Exercise and High-fat Feeding in Growth Restricted Mothers Differentially Modulates the Placental Nutrient Sensing, Vasculogenic and Stress Pathway of Second-generation Offspring

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This thesis is dedicated to God my father who has been a pillar of strength throughout. I also dedicate this work to my parents Elfigio and Fiona, my sisters Yananai and Ruvarashe who have been patient and loving throughout this very long journey.

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ABSTRACT

Fetoplacental growth and development depend on the placental insulin-like growth factor (IGF), nutrient transporter, angiogenesis, and stress systems. Dysregulation of these are observed in complicated pregnancies, such as those resulting in intrauterine growth restriction, which itself is linked to adult-onset disease and obesity. Pregnant growth restricted females are predisposed to gestational diabetes mellitus, highlighting the need for simple lifestyle interventions, such as moderate exercise, in women at risk of a complicated pregnancy to improve maternal and child health. The overall aim of thesis was to investigate the effects exercise and high-fat feeding has on second generation (F2) placentae of growth restricted females. Uteroplacental insufficiency surgery was performed to induce fetal growth restriction. Offspring consumed a chow or high-fat diet (43% kcal from fat) from weaning and were allocated to an exercise regime: remained sedentary (*Sedentary*), exercised prior to and during pregnancy (*Exercise*), or exercised only during pregnancy (*PregEx*). F2 placentae were collected at post-mortem to quantify expression of the IGF, nutrient transporters, angiogenesis, and stress systems. Maternal growth restriction deregulated placental systems of interest, which was likely aimed to increase nutrient exchange in male, but not female, fetuses. Maternal high-fat feeding, however, did not exacerbate these changes. *Exercise* increased fetal weight despite minimal alterations in placental systems of interest, which can be attributed to predictive programming to ensure fetal survival in conditions of increased maternal metabolic activity and varied nutrient supply. *PregEx*, on the other hand, profoundly altered placental systems of interest, which were likely aimed to increase nutrient exchange. This thesis highlights the placentas ability to adapt to positive maternal lifestyle interventions in women at risk of a complicated pregnancy, which could be key in preventing placentally-mediated transgenerational disease programming resulting in healthy children, although the benefits will likely depend on the timing of intervention initiation.

STATEMENT OF AUTHORSHIP

This thesis includes work by the author that has been published or accepted for publication as described in the text. Except where reference is made in the text of the thesis, this thesis contains no other material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Yeukai Mangwiro
15th December 2020

PREFACE

Chapter 3

Publication: Mangwiro YTM, Cuffe JSM, Briffa JF, Mahizir D, Anevska K, Jefferies AJ, Hosseini S, Romano T, Moritz KM and Wlodek ME (2018) Maternal exercise in rats upregulates the placental IGF-system with diet and sex-specific responses: minimal effects in mothers born growth restricted. *J Physiol*, 596(23), 5947-5964.

- Dr Kristina Anevska and Mr Andrew Jefferies performed the F0 bilateral uterine vessel ligation surgeries.
- I performed ~33% of the exercise with Dr Dayana Mahizir and Dr Kristina Anevska.
- I performed 30% of F1 mating's with Dr Dayana Mahizir and Dr Kristina Anevska.
- I performed ~25% of post-mortem placental collections with assistance from Mr Andrew Jefferies and Mrs Sogand Gravina (nee Hosseini).
- I sexed 25% of the fetuses via *Sry* qPCR with Mrs Sogand Gravina.
- I performed all placental mRNA/miRNA RNA extractions, cDNA synthesis, and qPCRs.
- I performed all protein extractions and Western blots.
- I performed ELISA analysis of F2 fetal plasma.
- Mrs Sogand Gravina (nee Hosseini) performed and analysed the fixed placentae for whole placental, labyrinth, and junctional zone cross-sectional areas.
- I performed all data analysis and interpretation and wrote the first full draft of the manuscript.
- Prof Mary Wlodek, Dr Jessica Briffa, Dr Tania Romano, Dr James Cuffe, and Prof Karen Moritz assisted with data analyses and provided critical revision of the manuscript.

Chapter 4

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- I performed 30% of F1 mating's with Dr Dayana Mahizir and Dr Kristina Anevska.

- I performed ~25% of post-mortem placental collections with assistance from Mr Andrew Jefferies and Mrs Sogand Gravina (nee Hosseini).
- I sexed 25% of the fetuses via *Sry* qPCR with Mrs Sogand Gravina.
- I performed all mRNA placenta RNA extractions, cDNA synthesis, and qPCRs.
- I performed all protein extractions and Western blots.
- Mrs Sogand Gravina performed and analysed the fixed placentae for glycogen cross-sectional area.
- I performed all data analysis and interpretation and wrote the first full draft of the manuscript.
- Prof Mary Wlodek, Dr Jessica Briffa, Dr Tania Romano, Dr James Cuffe, and Prof Karen Moritz assisted with data analyses and provided critical revision of the manuscript.

Chapter 5

Publication: Mangwiro YTM, Briffa JF, Mahizir D, Anevska K, Gravina S, Romano T, Moritz KM, and James SM Cuffe and Wlodek ME (2018) Maternal exercise and growth restriction in rats alters placental angiogenic factors and blood space area in a sex-specific manner. *Placenta*, 74, 47-54.

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- I performed all mRNA/miRNA placental RNA extractions, cDNA synthesis, and qPCRs.
- I performed all protein extractions and Western blots.
- Mrs Sogand Gravina performed and analysed the fixed placentae for placental blood spaces and labyrinth tissue area.
- I performed all data analysis and interpretation and wrote the first full draft of the manuscript.
- Prof Mary Wlodek, Dr Jessica Briffa, Dr Tania Romano, Dr James Cuffe, and Prof Karen Moritz assisted with data analyses and provided critical revision of the manuscript.

Chapter 6

Publication: Mangwiro YTM, Cuffe JSM, Vickers MH, Reynolds CM, Mahizir D, Anevska K, Gravina S, Romano T, Moritz KM, Briffa JF and Wlodek ME (2021) Maternal exercise alters rat fetoplacental stress response: minimal effects of maternal growth restriction and high-fat feeding. *Placenta*, 104, 57-70.

- All *Unstressed* data presented in the manuscript was generated as a direct result of my PhD aims, whereas the *Stressed* data was generated in collaboration with other laboratory projects that was not directly related to my PhD thesis.
- Dr Kristina Anevska and Mr Andrew Jefferies performed the F0 bilateral uterine vessel ligation surgeries.
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- I sexed 25% of the fetuses via *Sry* qPCR with Mrs Sogand Gravina.
- I performed all mRNA placental extractions, cDNA synthesis, and qPCRs.
- Mrs Sogand Gravina performed and analysed the fixed placentae for whole placental, labyrinth, and junctional zone cross-sectional areas.
- Mr Eric Thorstensen, Miss Laura Galante, and Ms Rachna Patel performed the liquid chromatography mass spectrometry and analysis under the guidance of Prof Mark Vickers and Dr Clare Reynolds.
- Dr Dayana Mahizir and Dr Kristina Anevska performed the maternal physiological measurements.
- I performed all data analysis and interpretation and wrote the first full draft of the manuscript.
- Prof Mary Wlodek, Dr Jessica Briffa, Dr Tania Romano, Dr James Cuffe, Prof Karen Moritz, Prof Mark Vickers, and Dr Clare Reynolds assisted with data analyses and provided critical revision of the manuscript.

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"*Train up a child in the way he should go, and when he is old, he will not depart from it*" - Proverbs 22 vs 6. To my parents Elfigio and Fiona, this thesis is the culmination of your training, the love, the patience, the tears, and frustrations over the years. You are both my inspiration and reminder that in life if for you to have something worth keeping you need to put in the work! Thank you for loving me unconditionally even in moments that I became so stressed and unbearable to be around. To my sweet sisters Yananai and Ruvarashe, you guys do not have to worry about your big sister anymore. Thank you for putting up with me and always showing me love and extending grace during my whole journey.

PUBLICATIONS, PRESENTATIONS AND AWARDS

Manuscripts

Published manuscripts

Mangwiro YTM, Cuffe JSM, Vickers MH, Reynolds CM, Mahizir D, Anevska K, Gravina S, Romano T, Moritz KM, Briffa JF & Wlodek ME. Maternal exercise alters rat fetoplacental stress response: minimal effects of maternal growth restriction and high-fat feeding. *Placenta*, 104, 57-70. doi: 10.1016/j.placenta.2020.11.006. Epub 2020 Nov 18. Chapter 6

Mangwiro YTM, Cuffe JSM, Mahizir D, Anevska K, Jefferies AJ, Gravina SS, Romano T, Moritz KM, Briffa JF & Wlodek ME (2019). Exercise initiated during pregnancy in rats born growth restricted alters placental mTOR and nutrient transporter expression. *J Physiol* 597(7), 1905-1918. doi: 10.1113/JP277227. Epub 2019 Mar 1. Chapter 4

Mangwiro YTM, Briffa JF, Mahizir D, Anevska K, Jefferies AJ, Hosseini SS, Romano T, Moritz KM, Cuffe JSM & Wlodek ME (2018). Maternal exercise and growth restriction in rats alters placental angiogenic factors and blood space area in a sex-specific manner. *Placenta*, 74, 47-54. doi: 10.1016/j.placenta.2018.12.005. Epub 2018 Dec 15. Chapter 5

Mangwiro YTM, Cuffe JSM, Briffa JF, Mahizir D, Anevska K, Jefferies AJ, Hosseini SS, Romano T, Moritz KM & Wlodek ME (2018). Maternal exercise in rats upregulates the placental IGF-system with diet and sex-specific responses: minimal effects in mothers born growth restricted. *J Physiol*, 596(23), 5947-5964. doi: 10.1113/JP275758. Epub 2018 Jul 26. Chapter 3

Manuscripts in preparation

Cuffe JSM, **Mangwiro YTM**, Zucchelli C, Moritz KM, Briffa JF, Wlodek ME & Wadley GD. The effect of endurance exercise in growth restricted rats on a high-fat diet alters placental oxidation status and telomere length.

Cuffe JSM, Briffa JF, **Mangwiro YTM**, Wadley GD, Moritz KM & Wlodek ME. Endurance exercise and maternal stress in growth restricted rats on a high-fat diet alters placental nutrient transport.

Cuffe JSM, **Mangwiro YTM**, Briffa JF, Moritz KM & Wlodek ME. Expression of novel miRNA transcripts in rat placenta from high-fat fed growth restricted females who performed endurance exercise.

Other manuscripts arising from collaborations during PhD

de Alwis N, Beard S, **Mangwiro YTM**, Binder NK, Kaitu'u-Lino TJ, Brownfoot FC, Tong S & Hannan NJ (2020). Pravastatin as the statin of choice for reducing pre-eclampsia-associated endothelial dysfunction. *Pregnancy Hypertens*, 20, 83-91.

Abstracts

Conference abstracts

Mangwiro YTM, Cuffe JSM, Mahizir D, Anevska K, Romano T, Moritz KM, Briffa JF & Wlodek ME. Maternal exercise alters male placental nutrient transporters with diet and maternal birth weight specific responses, Developmental Origins of Health and Disease Society of Australia and New Zealand, Sydney, Australia, July 2018. ***Poster***

Mangwiro YTM, Cuffe JSM, Briffa JF, Hosseini SS, Mahizir D, Anevska K, Romano T, Moritz KM & Wlodek ME. Exercise initiated during pregnancy reduces blood spaces in male associated placentae, despite increasing placental angiogenic markers, Australian Reproduction Update, Melbourne, Australia, November 2017. ***Invited oral***

Mangwiro YTM, Cuffe JSM, Briffa JF, Mahizir D, Anevska K, Romano T, Moritz KM & Wlodek ME. Sex-specific placental IGF-system adaptations to maternal exercise in growth restricted mothers, Australian Physiological Society, Melbourne, Australia, November 2017. ***Invited oral***

Mangwiro YTM, Cuffe JSM, Briffa JF, Hosseini SS, Mahizir D, Anevska K, Romano T, Moritz KM & Wlodek ME. Exercise initiated during pregnancy reduces blood spaces in males, despite increasing placental angiogenic markers, International Federation of Placenta Associations, Manchester, United Kingdom, August 2017. ***Poster***

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Mangwiro YTM, Briffa JF, Mahizir D, Anevska K, Jefferies AJ, Hosseini SS, Romano T, Moritz KM, Cuffe JSM & Wlodek ME. Differential effects of maternal growth restriction, high-fat feeding and exercise on the placental glucocorticoid barrier, Developmental Origins

of Health and Disease Society of Australia and New Zealand, Canberra, April 2017. ***Invited oral***

Mangwiro YTM, Mahizir D, Anevska K, Briffa JF, Jefferies AJ, Hosseini S, Richter VF, Cuffe JSM, Hryciw DH, Romano T, Moritz KM & Wlodek ME. The impact of exercise and high-fat feeding in growth restricted females on the placental IGF-system and nephron number in male fetuses, Australian Physiological Society, Adelaide, December 2016. ***Invited oral***

Mangwiro YTM, Mahizir D, Anevska K, Briffa JF, Jefferies AJ, Hosseini S, Cuffe JSM, Hryciw DH, Romano T, Moritz KM & Wlodek ME. Impact of growth restriction, high-fat diet and exercise on placental angiogenic and NOX4 mRNA in rats, Society for Reproductive Biology, Gold Coast, Australia, August 2016. ***Invited Oral***

Mangwiro YTM, Mahizir D, Anevska K, Briffa JF, Jefferies AJ, Hosseini S, Cuffe JSM, Hryciw DH, Romano T, Moritz KM & Wlodek ME. Impact of growth restriction, high-fat diet and exercise on placental IGF1 and let-7f-1 in rats, Developmental Origins of Health and Disease Society of Australia and New Zealand, Adelaide, Australia, June 2016. ***Poster***

Published abstracts

Mangwiro YTM, Cuffe JSM, Briffa JF, Hosseini SS, Mahizir D, Anevska K, Romano T, Moritz KM & Wlodek ME (2017). Exercise initiated during pregnancy reduces blood spaces in males, despite increasing placental angiogenic markers. *Placenta*, 57, 329.

Mangwiro YTM, Cuffe JSM, Briffa JF, Mahizir D, Anevska K, Romano T, Moritz KM & Wlodek ME (2017). Sex specific placental IGF-system adaptations to maternal exercise in growth restricted mothers. *Placenta*, 57, 242.

Mangwiro YTM, Cuffe JSM, Briffa JF, Mahizir D, Anevska K, Romano T, Moritz KM & Wlodek ME (2017). Sex-specific placental IGF-system adaptations to maternal exercise in growth restricted mothers, *Proc AuPS*, 123P.

Departmental Seminars

La Trobe University Research Progress Panel Seminar October 2016. Placental and maternal biomarkers: Exercise benefits for overweight female rats born small. ***Oral***

Cambridge University Department of Physiology, Development and Neuroscience Seminar September 2017. The impact of lifestyle interventions on the placenta in pregnancies complicated by maternal growth restriction. ***Invited oral***

Seminar for the Metabolic Research Laboratories at Cambridge University September 2017. The impact of lifestyle interventions on the placenta in pregnancies complicated by maternal growth restriction. ***Invited oral***

Postgraduate Scholarship

La Trobe University Postgraduate Research Scholarship (LTUPRS); March 2015 – July 2018.

Other Awards

YW Loke New Investigator Award ($500 USD), IFPA, Manchester, England, United Kingdom. August 30 - September 02, 2017.

Manchester University Travel award ($500 USD), IFPA, Manchester, England, United Kingdom. August 30 - September 02, 2017.

Travel Grant ($500 AUD), DOHaD ANZ, Canberra, NSW, Australia. April 5 - 6, 2017.

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TABLE OF CONTENTS

ABBREVIATIONS

Actb	β-actin
BCA	bicinchoninic acid assay
BM	basement membrane
BMI	body mass index
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
Crhr	corticotropin releasing hormone receptor
CTB	cytotrophoblasts
DNA	deoxyribonucleic acid
E	Embryonic
ELISA	enzyme linked immunosorbent assay
et al.	and associates
Exercise	exercise before and during pregnancy
F0	initial generation
F1	first generation
F2	second generation
Flt-1	fms related receptor tyrosine kinase 1
GDM	gestational diabetes mellitus
GLUT	glucose transporters
GR	glucocorticoid receptor
HFD	high-fat diet
HSD11β	11β-hydroxysteroid dehydrogenase
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGFR	insulin-like growth factor receptor
IUGR	intrauterine growth restriction
kcal	Kilocalorie
miRNA	micro ribonucleic acid
MR	Mineralocorticoid
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MVM	microvillous plasma membrane
NCD	non-communicable diseases
Nr3c	nuclear receptor subfamily 3 group c member
Ogt	o-linked n-acetylglucosamine transferase
PL	placental lactogens
Plgf	placental growth factor
PN	postnatal day
PregEx	exercise during pregnancy only
PRL	Prolactin
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RT	room temperature
Sedentary	Sedentary
Slc38a	solute carrier family 38 member
Slc3a	solute carrier family 3 member
Slc5a	solute carrier family 5 member
SMP	skim milk powder

SNAT	system a sodium dependent neutral amino acid transporter
Sry	sex determining region y
STB	Syncytiotrophoblast
Tbp	tata box binding protein
TBS	tris-buffered saline
TBST	tris-buffered saline with tween 20
UPI	uteroplacental insufficiency
Vegfa	vascular endothelial growth factor a
WKY	Wistar Kyoto

SYMBOLS

~	Approximately
β	Beta
=	equals
>	greater than
≥	greater than or equal to
<	less than
µ	Micro
%	Percent
±	plus, or minus

UNITS OF MEASUREMENT

°C	degrees Celsius
$\mu g.mL^{-1}$	micrograms per millilitre
µL	micro litre
$day.wk^{-1}$	days per week
g	Gram
hr	Hour
$hr.day^{-1}$	hours per day
kcal	Kilocalorie
$kcal.g^{-1}$	kilocalories per gram
kg	Kilogram
kg/m^2	kilograms per meter squared
$m.min^{-1}$	meters per minute
mg	Milligram
min	Minute
$min.day^{-1}$	minutes per day
$MJ.kg^{-1}$	millijoules per kilogram
mL	Millilitre
$pg.mL^{-1}$	picograms per millilitre
s	Second
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight
x g	relative centrifugal force

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Chapter 1 Review of the Literature

The placenta is a transient but key organ that functions as the only fetal-to-maternal interface during pregnancy. It actively regulates maternal physiology to maintain the optimal exchange of nutrients, gas, and waste between the mother and fetus, which is reliant on placental morphology as well as growth-related pathways and the nutrient transportation system. Interruption of normal placental function can compromise maternal health, inadvertently affecting the ability of the fetus to reach its genetically predisposed growth potential. Therefore, as a consequence of placental dysfunction, fetal intrauterine growth restriction (IUGR) occurs. IUGR accounts for 10% of pregnancies worldwide and is defined as a baby weighing less than the 10th percentile for their gestational age or a term birth weight of less than 2,500 g. In Western populations, uteroplacental insufficiency (UPI) is the main cause of IUGR (Hamilton *et al.*, 2015), which results in altered placental morphology (impaired blood vessel formation (Mayhew *et al.*, 2007) as well as dysregulated placental insulin-like growth factor (IGF) (Laviola *et al.*, 2005), nutrient transportation (Bell & Ehrhardt, 2002; Brett *et al.*, 2014), and stress (Briffa *et al.*, 2017) systems.

Previous research has demonstrated that first generation (F1) IUGR offspring are at a greater predisposition of developing a plethora of diseases, including cardiometabolic and renal diseases, obesity, and osteoporosis (Martin *et al.*, 2017), which is likely in part driven the placenta (Fowden *et al.*, 2008). These outcomes are sex-specific, whereby males have worse outcomes than females (Gallo *et al.*, 2012c; Gallo *et al.*, 2013; Cheong *et al.*, 2016b) even though both sexes present with the same organ deficits.

Pregnancy is the greatest physiological challenge a woman faces in her lifetime, which results in overt alterations to maternal physiology and metabolism that is in part mediated by the placenta. Therefore, it is not surprising that pregnancy in females who were themselves born from a restricted pregnancy (hereafter referred to as IUGR females) unmasks metabolic dysfunction, such as gestational diabetes mellitus (GDM) (Klebanoff *et al.*, 1999; Seghieri *et al.*, 2002; Zetterstrom *et al.*, 2007; Gallo *et al.*, 2012c; Mahizir *et al.*, 2020) , which does not occur in aged virgin counterparts; highlighting that pregnancy is a "second-hit" in IUGR females. Importantly, recent research has well established that diseases mothers have can be passed onto their children, likely via placental programming, in a sex-specific manner (Murray *et al.*, 2001; Moritz *et al.*, 2010). However, there is limited research investigating the F2

placental alterations in pregnant IUGR females that may be mediating this transgenerational disease transmission.

A major challenge to health in the 21st century is the obesity epidemic where, if the current trends continue, it is estimated that 20% of the world's population will be obese by 2030 (Kelly *et al.*, 2008). The increasing prevalence of obesity is fuelled by a sedentary lifestyle, increased consumption of nutritionally dense processed foods, and the boom in economic growth (Schmidhuber & Shetty, 2005). Most importantly, the chronic inflammation that is caused by obesity is associated with adverse placental outcomes, including altered fetoplacental growth pathways and deregulated nutrient sensing pathways; both of which result in adverse fetal growth (Heerwagen *et al.*, 2010; Higgins *et al.*, 2011; Catalano & deMouzon, 2015; Catalano & Shankar, 2017). As IUGR individuals are predisposed to obesity it is, therefore, likely that obesity in F1 IUGR females may unmask or exacerbate alterations in placental development and function, which will consequently alter F2 fetal growth and may program disease.

This highlights the necessity to devise effective and relatively simple interventions to improve maternal health and fetal outcomes in women at risk of pregnancy complications. The use of pharmaceutical therapeutics is limited during pregnancy due to their potential teratogenic effects on the growing fetus. As such lifestyle interventions that have beneficial physiological outcomes in both non-pregnant and pregnant women prove to be much more promising in ameliorating disease outcomes. In non-pregnant individuals it is well known that physical activity or exercise improves cardiometabolic and hypertensive outcomes as well as obesity induced insulin sensitivity and chronic inflammation (Bradley *et al.*, 2008; Yassine *et al.*, 2009). Additionally, exercise during pregnancy has beneficial effects that are both maternally and fetal encompassing. However, the full effects of maternal exercise on fetoplacental outcomes during pregnancy and long-term offspring health are yet to be fully elucidated.

Therefore, the scope of this thesis is to investigate the effect F1 maternal growth restriction has on F2 placental growth factor, nutrient transporter, angiogenesis, and stress-responsive pathways, which independently and/or collectively influence sex-specific fetoplacental outcomes. Furthermore, this thesis characterised whether these changes are exacerbated with high-fat feeding and if maternal exercise can prevent these placental changes.

1.1 The Placenta

The placenta is a transient organ that acts as the functional interface between the mother and fetus during pregnancy. The placenta plays a number of key roles in pregnancy that are pivotal in ensuring optimal fetal growth and organ development. Firstly, the placenta acts an anchor for the growing conceptus to the maternal endometrium, which is essential for pregnancy progression (Woods *et al.*, 2018). Secondly, the placenta supplies the fetus with appropriate nutrients and oxygen from the maternal circulation for optimal fetal growth (Gude *et al.*, 2004). Thirdly, the placenta acts as an immunological and protective barrier as it *i)* prevents the rejection of the conceptus by the maternal immune system, *ii)* prevents pathogens from crossing over the placenta to the developing fetus, and *iii)* enables passive immunity to the fetus via the transport of maternal antibodies (Mor & Cardenas, 2010). Lastly, the placenta can synthesise its own hormones, cytokines, and growth-related factors that are critical to the maintenance of pregnancy and the successful growth of the conceptus (Fowden *et al.*, 2009). It is through these functions that the placenta can modulate maternal metabolic outcomes and, importantly, fetal growth and development via the regulation of the maternal environment. This intimate relationship between the placenta and the maternal environment is initiated when the trophectoderm layer of the blastocyst comes into contact with the maternal endometrium during implantation. This initial contact stimulates a cascade of events, particularly the invasion of the trophoblast cells into the endometrium and the remodelling of the maternal endometrium vasculature, in order to allow embryo/fetal access to the maternal circulation for nutrient and oxygen exchange (Woods *et al.*, 2018).

1.1.1 Placental development

The human placenta is formed via interstitial implantation, which involves the complete embedding of the embryo within the maternal endometrium (Knofler *et al.*, 2019). Between days 5 to 6.5 post-fertilisation the blastocyst attaches to the uterine epithelium at the embryonic pole and the blastocyst trophectoderm layer commences differentiation and proliferation (Moore *et al.*, 2016), which results in the formation of two cell layers. The first layer is formed by the specialised inner cell layer of cytotrophoblast (CTB) cells and the second, outer cell layer, is produced by a group of CTB cells that lose their membrane and form a protoplasmic multinucleated mass by fusing their cytoplasm's to form the syncytiotrophoblast (STB) layer (**Figure 1.1a**). These early CTB and STB cells have been shown to differ phenotypically and functionally in comparison the population found in the late gestation mature placenta (Yabe *et al.*, 2016). The STB allows the apposition of the blastocyst (free of the zona pellucida) to the

maternal endometrium wall as this is the only embryonic tissue that comes into contact with maternal cells at this stage. At the site of apposition, the adhesion process of implantation commences ushered by the increased release of hormones, such as progesterone and cytokines, by the endometrium to increase blastocyst receptivity. Additionally, the STB allows for further intrusion into the maternal endometrium by eroding and invading the endometrial stroma through the degradation of the local extracellular matrix, increasing the anchorage of the blastocyst to the maternal endometrium (Pijnenborg *et al.*, 1981) (**Figure 1.1a**). Following adhesion, invasion of the blastocyst into the endometrium occurs with the formation of junctional complexes with the lateral borders of the endometrium epithelial cells by the STB. The STB basal lamina continuously releases cytokines to induce apoptosis within the endometrium and continues its mass expansion. As a result, approximately 9-10 days post-fertilisation, anastomoses occur to form sinusoidal blood-filled spaces known as lacunar (Bernirschke & Kaufmann, 1990; Smarason *et al.*, 1993), which are precursors of the uteroplacental circulation (Burton *et al.*, 1999). More lacunae form with further invasion of the STB into the endometrium and adjacent lacunae fuse to form lacunae networks (**Figure 1.1a**). Between 11- and 13-days post-fertilisation, CTB cells proliferate and form finger like projections that rapidly project into the STB to form villi (primary placental villi), which are bathed by the maternal blood in the lacunar (Bernirschke & Kaufmann, 1990; Smarason *et al.*, 1993). Shortly thereafter (between 15 to 16 days post-fertilization) the secondary villi are created, through transformation of the primary villi facilitated by the invasion of the extraembryonic mesenchymal cells (Bernirschke & Kaufmann, 1990). By 21 days post-fertilisation, the tertiary villi are formed via the differentiation of mesenchymal cells of the secondary villi into connective tissue forming a villous branching network as well as vascularization forming fetal blood capillaries that are connected to the embryo. In addition to the formation of chorionic villi, invasion of CTB cells into the extra villous trophoblast is a critical step in establishing the uteroplacental villous circulation. CTB invasion encourages the transformation of maternal spiral arteries from high to low resistance vessels facilitating increased placental and, hence, fetal blood flow. 21 days post-fertilisation, the anatomical arrangements of the primordial placenta are established, which allows for the exchange of oxygen and nutrients between mother and fetus. After these processes occur, a complex vascular network is established in the placenta that facilitates the production of hormones, nutrients, and waste exchange. The establishment of the placental vascular bed is completed by the end of the 18th week of pregnancy, with continued vascular remodelling occurring at an

exponential rate throughout the rest of gestation via the process of capillary loop elongation (Burton *et al.*, 2009).

The fully formed human placenta is a haemochorial type placenta that is discoid (circular) shaped with a diameter between 20-22 cm and a central thickness of 2.5 cm at term, which covers 15-30% of the endometrial surface. The fetal chorionic plate allows the insertion of the umbilical cord and contains chorionic vessels that form the villous tree, which carries fetal blood enriched with nutrients and oxygen to the fetus (Huppertz, 2008). The maternal placental surface is referred to as the basal plate and is a mixture of fetal extravillous trophoblasts and uterine decidua that are subdivided into cotyledons by the placental septa (Huppertz, 2008).
The accessibility of model organisms to modern research has enriched the field of biomedical science allowing a platform to study the fundamental physiological and pathological processes of the human body. While the function of the placenta is conserved in all mammalian species, it is important to consider that structural and endocrine differences exist between humans and animal models, which need to be considered when drawing conclusions from rodent studies in relation to human function. As this thesis utilized a rodent model, placentation in the rat is described in detail below.

1.1.2 Development of the rat placenta

In rodents, the blastomere is formed following fertilisation that comprises of the trophectoderm (becomes the placenta) and the inner cell mass (becomes the embryo). The blastomere moves towards the maternal endometrium and, with the release of cytokines and chemokines, it invades the maternal endometrium through the process of adhesion for implantation to occur (Sood *et al.*, 2006). The outer layer of the blastomere, the trophectoderm, is then further divided and replicated into two main layers: the mural trophectoderm and the polar trophectoderm. It is from the mural trophectoderm that the primary trophoblastic giant cells originate from following cell cycle arrest and differentiation. The polar trophectoderm, on the other hand, forms the chorion that differentiates into the trophoblast cells that, in conjunction with the mesoderm from the extraembryonic ectoderm, become the vascular endothelium of the labyrinth zone; the main site for nutrient exchange in the rodent (**Figure 1.1b**). From the polar trophectoderm an ectoplacental cone is formed that the spongiotrophoblast, trophoblastic giant cells, and glycogen cells originate from that become the basal, or junctional, zone of the placenta (**Figure 1.1b**). The glycogen cells form multiple cell masses that develop into islands of glycogen storage cells that mostly disappear prior to parturition. The maternal region of the

placenta is comprised of endometrial stromal cells, that form the decidua and the metrial gland. It is from the decidual cells that surrounded the blastomere that the primary decidual zone is formed, which is avascular and later forms a secondary decidual zone. This secondary decidual zone progressively degenerates and is reduced to a small thin layer with the mesometrial decidua cells at the base of the placenta, which is referred to as the decidua basalis and is an important site of maternal angiogenesis. Following the establishment of the placental layers, the placenta continually grows to meet the demands of the growing embryo. Similarly, to the human placenta, the fully formed rat placenta is discoid in shape and is of a hemochorial classification, more specifically hemotrichorial as it contains three cell layers: two STB layers and one CTB cell layer separating the maternal from the fetal capillaries.

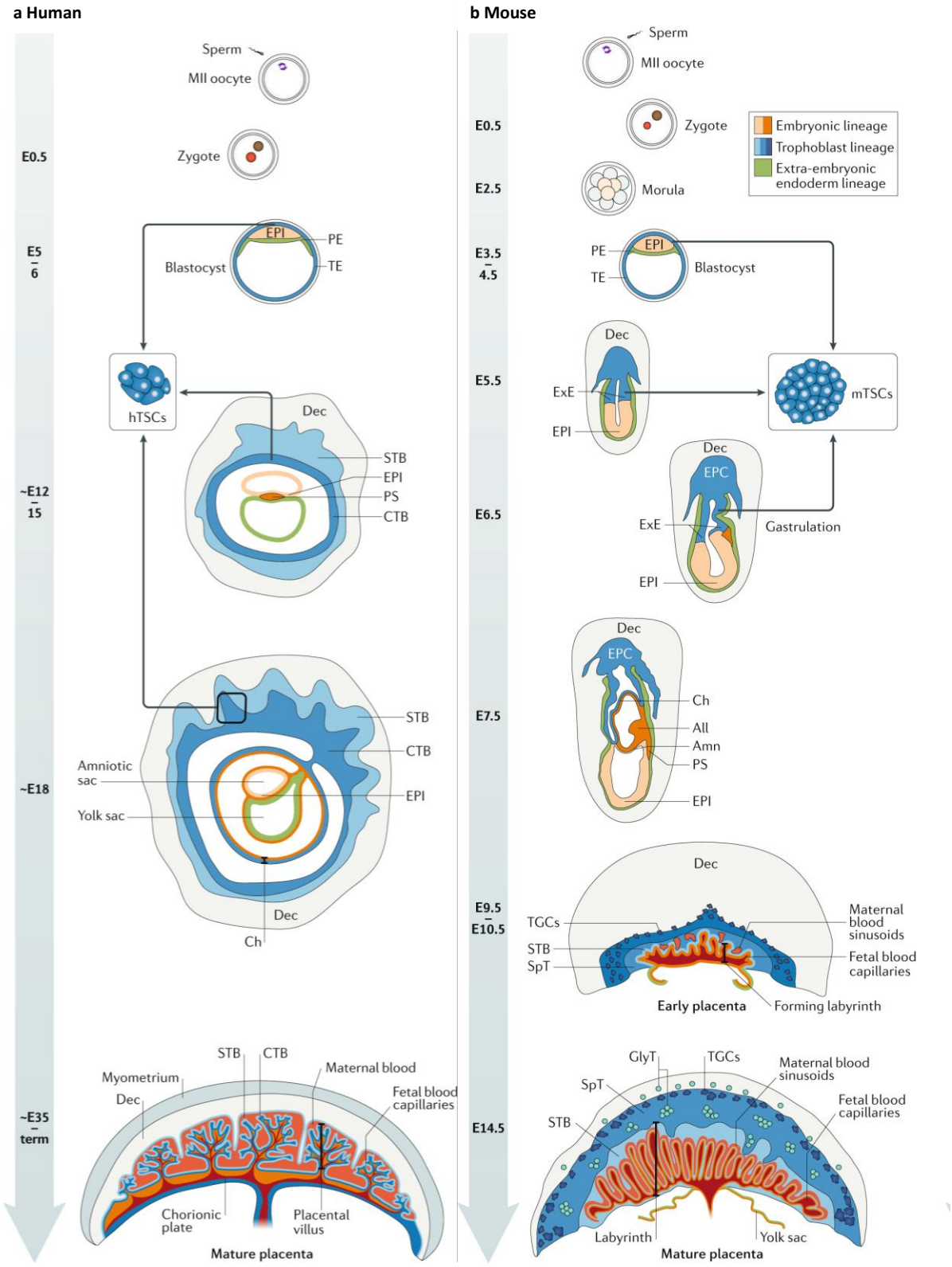


Figure 1. 1 Human and Rodent (mouse) placental development.

