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Low female birth weight and advanced maternal age programme alterations in next-generation blastocyst development

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1 **Low female birth weight and advanced maternal age program alterations in next**  
2 **generation blastocyst development**

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18 Short title: **Being born small alters the F2 blastocyst**

19

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24

25 **Abstract**

26           Low birth weight is associated with increased risk for adult disease development with  
27 recent studies highlighting transmission to subsequent generations. However, the mechanisms  
28 and timing of programming of disease transmission to the next generation remain unknown. The  
29 aim of this study was to examine the effects of low birth weight and advanced maternal age on  
30 second-generation preimplantation blastocysts. Uteroplacental insufficiency or sham surgery was  
31 performed in late gestation WKY pregnant rats, giving rise to first generation (F1) Restricted  
32 (born small) and Control offspring, respectively. F1 Control and Restricted females, 4 or 12  
33 months of age, were naturally mated with normal males. Second generation (F2) blastocysts  
34 from Restricted females displayed reduced expression of genes related to growth compared to F2  
35 Control ( $P<0.05$ ). Following 24h culture, F2 Restricted blastocysts had accelerated development,  
36 with increased total cell number, a result of increased trophoctoderm cells compared to Control  
37 ( $P<0.05$ ). There were alterations in carbohydrate and serine utilization in F2 Restricted  
38 blastocysts and F2 Restricted outgrowths from 4 month females, respectively ( $P<0.05$ ). F2  
39 blastocysts from aged Restricted females were developmentally delayed at retrieval, with  
40 reduced total cell number attributable to reduced trophoctoderm number with changes in  
41 carbohydrate utilization ( $P<0.05$ ). Advanced maternal age resulted in alterations in a number of  
42 amino acids in media obtained from F2 blastocyst outgrowths ( $P<0.05$ ). These findings  
43 demonstrate that growth restriction and advanced maternal age can alter F2 preimplantation  
44 embryo physiology and subsequent offspring growth.

45 Key Words: *preimplantation rat blastocyst, uteroplacental insufficiency, transgenerational*  
46 *transmission, growth restriction*

47

## 48 **Introduction**

49           Intrauterine growth restriction, characterized by birth weight below the 10<sup>th</sup> percentile for  
50 gestational age, complicates approximately 10% of all pregnancies in the Western world and is  
51 commonly caused by placental dysfunction during late gestation (Barker 1995; McMillen &  
52 Robinson 2005). Epidemiological studies have highlighted that being born small is linked with  
53 an increased disease risk not only for that individual, but to subsequent generations of offspring  
54 (Heijmans *et al.* 2008; Painter *et al.* 2008). It has been reported that in first generation (F1)  
55 women who themselves were undernourished *in utero* and born small, delivered smaller second  
56 generation (F2) babies with increased risk of developing hypertension, cardiovascular disease,  
57 impaired glucose tolerance and obesity in adult life (Heijmans *et al.* 2008; Painter *et al.* 2008).  
58 The pathways hypothesized to regulate transmission of disease risk include direct effects on  
59 germ cells from the F1 fetus, by inheritance through persistent epigenetic modifications of either  
60 of the parental germ cells, and/or abnormal pregnancy adaptations (Gallo *et al.* 2012a). However,  
61 it is unclear if early changes in F2 physiology can be detected.

62           The blastocyst stage represents an early developmental window where reprogramming  
63 can occur (Watkins *et al.* 2008a). Prior to implantation, the blastocyst moves through the female  
64 reproductive tract bathed in nutrients from the surrounding maternal microenvironment and is  
65 therefore sensitive to changes in secretions in the maternal milieu. The primary sources of energy  
66 for the embryo from fertilization to compaction are pyruvate, lactate and non-essential amino  
67 acids, while post compaction development, the utilization of glucose and both essential and non-  
68 essential amino acids become increasingly important (Hardy *et al.* 1989; Brison & Leese 1991;  
69 Lane & Gardner 1997; Gardner 1998). Alterations in metabolic control during pregnancy, as  
70 reported in late gestation F1 female growth restricted offspring (Gallo *et al.* 2012c), may be

71 apparent earlier in development, and alter nutrient availability to the developing F2 blastocyst  
72 prior to implantation. These changes may lead to subsequent alterations in growth and  
73 development of the fetus and placenta. The blastocyst comprises the inner cell mass (ICM) and  
74 the trophoctoderm (TE), which give rise to the fetus and extra-embryonic tissues and placenta,  
75 respectively. The placenta is an essential regulator of fetal growth during gestation, providing  
76 gas and nutrients to the developing fetus. Therefore, alterations caused by impaired TE  
77 development or function, may impact on placental development and therefore fetal nutrition and  
78 growth, leading to the programming of disease risk in resultant progeny.

79         Several studies document clear alterations in F1 preimplantation embryo physiology and  
80 development that contribute to subsequent F1 health when an insult is experienced by the mother  
81 (F0) during pregnancy. Exposure of preimplantation mouse embryos to gestational diabetes leads  
82 to reductions in intracellular glucose and glucose utilization, resulting from reductions in glucose  
83 uptake and expression of transporters at the mRNA and protein level in F1 preimplantation  
84 embryos (Moley *et al.* 1998). In a rat model of diabetes, high incidences of abnormal F1  
85 preimplantation blastocysts have been reported, where only 33% of blastocysts reached the  
86 expanded blastocyst stage with a 20% reduction in ICM cell numbers (Lea *et al.* 1996).  
87 Similarly, studies examining the effect of maternal low protein diet during early gestation (0-  
88 4.25 days) in rats, have reported F1 female offspring born of low birth weight, accelerated  
89 postnatal growth in both F1 male and female offspring, and the development of hypertension in  
90 F1 male offspring (Kwong *et al.* 2000). Assessment of the early stage F1 preimplantation  
91 blastocyst revealed a reduction in ICM number followed by reduced ICM and TE cell number at  
92 the expanding blastocyst stage due to slowed proliferation (Kwong *et al.* 2000). It was concluded

93 that due to a mildly hyperglycaemic and amino acid depleted maternal environment, maternal  
94 undernutrition may program metabolic stress within the blastocyst.

95 Abnormal glucose metabolism in the mouse blastocyst has been linked to decreased  
96 developmental and implantation potential (Lane & Gardner 1996). Provision of adequate glucose  
97 and amino acids in combination is beneficial to blastocyst development, ICM number and post-  
98 transfer fetal development, indicating that blastocyst nutrition is highly responsive to  
99 environmental metabolites. Amino acids and vitamins prevent metabolic perturbations and  
100 associated loss of viability of mouse blastocysts (Lane & Gardner 1998). Studies have  
101 highlighted that physiological changes occur within the preimplantation blastocyst to stabilize  
102 fetal growth and promote postnatal fitness in response to maternal low protein diet during  
103 pregnancy (Watkins *et al.* 2008b; Watkins *et al.* 2008c). The majority of embryo programming  
104 studies have however, focused on alterations in F1 embryos and F1 offspring health. Few studies  
105 have characterized alterations in embryo growth and development in the next (F2) generation.

