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Maternal exercise in rats upregulates the placental IGF-system with diet and sex-specific responses: minimal effects in mothers born growth restricted

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Running title - Placenta IGF-system following maternal exercise

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Keywords – growth restriction, placenta, fetal programming, exercise, IGF-system

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Key points

- The placental IGF-system is critical for normal fetoplacental growth, which is dysregulated following several pregnancy perturbations including uteroplacental insufficiency and maternal obesity. We report that the IGF-system was altered in placentae of mothers born growth restricted compared to normal birth weight mothers, with maternal diet- and fetal sex-specific responses.
- Additionally, we report increased body weight and plasma IGF1 concentrations in fetuses from Chow-fed normal birth weight mothers who exercised prior to and continued during pregnancy compared to Sedentary.
- Exercise initiated during pregnancy, on the other hand, resulted in placental morphological alterations and increased IGF1 and IGF1R protein expression, which may in part be modulated by reduced *Let 7f-1* miRNA abundance.
- Growth restriction of mothers before birth and exercise differentially regulate the placental IGF-system with diet- and sex-specific responses, which likely aims to improve fetoplacental growth and development, hence neonatal survival. This increased neonatal survival may prevent adult disease onset.

Abstract

The insulin-like growth factor (IGF) system regulates fetoplacental growth and plays a role in disease programming. Dysregulation of the IGF-system is implicated in several pregnancy perturbations associated with altered fetal growth, including intrauterine growth restriction and maternal obesity. Limited human studies have demonstrated that maternal exercise enhances fetoplacental growth and decreases cord IGF ligands, which may restore the

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placental IGF-system in complicated pregnancies. This study investigated the impact maternal exercise has on the placental IGF-system in placentae from mothers born growth restricted and if these outcomes are 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 (20 weeks) with placentae associated with F2 fetuses collected at E20. The placental IGF-system mRNA abundance and placental morphology was altered in mothers born growth restricted. *Exercise* increased fetal weight and Control plasma IGF1 concentrations, and decreased female placental weight. *PregEx* did not influence fetoplacental growth but increased placental IGF1 and IGF1R (potentially modulated by reduced *Let 7f-1* miRNA) and decreased placental IGF2 protein. Importantly, these placental IGF-system changes occurred with sex-specific responses. These data highlight that exercise differently influences fetoplacental growth and the placental IGF-system depending on maternal exercise initiation, which may prevent the transgenerational transmission of deficits and dysfunction.

Abbreviations list

ANOVA, analysis of variance; BMI, body mass index; ELISA, enzyme-linked immunosorbent assay; E, embryonic day; *Exercise*, Exercise before and during pregnancy; IGF1, insulin-like growth factor 1; IGF2, insulin-like growth factor 2; IGF1R, insulin-like growth factor 1 receptor; IGF2R, insulin-like growth factor 2 receptor; IGFBP3, insulin-like growth binding protein 3; mTOR, mammalian target of rapamycin; NBF, neutral buffered formalin; PN, postnatal day; *PregEx*, Exercise during pregnancy only; qPCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; SRY, sex-determining region y; WKY, Wistar Kyoto; Wk, Week.

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Introduction

The insulin-like growth factor (IGF) system is involved in a myriad of physiological pathways that promote fetoplacental growth and development by the binding of IGF ligands (IGF1 and IGF2) to their receptors (IGF1R and IGF2R). Specifically, either ligand binding to IGF1R activates pathways that promote cellular proliferation, differentiation and survival. IGF2, on the other hand, has a higher affinity to bind to IGF2R, where it promotes placental growth and increases IGF2 clearance (Han *et al.*, 1996). The IGF-system also consists of IGF binding proteins (IGFBPs) that have a higher affinity of binding IGF ligands than IGF receptors, thus sequestering their ability to act on their receptors. Recent studies demonstrate that the IGF-system, specifically IGF1 and IGF1R, can be modulated by the action of *Let 7f-1* miRNA. Specifically, increased *Let 7f-1* miRNA abundance is associated with reduced IGF1 and IGF1R protein expression (Hu *et al.*, 2014). IGF-system knockout mouse models clearly demonstrate the essential role of this system in fetoplacental growth, whereby *Igf1*$^{-/-}$, *Igf2*$^{-/-}$ and *Igf1r*$^{-/-}$ mice have a 40-55% reduction in birth weight (DeChiara *et al.*, 1990; Baker *et al.*, 1993; Liu *et al.*, 1993; Woods *et al.*, 1996; Lupu *et al.*, 2001).

Uteroplacental insufficiency is the leading cause of fetal growth restriction in Western populations, and impairs oxygen and nutrient delivery to the growing fetus. It is therefore not surprising that dysregulation of the IGF-system has been implicated in the pathogenesis of fetal growth restriction. Clinically, growth restricted human fetuses have lower IGF1, IGF2 and IGFBP3 cord blood concentrations, along with elevated IGFBP1 concentrations (Langford *et al.*, 1994; Spencer *et al.*, 1995), which is likely due to reduced placental development (Koutsaki *et al.*, 2011). Similar findings are reported in experimental animal models of growth restriction, whereby fetal plasma IGF1 and tissue specific IGF1 concentrations are reduced in naturally occurring growth restricted rabbits (Thakur *et al.*, 2000) and in several models of growth restricted sheep (Bauer *et al.*, 1995; Kind *et al.*, 1995; de Vrijer *et al.*, 2006; Gentili *et al.*, 2009). These data clearly highlight that dysregulation of the placental IGF-system following pregnancy perturbations influence fetoplacental development. However, limited studies have characterized if similar changes in the placental

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IGF-system are observed in placentae associated with the second (F2) generation whose mother was born small, which may contribute to the F2 cardiometabolic dysfunction we have previously reported in our animal model of uteroplacental insufficiency (Gallo *et al.*, 2012; Gallo *et al.*, 2013).

Concerningly, 40% of pregnancies in Australia are complicated by maternal overweight/obesity that has negative effects on maternal and fetal health, which is in part due to its chronic inflammatory state. Of importance, individuals that were born growth restricted have an increased susceptibility to developing obesity (Cottrell & Ozanne, 2008). Maternal obesity increases the risk of pregnancy complications, including gestational diabetes mellitus that is similarly observed in growth restricted individuals, and can alter fetoplacental growth and development (Leddy *et al.*, 2008; Higgins *et al.*, 2011). Maternal obesity in humans reduces cord blood IGFBP1 and IGFBP3 concentrations and increases cord IGF2 concentrations and birth weight (Jansson *et al.*, 2008; Hoyo *et al.*, 2012), which may in part be due to increased nutrient availability. In the mouse, maternal consumption of a high-fat/high-sugar diet increases placental *Igf2* mRNA abundance, increases placental nutrient transportation, and alters placental morphology (Sferruzzi-Perri *et al.*, 2013; Rosario *et al.*, 2016). It is therefore possible that maternal obesity in growth restricted females may further compound alterations in the placental IGF-system.

Physical activity is associated with a number of positive health benefits including improved cardiovascular and metabolic health (Petersen & Pedersen, 2005; Bruun *et al.*, 2006; O'Gorman *et al.*, 2006). Exercise during pregnancy in humans improves maternal cardiometabolic outcomes, reducing the incidences of gestational diabetes mellitus and enhances fetoplacental growth (Clapp *et al.*, 2000; Clapp *et al.*, 2002; Clapp, 2006), which may in part be due to the IGF-system (Vega *et al.*, 2011). It is important to note, however, that research on the impact maternal exercise has on fetoplacental growth is contradictory with other studies reporting no change in birth weight (Hopkins *et al.*, 2010), which

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highlights the need for additional studies. Only one study to date has characterized the IGF-system following maternal exercise, which reported reduced cord blood concentrations of IGF-ligands (IGF1 and IGF2) (Hopkins *et al.*, 2010). It is, however, important to note that the impact of exercise on maternal health and fetoplacental outcomes are dependent on the intensity, duration and timing of exercise initiation. Despite limited evidence of maternal exercise altering the placental IGF-system there is a large body of evidence that demonstrates that exercise in non-pregnant individuals can modulate the IGF-system (Borer, 1995; Raastad *et al.*, 2000; Turgut *et al.*, 2006). Therefore, it is possible that maternal exercise may restore changes in the placental IGF-system associated with mothers born small and following consumption of a high-fat diet, which could break the transgenerational disease cycle.

Therefore, this study first aimed to determine changes in the placental IGF-system of F2 fetuses whose mother was born growth restricted and the period of exercise initiation (prior to or during pregnancy) that is most beneficial in preventing these alterations. We next aimed to determine whether maternal high-fat diet consumption exacerbated these changes in the placental IGF-system associated with mothers born growth restricted within each exercise group. As previous studies have demonstrated that male and female associated placentae respond differently to several pregnancy perturbations, we additionally characterized whether these responses were different in male and female associated placentae (Cuffe *et al.*, 2014; Gardebjer *et al.*, 2014; Cuffe *et al.*, 2017).