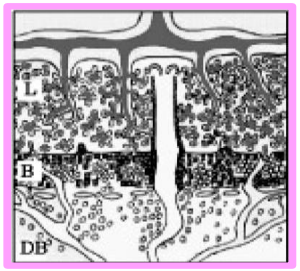
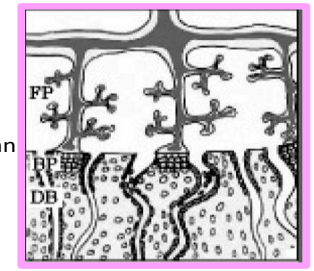
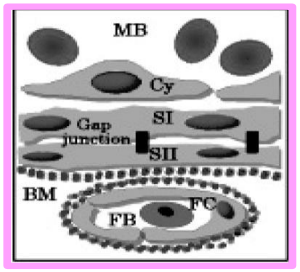
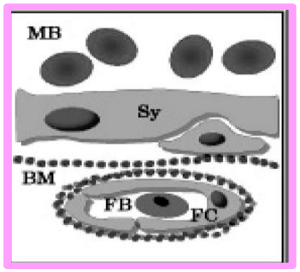
Illustration of mouse (a) and human (b) placental development throughout gestation adapted from Hemberger *et al.* (2020). All – allantois; Amn – amnion; Ch – chorion; CTB – cytotrophoblast; Dec – decidua; EPI – epiblast; EPC - ectoplacental cone; ExE – extraembryonic ectoderm; GlyT - glycogen trophoblast; hTSCs - human trophoblast stem cells mTSCs - mouse trophoblast stem cells; PE – primitive endoderm; PS – primitive streak; SpT – spongiotrophoblast; STB – syncytiotrophoblast; TE - outer trophectoderm; TGCs - trophoblast giant cells.

1.1.3 Comparison between the rat and human placenta

While rodent experimental models have been invaluable in evaluating the potential dangers that lie behind the administration of certain medications or drugs on the human reproductive system and their offspring when taken during pregnancy, not all researchers are in consensus with the use of rodents as models of human placental function due to concerns of evolutionary divergence. In addition to the structural differences in cells layers between rats and humans, there are differences in implantation with eccentric implantation occurring in the rat whereas interstitial implantation occurs in humans. The most important difference is in placental endocrine function, which limits the level at which we can cross compare between the rat and human placenta. Specifically, estrogen biosynthesis differs between humans and rats, which limits cross comparisons between chemically induced developmental aberrations or placental toxicity related to placental endocrine function. Differences also exist in the organisation of the vasculature within the rat placental labyrinth versus the human villous tree; however, functionally nutrient supply to the growing fetus is similar (**Table 1.1**). Despite these differences, the progression and succession of the main placental developmental events is similar and, therefore, the rat placenta is an appropriate model to investigate the impact of several experimental manipulations.

Table 1.1 Comparisons between the rat and human placenta.

Adapted from Furukawa *et al.* (2011).

	Rat	**Human**
Implant type	**Eccentric Implantation**	**Interstitial implantation**
Chorio-allantoic placenta	<u>Labyrinth Type</u> • Labyrinth Zone (LZ) • Syncytiotrophoblast • Cytotrophoblast • Basal Zone (B) • Spongiotrophoblast • Glycogen cell • Trophoblast giant cell • Decidual basalis (DB) 	Villous Type • Fetal Placenta (FP) • Syncytiotrophoblast • Basal plate (BP) • Cytotrophoblast cell column • Interstitial trophoblast • Decidua basalis(DB) 
Placental Barrier	Maternal blood (MB) ↓ Cytotrophoblast (Cy) ↓ Syncytiotrophoblast I (SI) ↓ Syncytiotrophoblast II(SII) ↓ Basement membrane (BM) ↓ Fetal Capillary (FC) ↓ Fetal Blood (FB) 	Maternal blood (MB) ↓ Syncytiotrophoblast I (Sy) ↓ Basement membrane (BM) ↓ Fetal Capillary (FC) ↓ Fetal Blood (FB) 
Trophoblast associated vascular remodeling	Vascular remodeling effected by endovascular trophoblasts in metrial gland	Vascular remodeling effected by endovascular and interstitial trophoblast in metrial gland
Placental steroidogenesis	The placental synthesizes androgen from cholesterol. Estrogen is produced in the ovary due to the absence of aromatase	The Placenta synthesizes pregnenolone from cholesterol. Estrogen is produced in the placenta after the fetal adrenal gland converts dehydroepiandrosterone due to the absence of CYP17
Glycoprotein hormone synthesis	Glycoprotein hormone is not produced due to not producing LH/HCG or chain	HCG is produced in the placenta

1.2 Factors Influencing Placental Development and Function

The process of placental formation is highly regulated, which involves pathways that mediate trophoblast proliferation, differentiation, and invasion that are influenced by a myriad of hormones and ligands (Demir *et al.*, 1997; Cochard, 2002; Demir *et al.*, 2007). Placental growth factor, angiogenesis, vasculogenesis, nutrient transportation, and stress pathways play a key role in modulating fetoplacental growth, especially during late gestation (**Figure 1.2**). It is, therefore, not surprising that these systems work in unison to facilitate normal fetal growth and development.

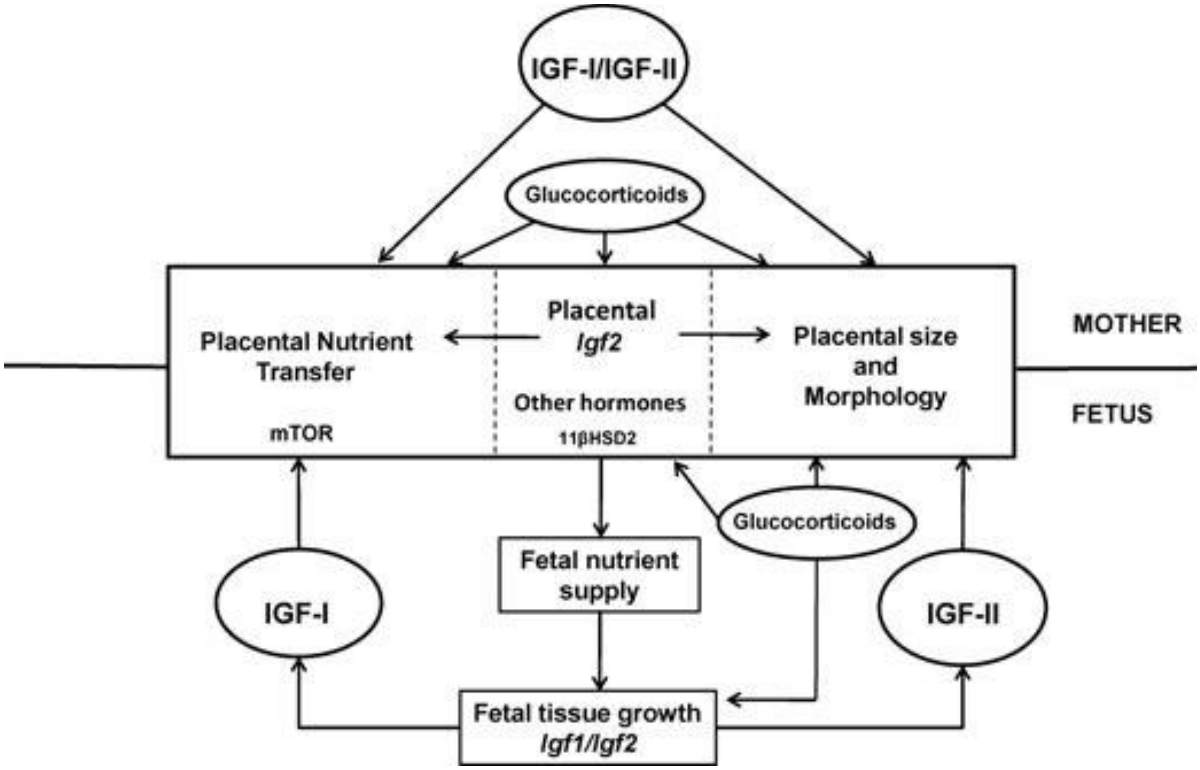


Figure 1. 2 Role of maternal, placental, and fetal influences on fetoplacental outcomes.

Schematic diagram demonstrating the regulation of placental and fetal outcomes by the growth factor, nutrient, and glucocorticoid pathways (Fowden *et al.*, 2009). 11βHSD2 - 11β-hydroxysteroid dehydrogenase type 2; IGF – insulin-like growth factor; mTOR – mammalian target of rapamycin.

1.2.1 Placental growth factors

The peptide hormones IGF1 and IGF2 mediate a number of mitogenic and metabolic processes by acting on their receptors (IGF1R and IGF2R), which are located on the surface of target cells and tissues (Baxter, 1991; Clemmons, 1991; Irwin *et al.*, 1999). As IGF1 and IGF2 are homologous single chain peptides there is an overlap in the binding of the ligands on the IGF receptors. Specifically, IGF1 has a higher affinity to its cognate receptor (IGF1R), whereas IGF2 can bind to IGF1R, at a comparable affinity to IGF1, and also its cognate receptor IGF2R (Baxter, 1991; Clemmons, 1991; Irwin *et al.*, 1999). These peptides, and their downstream signalling pathways, are important for normal fetal and placental development. Specifically, both peptides are synthesised by the placenta and fetus, and are essential for placental development, cell migration, and nutrient transfer to the growing fetus (Roberts *et al.*, 2001). The endocrine and autocrine actions of the IGF receptors have been demonstrated in both *in vivo* and *in vitro* models (DeChiara *et al.*, 1990; Baker *et al.*, 1993; Liu *et al.*, 1993; Woods *et al.*, 1996; Lupu *et al.*, 2001) where they play a key role in regulating fetal growth. Specifically, ablation of IGF1R in mice results in a 45% reduction in fetal growth (Woods *et al.*, 1996; Rother & Accili, 2000), which is primarily due to the actions of IGF1; where IGF1 knockdown reduces fetal, but not placental, weight. These studies suggest that IGF1, acting via IGF1R, plays a key role in modulating fetal growth, whereas IGF2 is primarily responsible for placental growth (Sibley *et al.*, 2004; Constancia *et al.*, 2005). This is further supported by a 24% reduction in placental weight in *Igf2*$^{-/-}$ mice as well as a reduction in labyrinth exchange barrier thickness (Constancia *et al.*, 2002; Sibley *et al.*, 2004; Fowden *et al.*, 2006a). IGF2 has also been established to play a key role in trophoblast invasion, likely acting via IGF2R (Harris *et al.*, 2011). Collectively these data suggest that the IGF ligands play a key role in the establishment of the placental bed, which is imperative for fetal growth and organ development (**Figure 1.3**).

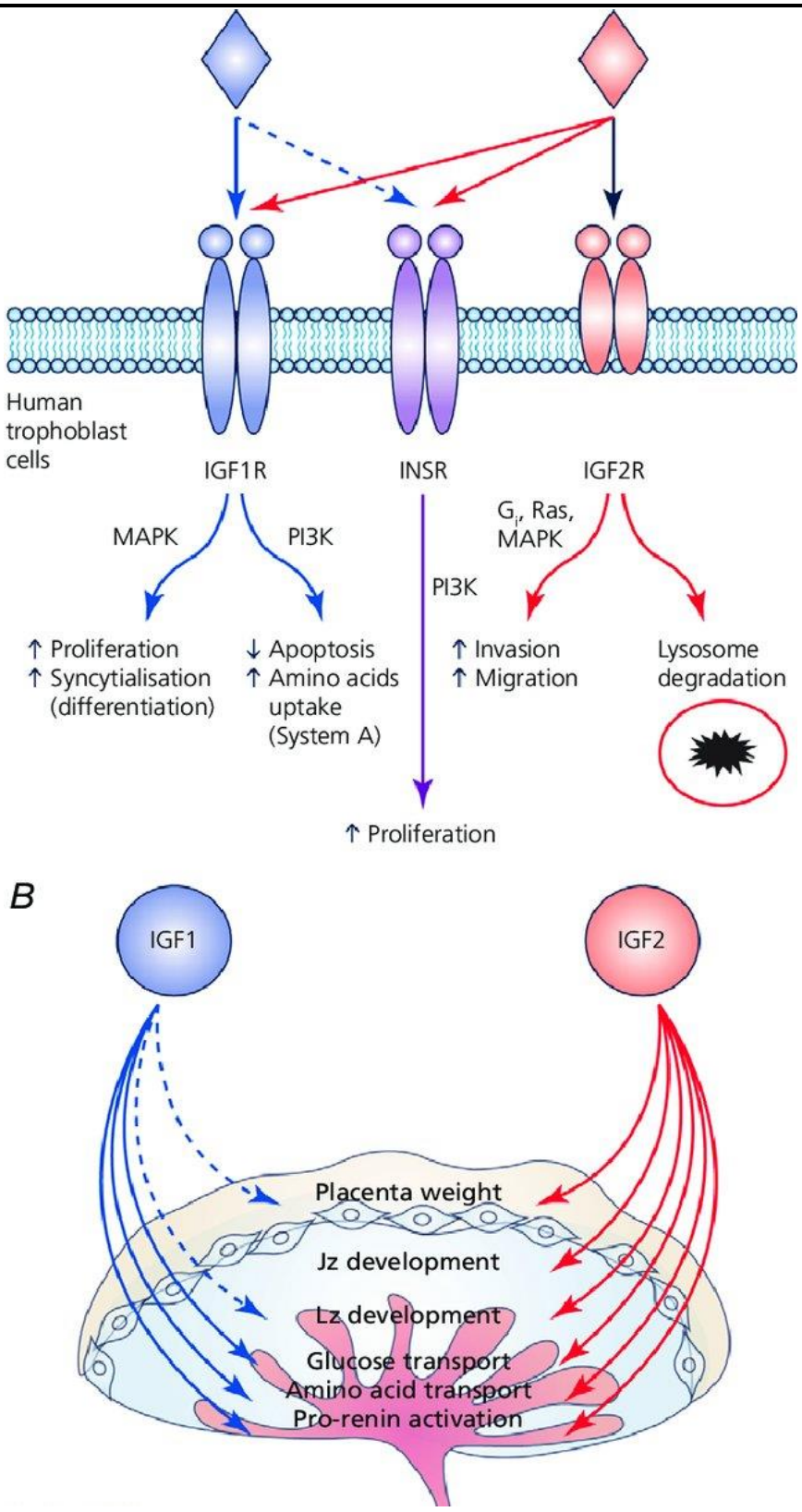


Figure 1. 3 IGF-system placental signalling.

Illustration of the insulin-like growth factor (IGF) system showing the proposed signalling pathways (A) and the effect of exogenous maternal IGFs on the rodent placenta (B) *in vivo*. Figure adapted from Sferruzzi-Perri *et al.* (2017). INSR – insulin receptor; JZ – Junctional zone; LZ – labyrinth zone; MAPK – mitogen activated protein kinase; PI3K - phosphatidylinositol-3-kinase.

1.2.2 Placental nutrient transporters

As the fetus is unable to source or synthesise its own nutrients for organ growth and development its nutrient supply is dependent on the efficiency of the placenta to facilitate nutrient transfer from the maternal circulation to the fetus. Placental nutrient transportation occurs via active and passive nutrient transporters located on the trophoblast membranes whose expression are dependent on maternal and fetal signals (Jansson, 2001; Baumann *et al.*, 2002) as well as nutrient metabolism by the placenta itself. As this thesis is investigating only glucose and amino acid nutrient transporters, they will be the focus of the below sections.

1.2.2.1 Glucose transport

Glucose is the primary energy substrate for the fetus during gestation and the fetus is entirely dependent on maternal glucose supply for survival (Baumann *et al.*, 2002). It is, therefore, not surprising that placental glucose uptake increases with advancing gestation to coincide with accelerating fetal growth. Glucose is transported into and across the placenta by facilitated carrier mediated diffusion via glucose transporters (GLUT) that occurs down a concentration gradient; with fetoplacental glucose influx and glucose availability being dependent on the expression and function of the placental glucose transporters. In humans, the microvillous plasma membrane (MVM) contains a higher proportion of glucose transporters allowing for the increased transfer rate of glucose into the STB, with a lower concentration of glucose transporters located on the basal membrane (BM). GLUT1 and GLUT3 are the primary glucose transporters in both human and rodent placentae, with GLUT1 expression increasing with advancing gestation (Ericsson *et al.*, 2005b) and GLUT3 expression is the highest during early gestation (Brown *et al.*, 2011). Studies demonstrate that the regulation of these glucose transporters differs across trimesters, which is likely, in part, due to changes in other ligand concentrations that have been shown to regulate GLUT1 and GLUT3 expression in *in vitro* cultured trophoblast cells, such as growth hormone (Ericsson *et al.*, 2005a), corticotropin-releasing hormone (Gao *et al.*, 2012) and IGF-1 (Baumann *et al.*, 2014). Steroid hormones and insulin have also been demonstrated to influence glucose transporter expression, which can influence placental and birth weight outcomes (Hahn *et al.*, 1999; Acevedo *et al.*, 2005; Lappas *et al.*, 2012). Furthermore, there is a higher concentration of GLUT1 on the MVM compared to the BM thus suggesting the rate limiting step of glucose transport occurs at the BM (Jansson *et al.*, 1993). Whereas the MVM of the SCTB is the primary location of GLUT3 expression (Brown *et al.*, 2011), thus allowing a higher and faster rate of glucose transport into the placenta as GLUT3 has a higher affinity to glucose compared to GLUT 1 (Simpson *et al.*, 2008).

1.2.2.2 Amino acid transport

In addition to glucose, amino acids are the building blocks required for the development of placental and fetal tissues (Lin *et al.*, 2014). Placental transport of amino acids is regulated by active transportation, which is regulated by a number of systems (Regnault *et al.*, 2002) as fetal concentrations of amino acids are higher than maternal concentrations. There at least 15 different amino acid transporter systems in human placentae, all of which have overlapping substrate specificity in both the MVM and BM. In particular, the system A sodium dependent neutral amino acid transporters (SNAT) are the most characterised of the amino acid transportation systems and are essential for fetal growth (**Figure 1.4**). The SNATs are highly polarized in the MVM and, importantly, their presence is essential for the transport of essential amino acids such as glycine. Of the SNATs, SNAT4 has been demonstrated by Desforges and colleagues to be highly expressed in MVM and villous tissues in the first trimester compared to the third trimester (Desforges *et al.*, 2009). Whilst in term placenta, SNAT1 is the major contributor of amino acid transportation (Desforges *et al.*, 2010).

1.2.2.3 Mammalian target of rapamycin

The expression of both glucose and amino acid transporters are largely regulated by the mammalian target of rapamycin (mTOR) signalling pathway, which is a master regulator of cell growth, insulin-like growth factors, amino acid and cellular metabolism pathways(Saxton & Sabatini, 2017). mTOR exits as two complexes, mTORC1 and mTORC2, that are located in the STB, with most studies focusing on mTORC1 as it regulates the placental nutrient sensing network to control cellular metabolism and growth (Howell & Manning, 2011). However, the role of mTORC2 is yet to be fully elucidated in placental nutrient transport and regulation. Interestingly, mTORC1 is stimulated by an array of upstream signalling systems including amino acids, glucose as well as growth factor systems and can modulate the expression of nutrient transporters (**Figure 1.5**) (Roos *et al.*, 2007). Specifically, the mTORC1 complex influences, at the post transcriptional level, the trafficking and cell surface availability of amino acid transports on the trophoblast (Rosario *et al.*, 2016a). mTOR and its downstream effector p70-S6 kinase 1 (p70S6k1) are believed to the regulatory link between the activity of system-L amino acid transporters and the concentration of nutrients and growth factors. Furthermore, the activation of mTOR via nutrient regulation has been also demonstrated to result in the inhibition of PI3-kinase signalling of insulin and IGF1 (Harrington *et al.*, 2005). Thus, highlighting that mTOR, more specifically mTORC1, is a key regulator in placental nutrient transportation.

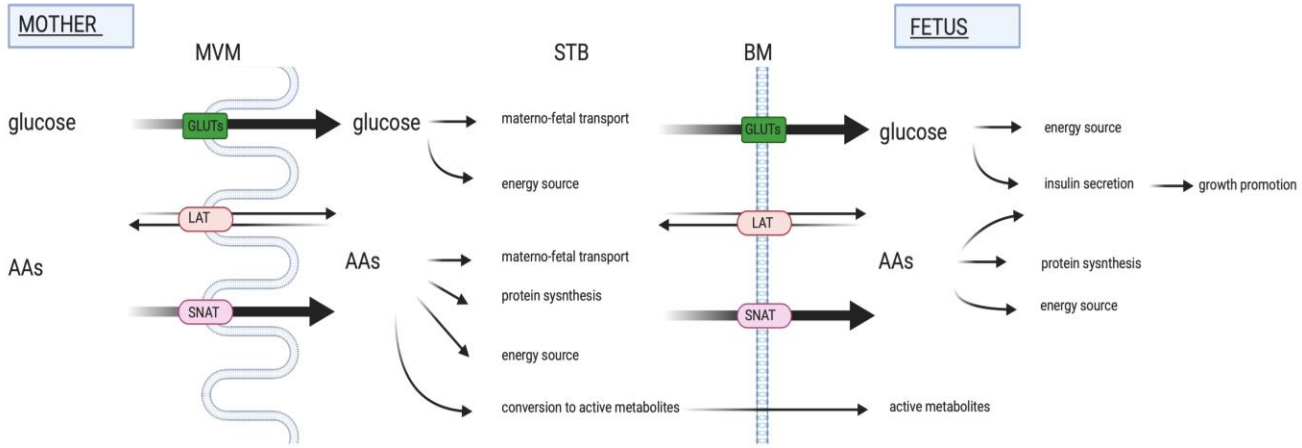


Figure 1. 4 Placental nutrient transport.

The syncytiotrophoblast (STB) expresses a number of transporters, including glucose (GLUT) and amino acid transporters. The system a sodium dependent neutral amino acid transporter (SNAT) are responsible for the transport of amino acids to the growing fetus. Figure adapted from Staud & Karahoda (2018) created with BioRender.com. AA – amino acid; BM – basal membrane; LAT - L-amino acid transporters; MVM - microvillous plasma membrane.

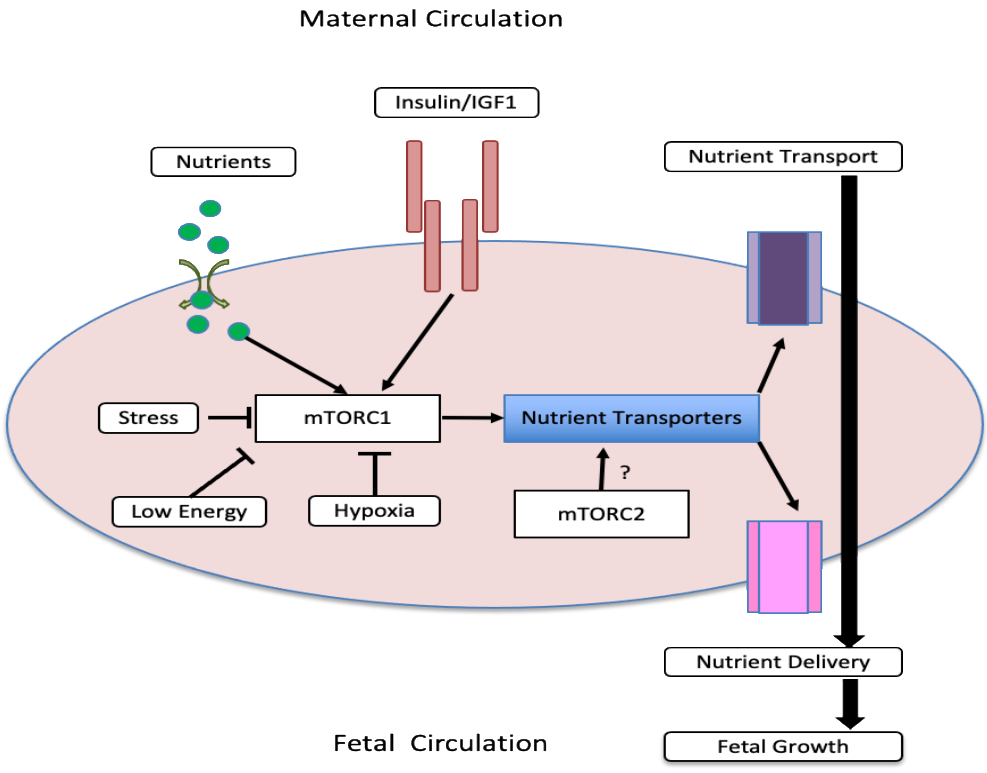


Figure 1. 5 Placental mTOR signalling.

Illustration of placental mammalian target of rapamycin (mTOR) modulation by its upstream regulators. Adapted from Roos *et al.* (2009) mTORC1, mechanistic target of rapamycin complex 1; mTORC2, mechanistic target of rapamycin complex 2; IGF1, insulin-like growth factor 1.

1.2.4 Placental glucocorticoid barrier

As previously mentioned, the placenta acts a protective barrier between the mother and fetus. The placental glucocorticoid barrier is comprised of, and is predominantly regulated by, the placental 11β-hydroxysteroid dehydrogenase (HSD11β) enzymes. HSD11β2 catalyses the conversion of cortisol (corticosterone in rodents) into cortisone (11-dehydrocorticosterone in rodents), which protects the fetus from the deleterious effects of excessively high maternally derived glucocorticoids. Whereas HSD11β1 plays the reductase role that involves the reconstitution of the inactive forms (cortisone/11-dehydrocorticosterone) of the steroids back into their active forms (cortisol/corticosterone). During late gestation, HSD11β2 expression increases almost 50-fold in humans and peaks at term (McTernan *et al.*, 2001), which is associated with the period of increased fetal growth. This finding is in contrast to what is observed in the rodent, whereby HSD11β2 expression is reduced within the labyrinth during late gestation (Burton *et al.*, 1996), which may appear to be counterintuitive as excess corticosteroids can negatively affect fetal growth. However, late gestation is the period in fetal development where the adrenals are mature and fully functional (Ishimoto & Jaffe, 2011) and fetal tissues (such as the kidneys and liver) express HSD11β2, which can regulate fetal steroid concentrations independently of the placenta (Stewart *et al.*, 1994). Therefore, this reduction in HSD11β2 expression in the rat placental labyrinth allows for increased maternal corticosterone transfer to the fetus to enable fetal organ maturation, whilst fetal expression of HSD11β2 maintains steroid concentrations at physiological conditions. Studies have additionally demonstrated that the glucocorticoid (GR) and mineralocorticoid (MR) receptors (NR3C1 and NR3C2, respectively) are regulated by HSD11β2 as well as maternal progesterone concentrations during late gestation. Specifically, *Hsd11b2*$^{-/-}$ mice have reduced *Nr3c1* abundance in the placenta and fetal brain (Mark *et al.*, 2009); suggesting that dysregulated HSD11β2 has the potential to affect both the placental and fetal steroid and glucocorticoid milieu. Furthermore, studies suggest that the role of HSD11β2 is not limited to glucocorticoid barrier function, whereby *Hsd11b2*$^{-/-}$ mice have impaired placental nutrient transportation with reduced fetoplacental weight (Wyrwoll *et al.*, 2009).

Overall, this collectively demonstrates the importance of the placenta in modulating fetal outcomes by facilitating an optimal supply of nutrients and growth factors, which are essential for fetal growth and development. Therefore, it is not surprising, that any disruptions in these highly regulated processes will alter fetal growth and development outcomes, which may program long-term disease.

1.3 Developmental Origins of Health and Disease

Genes and maternal lifestyle factors are key determinants of fetal growth and long-term offspring health (Schousboe *et al.*, 2003; Godfrey *et al.*, 2017). The fetal origins of health and disease hypothesis, introduced by David Barker and colleagues in 1989, proposes that insults during critical stages of fetal development program poor fetal growth and an increased susceptibility of the baby developing cardiometabolic and kidney diseases due to organ deficits and dysfunction (**Figure 1.6**) (Barker *et al.*, 1989a; Barker *et al.*, 1989b). Indeed, the Hertfordshire study was pivotal in the field, which identified that males with poor fetal and infant growth had an increased risk of developing ischemic heart disease (Barker *et al.*, 1989b); an outcome that has since been replicated by a plethora of epidemiological studies from different populations and ethnic groups (Hales & Barker, 1992; Hardy *et al.*, 2004). Experimental studies have since expanded on these findings to demonstrate that numerous maternal pregnancy insults (undernutrition, GDM, preeclampsia) impact on *in utero* growth and long-term offspring health in which the placenta is believed to play a key role (Barker *et al.*, 1990; Langley & Jackson, 1994; Woodall *et al.*, 1996b; Jansson & Lambert, 1999; Khan *et al.*, 2005).

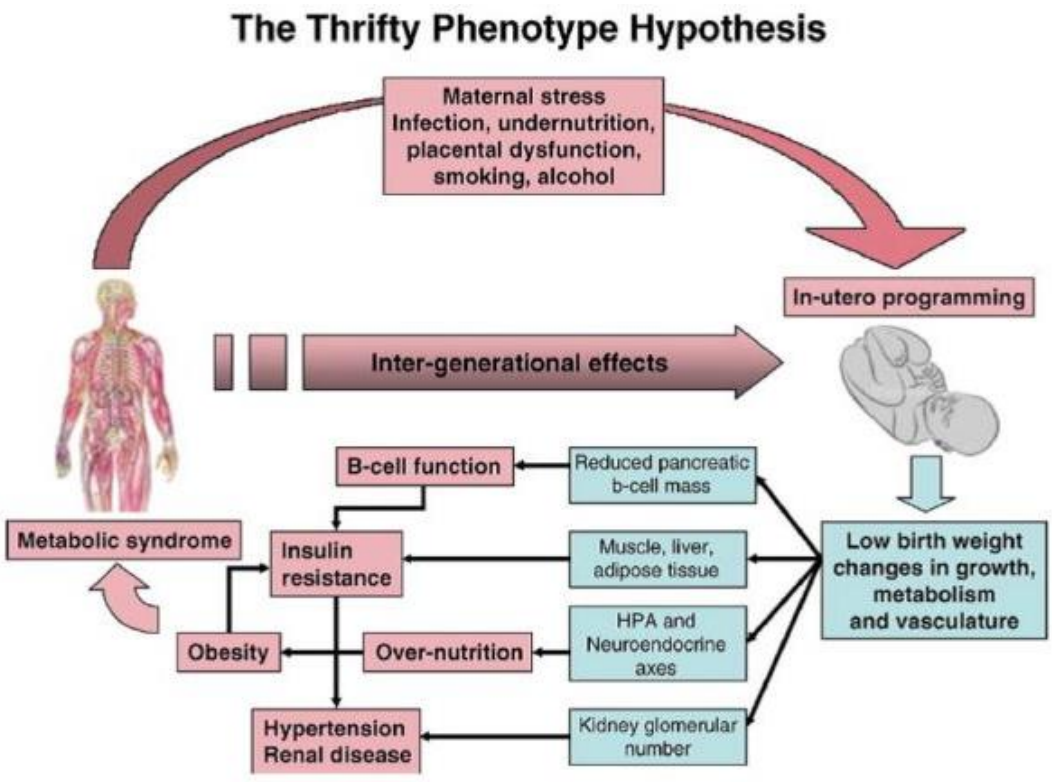


Figure 1. 6 Thrifty phenotype hypothesis.

Schematic illustrating the programming effects of a subpar maternal *in utero* environment of fetal weight and the link between a low birth weight and adult disease programming. Taken from Fernandez-Twinn & Ozanne (2006).

