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

3

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18

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31

32 Abstract

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

54 **Introduction**

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

63

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

122 **Materials and Methods**

123 **Animals and experimental design**

124 All animal procedures were performed in accordance with the UK Animals (Scientific
125 Procedures) Act 1986 with approval from the Local Ethics Committee of the University of
126 Nottingham. The experimental nutritional intervention has previously been described in detail
127 (Sebert *et al.* 2011). In brief, 28 Bluefaced Leicester cross Swaledale twin bearing sheep
128 (*ovis*) were individually housed at 100 days of gestation (dGA) and, at day 110 dGA,
129 randomly allocated to the experimental groups (Figure 1). All pregnancies continued
130 normally until term ($\sim 145 \pm 1$ days) and produced heterozygous twins. They included a
131 control group (C, n=9) that were fed to requirements through pregnancy (i.e. from 0.46
132 MJ/kg.BW^{0.75} at 110 days gestation, increasing to 0.72 MJ/kg.BW^{0.75} at dGA 130), whilst the
133 remaining 19 mothers were caloric restricted (R) and were pair-fed to 60% of control intake,
134 based on their body weight. All mothers were individually weighed once a week prior to
135 feeding in order that their total food requirements could be adjusted. From birth, the offspring
136 born to C mothers were then reared to promote accelerated (A) early postnatal growth (CA,
137 n=8, 4 males and 4 females), accomplished by only one twin being reared by its mother. The
138 offspring born to R mothers were reared to promote an accelerated (RA n=9, 2 males and 7
139 females) or a regular (RR, n=17) early postnatal growth rate, accomplished by being reared
140 together as twins. After weaning, all offspring were kept in a low activity environment until
141 17 months of age in order to promote obesity (O, 6 animals on 19 m², fed *ad libitum* on straw
142 nuts and a micronutrient supplement) with the exception of 9 RR offspring that were kept in a
143 normal physical activity environment, in order to remain lean (RRL, n=9, 5 males and 4
144 females, 6 animals on 1125 m², *ad libitum* access to grass and a micronutrient supplement;
145 RRO n=8; 2 males and 6 females; Figure 1). Discrepancies between the total number (n) of
146 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 (≥ 18 h) 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

172 (≥ 18 h) and injected intravenously with 0.5 g/kg glucose. Glucose and insulin concentrations
173 were measured in plasma samples before and at 10, 20, 30, 60, 90, and 120 minutes, after the
174 intravenous glucose (Gardner *et al.* 2005). The homeostatic model assessment for insulin
175 resistance (HOMA-IR) index was calculated by multiplication of glucose (mmol/L) and
176 insulin ($\mu\text{g/L}$) concentrations measured in fasted plasma (Wallace *et al.* 2004).

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

178 **age:** Total body fat was determined when the animal was sedated (intramuscular injection of
179 1.5 mg /kg ketamine with 0.1 mg /kg xylazine) and scanned in a transverse position using a
180 Lunar DPX-L (fast-detail whole body smartscan, GE Healthcare, Little Chalfont, UK). The
181 level of spontaneous physical activity in adulthood in their respective environments was
182 determined using uniaxial accelerometers (Actiwatch; Linton Instrumentation, Diss, UK).
183 Average total food intake was measured in 24h intervals over a 10 day period with all
184 animals kept in individual pens and with *ad libitum* access to feed, straw nuts (8.5 MJ/kg) and
185 concentrate pellets (12.5 MJ/kg).

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

192

193 **Laboratory analysis**

194 **Plasma metabolites and hormones**

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

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

199

200 **Analysis of the plasma metabolome**

201 Fasted heparin-treated plasma samples taken from mothers at dGA 130 were analyzed for a
202 wide spectrum of metabolites by liquid chromatography coupled to high resolution mass
203 spectrometry (LC-HRMS). Plasma was defrosted on ice and filtered by centrifugation
204 (Nanosep Omega, Pall, Port Washington, NY) to remove high molecular weight species,
205 proteins in particular (over 10kDa). Metabolomic LC-HRMS profiles were acquired from 15
206 μL of each filtered serum sample using an Agilent 1200TM HPLC system equipped with a 150
207 x 2.1 mm Uptisphere HDO-C₁₈ column with 3 μm particle size (Interchim, Montluçon,
208 France) coupled to a high resolution LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher
209 Scientific, Bremen, Germany) fitted with an electrospray source operated in the positive ion
210 mode. The detailed conditions applied both for the HPLC separation and mass spectrometric
211 signal acquisition were previously described (Courant *et al.* 2009; Alexandre-Gouabau *et al.*
212 2011). Quality control standards and samples were randomly included five times into the
213 sequence of injection.