Materials and Methods

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. Female Wistar-Kyoto (WKY) rats (8 wks of age) were acquired from the biological resource

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facility at the University of Melbourne and were housed in an environmentally-controlled room (19-22°C) under a 12 h light–dark cycle with *ad libitum* access to standard rat chow and water. Rats were mated and surgery was performed on day 18 of gestation (term = 22 days) as described previously (Wlodek *et al.*, 2005). 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 animals recovery), with 0.125% bupivacaine administered to the skin and muscle layers prior to closure. Pregnant females were randomly allocated to undergo uteroplacental insufficiency surgery, by bilateral uterine vessel ligation, (offspring termed Restricted) or sham surgery (offspring termed Control) and dams were allowed to deliver naturally at term. At weaning, on postnatal day 35 (PN35), litter mate F1 normal birth weight (Control) and growth restricted (Restricted) female were randomly allocated to a Chow (AIN93G; Specialty Feeds, Glen Forrest, WA, Australia) or High-fat diet (SF03-020 and SF01-028; Specialty Feeds) that were matched for micro- and macronutrients. At 16 weeks, F1 female offspring were further randomly allocated to one of the following exercise regimes: remained Sedentary, exercised before and during pregnancy (*Exercise*; from 16 to 24 weeks of age) or exercised only during pregnancy (*PregEx*; Sedentary prior to 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 as illustrated in Figure 1A. All animals were generated concurrently.

Exercise training

The exercise regime is outlined in Figure 1B. Briefly, starting at 16 weeks of age, F1 females allocated to the *Exercise* group exercised for 5 days/week on a motorized treadmill (Columbus Instruments, Columbus, OH, USA) followed by 2 days of rest. On the first day of training, rats allocated to the *Exercise* group ran for 20 mins at a speed of 15m/min. Each subsequent day an additional 10 min per day was added to the running time 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 were exercised for 60 min/day at a speed of 20 m/min, as previously described (Laker *et al.*, 2011; Laker *et al.*, 2012; Wadley *et al.*, 2016; Asif *et al.*, 2018). The day after mating

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(on E1), for *week 1 of pregnancy* rats were exercised for 50 min/day at a speed of 17 m/min, for *week 2 of pregnancy* (on E8) rats were exercised for 30 min/day at a speed of 13m/min and for *week 3 of pregnancy* (on E15) rats were exercised for 20 min/day at a speed of 11 m/min. Females allocated to the *PregEx* group remained *Sedentary* prior to mating and for the first week of pregnancy, and underwent exercise for 5 days/week on a motorized treadmill followed by 2 days of rest from E8 at the same intensities and durations as per the *Exercise* group. *Sedentary* rats were placed on a stationary treadmill for the same duration as the exercising rats. Rats were encouraged to run by blowing compressed air near the base of their tail.

Post-mortem

At E20, F1 females were anesthetized with a 1:1 mixed solution of Ketamine (100 mg/kg; Parnell Laboratories; Alexandria, NSW, Australia) and Ilium Xylazil (30 mg/kg; Troy Laboratories; Smithfield, NSW, Australia) and their uterus exposed. F2 fetuses were weighted, sexed by visual inspection of the anogenital distance and were then killed by decapitation. Fetal plasma was collected and pooled into litters, with fetal tails collected to verify fetal sex. The placentae were excised, weighted and fixed whole in 10% neutral buffered formalin (NBF) or separated into labyrinth and junctional regions then frozen immediately in liquid nitrogen and stored at -80°C for subsequent analysis. For tissue analyses (morphology and gene/protein expression) placentae associated with one male and one female from each litter were chosen with a fetal and placental weight closest to the litter average, with each sample representing a single animal (i.e. n = 1). The dam was then killed by cardiac puncture. Fetal sex was confirmed by qPCR to determine the presence/absence of the sex-determining region Y (SRY) in DNA extracted from fetal tails using a commercially available Taqman probe (Rn04224592_u1; NM_012772.1) (Life Technologies; Scoresby, VIC, Australia) as previously described (Cuffe *et al.*, 2012).

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Placental Morphology

Fixed placentae were processed into paraffin blocks, sectioned at 5µm and stained with haemotoxylin and eosin (n = 3 - 4 dams/group with 1 male and female analyzed/dam). Five sections per placenta were analyzed for whole placental, labyrinth and junctional zone cross-sectional areas using the Aperio ScanScope system (Aperio Technologies, Vista, CA, USA) and Image Scope software (Leica Microsystems, Mt Waverly, VIC, Australia).

Placental gene abundance

RNA and miRNA were extracted from the placental labyrinth (nutrient transport) region using the Precellys 24 homogenizer (Bertin Technologies; Aix en Provence, France) with CK14 ceramic beads using a commercially available kit with on-column DNase digestion (miRNA easy mini kit; Qiagen, Chadstone, VIC, Australia) (Cheong *et al.*, 2016). First strand cDNA was generated from 1 µg of RNA using the High Capacity cDNA kit (for mRNA; Life Technologies) and the Taqman MicroRNA Reverse Transcription Kit (for miRNA; Life Technologies) according to the manufacturer's instructions. qPCR was then conducted using Taqman mastermix (Life Technologies). PCR primers were purchased from Life Technologies for the following IGF-system targets of interest; *Igf1* (Rn00710306_m1; NM_178866.4), *Igf2* (Rn01454518_m1; NM_031511.2), *Igf1r* (Rn00583837_m1; NM_052807.2), *Igf2r* (Rn01636937_m1; NM_012756.1) and *Igfbp3* (Rn00561416_m1; NM_012588.2) mRNA as well as *Let7f-1* miRNA (Mm04238181_s1; NR_029731.1). To compensate for variations in RNA input amounts and reverse transcriptase efficiency, mRNA and miRNA abundance of the genes of interest were normalized to the geometric mean of two reference RNA or miRNA genes; TATA box binding protein (*Tbp*, Rn01455646_m1; NM_001004198.1) and β Actin (*Actb*, Rn00667869_m1; NM_031144.3) were selected for mRNA and *191* miRNA (Hs04231511_s1; NR_029690.1) and *U6* snRNA (001973; NR_004394) were selected for *Let 7f-1* miRNA. 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 sec. Relative changes in mRNA and miRNA

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abundance was quantified using the $2^{\Delta\Delta CT}$ method and reported in arbitrary units normalized to Control Sedentary Chow male values. *Tbp*, *Actb, miRNA 191* and *U6 snRNA* were not different between Treatments, Diets, Exercises or Sexes.

Protein extraction and Western blot analysis

Protein was extracted from 50 mg of placental labyrinth tissue using RIPA buffer (Cuffe *et al.*, 2011). 20 µg of protein lysate was loaded onto a 4-15% Tris-Glycine extended (TGX) Stain-Free gel (Bio-Rad Laboratories; Gladesville, NSW, Australia) and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories) (Gardebjer *et al.*, 2014). As it was not possible to include all samples on an individual gel, multiple gels were run concurrently with cross gel calibrators included. The *PregEx* samples were ran on a separate gel (with a Sedentary Chow Control male sample used an absolute control). Membranes were probed with antibodies against IGF1 (1:1000, Abcam; Melbourne, VIC, Australia), IGF2 (1:1000, Abcam) or IGF1R (1:1000, Abcam). Densitometric analysis was performed using a ChemiDoc MP with ImageLab Software (Bio-Rad Laboratories). Protein expression was normalized relative to Stain-Free total protein in each well (Parviainen *et al.*, 2013) allowing us to control for all of our experimental conditions and expressed as values relative to Control Sedentary Chow males. All gels contained a Control Sedentary Chow male sample for normalization.

Fetal plasma IGF1 analysis

IGF1 concentrations in pooled fetal plasma were analyzed using an enzyme-linked immunosorbent assay (ELISA) following the manufacturers protocol (R&D Systems, Minneapolis, MN, USA) with a minimum detection limit of 3.5 pg/ml, and intra- and inter-assay coefficients of variation of 4.3% and 6.0%, respectively.

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Statistical analysis

A two-way ANOVA was first conducted to identify differences between Treatment and Exercise within each Diet and Sex. If a main Exercise effect was present, a one-way ANOVA 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 effects within each Exercise using a Student's unpaired t-test and a one-way ANOVA determined Exercise effects in Control and Restricted groups. To determine differences between Diets, the data was split by Sex and Exercise and a two-way ANOVA conducted to report main Diet effects within Treatments in each Exercise. To identify any Sex differences, a Student's unpaired t-tests was used. There were minimal Diet- and Sex-specific effects. 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

Fetal and placental outcomes

Effects of F1 maternal growth restriction prior to birth on F2 fetal and placental outcomes: Chow-fed mothers that were growth restricted prior to birth had normal weight fetuses compared to the Chow-fed normal birth weight (Control) mothers (Figures 2A, 2B). Chow-fed mothers that were growth restricted prior to birth (Restricted) increased placental efficiency in F2 males from *Sedentary* mothers and decreased placental efficiency in F2 males from *Exercise* mothers compared to their respective Chow-fed normal birth weight (Control) mothers (Figure 2E; Student's t-test p=0.012), despite no changes in placental weight (Figure 2C). There were no changes observed in placental weight or placental efficiency in F2 females from mothers that were growth restricted prior to birth (Restricted) compared to normal birth weight (Control) mothers (Figure 2D and 2F).