1.4 Intrauterine Growth Restriction

Intrauterine growth restriction is defined as a fetal weight of less that the 10th percentile for gestational age, which is caused by a pathological process affecting the predetermined genetical growth of the fetus and often results in the fetus being born small (Lausman *et al.*, 2013). Being born small is defined as weighing less than the 10th percentile for gestational age (less than 2,500 g at term) or lower than 2 standard deviations below the population mean (Wollmann, 1998; Lee *et al.*, 2003), which accounts for ≥10% of births in Western populations (Hamilton *et al.*, 2015). There are two defined forms of fetal growth restriction: symmetrical and asymmetrical growth restriction. Symmetrical growth restriction occurs in the first and second trimester of pregnancy, which affects 20-30% of IUGR pregnancies and results in whole body growth restriction (Wollmann, 1998). This is due to an overall decrease in cellular proliferation in the fetuses body organ systems as a response to reduced nutrient flow during critical periods of cellular growth and differentiation. Asymmetrical growth restriction, on the other hand, occurs during late gestation (third trimester) and affects 70-80% of IUGR pregnancies, which is associated with a reduced ponderal index (Wollmann, 1998); the overall reduction in weight of every organ system in relation to the head (head sparing). Asymmetrically IUGR children are more likely to experience catch-up growth in early postnatal years and tend to have worse health outcomes in later life (Wollmann, 1998; Kramer *et al.*, 2014). A number of factors are associated with the aetiology of IUGR including genetics, smoking, alcohol consumption during pregnancy, drug abuse, maternal stress, and maternal malnutrition (Ng *et al.*, 2014). In developing countries, maternal undernutrition is the predominant cause of IUGR (Haggarty *et al.*, 2002; Jansson *et al.*, 2006), which epidemiological and experimental models have demonstrated causes fetal growth restriction through altered placental nutrient transport (Malandro *et al.*, 1996). Available evidence indicates that the placenta and fetus are much more sensitive to nutrient deficiencies, such as protein and micronutrients, during the perinatal and placental implantation period. For example, a study in rural Gambian women that go through an annual 'hungry' season demonstrated that this chronic negative energy balance reduces nutrient transfer to the growing fetus, which reduces birth weight by 200-300 g (Moore *et al.*, 2001). In rodent models, dietary manipulation has been utilised to characterise the IUGR outcomes associated with reduced maternal caloric intake. Specifically, maternal protein restriction (8.5% *vs* 19% protein diet) in rats throughout gestation reduces fetal weight and programs adult offspring hypertension (Woods *et al.*, 2001); a finding that has been replicated in numerous other animal models and

organ systems (Coan *et al.,* 2010;Jansson *et al.,* 2006;Holemans, 2003). Although models of maternal undernutrition have provided important evidence on the fetal outcomes associated with growth restriction, these models do not appropriately discuss what occurs in Western population as the major cause of IUGR is UPI, which is mediated by the placenta.

1.4.1 Uteroplacental insufficiency

UPI affects ~8% of pregnancies and is characterised by a reduction in nutrient and oxygen supply to the growing fetus, which arises due to incomplete trophoblast invasion during placentation leading to placental vessel maladaptation (Ogilvy-Stuart & Beardsall, 2020). Some causes of UPI include maternal genetics, smoking in pregnancy, maternal hypertension, undernutrition, and diabetes (Ganguly *et al.,* 2016). Of major concern is the fact that up to 60% of cases of UPI are idiopathic, thus making it difficult to diagnose prior to onset for earlier intervention (Hoffman & Bakketeig, 1984). As a consequence of the reduced nutrient and oxygen perfusion in the placenta fetal growth is stunted, which results in IUGR. The developmental adaptations that occur in response to the reduced nutrient and oxygen supply due to UPI result in the programming of diseases that present themselves in adult life. A major consequence of fetal growth restriction resulting from UPI is the increased incidence of adult diseases including cardiovascular disease, metabolic disease, and neurological dysfunction in IUGR infants (Scherjon *et al.,* 1993; Barker, 1999). The fetal adaptations to UPI primarily include the redirection of blood flow to major organs such as the heart, brain, and adrenals (Harding *et al.*, 2000). In humans UPI results in myocardial and adrenal hypertrophy, outcomes which have been also demonstrated in sheep models of UPI (Murotsuki *et al.*, 1996; Fouzas *et al.*, 2014). In the placenta, studies in humans during the third trimester indicate that placental blood flow (perfusion) is reduced in most pregnancies resulting in IUGR (Lunell *et al.*, 1979; Lunell & Nylund, 1992). With additional human functional and pathological studies demonstrating that in UPI placental vascular impedance is increased in 60% of cases and it is accompanied by pathophysiological placental changes including altered spiral artery remodelling and atherosis resulting in placental lesions (Gerretsen *et al.,* 1981). It is not, therefore, surprising that infants at the 3rd birth weight percentile are chronically hypoxic (Helwig *et al.,* 1996). Therefore, IUGR affected placentae due to UPI have altered growth factor expression, stress responses as well as pathological placental changes including altered spiral artery remodelling.

Although human studies provide insightful knowledge on the outcomes associated with UPI, they are limited in the extent by which interventions are possible to characterise pregnancy disease aetiology due to obvious ethical considerations. UPI is induced in animal models using numerous interventions, which reduces fetal growth and allows for the detailed investigation into the developmental programming of adult diseases (de Grauw *et al.*, 1986; Woodall *et al.*, 1996a; Wlodek *et al.*, 2007; Siebel *et al.*, 2008; Wlodek *et al.*, 2008; Wlodek *et al.*, 2009; Mazzuca *et al.*, 2010; Siebel *et al.*, 2010; Nassar *et al.*, 2012). Wigglesworth pioneered the surgical model of UPI by ligating the uterine vessels in rats to reduce blood flow and, consequently, oxygen and nutrients delivery to the growing fetus (Wigglesworth, 1964). Our laboratory has utilised the same model, where bilateral uterine vessel ligation surgery is performed during late gestation (day 18 of a 22-day gestation) in Wistar Kyoto (WKY) rats (Wlodek *et al.*, 2007; Siebel *et al.*, 2008; Wlodek *et al.*, 2008; Wlodek *et al.*, 2009; Mazzuca *et al.*, 2010; Siebel *et al.*, 2010). Our model mimics human UPI as the offspring are born 10-15% smaller (Wlodek *et al.*, 2005), present with organ deficits, and have sex-specific glucose intolerance, insulin resistance, high blood pressure, and osteoporosis (Wlodek *et al.*, 2007; Siebel *et al.*, 2008; Wlodek *et al.*, 2008; Wlodek *et al.*, 2009; Mazzuca *et al.*, 2010; Siebel *et al.*, 2010; Gallo *et al.*, 2012c; Cheong *et al.*, 2016b). Regardless of the underlying cause of fetal growth restriction, a common feature to them all is an inability of the placenta to transfer appropriate oxygen and nutrients to the fetus, either due to inadequate maternal intake (undernutrition) or placental dysfunction (UPI). This, therefore, highlights the key role the placenta plays in UPI and developmental programming.

1.4.2 Impact of intrauterine growth restriction on the placenta

Research has clearly demonstrated the association between placental size and fetal weight. Specifically, human studies demonstrate that pregnancies complicated by IUGR have reduced placental weight and volume from 12 weeks' gestation, which precedes a reduction in fetal and, hence, birth weight (Jansson *et al.*, 1986; Hafner *et al.*, 2003). This can, consequently, result in altered placental efficiency (a common marker of placental function), which is described as the grams of the fetus produced per gram of the placenta at birth (Wilson & Ford, 2001). In IUGR humans and animal models, placental efficiency is altered in a manner that is dependent on the model. Specifically, in rodent models of growth restriction UPI surgery and direct hypoxemia reduces placental efficiency, whereas protein and caloric restriction tends to increase placental efficiency (Doherty *et al.*, 2003; Eskild *et al.*, 2016). In malnutrition models, this increase in placental efficiency is likely the result of an adaptative mechanism to facilitate

increased nutrient transport, which may involve alterations in placental morphology aiming to improve fetal weight outcomes. These studies clearly demonstrate the importance of placental size in modulating fetal growth (Owens *et al.*, 1989; Wallace *et al.*, 2002; Anthony *et al.*, 2003) which is likely driven by dynamic changes in placental vascularisation, nutrient transportation, growth factor signalling, and the glucocorticoid barrier function.

1.4.1.1 Placental morphology and vascularisation in IUGR

Given the aforementioned reduction in fetal oxygen and nutrient supply in IUGR fetuses, it is not surprising that there are dynamic changes in the placental vasculature and structure, which likely plays a key role in the aetiology of UPI. Specifically, research has demonstrated that placentae of IUGR human babies have reduced STB area with increased exchange barrier thickness and decreased volume of villi and intervillous spaces (Mayhew *et al.*, 2003). In addition, using Doppler ultrasound, placental vascular resistance is increased in the umbilical artery resulting in reduced umbilical placental blood flow (Giles *et al.*, 1985), and the surface area of the fetal terminal villi and terminal capillary loops are reduced (Verma and Verma 2013) in IUGR fetuses. Histological analyses have indeed confirmed that these changes are likely due to incomplete remodelling of the maternal spiral arteries (Khong et al, 1986) and abnormal branching leading to a reduction in the terminal fetal capillary villi trees (Verma & Verma, 2013), which may be due to dysregulated apoptosis resulting in poor trophoblast invasion and remodelling of uterine spiral arteries (Smith *et al.*, 1997). Although placental endothelial cell apoptosis occurs with the natural progression of pregnancy, especially during late gestation, imbalances to injury and/or repair of the fetal villous tree will likely affect the overall effectiveness of placental function and, ultimately, impair placental blood supply. Indeed, a characteristic of IUGR is the absence of endovascular trophoblast invasion into the myometrium resulting in the inability to convert the maternal uterine vessels into low resistance vessels, which predisposes the placenta and, ultimately, fetus to hypoperfusion, hypoxia (Scifres & Nelson, 2009), and reduces nutrient transportation capacity. It is interesting to note, however, that the abnormal Doppler waveforms in pregnancies complicated by growth restriction appear to suggest that blood vessel morphology in itself is secondary to altered placental villi function. Whereby cord concentrations of venous oxygen concentrations are increased *in utero,* which is indicative of reduced oxygen extraction (Owens *et al.*, 1987) and suggests that reduced fetal oxygen extraction is more of an issue than reduced umbilical blood flow rate in pregnancies complicated by IUGR. This implies that placental growth factors and/or nutrient transport mechanisms, as opposed to alterations in placental morphology and vascularisation, are key contributing factors to the aetiology of fetal growth restriction.

1.4.1.2 Placental IGF-system in IUGR

Given its key role in fetal and placental growth, it is not surprising that the IGF-system has been implicated in the pathogenesis of fetal growth restriction. Clinically, IUGR human fetuses have lower cord blood concentrations of IGF1, IGF2 and IGFBP3 along with elevated IGFBP1

concentrations (Langford *et al.*, 1994; Spencer *et al.*, 1995), which are 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 IUGR rabbits (Thakur *et al.*, 2000) and in experimental models of IUGR 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 influence fetoplacental development.

1.4.1.3 Placental nutrient transportation in IUGR

In addition to dysregulated growth factor expression, placental nutrient transportation is also impaired in IUGR. Specifically, IUGR fetuses often present as hypoglycaemic(Economides & Nicolaides, 1989), which is likely in part due to dysregulated glucose transporter expression. Research in human placentae have reported no changes in placental GLUT1 expression, with increased GLUT3 expression on the maternal aspect of the placenta (Jansson *et al.*, 1993; Janzen *et al.*, 2013). As GLUT3 facilitates glucose transportation into, but not across, the placenta this suggest that increased placental glucose metabolism due to increased GLUT3 expression likely contributes to the fetal hypoglycaemia (Janzen *et al.*, 2013). Placental amino acid transportation has been extensively reviewed in pregnancies complicated by IUGR (Johansson *et al.*, 2003; Sibley *et al.*, 2005). Indeed, the hallmark characteristic of IUGR is reduced amino acid transport and activity, particularly in the System A family, which is associated with low fetal plasma amino acid concentrations (Lin *et al.*, 2012). Specifically, the expression of SNAT1, 2 and 4 are reduced at the microvillus plasma membrane in IUGR humans (Glazier *et al.*, 1997). As described previously (**Section 1.2.2**), a likely mechanism responsible for these changes in glucose and amino acid transporters is via mTORC1, which is downregulated in the placenta of IUGR humans (Roos *et al.*, 2007) and in animal models of altered fetal growth (Rosario *et al.*, 2017); thus likely affecting nutrient transporter expression and nutrient influx.

1.4.1.4 Placental glucocorticoid barrier and glucocorticoid receptors in IUGR

Despite the well documented role of fetal glucocorticoid exposure during late gestation to enhance fetal growth in normal pregnancies, at excessive concentrations it can be detrimental to fetal growth. Epidemiological and experimental studies of growth restriction have demonstrated that placental HSD11β2 expression and activity are reduced (Stewart *et al.*, 1995; Lindsay *et al.*, 1996; Murphy *et al.*, 2002), which would enhance maternal-to-fetal glucocorticoid transportation and impair fetal growth. This finding is likely, in part, driven by

the reduced placental oxygen concentrations in IUGR, a theory of which is supported by an *in vitro* study where maternal hypoxia reduced HSD11β2 activity in a placental cell line (Homan *et al.*, 2006). However, no studies to date have characterised the impact IUGR has on placental GR or MR expression. As the GR is a transcription factor regulator as well as a transcription factor itself, it is possible that it may be altered expression in IUGR placenta as it is a regulator of *Hsd11b2* gene abundance (Garbrecht & Schmidt, 2013). Therefore, alterations in the placental glucocorticoid barrier and/or GR may play a role in the development of IUGR.

1.4.2 Pregnancy in women born growth restricted

During pregnancy there are profound changes to maternal cardiorenal and metabolic systems, which are aimed to sustain the growing fetus and maintain the mother's health. However, in growth restricted females these adaptations are dysfunctional, which results in GDM (Gallo *et al.*, 2012b; Gallo *et al.*, 2012c; Gallo *et al.*, 2013; Tran *et al.*, 2013) and compromises fetal growth and development (Gallo *et al.*, 2012c). We, and others, have demonstrated that F2 offspring from F1 mothers born growth restricted have a fetal nephron deficit (gestation day (E) 20) that is restored by weaning, which may be linked to the high blood pressure in F2 males (Harrison & Langley-Evans, 2009; Gallo *et al.*, 2012c). Furthermore, these F2 male offspring are also glucose intolerant (Reusens & Remacle, 2001). Interestingly, despite no direct *in utero* insult affecting the growth and development of the F2 fetuses, it is likely that a mechanism associated with the F1 mother's growth restriction *in utero* is programming the transgenerational disease in her offspring. However, there is limited scientific evidence on whether these changes occur due to placentally programmed and/or maternally driven mechanisms. As the placenta plays a key role in fetal growth and development, it is likely that any perturbation the mother experienced during pregnancy may result in programmed placental changes during her own pregnancy to overcome the adverse *in utero* environment she was exposed to. However, it is not known whether these adaptations are beneficial or detrimental to the growth and development of her children.

1.4.2.1 Placental responses to F1 maternal growth restriction

Research suggests that a number of mechanisms are likely responsible for the intergenerational programming of F2 diseases, including genomics, somatotropic (IGF-system), placental vascular development (uteroplacental and fetoplacental), placental transport and glucocorticoid barrier mechanisms. Maternally and/or paternally imprinted placental genes impact on the conservation of maternal resources and the enhancement of nutritional resources to the fetus, respectively (Sferruzzi-Perri *et al.*, 2017), which is regulated by epigenetic mechanisms such

as DNA methylation, histone acetylation, and miRNA regulation of gene expression. Indeed, in the F0 pregnancy in which the original insult of IUGR occurs, somatotropic mechanism are altered, specifically the expression of the IGF-system including IGF1 and IGF2 and their receptors. As previously discussed in **Section 1.2.1**, the repression and/or overexpression of these factors is associated with altered placental development and function as well as fetal growth and programming. Alterations in the IGF-system is likely to also affect the mTOR nutrient sensing pathway and the resultantly linked nutrient transporter pathways (Jansson *et al.*, 2012). However, limited studies have characterized if similar changes in the placental IGF-system and/or nutrient transportation systems are observed in placentae associated with F2 offspring whose mother was born small. Additionally, while there is an abundance of research examining the impact growth restriction has on placental morphology and vascularisation as well as the placental glucocorticoid barrier, whether similar mechanisms and pathways are affected in F2 pregnancies is yet to be explored.

It is evident that the programming of disease in F1 offspring is driven by numerous mechanisms that can be associated with aberrant disease mechanisms in later life. However, it is clear from the lack of data that the role that these pathways play in the programming of disease in the F2 generation is yet to be elucidated. In addition to exploring the mechanism/s associated with the programming of disease in the F2 generation from mothers born growth restricted it is also important to understand the effect adverse and beneficial maternal lifestyle factors have on these placental systems.

1.5 Maternal Obesity

Approximately 30-60% of pregnant Australian women are overweight (body mass index [BMI] = 25-30 kg/m^2), with 30% of those women being classified as obese (BMI >30 kg/m^2) (Doherty *et al.*, 2006; McIntyre *et al.*, 2012; Cunningham & Teale, 2013; Dodd *et al.*, 2014; AIHW, 2017, 2019b). Importantly, as previously mentioned, individuals born growth restricted are more susceptible to obesity. Maternal obesity affects all stages of pregnancy including the process of conception whereby obese women have lower fertility and fecundity rates in both natural and artificial pregnancies. During pregnancy, obese women are more likely to have a complicated pregnancy, with increased risks of miscarriages, developing GDM, pregnancy-induced hypertension, preeclampsia, and thromboembolic events (Galtier-Dereure *et al.*, 2000). Additionally, obese women are at an increased risk of preterm birth as well as late gestational still birth. From the fetal perspective, pregnancies complicated by maternal obesity are at a higher risk of IUGR, delivery complications (including shoulder dystocia) and their children have an increased risk of developing obesity, cardiometabolic and renal diseases in adult life (Drake & Reynolds, 2010).

1.5.1 Impact of maternal obesity on the placenta

Maternal obesity increases maternal adiposity and exacerbates the insulin resistance and inflammation associated with pregnancy. As such it is understood that alterations in placental development and function are likely due to increased maternal and placental fat deposition and, consequently, increased concentrations of inflammasomes. Indeed, several studies have demonstrated that maternal obesity alters placental size, villous structure as well as the expression of nutrient transporters associated with placental function and fetal growth and development.

1.5.1.1 Placental morphology and vascularisation in maternal obesity

With increasing maternal BMI there is a simultaneous increase in average placental weight (Williams *et al.*, 1997; Swanson & Bewtra, 2008), which is despite the reduced placental proliferation in pregnancies affected by increased BMI in early gestation (Higgins *et al.*, 2013); suggesting there may be increased placental fat deposition. Numerous rodent models have been utilised to investigate the impact maternal obesity has on placental morphological and efficiency outcomes. Kim *et al.* (2014) demonstrated that obese rats fed a high-fat diet (HFD; 45% *vs* 10% kcal from fat) from 4 weeks of age reduces placental labyrinth zone thickness (Kim *et al.*, 2014), which is indicative of poor and/or reduced placental trophoblast division.

This change in placental barrier thickness can also alter placental blood flow, which may result in preeclampsia and IUGR when aberrant. Additionally, a study in obese mice fed an obesogenic diet (10% simple sugars with 20% animal fat (*wt*/*wt*)) supplemented with sweetened condensed milk (55% simple sugar, 8% fat (*wt*/*wt*)) three weeks prior to mating demonstrated that the proportion of fetal blood vessels in the labyrinth zone is reduced in female associated placentae (de Barros Mucci *et al.*, 2020). Although not statistically significant, Stuart and colleagues recently demonstrated that maternal obesity due to high-fat feeding (5.1 kcal.g^{-1} *vs* 4.7 kcal.g^{-1}) for 12 weeks prior to mating results in placentae with reduced microvessel density and increased umbilical artery and uterine resistance, which would reduce nutrient transportation capacity to the growing fetuses (Stuart *et al.*, 2018). Furthermore, the placentae respond in a sexually dimorphic manner whereby microvessel density was reduced by 30% in male associated placentae and 20% in female associated placentae (Stuart *et al.*, 2018). This characterisation of sex-specific abnormalities in response to suboptimal maternal conditions has become increasingly popular as it possesses the potential of mapping out sex-specific responses to disease and, potentially, treatments.

1.5.1.2 Placental IGF-system and nutrient transportation in maternal obesity

In both humans and animals, the structural placental changes characterised in maternal obesity are commonly linked to alterations in nutrient signalling and transport pathways. Studies in humans demonstrate that GLUT1 and SNAT gene and protein expression on the MVM are deregulated in pregnancies where maternal nutrient supply is increased, such as maternal obesity and GDM (Kuruvilla *et al.*, 1994; Jansson *et al.*, 2013), which is suspected to facilitate increased glucose and amino acid delivery to the fetus via the upregulation of transporter expression on the MVM surface (Jansson *et al.*, 2013). This is consistent with data from mice fed a high-fat/high-sugar (41% kcal fat with 20% sucrose solution) diet from 13 weeks of age where placental glucose and amino acid transportation are increased (Rosario *et al.*, 2015), with increased maternal plasma insulin. Importantly, as mTOR is a master regulator of placental nutrient transportation, its deregulation likely results in the observed fetal overgrowth (Jansson *et al.*, 2013; Rosario *et al.*, 2015) and may be due to upstream regulators, such as the IGF-system (Zoncu *et al.*, 2011). Indeed, serum IGF1 concentrations are elevated in obese pregnant women (Ramsay *et al.*, 2002; Jansson *et al.*, 2008; Jansson & Powell, 2013), which may promote fetal overgrowth as demonstrated in **Figure 1.7**. Collectively, all these changes would alter nutrient supply to the fetus, which could influence growth and development outcomes.

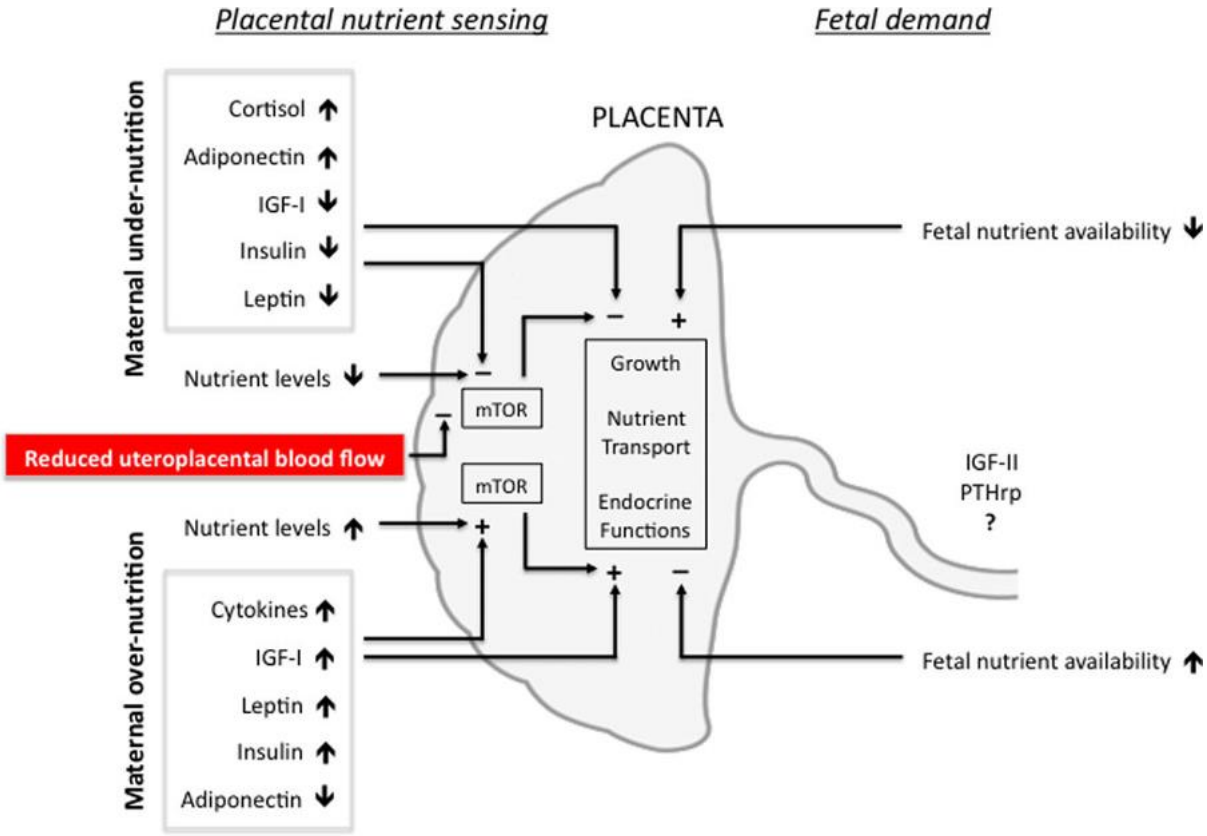


Figure 1. 7 Placental nutrient sensing pathway.

Illustration of placental nutrient sensing pathways in conditions of altered maternal nutrient supply. Adapted from Jansson & Powell (2013). IGF – insulin-like growth factor; mTOR – mammalian target of rapamycin; PTHrP - parathyroid hormone-related protein.

1.5.1.3 Placental glucocorticoid barrier in maternal obesity

The placental glucocorticoid barrier plays a crucial role in fetal growth, whereby deregulation can compromise fetal growth and program disease (Dy *et al.*, 2008). It is, therefore, not surprising that cortisol concentrations and the glucocorticoid barrier system are influenced by maternal obesity. Specifically, obesity in humans reduces maternal cortisol concentrations, which is associated with increased placental HSD11β2 expression and macrosomia at term (Stirrat *et al.*, 2014). Johns and team suggest that, based on their unpublished data, *Hsd11b2* abundance is positively correlated with maternal BMI and is particularly elevated in samples from obese women at term (Johns *et al.*, 2020). The increase in HSD11β2 may result in a reduction in fetal glucocorticoid exposure, thus contributing to the mechanisms behind fetal microsomia and the prolonged gestation commonly observed in obese women (O'Reilly, 2014). However, to this day the limited research completed needs to be replicated in both human and animal models in order for us to understand the mechanistic impact that obesity has on the placental glucocorticoid barrier. As the glucocorticoid barrier does not work in isolation, it is likely that maternal obesity imparts its programming changes via numerous mechanistic pathways, including the glucocorticoid barrier, to increase fetal growth.

Data presented thus far, collectively, demonstrate that the increased maternal nutrient supply in pregnancies complicated by obesity influences pathways and mechanisms that work by upregulating factors in favour of fetal growth. However, there is no available evidence demonstrating whether obesity in growth restricted women exacerbates the placental adaptations to pregnancy and/or programs worse adult health in F2 offspring, which requires further investigation. Nevertheless, one easily attainable method to improve overall maternal health following growth restriction and/or obesity is via positive lifestyle interventions, such as exercise.

1.6 Maternal Exercise

The benefits of exercise in disease prevention has been well defined in both human and animal studies. Studies in non-pregnant obese women present strong evidence that regular exercise improves insulin sensitivity (Winnick *et al.*, 2008), glucose tolerance (Fenicchia *et al.*, 2004), cardiac function (Figueroa *et al.*, 2007), reduces total body fat (Kondo *et al.*, 2006). Importantly, current research indicates that regular exercise during pregnancy improves overall maternal health. Specifically, a randomised clinical trial where women performed aerobic and muscular conditioning exercises reduced pregnancy weight gain and improved glucose tolerance (Cordero et al 2014), which reduced the incidence of GDM. Similar findings of reduced GDM risks with maternal exercise have also been reported in observational studies were women self-report their recreational physical activity, particularly during early pregnancy (Dempsey *et al.*, 2004a; Dempsey *et al.*, 2004b). However, the risk of GDM was not reduced if the mother commenced exercise in the second or third trimester of pregnancy (Barakat *et al.*, 2013); highlighting that the timing of exercise initiation plays a key role in maternal health outcomes. Exercise has also been demonstrated to reduce the incidence and improve the outcomes of preeclampsia, whereby preeclampsia incidence is reduced amongst physically active nulliparous women (Saftlas *et al.*, 2004). A case control study by Sorenson et al, revealed that in the first 20 weeks of pregnancy moderate-intensity recreational (physical) activity resulted in a 24% reduction in the risk of preeclampsia. Furthermore, there appeared to be a dose-related response with vigorous recreational activity resulting a greater reduction in preeclampsia risk by 54%, which was even greater if the mother engaged in vigorous recreational activity a year prior to conception. This association has also been demonstrated by Marcoux et al and Irwin (Marcoux *et al.*, 1989) where there was consistently a dose-dependent response with increased leisure-time physical activity and occupational physical activity (Irwin *et al.*, 1994) resulting in an even greater reduction in preeclampsia risk. These data, collectively, demonstrates the benefits of exercise during pregnancy on maternal outcomes, including preventing the onset of pregnancy complications.

Although there are numerous studies exploring the potential benefits and risks of exercise in healthy women during the periconceptual period as well as during pregnancy, there is a lack of consensus as to whether exercise during pregnancy and the changes it elicits in the maternal metabolism is beneficial or detrimental for the fetus, which may program long-term offspring health. Indeed, the impact maternal exercise has on the fetus, particularly birth weight, is conflicting with some studies reporting increased (Clapp *et al.*, 2000), others report decreased

(Hopkins *et al.*, 2010; Bisson *et al.*, 2017), and some report no changes (Bell *et al.*, 1995; Kardel & Kase, 1998) in birth weight. This lack of consensus is likely due to differences in the type, duration, intensity, and timing of exercise initiation. Furthermore, there is a lack of evidence as to whether exercise is beneficial for growth restricted and/or obese women and their fetus, as most exercise studies are in metabolically healthy women.

1.6.1 Impact of maternal exercise on the placenta

The functional mechanism of exercise on the body includes increased metabolic demand in skeletal muscle, which is associated with reduced oxygen and nutrient supply to visceral organs (Moghetti *et al.*, 2016). Although the placenta is transient, it is a visceral organ that is the only communication and barrier between the maternal metabolic environment and the fetus. Therefore, it is only logical that any beneficial fetal outcomes that may be linked to maternal exercise is modulated via the actions of the placenta. However, as exercise increases both the catabolic and anabolic processes that affect oxygen, nutrient, and hormonal (steroid) supply in the maternal system, this is likely to trigger adaptive placental mechanisms to ensure adequate/optimal fetal growth.

1.6.1.1 Placental morphology and vascularisation with maternal exercise

Clapp and colleagues demonstrated that regular exercise during early pregnancy stimulates placental growth in healthy women, which was evident by increased placental growth rates and volumes (Clapp, 2003) as well as increased volume of functional tissue (Jackson *et al.*, 1995). Furthermore, a study by Bergmann and Clapp in humans demonstrated that running throughout pregnancy increased villous vascular volume and cell proliferation (Bergmann *et al.*, 2004). The authors suggested that this increase in placental villous volume may be of clinical importance as increases in placental volume may prove favourable for the growing fetus to improve fetoplacental nutrient and oxygen transfer via increased exchange surface area. However, placental weight was not increased suggesting that exercise during pregnancy stimulates placental pathways that improve functional tissue development. In addition to enhancing placental growth rate and volumes, maternal exercise modulates placental vascular remodelling, whereby serum PLGF is increased and serum s-Flt-1 concentrations are reduced in women who performed chronic (before pregnancy and during) and acute (only during pregnancy) exercise compared to inactive women (Weissgerber *et al.*, 2010). From this study, it was concluded that exercising regularly during pregnancy results in a maternal proangiogenic serum profile during late gestation as no differences in angiogenic factors where characterised in the non-pregnant cohort, which may aim at facilitating placental vascularisation. However,

human studies characterising the effect of exercise on placental morphology are limited by subject numbers and consistency in exercise regimen; as the majority of these studies are dependent on patient self-reporting, which limits the extent to which we can infer that certain exercises are beneficial for placental and/or fetal development (Downs *et al.*, 2012). These limitations have been overcome by the use of animal models to demonstrate the effect exercise has on placental morphology. Specifically, a study in spontaneously hypertensive pregnant rats that swam 6 weeks prior to pregnancy and continued throughout pregnancy demonstrated that swimming exercise increases placental blood vessel percentage, which was associated with an increased area occupied by fetal vessels in the labyrinth (Abate *et al.*, 2012); indicative of enhanced placental vasculogenesis and/or angiogenesis. In line with this, voluntary wheel running in Sprague Dawley rats from 3 weeks of age increases circulating VEGF, decreases sFlt-1, and improves endothelium dependent vascular relaxation (Gilbert *et al.*, 2012). These data, collectively, demonstrate that exercise stimulates proangiogenic factors that likely aim to improve placental vascularisation and enhance nutrient transportation to the growing fetus via increased vascular perfusion.

