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Title:
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Date:
2019-04-01

Citation:
Mangwiro, Y. T. M., Cuffe, J. S. M., Mahizir, D., Anevska, K., Gravina, S., Romano, T., Moritz, K. M., Briffa, J. F. & Wlodek, M. E. (2019). Exercise initiated during pregnancy in rats born growth restricted alters placental mTOR and nutrient transporter expression. Journal of Physiology, 597 (7), pp.1905-1918. https://doi.org/10.1113/JP277227.

Persistent Link:
https://hdl.handle.net/11343/285625

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DOI: 10.1113/JP277227

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Exercise initiated during pregnancy in rats born growth restricted alters placental mTOR and nutrient transporter expression

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1113/JP277227.

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Running title – Exercise on placental nutrient transport

Keywords – exercise, fetal programming, growth restriction, nutrient transport, placenta

Key points

- Fetal growth is dependent on effective placental nutrient transportation, which is regulated by mTORC1 modulation of nutrient transporter expression. These transporters are dysregulated in pregnancies affected by uteroplacental insufficiency and maternal obesity.
- Nutrient transporters and mTOR were altered in placentae of mothers born growth restricted compared to normal birth weight dams, with maternal diet- and fetal sex-specific responses.
- Exercise initiated during pregnancy (*PregEx*) downregulated MTOR protein expression, despite an increase in mTOR activation in male associated placentae, and reduced nutrient transporter gene abundance, which was also dependent on maternal diet and fetal sex.
- Limited changes were characterised with exercise initiated before and continued throughout pregnancy (*Exercise*) in nutrient transporter and mTOR expression.
- Maternal exercise during pregnancy (*PregEx*) differentially regulated mTOR and nutrient transporters in a diet- and sex-specific manner, which likely aims to improve late gestational placental growth and neonatal survival.

Abstract

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Adequate transplacental nutrient delivery is essential for fetoplacental development. Intrauterine growth restriction and maternal obesity independently alter placental nutrient transporter expression. Although exercise is beneficial for maternal health, limited studies have characterized how the timing of exercise initiation influences placental nutrient transport. Therefore, this study investigated the impact of maternal exercise on placental mTOR and nutrient transporter expression in growth restricted mothers and if these outcomes were dependent on maternal diet or fetal sex. Uteroplacental insufficiency (*Restricted*) or sham (*Control*) surgery was induced on embryonic day (E) 18 in Wistar-Kyoto rats. F1 offspring were fed a Chow or High-fat diet from weaning and at 16 weeks were randomly allocated an exercise protocol; *Sedentary*, Exercised prior to and during pregnancy (*Exercise*), or Exercised during pregnancy only (*PregEx*). Females were mated with normal males (20 weeks) and F2 placentae collected at E20. *PregEx* reduced mTOR protein expression in all groups and increased mTOR activation in male associated placentae. *PregEx* decreased the expression of amino acid transporters in a diet and sex-specific manner. Maternal growth restriction altered mTOR and system A amino acid transporter expression in a sex and diet specific manner. These data highlight that maternal exercise initiated during pregnancy alters placental mTOR expression, which may directly regulate amino acid transporter expression, to a greater extent than exercise initiated prior to and continued during pregnancy, in a diet and fetal sex dependent manner. These findings highlight that the timing of exercise initiation is important for optimal placental function.

Abbreviations list

ANOVA, Analysis of variance; E, Embryonic day; Exercise, Exercise before and during pregnancy; GLUT1, Glucose transporter 1; GLUT3, Glucose transporter 3; IGF, Insulin-like growth factor; mTOR, Mammalian target of rapamycin; pMTOR, Phosphorylated mammalian target of rapamycin; PN, Postnatal day; PregEx, Exercise during pregnancy only; PregEx, Exercise during pregnancy only; qPCR, Quantitative polymerase chain reaction; RNA, ribonucleic acid; Slc2a1, Solute carrier family 2 member 1; Slc2a3, Solute carrier family 2 member 3; Slc38a1, Solute Carrier Family 38 Member 1; Slc38a2, Solute Carrier

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Family 38 Member 2; Slc38a4, Solute Carrier Family 38 Member 4; Slc5a1, solute carrier family 5 member 1; SRY, Sex-determining region Y; WKY, Wistar Kyoto; Wk, Week.

Introduction

The placenta modulates nutrient exchange between the mother and fetus to regulate fetal growth and development. Glucose is the primary energy substrate utilized by the developing fetus, with a third of placental glucose uptake being utilized by the placenta. Glucose is primarily transported across the placenta via the glucose transporters GLUT1 and GLUT3 (Fowden *et al.*, 2009), with emerging evidence of the presence of Na^+-dependent active glucose transporters (SLC5) in rabbit and human placentae (Kevorkova *et al.*, 2007), suggesting that they too may be contributing to placental glucose transportation. In addition to glucose, amino acids also play a key role in promoting fetal growth. The System A family of amino acid transporters (including SNAT1, 2 and 4) actively transfer small neutral amino acids across the placenta (Cetin *et al.*, 1992; Jansson, 2001). The expression of nutrient transporters in the placenta largely involves regulation by the mammalian target of rapamycin (mTOR) signaling pathway (Jansson *et al.*, 2012; Diaz *et al.*, 2014), which is a master regulator of cell growth, insulin-like growth factor production, nutrient transporter expression, and cellular metabolism via MTORC1 activation (Saxton & Sabatini, 2017).

In a number of pregnancy complications, including intrauterine growth restriction (IUGR), there is a shift in the balance of nutrient transportation across the placenta and a disruption in nutrient gradients, which ultimately impairs fetal growth (Jansson & Powell, 2006; Diaz *et al.*, 2014). IUGR affects 10% of pregnancies in the Western population (Hamilton *et al.*, 2015) and is primarily caused by impaired placental function, which often involves reduced placental glucose and amino acid transport. Cord blood concentrations of essential amino acids are decreased in growth restricted babies (Jansson *et al.*, 2012; Lin *et al.*, 2012; Diaz *et al.*, 2014; Dimasuay *et al.*, 2016) who also often present hypoglycaemic (Economides & Nicolaides, 1989; Jansson *et al.*, 2012; Diaz *et al.*, 2014); with both of these outcomes likely

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due to alterations in placental abundance and localization of glucose and amino acid transporters (Glazier *et al.*, 1997; Janzen *et al.*, 2013; Cuffe *et al.*, 2014; Gardebjer *et al.*, 2014a; Dimasuay *et al.*, 2016; Zhang *et al.*, 2016). Interestingly, mTOR signaling is also downregulated in the placenta of IUGR humans (Roos *et al.*, 2007; Jansson *et al.*, 2012; Diaz *et al.*, 2014; Fahlbusch *et al.*, 2015) as well as in animal models of reduced fetal growth (Jansson *et al.*, 2012; Dimasuay *et al.*, 2016; Zhang *et al.*, 2016; Mejia *et al.*, 2017; Rosario *et al.*, 2017). Animal studies have eloquently demonstrated that the disease burden of being born small is not limited to the first directly affected generation (F1), but can be transmitted across generations (Aerts & Van Assche, 2006; Gallo *et al.*, 2012; Gallo *et al.*, 2013; Cheong *et al.*, 2016b). However, no studies to date have characterized alterations in nutrient transporters or mTOR expression in placentae associated with F2 offspring of mothers that were growth restricted.