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Representative sections were taken from F2 female associated placentae whose F1 mother was of normal birth weight (Control) and growth restricted prior to birth (Restricted) who *Exercised* (Figure 3B) to highlight the effect F1 maternal growth restriction prior to birth has on placental morphology. F1 mothers that were growth restricted prior to birth and consumed a Chow diet, irrespective of Exercise, had increased total placental, junctional zone and labyrinth cross-sectional areas in F2 females compared to normal birth weight (Control) mothers (Figures 3D, 3F and 3H, respectively; two-way ANOVA). No differences in placental morphology was reported in F2 male associated placentae (Figures 3C, 3E and 3G).

F1 maternal exercise effects on F2 placental and fetal outcomes: Exercise in F1 Chow-fed mothers, irrespective of maternal birth weight, increased F2 male and female fetal weights compared to *Sedentary* and *PregEx* mothers, with no changes in F1 mothers fed a High-fat diet (Figures 2A and 2B; two-way ANOVA). Placental weight was reduced in F2 females whose F1 mother underwent *Exercise* (High-fat only) and *PregEx*, irrespective of maternal birth weight, compared to F1 *Sedentary* mothers (Figure 2D; two-way ANOVA). *Exercise* and *PregEx* in F1 Chow-fed normal birth weight (Control) mothers increased F2 male placental efficiency compared to F1 Chow-fed normal birth weight (Control) *Sedentary* mothers (Figure 2E; one-way ANOVA), with no exercise effects observed in F2 male associated placentae from F1 mothers that were growth restricted prior to birth. In F2 females, placental efficiency was increased with *Exercise* (High-fat mothers only) and *PregEx* (Chow and High-fat fed mothers), irrespective of maternal birth weight, compared to F1 *Sedentary* mothers (Figures 2F; one-way ANOVA).

Representative sections were taken from F2 male associated placentae whose F1 mother was *Sedentary* and *PregEx* (Figure 3A) to highlight exercise effects on placental morphology. *PregEx* increased placental and labyrinth zone cross-sectional areas in F2 males (Chow-fed mothers only) and F2 females (High-fat fed mothers only) (Figures 3C, 3D, 3G and 2H; two-way ANOVA), and increased junctional zone cross-sectional area in F2 males (High-fat fed mothers only; Figure 3E, two-way ANOVA), irrespective of maternal birth weight, compared to F1 *Sedentary* mothers.

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Fetal plasma IGF1 concentrations

Effects of F1 maternal growth restriction prior to birth on F2 fetal plasma outcomes: F1 mothers who were growth restricted prior to birth (Restricted) had F2 fetuses with reduced IGF1 plasma concentrations (pooled) in Chow-fed *Exercise* mothers (Figure 4; Student's unpaired t-test) compared to F1 normal birth weight (Control) Chow-fed mothers who *Exercised*. IGF1 plasma concentrations were not altered in F2 fetuses from mothers that were growth restricted prior to birth (Restricted) who consumed a High-fat diet.

F1 maternal exercise effects on F2 fetal plasma outcomes: Exercise in Chow-fed F1 normal birth weight (Control) mothers increased pooled F2 fetal IGF1 plasma concentrations compared to F1 *Sedentary* Chow-fed normal birth weight (Control) mothers, with no Exercise changes reported in mothers that were growth restricted prior to birth (Restricted, Figure 4; one-way ANOVA). Exercise in F1 mothers that consumed a High-fat diet did not alter pooled F2 fetal plasma IGF concentrations (Figure 4).

Placental IGF1

Effects of F1 maternal growth restriction prior to birth on F2 placental IGF1: F1 mothers that were growth restricted prior to birth (Restricted) and consumed a High-fat diet increased *Let7f-1* miRNA abundance in placentae of F2 males, irrespective of maternal exercise, compared to F1 normal birth weight (Control) mothers that consumed a High-fat diet (Figure 5A; two-way ANOVA), with no changes in IGF1 protein expression (Figure 5E). No changes in placental *Let 7f-1* miRNA, *Igf1* mRNA or IGF1 protein were detected in F2 female associated placentae whose mother was growth restricted prior to birth (Restricted) who consumed a Chow or High-fat diet compared to normal birth weight (Control) mothers (Figures 5B, 5D and 5F).

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F1 maternal exercise effects on F2 placental IGF1: PregEx in F1 High-fat fed mothers, irrespective of maternal birth weight, reduced *Let 7f-1* miRNA abundance in F2 male associated placentae compared to F1 *Sedentary* and *PregEx* High-fat fed mothers (Figure 5A; two-way ANOVA). In F2 female associated placentae, *Exercise* and *PregEx* in F1 Chow-fed mothers reduced *Let7f-1* miRNA abundance, irrespective of maternal birth weight, compared to F1 Chow-fed *Sedentary* mothers (Figure 5B; two-way ANOVA). *Exercise* in F1 mothers that were growth restricted prior to birth (Restricted) and consumed a High-fat diet had increased placental *Igf1* mRNA abundance in F2 males compared to Restricted *Sedentary* mothers (Figure 5C; one-way ANOVA), which did not translate to changes in IGF1 protein abundance (Figure 5E). Despite no changes in *Igf1* mRNA abundance in Chow-fed F2 male associated placentae (Figure 5C), *Exercise* and *PregEx* increased IGF1 protein expression, irrespective of maternal birth weight, compared to F1 Chow-fed *Sedentary* mothers (Figure 5E; two-way ANOVA). Similarly, despite no changes in *Igf1* mRNA abundance in F2 female associated placentae (Figure 5C), consumption of a High-fat diet in *PregEx* F1 mothers that were growth restricted prior to birth (Restricted) increased IGF1 protein expression in F2 females associated placentae compared to F1 *Sedentary* and *Exercise* Restricted mothers (Figure 5F; one-way ANOVA). With *PregEx* in High-fat fed normal birth weight (Control) mothers increasing IGF1 protein expression in F2 female associated placentae compared to *Exercise* in F1 normal birth weight (Control) mothers (Figure 5F).

Placental IGF1R

Effects of F1 maternal growth restriction prior to birth on F2 placental IGF1R: F1 mothers that were growth restricted prior to birth (Restricted) who consumed a Chow diet, irrespective of maternal exercise, had reduced *Igf1*r mRNA abundance in F2 male associated placenta compared to normal birth weight (Control) mothers (Figure 6A, two-way ANOVA). No effects were observed in F2 male associated placentae if their F1 mother was growth restricted prior to birth (Restricted) and consumed a High-fat diet (Figure 6A). In F2 female associated placentae, *PregEx* in F1 mothers growth restricted prior to birth (Restricted) that consumed a Chow diet reduced *Igf1r* mRNA abundance compared to *PregEx* in F1 Chow-fed

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normal birth weight (Control) mothers (Figure 6B; Student's unpaired t-test). No effects were observed in F2 female associated placentae if their F1 mother was growth restricted prior to birth (Restricted) and consumed a High-fat diet (Figure 6B). However, IGF1R protein expression was not affected by F1 mothers that were growth restricted prior to birth (Restricted; Figures 6C and 6D).

F1 maternal exercise effects on F2 placental IGF1R: No exercise effects were observed in *Igf1r* mRNA abundance in F2 male associated placentae whose F1 mother consumed a Chow or High-fat diet (Figure 6A). In F1 normal birth weight (Control) mothers on a Chow diet, *Exercise* reduced *Igf1r* mRNA abundance and *PregEx* increased *Igf1r* mRNA abundance in F2 females (Figure 6B; one-way ANOVA) compared to F1 normal birth weight (Control) *Sedentary* Chow-fed mothers. No exercise effects were observed in *Igf1r* mRNA abundance in F2 female associated placentae if their F1 mother consumed a High-fat diet (Figure 6B). Interestingly, *PregEx* increased IGF1R protein expression, irrespective of maternal birth weight, compared to *Sedentary* in F2 male and female associated placenta if their F1 mother consumed a Chow or High-fat diet (Figures 6C and 6D; two-way ANOVA).