106 Our established rat model of uteroplacental insufficiency mimics intrauterine growth  
107 restriction similar to that observed in the Western world (Wlodek *et al.* 2005; Wlodek *et al.*  
108 2008). Both F1 male and female offspring have organ deficits, but only F1 male offspring  
109 develop hypertension and metabolic dysfunction in adult life (Wlodek *et al.* 2008; Wadley *et al.*  
110 2008; Moritz *et al.* 2009). During late gestation, F1 female growth restricted offspring become  
111 glucose intolerant, develop glomerular hypertrophy and have modifications in uterine artery  
112 function (Mazzuca *et al.* 2010; Gallo *et al.* 2012c). Alterations in the intrauterine nutrient  
113 environment caused by glucose intolerance may compromise F2 embryonic and fetal  
114 development, and therefore program disease development in that generation, particularly  
115 affecting germ cell development. Altered fetal growth induced by uteroplacental insufficiency

116 programs F2 nephron and  $\beta$ -cell mass deficits, as well as hypertension and metabolic dysfunction  
117 in F2 offspring in the absence of low F2 birth weight (Anderson *et al.* 2006; Bertram *et al.* 2008;  
118 Torrens *et al.* 2008; Gallo *et al.* 2012c; Tran *et al.* 2013; Gallo *et al.* 2013). These studies  
119 highlight that the maternal metabolic environment may significantly impact the development of  
120 the blastocyst prior to implantation.

121         Increasing age has also been strongly associated with decreased fertility in humans,  
122 leading to recurrent pregnancy loss or complications (Gindoff & Jewelewicz 1986). Maternal  
123 age, particularly after 35 years, is also associated with decreased ovarian reserve and increased  
124 rates of aneuploidy and chromosomal abnormality in their oocytes and cleavage stage  
125 preimplantation embryos (Munne *et al.* 1995; Munne *et al.* 2007; Harton *et al.* 2013). Murine  
126 models are in agreement with these changes, as increasing maternal age is related to increased  
127 rates of embryo fragmentation, although rodents do not show the same dramatic decline as  
128 humans in reproductive performance (Jurisicova *et al.* 1998). Maternal age, in both species, is  
129 however related to gestational factors such as increased placental weight (Haavaldsen *et al.*  
130 2011; Gallo *et al.* 2012b). In recent studies of advanced maternal age, we have demonstrated that  
131 this parameter leads to similar placental weight increase at embryonic (E) day 20, followed by  
132 reduced litter size and birth weight (Gallo *et al.* 2012b). However, the effect of advanced  
133 maternal age on next generation blastocyst morphology, carbohydrate utilization, outgrowth  
134 potential and amino acid utilization has yet to be addressed.

135         Therefore, the aims of this study were to assess blastocyst growth and development,  
136 metabolism and function of F2 preimplantation rat blastocysts derived from F1 growth restricted  
137 females induced by uteroplacental insufficiency, and to examine the effect of age on  
138 preimplantation blastocyst development and viability. We propose that adverse pregnancy

139 adaptations, such as glucose intolerance and glomerular hypertrophy, in female offspring born  
140 small would lead to alterations in the development and metabolism of their preimplantation  
141 blastocysts, which may impact growth and development of offspring in the next generation.

142

## 143 **Materials and Methods**

144 Unless otherwise stated, chemicals were from Sigma, St Louis, MO, USA.

### 145 *Mating and animal generation*

146 This study was approved by The University of Melbourne Animal Ethics  
147 Experimentation Committee prior to all experimental procedures (AEC 1112128). A vaginal  
148 impedance reader (model MK-10B, Mukomachi Kikai, Osaka, Japan) was used to determine the  
149 time of estrus for mating as described previously (Wlodek *et al.* 2005; Wlodek *et al.* 2008).  
150 Female WKY rats (F0) were mated between 18-24 weeks of age. On day 18 of gestation, rats  
151 underwent sham (Control) or bilateral uterine vessel ligation (Restricted) surgery (Wlodek *et al.*  
152 2005; Wlodek *et al.* 2008). Rats gave birth naturally at term (day 22) to F1 Control and Growth  
153 Restricted offspring (born small), respectively. Post weaning, day 35, F1 females were removed  
154 from F0 mothers and housed with 1-2 others females until the time of mating. Body weights and  
155 dimensions were measured in F1 females at day 1, day 7, day 35, 2 months, 3 months and 4  
156 months of age (Wlodek *et al.* 2007).

157 F1 Control and Restricted females, at 4 months (n=10/group) or 12 months (aged line)  
158 (n=10/group) at estrus, were naturally mated with normal (F0) males. For all experimental  
159 groups, 1 female per litter was used. Three sets of females were generated for gene analyses  
160 (n=6/group; performed in the 4 month cohort only), blastocyst morphology and carbohydrate  
161 utilization (n=6/group) and outgrowth and amino acid utilization (n=6/group). Mating success  
162 was confirmed by the presence of a vaginal plug after mating, and the following morning was  
163 considered day 0.5 of pregnancy. All female body weights and dimensions were measured prior  
164 to mating and at post mortem (day 4.5 of pregnancy) (Wlodek *et al.* 2005; Wlodek *et al.* 2008)

165 after they were euthanized by CO<sub>2</sub> inhalation. Plasma insulin concentrations were measured in  
166 duplicate using a rat insulin radioimmunoassay kit (Millipore, Abacus ALS, Brisbane, QLD,  
167 Australia) (Tran *et al.* 2013). Plasma glucose concentrations were measured in duplicate using a  
168 scaled-down version of the enzymatic fluorometric analysis (Tran *et al.* 2013).

### 169 *RT<sup>2</sup> profiler PCR array analysis*

170 Total RNA was extracted from three independent biological replicates of blastocysts  
171 using the Roche<sup>®</sup> Total RNA Isolation Kit (Roche, Dee Why, NSW, Australia) according to the  
172 manufacturer instructions [performed in the 4 month cohort only](#). There were n=6 females/group  
173 and 10-20 blastocysts were pooled from 2 mothers resulting in n=3 independent biological  
174 replicates/group. Briefly, samples were lysed and bound to a silica-based filter where they were  
175 treated with RNase-free DNase I (Roche, Dee Why, NSW, Australia), then washed with the kit  
176 buffer prior to elution in 50µl of the kit elution buffer. RNA concentration was assessed using a  
177 NanoDrop<sup>™</sup> absorbance spectrophotometer (ND1000, Thermo Scientific, Waltham, MA, USA)  
178 and RNA integrity was evaluated with the Agilent 2100 Bioanalyzer using a RNA 6000 Nano  
179 Assay Kit (Agilent Technologies, Santa Clara, CA, USA). RNA was converted to cDNA using  
180 the RT<sup>2</sup> First Strand cDNA synthesis kit (Qiagen, Chadstone, VIC, Australia), and amplified  
181 using a RT<sup>2</sup> PreAMP cDNA synthesis kit (Rat mTOR Signalling PCR array and Rat Insulin  
182 Signalling Pathway PCR array, Qiagen) according to manufacture instructions. Gene expression  
183 of mTOR (84 genes in total, supplementary table 1) and insulin signalling (84 genes in total,  
184 supplementary table 1) pathways were analysed on respective RT<sup>2</sup> Profiler Arrays (Qiagen).  
185 Amplification was performed on an ABI Vii7 (Applied Biosystems Life Technologies,  
186 Mulgrave, Victoria, Australia) with resultant gene expression analysed using Web-based PCR  
187 Array Data Analysis software ([www.SAbiosciences.com](http://www.SAbiosciences.com)). [All significant gene expression data,](#)

188 summarized in the PI3K, PKB/AKT, growth and motility and metabolism and inflammation  
189 pathways, are presented and includes other relevant genes in the respective pathways.