1.6.1.2 Placental IGF-system and nutrient transportation with maternal exercise

Both human and animal studies in non-pregnant populations have well demonstrated that exercise enhances systemic IGF concentrations (Schwarz *et al.*, 1996; Giesel *et al.*, 2009; Stein *et al.*, 2018). Specifically, a combination of endurance and resistance training exercise increased total IGF1 concentrations in non-pregnant women (Gregory *et al.*, 2013). Similar outcomes are also characterised in males following high resistance training where circulating IGF1 concentrations are increased by 19% (Poehlman *et al.*, 1994). Despite these studies clearly linking exercise to enhancing the IGF-system, there is limited data examining the impact exercise has on the placental IGF-system and its potential benefits on fetoplacental growth and development. A study by Turgut and associates identified that treadmill exercise (20 $m.min^{-1}$ for 20 $min.day^{-1}$ with electric shock stimulus) initiated from the first day of pregnancy and continued until day 20 in Wistar rats increases maternal IGF1 and IGFBP3 plasma concentrations at E20, but reduced fetal weight (Turgut *et al.*, 2006). It is likely that the exercise intensity maintained throughout pregnancy was the cause of the reduced fetal and liver weights as the intensity was considered moderate and, according to the Royal Australian and New Zealand College of Obstetrics and Gynaecologists, pregnant women should try and accumulate 150-300 mins of moderate intensity exercise each week (20-40 $min.day^{-1}$) or 2 alternated days of high intensity exercise with a reduction in intensity with advancing gestation

(RANZCOG, 2019). However, this increase in the IGF-system suggests that, despite the already increased maternal metabolic demand induced by the exercise and changes associated with pregnancy, pregnant rats respond to exercise in a similar manner to non-pregnant humans. This increase in IGF1 concentrations may be an attempt to promote fetal growth and development in a high maternal energy demanding environment, given IGF1s essential role in fetal growth and development as well as its association in modulating nutrient transportation pathways in conditions of moderate physical activity or exercise (Turgut *et al.*, 2006).

As exercise may induce some degree of placental hypoxia, due to periods of reduced uteroplacental blood flow, it is possible that placental glucose transfer via IGF1 regulation occurs similarly to what is observed in hypoxia (Baumann *et al.*, 2002). In addition, as exercise upregulates GLUT4 expression in non-pregnant individuals it is possible that a similar upregulation occurs in the placenta, which would facilitate increased uptake of glucose from the fetus into the placenta for energy usage (Hawley *et al.*, 2011). Given the previously described confounding results with regards to birth weight outcomes following exercise, it is important to consider the role placental amino acid transportation play, which is essential for appropriate fetal growth and development. Most of the current knowledge of the effect exercise has on amino acid transporter expression is from non-pregnant human and animal models. In a study by Drummond and associates, resistance exercise in young and old non-pregnant humans increased skeletal muscle amino acid transportation via SLC38A2 (SNAT2) (Drummond *et al.*, 2011). Importantly, the authors suggested that mTORC1 may, in part, be responsible for this upregulation (Drummond *et al.*, 2011), which could enhance placental nutrient transporter expression, as described in **Section 1.2.2.3**. In the only human study characterising nutrient transportation following physical activity during pregnancy that we are aware of, by Brett et al, physical activity during the second trimester reduced placental IGF1 and increased SNAT2 transporter expression (without changes in SNAT1) and altered mTOR expression (Brett *et al.*, 2015). However, the measurement of physical activity in the subjects was based on data collected using an accelerometer for which movement at >21.43 min at moderate to vigorous intensity was categorized as the exercise group. Given the range of exercise intensities, the varied physical activity durations times, and the dependency of self-reporting and recall of maternal nutrient intake it is prudent to note that although this study pioneered the exploration into the effects of maternal exercise on nutrient partitioning and energy balance the interpretation of the data is limited. Despite the limited investigation into the impact exercise has on placental outcomes, it is likely that exercise can ameliorate the

detrimental impact of adverse maternal health, which may improve maternal and offspring outcomes (**Figure 1.8**).

1.6.2 Effects of maternal exercise on the placenta in complicated pregnancies

Physical activity improves maternal health in both non-pregnant and pregnant populations, including in individuals born small and obese. Aerobic training four times a week at 55% VO_2 max decreases body fat mass in obese non pregnant women, which also reduced the insulinogenic index and plasma cholesterol (Despres *et al.*, 1991). In both males and females, the risk of developing metabolic syndrome, which is increased in obesity and growth restricted individuals, is inversely related with increased physical activity. Furthermore, a number of studies demonstrate that the exercise type and intensity play a key role in beneficial outcomes, such as weight loss and reduced risk of type-II diabetes mellitus (Church, 2011). In growth restricted individuals, moderate or frequent exercise in elderly males and females (65-75 years old) lowers rates of glucose intolerance (Eriksson *et al.*, 2004) and in adolescence physical activity decreases the risk of insulin resistance (Eriksson *et al.*, 2004; Ortega *et al.*, 2011; Gatford *et al.*, 2014). Animal studies have further supported this outcome whereby 4 weeks of treadmill exercise (20 $m.min^{-1}$ for 60 $min.day^{-1}$) in male growth restricted offspring during juvenile life in rats restores β-cell mass in adulthood (Laker *et al.*, 2011), which may be beneficial for long-term metabolic health. However, there is limited evidence as to the impact exercise has on maternal health in female growth restricted rats. A study by Corvino and colleagues demonstrated that swimming exercise initiated a month prior to mating and continued throughout pregnancy for three days a week for 30 mins in growth restricted females results in improved maternal glucose tolerance, reduced adiposity and, importantly, increased fetal (F2) offspring organ weights (Corvino *et al.*, 2015). These data demonstrate that exercise has similar positive outcomes in the growth restricted population, which may prevent the development of pregnancy complications, such as GDM. However, exercise prior to conception may be more beneficial than exercise initiated after the first trimester of pregnancy, once the placenta is already established, in improving maternal and fetoplacental outcomes.

While exercise in growth restricted and obese women has many positive effects on maternal health, there is no evidence as to the placental changes that may occur to modulate maternal and fetal outcomes. As the placenta is sexually dimorphic, it is also likely that the placental specific responses to maternal exercise will be fetal sex dependent, which warrants further investigation, especially as treatment options to improve fetal outcomes may be sex specific.

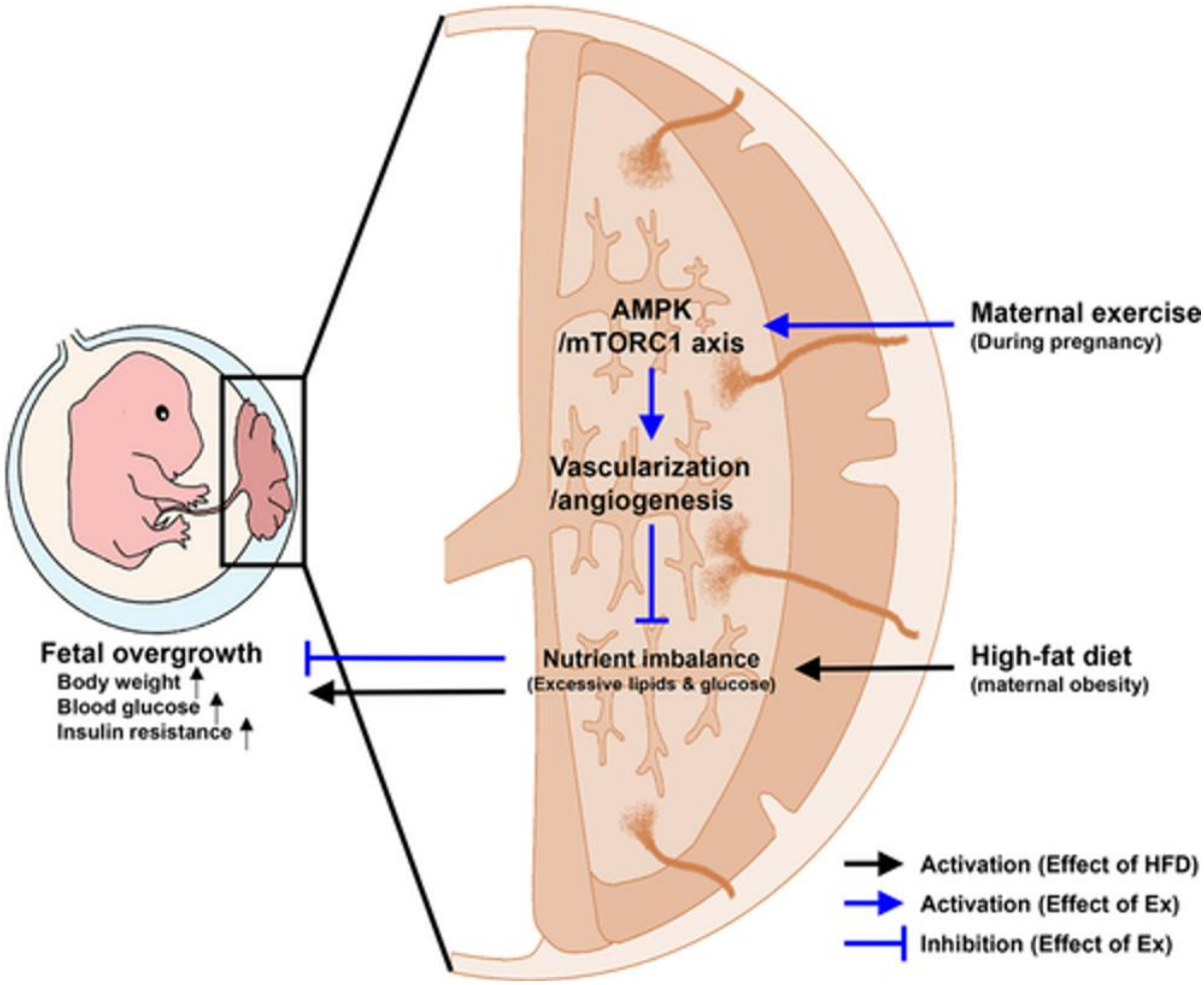


Figure 1. 8 Schematic of potential mechanisms of exercise in pregnancy.

Exercise (Ex) in pregnancy may alter placental vascularisation in order to inhibit the aberrant effects of nutrient imbalances due to a maternal high fat diet (HFD). Figure adapted from Son *et al.* (2019). AMPK - Adenosine monophosphate activated protein kinase; mTORC1 – mammalian target of rapamycin complex 1.

1.7 Overall Thesis Aims

The overall aim of this thesis was to investigate the effect F1 maternal growth restriction has on the placentae of F2 offspring, which may predispose these offspring to poor cardiometabolic health. Moreover, I aimed to examine whether maternal high-fat feeding exacerbated these outcomes and the timing of exercise initiation that is most beneficial in improving any negative outcomes associated with maternal growth restriction and high-fat feeding.

Chapter 3

The overall aim of Chapter 3 was to determine changes in the placental labyrinth IGF-system of F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to identify if maternal high-fat feeding exacerbated any changes in the F2 placental IGF-system within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

Specific aims were to assess the following:

i) if maternal growth restriction prior to birth programs alterations in F2 fetoplacental growth or the placental labyrinth IGF-system;
ii) if maternal exercise initiation prior to and/or during pregnancy attenuates outcomes;
iii) if maternal high-fat feeding exacerbates outcomes;
iv) if there are any sex-specific differences.

It was hypothesized that the placental labyrinth IGF-system of F2 offspring whose mother was born growth restricted (*Restricted*) would be deregulated in a manner dependent on the period of exercise initiation (prior to or during pregnancy). It was predicted that maternal high-fat feeding would exacerbate these outcomes and that *Exercise* would improve F2 placental and fetal outcomes to a greater extent than exercise initiated only during pregnancy (*PregEx*).

Chapter 4

The overall aim of Chapter 4 was to determine changes in placental labyrinth nutrient transporter expression in F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to identify if maternal high-fat feeding exacerbated any alterations within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

Specific aims were to assess the following:

i) if maternal growth restriction prior to birth alters F2 labyrinth nutrient transporter and mTOR expression;
ii) if maternal exercise initiation prior to and/or during pregnancy attenuates outcomes;
iii) if maternal high-fat feeding exacerbates outcomes;
iv) if there are any sex-specific differences.

It was hypothesized that F2 offspring from *Restricted* mother's would have reduced nutrient transporter expression compared to F2 offspring from normal birth weight mothers (*Control*). It was predicted that maternal high-fat feeding would exacerbate these outcomes and that *Exercise* would improve F2 fetal outcomes to a greater extent than *PregEx*.

Chapter 5

The overall aim of Chapter 5 was to determine changes in placental labyrinth angiogenesis in F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to characterize if these outcomes are exacerbated by maternal high-fat feeding within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

Specific aims were to assess the following:

i) if maternal growth restriction prior to birth alters F2 labyrinth vasculogenic and/or angiogenic expression;
ii) if maternal exercise initiation prior to and/or during pregnancy attenuates outcomes;
iii) if maternal high-fat feeding exacerbates outcomes;
iv) if there are any sex-specific differences.

It was hypothesized that F2 offspring from *Restricted* mothers would have aberrant expression of vasculogenic and/or angiogenic factors compared to offspring from *Control* mothers. It was predicted that maternal high-fat feeding would exacerbate these outcomes and that *Exercise* would improve F2 fetal outcomes to a greater extent than *PregEx*.

Chapter 6

The overall aim of Chapter 6 was to determine changes in the placental labyrinth glucocorticoid barrier in F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to identify if maternal high-fat feeding exacerbated any alterations

within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

Specific aims were to assess the following:

i) if maternal growth restriction prior to birth alters the F2 labyrinth glucocorticoid barrier;
ii) if maternal exercise initiation prior to and/or during pregnancy attenuates outcomes;
iii) if maternal high-fat feeding exacerbates outcomes;
iv) if there are any sex-specific differences.

It was hypothesized that F2 offspring from *Restricted* mothers would have an aberrant expression of glucocorticoid barrier genes compared to offspring from *Control* mothers. It was predicted that maternal high-fat feeding would exacerbate these outcomes and that *Exercise* would improve F2 fetal outcomes to a greater extent than *PregEx*.

Chapter 2 Material and Methods

2.1 Study Overview

All animal experiments were approved by the University of Melbourne's Animal Ethics Committee (AEC #1212639) and conducted in accordance with the National Health and Medical Research Council of Australia's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004) and Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes (NHMRC, 2008). WKY rats were provided by the Biological Research Facility at the University of Melbourne and housed in stainless steel open top cages in an environmentally controlled room at 22°C with a 12-hr light/dark cycle and 45-55% relative humidity in the Biological Research Facility at the University of Melbourne. All rats had *ad libitum* access to standard chow consisting of 19% protein, 76.4% carbohydrate and 4.6% fat (Specialty feeds; Glen Forrest, WA, Australia) and tap water.

2.1.1 Experimental timeline

In brief, 18-week-old F0 female WKY rats were mated with breeder males. At E18 pregnant rats were exposed to sham or UPI surgery to generate F1 *Control* and *Restricted* offspring, respectively. Following surgery rats gave birth at term (22 days) and at five wks (postnatal day 35 (PN35)) F1 *Control* and *Restricted* females were randomly allocated to either a Chow or HFD diet throughout the study. At 16 wks, F1 females were further randomly allocated to one of three exercise regimes: *Sedentary*, *Exercise*, or *PregEx* and mated with breeder males. On E20, post-mortem was performed on pregnant rats as well as the F2 male and female fetuses, with placentae and fetal plasma collected (**Figure 2.1**). The experimental timeline and experimental groups (n= 8-12 litters/group) are summarised in **Figure 2.1** and **Figure 2.2**, respectively.

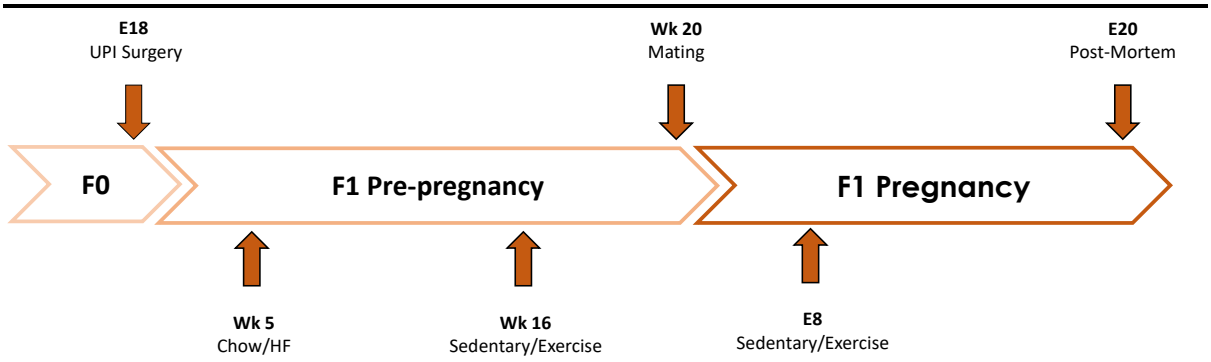


Figure 2. 1 Experimental timeline

F0 females were mated with breeder males and on day 18 of pregnancy (E18) were exposed to uteroplacental insufficiency (UPI) or sham surgery to generate F1 *Restricted* and *Control* offspring, respectively, and delivered naturally at term. First generation (F1) *Restricted* and *Control* female offspring were randomly allocated to a Chow or a High-fat (HF) diet from 5 weeks of age, and at 16 weeks were further randomly allocated to an *Exercise* or *Sedentary* group. F1 females were mated with a breeder male at 20 weeks. On E8, *Sedentary* dams were further randomly allocated to remain *Sedentary* or undergo exercise during pregnancy. Post-mortems were performed on E20 with fetal plasma and placentae collected for analysis in this thesis.

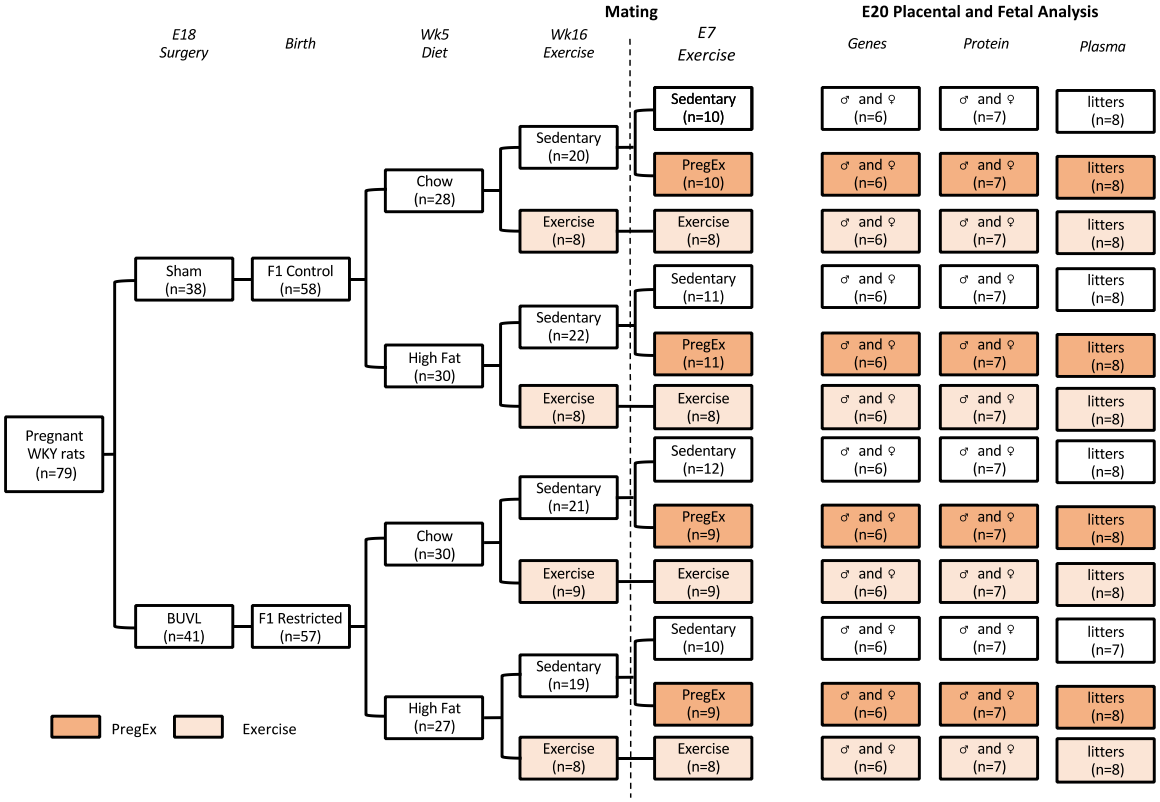


Figure 2. 2 Experimental groups and animal allocations

Female Wistar Kyoto (WKY) rats were mated with normal males and, at embryonic day (E) 18, they underwent either Sham surgery (*Control* offspring) or bilateral uterine vessel ligation (BUVL) surgery (*Restricted* offspring). On postnatal day (PN) 35 the Control and Restricted first generation (F1) females were allocated to either a Chow or high-fat diet (HFD) diet and then, at 16 weeks of age, were further allocated to one of two exercise regimes; to remain *Sedentary* or begin treadmill exercise (*Exercise*). Females were then mated with a breeder male and those in the *Sedentary* group where further allocated to either remain *Sedentary* or to begin exercise in the second week of pregnancy (*PregEx*). This resulted in 12 experimental groups from which all placental labyrinth tissue was separated by sex where analysed for gene and protein analysis. Fetal plasma concentrations where also analysed.

2.2 Generation of Offspring

2.2.1 Mating

F0 virgin female rats were examined to identify whether they were in the appropriate estrous cycle for mating using a vaginal impedance meter (model MK-10B; Mukomachi Kikai, Osaka, Japan). A reading of >7 kΩ mid-afternoon suggested that the rat is in proestrus and would likely enter estrous overnight. Two proestrus virgin females were housed with one male breeder overnight for mating. The presence of sperm in the vaginal smear taken the following morning was considered the first day of pregnancy (E1) (O'Dowd *et al.*, 2008).

2.2.2 Uteroplacental insufficiency surgery

F0 pregnant rats were weighed and their abdomen examined on E18 to confirm pregnancy. They were then randomly allocated to a *Control* (sham) or *Restricted* (UPI) surgery group. Bilateral uterine vessel ligation (artery and vein) surgery was used to induce UPI in the *Restricted* group, which reduces oxygen, nutrient, and blood supply to the developing fetus resulting in fetal growth restriction (Wlodek *et al.*, 2005; Wlodek *et al.*, 2007; Siebel *et al.*, 2008). F0 female rats were anaesthetized with 4% isoflurane (Baxter Healthcare; Old Toongabbie, NSW, Australia) 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). Corneal and pedal reflexes were used as indicator of animal consciousness, where the absence of either reflex indicates that the animal is completely unconscious, and the surgery could proceed. The abdomen was shaved with clippers and cleaned three times with 4% chlorohexidine and 80% EtOH. A 2-3 cm incision was then made in the skin and muscle layers at the midline using sharp sterilised scissors. Following the incision, the right and left uterine horns were carefully taken out of the abdominal cavity and placed onto gauze soaked in sterile saline (0.9% NaCl: Baxter Healthcare). Sterile saline was used throughout the surgical procedure to flush the exposed uterus in order to keep it moist. Using sterile 4-0 silk suture (Ethicon; Piscataway, NJ, USA), the cervical end of the left and right uterine arteries and veins were ligated (**Figure 2.3**). Following vessel ligation, both uterine horns were carefully placed back into the abdominal cavity. The muscle wall layer was sutured using 4-0 chromic catgut (Johnson & Johnson Medical; North Ryde, NSW, Australia) and the skin layer was sutured using individual stiches with 4-0 nylon suture. Animals allocated to the sham surgery underwent the same procedure as the *Restricted* females, except the uterine vessel ligation was not performed. Following surgery 0.125% bupivacaine (AstraZeneca; Cambridge, CBE, United Kingdom) was

administered to the skin and muscle layers prior to closure. Females were then individually housed with nesting material and allowed to deliver naturally undisturbed at term.

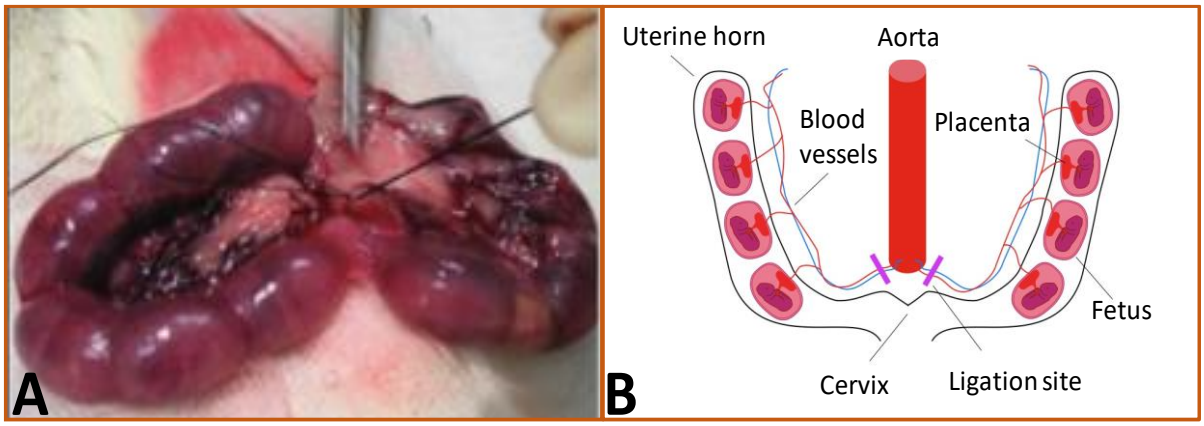


Figure 2. 3 Bilateral uterine vessel ligation surgery

Photograph and schematic were used with the permission of Prof Wlodek and Sam van der Linde.

2.2.3 F1 allocation to diet protocol

At weaning (PN35), litter mate F1 *Control* and *Restricted* females were separated from their mothers and randomly allocated to a Chow or HFD. The females in the Chow group consumed a commercially available pellet diet *ad libitum* (AIN93G; Specialty Feeds, Glen Forrest, WA, Australia; **Appendix 1**). Females allocated to the HFD consumed *ad libitum* a mix of commercially sourced diets based on the AIN93G diet (SF03-020 and SF01-028; Specialty Feeds: **Appendices 2 and 3**) mixed with the chow diet (Raipuria *et al.*, 2015). All diets were matched for micro- and macronutrients (**Table 2.1**).

Table 2. 1 Nutritional parameters for Chow (AING93) and High-fat (SF03-020 and SF01-028) diets.

Calculated Nutritional Parameters	AING93	SF03-020	SF01-028
Protein	19.40%	19.40%	19.00%
Total Fat	7.00%	23.00%	22.60%
Total Carbohydrate	56.90%	56.90%	56.90%
Crude Fibre	4.70%	4.70%	4.70%
AD Fibre	4.70%	4.70%	4.70%
Digestible energy	16.1 $MJ.kg^{-1}$	20 $MJ.kg^{-1}$	19 $MJ.kg^{-1}$
% Total calculated digestible energy from lipids	16.00%	43.00%	43.00%
% Total calculated digestible energy from protein	21.00%	17.00%	17.00%

2.2.4 F1 exercise protocol

At 16 weeks of age, F1 females were allocated to either an *Exercise* or *Sedentary* group. The *Exercise* group exercised for 5 day.week^{-1} on a motorized treadmill (Columbus Instruments, Columbus, OH, USA) followed by 2 rest days. On the first day of training, rats ran for 20 mins at a speed of 15 m.min^{-1} with additional 10 mins added to the running time each 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^{-1} at a speed of 20 m.min^{-1}, as indicated in **Figure 2.4** and previously described (Laker *et al.*, 2011; Laker *et al.*, 2012; Wadley *et al.*, 2016; Asif *et al.*, 2018; Mangwiro *et al.*, 2018). At 20 weeks of age, F1 females were mated with breeder males acquired from the Biological Resource Facility at the University of Melbourne as described in **Section 2.2.1**. The day after mating (on E1), for *week 1 of pregnancy*, females in the *Exercise* group exercised for 50 min.day^{-1} at a speed of 17 m.min^{-1}. For *week 2 of pregnancy* (on E8) rats exercised for 30 min.day^{-1} at a speed of 13 m.min^{-1} and for *week 3 of pregnancy* (on E15) rats exercised for 20 min.day^{-1} at a speed of 11 m.min^{-1}. On E8, upon confirmation of pregnancy, females in the *Sedentary* group were further randomly allocated to either remain *Sedentary* or were allocated the *PregEx* group. Females in the *PregEx* group remained *Sedentary* prior to mating and for the first week of pregnancy and underwent exercise from E8 at the same intensities and durations as per the *Exercise* group. Rats were encouraged to run by blowing compressed air near the base of their tail. *Sedentary* rats were placed on a stationary treadmill for the same duration as the exercising rats (**Figure 2.4**).

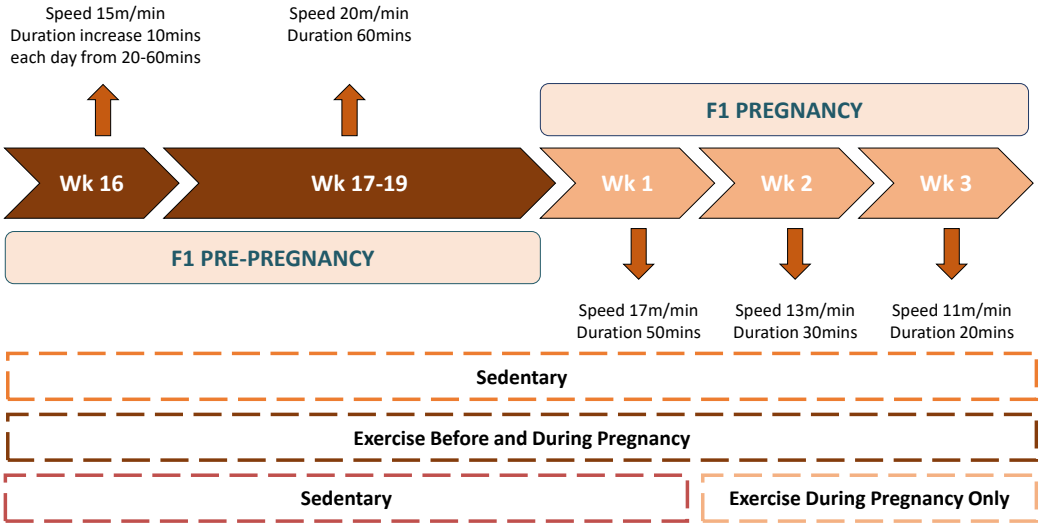


Figure 2. 4 Flow of the exercise protocol.

F1 females were allocated to either remain *Sedentary* or to exercise before and during pregnancy. Rats underwent treadmill exercise from 16 weeks of age (Week 16) at a speed of 15 m/min for 20 min/day with an additional 10 mins added per day to a max of 60 min, followed by two rest days. Females continued to exercise at 20m/min for 60 mins until pregnancy confirmation, where exercise intensity and duration gradually decreased. A subset of *Sedentary* rats began exercise from the second week of pregnancy.

2.2.5 F1 post-mortem and F2 tissue collection

On the morning of E20 post-mortems were performed on all rats. Dams were administered an overdose of 1:1 mixed solution of Illium Xylazil-20 (30 $mg.kg^{-1}$; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia) and Ketamine (100 $mg.kg^{-1}$; Parnell Laboratories Pty Ltd, Alexandria, NSW, Australia) via intraperitoneal injection. Upon confirmation of unconsciousness (corneal and pedal reflexes), a vertical midline incision was made from the sternum to the lower abdomen of the pregnant rat to expose the left and right uterine horns. Fetuses were visually sexed via the anogenital distance with fetal blood collected and pooled within litters by decapitation using capillary tubes. Each fetus and placentae were removed from the uterus and individually weighed. Using fine forceps, the placentae were separated into the junctional zone (identified by its pale appearance due to the absence of fetal blood vessels) and the labyrinth zone (identified by the rich presence of fetal blood vessels). These placental regions where snap frozen in liquid nitrogen or fixed in 10% neutral buffered formalin for further analysis. Fetal tails were collected individually and snap frozen in liquid nitrogen to verify fetal sex using *Sry* quantitative polymerase chain reaction (qPCR) analysis. Maternal blood was collected via cardiac puncture using heparinised syringes. Maternal and fetal blood were centrifuged at 3,000 *x g* at 4°C for 15 mins, with plasma collected and stored at -20°C for subsequent analyses. F1 birth weights as well as maternal weights prior to and during pregnancy are includes in Appendices 2-4.

2.3 F2 Fetal Sex Determination

2.3.1 DNA extraction

To ensure accurate visual sexing of the fetuses, 2-3 mm of excised fetal tails were incubated overnight in DNA lysis buffer (77 µM KCl, 10 mM Tris (pH 8.3), 2 mM $MgCl_2$, 0.1% *w/v* Gelatine, 0.005% *v/v* NP-40, 0.005% *v/v* Tween-20, 0.1% Proteinase K) at 55°C as described previously (Cuffe *et al.*, 2012). Protease K was then deactivated by heating the samples to 95°C for 15 mins. The samples were vortexed then centrifuged at 13,200 *x g* for five mins at room temperature (RT). The supernatant was collected and transferred to Eppendorf tubes for a DNase clean-up step where 40 µl 6M ammonium acetate (Sigma-Aldrich; Castle Hill, NSW, Australia) was added to each sample, vortexed and incubated on ice for 15 mins. The sample were then centrifuged at 13,200 *x g* for ten mins at 4°C. The supernatant was collected and 100 µl isopropanol (Sigma-Aldrich) was added then vortexed and centrifuged at 13,200 *x g* for three mins at RT. The isopropanol was removed, and the pellet washed in 50 µl 70% EtOH, vortexed and centrifuged at 13,200 *x g* for one min at RT. EtOH was removed and the pellet was then resuspended in 20 µl DNA lysis buffer at 55°C for 15 mins and stored at -20°C for subsequent qPCR analyses.