Placental IGF2

Effect of F1 maternal growth restriction prior to birth on F2 placental IGF2: Exercise in F1 mothers growth restricted prior to birth (Restricted) that consumed a Chow diet reduced *Igf2* mRNA abundance in F2 male associated placentae compared to F1 Chow-fed normal birth weight (Control) mothers that *Exercised* (Figure 7A; Student's unpaired t-test). Additionally, *PregEx* in F1 mothers growth restricted prior to birth (Restricted) who consumed a Chow diet increase *Igf2* mRNA abundance in F2 male associated placentae compared to *PregEx* in F1 Chow-fed normal birth weight (Control) mothers (Figure 7A; Student's unpaired t-test). No effects were observed in *Igf2* mRNA abundance in F2 female associated placentae if their F1 mother was growth restricted prior to birth (Restricted) and consumed a Chow or High-fat diet (Figure 7B). Consumption of a High-fat diet in F1 mothers growth restricted prior to

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birth (Restricted) increased *Igf2* mRNA abundance in F2 male and female associated placentae, irrespective of maternal exercise, compared to F1 normal birth weight (Control) mothers (Figures 7A and 7B; two-way ANOVA). No effects were observed in IGF2 protein expression in F2 male or female associated placentae if their F1 mother was growth restricted prior to birth (Restricted) and consumed a Chow or High-fat diet (Figures 7C and 7D).

F1 maternal exercise effects on F2 placental IGF2: In F2 Chow-fed mothers, *Exercise* in mothers growth restricted prior to birth (Restricted) caused a reduction in placental *Igf2* mRNA abundance compared to *Sedentary* and *PregEx* F1 Chow-fed mothers that were growth restricted prior to birth (Restricted; Figure 7A, one-way ANOVA). *Exercise* in normal birth weight (Control) mothers increased *Igf2* mRNA abundance in F2 male associated placentae compared to *PregEx* in F1 Chow-fed normal birth weight (Control) mothers (Figure 7A, one-way ANOVA). In F2 female associated placentae, *PregEx*, irrespective of maternal birth weight, reduced *Igf2* mRNA abundance compared to F1 Chow-fed *Sedentary* and *Exercise* mothers (Figure 7B, two-way ANOVA). No exercise effects were observed in F2 male or female associated placentae, irrespective of maternal birth weight, if their F1 mother consumed a High-fat diet (Figure 7A and 7B).

Exercise in F1 Chow-fed mothers, irrespective of maternal birth weight, increased placental IGF2 protein expression in F2 male associated placentae compared to F2 Chow-fed mothers that were *Sedentary* or *PregEx* (Figure 7C, one-way ANOVA). Similarly, *Exercise* in F1 normal birth weight (Control) mothers that consumed a High-fat diet increased IGF2 protein expression in F2 male associated placentae compared to F1 normal birth weight (Control) mothers that were *Sedentary* or *PregEx* (Figure 7C, one-way ANOVA). In F2 female associated placentae, *Exercise* in F1 Chow-fed mothers, irrespective of maternal birth weight, increased IGF2 protein expression compared to F1 Chow-fed *Sedentary* mothers (Figures 7D, two-way ANOVA). Whereas, *PregEx* in F1 High-fat mothers, irrespective of maternal birth weight, reduced IGF2 protein expression in F2 female associated placentae compared to F1 High-fat fed mothers that were *Sedentary* or *PregEx* (Figure 7D, one-way ANOVA).

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Placental IGF2R

Effect of F1 maternal growth restriction prior to birth on placental IGF2R: F1 mothers growth restricted prior to birth (Restricted) that consumed a Chow diet reduced *Igf2r* mRNA abundance in F2 male associated placentae, irrespective of maternal exercise, compared to Chow-fed F1 normal birth weight (Control) mothers (Figure 8A; two-way ANOVA). *PregEx* in mothers that were growth restricted prior to birth (Restricted) and consumed a High-fat diet caused a reduction in *Igf2r* mRNA abundance in F2 female associated placentae compared to *PregEx* in Chow-fed F1 normal birth weight (Control) mothers (Figure 8B; Student′s unpaired t-test). No effects were observed in F2 male or female associated placentae, irrespective of maternal exercise, if their F2 mother was growth restricted prior to birth (Restricted) and consumed a High-fat diet (Figures 8A and 8B).

F1 maternal exercise effects on F2 placental IGF2R: PregEx, irrespective of maternal birth weight, increased *Igf2*r mRNA abundance in Chow-fed F2 male associated placentae compared to Chow-fed F1 mothers that were *Sedentary* or *Exercised* (Figure 8A; one-way ANOVA). With High-fat feeding, *PregEx* in F1 normal birth weight (Control) mothers increased *Igf2r* mRNA abundance in F2 male associated placentae compared to F1 normal birth (Control) weight *Sedentary* mothers (Figure 8A, one-way ANOVA). *PregEx* in F1 mothers that were growth restricted prior to birth (Restricted) and consumed a High-fat diet caused an increase in *Igf2r* mRNA abundance in F2 male associated placentae compared to *Sedentary* and *Exercised* F1 mothers growth restricted prior to birth (Restricted; Figure 8A, one-way ANOVA). In F2 female associated placenta from F1 normal birth weight (Control) mothers that consumed a Chow diet, *Igf2r* mRNA abundance was increased compared to F1 normal birth weight (Control) mothers that were *Sedentary* or who *Exercised* (Figure 8B, one-way ANOVA). High-fat feeding in *PregEx* F1 mothers, irrespective of maternal birth weight, increased *Igf2r* mRNA abundance in F2 female associated placentae compared to *Sedentary* F1 mothers (Figure 8B; one-way ANOVA).

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Placental *IGFBP3*

No effects were observed in *Igfbp3* mRNA abundance in F2 male or female associated placentae if their mother was growth restricted prior to birth (Restricted) or exercised on either diet (Chow and High-fat; Figures 8C and 8D).

Discussion

This study has, for the first time, demonstrated that the placental IGF-system is independently influenced by maternal birth weight and exercise. Furthermore, these responses are dependent upon the maternal diet and fetal sex. We have previously demonstrated that F1 mothers born growth restricted, who develop glucose intolerance only during pregnancy, transmit β-cell deficits to F2 male offspring (Cheong *et al.*, 2016), which may increase their susceptibility to metabolic disease with additional lifestyle challenges. This disease transmission is likely, in part, due to placental programming, which this study highlights may not be due to changes in the placental IGF-system. Given that exercise is beneficial for maternal and fetal health, it is possible that maternal exercise in 'at risk' women may prevent the transgenerational transmission of disease, which requires further investigation. However, based on the current study it is likely that any benefits of exercise is not due to improvements in the placental IGF-system, as we report minimal exercise effects in F1 mothers born growth restricted or following high-fat feeding. Additionally, as growth restricted women are susceptible to becoming obese, which is independently linked to poor childhood health and increased adult disease susceptibility, changes in the placental IGF-system in F1 mothers that were born growth restricted may be more adversely impacted by maternal obesity.

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Impact of F1 maternal growth restriction prior to birth

Several studies have demonstrated that the IGF-system is dysregulated following fetal growth restriction due to both uteroplacental insufficiency (Laviola *et al.*, 2005) and maternal undernutrition (Coan *et al.*, 2010). This study has, for the first time, demonstrated that this system is similarly dysregulated in placentae of the next generation (F2), which likely contributes to the transgenerational disease programming we have previously reported (Gallo *et al.*, 2012; Gallo *et al.*, 2013; Cheong *et al.*, 2016). In the current study F2 male associated placentae from F1 Chow-fed mothers born growth restricted had reduced *Igf2r* mRNA abundance compared to F1 normal birth weight mothers, which may increase placental IGF2 abundance. As placental IGF2 regulates nutrient handling or partitioning by influencing placental labyrinth morphology and nutrient transport efficiency (Constancia *et al.*, 2002), this likely explains the increased placental efficiency in *Sedentary* Chow-fed F1 mothers born growth restricted compared to *Sedentary* normal birth weight mothers, which is maintaining normal F2 fetoplacental growth. It thus appears that F2 male fetuses of Chow-fed F1 mothers born growth restricted have an intrinsic adaptation that aims to normalize F2 fetal growth and development by optimizing placental efficiency through IGF2 signaling, by reducing *Igf2r* mRNA abundance. If this change in *Igf2r* mRNA abundance results in increased protein expression it would limit the amount of placental IGF2 available to bind to IGF1R thus inhibiting growth of the F2 fetoplacental unit (Wylie *et al.*, 2003; Harris *et al.*, 2011). Despite similar alterations in IGF2 signaling in F2 male associated placentae of F1 mothers born growth restricted that *Exercised*, this adaptation is inadequate as placental efficiency is reduced compared to *Exercise* in Chow-fed F1 normal birth weight mothers, which is likely due to the high maternal metabolically demanding environment. Specifically, *Exercise* in Chow-fed F1 mothers born growth restricted would result in the reallocation of nutrients to favor the maternal metabolic system (Mottola & Christopher, 1991), reducing placental nutrient transport capacity (and hence placental efficiency) and could explain the reduced pooled F2 fetal plasma IGF1 concentrations due to impaired placental IGF1 secretion (as IGF1 protein expression is increased), which requires further investigations. Therefore, this reduction in placental nutrient uptake capacity by F2 male fetuses, whose mother was born growth restricted and *Exercised*, may compromise F2 male fetal development via the IGF

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pathway. However, additional studies are required to identify if this compromises birth weight and long-term offspring health. Given that maternal obesity is associated with excess nutrition, it is not surprising that minimal alterations in the IGF-system were found in F2 male associated placentae of High-fat fed F1 mothers born growth restricted.