#### 190 *Blastocyst flushing and culture*

191 Day 4.5 blastocysts were retrieved from intact uteri by flushing with lab-made MOPS  
192 buffered G2 medium modified for the rat by increasing the osmolality to 310 mOsmol/kg using  
193 NaCl, as described previously (n=30-60 blastocysts/group from n=6 females/group) (Oh *et al.*  
194 1998; Gardner & Lane 2014). Blastocysts from F1 Control and Restricted females were then  
195 cultured in lab-made NaHCO<sub>3</sub>-buffered G2 medium (Gardner & Lane 2014) also with modified  
196 osmolality, supplemented with 5mg mL<sup>-1</sup> human serum albumin (HSA, Vitrolife, Västra  
197 Frölunda, Göteborg, Sweden) under paraffin oil (Ovoil, Vitrolife, Västra Frölunda, Göteborg,  
198 Sweden) and housed in a dual gas incubator (ThermaForma, Mariette, OH, USA) at 37°C, in an  
199 atmosphere of 6% CO<sub>2</sub>, 5% O<sub>2</sub> and 89% N<sub>2</sub>, for 24h (Oh *et al.* 1998; Lane & Gardner 2004;  
200 Gardner & Lane 2007). F2 blastocysts were scored for stage of development at flushing and  
201 again after the 24h culture period, according to a numerical grade based on blastocyst  
202 morphology (grade 3, early blastocyst, grade 4, expanded blastocyst, grade 5, hatching  
203 blastocyst, grade 6, fully hatched blastocyst) (Gardner *et al.* 2000). Following 24h culture,  
204 preimplantation blastocysts were prepared for differential nuclear staining or outgrowth culture.

#### 205 *Differential nuclear staining*

206 Total cell number, and the proportion of TE and ICM cells were assessed in F2 rat  
207 blastocysts using a modification of a previously described method (n=30-60 blastocysts/group  
208 from n=6 females/group) (Handyside & Hunter 1984; Hardy *et al.* 1989). Briefly, blastocysts  
209 were incubated in pronase, (Sigma, 0.5% in GMOPS, 5min at 37°C) to remove the zona

210 pellucida, then incubated in picrylsulfonic acid (Sigma, 10min at 37°C) and washed in GMOPS  
211 with 5mg mL<sup>-1</sup> HSA. Embryos were then incubated in anti-dinitrophenol (Sigma, 10min at 37°C)  
212 followed by washing in GMOPS supplemented with 5mg mL<sup>-1</sup> HSA. Complement mediated  
213 lysis was performed by a short incubation in guinea pig serum (IMVS, Adelaide, SA, Australia,  
214 10min) prior to transfer to a bisbenzimidazole solution of 0.1mg mL<sup>-1</sup> (Hoescht 33342, Sigma).  
215 Blastocysts were then mounted in glycerol and nuclei counted under UV light (filter) using an  
216 inverted microscope (TS100-F, Nikon, Yamato, Kanagawa, Japan).

#### 217 *Blastocyst glucose consumption and lactate production quantification*

218 Spent culture medium samples were stored at -80°C following 24h incubation with  
219 individual F2 blastocysts (n=30-60 blastocysts/group from n=6 females/group). Glucose and  
220 lactate concentrations were measured using enzyme-linked assays described by Gardner and  
221 Leese (Gardner & Leese 1987; Gardner 2007). F2 blastocysts were first incubated in 4µl drops  
222 of incubation medium, comprising of HCO<sub>3</sub><sup>-</sup> buffered G2 formulated with 0.5mM glucose as the  
223 sole substrate and no added lactate or pyruvate. Following the 24h incubation, glucose  
224 consumption and lactate production were quantitated per embryo using a NanoDrop<sup>TM</sup>  
225 fluorospectrometer (ND3300, Thermo Scientific, Waltham, MA, USA) (expressed in mol) and  
226 normalized to the number of cells per embryo to account for differences in blastocyst cell  
227 numbers. Glycolytic activity (lactate:glucose percent ratio) of each embryo was calculated on the  
228 assumption that 2 mol of lactate are formed from 1 mol of glucose (Lane & Gardner 1996).

#### 229 *Blastocyst outgrowth culture*

230 Outgrowths in F2 blastocysts were performed as described previously (n=10-40  
231 blastocysts/group from n=6 females/group) (Hannan *et al.* 2011). Flat bottomed 96-well plates

232 (BD Biosciences, San Jose, CA, USA) were coated with fibronectin (10 $\mu$ g/ml; Sigma) and  
233 incubated with 4mg/ml bovine serum albumin (BSA, Sigma) for 1h. Wells were washed and  
234 filled with 150 $\mu$ l of a modified G2 medium supplemented with 5mg mL<sup>-1</sup> HSA (Vitrolife, Västra  
235 Frölunda, Göteborg, Sweden) and equilibrated at 37°C under paraffin oil for 3h. Hatched  
236 blastocysts were placed individually into coated wells and incubated for a period of 66h.  
237 Blastocyst outgrowth was examined through the acquisition of images taken at 10x  
238 magnification at 4, 18, 23, 28, 42, 47, 52 and 66h time-points during culture, using an inverted  
239 microscope equipped with a heated stage at 37°C (Eclipse TS100-F, Nikon, Yamato, Kanagawa,  
240 Japan). The area of outgrowth was measured in each image using NIS Elements BR 3.00, SP7  
241 Laboratory Imaging software (Nikon, Yamato, Kanagawa, Japan). All images were analysed at  
242 matching magnification (x10). The average area of outgrowth was calculated for each treatment  
243 and repeated three times. At the completion of culture (66h post-transfer), 100 $\mu$ l of outgrowth  
244 media was collected for NMR analysis.

#### 245 *Metabolomic analysis of culture media*

246 Outgrowth media was analysed for carbohydrate and amino acid composition using <sup>1</sup>H-  
247 NMR spectroscopy (n=10-40 blastocysts/group from n=6 females/group). 140 $\mu$ l of chilled  
248 methanol was added to 70 $\mu$ l aliquots of spent outgrowth medium from F2 Control and Restricted  
249 blastocyst cultures, and incubated on ice for 15min (Sheedy *et al.* 2010; Gook *et al.* 2014).  
250 Samples were then centrifuged at 5000g for 15min. A 160 $\mu$ l aliquot of supernatant was collected  
251 and dried under speed vacuum for 12h at 45°C. Samples were then resuspended in 540 $\mu$ l of  
252 200mM of trisodium phosphate in deuterium oxide (Na<sub>3</sub>PO<sub>4</sub>.D<sub>2</sub>O, titrated to pH7 with DCl)  
253 (Sigma and Cambridge Isotope Laboratories Inc., Andover, MA, USA). An additional 60 $\mu$ l of  
254 5mM 2,2-dimethylsilyl-2-propane sulfonic acid (DSS.D<sub>2</sub>O, Cambridge) was added as a standard

255 for determining concentrations of media components. The 600 $\mu$ l final sample volume was added  
256 to 5mm 507 grade glass NMR tubes (Wilmad LabGlass, Vineland, NJ, USA) prior to spectral  
257 acquisition.

