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

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

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Abstract

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

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

Introduction

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

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

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

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

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

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

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

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

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

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

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

Materials and Methods

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

Mating and animal generation

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

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

after they were euthanized by CO_2 inhalation. Plasma insulin concentrations were measured in duplicate using a rat insulin radioimmunoassay kit (Millipore, Abacus ALS, Brisbane, QLD, Australia) (Tran *et al.* 2013). Plasma glucose concentrations were measured in duplicate using a scaled-down version of the enzymatic fluorometric analysis (Tran *et al.* 2013).

RT^2 profiler PCR array analysis

Total RNA was extracted from three independent biological replicates of blastocysts using the Roche® Total RNA Isolation Kit (Roche, Dee Why, NSW, Australia) according to the manufacturer instructions performed in the 4 month cohort only. There were n=6 females/group and 10-20 blastocysts were pooled from 2 mothers resulting in n=3 independent biological replicates/group. Briefly, samples were lysed and bound to a silica-based filter where they were treated with RNase-free DNase I (Roche, Dee Why, NSW, Australia), then washed with the kit buffer prior to elution in 50µl of the kit elution buffer. RNA concentration was assessed using a NanoDrop™ absorbance spectrophotometer (ND1000, Thermo Scientific, Waltham, MA, USA) and RNA integrity was evaluated with the Agilent 2100 Bioanalyzer using a RNA 6000 Nano Assay Kit (Agilent Technologies, Santa Clara, CA, USA). RNA was converted to cDNA using the RT^2 First Strand cDNA synthesis kit (Qiagen, Chadstone, VIC, Australia), and amplified using a RT^2 PreAMP cDNA synthesis kit (Rat mTOR Signalling PCR array and Rat Insulin Signalling Pathway PCR array, Qiagen) according to manufacture instructions. Gene expression of mTOR (84 genes in total, supplementary table 1) and insulin signalling (84 genes in total, supplementary table 1) pathways were analysed on respective RT^2 Profiler Arrays (Qiagen). Amplification was performed on an ABI Viia7 (Applied Biosystems Life Technologies, Mulgrave, Victoria, Australia) with resultant gene expression analysed using Web-based PCR Array Data Analysis software (www.SAbiosciences.com). All significant gene expression data,

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

Blastocyst flushing and culture

Day 4.5 blastocysts were retrieved from intact uteri by flushing with lab-made MOPS buffered G2 medium modified for the rat by increasing the osmolality to 310 mOsmol/kg using NaCl, as described previously (n=30-60 blastocysts/group from n=6 females/group) (Oh *et al.* 1998; Gardner & Lane 2014). Blastocysts from F1 Control and Restricted females were then cultured in lab-made $NaHCO_3$-buffered G2 medium (Gardner & Lane 2014) also with modified osmolality, supplemented with 5mg mL^{-1} human serum albumin (HSA, Vitrolife, Västra Frölunda, Göteburg, Sweden) under paraffin oil (Ovoil, Vitrolife, Västra Frölunda, Göteburg, Sweden) and housed in a dual gas incubator (ThermaForma, Mariette, OH, USA) at 37ºC, in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2, for 24h (Oh *et al.* 1998; Lane & Gardner 2004; Gardner & Lane 2007). F2 blastocysts were scored for stage of development at flushing and again after the 24h culture period, according to a numerical grade based on blastocyst morphology (grade 3, early blastocyst, grade 4, expanded blastocyst, grade 5, hatching blastocyst, grade 6, fully hatched blastocyst) (Gardner *et al.* 2000). Following 24h culture, preimplantation blastocysts were prepared for differential nuclear staining or outgrowth culture.

Differential nuclear staining

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

pellucida, then incubated in picrylsulfonic acid (Sigma, 10min at 37ºC) and washed in GMOPS with 5mg mL^{-1} HSA. Embryos were then incubated in anti-dinitrophenol (Sigma, 10min at 37ºC) followed by washing in GMOPS supplemented with 5mg mL^{-1} HSA. Complement mediated lysis was performed by a short incubation in guinea pig serum (IMVS, Adelaide, SA, Australia, 10min) prior to transfer to a bisbenzimide solution of 0.1mg mL^{-1} (Hoescht 33342, Sigma). Blastocysts were then mounted in glycerol and nuclei counted under UV light (filter) using an inverted microscope (TS100-F, Nikon, Yamato, Kanagawa, Japan).