As several studies report sex-specific responses in the placenta following several pregnancy perturbations (Clifton, 2005; Cuffe *et al.*, 2011; Cuffe *et al.*, 2012), it is not surprising that placentae of F2 fetuses responded differently if their F1 mother was born growth restricted. Specifically, placentae of F2 female fetuses whose F1 mother was born growth restricted and consumed a Chow diet have significant morphometric adaptations, which likely ensures normal fetoplacental growth by increasing hormone and nutrient storage (junctional zone) and nutrient transportation (labyrinth). These morphological changes would facilitate increased nutrient delivery to the F2 female fetus, which may influence fetal weight; however no changes in F2 female fetal weight were reported. It is possible that the reduction in pooled IGF1 plasma concentrations measured in F2 fetuses of F1 mothers born growth restricted who *Exercised* may be due to impaired fetal and/or placental nutrient availability in F2 male fetuses and is not a true representation of what occurred in each sex, which may mask any subtle differences between sexes. Thus, additional studies are required to quantify sex-specific responses in F2 plasma IGF1 concentrations during pregnancy and at birth along with placental nutrient transporter expression.

Impact of F1 maternal exercise

To our knowledge we are the first to report dynamic changes in F2 fetal plasma IGF1 concentrations following maternal exercise and to identify that changes in the placental IGF-system are dependent on timing of exercise initiation. Similar to studies in humans who perform weight bearing exercise prior and during pregnancy (Clapp *et al.*, 2002) *Exercise* in F1 Chow-fed mothers, irrespective of maternal birth weight, increased F2 fetal weight, which appears to be caused by different mechanisms for each sex. Specifically, in F2 male

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associated placentae, *Exercise* in F1 Chow-fed mothers increased the expression of IGF ligands (IGF1 and IGF2), which stimulates fetoplacental growth by increasing placental nutrient transport capacity (Fowden, 2003) and through their receptor specific signaling cascades by stimulating growth, differentiation and proliferation (Chitnis *et al.*, 2008). This potential increase in placental nutrient transportation following *Exercise* in F1 Chow-fed mothers may explain the increased pooled IGF1 concentrations in F2 fetuses of normal birth weight mothers, but not in mothers born growth restricted, due to the aforementioned reduced F2 male placental efficiency, which requires further investigation. In F2 female associated placentae, however, the increased F2 fetal weight in Chow-fed F1 mothers is likely attributed to the increased placental IGF2 protein expression, which is known to increase fetal growth via regulation of placental morphology and nutrient partitioning (Constancia *et al.*, 2002; Kent *et al.*, 2012). Nevertheless, it remains to be determined whether this increased F2 female fetal weight in Chow-fed mothers is beneficial or detrimental to birth weight and/or long-term offspring health, especially as maternal High-fat feeding did not alter fetal weight.

Interestingly, more dynamic changes in the placental IGF-system were reported following *PregEx* compared to *Exercise*, which may be due to the exercise being initiated after implantation and the placenta was required to adapt to the high metabolically demanding environment to ensure normal F2 fetoplacental growth. In line with this suggestion *PregEx*, irrespective of maternal birth weight, increased placental IGF receptors (IGF1R and *Igf2r*) and IGF1 protein expression in both F2 male and female associated placentae, which would aim at increasing nutrient delivery to the fetus (Sferruzzi-Perri *et al.*, 2006). This finding is consistent with studies in humans that underwent low-intensity exercise during mid-gestation where placental vascular volume and surface area are increased, indicating a placental adaptation to increase nutrient transfer via increased blood flow (Jackson *et al.*, 1995). It is interesting to note that these increases in IGF1 and IGF1R protein expression in *PregEx* may be due to reduced *Let 7f-1* miRNA abundance compared to *Sedentary* in F2 male (High-fat diet; IGF1R only) and female (Chow diet) associated placentae. This is of interest as *Let 7f-1* miRNA is a known regulator of IGF1 and IGF1R gene and protein expression (Hu *et al.*,

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2014). To our knowledge, this is the first study to demonstrate that *Let 7f-1* miRNA is increased in F2 male associated placentae from F1 mothers that were born growth restricted who consumed a High-fat diet and that *Let 7f-1* miRNA is reduced following exercised prior to and during pregnancy (F2 male and female associated placenta whose mother consumed a high-fat and chow diet, respectively) or during pregnancy only (F2 female associated placentae whose mother consumed a chow diet). However, as changes in *Let 7f-1* miRNA abundance did not always correlate with alterations in IGF1 or IGF1R gene/protein expression it suggests that other post-transcriptional regulators may be involved in the regulation of the placental IGF-system in the current study. As such future studies should characterize other *Let 7* cluster miRNA as potential modulators of IGF1/IGF1R regulation in F2 placentae of F1 mothers born growth restricted and following maternal exercise and high-fat feeding.

Surprisingly both *Exercise* (High-fat fed mothers only) and *PregEx* (Chow and High-fat feeding) reduced F2 female associated placental weight, irrespective of maternal birth weight, compared to F1 *Sedentary* mothers, the mechanisms by which are dependent on the timing of exercise initiation. Specifically, the reduction in F2 placental IGF2 protein expression and increased *Igf2r* mRNA abundance following *PregEx* in High-fat fed mothers, irrespective of maternal birth weight, would limit the amount of placental IGF2 binding IGF1R, thus reducing F2 placental weight. *Exercise* in High-fat fed mothers, on the other hand, reduces F2 placental weight independently of the IGF-system and is likely due to another pathway, such as placental growth factor. Despite *PregEx* in F1 High-fat fed mothers reducing F2 IGF2 protein expression (mothers born growth restricted only) and increasing *Igf2r* mRNA abundance in F2 placentae associated with males, junctional zone cross-sectional area was increased, irrespective of maternal birth weight, with no change in placental weight, which may be an adaptation to increase nutrient storage or increase placental hormone production to facilitate normal fetoplacental growth (Burton & Fowden, 2012). However, additional studies are required to characterise alterations in placental nutrient handling and nutrient partitioning following maternal exercise.

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Study limitations

A strength of the current study is that it allows the direct comparison of the impact F1 maternal growth restriction and maternal diet consumption have on the placental IGF-system in both male and female F2 fetuses, thus improving our understanding of the impact these factors have on the transgenerational programming of disease. However it should be noted that to address this research question required the generation of a large number of groups and, as such, the sample sizes used throughout limits the power of the analysis to be able to statistically compare how these parameters (treatment, exercise, diet and fetal sex) in combination influence the placental IGF-system (i.e. four-way ANOVA). Instead analysis prioritized each of the major effects in isolation.

Conclusion

This study demonstrates that the placental IGF-system is differentially regulated in F1 mothers growth restricted prior to birth and following maternal exercise, with responses dependent on maternal diet- and fetal sex, which likely aims to improve F2 fetoplacental growth in the face of adverse *in utero* environments. F2 fetuses of F1 mothers born growth restricted have structural placental alterations that would facilitate increased nutrient delivery (females) and increase placental IGF2 signaling (males) both of which would promote fetoplacental growth. However, this adaptation in F2 males is inadequate if the growth restricted mother consumes a Chow diet and *Exercises* as fetoplacental efficiency is impaired. Therefore, F2 female fetuses of mothers born growth restricted are able to withstand additional pregnancy challenges that can influence fetoplacental growth via the placental IGF-system and may explain why F2 males have compromised organ development (Cheong *et al.*, 2016).

Maternal exercise, specifically *PregEx*, resulted in profound changes in the placental IGF-system that increases the expression of IGF ligands and their receptors to maintain normal

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fetoplacental growth, which mostly occurred in both mothers with a normal and small birth weight. This adaptation is however inadequate in F2 female associated placentae whereby placental weight is reduced due to limited IGF2 availability (Chow-fed mothers) or in a manner that is independent of the IGF-system (High-fat fed mothers). Importantly, reductions in *Let 7f-1* miRNA abundance with *PregEx* may play a role in the regulation of the placental IGF-system as it coincided with increased IGF1 (female associated placenta whose mother consumed a chow diet) and IGF1R (male and female associated placenta whose mother consumes a high-fat and chow diet, respectively). However, the exact effects these alterations in the placental IGF-system following maternal exercise has on F2 offspring birth weight, development and long-term health is unknown and future studies are required.

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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.H. performed all experiments. D.M., K.A. and A.J.J performed the animal work, with assistance from Y.T.M.M. Y.T.M.M., J.S.M.C., J.F.B. and S.H. 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.

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Figure legends

Figure 1: Flow chat of experimental exercise and diet protocol

(A) Flow chart of the experimental protocol indicating the allocation of pregnant rats between the different treatments, diet and exercise groups. (B) Flow chart of exercise protocol. E- embryonic day; HFD- high fat diet; m/min- meters per minute; min/day- minutes per day; UPI- uteroplacental insufficiency; Wk- week.