214

215 **Metabolomic data processing**

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

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

226

227 **Gene expression measurements**

228 Offspring hypothalami were homogenized and RNA isolated, using the RNeasy Plus kit
229 (Qiagen, Hilden, Germany). An aliquot of 4 μ g of RNA was reverse transcribed with the
230 High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). The
231 resulting cDNA was amplified in a real-time thermocycler (Quantica, Techne, Burlington,
232 NJ, USA) using a SYBR green system in Taq polymerase reaction mix (Absolute blue QPCR
233 SYBR green, Thermo Scientific, Epsom, UK). Specificity of primers was confirmed by
234 sequencing PCR product (Supplementary Information Table 1). Hypothalamic gene
235 expression was assessed for the following pathways: a) orexigenic neurotransmitters:
236 neuropeptide Y (*NPY*); agouti-related peptide (*AGRP*), b) insulin and leptin signaling: protein
237 tyrosine phosphatase non-receptor type 1 (*PTP1B*); suppressor of cytokine signaling 3
238 (*SOCS3*); insulin receptor (*IR*); and leptin receptor (*OBRB*), c) intracellular energy signaling:
239 AMP-activated kinase (*AMPKA2*); mammalian target of rapamycin (*MTOR*); and fat mass
240 and obesity-related gene (*FTO*), d) cortisol regulation: glucocorticoid receptor (*GCR*);
241 corticotropin releasing hormone (*CRH*); and arginine vasopressin (*AVP*). Ribosomal RNA
242 *18S* showed a stable expression and was used as a housekeeping gene. Gene expression was
243 calculated by using the $2^{-\Delta\Delta C_t}$ 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.

268 **For non-metabolomic outcomes:** Statistical analysis of the data was performed using
269 PASW[®] statistics software (v 17.02, IBM, Chicago, USA). Kolmogorov-Smirnoff tests were
270 realized on every parameter analyzed to determine the Gaussian distributions of the variables.
271 The influence of maternal nutrition (CAO vs. RAO), early postnatal growth (RAO vs. RRO),
272 and obesogenic environment (RRL vs. RRO) were determined, according to parametric
273 distribution, using ANOVA with a pairwise a priori test or Mann-Whitney U tests. Of the
274 metabolites and hormones measured over 24 hours, changes over time were tested with the
275 use of a paired t-test. Data is expressed as mean values with their standard errors. To address
276 the limitations of multiple testing, statistical trend was accepted with a 95% interval of
277 confidence ($P < 0.05$) and significance was accepted with a confidence interval of 99%
278 ($P < 0.01$). Correlations were tested with the non-parametric Spearman's test and slope of the
279 correlation was reported on the linear fit. Each variable was tested for sex. Body weight and
280 fat mass are known to differ, in absolute scale, between male and female sheep (Bloor *et al.*
281 2013) thus sex-specific Z-score transformation was used prior to analyses. Specifically, we
282 saw no indication of a difference in male and female offspring in glucose homeostasis, which
283 is consistent with earlier studies (Gardner *et al.* 2005). Moreover, comparison for each
284 variable between groups for females only demonstrated similar outcomes, although without
285 reaching statistical significance, hence data for each sex were combined for further analyses
286 and greater statistical power.
287