258 Samples were analysed on a 600MHz Bruker Avance US<sup>2</sup> (Bruker BioSpin Pty. Ltd.  
259 Alexandria, NSW, Australia) spectrometer equipped with a 5mm triple resonance cryoprobe.  
260 Samples were locked to deuterium (D<sub>2</sub>O) and gradient shimmed. The 90° pulse width was  
261 calibrated and receiver gain optimized for each sample. Spectra were collected over 64k data  
262 points and 256 scans. A 1-dimensional Nuclear Overhauser spectrometry pulse sequence with  
263 presaturation (noesypr1d) for water suppression was used (recycle delay-90°- $\tau$ -90°-  $\tau_m$ -90°-  
264 acquire FID), with a recycle delay of 1.5sec, mixing time ( $\tau_m$ ) of 50msec and transmitter  
265 frequency offset of 2848Hz. Spectra were Fourier transformed and phase corrected, and  
266 calibrated to the DSS signal at 0.00ppm. A line broadening factor of 0.5Hz was applied to all  
267 spectra. Processed NMR spectra were imported into the Chenomx NMR Suite v. 5.1 (Chenomx  
268 Inc., Edmonton, AB, Canada) and media components were identified and quantified using the  
269 600MHz compound library. The NMR data acquisition and processing parameters were selected  
270 to maintain compatibility with the Chenomx metabolite library as described previously (Sheedy  
271 *et al.* 2010).

## 272 *Statistical analyses*

273 All developmental, gene expression and microfluorescence data were analysed using a  
274 two-way ANOVA (SPSS Inc., Chicago, Illinois, USA) to determine main effects of experimental  
275 groups. If a significant interaction was detected, Mann-Whitney U tests (SPSS Inc., Chicago,  
276 Illinois, USA) were performed for *post hoc* comparisons. For all NMR data, metabolite  
277 concentrations were normalized by blastocyst outgrowth area (Constant Sum normalization) to

278 correct for differences in metabolic flux due to blastocyst size (Webb-Robertson *et al.* 2005;  
279 Craig *et al.* 2006). As outgrowths can have bi/multi-nucleated cells, cell number estimation may  
280 be inaccurate. A data matrix of quantified metabolites from <sup>1</sup>H-NMR spectral analysis of all  
281 samples was created. Scedasticity was corrected using log<sub>10</sub> transformation. All data were  
282 analysed using a two-way ANOVA (SPSS Inc., Chicago, Illinois, USA) to determine main  
283 effects of experimental groups. If a significant interaction was detected, Mann-Whitney U tests  
284 (SPSS Inc., Chicago, Illinois, USA) were performed for *post hoc* comparisons. In addition,  
285 Mann-Whitney U tests (SPSS Inc., Chicago, Illinois, USA) were conducted to determine  
286 significant interactions between experimental groups F2 Control and Restricted outgrowth media  
287 samples from 4 and 12 month F1 females. Metabolite concentrations are presented as μM/(pixel<sup>2</sup>  
288 x 10<sup>3</sup>) ± SEM with metabolite concentrations presented as production (positive values) or  
289 consumption (negative values) relative to the control media sample. All data are presented as  
290 means ± SEM and P<0.05 was considered statistically significant.

291

## 292 **Results**

### 293 *Uteroplacental insufficiency impacts physiology of F1 females*

294 F1 growth restricted female offspring arising from F0 mothers that underwent bilateral  
295 uterine vessel ligation surgery were born small and remained 7-13% smaller at day 7 and day 14  
296 compared to F1 Control offspring ( $P < 0.05$ ; Table 1). The 12 month Control and Restricted  
297 cohort had a slight increase in body weight at day 35 and 2 months of age compared to the 4  
298 month cohort but there were no differences in body weight between F1 Control and Restricted  
299 females at 3 months and 4 months of age in the 4 or 12 month cohort (Table 1). At post mortem,  
300 in the 4 and 12 month cohort, there were no differences in relative heart, liver, pancreas, dorsal  
301 fat, uterus and ovary weights between F1 Control and Restricted females, however kidney and  
302 mammary weight were reduced in F1 Restricted females ( $P < 0.05$ ; Table 1). In the 12 month  
303 cohort compared to the 4 month cohort, regardless of growth restriction, F1 females had  
304 increased heart, kidney, liver and ovary weight ( $P < 0.05$ ; Table 1). At mating, in the 4 and 12  
305 month cohort, no differences in maternal age, weight, basal plasma glucose, basal plasma insulin,  
306 mating success rate or the number of blastocysts collected were observed between F1 Control  
307 and Restricted females (Table 2). F1 females in the 12 month cohort, had an increase in body  
308 weight and age compared to F1 females in the 4 month cohort ( $P < 0.05$ ; Table 2). These results  
309 were collected from females allocated to the cohort of blastocyst morphology and carbohydrate  
310 utilization measures.

### 311 *Growth restriction of F1 females alters F2 blastocyst physiology*

312 The morphological stage of blastocyst development upon retrieval at day 4.5 of gestation  
313 was not significantly different between F2 Control and Restricted blastocysts from 4 month

314 females (Figure 1A). However, following 24h incubation *in vitro*, F2 Restricted blastocysts, from  
315 4 month old females, displayed accelerated development, with a higher proportion of blastocysts  
316 developing to both the hatching and fully hatched blastocyst stages compared with Control  
317 blastocysts, which remained at the hatching stage ( $P<0.05$ ; Figure 1B). F2 Restricted blastocysts  
318 collected from 12 month old (aged) F1 females were delayed in their development, with mostly  
319 early blastocysts observed on day 4.5 compared to Control females, with collection of mostly  
320 expanded blastocysts after flushing ( $P<0.05$ ; Figure 1A). Following 24h *in vitro* culture, there  
321 were no differences in development to the hatching blastocyst stage between F2 Control and  
322 Restricted blastocysts from 12 month females (Figure 1B). F2 blastocysts from F1 aged females,  
323 however, were more advanced (developmental stage 5-6), irrespective of growth restriction,  
324 compared to blastocysts from 4 month old females (developmental stage 4-5,  $P<0.05$ ; Figure  
325 1B).

326 This growth acceleration in F2 Restricted blastocysts from 4 month females was  
327 associated with greater total cell number, compared to F2 Control blastocysts, over the same 24h  
328 period ( $P<0.05$ ; Figure 2A). There was a greater TE cell number (11%,  $P<0.05$ ; Figure 2B),  
329 while ICM cell number remained constant (Figure 2C). The proportion of ICM:TE was not  
330 different (data not shown). A lower total cell number and TE cell number was observed in F2  
331 Restricted blastocysts from aged F1 Restricted females versus F2 Control blastocysts (16-19%,  
332  $P<0.05$ ; Figure 2A, 2B). F2 Control blastocysts from aged F1 females had a higher number of  
333 total and TE cells compared to control blastocysts from 4 month old females ( $P<0.05$ ; Figure 2A,  
334 2B). No differences in ICM cell number (Figure 2C), or the ratio of ICM:TE cells (data not  
335 shown) were detected between F2 Control or Restricted blastocysts from either age group.