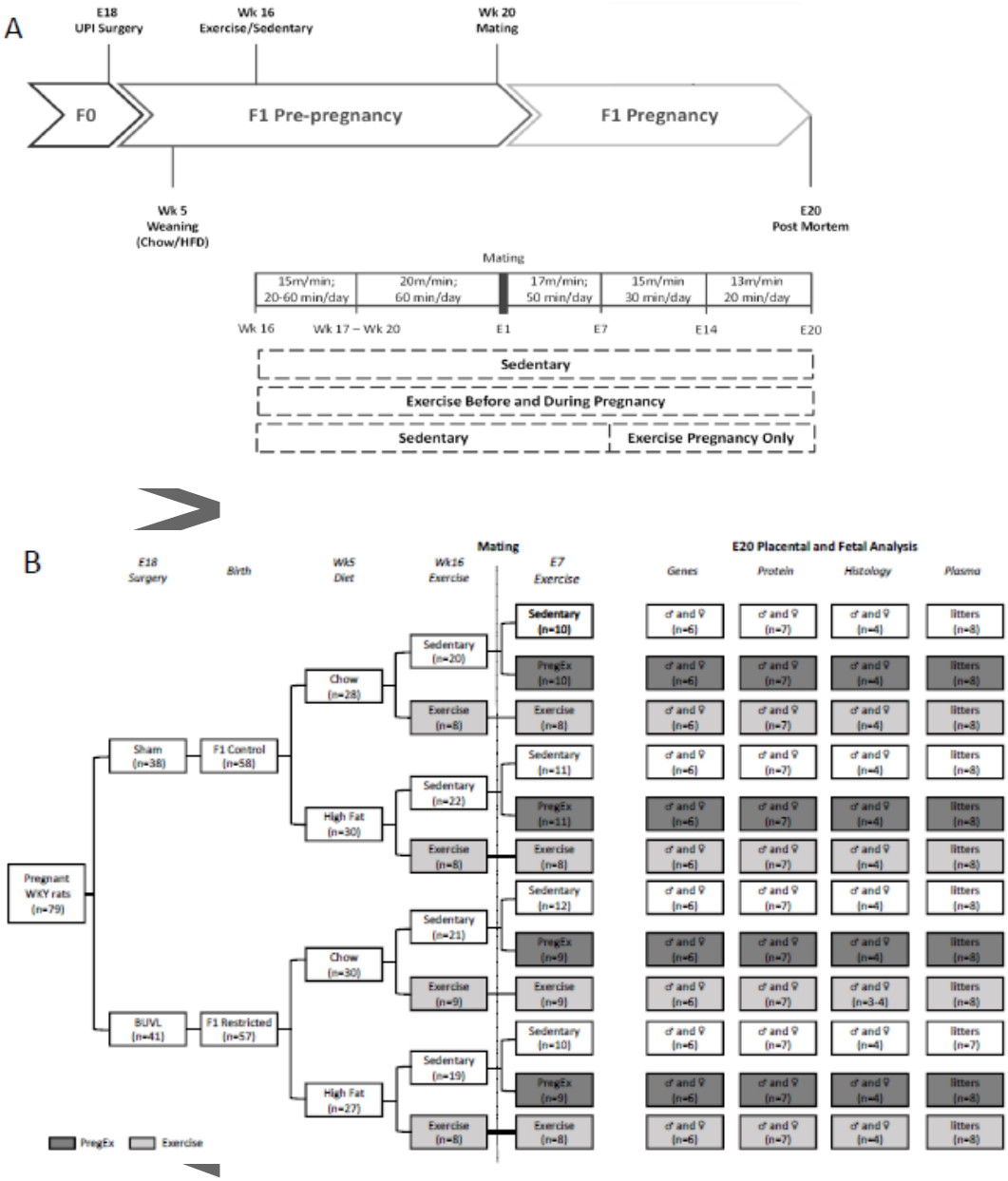

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Figure 2: Fetal and placental weights

Fetal (A and B) and placental (C and D) weight along with Fetal: weight (E and F) (n= 8-12 litters in each group) for male and female fetuses whose mothers were Control (open bars) or Restricted (black bars) that consumed a Chow (left panel) of High-fat diet (right panel). Data were analysed by a two-way ANOVA 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'.

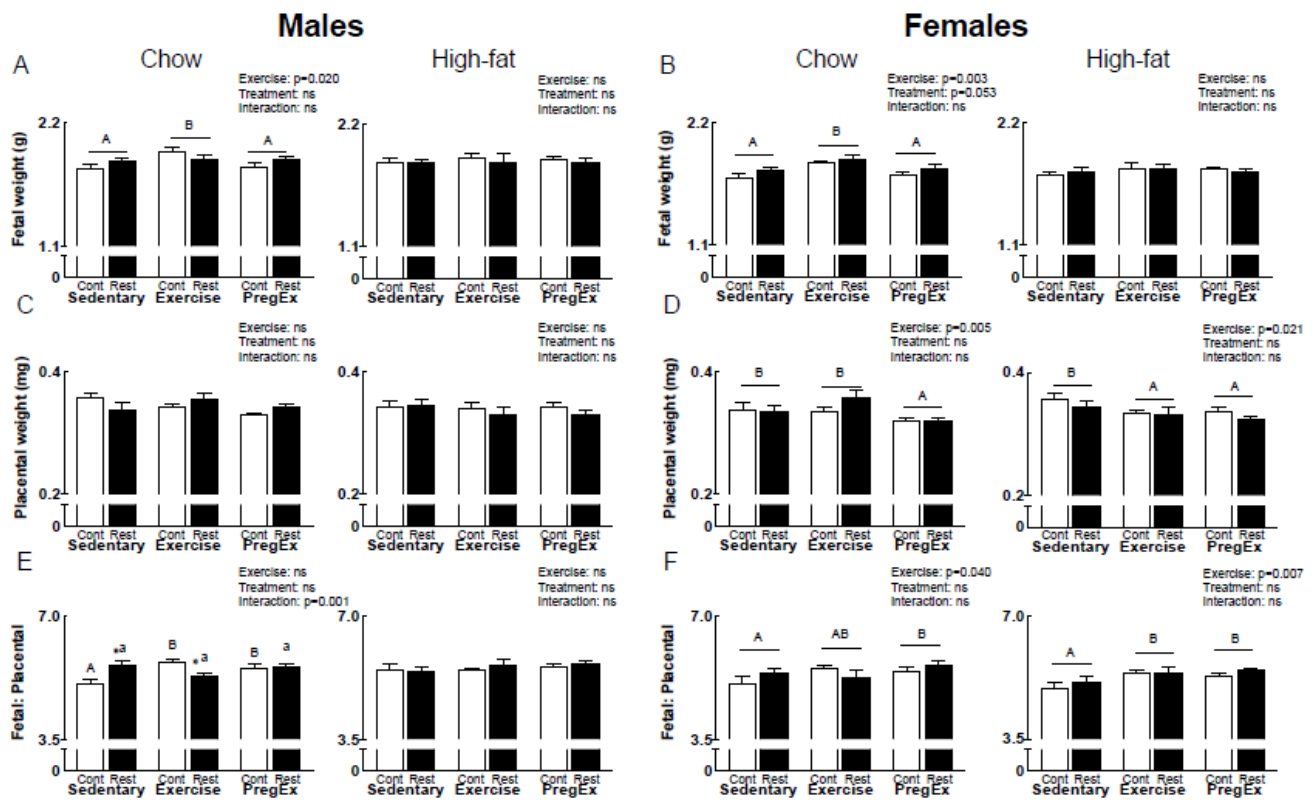

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Figure 3: Placental histology parameters

Representative whole placental images demonstrating morphological changes following *PregEx* compared to *Sedentary* (male placentae from Chow-fed mothers) (A) and in Restricted compared to Control (B) placentae (female placentae from *Exercise* mothers). Whole placental (C and D), junctional zone (E and F) and labyrinth (G and H) cross-sectional areas 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) (n= 3-4 in each group/sex n = 1 representing 1 pup from 1 litter). Data were analysed by a two-way ANOVA and presented as mean ± SEM. Differences across exercises are denoted by different letters where 'A' is different to 'B', but not 'AB'.