2.3.2 Sry qPCR

qPCR was conducted on purified fetal genomic DNA using TaqMan as the fluorescent agent and a commercially available TaqMan probe for the sex-determining region Y (*Sry*; Rn04224592_u1; NP_036904.1) (Life Technologies). Samples were heated to 50°C for two mins for activation and then heated to 95°C for ten mins then 40 cycles of 95°C for 15 s and 60°C for 60 s for qPCR using the Bio-Rad CFX manager (Bio-Rad; Gladesville, NSW, Australia). Fetal sex was determined from the amplification plot, with an amplification occurring before 30 cycles indicating a male fetus (**Figure 2.5**).

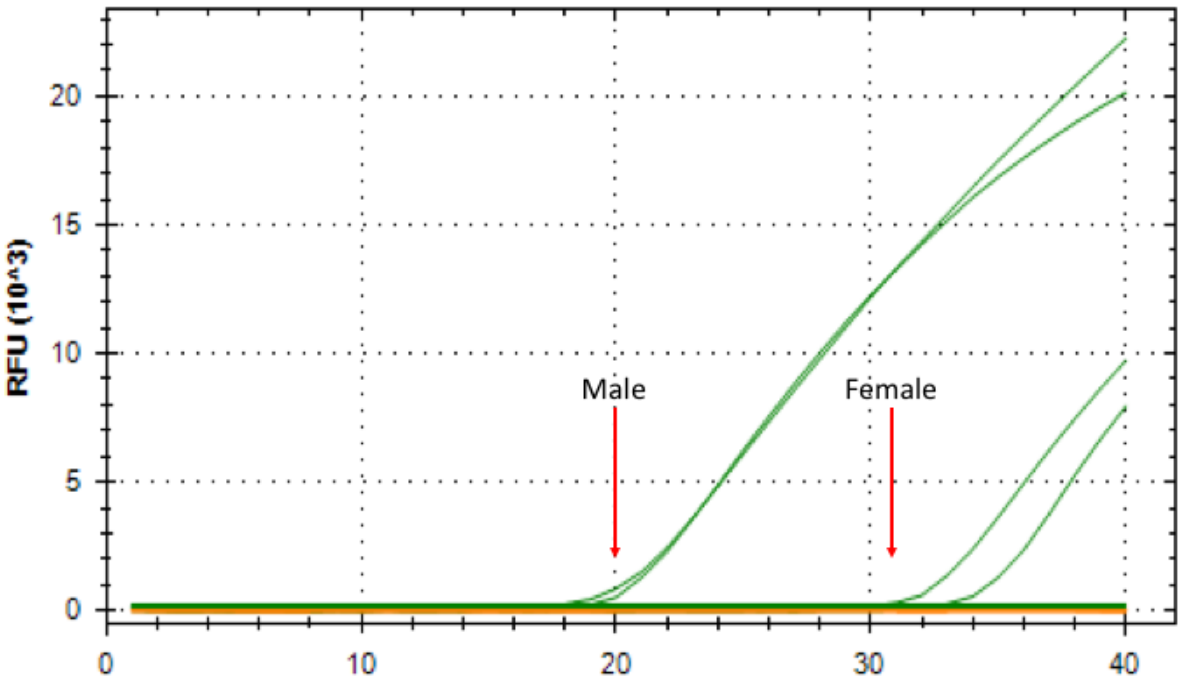


Figure 2. 5 Sry amplification plot

Amplification plot of sex-determining region (*Sry*). Male pup amplification occurs before 30 cycles and female amplification occurs after 30 cycle due to Sox3 gene homology with SRY.

2.4 F2 Labyrinth qPCR

2.4.1 Labyrinth RNA Extraction

Following sex determination, RNA was extracted from 50 mg labyrinth tissue from one male and one female fetus per litter (n=6 male and female placentae per experimental group). RNA was extracted by disrupting the cell membranes with homogenization using the Cryolys Precellys 24 homogenizer (Bertin Technologies; Aix en Provence, France) with CK14 ceramic beads (Bertin Technologies) in Qiazol reagent (Qiagen) as per manufacturer's instructions using a commercially available kit (Qiagen; miRNeasy kit). Labyrinth tissues were homogenised in 700 µl Qiazol at 5,500 *x g* for ten s three times with a 15 s break in-between cycles and incubated at RT for three mins to allow for the foam to subside. 140 µl Chloroform (Sigma-Aldrich) was added to the homogenate and shaken vigorously for 15 s and incubated at RT for three mins. The homogenate was then centrifuged at 12,000 *x g* for 15 min at 4°C, which separates the sample into three phases: an upper colourless aqueous phase containing RNA, a white interphase containing DNA, and a red organic phase containing protein and other organic tissue substrates. The aqueous phase was transferred to a new collection tube and 1.5 volume of 100% EtOH was added and the samples mixed. The sample was added to a spin column and centrifuged at 8,000 *x g* for 15 s at RT. To extract total RNA, including small non-coding RNAs, 350 µl Buffer RWT (Qiagen) was added to the spin column and the samples were centrifuged at 8,000 *x g* for 15 s at RT. The RNA then underwent DNase digestion, where 80 µl DNase incubation mix was directly added to the RNeasy column and incubated at RT for 15 mins. Following incubation, 350 µl RWT buffer was added and centrifuged at 8,000 *x g* for 15 s at RT and the column was then washed twice with 500 µl Buffer RPE (Qiagen). 50 µl RNase-free water was then added directly to the column and was centrifuged for one min at 8,000 *x g* to elute total RNA. RNA concentrations were determined using a Nanodrop (Thermo Fisher Scientific; Scoresby, VIC, Australia). The 260/280 ratio was used to assess the RNA purity, with a value of 2 indicating pure RNA, and the 230/260 ratio indicated pure nucleic acids, with any value below 2 indicating contamination. RNA was stored at -80°C for subsequent analysis.

2.4.2 cDNA synthesis

2.4.2.1 mRNA cDNA synthesis

To convert mRNA to cDNA the high-capacity cDNA reverse transcription kit (Applied Biosystems) was used according to the manufacturer's instructions. Briefly, 10 µl 2X reverse

transcription master mix (Applied Biosystems) was added to each Eppendorf tube containing 1 µg RNA in 10 µl RNAse free water (Qiagen). To generate cDNA, the reaction was activated by heating samples to 25°C for ten mins and were then were heated to 37°C for 2 hrs to generate first strand cDNA followed by heating to 87°C for five mins to deactivate the reaction using the palm cycler (Cobett Life Science; Mortlake, NSW, Australia). Samples were topped up to 200 µl with RNAse/DNase free water (1:5 dilution; Qiagen) and the cDNA stored at -20°C.

2.4.2.2 miRNA cDNA synthesis

For miRNA transcription, the TaqMan micro-RNA reverse transcription kit (Thermo Fisher Scientific) was used following the manufacturer's instructions. Briefly, 5X small RNA specific stem-loop reverse transcription primers specific to the miRNA targets of interest were added to a working stock solution using 0.1X TE buffer (Thermo Fisher Scientific) to create an RT miRNA primer pool. 7 µl reverse transcription master mix was added to 1 µg RNA in 5µl and vortexed. 12 µl reverse transcription reaction was then added to an Eppendorf tube and 3 µl 5X reverse transcription primer pool was added to the reverse transcription reaction tube. The 15µl reaction was completed using the palm cycler (Corbett Life Science) and was initially held at 16°C for 30 mins, 42°C for 30 mins and finally 85°C for five mins to deactivate the reaction. Samples were topped up to 180 µl using RNAse/DNase free water (1:12 dilution; Qiagen).

2.4.3 qPCR

qPCR of key mRNA and miRNA targets in the pathways of interest (IGF-system, nutrient, vasculogenesis and stress placental pathways) were then conducted using commercially available TaqMan probes (**Table 2.2** and **Table 2.3**, respectively) and TaqMan universal gene expression master mix (Thermo Fisher Scientific), in line with the MIQE guidelines (Bustin *et al.*, 2009). 2.5 µl cDNA was plated in duplicate on 384-well PCR plates (Bio-Rad) with 7.5 µl master mix using a pipetting robot (LTF Labortechnik; Wasserburg, Bodensee, Germany). To compensate for variations in RNA concentrations and cDNA conversion efficiency, target mRNA (**Table 2.2**) and miRNA (**Table 2.3**) genes of interest were normalised to the housekeeping genes TATA box binding protein (*Tbp*) and β Actin (*Actb*) for mRNA analyses and *191 miRNA* and *U6 snRNA* for miRNA analyses. Samples were heated to 50°C for two mins and held at 95°C for ten mins. qPCR reactions were then ran for 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative changes in mRNA and miRNA abundance were quantified using the $2^{-\Delta\Delta CT}$ method and were normalised to male samples from *Sedentary-Control*-Chow dams and presented in arbitrary units.

Table 2. 2 mRNA targets.

Commercially available TaqMan probes of the mRNA targets of interest.

Gene	Accession Number
Igf1	Rn00710306_m1; NM_178866.4
Igf2	Rn01454518_m1; NM_031511.2
Igf1r	Rn00583837_m1; NM_052807.2
Igf2r	Rn01636937_m1; NM_012756.1
Igfbp3	Rn00561416_m1; NM_012588.2
Vegfa	Rn01511602_m1, NM_001110333.2
Flt-1	Rn01409533_m1; NM_019306.1
Plgf	Rn00585926_m1; NM_053595.2
Mtor	Rn00693900_m1; NM_019906.1
Slc2a3	Rn01640916_m1; NM_017102.2
Slc2a1	Rn01417099_m1; NM_138827.1
Slc5a1	Rn01640634_m1; NM_013033.2
Slc38a1	Rn00593696_m1; NM_138832.1
Slc38a2	Rn00710421_m1; NM_181090.2
Slc38a4	Rn00590667_m1; NM_130748.1
Ogt	Rn00820779_m1; NM_017107.2
Nr3c1	Rn00561369_m1; NM_012576.2
Nr3c2	Rn00565562_m1; NM_013131.1
Hsd11b1	Rn00567167_m1; NM_017080.2
Hsd11b2	Rn04341420_g1; NM_017081.2
Crhr1	Rn00578611_m1; XM_006247542
Tbp	Rn01455646_m1; NM_001004198.1
Actb	Rn00667869_m1; NM_031144.3

Table 2. 3 miRNA targets.

Commercially available TaqMan probes of the miRNA targets of interest.

miRNA	Accession Number
Let7f-1	462250_mat; MIMAT0017089
miRNA27a	000408; MIMAT0000799
miRNA 191	002299; MIMAT0000866
U6 snRNA	001973; NR_004394

2.5 F2 Labyrinth Protein Analysis

2.5.1 Protein extraction

50 mg of placental labyrinth tissues were cut and added to Eppendorf tubes with 500 µl radioimmunoprecipitation assay buffer (RIPA; Cell Signalling Technologies) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Each sample was homogenised for three bursts of 20 s using a hand-held homogenizer and placed onto a Rotary tube mixer for two hrs with end-to-end rotation at 4°C. The samples were then centrifuged at 11,000 *x g* for ten mins at 4°C and the supernatant collected and transferred to a new Eppendorf tube, which was stored at -80°C for subsequent analysis.

To assess the protein concentration in the extracted labyrinth tissues, a bicinchoninic acid (BCA) assay (Pierce) was performed. Briefly, 5 µl labyrinth supernatant was diluted with 95 µl RIPA buffer (1:20 dilution) and vortexed. 25 µl of the diluted sample was then added to a 96-well plate in triplicate. 25 µl of bovine serum albumin (BSA) protein standards (20-2000 $\mu g.mL^{-1}$) were also added to the plate in triplicate to generate a standard curve. 200 µl of working reagent (ten mL BCA Reagent A and 200 µl BCA Reagent B) was added to each well and the plate was covered and incubated at 37°C for 30 mins. Following incubation, the plate was read using the xMark Microplate Absorbance Spectrophotometer (Bio-Rad) at 562 nm. A standard curve was generated using the protein standards on the plate, which was used to determine protein concentrations in the samples.

2.5.2 Labyrinth Western blotting

Western blot analysis was carried out using equal concentrations (30 µg) of labyrinth protein lysate. To denature the protein, 10 µl of the samples containing 30 µg protein were suspended in 10% *v/v* Laemmli Sample buffer (90 µl Laemmli loading buffer and 10 µl beta-mercaptoethanol). The reaction was vortexed and placed into a Digital Dry Block heater (Ratek; Boronia, Victoria, Australia) for five mins at 95°C, and were centrifuged following denaturing. In the first lane of each 15 lane 4-20% Tris-HCL gel (Bio-Rad), 1 µl of precision plus protein dual colour standard ladder (Bio-Rad) was added and the other lanes contained samples of interest, with a male *Control*-Chow-*Sedentary* sample ran on each gel for normalization across gels. The gel was then electrophoresed in Running buffer (25 mM Tris-base, 192 mM glycine, 0.1% *w/v* SDS; pH 8.3) at 200V for 35 mins.

Following electrophoresis, the stain free gel was carefully removed from its cassette and soaked in Running Buffer before being activated using the Chemidoc MP Imaging system (Bio-Rad). After gel activation, the gel was then placed in running buffer before being gently transferred to a nitrocellulose membrane. A Trans-Blot Turbo Mini Nitrocellulose Transfer Pack (Bio-Rad) was used in order to transfer the proteins from the gel. Once placed onto the membrane, slight pressure was applied to remove any air bubbles that may interfere with the transfer process. Proteins were transferred onto a nitrocellulose blotting membrane using the Turbo Transfer System (Bio-Rad) for 7 mins at 50 mA, with alternating voltages for a mixed molecular weight protein transfer. Following successful protein transfer the blotting membrane was transferred to a 50ml centrifugation tube containing 15 mL Tris–Buffered Saline (TBS; 20 mM Tris-base, 0.1 M NaCl, 2.7 mM KCl at pH 7.5) and placed on a roller tube Mixer (Ratek) for five mins at RT. The TBS was discarded and 25 mL 5% *w*/*v* skim milk powder (SMP) (5% *w*/*v* SMP, 0.1% *v*/*v* TBS with Tween-20 (TTBS)) was added to the tube to block non-protein bound regions on the membrane on a roller tube mixer for one hr. Following the blocking, the skim milk solution was discarded, and the membrane was washed for five mins three times using 0.1% TTBS (20 mM Tris-base, 0.1 M NaCl, 2.7 mM KCl, 0.01% *v/v* Tween-20; pH 7.5). After the washes, a primary antibody with a cross-reactivity to rats was added to the membrane that was specific to each of the proteins of interest (**Table 2.4**), which was diluted in 5 mL 5% SMP and incubated overnight at 4°C on a roller tube mixer. After incubation, the solution was discarded, and the nitrocellulose blotting membrane was rinsed three times for five mins each using 15 mL 0.05% TTBS (20 mM Tris-base, 0.1 M NaCl, 2.7 mM KCl, 0.05% *v/v* Tween-20; pH 7.5). Following the final rinse, a secondary antibody, specific to the animal the primary

antibody was raised in, was added to 5 mL 5% SMP and was left to incubate at RT on the roller Tube Mixer for one hr. Following incubation, the solution contents were discarded, and the nitrocellulose membrane was again washed three times for five mins using 15 mL 0.05% TTBS.

To visualise the proteins of interest a 1:1 Clarity Western ECL Blotting substrate solution (Bio-Rad) made consisting of 600 µl of Peroxide reagent and 600 µl of Luminol/Enhancer reagent. The detection solution was added to the tube containing the nitrocellulose membrane and was placed on a roller tube mixer at RT for five mins. To visualise the proteins of interest, Image Lab software was used (Bio-Rad) and chemiluminescence detected using the Chemidoc MP Imaging system (Bio-Rad). Quantification of the data was analysed by densitometry using Image lab software (Bio-Rad). Each band detected in the samples of interest were normalised to the total protein detected in each lane as a loading control using stain-free technology (Parviainen *et al.*, 2013; Mangwiro *et al.*, 2018).

Table 2. 4 F2 Labyrinth Western Blot Antibodies.

Primary and secondary antibody names and concentrations used for Western blots.

Primary Antibody	Concentration	Supplier	Secondary Antibody	Concentration	Supplier
IGF1	1:1000	Abcam	Anti-mouse IgG, HRP linked antibody	1:1000	Cell Signalling Technology
IGF2	1:1000	Abcam	Anti-rabbit IgG, HRP linked antibody	1:1000	Cell Signalling Technology
IGF1R	1:1000	Abcam	Anti-rabbit IgG, HRP linked antibody	1:1000	Cell Signalling Technology
VEGF	1:1000	Santa Cruz Biotechnology	Anti-mouse IgG, HRP linked antibody	1:1000	Santa Cruz Biotechnology
VEGF1R	1:1000	Santa Cruz Biotechnology	Anti-mouse IgG, HRP linked antibody	1:1000	Santa Cruz Biotechnology
PLGF	1:1000	Santa Cruz Biotechnology	Donkey anti-goat IgG, HRP linked antibody	1:1000	Santa Cruz Biotechnology
MTOR	1:1000	Cell Signalling Technology	Anti-rabbit IgG, HRP linked antibody	1:1000	Cell Signalling Technology
p-MTOR (Ser2448)	1:1000	Cell Signalling Technology	Anti-rabbit IgG, HRP linked antibody	1:1000	Cell Signalling Technology
GLUT3	1:1000	Santa Cruz Biotechnology	Anti-mouse IgG, HRP linked antibody	1:1000	Santa Cruz Biotechnology

2.6 F2 Fetal Plasma IGF1 ELISA

To investigate changes in F2 fetal plasma IGF1 concentrations, an enzyme linked immunosorbent assay (ELISA) was performed following the manufacturers protocol (R&D Systems, Minneapolis, MN, US). Briefly, 50 µl of calibrator diluent was added to each well on the precoated assay plate and 50 µl of standards, control, or pooled litter fetal plasma samples were added to the wells (1:10 dilution) in duplicate and incubated at RT for two hrs. Following incubation, the contents of the wells were discarded, and the wells were washed five times using 200 µl Wash Buffer and, following the last wash, the plate was inverted and blotted onto clean paper towel to ensure complete removal of Wash Buffer. 100 µl Mouse/Rat conjugate was added to each well and incubated on a shaker mixer for two hrs at RT. Following incubation, the contents were discarded, and the wells washed five times using Wash Buffer then blotted dry on paper towel. 100 µl Substrate Solution was then added to each well and incubated for 30 mins in a foil lined pouch. After incubation, 100 µl Stop Solution was added to each well to stop the reaction. The absorbance of each sample was then measured using the xMark Microplate Absorbance Spectrophotometer using Microplate Manager 6 software at 450 nm and a correction wavelength of 570 nm to remove background. The concentration of IGF1 in the fetal plasma was determined by comparing sample absorbances to the standard curve. The intra-assay and inter-assay comparisons were 4.3% and 6.0% respectively, with minimum assay sensitivity of 3.5 $pg.mL^{-1}$.

2.7 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 a main Treatment effect was present, the two-way ANOVA provided the p-value for the comparison between *Control* and *Restricted* groups. 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 with a Duncan's post-hoc test 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 was conducted to report main Diet effects within each Exercise. If an interaction was observed, the data was further split to identify Diet effects within each Treatment using a Student's unpaired t-test. To identify any Sex differences within each experimental group, a Student's unpaired t-tests was used. 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 $p < 0.05$.

Chapter 3 Maternal exercise in rats upregulates the placental IGF-system with diet and sex-specific responses: minimal effects in mothers born growth restricted

Results of this study are presented in its published form, following expanded aims, hypotheses, and methodologies used in the study

Publication: Mangwiro YTM, Cuffe JSM, Briffa JF, Mahizir D, Anevska K, Jefferies AJ, Hosseini S, Romano T, Moritz KM and Wlodek ME (2018) Maternal exercise in rats upregulates the placental IGF-system with diet and sex-specific responses: minimal effects in mothers born growth restricted. *J Physiol*, 596(23), 5947-5964.

3.1 Aims and Hypotheses

The overall aim of this study was to determine changes in the placental IGF-system of F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to identify if maternal high-fat feeding exacerbated the changes in the F2 placenta IGF-system within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

Specific aims were to assess the following:

i) if maternal growth restriction prior to birth programs alterations in F2 fetoplacental growth or the placental labyrinth IGF-system;
ii) if maternal exercise initiation prior to and/or during pregnancy attenuates outcomes;
iii) if maternal high-fat feeding exacerbates outcomes;
iv) if there are any sex-specific differences.

It was hypothesized that the placental labyrinth IGF-system of F2 offspring from *Restricted* would be deregulated in a manner dependent on the period of exercise initiation (prior to or during pregnancy). It was predicted that maternal high-fat feeding would exacerbate these outcomes and that *Exercise* would improve F2 placental and fetal outcomes to a greater extent than *PregEx*.

3.2 Methods

Methodologies used in this study have been described in Chapter 2 and are expanded from the published version (**Section 3.3**) in subsequent sections. The current study utilises qPCR, Western blotting, and ELISA experiments in WKY rats that underwent bilateral uterine vessel ligation that were allocated one of two diets and underwent treadmill exercise.

3.2.1 Animals

UPI (*Restricted*) or sham (*Control*) surgery was induced on E18 in WKY rats. F1 offspring were fed a Chow or HFD from weaning, and at 16 weeks were randomly allocated an exercise protocol; *Sedentary*, *Exercise* or *PregEx* as described in **Section 2.2**. Females were mated (20 weeks) with placentae associated with F2 fetuses collected at E20, weighed, and stored at -80°C. Fetal plasma was pooled within litters and stored at -20°C.

3.2.2 Placental gene abundance

Total RNA was isolated from 50 mg of labyrinth tissue as described in **Section 2.5**. qPCR was performed to quantify the abundance of the following genes of interest; *Igf1*, *Igf2*, *Ig1r*, *Igf2r* and *Igfbp3* (**Table 2.2**) and *Let7f-1* miRNA (**Table 2.3**).

3.2.3 Western blotting

Protein was isolated from 50 mg labyrinth tissue as described in **Section 2.5**. Western blotting was then performed to determine changes in IGF1, IGF2 and IGF1R (**Table 2.4**).

3.2.4 Fetal plasma analysis

F2 fetal plasma IGF1 concentration was determined via ELISA as described in **Section 2.6**.

3.2.5 Statistical analysis

The published work (**Section 3.3**) contains the statistical analysis performed for this Chapter.

3.3 Published Manuscript

J Physiol 596.23 (2018) pp 5947–5964

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

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Key points

- The placental insulin-like growth factor (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 that exercised prior to and continued during pregnancy compared to sedentary mothers.
- 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, probably as a means to improve fetoplacental growth and development, and 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

Yeukai Mangwiro is a PhD candidate in the Department of Physiology, Anatomy and Microbiology at La Trobe University, Melbourne, Australia, in collaboration with the Fetal, postnatal & adult physiology & disease laboratory of Professor Mary Wlodek at the University of Melbourne. Her research is centred on the effects of exercise during pregnancy on F2 fetal and placental outcomes including the growth factor system from mothers born growth restricted exposed to a high-fat diet. She plans to continue her research in the programming field, researching methods to better understand the effects of lifestyle interventions on placental programming in complicated pregnancies.

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and maternal obesity. Limited human studies have demonstrated that maternal exercise enhances fetoplacental growth and decreases cord IGF ligands, which may restore the 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.

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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), probably 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 influences fetoplacental development. However, limited studies have characterized if similar changes in the placental 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, 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 several 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 incidence of gestational diabetes mellitus and enhancing fetoplacental growth (Clapp *et al.* 2000, 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 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). However, it is 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, 2017; Gardebjer *et al.* 2014).

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 weeks of age) were acquired from the biological resource 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 oxygen flow (reduced to 3.2% isoflurane and 250 ml/min oxygen flow when suturing to aid in the animal's 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) females 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 embryonic day (E)7 to E19]. At 20 weeks of age, F1 females were mated with normal males as illustrated in Fig. 1*A*. All animals were generated concurrently.

Exercise training

The exercise regime is outlined in Fig. 1*B*. 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 min at a speed of 15 m/min. On 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 min. On *day 1 of week*

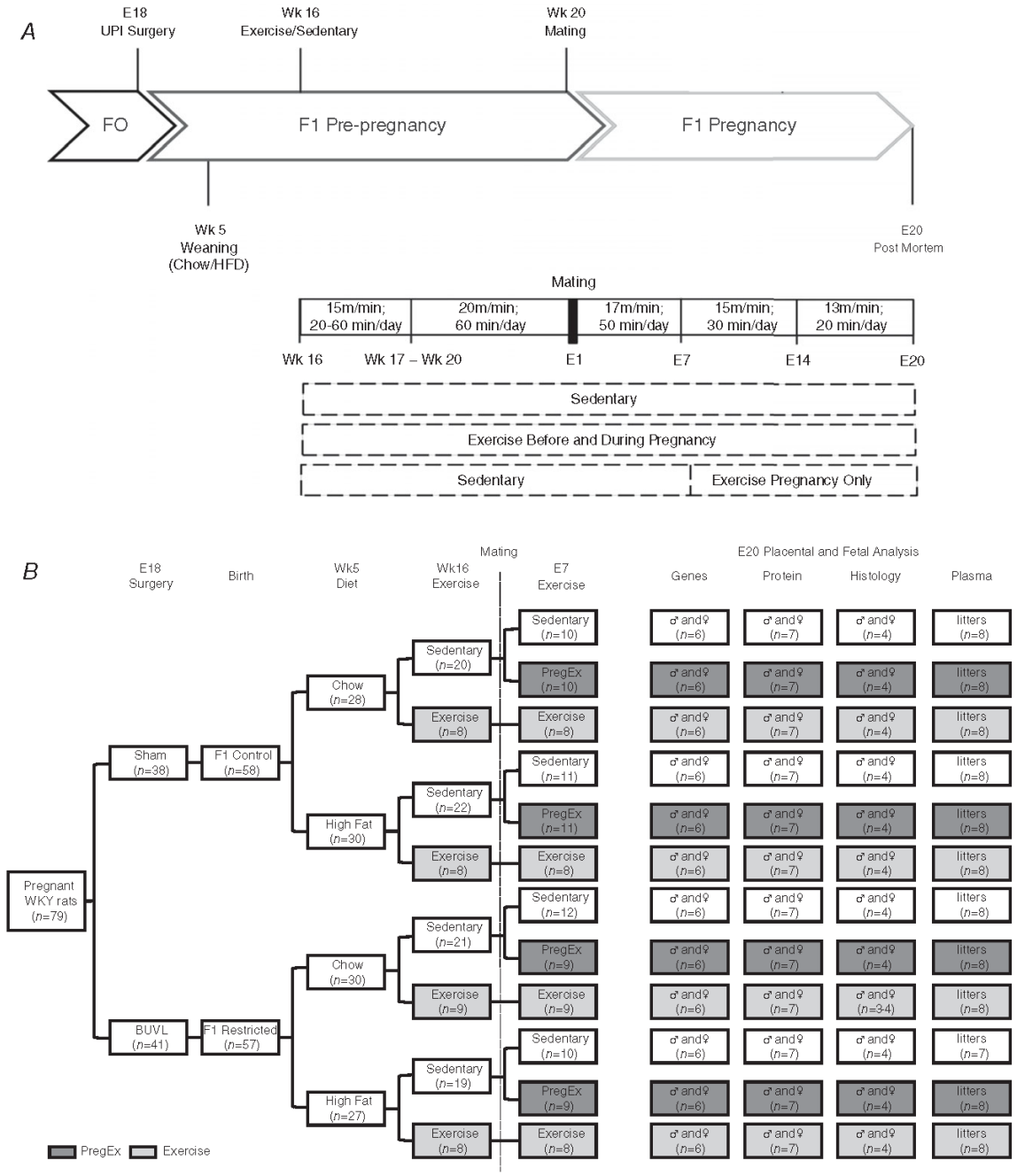

Figure 1. Flow chart of experimental exercise and diet protocol
A, flow chart of the experimental protocol indicating the allocation of pregnant rats between the different treatments, diet and exercise groups. *B*, flow chart of exercise protocol. E, embryonic day; HFD, high-fat diet; UPI, uteroplacental insufficiency; Wk, 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, 2012; Wadley *et al.* 2016; Asif *et al.* 2018). The day after mating (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 13 m/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 anaesthetized 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 weighed, sexed by visual inspection of the anogenital distance and then killed by decapitation. Fetal plasma was collected and pooled into litters, with fetal tails collected to verify fetal sex. The placentae were excised, weighed 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 quantitative PCR (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).

Placental morphology

Fixed placentae were processed into paraffin blocks, sectioned at 5 μm and stained with haemotoxylin and eosin (n = 3–4 dams/group with one male and female analysed per dam). Five sections per placenta were analysed 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 a 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 min, then qPCRs were run for 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative changes in mRNA and miRNA 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). Twenty micrograms 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 as 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 analysed using an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's 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.

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 were 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 were 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*-test 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 (Fig. 2*A* and *B*). 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 (Fig. 2*E*; Student's *t*-test $P = 0.012$), despite no changes in placental weight (Fig. 2*C*). 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 (Fig. 2*D* and *F*).

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) that *Exercised* (Fig. 3*B*) 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 (Fig. 3*D*, *F* and *H*, respectively; two-way ANOVA). No differences in placental morphology was reported in F2 male-associated placentae (Fig. 3*C*, *E* and *G*).

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 (Fig. 2*A* and *B*; 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 (Fig. 2*D*; 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 (Fig. 2*E*; 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 (Fig. 2*F*; one-way ANOVA).

Representative sections were taken from F2 male-associated placentae whose F1 mother was *Sedentary* and *PregEx* (Fig. 3*A*) 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) (Figs 3*C*, *D*, *G* and 2H; two-way

ANOVA), and increased junctional zone cross-sectional area in F2 males (High-fat fed mothers only; Fig. 3*E*, two-way ANOVA), irrespective of maternal birth weight, compared to F1 *Sedentary* mothers.

Fetal plasma IGF1 concentrations

Effects of F1 maternal growth restriction prior to birth on F2 fetal plasma outcomes. F1 mothers that were growth restricted prior to birth (Restricted) had F2 fetuses with reduced IGF1 plasma concentrations (pooled) in Chow-fed *Exercise* mothers (Fig. 4; Student's unpaired *t*-test) compared to F1 normal birth weight (Control) Chow-fed mothers that *Exercised*. IGF1 plasma concentrations were not altered in F2 fetuses from mothers that were growth restricted prior to birth (Restricted) that 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, Fig. 4; one-way ANOVA). Exercise in F1 mothers that consumed a High-fat diet did not alter pooled F2 fetal plasma IGF concentrations (Fig. 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 had 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 (Fig. 5*A*; two-way ANOVA), with no changes in IGF1 protein expression (Fig. 5*E*). No changes in placental *Let 7f-1* miRNA, *Igf1* mRNA or IGF1 protein

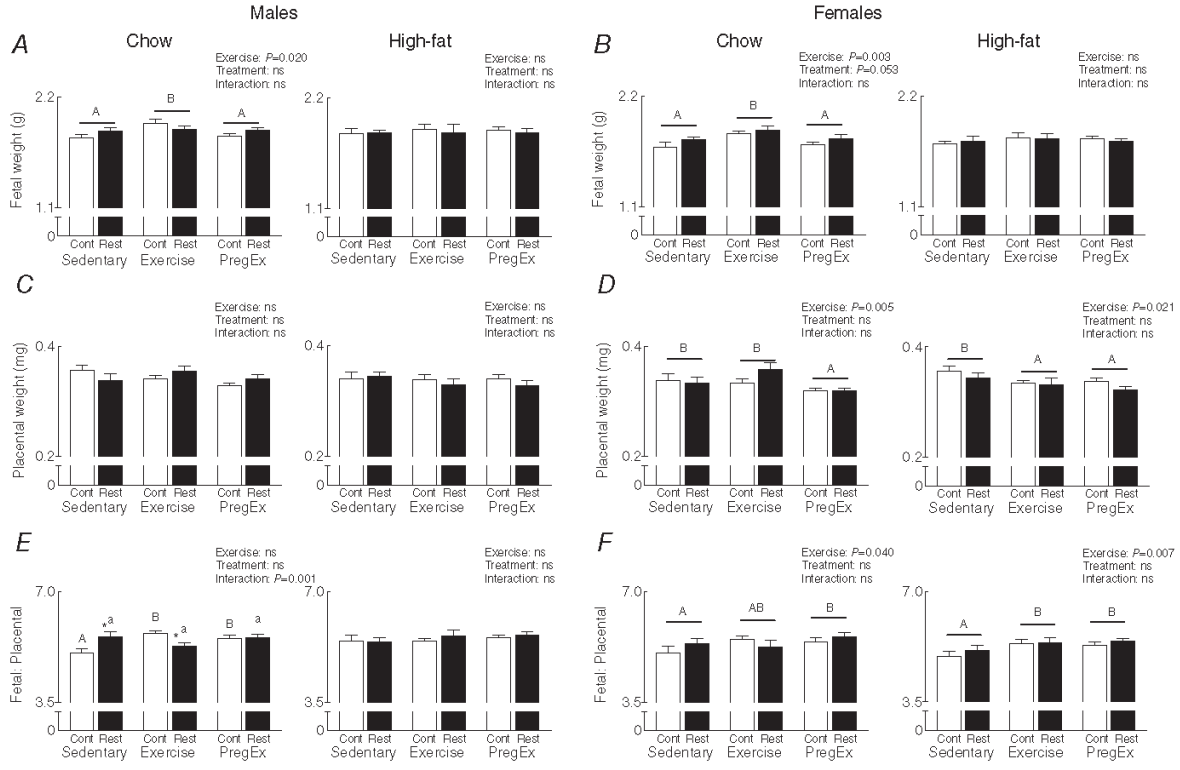

Figure 2. Fetal and placental weights
Fetal (*A* and *B*) and placental (*C* and *D*) weight along with fetal-placental ratio (*E* and *F*) (n = 8–12 litters in each group) for male and female fetuses whose mothers were Control (open bars) or Restricted (black bars) and consumed a Chow (left panel) or 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 from 'b/B' but not 'ab/AB'.

were detected in F2 female-associated placentae whose mother was growth restricted prior to birth (Restricted) that consumed a Chow or High-fat diet compared to normal birth weight (Control) mothers (Fig. 5*B*, *D* and *F*).