336 No differences were observed between F2 Control and Restricted blastocysts from 4  
337 month females after the 24h incubation, in either glucose consumption per hour, or when  
338 normalized to total cell number (Figure 3A, 3B). However, a significant increase was detected in  
339 lactate production by F2 Restricted blastocysts from 4 month females compared to F2 Control  
340 blastocysts ( $P < 0.05$ ; Figure 3C), although this difference was not present when lactate  
341 production was expressed per cell. Glycolytic rate was not significantly different between F2  
342 Control or Restricted blastocysts from 4 month old females (Figure 3E, 3F). An increase in  
343 glucose consumption per blastocyst was observed in F2 Restricted blastocysts from aged F1  
344 females, compared to the 12 month Control and 4 month Restricted groups at 24h ( $P < 0.05$ ;  
345 Figure 3A). This difference was maintained when glucose consumption was normalized to total  
346 cell number ( $P < 0.05$ ; Figure 3B). Neither lactate consumption nor glycolytic rate was  
347 significantly different between F2 Restricted and Control blastocysts from aged F1 females  
348 (Figure 3C-3F). However, lactate production per blastocyst and per cell were both higher in the  
349 maternal 12 month cohort compared to the maternal 4 month cohort ( $P < 0.05$ ; Figure 3C, 3D).

350 Following outgrowth culture, all F2 blastocysts attached regardless of their maternal  
351 origin (from either 4 or 12 month F1 females). F2 blastocyst outgrowth area did not significantly  
352 differ between F2 Control and Restricted groups from 4 and 12 month F1 females, at any of the  
353 time-points assessed (Figure 4). There were no alterations to carbohydrate utilization between F2  
354 Control and Restricted outgrowths. Analysis of amino acid utilization by F2 Restricted  
355 outgrowths from 4 month females revealed an increase in lysine and serine consumption, and a  
356 increased production in histidine and glutamate compared to Controls ( $P < 0.05$ ; Table 3).

357 Glucose and lactate production were increased in outgrowths from F2 Control and  
358 Restricted blastocysts from aged females, while pyruvate production was reduced when

359 compared to blastocysts from 4 month old females at the end of outgrowth culture (P<0.05;  
360 Table 3). Aspartate and serine consumption were increased in F2 Restricted outgrowths from 12  
361 month females compared to Control (P<0.05; Table 3). Production of the amino acids isoleucine,  
362 methonine, glycine and proline were increased, while cystine production was reduced (P<0.05;  
363 Table 3) in outgrowths from 12 month females. There was reduced consumption of arginine,  
364 leucine, valine and tyrosine in outgrowths from 12 month females (P<0.05; Table 3).

### 365 *Growth restriction of F1 females alters F2 blastocyst gene expression dynamics*

366 Expression of genes involved in growth and insulin signalling pathways were  
367 significantly reduced in day 4.5 F2 Control and Restricted blastocysts from 4 month females  
368 (P<0.05; Figure 5; total of 168 genes targeted). Genes involved in PI3K signalling, *Pik3ca* and  
369 *Pik3cb*, had reduced expression in F2 Restricted blastocysts compared to controls (26-32%,  
370 P<0.05; Figure 5A). Energy sensing pathway genes, *Prkag1*, *Prkcb*, *Tsc1*, *Rraga*, *Mtor* and *Ulk1*,  
371 involved in PKB/Akt signalling, were significantly reduced in F2 Restricted blastocysts (30-  
372 97%, P<0.05; Figure 5B). Growth and motility gene expression of  $\beta$ -actin and *Prl* were reduced  
373 (31-53%, P<0.05; Figure 5C) however, *Rras* gene expression was significantly increased in F2  
374 Restricted blastocysts compared to controls (110%, P<0.05; Figure 5C). F2 Restricted  
375 blastocysts also displayed reduced expression of genes involved in metabolism, *Ldha* and *Ucp1*,  
376 (38-77%, P<0.05; Figure 5D) compared to maternal 4 month Control blastocyst levels.

377

378 **Discussion**

379           This study identifies alterations in the expression of genes involved in growth and  
380 development pathways, accompanied by increased proliferation of the trophoctoderm, altered  
381 lactate metabolism and amino acid consumption of serine by F2 blastocysts arising from females  
382 born small due to intrauterine growth restriction. Advanced maternal age alters F2  
383 preimplantation blastocyst growth and development with alterations in blastocyst cell number as  
384 well as altered carbohydrate and amino acid metabolism in arginine, isoleucine, leucine  
385 methionine, valine, cysteine, glycine, proline and tryosine, compared to 4 month old females.  
386 *These data demonstrate that the next generation preimplantation blastocyst is programmed via*  
387 *the maternal line of transmission and age. These alterations in blastocyst development and*  
388 *function may represent a mechanistic pathway contributing to the altered cardiovascular and*  
389 *metabolic phenotype of next generation F2 offspring that we have reported ([Gallo et al. 2012b](#);*  
390 *[Gallo et al. 2012c](#); [Gallo et al. 2013](#)).*

391 *Uteroplacental insufficiency does not impact reproductive fitness of F1 females*

392           The phenomenon of catch-up growth, with or without the combination of low birth  
393 weight, is known to program disease development in offspring during adult life (Eriksson *et al.*  
394 2001). In the present study, F1 growth restricted females were born small and displayed catch-up  
395 growth to reach a similar weight as Control animals, consistent with epidemiological and  
396 experimental evidence (McMillen & Robinson 2005). F1 growth restricted females have normal  
397 reproductive potential and basal glucose and insulin levels with unknown metabolic function.  
398 However, this is not indicative of changes that may be occurring in the uterine circulation and  
399 lumen. We have also previously reported no alterations in F2 litter size, which is indicative of

400 maintenance of pregnancy by F1 growth restricted females (Gallo *et al.* 2012c). While these  
401 experiments demonstrate that there are no differences in birth weight of F2 Restricted offspring,  
402 cellular deficits and programming of metabolic disease and hypertension occur in a gender-  
403 specific manner in F2 offspring (Wlodek *et al.* 2008; Moritz *et al.* 2009; Mazzuca *et al.* 2010;  
404 Gallo *et al.* 2012c). Importantly, we have previously demonstrated loss of glucose tolerance  
405 during late gestation in F1 growth restricted mothers at 4 months of age (Mazzuca *et al.* 2010;  
406 Gallo *et al.* 2012c). The onset and timing of these alterations may impact on next generation  
407 embryonic and fetal development.

#### 408 *Accelerated growth in F2 blastocysts*

409 F2 Restricted blastocysts accelerate growth when placed into *in vitro* culture, 24h after  
410 retrieval. This growth was associated with greater TE cell number, which may be responsible for  
411 the increase in lactate production. Significantly, over-production of lactate alone has been  
412 correlated with lower implantation potential in mouse blastocysts grown *in vitro* (Lane &  
413 Gardner 1996). Previous studies using rat embryos have indicated that nutrient imbalances can  
414 impact rat embryo metabolism and alters normal cellular function (Lane & Gardner 1998).  
415 Adaptation to the *in vitro* culture environment is reported to be associated with a higher ratio of  
416 TE to ICM, and as a result reduced viability (Hurst *et al.* 1993). Accelerated blastocyst and  
417 increased total cell number attributed to greater TE cell number, may therefore be indicative of  
418 an adaptation to the culture environment in order to maintain the integrity of the ICM.