Research has well established that being born growth restricted increases the risk of becoming obese in adulthood, with maternal obesity being associated with altered placental function and adverse fetal and maternal outcomes (Hediger *et al.*, 1998; Parsons *et al.*, 2001; Boney *et al.*, 2005; Catalano & Ehrenberg, 2006). Maternal obesity in mice increases placental GLUT1 expression and glucose clearance (Jones *et al.*, 2009). Placental SNAT2 and SNAT4 expression and activity are also increased in mouse models of obesity due to high-fat feeding (Jones *et al.*, 2009) and a cafeteria diet (Gaccioli *et al.*, 2013), changes of which are likely due to increased MTORC1 activation (Jansson *et al.*, 2012; Gaccioli *et al.*, 2013) resulting in the increased fetal weight. The benefits of exercise on general health and as an obesity preventative are well known (Ross *et al.*, 2000; Brett *et al.*, 2015). However, limited research has characterized the impact exercise has on pregnancy outcomes complicated by maternal growth restriction and a second hit of maternal high-fat feeding. Furthermore, it is unknown if exercise initiated for the first-time during pregnancy is beneficial or detrimental for the developing fetus.

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We recently demonstrated that the insulin-like growth factor system (IGF-system) is dysregulated in placentae from F2 offspring of mothers born growth restricted (Mangwiro *et al.*, 2018). A key finding of this study was that maternal exercise initiated before and continued pregnancy throughout (*Exercise*), but not exercise initiated in the final two thirds of pregnancy (*PregEx*), increased fetal weight (Mangwiro *et al.*, 2018). Importantly, mRNA and protein regulation of the IGF-system were significantly altered with *PregEx*, but not *Exercise*, suggesting a placental adaptive response to alterations in the maternal metabolic system (Mangwiro *et al.*, 2018). As previous studies have demonstrated that maternal metabolic status influences the placental IGF-system and nutrient transportation (Fowden *et al.*, 2009), it is likely that the placental nutrients transporters will similarly be dysregulated as per the IGF-system in our model.

Therefore, in the present study we first aimed to characterize the placental nutrient transporter and mTOR changes in the rat labyrinth zone of F2 fetuses from mothers that were growth restricted at birth and the period of exercise initiation (Prior to (*Exercise*) or during pregnancy (*PregEx*)) that is most beneficial in preventing these alterations. We next aimed to determine whether maternal high-fat feeding exacerbated any alterations in nutrient transporter expression within each exercise group. Finally, we determined any sex-specific responses within each experimental group, as male and female offspring respond differently to the same *in utero* environment (Di Renzo *et al.*, 2007).

Materials and Methods

Ethical approval and animals

All experiments were approved by The University of Melbourne's animal experimentation ethics sub-committee (AEC: 1212639) following the National Health and Medical Research Councils (NHMRC) Australian code for the care and use of animals for scientific purposes.

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The authors understand the ethical principles under which The Journal of Physiology operates and confirm that this work meets the standards of the journal's animal ethics checklist.

Eight week old female Wistar-Kyoto (WKY) rats were acquired from the biological resource facility at the University of Melbourne and provided with standard rat chow and water *ad libitum.* Throughout the duration of the study, all rats were housed under environmentally controlled conditions (19-22°C) with a 12 h light–dark cycle. Female rats were mated overnight with normal males and underwent uteroplacental insufficiency surgery on day 18 of gestation (term = 22 days) as described previously (Wlodek *et al.*, 2005). The protocol for this animal work is consistent with current guidelines in the field (Dickinson *et al.*, 2016; Morrison *et al.*, 2018). Briefly, F0 female rats were anaesthetized with 4% isoflurane and 650 $ml.min^{-1}$ oxygen flow (reduced to 3.2% isoflurane and 250 $ml.min^{-1}$ oxygen flow when suturing to aid in the animal's recovery). Uteroplacental insufficiency was then induced by bilateral uterine vessel ligation (offspring termed *Restricted*) or sham (offspring termed *Control*) surgery and dams were allowed to deliver naturally at term. F1 *Control* and *Restricted* females were weaned from their mothers on postnatal day 35 (PN35) and were randomly allocated to either a Chow (AIN93G; Specialty Feeds, Glen Forrest, WA, Australia) or a selection of two High-fat diets (SF03-020 and SF01-028; Specialty Feeds) that were matched for micro- and macronutrients. At 16 weeks, F1 females where further randomly allocated to one of the following exercise groups: *Sedentary*, exercised before and during pregnancy (*Exercise*; from 16 to 24 weeks of age) or exercised only during pregnancy (*PregEx*; Sedentary prior to mating and in the first week of pregnancy, then exercised from E7 to E19). At 20 weeks of age, F1 females were mated with normal males (Mangwiro *et al.*, 2018). For an overview of animal allocations with sample sizes please refer to Figure 1.

Exercise training

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For the entirety of their exercise regime, F1 females exercised 5 days/week on a motorized treadmill followed by 2 days of rest (Columbus Instruments, Columbus, OH, USA) and were encouraged to run by blowing compressed air near the base of their tail. On the first day of training, rats allocated to the *Exercise* group ran for 20 mins at 15m/min, with an additional 10 min per day applied on each subsequent day until on *day 5 of week 1* the rats were exercised for 60 mins. On *day 1 of week 2* and thereafter until mating, the rats exercised for 60 min/day at 20 m/min, as previously described (Laker *et al.*, 2011; Laker *et al.*, 2012; Wadley *et al.*, 2016; Asif *et al.*, 2017; Mangwiro *et al.*, 2018). The day after mating, for *week 1 of pregnancy* rats were exercised for 50 mins at 17 m/min, for *week 2 of pregnancy* they exercised for 30 mins at 13m/min and for *week 3 of pregnancy* they exercised for 20 mins at 11 m/min. Females allocated to the *PregEx* group remained Sedentary prior to mating and for the first week of pregnancy, and underwent exercise from *week 2 of pregnancy* as per the *Exercise* group. *Sedentary* rats were placed on a stationary treadmill for the same duration as the exercising rats. The exercise protocol is presented in a schematic in our recent publication (Mangwiro *et al.*, 2018).

Post-mortem

At E20, F1 females were anesthetized (100 mg/kg Ketamine- (Parnell Laboratories Pty Ltd, Alexandria, NSW, Australia) and 30 mg/kg Ilium Xylazil (Troy Laboratories Pty Ltd, Smithfield, NSW, Australia)) and their uterus exposed. F2 fetuses were weighed, sexed (visual inspection of the ano-genital distance) and killed by decapitation. Fetal tails were collected to verify fetal sex by qPCR of the sex-determining region Y (SRY) using a commercially available Taqman probe (Rn04224592_u1; NM_012772.1) (Life Technologies; Scoresby, VIC, Australia) as previously described (Cuffe *et al.*, 2012; Mangwiro *et al.*, 2018). The placentae were excised, weighed and fixed whole in 10% neutral buffered formalin or separated into regions (labyrinth and junctional zones) and frozen immediately in liquid nitrogen and stored at -80°C.Placentae associated with one male and one female from

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each litter were chosen for analyses, with each sample representing a single animal (i.e. n = 1). The dam was then killed by cardiac puncture.