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 (Fig. 5*A*; 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 (Fig. 5*B*; 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 (Fig. 5*C*; one-way ANOVA), which did not translate to changes in IGF1 protein abundance (Fig. 5*E*). Despite no changes in *Igf1* mRNA abundance in Chow-fed F2 male-associated placentae (Fig. 5*C*), *Exercise* and *PregEx* increased IGF1 protein expression, irrespective of maternal birth weight,

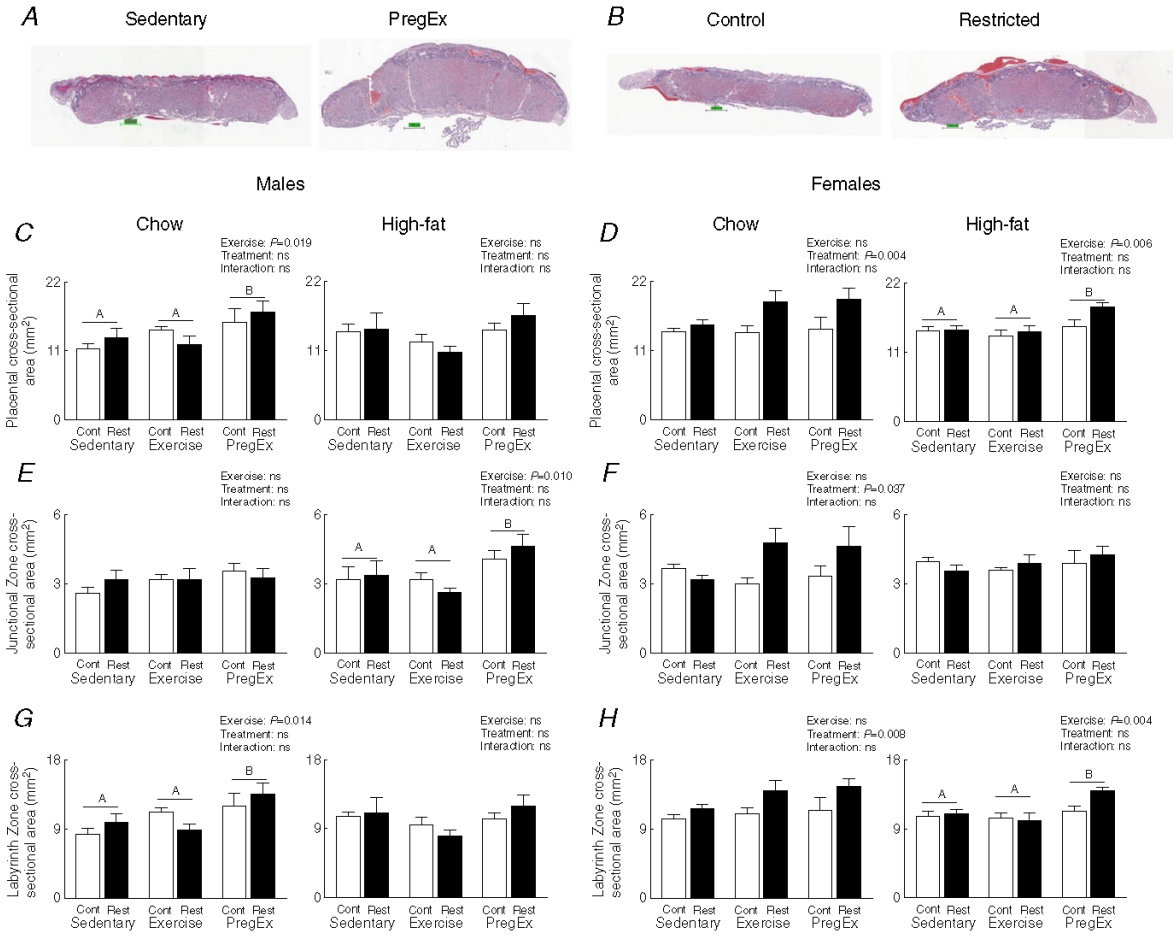

Figure 3. Placental histology parameters
Representative whole placental images demonstrating morphological changes following *PregEx* compared to *Sedentary* (male placentae from Chow-fed mothers) (*A*) and in Restricted compared to Control (*B*) placentae (female placentae from *Exercise* mothers). 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) and consumed a Chow (left panel) or High-fat diet (right panel) (n = 3–4 in each group/sex n = 1 representing one pup from one 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 from 'B', but not 'AB'. [Colour figure can be viewed at wileyonlinelibrary.com]

compared to F1 Chow-fed *Sedentary* mothers (Fig. 5*E*; two-way ANOVA). Similarly, despite no changes in *Igf1* mRNA abundance in F2 female-associated placentae (Fig. 5*C*), consumption of a High-fat diet in *PregEx* F1 mothers that were growth restricted prior to birth (Restricted) increased IGF1 protein expression in F2 female-associated placentae compared to F1 *Sedentary* and *Exercise* Restricted mothers (Fig. 5*F*; one-way ANOVA). With *PregEx* in High-fat fed normal birth weight (Control) mothers, we observed increasing IGF1 protein expression in F2 female-associated placentae compared to *Exercise* in F1 normal birth weight (Control) mothers (Fig. 5*F*).

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) and 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 (Fig. 6*A*, 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 (Fig. 6*A*). 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 normal birth weight (Control) mothers (Fig. 6*B*; 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 (Fig. 6B). However, IGF1R protein expression was not affected by F1 mothers that were growth restricted prior to birth (Restricted; Fig. 6*C* and *D*).

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 (Fig. 6*A*). 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 (Fig. 6*B*; 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 (Fig. 6*B*). 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 (Fig. 6*C* and *D*; 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* (Fig. 7*A*; Student's unpaired *t*-test). Additionally, *PregEx* in F1 mothers growth restricted prior to birth (Restricted) that consumed a Chow diet increased *Igf2* mRNA abundance in F2 male-associated placentae compared to *PregEx* in F1 Chow-fed normal birth weight (Control) mothers (Fig. 7*A*; Student's unpaired *t*-test).

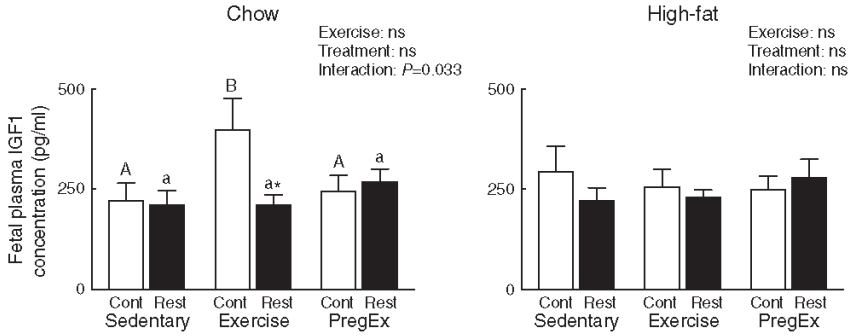

Figure 4. Fetal IGF1 concentrations
Pooled plasma IGF1 concentrations in fetuses from Chow (left) and High-fat (right) fed mothers (n = 8–10 litters in each group). Data were analysed by a two-way ANOVA and presented as mean ± SEM, where 'ns' is not significant. *P<0.05 *vs.* Control and differences across exercises are denoted by different letters where 'a/A' is different from 'b/B'.

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 (Fig. 7*B*). Consumption of a High-fat diet in F1 mothers growth restricted prior to 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 (Fig. 7*A* and *B*; 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 (Fig. 7*C* and *D*).

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; Fig. 7*A*, 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 (Fig. 7*A*, 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 (Fig. 7*B*, two-way ANOVA). No exercise effects were

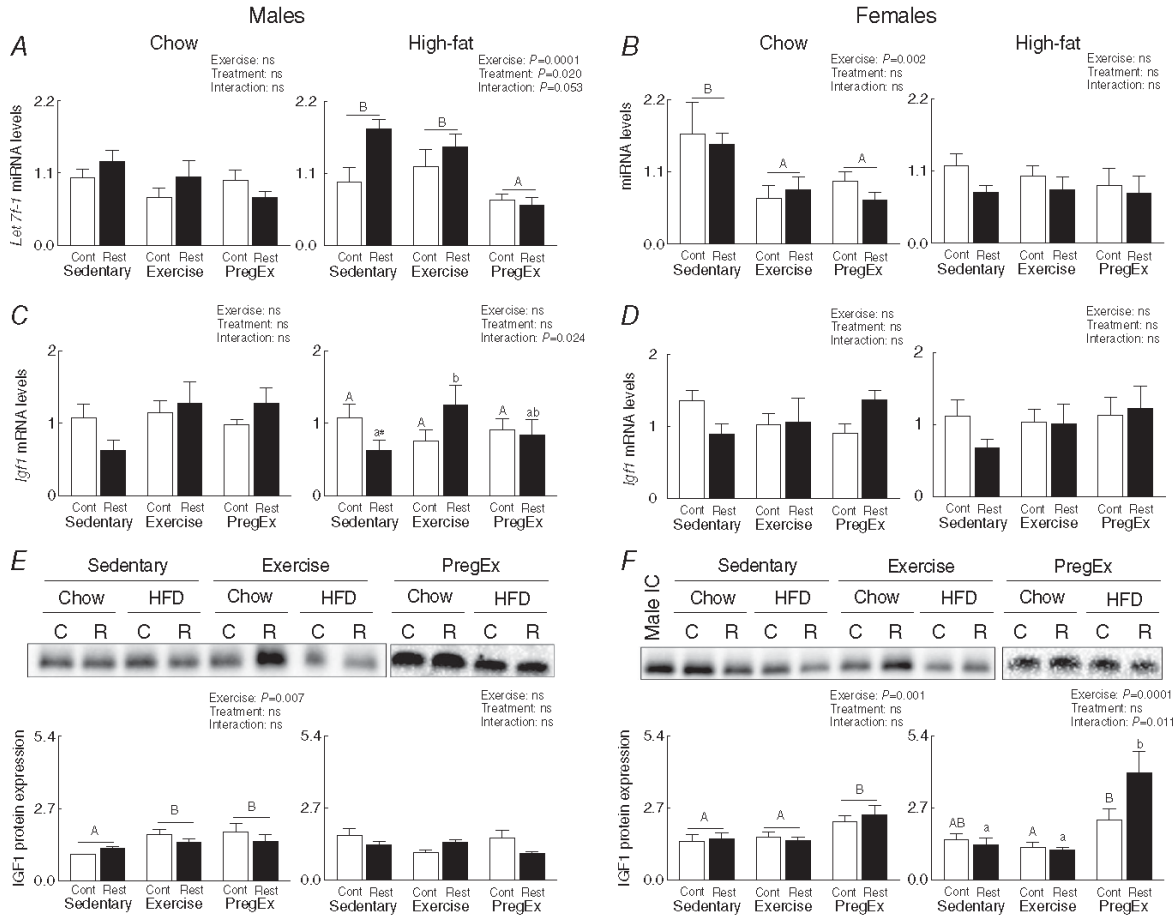

Figure 5. Placental IGF1 expression
Let 7f-1 miRNA abundance (*A* and *B*), *Igf1* mRNA abundance (*C* and *D*) ($n = 6$ in each group/sex $n = 1$ representing one pup from one litter) and IGF1 protein expression (*E* and *F*) ($n = 6$–7 in each group/sex $n = 1$ representing one pup from one litter) in male- and female-associated placentae whose mothers were Control (open bars) or Restricted (black bars) and 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 from 'b/B' but not 'ab/AB'.

observed in F2 male- or female-associated placentae, irrespective of maternal birth weight, if their F1 mother consumed a High-fat diet (Fig. 7*A* and *B*).

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* (Fig. 7*C*, 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* (Fig. 7*C*, 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 (Fig. 7*D*, two-way ANOVA). By contrast, *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* (Fig. 7*D*, one-way ANOVA).

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 (Fig. 8*A*; 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 (Fig. 8*B*; 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 (Fig. 8*A* and *B*).

F1 maternal exercise effects on F2 placental IGF2R. *PregEx*, irrespective of maternal birth weight, increased

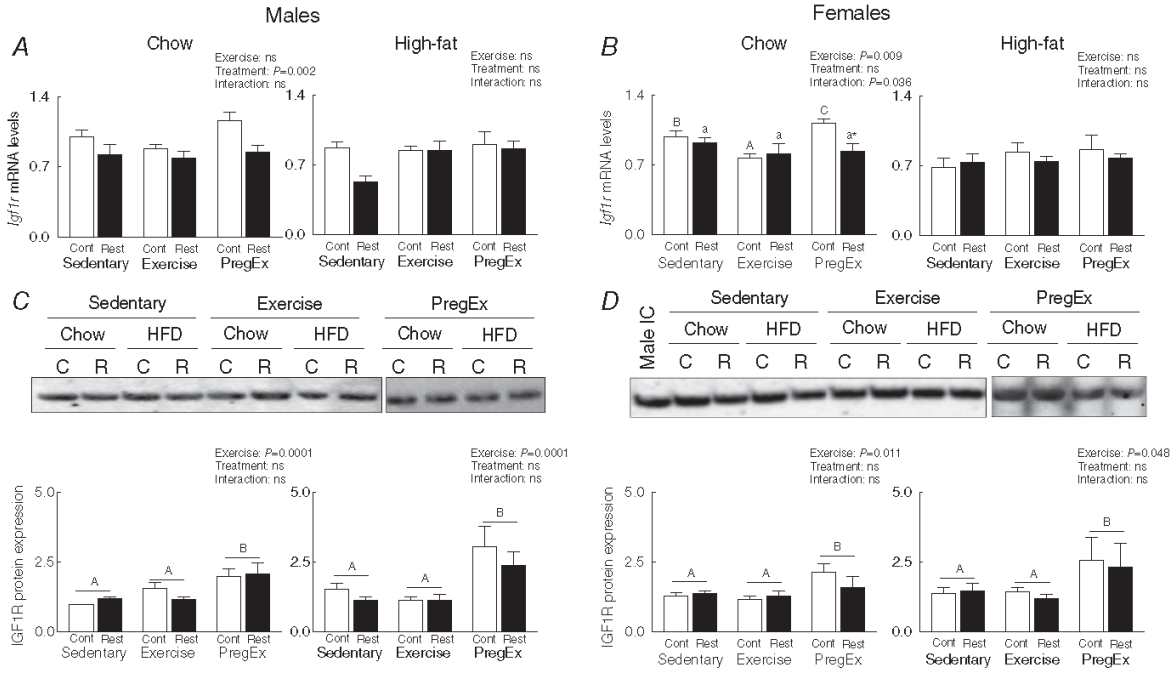

Figure 6. Placental IGF1R expression
IGF1R mRNA abundance (*A* and *B*) ($n = 6$ in each group/sex $n = 1$ representing one pup from one litter) and protein expression (*C* and *D*) (n =6–7 in each group/sex $n = 1$ representing one pup from one litter) in male- and female-associated placentae whose mothers were Control (open bars) or Restricted (black bars) and 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 from 'b/B' and 'c/C' but not different from 'ab/AB'.

*Igf2*r mRNA abundance in Chow-fed F2 male-associated placentae compared to Chow-fed F1 mothers that were *Sedentary* or *Exercised* (Fig. 8*A*; 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 (Fig. 8*A*, 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; Fig. 8*A*, 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 that *Exercised* (Fig. 8*B*, 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 (Fig. 8*B*; one-way ANOVA).

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; Fig. 8*C* and *D*).

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, and that 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, to be due to placental programming, which this study highlights may not be due to changes in the placental IGF system. Given that exercise is

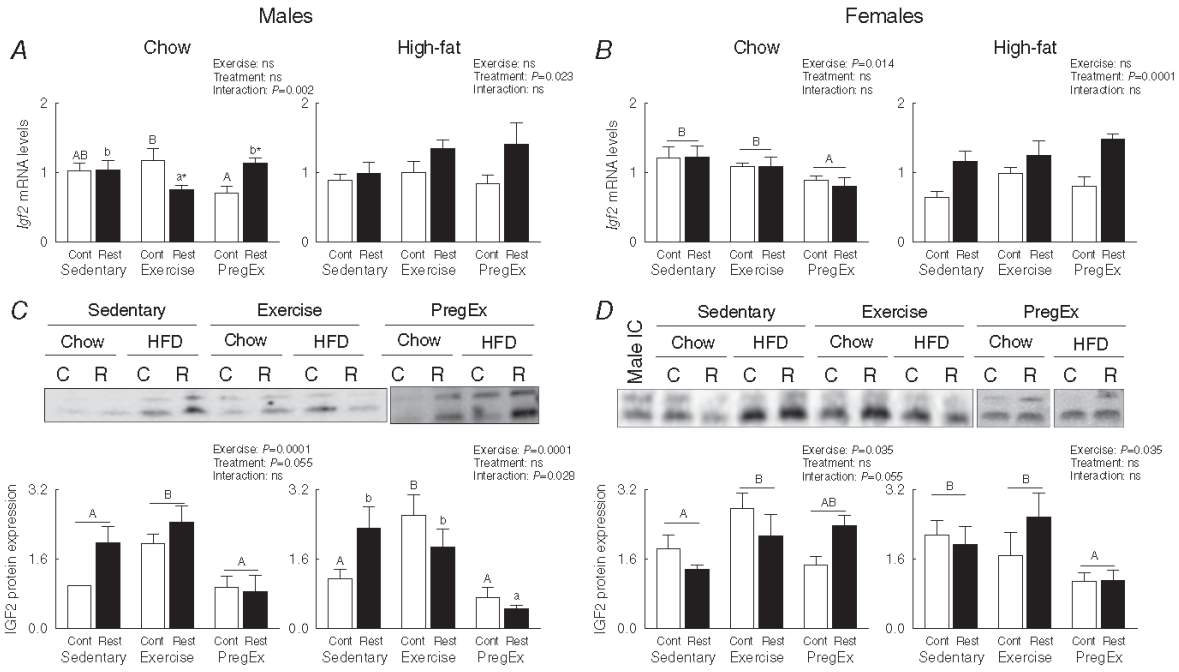

Figure 7. Placental IGF2 expression
IGF2 mRNA abundance (*A* and *B*) ($n = 6$ in each group/sex $n = 1$ representing one pup from one litter) and protein expression (*C* and *D*) ($n = 6$–7 in each group/sex $n = 1$ representing one pup from one litter) in male- and female-associated placentae whose mothers were Control (open bars) or Restricted (black bars) and 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 from 'b/B' and 'c/C' but not different to 'ab/AB'.

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 are 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.

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 probably contributes to the transgenerational disease programming we have previously reported (Gallo *et al.* 2012, 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 probably 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 signalling, 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 signalling 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 probably 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 favour the maternal metabolic system (Mottola & Christopher, 1991), reducing placental nutrient transport capacity (and hence placental efficiency) and

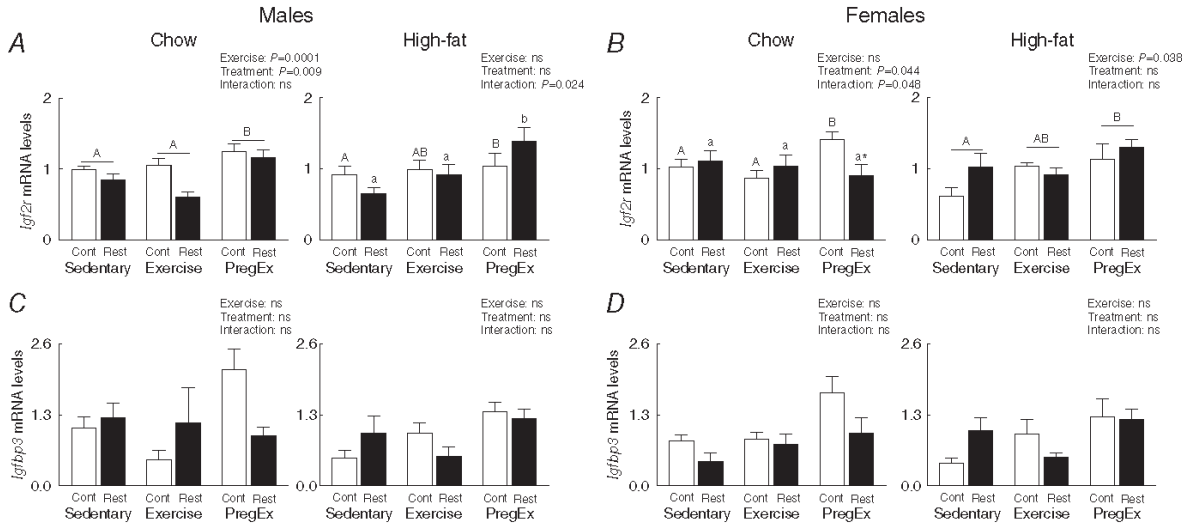

Figure 8. Placental *Igf2r* and *Igfbp3* mRNA abundance
Igf2r (*A* and *B*) and *Igfbp3* (*C* and *D*) mRNA abundance in male- and female-associated placentae whose mothers were Control (open bars) or Restricted (black bars) and consumed a Chow (left panel) or High-fat diet (right panel). Data were analysed by a two-way ANOVA and presented as mean ± SEM, where 'ns' is not significant ($n = 6$ in each group/sex $n = 1$ representing one pup from one litter). *$P < 0.05$ *vs.* Control and differences across exercises are denoted by different letters where 'a/A' is different from 'b/B' but not 'ab/AB'.

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 investigation. 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 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, 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 probably 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 that *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 the 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-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 signalling 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 to be due 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 the aim of increasing nutrient delivery to the fetus (Sferruzzi-Perri *et al.* 2006). This finding is consistent with studies in humans who 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.* 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 and 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, this 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 miRNAs 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 of 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, by contrast, reduces F2 placental weight independently of the IGF system and is probably 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 characterize alterations in placental nutrient handling and nutrient partitioning following maternal exercise.

Study limitations

A strength of the current study is that it allows direct comparison of the impact that 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 born growth restricted and following maternal exercise, with responses dependent on maternal diet and fetal sex, which probably 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 signalling (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 fetoplacental growth, which mostly occurred in both mothers with a normal and small birth weight. However, this adaptation is 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 that these alterations in the placental IGF system following maternal exercise have on F2 offspring birth weight, development and long-term health are unknown and future studies are required.

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Additional information

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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Chapter 4 Exercise initiated during pregnancy in rats born growth restricted alters placental mTOR and nutrient transporter expression

Results of this study are presented in its published form, following expanded aims, hypotheses, and methodologies used in the study

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4.1 Aims and Hypotheses

The overall aim of this study was to determine changes in the placental labyrinth nutrient transporter expression in F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to identify if maternal high-fat feeding exacerbated the alterations within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

Specific aims were to assess the following:

i) if maternal growth restriction prior to birth alters F2 labyrinth nutrient transporter and mTOR expression;
ii) if maternal exercise initiation prior to and/or during pregnancy attenuates outcomes;
iii) if maternal high-fat feeding exacerbates outcomes;
iv) if there are any sex-specific differences.

It was hypothesized that F2 offspring from *Restricted* mother's would have reduced nutrient transporter expression compared to F2 offspring from normal birth weight mothers (*Control*). It was predicted that maternal high-fat feeding would exacerbate these outcomes and that *Exercise* would improve F2 fetal outcomes to a greater extent than *PregEx*.

4.2 Methods

Methodologies used in this study have been described in Chapter 2 and expanded from the published version (**Section 4.3**) in subsequent sections. The current study utilises qPCR and Western blotting experiments in WKY rats that underwent bilateral uterine vessel ligation that were allocated one of two diets and underwent treadmill exercise.

4.2.1 Animals

UPI (*Restricted*) or sham (*Control*) surgery was induced on E18 in WKY rats. F1 offspring were fed a Chow or HFD from weaning, and at 16 weeks were randomly allocated an exercise protocol; *Sedentary*, *Exercise* or *PregEx* as described in **Section 2.2**. Females were mated (20 weeks) with placentae associated with F2 fetuses collected at E20, weighed, and stored at -80°C.

4.2.2 Placental gene abundance

Total RNA was isolated from 50 mg of labyrinth tissue as described in **Section 2.4**. qPCR was performed to quantify the abundance of the following genes of interest; *Slc2a1*, *Slc2a3*, *Slc5a1*, *Sl38a1*, *Slc38a2*, *Slc38a4* and *Mtor* (**Table 2.2**).

4.2.3 Western blotting

Protein was isolated from 50 mg labyrinth tissue as described in **Section 2.5**. Western blotting was then performed to determine changes in GLUT3, MTOR and p-MTOR (Ser 2448) (**Table 2.4**).

4.2.4 Statistical analysis

The publication (**Section 4.3**) contains the statistical analysis performed for this Chapter.

4.3 Published Manuscript

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

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Key points

- Fetal growth is dependent on effective placental nutrient transportation, which is regulated by mammalian target of rapamycin (mTOR) complex 1 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 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 characterized with exercise initiated before and continued throughout pregnancy in nutrient transporter and mTOR expression.
- Maternal exercise during pregnancy differentially regulated mTOR and nutrient transporters in a diet- and sex-specific manner, which likely aimed to improve late gestational placental growth and neonatal survival.

Abstract 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 mechanistic target of rapamycin (mTOR) and nutrient transporter expression in growth restricted mothers and

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J. F. Briffa and Mary E. Wlodek are co-senior authors.

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whether these outcomes were dependent on maternal diet or fetal sex. Uteroplacental insufficiency or sham 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 to an exercise protocol: sedentary, exercised prior to and during pregnancy, or exercised during pregnancy only. Females were mated with normal males (20 weeks) and F2 placentae collected at E20. Exercise during pregnancy only, reduced mTOR protein expression in all groups and increased mTOR activation in male associated placentae. Exercise during pregnancy only, 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.

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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) signalling 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 mTOR complex 1 (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), which also often present with hypoglycaemia (Economides & Nicolaides, 1989; Jansson *et al.* 2012; Diaz *et al.* 2014), with both of these outcomes likely 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.* 2014; Dimasuay *et al.* 2016; Zhang *et al.* 2016). Interestingly, mTOR signalling 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, 2013; Cheong *et al.* 2016*a*). 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.

We recently demonstrated that the insulin-like growth factor (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 throughout pregnancy (termed '*Exercise*'), but not exercise initiated in the final two-thirds of pregnancy (termed '*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 nutrient 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).

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 Council's *Australian Code for the Care and Use of Animals for Scientific Purposes*. 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 were 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, see Fig. 1.

Exercise training

For the entirety of their exercise regime, F1 females exercised 5 days per week on a motorized treadmill (Columbus Instruments, Columbus, OH, USA) followed by 2 days of rest 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 min at 15 m·min^{-1}, 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 min. On day 1 of week 2 and thereafter until mating, the rats exercised for 60 min·day^{-1} at 20 m·min^{-1}, as previously described (Laker *et al.* 2011, 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 min at 17 m·min^{-1}, for week 2 of pregnancy they exercised for 30 min at 13 m·min^{-1} and for week 3 of pregnancy they exercised for 20 min at 11 m·min^{-1}. 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 diagram in our recent publication (Mangwiro *et al.* 2018).

Post mortem

At E20, F1 females were anaesthetized (100 mg·kg^{-1} ketamine (Parnell Laboratories Pty Ltd, Alexandria, NSW, Australia) and 30 mg·kg^{-1} Ilium Xylazil (xylazine; 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, Victoria, 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 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 (5 μm) 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 cell cross-sectional area using the Aperio ScanScope system (Aperio Technologies, Vista, CA, USA) and Image Scope software (Leica Microsystems, Mt Waverly, Victoria, Australia), as described previously (Gardebjer *et al.* 2014).

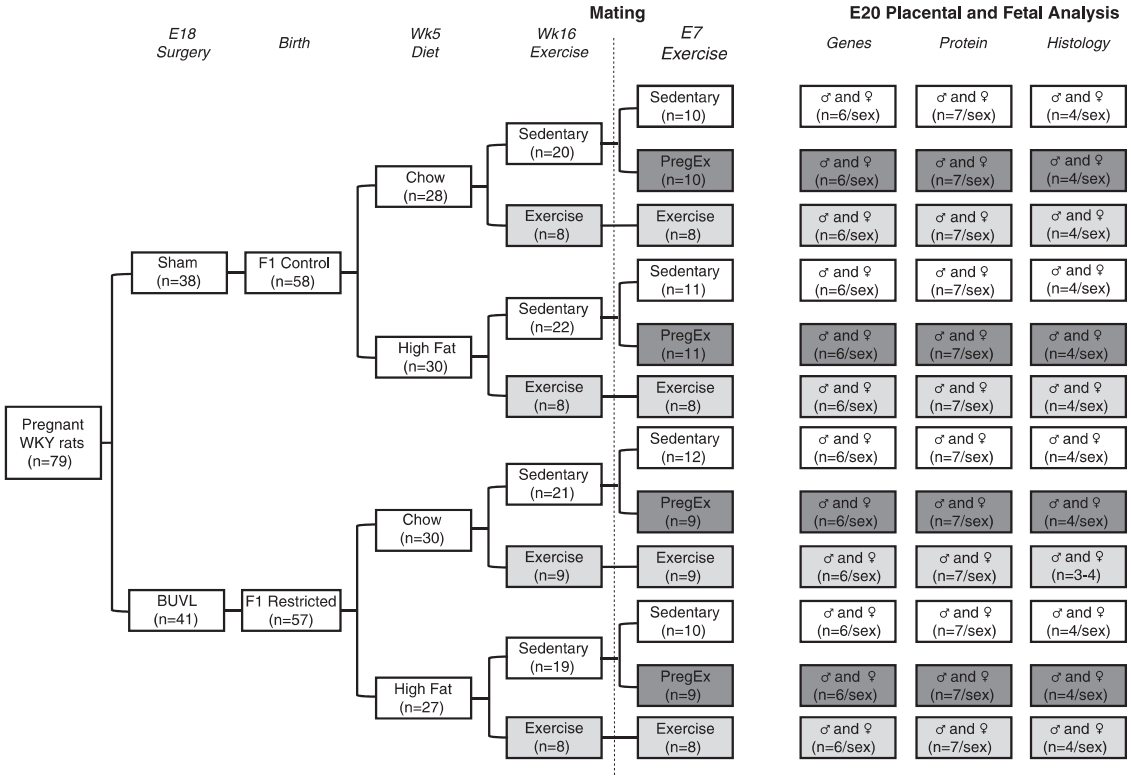

Figure 1. Study design and sample sizes
Flow chart of the experimental protocol indicating the allocation of pregnant rats within the different treatments, diet and exercise groups along with the sample size per experimental outcome. Exercise, exercise prior to pregnancy and continued throughout gestation; E, embryonic day; F1, first generation; *n*, sample size; PregEx, exercise initiated during the second week of gestation; Wk, week.