419 When F2 Restricted blastocysts were placed in outgrowth culture, alterations in  
420 carbohydrate and amino acid metabolism were observed. Outgrowth of blastocysts revealed  
421 increases in the consumption of amino acids, specifically serine and lysine consumption, which  
422 may reflect the metabolic changes detected at 24h, due to increased lactate secretion, probably

423 from TE cells. Serine consumption has been shown to act on intracellular growth factors and  
424 extracellular matrix proteins to favour correct formation and function in placental cells (De *et al.*  
425 2004). Regulation of serine utilisation may be required for the initial short-term increased growth  
426 of F2 Restricted blastocysts immediately following flushing, which is then maintained and  
427 stabilized at later time-points, reflected in no differences in outgrowth area of F2 blastocysts.  
428 Such alterations to blastocyst metabolism and amino acid profiles that drive cell proliferation and  
429 differentiation would have significant implications for the development of the nutrient transport  
430 systems within the developing placenta, especially during trophoblast differentiation from the TE  
431 (Fowden *et al.* 2008). Glutamate production was increased, which may reflect reduced  
432 intracellular stores within the blastocyst. Glutamate been reported to be important for placental  
433 and fetal metabolism as it generates NADPH for placental fatty acid steroid synthesis (Moores,  
434 Jr. *et al.* 1994; Vaughn *et al.* 1995). Human intrauterine growth restriction studies are  
435 characterized by reduced fetal plasma concentrations of essential amino acids such as valine,  
436 leucine, isoleucine and lysine (Cetin *et al.* 1988; Avagliano *et al.* 2012) A reduction in uptake of  
437 lysine in the placenta due to reduced system A amino acid transport activity has also been  
438 demonstrated in pregnancies complicated by intrauterine growth restriction (Dicke & Henderson  
439 1988; Jansson *et al.* 1998).

440 [It is not known whether alterations in amino acids levels in the F1 Restricted uterine](#)  
441 [environment exist, which may subject the F2 Restricted blastocyst to an altered nutrient](#)  
442 [environment.](#) Low nutrient availability or absorption can alter signalling pathways, which will  
443 impact on growth and development of the preimplantation blastocyst (Hardy *et al.* 1989; Brison  
444 & Leese 1991; Lane & Gardner 1997; Gardner 1998). Conversely, transition from low to high  
445 nutrient availability can trigger an adaptive response. Amino acids are also required for TE

446 motility through the activation of mTOR (mammalian target of rapamycin)-dependent signal  
447 cascades (Martin & Sutherland 2001; Martin *et al.* 2003). Regulated by amino acids, the mTOR  
448 pathway is responsible for cell growth, proliferation, motility, protein synthesis and transcription.  
449 The day 4.5 F2 Restricted blastocysts exhibited significant reductions in *Pi3k* genes, kinases and  
450 binding genes (*Prk's* and *Tsc1*), *Mtor* and growth related genes (*Ulk1* and *Prl*) in the mTOR  
451 growth pathway. The downstream events of PI3K signalling are mediated by serine/threonine  
452 protein kinase B (PKB/Akt) (Datta *et al.* 1999; Han & Carter 2001; Blume-Jensen & Hunter  
453 2001). Loss or reduction in PKB activity has been related to defects in growth and development  
454 of the placenta, placental insufficiency, and results in impaired fetal growth (Yang *et al.* 2003).  
455 Reduced mTORC1 activity was reported in F1 mouse blastocysts from mothers subjected to  
456 maternal undernutrition (0-4.25 days) during pregnancy (Eckert *et al.* 2012). As gene expression  
457 analysis was performed on blastocysts retrieved from F1 growth restricted females, this clearly  
458 highlights pre-existing (epi)genetic alterations that could affect blastocyst metabolism. Limited  
459 availability of blastocyst material precluded validation of alterations in protein expression.  
460 Epigenetic modifications to F1 germ cells or alterations to F1 germ cells, due to the initial insult  
461 of growth restriction, may directly impact F2 blastocyst growth and development and  
462 consequently placental and fetal growth.

#### 463 *Age-related alterations in F2 blastocysts*

464 'Second-hits', such as advanced maternal age, are known to highlight growth restriction  
465 phenotypes in female offspring (Nenov *et al.* 2000). However, we have demonstrated that with  
466 advanced maternal age, basal glucose homeostasis, ovarian function and reproductive potential  
467 remain normal in the rat (Gallo *et al.* 2012b). We have previously reported that advanced  
468 maternal age, regardless of growth restriction, results in reduced F2 fetal weight (E20) and litter

469 size (Gallo *et al.* 2012b). These data indicate that although mating success appears normal in  
470 aged females, it is not necessarily a reflection on ovarian function and oocyte, since fetal loss can  
471 also be attributed to inadequate placental function.

472 F1 growth restricted female rats, with advanced age at conception, produced blastocysts  
473 with delayed morphological development and lower cell numbers, which is likely to be attributed  
474 to lower TE cell number. The same blastocysts also showed an unusual adaptive metabolic  
475 response, this time reflected in an increased glucose uptake, but no change in lactate output. This  
476 is curious, since increased glucose uptake correlates positively with blastocyst implantation  
477 potential in the mouse and human (Gardner *et al.* 2011). In our case, the higher glucose  
478 consumption in blastocysts with lower TE cell numbers is potentially a very sudden adaptive  
479 response to meet the demands of timely blastocoel expansion. Indeed, the speed of blastocoel  
480 fluid accumulation is positively correlated with glucose uptake in rat blastocysts (Brison & Leese  
481 1994). Furthermore, glucose uptake may lead to increased glucose accumulation in the blastocoel  
482 itself, rather than be metabolized (Brison *et al.* 1993) which would explain the lack of change in  
483 lactate production. The glucose adaptation may also be reflective of the sustained alterations to  
484 carbohydrate and amino acid metabolism seen during blastocyst outgrowth. Of note is the  
485 reversal of aspartate levels from its production in the aged restricted blastocyst outgrowths, to its  
486 consumption in the Controls. Aspartate is highly consumed by mouse blastocysts, and blockage  
487 of this amino acid from entering the citric acid cycle has been reported to lead to impaired fetal  
488 development (Mitchell *et al.* 2009).

489 Altered carbohydrate metabolism has likewise been linked to abnormalities in fetal  
490 development and in some cases loss of pregnancy in both humans and murine models (Jurisicova  
491 *et al.* 1998; Harton *et al.* 2013). Studies have highlighted that oocytes and blastocysts from older

492 females present with chromosomal abnormalities or maladaptation of the embryo in the absence  
493 of chromosome abnormalities (Munne *et al.* 1995; Munne *et al.* 2007; Harton *et al.* 2013), which  
494 may explain the reduction in F2 litter size from aged F1 female rats that we have previously  
495 reported (Gallo *et al.* 2012b). Furthermore, DNA damage has also been correlated with deficits  
496 in metabolic activity in preimplantation embryos (Sturmeiy *et al.* 2009). Collectively, these data  
497 are indicative of cell stress responses, and highlight that F2 blastocysts from aged females  
498 require a greater amount of time to adapt, depending of the degree of physiological deficit.  
499 Expression of genes related to growth, development and metabolism may have also been  
500 exacerbated by advanced maternal age, however future studies and new cohorts are required to  
501 establish this. [Future studies characterising maternal metabolic status and uterine fluid](#)  
502 [composition in early pregnancy in aged females born of normal birth weight and born small may](#)  
503 [provide mechanistic insight into age-reported effects](#)

#### 504 *Transgenerational transmission of growth restriction*

505 The maternal uterine microenvironment and alterations to pregnancy adaptations, can  
506 program the inheritance of diseases to the next generation (Gallo *et al.* 2012a). How the maternal  
507 environment influences the entirety of embryonic, fetal and placental growth, development and  
508 function, remains unknown. Future studies need to focus on alterations to pregnancy adaptations  
509 during all stages from conception to term. Comprehensive biochemical characterization of F1  
510 follicular and uterine fluid will be necessary to identify key nutrients which may impact on  
511 oocyte and embryo quality and program developmental competence.

