted	3.6 ± 0.6	2.3 ± 0.2 *
2H-fed	4.3 ± 0.9	7.0 ± 0.4 **
Change	1.4 ± 1.0	4.7 ± 0.5 **
NEFA (mmol/L)		
Fasted	0.34 ± 0.05	0.85 ± 0.08 ***
2H-fed	0.74 ± 0.12	0.65 ± 0.09
Change	0.50 ± 0.05	-.31 ± 0.12 ***
Triglycerides (mmol/L)		
Fasted	0.32 ± 0.01	0.33 ± 0.01
2H-fed	0.30 ± 0.02	0.32 ± 0.01
Change	-0.02 ± 0.03	-0.01 ± 0.02
Fasted insulin (µg/L)	0.3 ± 0.01	0.3 ± 0.01
Fasted leptin (ng/ml)	1.5 ± 0.3	1.0 ± 0.1
Fasted cortisol (nmol/L)	17 ± 3	22 ± 3

B

Metabolites (LC-HRMS)	M	Fold change	*P*
o-Acetyl-carnitine $[M+H]^+$	203.11	1.8 ± 0.2	0.0003
o-Acetyl-carnitine [M+Na]	203.11	2.7 ± 0.4	0.0008
o-Acetyl-carnitine $[2M+H]^+$	203.11	2.9 ± 0.3	0.0011
Tryptophane $[M-NH_3+H]^+$	204.09	2.9 ± 0.3	0.0011
Phenylalanine $[M+H]^+$	165.08	1.2 ± 0.1	0.0085

Table 2

Maternal diet	**Control**	**Nutrient restricted**		
Growth from birth to weaning	**Accelerated**		**Restricted**	
Phenotype from weaning	**Obese**			**Lean**
Group	**CAO**	**RAO**	**RRO**	**RRL**
Birth weight (kg)	5.0 ± 0.2 [c]	4.0 ± 0.1 [d]		
Weight gain, 0 to 81 days (kg/kg)	6.6 ± 0.3 [c]	7.5 ± 0.5 [d]	6.1 ± 0.3 [c]	
Weight 7 months (z-score)	0.44 ± 0.35	0.27 ± 0.31	-0.07 ± 0.16	-0.60 ± 0.33
Weight 17 months (z-score)	0.65 ± 0.17	0.37 ± 0.25	0.38 ± 0.32 [c]	-1.24 ± 0.12 [d]
Relative body fat (z-score)	0.04 ± 0.29	0.37 ± 0.38	0.12 ± 0.34	-0.52 ± 0.31
Physical activity (counts/24h)	121 ± 13	166 ± 16	178 ± 54 [c]	550 ± 40 [d]
Food intake (MJ/kg/d)	0.32 ± 0.01	0.32 ± 0.01	0.29 ± 0.04 [a]	0.26 ± 0.02 [b]
Fasted TG (mg/dL)	0.18 ± 0.02 [a]	0.12 ± 0.03 [b]	0.12 ± 0.02	0.16 ± 0.02
Fasted NEFA (mmol/L)	0.65 ± 0.06	0.49 ± 0.07	0.48 ± 0.08	0.38 ± 0.05

Table 3

	CAO	RAO	RRO	RRL
At 7 months of age				
Glucose AUC (mmol/L)	1167 ± 46	1070 ± 74	1252 ± 93 [a]	1015 ± 77 [b]
Insulin AUC (µg/L)	45 ± 12	67 ± 13 [a]	37 ± 7 [b]	23 ± 6
HOMA-IR	0.61 ± 0.05	0.66 ± 0.07	0.52 ± 0.08	0.70 ± 0.21
At 16 months of age				
Glucose AUC (mmol/L)	1302 ± 36	1226 ± 60	1215 ± 78	1148 ± 46
Insulin AUC (µg/L)	53 ± 14 [a]	94 ± 14 [b]	67 ± 15	22 ± 5
HOMA-IR	0.92 ± 0.08 [c]	1.17 ± 0.05 [d]	1.00 ± 0.06 [c]	0.99 ± 0.03

Table 4

Pathway	Gene	CAO (n=5)	RAO (n=8)	RRO (n=7)	RRL (n=8)	Effect of maternal diet	Effect of postnatal growth	Effect of obesity
Orexigenic neurotransmitters	*NPY*	3.0±0.7	4.4±0.9	2.9±0.4	10.5±2.5	NS	NS	*P*=0.019
	AGRP	1.2±0.5	1.9±0.3	1.5±0.3	1.6±0.3	NS	NS	NS
Insulin and leptin signalling	*PTP1B*	1.3±0.2	1.9±0.2	1.9±0.3	1.2±0.2	(*P*=0.051)	NS	*P*=0.031
	SOCS3	5.9±1.1	8.5±1.3	9.3±2.0	7.4±1.4	NS	NS	NS
	IR	0.7±0.1	1.1±0.2	1.4±0.3	0.9±0.2	NS	NS	NS
	OBRB	6.5±0.6	7.0±0.8	6.7±1.3	7.5±0.9	NS	NS	NS
Intracellular energy signalling	*AMPKA2*	2.5±0.2	4.1±0.4	4.3±0.5	3.0±0.4	*P*=0.008	NS	*P*=0.014
	MTOR	1.2±0.1	1.9±0.2	2.2±0.2	1.3±0.1	*P*=0.014	NS	*P*=0.003
	FTO	8.0±0.7	12.1±1.1	12.3±0.9	9.4±1.1	*P*=0.016	NS	(*P*=0.053)
Cortisol regulation	*GCR*	1.1±0.1	1.5±0.1	1.6±0.2	1.1±0.2	NS	NS	*P*=0.038
	CRH	0.7±0.1	1.2±0.2	1.1±0.2	1.0±0.2	*P*=0.048	NS	NS
	AVP	1.5±0.2	2.8±0.3	2.4±0.6	2.7±0.6	*P*=0.007	NS	NS

Table 5

		NPY			*AGRP*		
	Group	**Slope**	**Spearman's ρ**	**p-value**	**Slope**	**Spearman's ρ**	**p-value**
Insulin	**CAO**	-0.02	-0.50	0.39	-47.3 [a)]	-0.72	0.19
	RAO	-0.0015	-0.21	0.61	-9.33 [a)]	-0.45	0.26
	RRO	-0.0026	-0.52	0.25	-14.7 [a)]	-0.64	0.12
	RRL	-0.01	-0.41	0.42	-15.0 [a)]	-0.12	0.83
Leptin	**CAO**	-19.8 [a)]	-0.48	0.39	-5.89 [b)]	-0.74	0.19
	RAO	-6.9 [a)]	-0.88	0.004 [**]	-1.93 [b)]	-0.71	0.047 [*]
	RRO	-1.7 [a)]	-0.04	0.90	-3.71 [b)]	-0.79	0.036 [*]
	RRL	+40.2 [a)]	0.33	0.42	-2.37 [b)]	-0.31	0.46