Placental Morphology

Fixed placentae were processed into paraffin blocks, sectioned (5μm) and stained with haematoxylin and eosin (n = 3 - 4 dams/group with 1 male and female analysed/dam). Five sections per placenta were analysed for glycogen cells cross-sectional area using the Aperio ScanScope system (Aperio Technologies, Vista, CA, USA) and Image Scope software (Leica Microsystems, Mt Waverly, VIC, Australia), as described previously (Gardebjer *et al.*, 2014b).

Placental gene abundance

RNA was extracted from 50 mg placental labyrinth using a commercially available kit (miRNA easy mini kit; Qiagen, Chadstone, VIC, Australia) and the Precellys 24 homogenizer (Bertin Technologies; Aix en Provence, France) with CK14 ceramic beads, as previously described (Cheong *et al.*, 2016a; Mangwiro *et al.*, 2018). First strand cDNA was generated from 1 μg RNA using the High Capacity cDNA kit (Life Technologies, Mulgrave, VIC, Australia). qPCR was then conducted using Taqman master mix (Life Technologies), in line with the MIQE guidelines (Bustin *et al.*, 2009). PCR primers where purchased for *Mtor* and the following nutrient transporter genes of interest (Life Technologies); *Slc2a*1 (Rn01417099_m1; NM_138827.1)*, Slc2a*3 (Rn00567331_m1; NM_017102.2), *Slc5a*1 (Rn01640634_m1; NM_013033.2), *Slc38a*1 (Rn00593696_m1; NM_138832.1), *Slc38a*2 (Rn00710421_m1; NM_181090.2), *Slc38a*4 (Rn00590667_m1; NM_130748.1) and *Mtor* (Rn00693900_m1; NM_019906.1). mRNA abundance of the genes of interest were normalized to the geometric mean of TATA box binding protein (*Tbp*, Rn01455646_m1; NM_001004198.1) and β Actin (*Actb*, Rn00667869_m1; NM_031144.3) to compensate for

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variations in RNA input amounts and reverse transcriptase efficiency;. HotStart DNA Taq Polymerase was activated by heating the mixture to 95°C for 10 mins, then qPCR reactions were run for 40 cycles of 95°C for 15 sec and 60°C for 60 seconds. Relative changes in mRNA abundance was quantified using the $2^{\Delta\Delta CT}$ method and reported in arbitrary units normalized to *Control Sedentary* Chow male values. *Tbp* and *Actb* were not different between Treatments (maternal birth weights), Exercises, Diets or Sexes.

Protein extraction and Western blot analysis

Protein was extracted from 50 mg placental labyrinth tissue using RIPA buffer (Cuffe *et al.*, 2011; Mangwiro *et al.*, 2018). 20 µg of lysate was loaded onto a 4-15% Tris-Glycine extended (TGX) Stain-Free gel (Bio-Rad Laboratories; Gladesville, NSW, Australia) for Western blotting (Mangwiro *et al.*, 2018). Nitrocellulose membranes were probed with antibodies against GLUT3 (1:1000, Santa Cruz Biotechnology; Dallas, Texas, USA), MTOR (1:1000, Cell Signaling Technology, Arundel, QLD, Australia) and pMTOR (Ser2448) (1:1000, Cell Signaling Technology). Densitometric analysis was performed using a ChemiDoc MP with ImageLab Software (Bio-Rad Laboratories). Protein expression of interest was normalized relative to Stain-Free total protein (Parviainen *et al.*, 2013; Mangwiro *et al.*, 2018) and expressed as values relative to *Control Sedentary* Chow males. All gels contained a *Control Sedentary* Chow male sample for normalization (Mangwiro *et al.*, 2018). To determine alterations in mTOR activation the pMTOR (Ser2448)-to-total MTOR ratio was calculated.

Statistical analysis

As described previously (Mangwiro *et al.*, 2018), a two-way analysis of variance (ANOVA) was first conducted to identify differences between Treatment (maternal birth weight) and Exercise within each Diet and Sex. If a main Exercise effect was present, a one-way ANOVA

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with a Duncan's post-hoc test was used to identify Exercise differences. If an interaction was observed, the data was further split to identify Treatment (maternal birth weight) effects within each Exercise regime using a Student's unpaired t-test and a one-way ANOVA determined Exercise effects within *Control* and *Restricted* groups. To determine any differences between Diets, the data was split by Sex and Exercise and a two-way ANOVA conducted to report main Diet effects within each exercise regime. To identify any sex-specific differences, a Student's unpaired t-tests was used to determine differences between male and female associated placentae within each experimental group. As there were minimal Diet- and Sex-specific effects, we will only draw reference to major changes of importance in the results. Our statistical approach is consistent with current guidelines in the field (Dickinson *et al.*, 2016; Morrison *et al.*, 2018) and our recent publication (Mangwiro *et al.*, 2018). ANOVA statistical analysis was performed using SPSS Statistics 22 (IBM; St Leonards, NSW, Australia) and Student's unpaired t-tests were performed using Excel (Microsoft; North Ryde, NSW, Australia). All data are presented as means ± SEM and statistical significance was set at $P < 0.05$.

Results

Placental mTOR expression

Male associated placentae: Maternal growth restriction (*Restriction*) in *Sedentary* and *Exercised* dams increased *Mtor* mRNA abundance in male associated placentae if their mother consumed a High-fat, but not Chow, diet (Table 1; Student's unpaired t-test, $P < 0.02$) compared to respective *Controls*. In Chow-fed dams, *Exercise* increased total MTOR protein expression whereas *PregEx* decreased total MTOR protein expression in *Restricted* dams (Figure 2A) compared to *Restricted Sedentary* dams. Despite these exercise-specific alterations in total MTOR protein expression, *Restriction* increased pMTOR (Ser2448) protein expression regardless of maternal exercise regime (Figure 2A). However, no maternal birth weight effects were observed in mTOR activation (Figure 2B).

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In Chow-fed dams, *Exercise* increased *Mtor* mRNA abundance in male associated placentae compared to *Sedentary* mothers (Table 1; one-way ANOVA). Whereas in *Restricted* High-fat fed dams, *Mtor* mRNA abundance was increased with *Exercise* and reduced with *PregEx* compared to *Restricted Sedentary* mothers (Table 1; one-way ANOVA). *PregEx* reduced total MTOR protein expression in Chow (*Restricted* only) and High-fat fed dams compared to *Sedentary* (Figure 2A; one-way ANOVA). Whereas, *Exercise* and *PregEx* reduced pMTOR (Ser2448) protein expression only in High-fat fed dams compared to *Sedentary* (Figure 2A; one-way ANOVA); changes of which were largely driven by the *Restricted* groups (-19% vs. -72% for *Exercise* and -89% vs. -80% for *PregEx* in Control and Restricted groups, respectively). Interestingly, *PregEx* in Chow and High-fat fed mothers increased mTOR activation in male associated placentae compared to *Sedentary* (Figure 2B; one-way ANOVA).