512 This study has illustrated that the F2 blastocyst, derived via the maternal line of growth  
513 restriction, have alterations in development and metabolism, which has the potential to alter the  
514 course of both placental and fetal function and development. A number of mechanisms for

515 transgenerational transmission of diseases have been proposed. These include direct exposure of  
516 germ cells from the F1 fetus, inheritance through persistent epigenetic modifications of parental  
517 germ cells and/or abnormal pregnancy adaptations, which include the development of glucose  
518 intolerance during late gestation (Gallo *et al.* 2012a). Also, the maternal environment could  
519 metabolically program the embryo via reproductive tract factors, impacting directly on embryo  
520 function and implantation events. In order to address how true transgenerational transmission  
521 may occur, a number of studies can therefore be applied in light of this new data. These include  
522 analyses of the F2 Restricted blastocyst derived via the paternal line of transmission, as it would  
523 delineate between epigenetic modifications and direct exposure of F1 germ cells to direct  
524 influences of the maternal environment and, importantly, characterizing the F3 offspring in order  
525 to verify true transgenerational transmission.

526

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719 **Figure Legends**

720 **Fig. 1. F2 Blastocyst Grade.** F2 blastocysts at A) retrieval (day 4.5) and B) following 24h  
721 culture from 4 and 12 month Control and Restricted cohorts. All data are presented as mean  $\pm$   
722 SEM, n=30-60 blastocysts/group from n=6 females/group. P<0.05 vs Control group (main  
723 effect). P<0.05 vs 4 month cohort (main effect).  $\delta$ P<0.05 vs Control group (following significant  
724 interaction).  $\gamma$ P<0.05 vs 4 month cohort (following significant interaction). \*P<0.05 vs Control.

725 **Fig. 2. F2 Blastocyst Nuclei Number.** F2 blastocysts A) total nuclei number, B) trophectoderm  
726 nuclei number and C) inner cell mass nuclei number following 24h culture from 4 and 12 month  
727 F1 Control and Restricted cohorts. All data are presented as mean  $\pm$  SEM, n=30-60  
728 blastocysts/group from n=6 females/group. P<0.05 vs 4 month cohort (main effect).  $\delta$ P<0.05 vs  
729 Control group (following significant interaction).  $\gamma$ P<0.05 vs 4 month cohort (following  
730 significant interaction).

731 **Fig. 3. F2 Carbohydrate Consumption.** F2 blastocysts A) glucose consumption per hour per  
732 blastocyst, B) glucose consumption per hour per cell, C) lactate production per hour per  
733 blastocyst, D) lactate production per hour per cell, E) glycolytic rate per hour per blastocyst and  
734 F) glycolytic rate per hour per cell following 24h culture from 4 and 12 month F1 Control and  
735 Restricted cohorts. All data are presented as mean  $\pm$  SEM, n=30-60 blastocysts/group from n=6  
736 females/group. P<0.05 vs Control group (main effect). P<0.05 vs 4 month cohort (main effect).  
737  $\delta$ P<0.05 vs Control group (following significant interaction).  $\gamma$ P<0.05 vs 4 month cohort  
738 (following significant interaction). \*P<0.05 vs Control.

739 **Fig. 4. F2 Blastocyst Outgrowth.** Measurements performed on F2 blastocysts (66h outgrowth  
740 culture) following 24h culture from 4 and 12 month F1 Control and Restricted cohorts. Open

741 circles represent F2 Control blastocysts from 4 month F1 Control females, Closed circles  
742 represent F2 Restricted blastocysts from 4 month F1 Restricted females, Grey open circles  
743 represent F2 Control blastocysts from 12 month F1 Control females and Grey closed circles  
744 represent F2 Restricted blastocysts from 12 month F1 Restricted females. All data are presented  
745 as mean  $\pm$  SEM, n=10-40 blastocysts/group from n=6 females/group.

746 **Fig. 5. F2 Day 4.5 Gene Expression.** Measurements performed on F2 blastocysts from 4 month  
747 F1 Control and Restricted females. A) *Pi3k* signalling, B) *Pkb/Akt* signalling, C) growth and  
748 motility and D) energy metabolism and inflammation relative gene expression to control level (1,  
749 dotted line). All data are presented as mean  $\pm$  SEM. There were n=6 females/group and 10-20  
750 blastocysts were pooled from 2 mothers resulting in n=3 independent biological replicates/group.  
751 . \*P<0.05 vs Control, \*\*P<0.01 vs Control and \*\*\*P<0.001 vs Control.

752

**Table 1. F1 Female Body and Organ Weights.**

	4 Month Cohort		12 Month Cohort		2-way ANOVA		
	Control	Restricted	Control	Restricted	Group	Cohort	Interaction
<i>Body Weight (g)</i>							
<b>Day 1</b>	4.27 ± 0.08	3.73 ± 0.10	4.49 ± 0.08	3.65 ± 0.09	0.0001	NS	NS
<b>Day 7</b>	10.69 ± 0.34	8.72 ± 0.67	10.29 ± 0.63	9.01 ± 0.67	0.017	NS	NS
<b>Day 14</b>	22.61 ± 0.67	19.45 ± 1.52	23.19 ± 0.84	21.18 ± 1.07	0.044	NS	NS
<b>Day 35</b>	69.07 ± 1.45	65.32 ± 3.23	79.29 ± 2.53	74.91 ± 2.27	NS	0.0001	NS
<b>2 Months</b>	147.12 ± 2.13	146.71 ± 5.76	165.11 ± 6.20	156.87 ± 6.83	NS	0.012	NS
<b>3 Months</b>	199.35 ± 4.01	189.41 ± 4.63	206.52 ± 6.88	197.03 ± 6.40	NS	NS	NS
<b>4 Months</b>	214.98 ± 2.91	205.07 ± 5.92	221.17 ± 4.79	216.79 ± 5.62	NS	NS	NS
<i>Relative Organ Weight (%)</i>							
<b>Heart</b>	0.42 ± 0.01	0.39 ± 0.01	0.99 ± 0.02	0.37 ± 0.01	NS	0.002	NS
<b>Kidney</b>	0.67 ± 0.01	0.64 ± 0.01	0.65 ± 0.02	0.61 ± 0.02	0.008	0.016	NS
<b>Liver</b>	4.60 ± 0.18	4.44 ± 0.08	4.02 ± 0.27	3.97 ± 0.24	NS	0.003	NS
<b>Pancreas</b>	0.26 ± 0.01	0.31 ± 0.04	0.73 ± 0.06	0.32 ± 0.04	NS	NS	NS
<b>Mammary</b>	0.25 ± 0.03	0.17 ± 0.03	0.32 ± 0.07	0.18 ± 0.03	0.015	NS	NS
<b>Dorsal Fat</b>	1.21 ± 0.06	1.15 ± 0.04	4.02 ± 0.94	1.51 ± 0.14	NS	NS	NS
<b>Uterus</b>	0.14 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	NS	NS	NS
<b>Ovary</b>	0.11 ± 0.01	0.11 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	NS	0.0001	NS

753

F1 body weight (birth to 4 months of age) and organ weights at postmortem (relative to body weight; day 4.5 of pregnancy) from 4 and 12 month F1

754

Control and Restricted cohort. All data are presented as mean ± SEM, n=10/group. P&lt;0.05 vs Control group (main effect). P&lt;0.05 vs 4 month cohort

755

(main effect).