Blastocyst glucose consumption and lactate production quantification

Spent culture medium samples were stored at -80°C following 24h incubation with individual F2 blastocysts (n=30-60 blastocysts/group from n=6 females/group). Glucose and lactate concentrations were measured using enzyme-linked assays described by Gardner and Leese (Gardner & Leese 1987; Gardner 2007). F2 blastocysts were first incubated in 4µl drops of incubation medium, comprising of HCO_3^- buffered G2 formulated with 0.5mM glucose as the sole substrate and no added lactate or pyruvate. Following the 24h incubation, glucose consumption and lactate production were quantitated per embryo using a NanoDrop™ fluorospectrometer (ND3300, Thermo Scientific, Waltham, MA, USA) (expressed in mol) and normalized to the number of cells per embryo to account for differences in blastocyst cell numbers. Glycolytic activity (lactate:glucose percent ratio) of each embryo was calculated on the assumption that 2 mol of lactate are formed from 1 mol of glucose (Lane & Gardner 1996).

Blastocyst outgrowth culture

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

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

Metabolomic analysis of culture media

Outgrowth media was analysed for carbohydrate and amino acid composition using ^{1}H-NMR spectroscopy (n=10-40 blastocysts/group from n=6 females/group). 140µl of chilled methanol was added to 70µl aliquots of spent outgrowth medium from F2 Control and Restricted blastocyst cultures, and incubated on ice for 15min (Sheedy *et al.* 2010; Gook *et al.* 2014). Samples were then centrifuged at 5000g for 15min. A 160µl aliquot of supernatant was collected and dried under speed vacuum for 12h at 45ºC. Samples were then resuspended in 540µl of 200mM of trisodium phosphate in deuterium oxide ($Na_3PO_4.D_2O$, titrated to pH7 with DCl) (Sigma and Cambridge Isotope Laboratories Inc., Andover, MA, USA). An additional 60µl of 5mM 2,2-dimethylsilyl-2-propane sulfonic acid ($DSS.D_2O$, Cambridge) was added as a standard

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

Samples were analysed on a 600MHz Bruker Avance US^2 (Bruker BioSpin Pty. Ltd. Alexandria, NSW, Australia) spectrometer equipped with a 5mm triple resonance cryoprobe. Samples were locked to deuterium (D_2O) and gradient shimmed. The 90º pulse width was calibrated and receiver gain optimized for each sample. Spectra were collected over 64k data points and 256 scans. A 1-dimensional Nuclear Overhauser spectrometry pulse sequence with presaturation (noesypr1d) for water suppression was used (recycle delay-90º-τ-90º- τ_m-90º-acquire FID), with a recycle delay of 1.5sec, mixing time (τ_m) of 50msec and transmitter frequency offset of 2848Hz. Spectra were Fourier transformed and phase corrected, and calibrated to the DSS signal at 0.00ppm. A line broadening factor of 0.5Hz was applied to all spectra. Processed NMR spectra were imported into the Chenomx NMR Suite v. 5.1 (Chenomx Inc., Edmonton, AB, Canada) and media components were identified and quantified using the 600MHz compound library. The NMR data acquisition and processing parameters were selected to maintain compatibility with the Chenomx metabolite library as described previously (Sheedy *et al.* 2010).

Statistical analyses

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

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

Results

Uteroplacental insufficiency impacts physiology of F1 females

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

Growth restriction of F1 females alters F2 blastocyst physiology

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

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

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

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

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

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

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

Growth restriction of F1 females alters F2 blastocyst gene expression dynamics

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

Discussion

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

Uteroplacental insufficiency does not impact reproductive fitness of F1 females

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

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

Accelerated growth in F2 blastocysts

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

When F2 Restricted blastocysts were placed in outgrowth culture, alterations in carbohydrate and amino acid metabolism were observed. Outgrowth of blastocysts revealed increases in the consumption of amino acids, specifically serine and lysine consumption, which 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 rapamycine)-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/theronine
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

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

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

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

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

Transgenerational transmission of growth restriction

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

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

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

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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 ±
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 ± 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 ± 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 ± 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 ± 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.

Table 1. F1 Female Body and Organ Weights.

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

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

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^{-1})	17.51 ± 2.21	17.61 ± 2.31	13.72 ± 1.08	10.21 ± 1.42	NS	NS	NS
Plasma Insulin (ng.ml^{-1})	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

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 month cohort (main effect).

Table 3. F2 Outgrowth Blastocyst Nutrient Utilization.

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

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

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