Female associated placentae: Mtor gene abundance was increased in female associated placentae whose *Restricted* mother consumed a High-fat, but not Chow, diet (Table 1; one-way ANOVA), which was largely driven by the increase in the *Sedentary* (+141%) and *Exercise* (+58%) groups. No maternal birth weight effects were identified in total MTOR and pMTOR (Ser2448) protein expression or mTOR activation (Figure 2C and 2D).

Compared to *Sedentary* dams, *PregEx* reduced *Mtor* mRNA abundance in female associated placentae whose mother consumed a High-fat, but not Chow, diet (Table 1; one-way ANOVA). Total MTOR protein expression was reduced with maternal *Exercise* (High-fat only) and *PregEx* in female associated placentae (Figure 2C; one-way ANOVA). Whereas, *Exercise* and *PregEx* reduced pMTOR (Ser2448) protein expression in Chow-fed mothers compared to *Sedentary* (Figure 2C; one-way ANOVA); changes of which were largely driven by the *Control* groups (-47% vs. -37% for *Exercise* and -72% vs. -58% for *PregEx* in *Control* and *Restricted* groups, respectively). In High-fat mothers, *PregEx* alone reduced pMTOR (Ser2448) protein expression compared to *Sedentary* (Figure 2C; one-way ANOVA). No maternal exercise effects were observed on mTOR activation (Figure 2D).

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Placental System A transporters

Male associated placentae: Maternal growth restriction increased *Slc38a*1 (*Sedentary* and *Exercise*) and *Slc38a*2 (*Exercise*) mRNA abundance in the High-fat, but not Chow, fed mothers compared to *Control* counterparts in males (Figures 3A and 3B; Student′s unpaired t-test, $P < 0.0002$). No maternal growth restriction effects were observed in *Slc38a*4 mRNA abundance (Figure 3C).

Exercise in Chow-fed mothers increased *Slc38a*1 and *Slc38a*2 mRNA abundance (Figures 3A and 3B; one-way ANOVA), whereas *PregEx* reduced *Slc38a*4 mRNA (Figure 3C, one-way ANOVA) in male associated placentae compared to *Sedentary*. In *Restricted* High-fat fed dams *Exercise* increased and *PregEx* decreased *Slc38a*1 and *Slc38a*2 mRNA abundance compared to *Restricted Sedentary* mothers (Figures 3A and 3B; one-way ANOVA). Additionally, *PregEx* in *Control* High-fat fed dams reduced *Slc38a*1 and *Slc38a*2 mRNA compared to *Sedentary Control* mothers (Figure 3B; one-way ANOVA). *Exercise* and *PregEx* in High-fat fed dams reduced *Slc38a*4 mRNA abundance in male associated placentae compared to *Sedentary* (Figure 3C; one-way ANOVA).

*Female associated placentae: Slc38a*1 and *Slc38a*2 mRNA abundance was increased in female associated placenta from *Restricted* High-fat, but not Chow, fed mothers compared to *Control* mothers (Figures 3D and 3E; two-way ANOVA), which was largely driven by increased *Slc38a*1 gene abundance in *Sedentary* (+179%) and *Exercise* (+41%) dams and increased *Slc38a*2 gene abundance in the *Sedentary* (+95%) and *PregEx* (+73%) dams. Whereas, *Slc38a*4 mRNA abundance were reduced in *Restricted* High-fat, but not Chow, fed mothers that *Exercised* compared to *Exercised Control* mothers (Figure 3F; Student′s unpaired t-test, $P = 0.02$).

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No exercise effects were observed in *Slc38a*1*, Slc38a*2 or *Slc38a*4 mRNA abundance, compared to *Sedentary*, in female associated placentae from Chow-fed mothers (Figure 3). In High-fat fed mothers, *PregEx* reduced *Slc38a*1 and *Slc38a*2 mRNA abundance compared to *Sedentary* (Figures 3D and 3E; one-way ANOVA). Neither *Exercise* nor *PregEx* altered *Slc38a*4 mRNA abundance, compared to *Sedentary*, in High-fat mothers (Figure 3F).

Placental glucose transporters

Male associated placentae: No maternal birth weight effects were characterized in *Slc2a*1, *Slc2a*3 and *Slc5a*1 mRNA abundance (Figure 4A and Table 1) or GLUT3 protein expression (Figure 4B) in male associated placentae on either diet.

No maternal exercise effects were reported in *Slc2a*1 (Figure 4A) and *Slc5a*1 (Table 1) mRNA abundance in male associated placentae. However, *PregEx* in Chow, but not High-fat, fed mothers increased *Slc2a*3 mRNA abundance (Table 1; one-way ANOVA) and GLUT3 protein expression (Figure 4B; one-way ANOVA) compared to *Sedentary* mothers.

Female associated placentae: No maternal birth weight effects were observed in *Slc2a*1 (Figure 4C) and *Slc5a*1 (Table 1) mRNA abundance in female associated placentae. Maternal growth restriction increased *Slc2a*3 mRNA abundance in *Sedentary* High-fat, but not Chow, fed dams compared to *Sedentary Control* (Table 1; Student′s t-test, p=0.020), however this did not translate to alterations in GLUT3 protein expression (Figure 4D).

No maternal exercise effects were reported in *Slc2a*1 (Figure 4C) and *Slc5a*1 (Table 1) mRNA abundance. *PregEx* (Chow only) and *Exercise* (High-fat only) increased *Slc2a*3 mRNA abundance in *Control*, but not *Restricted*, dams compared to *Control Sedentary* (Table 1; one-way ANOVA). However, these alterations in *Slc2a*3 mRNA abundance did not translate to alterations in GLUT3 protein expression (Figure 4D).

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Placental glycogen cell cross-sectional area

Male associated placentae: No maternal birth weight effects were reported in placental glycogen cross-sectional area in male associated placentae (Figure 5A). However, *PregEx* in High-fat, but not Chow, fed dams increased glycogen cell cross-sectional area in male associated placentae compared to *Sedentary* (Figure 5A; one-way ANOVA).

Female associated placentae: No maternal birth weight or exercise effects were observed in glycogen cross-sectional area in female associated placentae (Figure 5B); despite an ~84% increase in glycogen cross-sectional area in *Restricted* dams that *Exercised* compared to *Control* dams that *Exercised* on either diet.