288 **Results**289 **Mothers**

290 Relative to their weight at the beginning of the caloric restriction, R mothers gained less
291 weight up to term compared with C mothers (Figure 2A). At 130 dGA, plasma glucose was
292 reduced in fasted R mothers but there was a greater increase after feeding (Table 1A). Plasma
293 NEFA concentrations were higher in fasted R mothers but did not differ between groups after
294 feeding, whilst plasma triglycerides, cortisol, insulin, and leptin were unaffected by maternal
295 diet (Table 1A). Metabolomic analysis showed a specific biological signature associated with
296 the caloric restricted mothers, with a strong overall difference between the groups (OPLS-DA
297 model of all the 2629 [m/z, rt] features detected, using 2 latent factors for maternal plasma
298 metabolomic profiles (describing 43% of variable information); C mothers n=7 and R
299 mothers n=17; Validation parameters: R^2X (cum) = 0.434, R^2Y (cum) = 0.999, Q^2 (cum) =
300 0.994, permutation test (n = 100) with R^2 intercept = 0.331 and Q^2 intercept=-0.333, ANOVA
301 P -value = 9.9×10^{-19}), and of the 2629 detected features constituting these metabolomic
302 profiles, 133 differed significantly ($P < 0.01$). Of these, 95 were upregulated with a fold
303 change (expressed as a ratio of the mean abundance in R group compared to the mean
304 abundance in the C group) of >1.4 and only 7 were downregulated with a fold change of
305 <0.71 . Due to species-specific technical constraints, only five of these compounds could be
306 precisely identified (Table 1B) as phenylalanine, tryptophan, and three forms of o-acetyl-
307 carnitine which were all upregulated in R mothers.

308

309 **Offspring**

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

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

319 **Insulin sensitivity:** At 7 months of age, the glucose AUC during the GTT was higher in
320 obese compared to lean animals (RRO vs RRL, Figure 3 and Table 3). RAO offspring
321 showed twice the insulin response to a standard glucose challenge in comparison to RRO. By
322 16 months of age, glucose AUC did not differ between groups but the insulin response during
323 the GTT was significantly higher in RAO compared to CAO ($P<0.05$), as was the HOMA-IR,
324 an index of insulin resistance, which was also higher in RAO than in the RRO offspring
325 ($P<0.01$).

326 **Effect of feeding on plasma profiles of leptin and cortisol:** Prior to the feeding challenge,
327 plasma leptin was higher in RAO than in RRO (Figure 4A). In the RAO group, plasma leptin
328 initially declined on feeding ($P<0.05$), to gradually increase between 8 and 24 h after feeding
329 ($P<0.05$). This effect was not observed in any other group as plasma leptin remained
330 unchanged. Plasma glucose and insulin differed between RRO and RRL animals during the
331 24h of measurements ($P<0.05$) but not between groups raised in an obesogenic environment
332 (Figure 4B and 4C). Plasma cortisol both peaked 4 hours after feeding and was highest in
333 RAO compared with CAO offspring, a difference that persisted until at least 8 h after feeding
334 (Figure 4D).

335 **Hypothalamic gene expression:** Expression of appetite regulatory genes was unchanged,
336 whilst *AMPKA2*, *MTOR*, and *FTO* were all higher in RAO compared to CAO groups (Table
337 4). A statistically significant negative correlation between anorexigenic circulating hormones
338 insulin and leptin and the expression of orexigenic genes *NPY* and *AGRP* was observed in
339 RAO and RRO, but not CAO or RRL groups. Taken together, these different relationships
340 suggest a potential change in insulin and leptin sensitivity within the hypothalamus after
341 IUGR (Table 5). Gene expression of both *AVP* and *CRH* was higher in RAO offspring as
342 compared to CAO. Expression of *NPY* was three times higher and of *PTP1B*, *AMPKA2*,
343 *MTOR*, and *GCR* was lower in the RRL offspring as compared to RRO. Importantly,
344 postnatal growth rate (RAO vs RRO) did not have any effect on hypothalamic gene
345 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

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

378

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

388

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

396 challenges than obese animals, at least in terms of NEFA, insulin, and leptin (Sebert *et al.*
397 2009). One hypothalamic outcome of IUGR was increased expression of genes involved in
398 energy sensing, which were also higher in the offspring reared within an obesogenic,
399 compared with a lean environment (RRO vs RRL). In the lean IUGR group, the expression
400 for those genes was reduced to values very similar to obese controls, even though *FTO* is
401 known to be more highly expressed in obese than lean sheep (Sebert *et al.* 2010). This further
402 suggests that IUGR has a long-term effect which is not fully corrected with exposure to a
403 high activity environment. However, these assumptions will need to be tested further with a
404 more appropriate control group and in both male and female offspring.