756

757 **Table 2. F1 Maternal Parameters at Mating.**

	4 Month Cohort		12 Month Cohort		2-way ANOVA		
	Control	Restricted	Control	Restricted	Group	Cohort	Interaction
Age (weeks)	21.1±1.35	20 ± 0.91	52 ± 1.02	52 ± 1.53	NS	P<0.0001	NS
Weight (g)	228.31 ± 2.43	217.21 ± 3.01	249.98 ± 9.99	259.58 ± 6.97	NS	P<0.0001	NS
Plasma Glucose (mmol.l <sup>-1</sup> )	17.51 ± 2.21	17.61 ± 2.31	13.72 ± 1.08	10.21 ± 1.42	NS	NS	NS
Plasma Insulin (ng.ml <sup>-1</sup> )	1.65 ± 0.17	2.35 ± 0.41	2.03 ± 0.33	1.89 ± 0.37	NS	NS	NS
Mating Success Rate	100%	100%	100%	100%	NS	NS	NS
Blastocysts Collected	8 ± 0.41	6 ± 0.65	7 ± 1.85	5 ± 1.03	NS	NS	NS

758 Measurements were performed in 4 and 12 month Control and Restricted cohorts. All data are presented as mean ± SEM, n=10/group. P<0.05 vs 4  
 759 month cohort (main effect).

760

**Table 3. F2 Outgrowth Blastocyst Nutrient Utilization.**

Metabolite (pmol/blastocyst/hr/(pixels <sup>2</sup> )10 <sup>3</sup> )	4 Month Cohort		12 Month Cohort		2-way ANOVA		
	Control	Restricted	Control	Restricted	Group	Cohort	Interaction
<b>Glucose</b>	-28.72 ± 8.15	-20.15 ± 5.64	0.18 ± 1.49 <sup>#</sup>	1.09 ± 1.20 <sup>#</sup>	NS	P=0.003	NS
<b>Lactate</b>	-22.26 ± 8.08	-15.42 ± 6.62	8.74 ± 1.87 <sup>#</sup>	10.08 ± 2.12 <sup>#</sup>	NS	P=0.001	NS
<b>Pyruvate</b>	0.41 ± 0.10	0.30 ± 0.09	0.05 ± 0.02 <sup>#</sup>	0.03 ± 0.02 <sup>#</sup>	NS	P=0.004	NS
<i>Essential Amino Acids</i>							
<b>Arginine</b>	-2.94 ± 1.26	-3.64 ± 0.61	-0.55 ± 0.90	-1.28 ± 0.38 <sup>#</sup>	NS	P=0.048	NS
<b>Histidine</b>	-0.44 ± 0.20	0.26 ± 0.22 <sup>δ</sup>	0.19 ± 0.16 <sup>γ</sup>	-0.03 ± 0.05	NS	NS	P=0.049
<b>Isoleucine</b>	-1.58 ± 0.41	-1.67 ± 0.30	0.07 ± 0.24 <sup>#</sup>	0.14 ± 0.08 <sup>#</sup>	NS	P=0.0001	NS
<b>Leucine</b>	-1.79 ± 0.54	-1.73 ± 0.28	-0.01 ± 0.13 <sup>#</sup>	-0.02 ± 0.08 <sup>#</sup>	NS	P=0.001	NS
<b>Lysine</b>	-0.26 ± 0.90	-3.13 ± 0.43 <sup>*</sup>	-0.03 ± 0.28	-0.11 ± 0.09 <sup>#</sup>	NS	NS	NS
<b>Methionine</b>	-0.27 ± 0.09	-0.20 ± 0.05	0.08 ± 0.06 <sup>#</sup>	0.08 ± 0.04 <sup>#</sup>	NS	P=0.001	NS
<b>Phenylalanine</b>	-0.75 ± 0.21	-0.19 ± 0.20	-0.10 ± 0.15	-0.19 ± 0.07	NS	NS	NS
<b>Theronine</b>	0.29 ± 0.26	0.40 ± 0.26	0.47 ± 0.52	0.80 ± 0.29	NS	NS	NS
<b>Valine</b>	-1.76 ± 0.51	-1.48 ± 0.27	-0.10 ± 0.15 <sup>#</sup>	-0.07 ± 0.06 <sup>#</sup>	NS	P=0.002	NS
<i>Non-Essential Amino Acids</i>							
<b>Alanine</b>	-0.10 ± 0.25	0.26 ± 0.15	0.30 ± 0.11	0.13 ± 0.10	NS	NS	NS
<b>Asparagine</b>	0.22 ± 0.26	0.18 ± 0.35	0.02 ± 0.08	0.06 ± 0.09	NS	NS	NS
<b>Aspartate</b>	0.22 ± 0.36	0.94 ± 0.54	0.09 ± 0.14	-0.23 ± 0.05 <sup>*#</sup>	NS	NS	NS
<b>Cystine</b>	1.12 ± 0.29	1.30 ± 0.41	0.18 ± 0.14 <sup>#</sup>	0.13 ± 0.07	NS	P=0.007	NS
<b>Glutamate</b>	-0.83 ± 0.30	0.16 ± 0.31 <sup>*</sup>	0.15 ± 0.10	0.15 ± 0.12	NS	NS	NS
<b>Glycine</b>	-4.26 ± 1.29	-5.77 ± 1.84	1.47 ± 1.41 <sup>#</sup>	1.72 ± 0.57 <sup>#</sup>	NS	P=0.0001	NS
<b>Proline</b>	-3.10 ± 0.86	-0.42 ± 0.91	1.18 ± 0.40 <sup>#</sup>	0.64 ± 0.17	NS	P=0.008	NS
<b>Serine</b>	0.81 ± 0.27	-0.12 ± 0.39	0.13 ± 0.20	-0.50 ± 0.14 <sup>*</sup>	P=0.036	NS	NS
<b>Tyrosine</b>	-0.40 ± 0.14	-0.61 ± 0.20	-0.05 ± 0.09	-0.17 ± 0.03	NS	P=0.03	NS

762 Measurements were performed in F2 blastocysts from 4 and 12 month Control and Restricted cohorts. Metabolite concentrations presented as  
763 production (positive values) or consumption (negative values) relative to the media only sample. All data are presented as mean ± SEM; n=10-40  
764 blastocysts/group from n=6 females/group. P<0.05 vs 4 month cohort (main effect), <sup>δ</sup>P<0.05 vs Control group (following significant interaction) and  
765 <sup>γ</sup>P<0.05 vs 4 month cohort (following significant interaction). \*P<0.05 vs Control and <sup>#</sup>P<0.05 vs 4 month cohort.