Discussion

This study has, for the first time, demonstrated that nutrient transporter expression in the placental labyrinth is independently altered by maternal birth weight and exercise, outcomes of which are dependent on fetal sex and the maternal diet. We have recently demonstrated, in the same model, that the F2 disease transmission associated with mothers born growth restricted (Cheong *et al.*, 2016a) may be partly due to alterations in the placental IGF-system (Mangwiro *et al.*, 2018). Here we demonstrate a potential role of the placental nutrient transport system in this disease transmission and propose that disease programming outcomes may be as a result of a complex interplay between placental nutrient transportation and growth factor signaling. Although exercise initiated prior to pregnancy (*Exercise*) is beneficial for maternal and fetal health, the results of our current and previous (Mangwiro *et al.*, 2018) studies highlight that any benefits of exercise may be independent of alterations in placental IGF-signaling and/or nutrient transportation. In contrast, we identified a number of placental alterations that occurred in response to exercise initiated during pregnancy (*PregEx*) independent of maternal birth weight. Although in the current study we were unable to demonstrate that maternal high-fat feeding exacerbates placental outcomes in mothers that

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were growth restricted, placental nutrient transporters and mTOR responded differentially depending on the maternal diet, suggesting that maternal growth restriction, exercise, diet and sex have independent effects on this placental system that may interact to impact overall outcomes.

Impact of F1 maternal growth restriction prior to birth

Placental nutrient transporter expression alters throughout pregnancy, which increase in abundance towards term, due to maternal insulin resistance, to facilitate increased nutrient transfer to the growing fetus (Hay, 2006). Jansson and Powell have extensively studied and reviewed the extent to which nutrient transporters are altered in pregnancies complicated by IUGR and maternal obesity (Jansson *et al.*, 1993; Jansson *et al.*, 2006; Jansson & Powell, 2006; Jones *et al.*, 2009). In brief, growth restriction in F1 placentae is associated with a myriad of changes in placental nutrient transporter protein expression and gene abundance (Jansson & Powell, 2000, 2006). These changes are, however, pertaining to programming in the F1 placenta that is directly affected by IUGR. This study was the first to characterize alterations in placental nutrient transporter expression in F2 placentae from F1 *Restricted* mothers. To our surprise, we report minimal changes in nutrient transporters in male and female associated placentae of F1 *Restricted* mothers, despite our previous study reporting reductions in *Slc2a*1 and *Slc38a*2 gene abundance (Briffa *et al.*, 2017). This may be due to programming adaptations in the current model that did not occur previously or interactions between other factors, such as stress, which was investigated in the previous model (Briffa *et al.*, 2017). Nevertheless, with limited differences in nutrient transporter expression in placentae from *Restricted* mothers these data suggest that alterations in nutrient transportation are not likely the sole driver of the transgenerational programming of cardiometabolic disorders we have previously reported (Gallo *et al.*, 2012; Gallo *et al.*, 2013; Cheong *et al.*, 2016a).

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Previous studies have well established that maternal obesity increases the expression of glucose and system A amino acid transporters, which is associated with fetal macrosomia (Jones *et al.*, 2009; Jansson *et al.*, 2013) as fetal growth, particularly during late gestation, is dependent on amino acid supply from the mother. Consistent with this finding, we report in male and female associated placentae that high-fat feeding in *Restricted* mothers increases *Slc38a*1 (*Sedentary* and *Exercise* in males) and *Slc38a*2 (*Exercise* in males) gene abundance, with reductions in *Slc38a*4 gene abundance in female associated placentae whose mother *Exercised*. These alterations in amino acid transporters are likely in response to the increased nutrient availability associated with maternal high-fat feeding, which is aimed at increasing fetal growth.

Impact of F1 maternal exercise

To the best of our knowledge this is the first study to demonstrate the effects of maternal endurance exercise on mTOR and nutrient transporter expression in the placental labyrinth with diet- and sex-specific responses. One study to date has characterized that prenatal exercise in humans increased *Slc38a*2 gene abundance, with no changes in *Slc2a*1, *Slc38a*1, *Slc38a*4 or *Mtor* gene abundance (Brett *et al.*, 2015). In this previous study by Brett *et al*, maternal activity was not assessed prior to pregnancy, so it is unclear whether these effects are due to continuous exercise prior to and throughout pregnancy or during pregnancy only. Nevertheless, this finding is somewhat similar to our study, whereby *Exercise* increased *Slc38a*1 and *Slc38a*2 gene abundance in male associated placentae whose mother consumed a Chow and High-fat (only in *Restricted* mothers) diet and reduced *Slc38a*4 gene abundance in High-fat fed dams. This increased amino acid transportation may, in part, explain the increased male fetal weight in Chow-fed mothers that *Exercised* we previously reported (Mangwiro *et al.*, 2018). Interestingly, in female associated placentae no changes in nutrient transporter expression were observed with maternal *Exercise* apart from a reduction in MTORC1 expression in Chow-fed dams, which may in part be an adaption to prevent the fetal overgrowth we previously reported (Mangwiro *et al.*, 2018).

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In contrast to the subtle effects of *Exercise* on mTOR expression, *PregEx* had a consistent effect on reducing mTOR gene and protein expression. *PregEx* increased mTOR activation only in male associated placentae and reduced amino acid transporter expression. These effects highlight that if exercise is initiated during pregnancy placental function may be perturbed, which could have unfavourable consequences for the fetus; although further studies are required to characterize alterations to fetal organ development. In the current study, the reduction in total MTOR protein in both sexes, regardless of activation, is an indication of an altered nutrient sensing and regulating pathway. Exercise during pregnancy redirects oxygen and nutrient outflow to the maternal system and, although this may be beneficial for maternal outcomes, fetal growth is compromised (Clapp, 2003). Therefore, these data likely suggest that *PregEx* reduces total MTOR protein expression due to reduced oxygen content, which is known to modulate MTORC1 activity (Yung *et al.*, 2012; Vaughan *et al.*, 2015; Capobianco *et al.*, 2016). Although the current study did not result in reduced fetal weight (Mangwiro *et al.*, 2018) it is evident that the *PregEx* creates a complex interplay between the mTOR pathway and nutrient transporter expression that may result in a reduction in fetal and placental amino acid availability, which may consequently alter fetal outcomes such as body composition and term birth weight. The introduction of endurance exercise during the second week of pregnancy may negatively alter maternal nutrient resource allocation, favouring the mothers need to meet skeletal muscle energy demands rather than the fetoplacental unit for upregulated amino acid transport aimed to maintain fetal growth. In addition, insulin sensitivity is known to be enhanced by endurance exercise and in the process also reduces insulin secretion (Calegari *et al.*, 2011). Thus, *PregEx* in particular may result in a reduction in placental insulin receptor mediated mTOR activation as a consequence of increased peripheral insulin sensitivity in the mother.

The effects of *PregEx* on glucose transport and storage were less overt than the effects on amino acid transport. The increased GLUT3 protein expression in male associated placentae

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from Chow-fed mothers suggests an adaptation to increase placental glucose uptake in a high energy demanding environment to facilitate normal fetal growth (Mangwiro *et al.*, 2018). Of interest, we only reported alterations in placental glycogen content in male associated placentae if their mother underwent *PregEx* and consumed a high-fat diet. This finding suggests an adaptation in these males to increase glycogen storage in response to a nutrient rich environment, which is likely in case any additional perturbation occurs to facilitate normal fetal growth. We have previously demonstrated in other animal models of programmed disease that increased glycogen accumulation is associated with changes in glucose transporter expression within the junctional zone of the placenta (Gardebjer *et al.*, 2014b). As glycogen cells are located within the junctional zone, rather than the labyrinth zone examined in this study, further analysis should investigate alterations in glucose transporter expression within this region.