Placental gene abundance

RNA was extracted from 50 mg placental labyrinth using a commercially available kit (miRNeasy Mini Kit; Qiagen, Chadstone, Victoria, Australia) and the Precellys 24 homogenizer (Bertin Technologies; Aix en Provence, France) with CK14 ceramic beads, as previously described (Cheong *et al.* 2016*b*; Mangwiro *et al.* 2018). First strand cDNA was generated from 1 μg RNA using the High Capacity cDNA kit (Life Technologies, Mulgrave, Victoria, Australia). qPCR was then performed using Taqman master mix (Life Technologies), in line with the MIQE guidelines (Bustin *et al.* 2009). PCR primers were purchased for *Mtor* and the following nutrient transporter genes of interest (Life Technologies); *Slc2a1* (Rn01417099_m1; NM_138827.1), *Slc2a3* (Rn00567331_m1; NM_017102.2), *Slc5a1* (Rn01640634_m1; NM_013033.2), *Slc38a1* (Rn00593696_m1; NM_138832.1), *Slc38a2* (Rn00710421_m1; NM_181090.2), *Slc38a4* (Rn00590667_m1; NM_130748.1) and *Mtor* (Rn00693900_m1; NM_019906.1). mRNA abundance of the genes of interest was 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 variations in RNA input amounts and reverse transcriptase efficiency. HotStart DNA Taq Polymerase was activated by heating the mixture to 95°C for 10 min, then qPCR reactions were run for 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative change 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, TX, USA), mTOR (1:1000, Cell Signalling Technology, Arundel, Queensland, Australia) and p-mTOR (Ser2448) (1:1000, Cell Signalling 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 p-mTOR (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 with a Duncan's *post hoc* test was used to identify Exercise differences. If an interaction was observed, the data were further split to identify Treatment (maternal birth weight) effects within each Exercise regime using 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 were 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, Student's unpaired *t* test 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 refer 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 Corp., Armonk, NY, USA) and Student's unpaired *t* test was performed using Microsoft Excel. 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 a 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 (Fig. 2*A*) compared to *Restricted Sedentary* dams. Despite these exercise-specific alterations in total mTOR protein expression, Restriction increased p-mTOR (Ser2448) protein expression regardless of maternal exercise regime (Fig. 2*A*). However, no maternal birth weight effects were observed in mTOR activation (Fig. 2*B*).

In Chow-fed dams, *Exercise* increased *Mtor* mRNA abundance in male associated placentae compared to *Sedentary* mothers (Table 1; one-way ANOVA), whereas

Table 1. *Mtor*, *Slc2a3* and *Slc5a1* 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)

									Two-way ANOVA *P*-value		
Gene			Sedentary		Exercise		PregEx		Treatment	Exercise	Interaction
Mtor											
Male	Chow	Control	1.06 ± 0.15	A	1.64 ± 0.27	B	0.44 ± 0.06	A	ns	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	0.0001	0.0001	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	AB	2.91 ± 1.27	B	0.73 ± 0.19	A	ns	0.018	ns
		Restricted	1.75 ± 0.39		2.10 ± 0.40		0.73 ± 0.14				
	High-fat	Control	0.88 ± 0.14	B	1.02 ± 0.13	B	0.43 ± 0.04	A	0.009	0.003	ns
		Restricted	2.12 ± 0.48		1.61 ± 0.42		0.52 ± 0.03				
Slc2a3											
Male	Chow	Control	1.09 ± 0.22	A	1.47 ± 0.17	AB	1.99 ± 0.34	B	ns	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	0.040
		Restricted	1.63 ± 0.30	A	1.23 ± 0.19	A	1.16 ± 0.24	A			
	High-fat	Control	0.65 ± 0.14	a	1.39 ± 0.12	b	1.16 ± 0.27	ab	ns	0.030	0.030
		Restricted	1.43 ± 0.25*	AB	1.09 ± 0.20	A	1.93 ± 0.30	B			
Slc5a1											
Male	Chow	Control	1.31 ± 0.32		1.12 ± 0.41		0.21 ± 0.03		ns	ns	ns
		Restricted	0.49 ± 0.19		2.10 ± 1.15		0.17 ± 0.05				
	High-fat	Control	0.16 ± 0.08		0.21 ± 0.05		0.91 ± 0.42		ns	ns	ns
		Restricted	1.46 ± 0.75		0.25 ± 0.10		0.32 ± 0.16				
Female	Chow	Control	1.94 ± 0.95		1.15 ± 0.41		0.14 ± 0.03		ns	ns	ns
		Restricted	0.80 ± 0.29		1.39 ± 0.72		0.75 ± 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				

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 from 'b/B' but not 'ab/AB', with lowercase letters denoting Control and uppercase letters denoting Restricted dams.

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* (Fig. 2*A*; one-way ANOVA), whereas *Exercise* and *PregEx* reduced p-mTOR (Ser2448) protein expression only in High-fat fed dams compared to *Sedentary* (Fig. 2*A*; 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* (Fig. 2*B*; 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 p-mTOR (Ser2448) protein expression or mTOR activation (Fig. 2*C* and *D*).

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 (Fig. 2*C*; one-way ANOVA), whereas *Exercise* and *PregEx* reduced p-mTOR (Ser2448) protein expression in Chow-fed mothers compared to *Sedentary* (Fig. 2*C*; 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 p-mTOR (Ser2448) protein expression compared to *Sedentary* (Fig. 2*C*; one-way ANOVA).

No maternal exercise effects were observed on mTOR activation (Fig. 2*D*).

Placental system A transporters

Male associated placentae. Maternal growth restriction increased *Slc38a1* (*Sedentary* and *Exercise*) and *Slc38a2* (*Exercise*) mRNA abundance in the High-fat, but not Chow, fed mothers compared to *Control* counterparts in males (Fig. 3*A* and *B*; Student's unpaired *t* test, $P < 0.0002$). No maternal growth restriction effects were observed in *Slc38a4* mRNA abundance (Fig. 3*C*).

Exercise in Chow-fed mothers increased *Slc38a1* and *Slc38a2* mRNA abundance (Fig. 3*A* and *B*; one-way ANOVA), whereas *PregEx* reduced *Slc38a4* mRNA (Fig. 3*C*, one-way ANOVA) in male associated placentae compared to *Sedentary*. In *Restricted* High-fat fed dams, *Exercise* increased and *PregEx* decreased *Slc38a1* and *Slc38a2* mRNA abundance compared to *Restricted Sedentary* mothers (Fig. 3*A* and *B*; one-way ANOVA). Additionally, *PregEx* in *Control* High-fat fed dams reduced *Slc38a1* and *Slc38a2* mRNA compared to *Sedentary Control* mothers (Fig. 3*B*; one-way ANOVA). *Exercise* and *PregEx* in High-fat fed dams reduced *Slc38a4* mRNA abundance in male associated placentae compared to *Sedentary* (Fig. 3*C*; one-way ANOVA).

Female associated placentae. *Slc38a1* and *Slc38a2* mRNA abundance was increased in female associated placenta from *Restricted* High-fat, but not Chow, fed mothers compared to *Control* mothers (Fig. 3*D* and *E*; two-way ANOVA), which was largely driven by increased *Slc38a1* gene abundance in *Sedentary* (+179%) and *Exercise* (+41%) dams and increased *Slc38a2* gene abundance in the *Sedentary* (+95%) and *PregEx* (+73%) dams. Whereas *Slc38a4* mRNA abundance was reduced in *Restricted* High-fat, but not Chow, fed mothers that *Exercised* compared to *Exercised Control* mothers (Fig. 3*F*; Student's unpaired *t* test, $P = 0.02$).

No exercise effects were observed in *Slc38a1*, *Slc38a2* or *Slc38a4* mRNA abundance, compared to *Sedentary*, in female associated placentae from Chow-fed mothers (Fig. 3). In High-fat fed mothers, *PregEx* reduced *Slc38a1* and *Slc38a2* mRNA abundance compared to *Sedentary* (Fig. 3*D* and *E*; one-way ANOVA). Neither *Exercise* nor

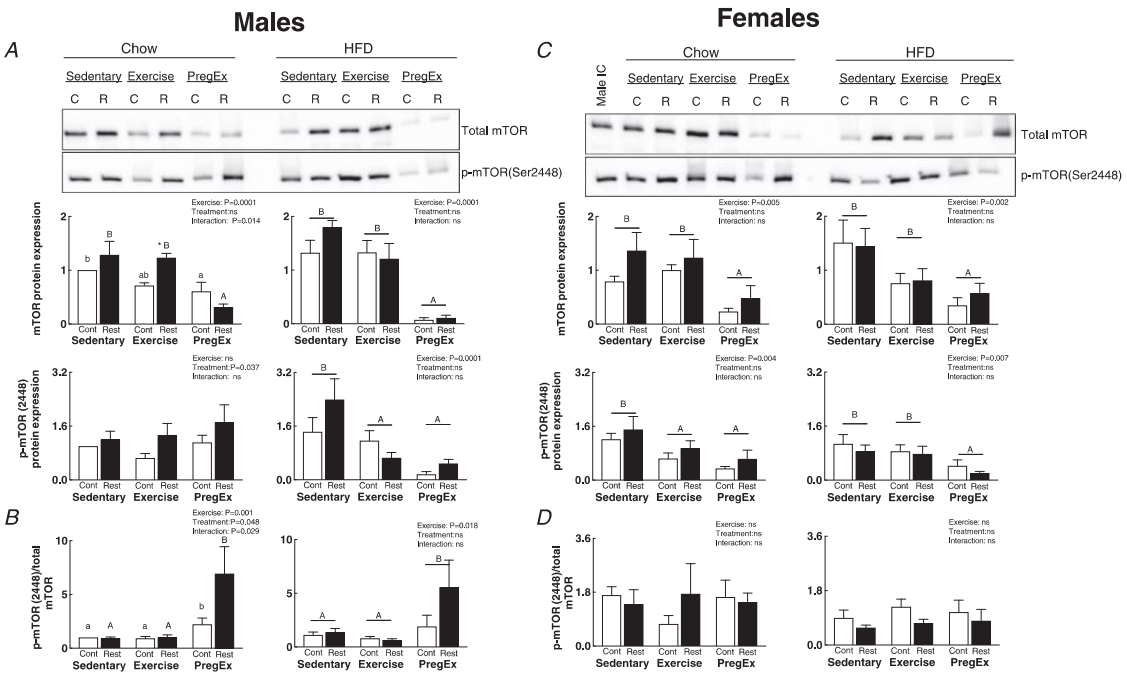

Figure 2. Placental mTOR and p-mTOR expression
Total and p-mTOR protein expression (*A* and *C*) and p-mTOR/total mTOR ratio (*B* and *D*) in male and female associated placentae whose mothers were Control (C; open bars) or Restricted (R; filled bars) that consumed a Chow (left panel) or High-fat diet (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 from 'b/B', with lowercase letters denoting Control and uppercase letters denoting Restricted dams.

PregEx altered *Slc38a4* mRNA abundance, compared to *Sedentary*, in High-fat mothers (Fig. 3*F*).

Placental glucose transporters

Male associated placentae. No maternal birth weight effects were characterized in *Slc2a1*, *Slc2a3* and *Slc5a1* mRNA abundance (Fig. 4*A* and Table 1) or GLUT3 protein expression (Fig. 4*B*) in male associated placentae on either diet.

No maternal exercise effects were reported in *Slc2a1* (Fig. 4*A*) and *Slc5a1* (Table 1) mRNA abundance in male associated placentae. However, *PregEx* in Chow, but not High-fat, fed mothers increased *Slc2a3* mRNA abundance (Table 1; one-way ANOVA) and GLUT3 protein expression (Fig. 4*B*; one-way ANOVA) compared to *Sedentary* mothers.

Female associated placentae. No maternal birth weight effects were observed in *Slc2a1* (Fig. 4C) and *Slc5a1* (Table 1) mRNA abundance in female associated placentae. Maternal growth restriction increased *Slc2a3* 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 (Fig. 4*D*).

No maternal exercise effects were reported in *Slc2a1* (Fig. 4*C*) and *Slc5a1* (Table 1) mRNA abundance. *PregEx* (Chow only) and *Exercise* (High-fat only) increased *Slc2a3* mRNA abundance in *Control*, but not *Restricted*, dams compared to *Control Sedentary* (Table 1; one-way ANOVA). However, these alterations in *Slc2a3* mRNA abundance did not translate to alterations in GLUT3 protein expression (Fig. 4*D*).

Placental glycogen cell cross-sectional area

Male associated placentae. No maternal birth weight effects were reported in placental glycogen cell cross-sectional area in male associated placentae (Fig. 5*A*). However, *PregEx* in High-fat, but not Chow, fed dams increased glycogen cell cross-sectional area in male

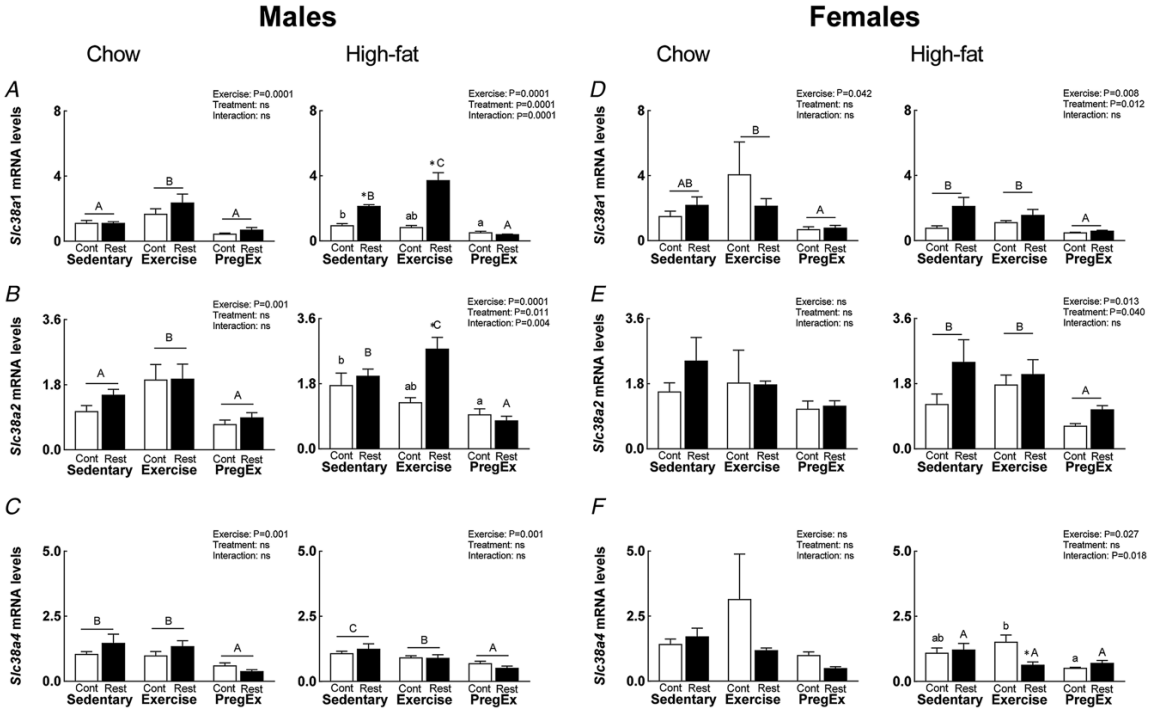

Figure 3. Placental system A amino acid transporter expression
Slc38a1 (*A* and *D*), *Slc38a2* (*B* and *E*) and *Slc38a4* (*C* and *F*) mRNA abundance in male and female associated placentae whose mothers were Control (open bars) or Restricted (filled bars) that consumed a Chow (left panel) or High-fat 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 from 'b/B' but not 'ab/AB', with lowercase letters denoting Control and uppercase letters denoting Restricted dams.

associated placentae compared to *Sedentary* (Fig. 5*A*; one-way ANOVA).

Female associated placentae. No maternal birth weight or exercise effects were observed in glycogen cell cross-sectional area in female associated placentae (Fig. 5*B*); despite an ∼84% increase in glycogen cell 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.* 2016*b*) may be partly due to alterations in the placental IGF system (Mangwiro *et al.* 2018). Here we

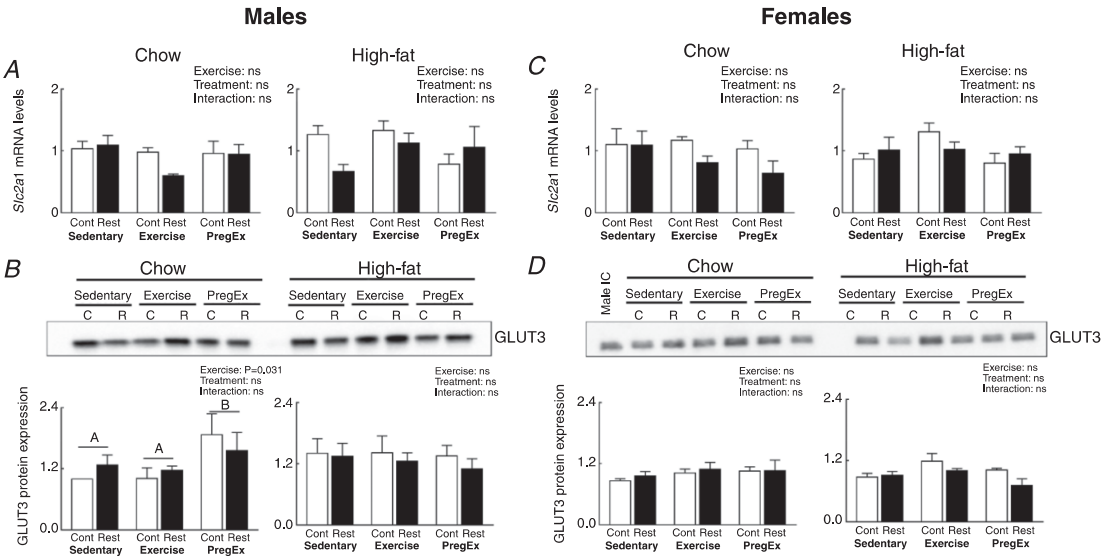

Figure 4. Placental GLUT3 expression and glycogen cell cross-sectional area
Slc2a1 (*A* and *C*) mRNA abundance and GLUT3 protein expression (*B* and *D*) in male and female associated placentae whose mothers were Control (C; open bars) or Restricted (R; filled bars) that consumed a Chow- (left panel) 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 from 'B'.

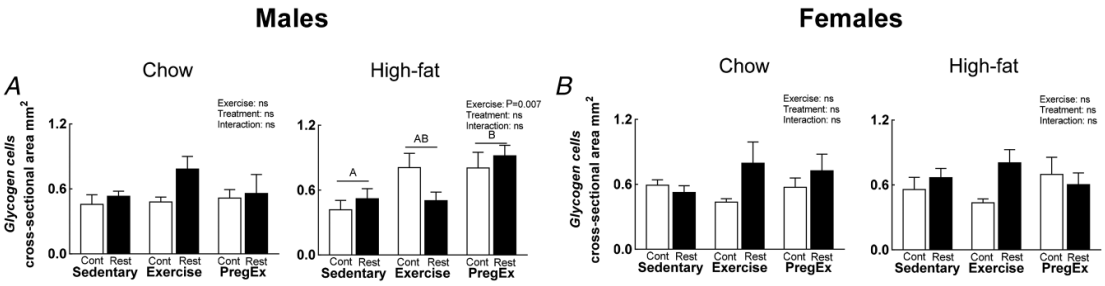

Figure 5. Placental glycogen cell cross-sectional area
Glycogen cell cross-sectional area in male (*A*) and female (*B*) associated placentae whose mothers were Control (open bars) or Restricted (filled bars) that consumed a Chow (left panel) or High-fat diet (right panel). Data were analysed by a two-way 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 from 'B' but not different from 'AB'.

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 signalling. 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 signalling 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 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, increasing 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, 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, however, pertain 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 *Slc2a1* and *Slc38a2* 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 study (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, 2013; Cheong *et al.* 2016*b*).

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 *Slc38a1* (*Sedentary* and *Exercise* in males) and *Slc38a2* (Exercise in males) gene abundance, with reductions in *Slc38a4* 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 increases *Slc38a2* gene abundance, with no changes in *Slc2a1*, *Slc38a1*, *Slc38a4* 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 *Slc38a1* and *Slc38a2* gene abundance in male associated placentae whose mother consumed a Chow and High-fat (only in *Restricted* mothers) diet and reduced *Slc38a4* 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* that 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 adaptation to prevent the fetal overgrowth we previously reported (Mangwiro *et al.* 2018).

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 *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 mother's 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 from Chow-fed mothers suggests there is 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.* 2014). 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 that were 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 p-mTOR 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 placental nutrient transport following maternal exercise have on offspring birth weight and long-term offspring health are unknown and require further investigation.

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Additional information

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 have read and approved

the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Chapter 5 Maternal exercise and growth restriction in rats alters placental angiogenic factors and blood space area in a sex-specific manner

Results of this study are presented in its published form, following expanded aims, hypotheses, and methodologies used in the study

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5.1 Aims and Hypotheses

The overall aim of this study was to determine changes in placental labyrinth angiogenesis in F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to characterize if these outcomes are exacerbated by maternal high-fat feeding within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

Specific aims were to assess the following:

i) if maternal growth restriction prior to birth alters F2 labyrinth vasculogenic and/or angiogenic expression;
ii) if maternal exercise initiation prior to and/or during pregnancy attenuates outcomes;
iii) if maternal high-fat feeding exacerbates outcomes;
iv) if there are any sex-specific differences.

It was hypothesized that F2 offspring from *Restricted* mothers would have aberrant expression of vasculogenic and/or angiogenic factors compared to offspring from *Control* mothers. It was predicted that maternal high-fat feeding would exacerbate these outcomes and that *Exercise* would improve F2 fetal outcomes to a greater extent than *PregEx*.

5.2 Methods

Methodologies used in this study have been described in Chapter 2 and expanded from the published version (**Section 5.3**) in subsequent sections. The current study utilises experiments in WKY rats that underwent bilateral uterine vessel ligation that were allocated one of two diets and underwent treadmill exercise and contains qPCR and Western blotting experiments.

5.2.1 Animals

UPI (*Restricted*) or sham (*Control*) surgery was induced on E18 in WKY rats. F1 offspring were fed a Chow or HFD from weaning, and at 16 weeks were randomly allocated an exercise protocol; *Sedentary*, *Exercise* or *PregEx* as described in **Section 2.2**. Females were mated (20 weeks) with placentae associated with F2 fetuses collected at E20, weighed, and stored at -80°C.

5.2.2 Placental gene abundance

Total RNA was isolated from 50 mg of labyrinth tissue as described in **Section 2.4**. qPCR was performed to quantify the abundance of the following genes of interest; *Vegfa*, *Flt-1* and *Plgf1* mRNA (**Table 2.2**) and *miRNA27a* (**Table 2.3**).

5.2.3 Western blotting

Protein was isolated from 50 mg labyrinth tissue as described in **Section 2.5**. Western blotting was then performed to determine changes in VEGF, FLT-1 and PLGF (**Table 2.4**).

5.2.4 Statistical analysis

The published work (**Section 5.3**) contains the statistical analysis performed for this Chapter.

5.3 Published Manuscript

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Maternal exercise and growth restriction in rats alters placental angiogenic factors and blood space area in a sex-specific manner

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Vasculogenesis

ABSTRACT

Fetal growth and development are dependent on adequate placental nutrient transfer. The surface area of the placental villous network is a key determinant of nutrient exchange, which is regulated by vasculogenic and angiogenic factors. These factors are altered by intrauterine growth restriction (IUGR) and maternal obesity in both the first (F1) and second (F2) generations. We investigated the impact of endurance exercise in IUGR dams fed a High-fat diet on placental vasculogenesis and angiogenesis. 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 further allocated an exercise protocol; *Sedentary*, Exercised prior to and during pregnancy (*Exercise*), or Exercised during pregnancy only (*PregEx*). Females were mated (20 weeks) and F2 placentae collected at E20. Maternal *Restriction*, High-fat feeding and *Exercise* had a minimal impact on placental regulators of vasculogenesis and angiogenesis. However, *Restriction* increased placental labyrinth tissue area in Chow-fed dams. *PregEx* induced overt adaptations, including increased VEGFA and decreased PLGF protein expression, and reduced blood space area. These alterations were sex-dependent and associated with alterations in *miRNA27a*, a known regulator of VEGF translation. These data highlight that maternal exercise initiated during pregnancy (*PregEx*) causes alterations in placental vasculogenesis and angiogenesis in a sex-dependent manner, with minimal *Restriction* and maternal diet effects. However, further investigation is required to determine if these adaptations are beneficial or harmful for maternal and fetoplacental outcomes.

1. Introduction

Fetal growth and development are highly dependent on placental transport of nutrients, oxygen and waste products, which is dependent on the establishment of an effective placental vascular network. The formation of this vascular network is regulated by pro-vasculogenic and angiogenic factors, such as the vascular endothelial growth factor family of proteins (VEGFA) and placental growth factor (PlGF), that elicit their effect by binding to VEGFR-1 (Flt-1) and VEGFR-2 (KDR) receptors [1,2]. VEGF binding to its receptors stimulates differentiation and maturation of the placental villous bed, including the differentiation of precursor cells into endothelial cells during vasculogenesis [3,4]. Studies in *Plgf*$^{-/-}$ mice demonstrate the importance of PlGF during placental blood vessel development, where these mice have reduced implantation sites and placental vascular branching as well as increased blood spaces [5]. Recent studies demonstrate that VEGFA expression can be modulated by micro RNAs (miRNA), including *miRNA27a* [6], which may implicate this miRNA in altered vasculogenesis and/or angiogenesis in complicated pregnancies.

Placental insufficiency is the most common cause of intrauterine growth restriction (IUGR) affecting 10% of pregnancies [7] and is characterized by aberrant changes in placental blood vessel morphology (decreased placental villi vascular density and impaired branching angiogenesis) as well as alterations in vasculogenic and angiogenic pathways [8–11]. Animal studies have reported similar outcomes in hypoxia induced IUGR mice, whereby placental labyrinth

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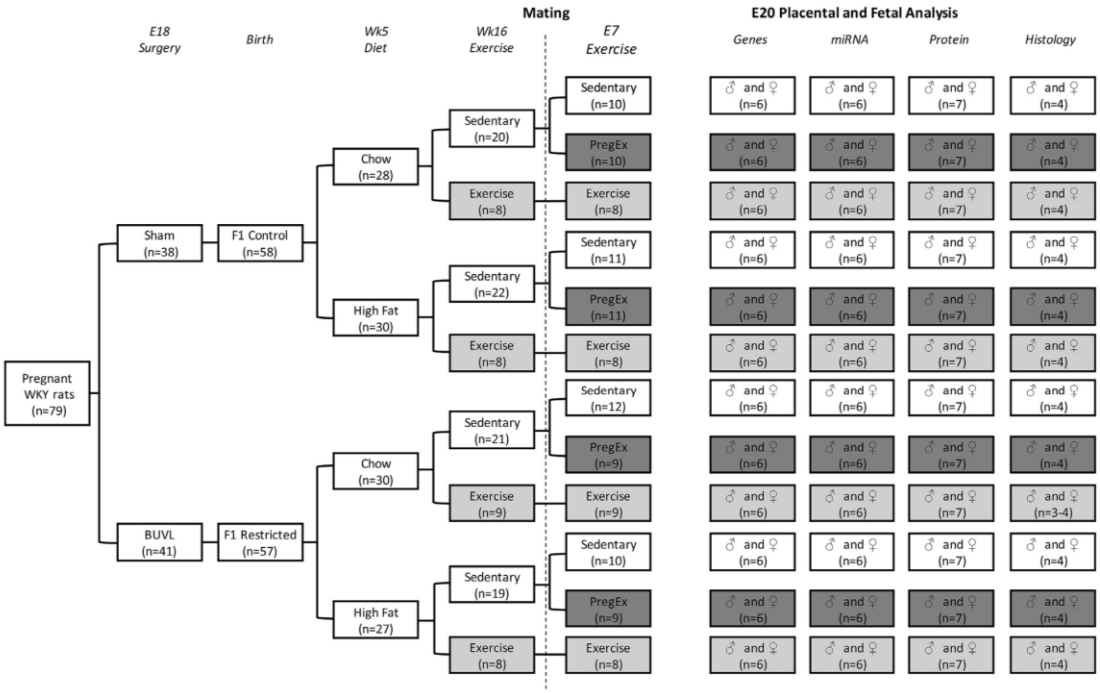

Fig. 1. Flow chart of experimental groups and sample sizes Flow chat of animal group allocation and sample numbers for gene, miRNA, protein and histological analysis. BUVL - bilateral uterine vessel ligation; F1 – first generation; E – embryonic day; Wk – week; n – sample size; Exercise – exercise initiated prior to and continued throughout pregnancy; PregEx – remained sedentary prior to and in the first week of pregnancy than exercised from week 2 of gestation.

blood space area is reduced in association with altered angiogenic factor expression [12]. The long-term impact of IUGR on offspring disease is well characterized in the first generation (F1), with a number of recent studies demonstrating intergenerational transmission of programmed non-communicable disease from the F1 to the F2 generation [13–16]. However, the impact of F1 growth restriction on F2 placental vascular development is uncharacterized.

Obesity during pregnancy is independently associated with aberrations in placental angiogenesis and vascularization [17,18]. Therefore, it is not surprising that one factor that may contribute to intergenerational disease transmission is maternal obesity, a phenotype that has been reported in both IUGR human populations and animal models [19]. This propensity for obesity in IUGR offspring is likely due to their increased preference for high-fat and/or high-calorie foods [20,21]. Therefore, females born small that develop obesity prior to pregnancy may have F2 fetuses with impaired placental vascularization, which may compromise fetal growth. Obese women are advised to lose weight prior to pregnancy to limit the negative impact obesity may have on the developing fetus, with exercise being an important lifestyle intervention to improve health outcomes [22]. However, approximately 40–50% of pregnancies are unplanned [23,24] and so for many pregnancies exercise may be initiated for the first time after pregnancy recognition.

Few studies have investigated the impact of exercise during pregnancy on placental formation [25] with only one study demonstrating that beginning regular exercise during pregnancy and continuing throughout pregnancy stimulates an increased rate of third trimester placental volume growth as well as increased placental size and villous volumes [26]. Furthermore, no studies have compared the impact of initiating exercise before pregnancy and continuing throughout pregnancy (*Exercise*) versus initiating exercise in the second third of pregnancy (*PregEx*) on placental vasculogenesis or angiogenesis.

Therefore, the current study aimed to characterize the expression of vasculogenesis and angiogenesis mediators as well as blood space area in placenta of F2 fetuses from mothers that were growth restricted at birth. This study also aimed to characterize the impact of a High-fat diet (HFD) on regulators of placental vascular development and to determine if *Exercise* or *PregEx* could ameliorate any negative outcomes or have independent harmful effects. As placental deficits have been shown to occur in a sex-specific manner [27,28], placentae of male and female fetuses were analysed separately.

2. Materials and methods

2.1. Animals

The University of Melbourne's animal experimentation ethics subcommittee (AEC: 1212639) approved all experiments, 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 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 access to standard rat chow and water *ad libitum*. Rats were mated with normal males and on day 18 of gestation (E18; term = 22 days) uteroplacental insufficiency surgery was performed as described previously [29,30]. 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 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 as previously published [31]. F1 *Control* and *Restricted* females were weaned from their mothers on postnatal day 35 (PN35) and were randomly allocated to either a Chow (AIN93G; 16% kcal fat

and 16.1 MJ/kg digestible energy) or a selection of two High-fat diets (SF03-020 and SF01-028; 43% kcal fat with 20 MJ/kg and 19.9 MJ/kg digestible energy respectively)) that were matched for micro- and macronutrients Specialty Feeds, Glen Forrest, WA, Australia. At 16 weeks, F1 female offspring where further randomly allocated to either one of the following exercise groups: remained *Sedentary*, *Exercise* (16–24 weeks of age) or *PregEx* (*Sedentary* prior to and in the first week of pregnancy, then exercised from E7 to E19). At 20 weeks of age, F1 females where mated with normal males [31]. Animal experimental group and sample numbers are presented in Fig. 1.

2.2. Exercise training

The Exercise protocol utilized in this study is as previously described [31–35]. Briefly, F1 females were exercised 5 days/week followed by 2 days of rest on a motorized treadmill (Columbus Instruments, Columbus, OH, USA). Rats allocated to the *Exercise* group ran for 20 min at a speed of 15 m/min on the initial training day. Each subsequent day, an additional 10 min per day were applied to the running time until on *day 5 of week 1* the rats were exercised for a maximum of 60 min. *Beginning* of *week 2* and thereafter until mating, rats were exercised for 60 min/day at a speed of 20 m/min. The day after mating and for *week 1 of pregnancy,* rats were exercised for 50 min/day at a speed of 17 m/min. During *week 2 of pregnancy,* rats were exercised for 30 min/day at a speed of 13 m/min and during *week 3 of pregnancy,* 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 in *week 2* and *week 3 of pregnancy* at the same intensities and durations as per the *Exercise* group. *Sedentary* rats were placed onto a stationary treadmill for the same duration as the exercising rats. Rats from the exercise groups were encouraged to run by blowing compressed air near the base of their tail. The exercise protocol is clearly presented in a schematic in our recent publication [31].

2.3. Post-mortem

As previously described, post-mortems were carried out at E20 of pregnancy, F1 females where anesthetized with a 1:1 mixed solution of Ketamine-100 mg/kg (Parnell Laboratories Pty Ltd, Alexandria, NSW, Australia) and Ilium Xylazil-30 mg/kg (Troy Laboratories Pty Ltd, Smithfield, NSW, Australia) and their uterus exposed [31]. F2 fetuses were weighed, sexed by visual inspection of the anogenital distance and were then killed by decapitation. Fetal tails were collected to verify sex by qPCR to determine the presence/absence of the sex-determining region Y (SRY) in extracted DNA using a commercially available Taqman probe (Rn04224592_u1; NM_012772.1) (Life Technologies; Scores by, VIC, Australia) as previously described [31,36]. The placentae were excised, weighed and fixed whole in 10% neutral buffered formalin or separated into labyrinth and junctional zone regions and frozen immediately in liquid nitrogen and stored at −80 °C for subsequent analysis. For tissue analyses, placentae associated with one male and one female from each litter were chosen, with each sample representing a single animal (i.e. n = 1). The dam was then killed by cardiac puncture.