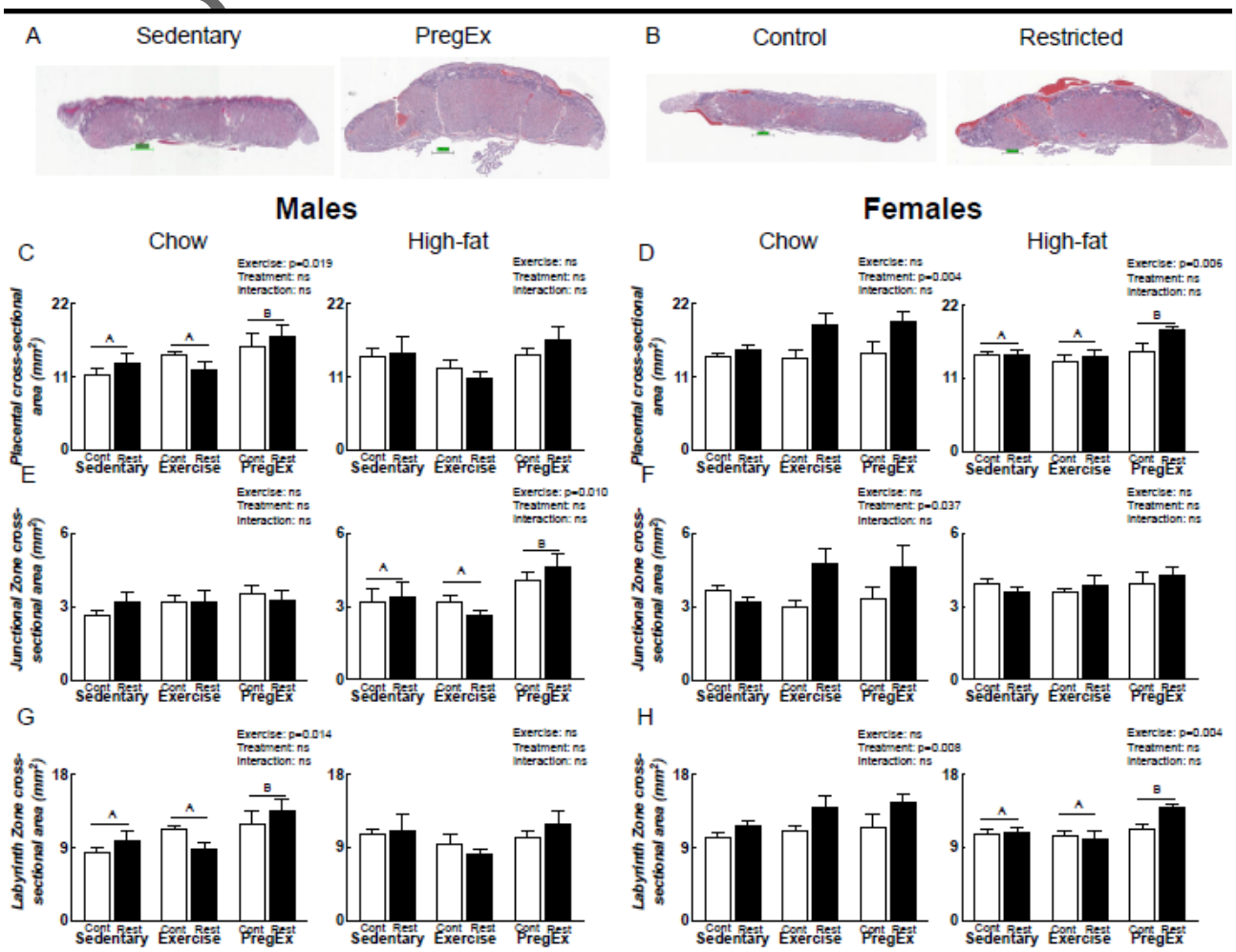

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Figure 4: Fetal IGF1 concentrations

Pooled plasma IGF1 concentrations in fetuses from Chow (left) and High-fat (right) fed mothers (n= 8-10 litters in each group). Data were analyzed by a two-way ANOVA 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'.

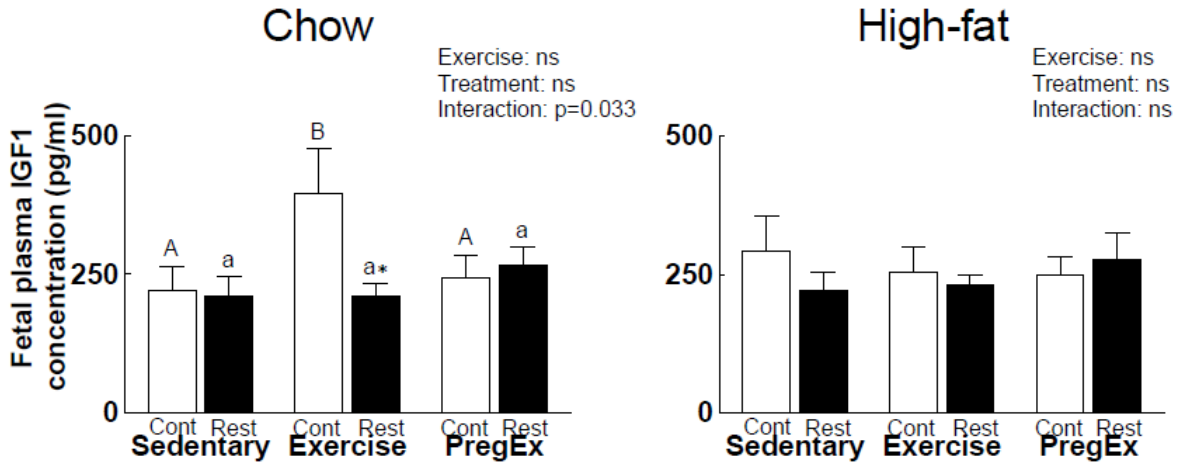

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Figure 5: Placental IGF1 expression

Let 7f-1 miRNA abundance (A and B), *Igf1* mRNA abundance (C and D) (n=6 in each group/sex n = 1 representing 1 pup from 1 litter) and IGF1 protein expression (E and F) (n=6-7 in each group/sex n = 1 representing 1 pup from 1 litter) 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 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'.

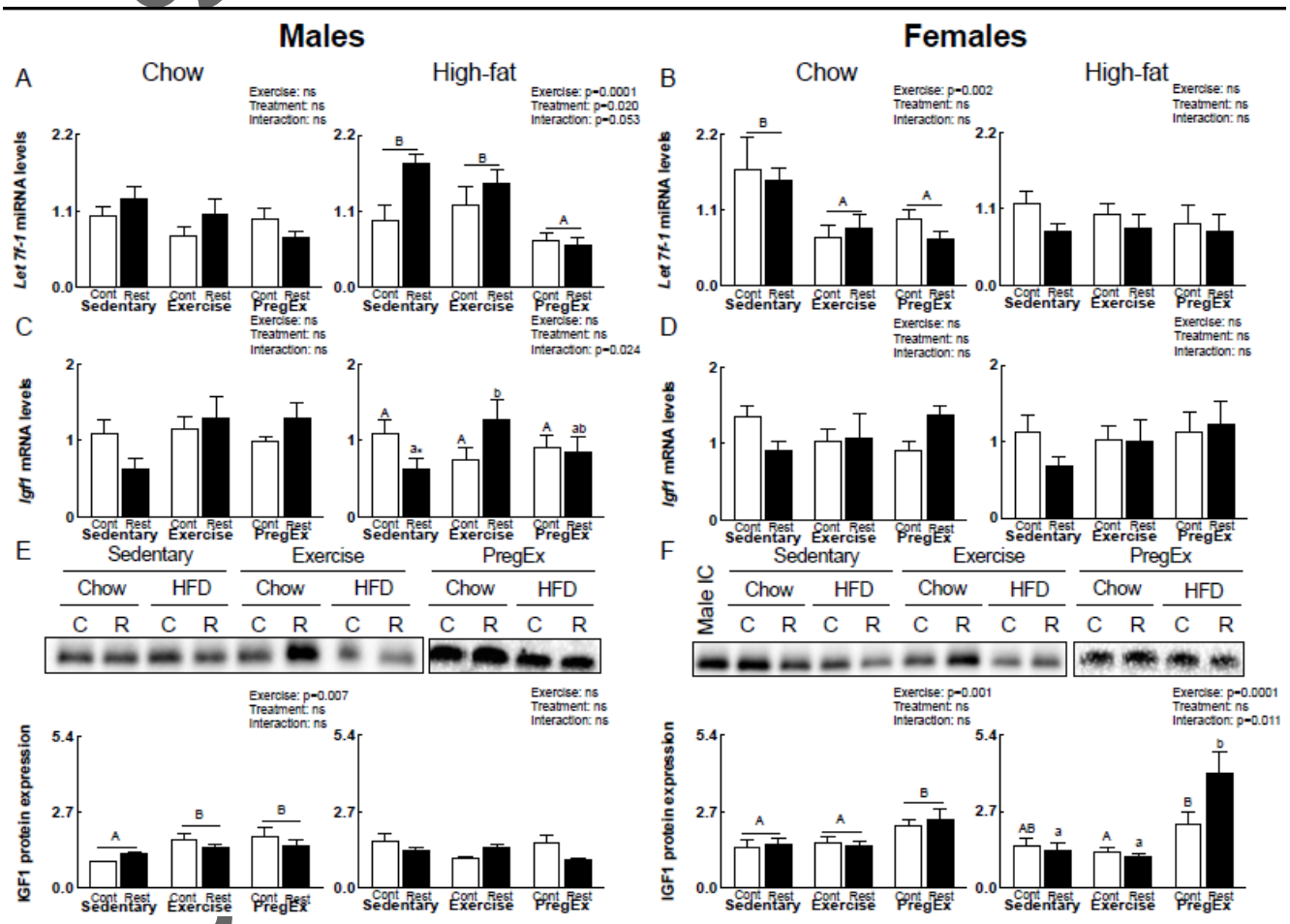

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Figure 6: Placental IGF1R expression

IGF1R mRNA abundance (A and B) (n=6 in each group/sex n = 1 representing 1 pup from 1 litter) and protein expression (C and D) (n=6-7 in each group/sex n = 1 representing 1 pup from 1 litter) 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 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' and 'c/C' but not different to 'ab/AB'.