Conclusion

This study demonstrates that nutrient transporter and mTOR expression are differentially regulated in F2 placentae from *Restricted* mothers. Most importantly it is the first time mTOR has been reported to be dynamically altered by maternal exercise, fetal sex and the maternal diet in F2 placentae of mothers′ growth restricted prior to birth. The minimal changes reported in placentae from *Restricted* mothers suggest other pathways may be responsible for transmitting disease across generations, some of which we describe in our recent publication (Mangwiro *et al.*, 2018). Only male associated placentae from *Restricted* mothers have upregulated pMTOR protein expression, a change of which, in the absence of any other nutrient transporter changes, may be an adaptation to improve fetoplacental growth through other pathways such as placental vascular remodelling. In contrast, maternal exercise, particularly *PregEx*, was shown to have profound effects on placental nutrient transporter expression, regardless of maternal birth weight. Specifically, *PregEx* downregulated mTOR expression and system A transporter gene abundance, a potential mechanism to avoid fetal overgrowth. However, the exact effects these alterations in

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placental nutrient transport following maternal exercise have on offspring birth weight and long-term offspring health is unknown and requires further investigation.

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Competing Interests

The authors declare no conflicts of interest.

Author Contributions

M.E.W. and K.M.M. designed the study. Y.T.M.M., J.S.M.C., J.F.B. and S.G. performed all experiments. D.M. and K.A. performed the animal work, with assistance from Y.T.M.M. Y.T.M.M., J.S.M.C., J.F.B. and S.G. analysed the data. All authors participated in the interpretation of the results and contributed to writing the manuscript. All authors approved the submission of this version to the Journal of Physiology.

Acknowledgements

The authors would like to thank Mr Andrew Jefferies for his assistance with animal surgeries and post-mortems.

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Funding

This research was supported by the National Health and Medical Research Council (NHMRC) of Australia (M.E.W.; 1045602) and a 2013 Diabetes Australia Research Trust Research Project (M.E.W). J.F.B. holds a Faculty of Medicine, Dentistry and Health Science Postdoctoral Fellowship at the University of Melbourne. Y.T.M.M. holds and K.A. held a La Trobe University Post Graduate Award and D.M. held a Malaysia Government Scholarship.

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Figure legends

Figure 1: Study design and sample sizes

Flow chart of the experimental protocol indicating the allocation of pregnant rats within the different treatments, diet and exercise groups along with the sample size per experimental outcome. F1 = first generation; Exercise = exercise prior to pregnancy and continued throughout gestation; PregEx = exercise initiated during the second week of gestation; n = sample size; E = embryonic day; Wk = week.

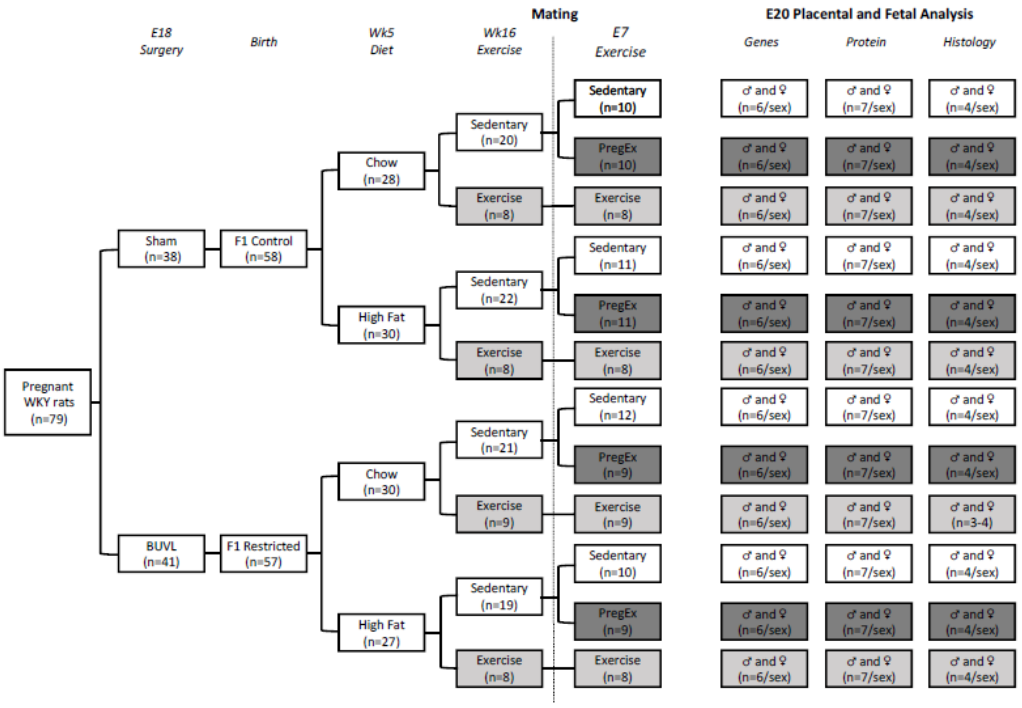


Figure 1

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Figure 2: Placental MTOR and pMTOR expression

Total and pMTOR protein expression (A and C) and pMTOR/total MTOR ratio (B and D) in male and female associated placentae whose mothers were *Control* (C; open bars) or *Restricted* (R; black bars) that consumed a Chow (left panel) or High-fat diet (right panel). Data were analysed by a two-way ANOVA identifying differences based on Treatment (maternal birth weight) and Exercise, and presented as mean ± SEM where 'ns' is not significant (n = 6 in each group/sex with n = 1 representing 1 pup from 1 litter). *p<0.05 vs *Control* and differences across exercises are denoted by different letters where 'a/A' is different to 'b/B', with lower case letters denoting *Control* and upper-case letters denoting *Restricted* dams.