405

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

417

418 All offspring raised in an obesogenic environment became equally obese irrespective of their
419 *in utero* diet, and this may reflect the more physiological, long term exposure we adopted to
420 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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574

575 **Figure Titles**

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

585

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

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

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

593

594 **Figure 3: Influence of fetal intrauterine growth restriction, accelerated postnatal**
595 **growth, and obesity on the onset of insulin resistance in the offspring.** Time course of
596 changes in plasma glucose (continuous line) and insulin (dashed line) following an intra-
597 venous glucose injection at (A) 7 (i.e. puberty) and (B) 16 months of age (i.e. young
598 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 \pm 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 \pm 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.

618 **Table Titles**

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

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

630

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

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

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

640

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 \pm 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 \pm 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 $\alpha 2$; *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

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

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

672 *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

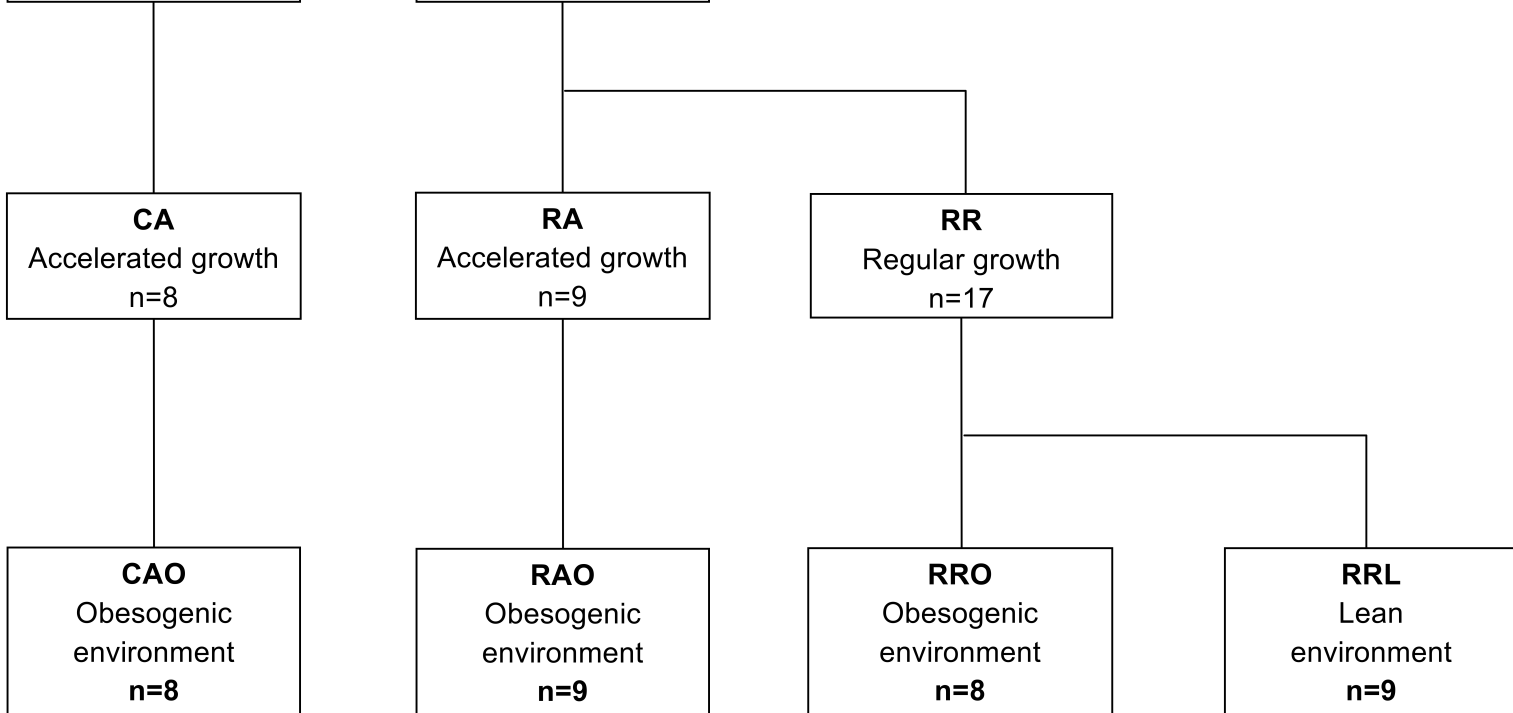
Control vs. IUGR

Suckling nutrition

Accelerated vs. regular early postnatal growth

Post-weaning

Obese vs. Lean



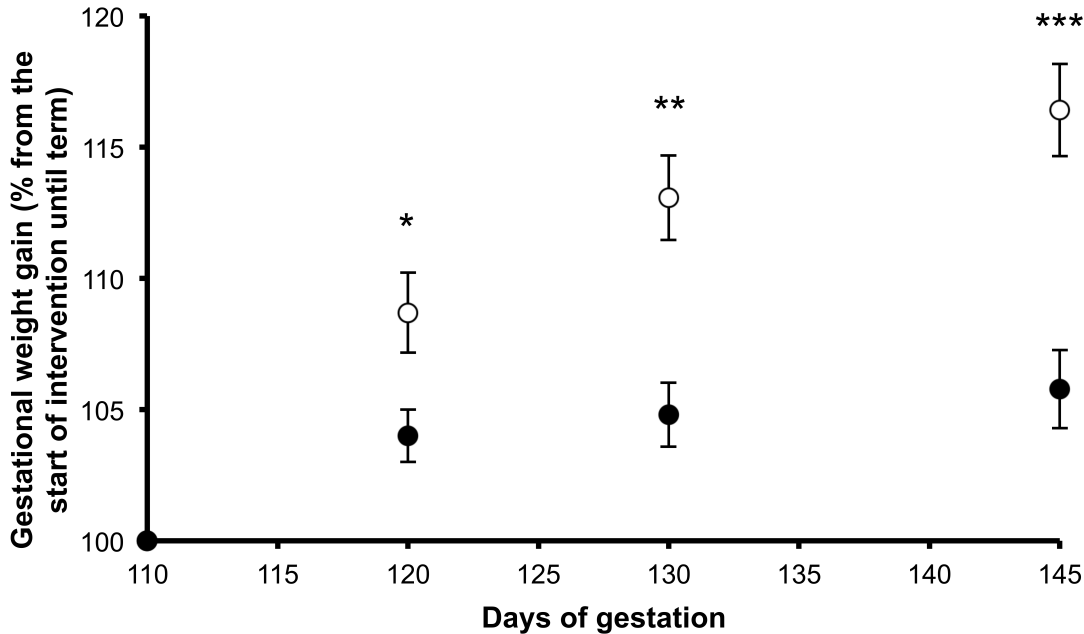
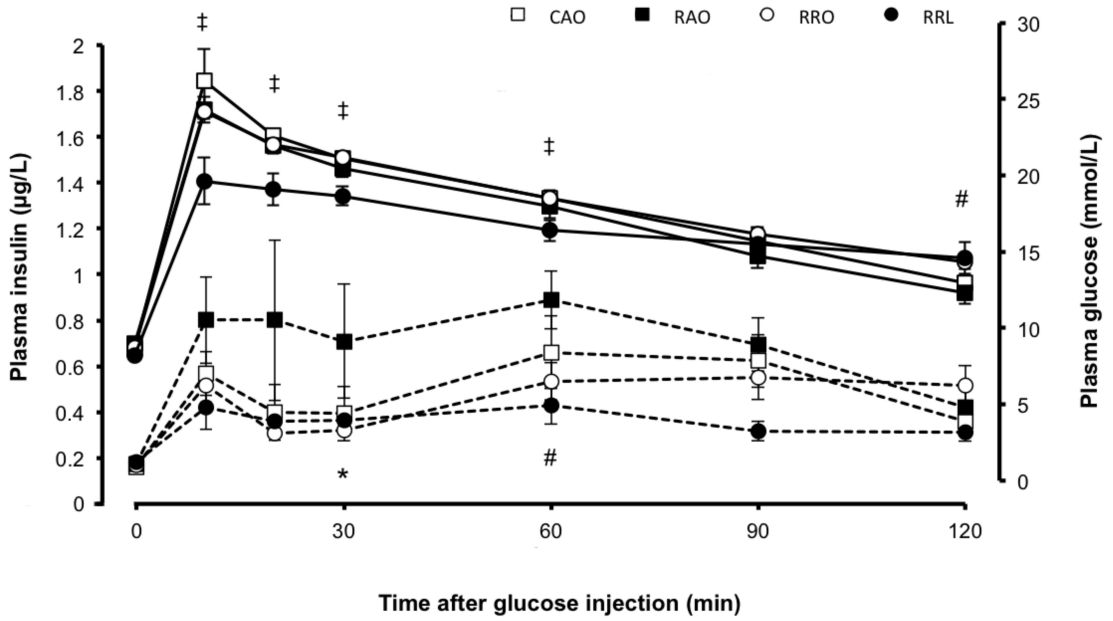