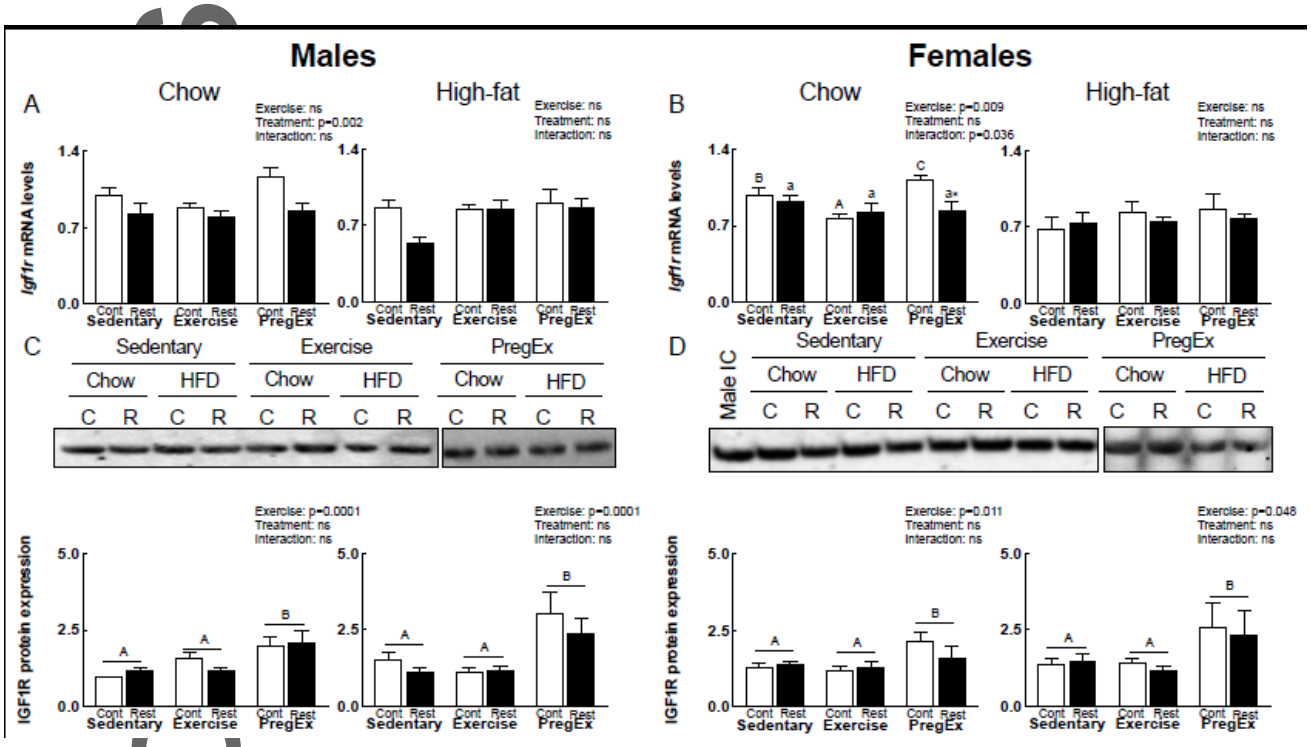

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Figure 7: Placental IGF2 expression

IGF2 mRNA abundance (A and B) (n=6 in each group/sex n = 1 representing 1 pup from 1 litter) and protein expression (C and D) (n=6-7 in each group/sex n = 1 representing 1 pup from 1 litter) 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 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' and 'c/C' but not different to 'ab/AB'.

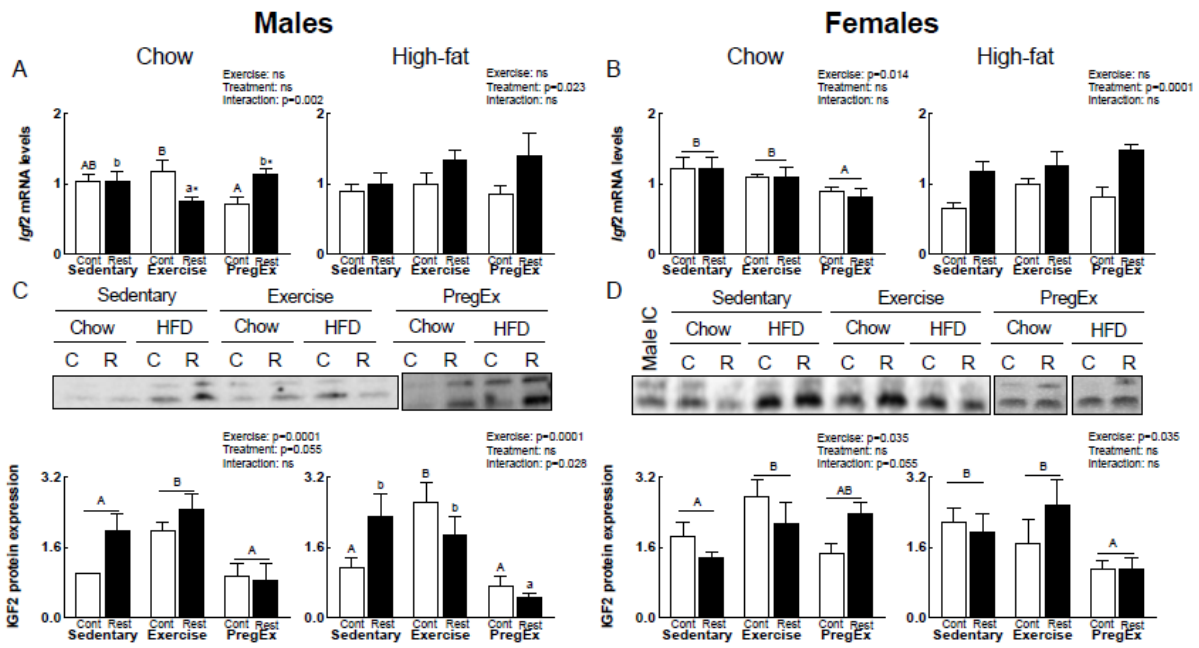

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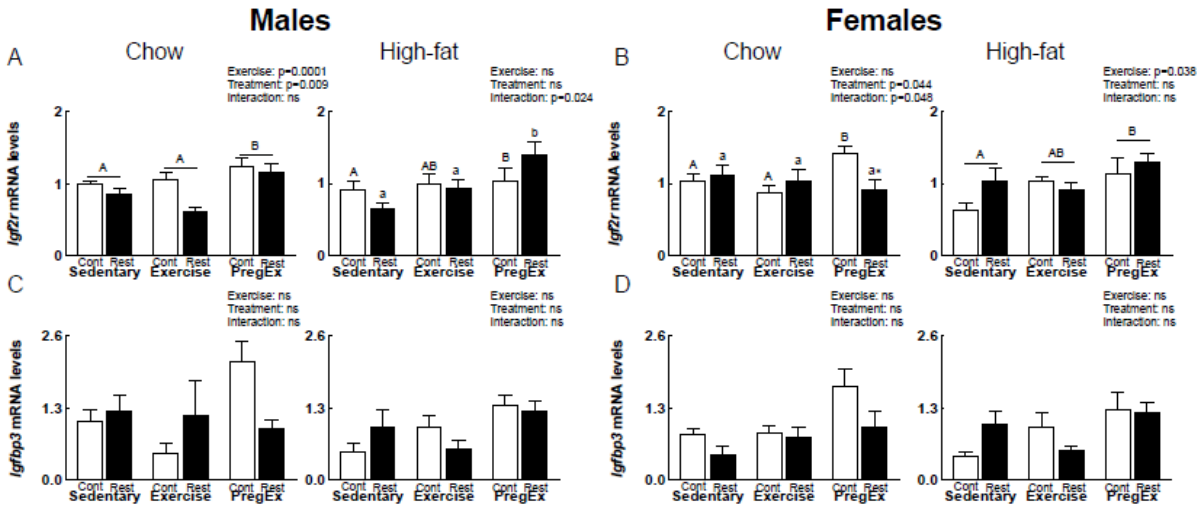
Figure 8: Placental *Igf2r* and *Igfbp3* mRNA abundance

Igf2r (A and B) and *Igfbp3* (C and D) 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 and presented as mean ± SEM, where 'ns' is not significant (n=6 in each group/sex 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'.



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