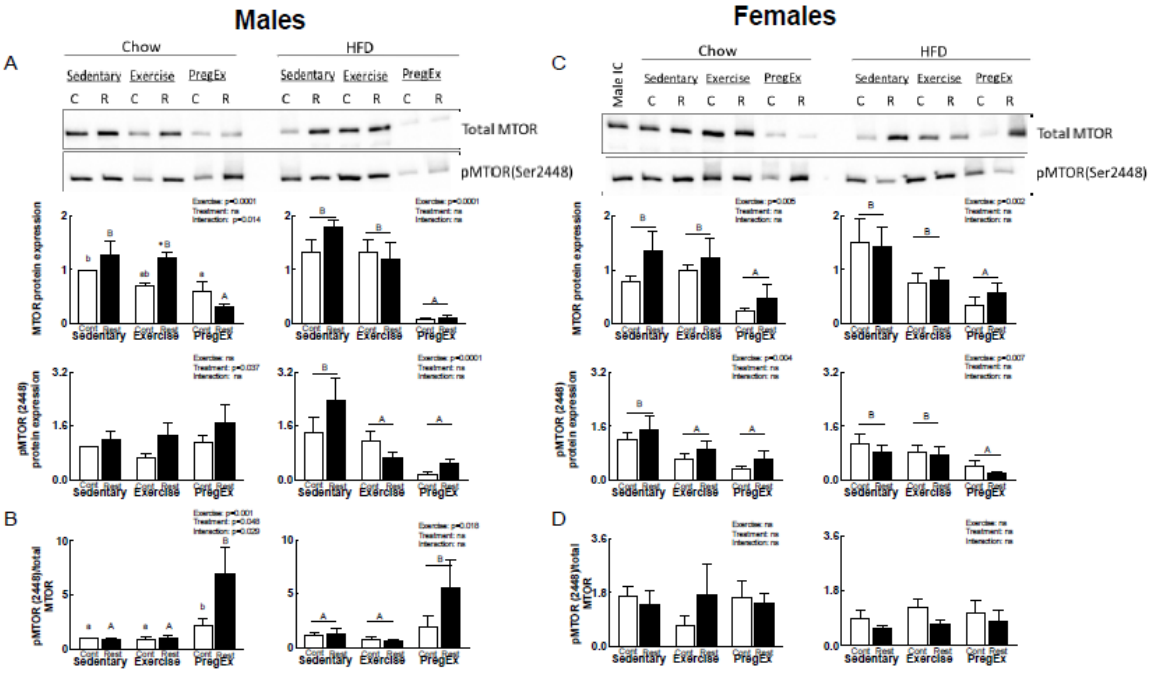


Figure 2

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Figure 3: Placental system A amino acid transporter expression

*Slc38a*1 (A and D), *Slc38a*2 (B and E) and *Slc38a*4 (C and F) mRNA abundance in male and female associated placentae whose mothers were *Control* (open bars) or *Restricted* (black bars) that consumed a Chow (left panel) or High-fat diet (right panel). Data were analysed by a two-way ANOVA identifying differences based on Treatment (maternal birth weight) and Exercise, and presented as mean ± SEM where 'ns' is not significant (n = 6 in each group/sex with n = 1 representing 1 pup from 1 litter). *p<0.05 vs *Control* and differences across exercises are denoted by different letters where 'a/A' is different to 'b/B' but not 'ab/AB', with lower case letters denoting *Control* and upper-case letters denoting *Restricted* dams.

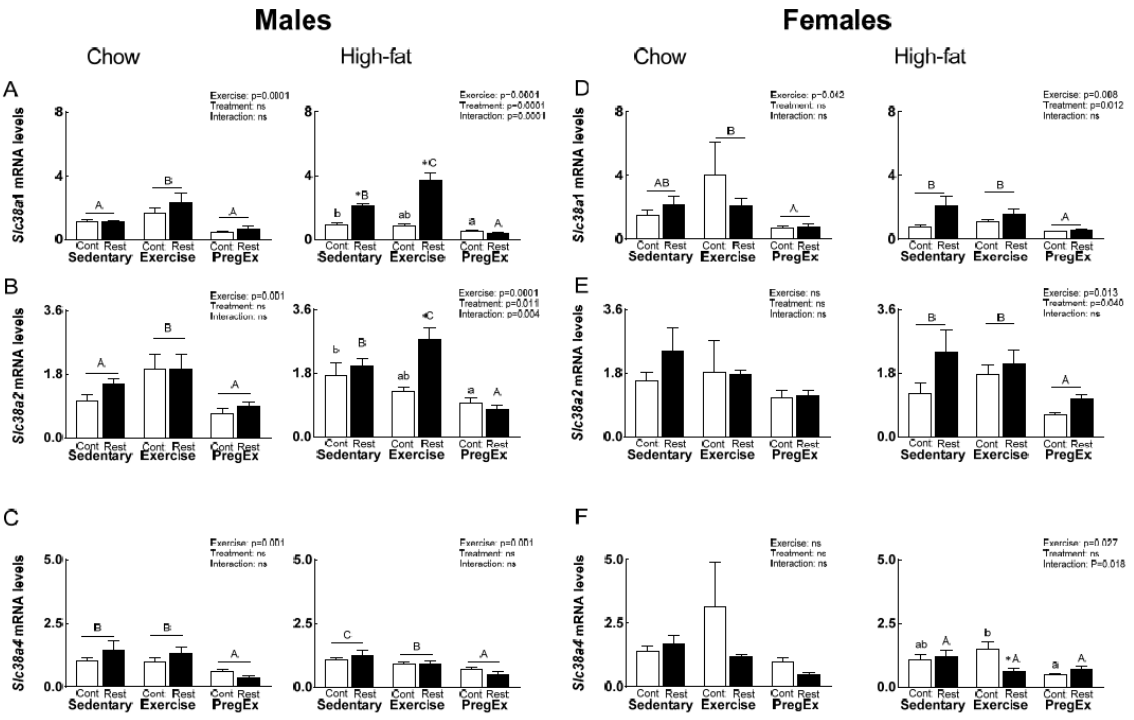


Figure 3

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Figure 4: Placental GLUT3 expression and glycogen cell cross-sectional area

*Slc2a*1 (A and C) mRNA abundance and GLUT3 protein expression (B and D) in male and female associated placentae whose mothers were *Control* (C; open bars) or *Restricted* (R; black bars) that consumed a Chow (left panel) or High-fat diet (right panel). Data were analysed by a two-way ANOVA identifying differences based on Treatment (maternal birth weight) and Exercise, and presented as mean ± SEM where 'ns' is not significant (n = 6-7 in each group/sex with n = 1 representing 1 pup from 1 litter). Differences across exercises are denoted by different letters where 'A' is different to 'B'.