Figure 2



B. 16 months of age

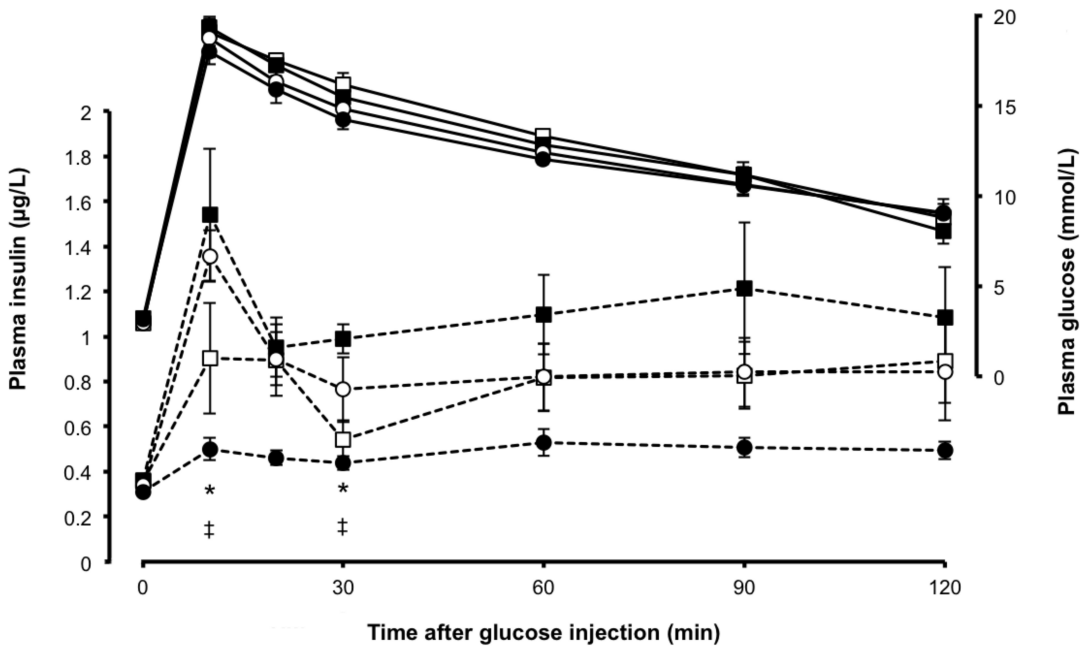
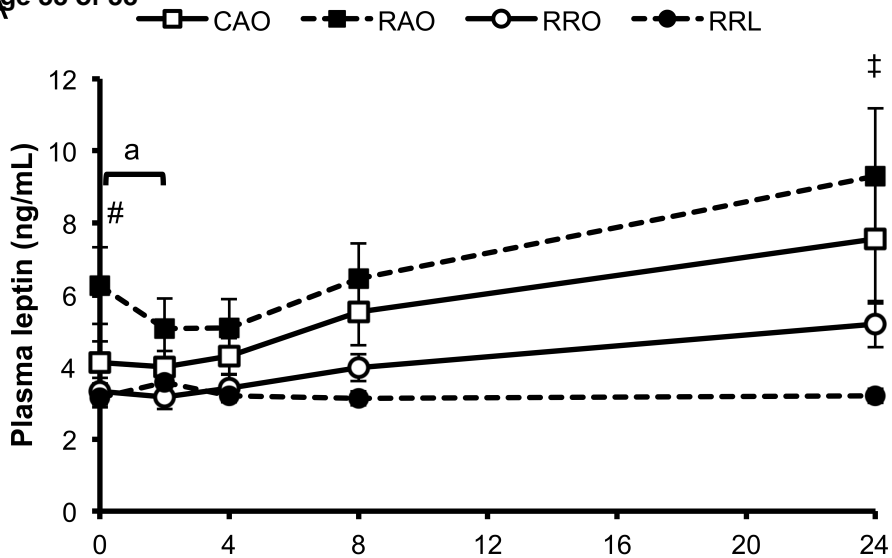


Figure 3

A



B

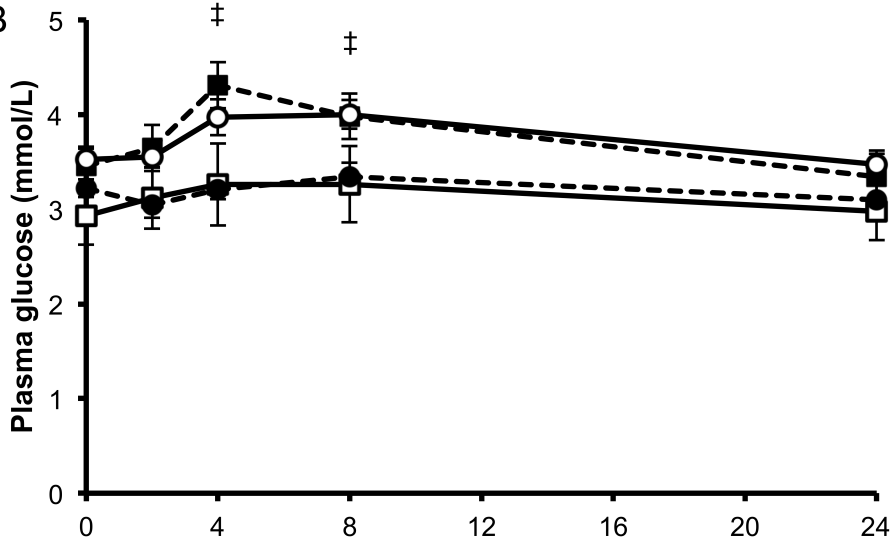


Table 1

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	-0.31 ± 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-NH₃+H]⁺	204.09	2.9 ± 0.3	0.0011
Phenylalanine [M+H]⁺	165.08	1.2 ± 0.1	0.0085

Table 2

<i>Maternal diet</i>	Control	Nutrient restricted		
<i>Growth from birth to weaning</i>	Accelerated		Restricted	
<i>Phenotype from weaning</i>	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	<i>NPY</i>	3.0±0.7	4.4±0.9	2.9±0.4	10.5±2.5	NS	NS	<i>P</i> =0.019
	<i>AGRP</i>	1.2±0.5	1.9±0.3	1.5±0.3	1.6±0.3	NS	NS	NS
Insulin and leptin signalling	<i>PTP1B</i>	1.3±0.2	1.9±0.2	1.9±0.3	1.2±0.2	(<i>P</i> =0.051)	NS	<i>P</i> =0.031
	<i>SOCS3</i>	5.9±1.1	8.5±1.3	9.3±2.0	7.4±1.4	NS	NS	NS
	<i>IR</i>	0.7±0.1	1.1±0.2	1.4±0.3	0.9±0.2	NS	NS	NS
	<i>OBRB</i>	6.5±0.6	7.0±0.8	6.7±1.3	7.5±0.9	NS	NS	NS
Intracellular energy signalling	<i>AMPKA2</i>	2.5±0.2	4.1±0.4	4.3±0.5	3.0±0.4	<i>P</i> =0.008	NS	<i>P</i> =0.014
	<i>MTOR</i>	1.2±0.1	1.9±0.2	2.2±0.2	1.3±0.1	<i>P</i> =0.014	NS	<i>P</i> =0.003
	<i>FTO</i>	8.0±0.7	12.1±1.1	12.3±0.9	9.4±1.1	<i>P</i> =0.016	NS	(<i>P</i> =0.053)
Cortisol regulation	<i>GCR</i>	1.1±0.1	1.5±0.1	1.6±0.2	1.1±0.2	NS	NS	<i>P</i> =0.038
	<i>CRH</i>	0.7±0.1	1.2±0.2	1.1±0.2	1.0±0.2	<i>P</i> =0.048	NS	NS
	<i>AVP</i>	1.5±0.2	2.8±0.3	2.4±0.6	2.7±0.6	<i>P</i> =0.007	NS	NS

Table 5

	Group	<i>NPY</i>			<i>AGRP</i>		
		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