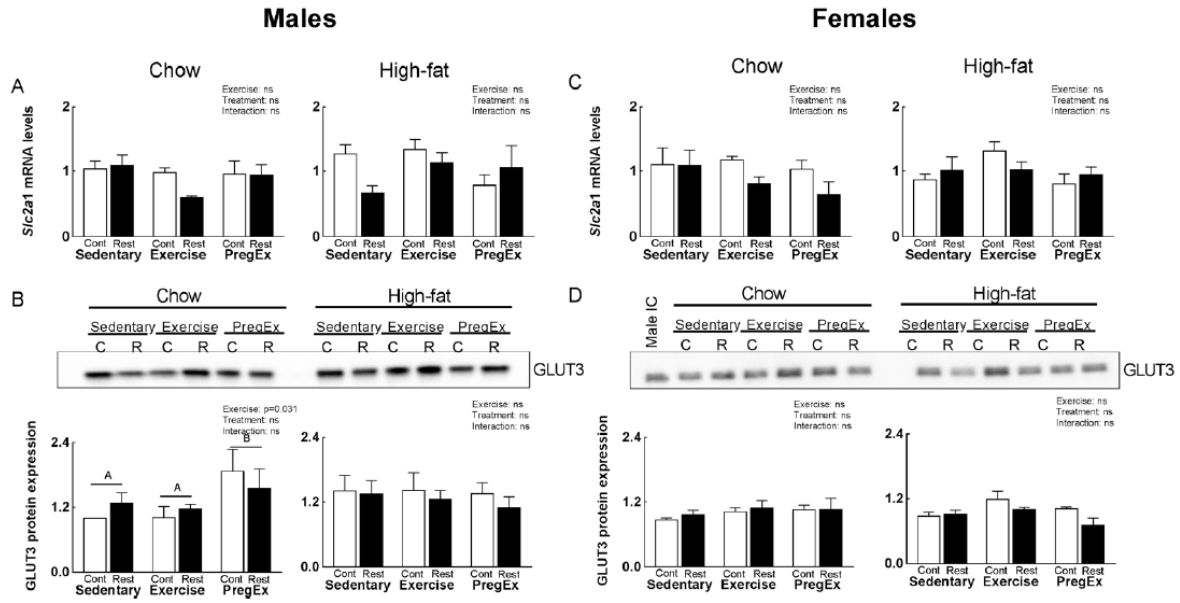


Figure 4

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Figure 5: Placental glycogen cell cross-sectional area

Glycogen cells cross-sectional area in male (A) and female (B) associated placentae whose mothers were *Control* (open bars) or *Restricted* (black bars) that consumed a Chow (left panel) or High-fat diet (right panel). Data were analysed by a two-way ANOVA identifying differences based on Treatment (maternal birth weight) and Exercise, and presented as mean ± SEM where 'ns' is not significant (n = 3-4 in each group/sex with n = 1 representing 1 pup from 1 litter). Differences across exercises are denoted by different letters where 'A' is different to 'B' but not different to 'AB'.

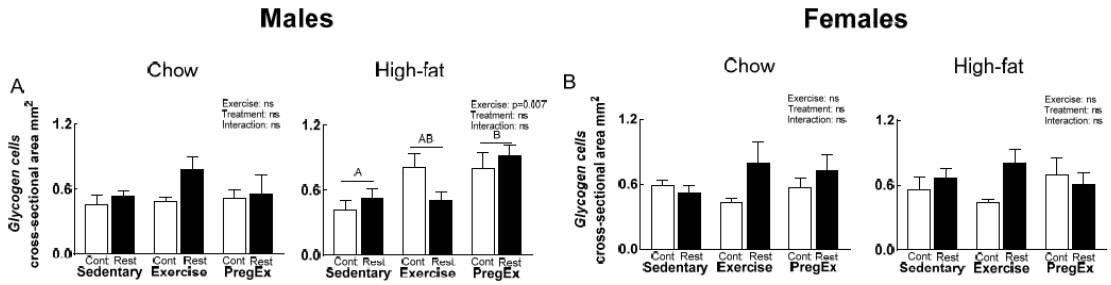


Figure 5

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Table 1. *Mtor*, *Slc2a*3 and *Slc5a*1 mRNA abundance in male and female associated placentae from *Control* and *Restricted* mothers on a Chow or High-fat diet at E20 (n=6 in each group/sex n = 1 representing 1 pup from 1 litter). Data were analysed by a two-way ANOVA identifying differences based on Treatment (maternal birth weight) and Exercise and presented as mean ± SEM, where 'NS' is not significant. *p<0.05 vs *Control* and differences across exercises are denoted by different letters where 'a/A' is different to 'b/B' but not 'ab/AB', with lower case letters denoting *Control* and upper-case letters denoting *Restricted* dams.

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Gene *Motor*			Sedentary		Exercise		PregEx		Two-way ANOVA: Treatment	Two-way ANOVA: Exercise	Two-way ANOVA: Interaction
Male	Chow	Control	1.06 ± 0.15	**A**	1.64 ± 0.27	**B**	0.44 ± 0.06	**A**	NS	**p=0.0001**	NS
		Restricted	1.05 ± 0.08		2.32 ± 0.54		0.64 ± 0.13				
	High-fat	Control	1.23 ± 0.30	**a**	0.74 ± 0.13	**a**	0.60 ± 0.10	**a**	**p=0.0001**	**p=0.0001**	**p=0.0001**
		Restricted	2.23 ± 0.22*	**B**	3.22 ± 0.44*	**C**	0.46 ± 0.06	**A**			
Female	Chow	Control	1.45 ± 0.29	**A**	2.91 ± 1.27	**B**	0.73 ± 0.19	**A**	NS	**p=0.018**	NS
		Restricted	1.75 ± 0.39	**B**	2.10 ± 0.40		0.73 ± 0.14				
	High-fat	Control	0.88 ± 0.14	**B**	1.02 ±	**B**	0.43 ±	**A**	**p=0.009**	**p=0.003**	NS

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					0.13		0.04				
		Restricted	2.12 ± 0.48		1.61 ± 0.42		0.52 ± 0.03				
Slc2a3											
Male	Chow	Control	1.09 ± 0.22		1.47 ± 0.17		1.99 ± 0.34				
				A		**AB**		**B**	NS	**p=0.047**	NS
		Restricted	1.21 ± 0.20		0.97 ± 0.12		1.35 ± 0.19				
	High-fat	Control	1.44 ± 0.31		1.59 ± 0.14		1.24 ± 0.23				
									NS	NS	NS
		Restricted	0.99 ± 0.09		0.94 ± 0.15		1.45 ± 0.10				
Female	Chow	Control	1.14 ± 0.15	**a**	1.61 ± 0.16	**ab**	1.76 ± 0.20	**b**			
									NS	NS	**p=0.040**
		Restricted	1.63 ± 0.30	**A**	1.23 ± 0.19	**A**	1.16 ± 0.24	**A**			

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	High-fat	Control	0.65 ± 0.14	**a**	1.39 ± 0.12	**b**	1.16 ± 0.27	**ab**			
		Restricted	1.43 ± 0.25*	**A B**	1.09 ± 0.20	**A**	1.93 ± 0.30	**B**	NS	**p=0.030**	**p=0.030**
Slc5a1											
Male	Chow	Control	1.31 ± 0.32		1.12 ± 0.41		0.21 ± 0.03				
		Restricted	0.49 ± 0.19		2.10 ± 1.15		0.17 ± 0.05		NS	NS	NS
	High-fat	Control	0.16 ± 0.08		0.21 ± 0.05		0.91 ± 0.42				
		Restricted	1.46 ± 0.75		0.25 ± 0.10		0.32 ± 0.16		NS	NS	NS
Female	Chow	Control	1.94 ± 0.95		1.15 ± 0.41		0.14 ± 0.03				
		Restric	0.80 ±		1.39 ±		0.75 ±		NS	NS	NS

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	ted	0.29	0.72	0.26			
High-fat	Control	0.67 ± 0.30	1.59 ± 0.96	0.31 ± 0.13	NS	NS	NS
	Restricted	1.60 ± 0.64	0.68 ± 0.22	0.38 ± 0.17			

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