2.4. Placental morphology

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

2.5. Placental gene abundance

miRNA and RNA 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 (miRNA easy mini kit; Qiagen, Chadstone, VIC, Australia) as previously described [31,37]. First strand cDNA was generated from 1 μg of RNA using the High Capacity cDNA kit (Life Technologies, Mulgrave, VIC, Australia) 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 where purchased from Life Technologies for the following vasculogenic and angiogenic genes; *Vegfa* (Rn01511602_m1; NM_001110333.2), *Flt-1* (Rn01409533_m1; NM_019306.1) and *Plgf* (Rn00585926_m1; NM_053595.2) as well as for *miRNA27a* (000408; MIMAT0000799). To compensate for variations in RNA or miRNA input amounts and reverse transcriptase efficiency, mRNA and miRNA abundance of the genes of interest were normalized to the geometric mean of two housekeeping genes; TATA box binding protein (*Tbp*, Rn01455646_m1; NM_001004198.1) and β-Actin (*Actb*, Rn00667869_m1; NM_031144.3) for mRNA and 191 miRNA (Hs04231511_s1; NR_029690.1) and *U6* snRNA (001973; NR_004394) were selected for normalization of *miRNA27a*. HotStart DNA Taq Polymerase was activated by heating the mixture to 95 °C for 10 min, then 'real - time' PCR reactions were run for 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Relative changes in mRNA and miRNA abundance was quantified using the $2^{-\Delta\Delta CT}$ method and reported in arbitrary units normalized to *Control Sedentary* Chow male values. *Tbp, Actb,* 191 miRNA and *U6* snRNA were not different between Treatments, Diets, Exercises or Sexes.

2.6. Protein extraction and western blot analysis

Protein was extracted from 50 mg of placental labyrinth tissue using RIPA buffer [28] and was subjected to SDS-PAGE and Western blotting. 20 μg of 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) [31]. Membranes were probed with antibodies against VEGF, FLT-1 and PLGF (1:1000, Santa Cruz Biotechnology; Dallas, Texas, USA). 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 [38] and expressed as values relative to *Control Sedentary* Chow males [31]. All gels contained a *Control Sedentary* Chow male sample for normalization.

2.7. Statistical analysis

A two-way analysis of variance (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 regime using a Student's unpaired *t*-test and a one-way ANOVA determined Exercise effects in *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. 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

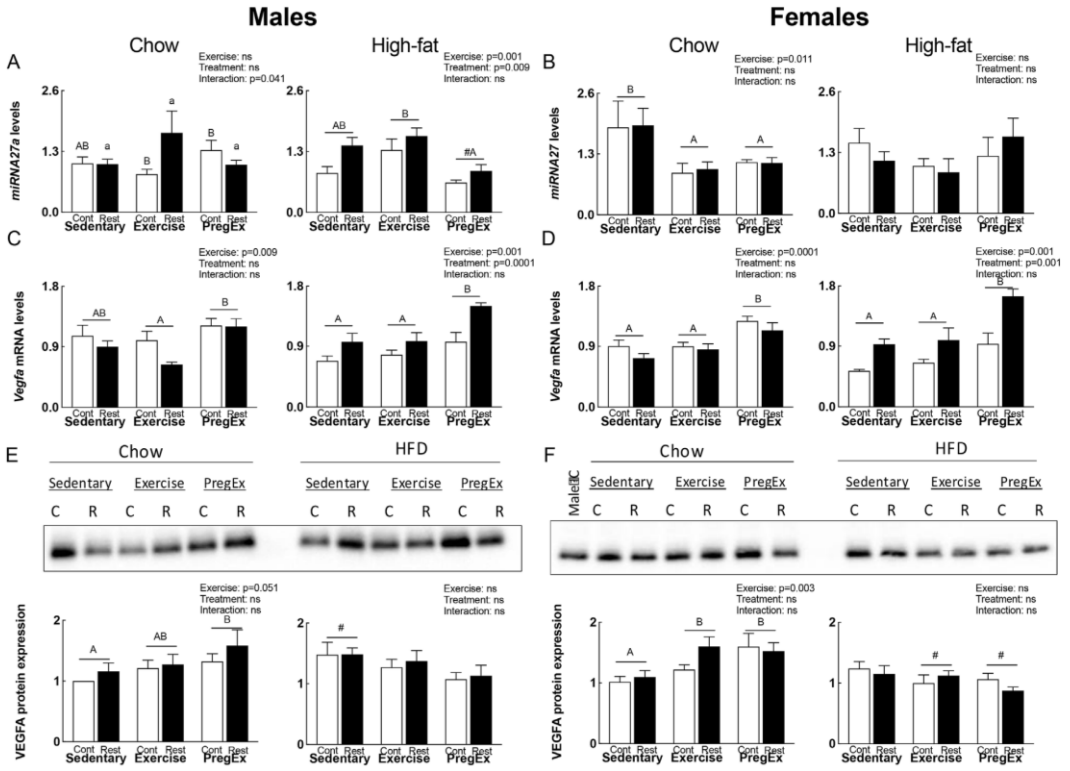

Fig. 2. VEGFA and *miRNA27a* labyrinth expression *miRNA27a* abundance (A and B) and VEGFA gene (C and D) and protein (E and F) expression (n = 6 in each group/sex; n = 1 representing 1 pup from 1 litter) in male and female associated placenta whose mothers were *Control* (open bars) or *Restricted* (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 Chow and differences across exercises are denoted by different letters where 'a/A' is different to 'b/B' but not 'ab/AB'.

statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of maternal growth restriction

Maternal growth restriction (*Restricted*) compared to normal birth weight (*Control*) dams upregulated *Vegfa* mRNA abundance in placentae from HFD, but not Chow, in males (+42%) and females (+68%) (Fig. 2C and D; two-way ANOVA). However, this increase in mRNA abundance did not translate to alterations in VEGFA protein (Fig. 2E and F; two-way ANOVA). Male placentae from HFD *Restricted* dams had increased *miRNA27a* abundance (+41%) compared to males from HFD *Control* dams regardless of maternal exercise (Fig. 2A; two-way ANOVA), with no changes in females. Placental Flt-1 (Fig. 3) and PlGF (Fig. 4) mRNA and protein expression were unaffected by maternal growth restriction. Blood space cross-sectional area was decreased in male associated placentae whose Chow-fed mother was *Restricted* (−15%) compared to Chow-fed *Control*, suggesting altered vascular development (Fig. 5A; two-way ANOVA). Conversely, labyrinth tissue cross-sectional area was increased in Chow-fed *Restricted* dams in male (+26%) and female (+15%) associated placentae compared to *Control* (Fig. 5C and D; two-way ANOVA).

3.2. Effects of maternal diet

Maternal High-fat feeding had minimal effects on placental regulators of vasculogenesis and angiogenesis. High-fat feeding in *Sedentary* dams increased VEGFA (+37%) and PLGF (+83%) protein expression in male associated placentae compared to Chow-fed *Sedentary* dams (Figs. 2E and 4C; two-way ANOVA). Whereas, *Exercise* and *PregEx* in HFD dams downregulated VEGFA (−25% and −38%, respectively) and FLT-1 (*PregEx* only; −55%) protein expression in female associated placentae (Figs. 2F and 3D; two-way ANOVA), and *PregEx* reduced *miRNA27a* abundance (−35%) in male-associated placentae compared to their respective Chow-fed counterparts (Fig. 2A; two-way ANOVA). High-fat feeding did not alter placentae blood space or labyrinth tissue cross-sectional areas (Fig. 5).

3.3. Effects of Exercise

Exercise had minimal effects on vasculogenic and angiogenic expression in both male and female associated placentae, regardless of maternal birth weight or diet. Interestingly, *miRNA27a* abundance was downregulated by *Exercise* (−50%) compared to *Sedentary* in female associated placentae from Chow-fed mothers (Fig. 2B; one-way ANOVA), which coincided with increased VEGFA protein expression (+33%, Fig. 2F; one-way ANOVA) despite no alterations in *Vegf* mRNA abundance (Fig. 2D). *Exercise* did not alter Flt-1 gene or protein abundance (Fig. 3), but decreased PLGF protein expression (−33%) in placentae associated with male fetuses from HFD dams compared to HFD *Sedentary* dams (Fig. 4C; one-way ANOVA). *Exercise* did not alter blood or labyrinth tissue cross-sectional areas (Fig. 4).

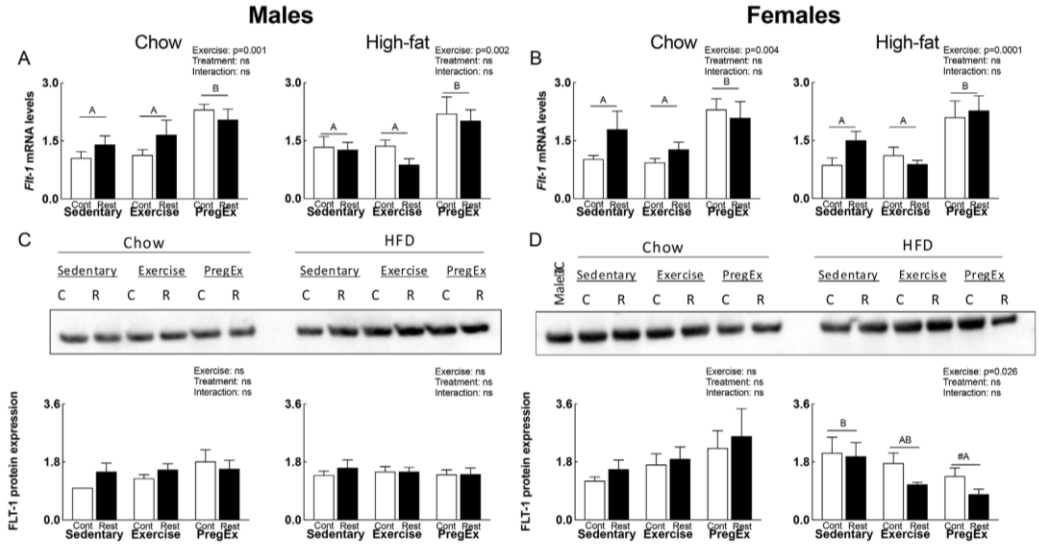

Fig. 3. Flt-1 labyrinth expression Flt
-1 gene (A and B) and protein (C and D) expression (n = 6 in each group/sex; n = 1 representing 1 pup from 1 litter) in male and female associated placenta whose mothers were *Control* (open bars) or *Restricted* (black bars) that consumed a Chow (left panel) or 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 Chow and differences across exercises are denoted by different letters where 'A' is different to 'B' but not 'AB'.

3.4. Effects of PregEx

PregEx upregulated *Vegfa* (Fig. 2C and D; one-way ANOVA), *Flt-1* (Fig. 3A and B; one-way ANOVA) and *Plgf* (Fig. 4A and B; one-way ANOVA) gene abundance in male (+50% (High-fat only), +70% and +62%, respectively) and female (+63%, +71% and +37%, respectively) associated placentae compared to *Sedentary*, regardless of maternal diet. *PregEx* upregulated VEGFA protein expression in Chow-fed male (+34%) and female (+48%) associated placentae compared to Chow-fed *Sedentary* dams (Fig. 2E and F; one-way ANOVA), which coincided with a reduction in *miRNA27a* abundance only in females (−41%, Fig. 2B; one-way ANOVA). Interestingly, despite *PregEx* increasing *Flt*-1 mRNA abundance in all groups, FLT-1 protein expression was decreased only in female associated placentae from HFD dams compared to HFD *Sedentary* dams (−47%, Fig. 3D; one-way ANOVA). Similarly, despite *PregEx* increasing *Plgf* mRNA abundance in all groups,

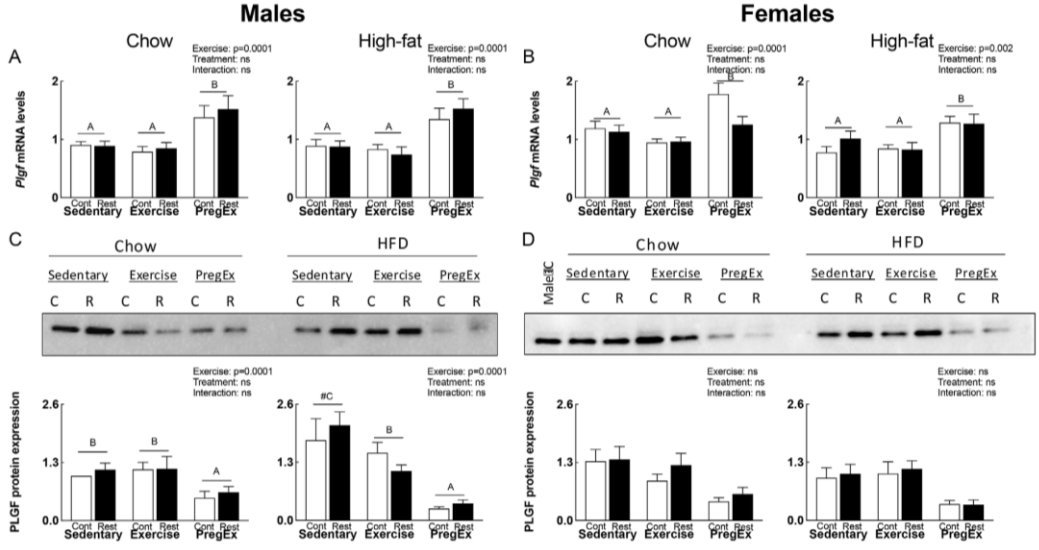

Fig. 4. PlGF labyrinth expression PlGF gene (A and B) and protein (C and D) expression (n = 6 in each group/sex; n = 1 representing 1 pup from 1 litter) in male and female associated placenta whose mothers were *Control* (open bars) or *Restricted* (black bars) that consumed a Chow (left panel) or High-fat 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 Chow and differences across exercises are denoted by different letters where 'A' is different to 'B' and 'C'.

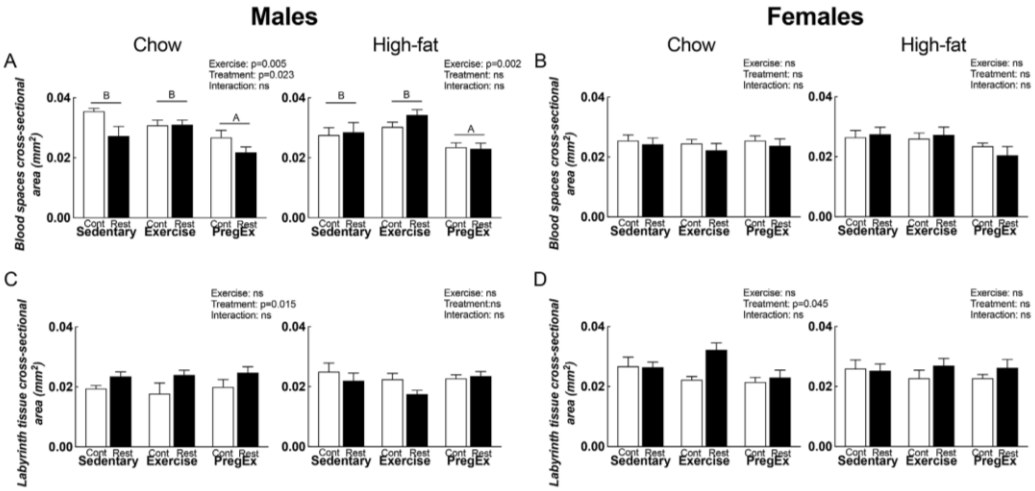

Fig. 5. Labyrinth blood and tissue cross-sectional areas Blood space (A and B) and tissue (C and D) cross-sectional areas (n = 3–4 in each group/sex; n = 1 representing 1 pup from 1 litter) in male and female associated placentae whose mothers were *Control* (open bars) or *Restricted* (black bars) that 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. Differences across exercises are denoted by different letters where 'A' is different to 'B'.

PLGF protein expression was decreased by *PregEx* in male, but not female, associated placentae compared to *Sedentary* (−66%, Fig. 4C; one-way ANOVA). *PregEx* reduced placental blood space area in placentae of male fetuses compared to placentae from *Sedentary* dams (−20%, Fig. 5A; one-way ANOVA), which is indicative of increased vascular branching.

4. Discussion

It is well accepted that mothers born small have an increased risk of developing obesity and transmitting growth restriction, as well as its associated comorbidities, onto subsequent generations. This study has demonstrated for the first time that maternal growth restriction and High-fat feeding independently dysregulate F2 placental blood and labyrinth tissue areas as well as markers of vasculogenesis and angiogenesis. We additionally demonstrated that markers of placental vasculogenesis and angiogenesis are differentially altered depending on the timing of exercise initiation. Specifically, *Exercise* had minimal effects on regulators of placental vasculogenesis, whereas *PregEx* perturbed multiple regulators of vasculogenesis and angiogenesis and decreased labyrinth blood space area. Currently, it is unclear if the placental vascular adaptations to *PregEx* are beneficial or harmful to fetal outcomes, but with such overt changes they are likely to impact long-term offspring health. This study highlights the need to further investigate if exercise initiation during pregnancy is beneficial or detrimental to offspring health despite its well characterized benefits on maternal health, especially in women at risk of a complicated pregnancy. The current results highlight that promotion of a healthy lifestyle long before conception should be a key health objective to optimize pregnancy success.

4.1. Maternal growth restriction effects

Growth restricted pregnancies are often associated with altered vasculogenic and angiogenic factors, which likely result in the aberrant placental vasculature development observed [39]. However, no studies to date have characterized if similar alterations in placental vascular development are observed in F2 placentae. We demonstrate for the first time that increases in labyrinth tissue area in Chow-fed *Restricted* mothers was independent of alterations in pro-vasculogenic or angiogenic markers in the placenta. This increased labyrinth tissue area, at least in female associated placentae, may be attributed to a general increase in placental size within these groups, as we recently published that total placental and labyrinth cross-sectional areas are increased *Restricted* dams [31]. Given that any impact of F1 growth restriction on F2 placental development is likely due to altered maternal physiology or epigenetic processes, it is also possible that the alterations in placental labyrinth tissue area at E20 are caused by disruption to key processes that may have occurred earlier in pregnancy. Specifically, studies in humans have demonstrated that pregnant women who were born small have an increased risk of developing severe preeclampsia [40], which is caused by impaired vascular remodeling during early gestation [41]. It is therefore possible that similar impairments in early placental vascular development occurred in *Restricted* dams in the current study, which do not result in overt maternal cardiovascular disease as previously characterized [42].

4.2. Maternal High-fat diet effects

Previous studies in overweight or obese human mothers and pregnant rodents have demonstrated altered placental vascular structure and pro-angiogenic factor expression [18,43]. Here we demonstrate that maternal High-fat feeding increases VEGFA and PLGF protein expression in male associated placentae from *Sedentary* mothers, a finding that is similar to mouse models of HFD induced gestational diabetes mellitus [44]. This increase in VEGFA and PLGF protein expression associated with High-fat feeding in male associated placentae is likely an adaptation to favor placental angiogenesis and may be related to alterations in oxidative stress associated with high-fat feeding [45]. Whereas, in female associated placentae maternal High-fat feeding in *Exercise* and *PregEx* dams reduced VEGFA and PLGF (*PregEx* only) protein expression, a mechanism that would likely impair angiogenesis, although no alterations in placental cross-sectional areas were observed [31]. Thus, our studies highlight sex dependent alterations in placental vasculogenic and angiogenic factor expression in response to maternal diet and exercise.

4.3. Impact of maternal Exercise

Previous studies in humans have demonstrated that regular exercise

initiated before and continued throughout pregnancy was associated with increased PlGF as well as reduced sFlt-1 and soluble endoglin (sEng) plasma concentrations in late gestation [46], which is indicative of enhanced placental angiogenesis. In contrast to these findings, and our initial hypothesis, *Exercise* had minimal effects on placental vasculogenesis and angiogenesis, which likely reflects the formation of the placenta in an environment that has adequately adapted to alterations in oxygen tension and blood flow that accompany exercise [47]. Of interest, we characterize a potential regulatory role of *miRNA27a* on placental vasculogenesis and angiogenesis in female associated placentae from Chow-fed dams. We report that a reduction in *miRNA27a* in response to *Exercise* and *PregEx* matched a reciprocal increase in VEGFA protein expression, an association that was recently validated in rat hippocampal tissue [6]. The role of miRNAs in regulating placental vasculogenesis and angiogenesis are yet to be fully explored. However, the current findings suggest a potential mechanism that may help to explain the sex- and treatment-specific alterations in key vasculogenic and angiogenic factors, such as VEGFA.

4.4. Impact of maternal PregEx

Unlike the modest changes characterized in the *Exercise* groups, *PregEx* clearly has a consistent impact on both the gene and protein expression of key placental vasculogenic and angiogenic proteins. A change of which is especially critical given that 40–50% of pregnancies are unplanned [23,24] and clinical interventions in complicated pregnancies including lifestyle changes such as exercise to improve maternal health, often occur after the placenta has developed; highlighting that it may have drastic impacts on short and long-term offspring health and development. Specifically, VEGFA protein expression was upregulated by *PregEx* in placentae from mothers on a Chow-fed diet with no change in FLT-1 protein expression, which may be an attempt to increase vascular branching to improve placental surface area for nutrient exchange. It is possible that the introduction of exercise during the second week of pregnancy may stimulate vascular branching and/or growth via VEGFA through a hypoxia-mediated mechanism. Specifically, during exercise the redistribution of maternal blood and nutrients may create intermittent periods of placental hypoxia that are sufficient to stimulate the upregulation of VEGFA, which may be an adaption to increase fetal nutrient availability especially during late gestation [48]. Studies in mice have demonstrated that hypoxia initiated at a similar stage of gestation (from the second week of pregnancy and continued until the end of pregnancy) alters placental vasculogenesis in association with alterations to VEGFR-2 [12] and less well characterized regulators of vascular branching such as the renin angiotensin system [49]. This supports the possibility that the effects of *PregEx* may be due to hypoxic alterations. Of interest, in human studies of maternal exercise there is an increase in placental functional volume as well as an increase in placental terminal villi [26]. This finding is consistent with the decreased placental blood space area we reported in male associated placentae, which is inversely related to vascular branching. We propose that the initiation of exercise during pregnancy activates VEGFA stimulated branching angiogenesis, impairing PLGF non-branching angiogenesis, which is likely in attempt to improve fetal and birth outcomes. This highlights that exercise initiated during pregnancy stimulates late gestational placental responses that may alter placental vascular development. It is difficult to determine if such adaptations improve or impair fetal development, and hence long-term disease risk, given that fetal weight was not reduced as we have previously reported [31].

5. Conclusion

This study demonstrates that maternal exercise differentially regulates F2 placental vasculogenic and angiogenic factor expression and blood space area, with minimal changes due to maternal birth weight and diet. The minimal changes reported in response to maternal growth restriction suggests additional placental factors likely mediate the previously characterized alterations in fetoplacental outcomes in this group. Importantly we demonstrated that maternal exercise, particularly *PregEx*, has profound effects on placental vasculogenic and angiogenic factor expression and blood space area. These changes suggest a potentially late adaptive mechanism targeted towards maintaining fetal growth in a highly metabolically demanding environment. However, how these alterations in vasculogenic and angiogenic factors affects F2 offspring birth weight and long-term health risks is still unknown and requires further investigation.

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Conflicts of interest

The authors declare no conflicts of interest.

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Chapter 6 Maternal exercise alters rat fetoplacental stress response: minimal effects of maternal growth restriction and high-fat feeding

Results of this study are presented in its submitted form, following expanded aims, hypotheses, and methodologies used in the study.

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6.1 Aims and Hypotheses

The overall aim of this study was to determine changes in the placental labyrinth glucocorticoid barrier in F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to identify if maternal high-fat feeding exacerbated any alterations within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

Specific aims were to assess the following:

i) if maternal growth restriction prior to birth alters the F2 labyrinth glucocorticoid barrier;
ii) if maternal exercise initiation prior to and/or during pregnancy attenuates outcomes;
iii) if maternal high-fat feeding exacerbates outcomes;
iv) if there are any sex-specific differences.

It was hypothesized that F2 offspring from *Restricted* mothers would have an aberrant expression of glucocorticoid barrier genes compared to offspring from *Control* mothers. It was predicted that maternal high-fat feeding would exacerbate these outcomes and that *Exercise* would improve F2 fetal outcomes to a greater extent than *PregEx*.

6.2 Methods

Methodologies used in this study have been described in Chapter 2 and expanded from the submitted version (**Section 5.3**) in subsequent sections. The current study utilises qPCR and liquid chromatography mass spectrometry experiments in fetal and placental samples from WKY rats that underwent bilateral uterine vessel ligation that were allocated one of two diets, allocated to a non-physiological measures group (*Unstressed*) or a physiological measures group (*Stressed*) and underwent treadmill exercise. As mentioned in the **Preface**, only the *Unstressed* analysis formed a direct part and aim of this thesis with the *Stressed* data being performed in collaboration with other laboratory members outside of my PhD.

6.2.1 Animals

UPI (*Restricted*) or sham (*Control*) surgery was induced on E18 in WKY rats. F1 offspring were fed a Chow or HFD from weaning, and at 16 weeks were randomly allocated an exercise protocol; *Sedentary*, *Exercise* or *PregEx* as described in **Section 2.2**. Females were mated (20 weeks) with placentae associated with F2 fetuses collected at E20, weighed, and stored at -80°C.

6.2.2 Placental gene abundance

Total RNA was isolated from 50 mg of labyrinth tissue as described in **Section 2.4**. qPCR was performed to quantify the abundance of the following genes of interest; *Ogt*, *Nr3c1*, *Nr3c2*, *Hsd11b1*, *Hsd11b2* and *Crhr1* (**Table 2.2**).

6.2.3 Statistical analysis

The submitted manuscript in *Placenta* (**Section 6.3**) contains the statistical analysis performed for this Chapter.

6.3 Published Manuscript

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Maternal exercise alters rat fetoplacental stress response: Minimal effects of maternal growth restriction and high-fat feeding

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ABSTRACT

Introduction: Fetal growth restriction complicates 10% of pregnancies and increases offspring (F1) risk of metabolic disorders, including obesity and gestational diabetes mellitus (GDM). This disease predisposition can be passed onto the next generation (F2). Importantly, the risk of pregnancy complications in obese women can be exacerbated by a stressful pregnancy. Exercise can reduce adiposity and improve health outcomes in obese women and those with GDM. This study investigated the impacts of maternal growth restriction, obesity, exercise, and stress on fetal and placental endocrine function.

Methods: Uteroplacental insufficiency (*Restricted*) or sham (*Control*) surgery was induced on embryonic day (E) 18 in F0 Wistar-Kyoto rats. F1 offspring were fed a Chow or High-fat (HFD) diet from weaning and, at 16 weeks, were randomly allocated an exercise protocol; *Sedentary*, Exercised prior to and during pregnancy (*Exercise*), or Exercised only during pregnancy (*PregEx*). Females were mated and further randomly allocated to either undergo (*Stress*), or not undergo (*Unstressed*), physiological measurements during pregnancy. On E20, F2 fetal plasma (steroid hormones), tissues (brain, liver), and placentae (morphology, stress genes) were collected.

Results: Maternal growth restriction and high-fat feeding had minimal impact on fetoplacental endocrine function. *PregEx* and *Exercise* increased cross-sectional labyrinth and junctional zone areas. *PregEx,* but not *Exercise*, increased fetal deoxycorticosterone concentrations and reduced placental *Hsd11b2* and *Nr3c2* gene abundance. Maternal stress increased fetal corticosterone concentrations in *Sedentary* HFD dams and increased placental cross-sectional areas in *PregEx* mothers.

Discussion: PregEx and *Stress* independently dysregulates the endocrine status of the developing fetus, which may program future disease.

1. Introduction

The placenta regulates fetal exposure to glucocorticoids by converting active glucocorticoids into their inactive metabolites via 11β-hydroxysteroid dehydrogenase type 2 (HSD11β2) [1], creating an effective gradient between the mother and fetus when glucocorticoids are at physiological levels. Excessive maternal glucocorticoids can, however, exceed the barriers functional capacity and allow an influx of glucocorticoids into the fetal circulation, which can impair fetal growth and program offspring disease [2–5]. While the placenta metabolizes glucocorticoids to reduce their impact on the fetus, it also synthesizes steroids, namely progesterone and estrogen. Although elevated progesterone concentrations are important for maintaining a healthy pregnancy [6], it is also a precursor for maternal glucocorticoid synthesis. Therefore, maternal stress may alter maternal progesterone concentrations [7,8]. Placental estrogen is also important for fetal hypothalamic

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pituitary-adrenocortical (HPA) axis development during mid- and late-gestation [9]. Therefore, understanding the complex interplay between maternal glucocorticoids and fetoplacental steroid hormone metabolism is imperative in understanding the effects of maternal stressors on fetal growth and the programming of offspring disease.

Intrauterine growth restriction (IUGR) is a result of impaired placental function, which affects 10% of live births in the Western world [10]. Increasing human and experimental evidence links IUGR to adult onset metabolic and cardiorenal disorders and obesity [11–16]. Despite males being at a greater risk than females [17], physiologically challenging events (such as pregnancy and stress) in IUGR females can act as a "second-hit" that may unmask programmed endocrine disorders, such as gestational diabetes mellitus (GDM) [18], which likely programs second generation (F2) cardiometabolic dysfunction in male, but not female, offspring [19–22]. We have recently demonstrated that this F2 disease burden may be due to sex-specific alterations in the placental insulin-like growth factor [23], nutrient transporter [24], and angiogenesis [25] pathways. Importantly, we have previously demonstrated that maternal growth restriction and stress independently dysregulates the fetoplacental stress system in placental samples [26]. However, the previous work utilized pooled placental samples and therefore the impact of sex-specific differences in the fetoplacental stress system on disease transmission remains to be elucidated.

Obesity independently increases cortisol secretion and alters the HPA-axis [27]. In humans, maternal obesity is linked with anxiety and depression due to elevated maternal cortisol and altered placental nutrient and stress genes [28]. Lifestyle interventions, such as exercise, are known to improve maternal metabolic health and fetoplacental outcomes [29,30], however the impact exercise has on fetal and placental stress pathways is largely unknown. To our knowledge, no studies have characterized the impact maternal exercise has on the fetoplacental stress pathway in obese growth restricted mothers or its programming of offspring disease.

Therefore, the current study first aimed to determine changes in the placental glucocorticoid barrier and fetal plasma steroid concentrations in 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 within each stress and dietary intervention. We next aimed to determine whether *i)* maternal high-fat diet consumption, or *ii)* maternal stress exacerbated these changes in the fetal stress-system associated with maternal growth restriction within each exercise group. As the placenta is sexually dimorphic [31], male and female placentae were analyzed separately in order to evaluate potential sex-specific programming effects and to expand on the findings of our previous study [26].

2. Materials and methods

2.1. 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 Australian code for the care and use of animals for scientific purposes. Eight-week-old female Wistar-Kyoto rats were acquired from the Biological Resource 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. Female rats were mated with breeder males and on day 18 of gestation (term = 22 days) uteroplacental insufficiency surgery was performed [32]. Briefly, F0 female rats were anaesthetized with 4% isoflurane and 650 ml min^{-1} oxygen flow. Uteroplacental insufficiency was then induced by bilateral uterine vessel ligation (offspring termed *Restricted*) or sham (offspring termed *Control*) surgery and dams delivered naturally at term. On postnatal day 35, F1 *Control* and *Restricted* females were randomly allocated to a semi-purified control (Chow) (AIN93G; Specialty Feeds, Glen Forrest, WA, Australia) or a selection of two high-fat (SF03-020 and SF01-028; Specialty Feeds; 43% kcal fat with 20 MJ/kg and 19.9 MJ/kg digestible energy, respectively) diets that were matched for micro- and macro-nutrients. At 16 weeks, F1 females were further randomly allocated to one of the following exercise groups: *Sedentary*, exercised before and during pregnancy (*Exercise*), or exercised only during pregnancy (*PregEx*). At 19 weeks of age, females where allocated to either undergo (*Stress*), or not undergo (*Unstressed*), physiological measurements and were mated with breeder males at 20 weeks of age (Fig. 1). Animals were evenly allocated to experimental groups over time to control for time and/or cohort effects that may arise due to block allocations.

2.2. Exercise training

For the duration of their exercise regime F1 females exercised on a motorized treadmill (Columbus Instruments, Columbus, OH, USA) for 5 days/week followed by 2 rest days. On the first training day rats allocated to the *Exercise* group ran for 20 min at 15 m/min with an additional 10 min/day applied to each subsequent day until on *day 5 of week 1* the rats exercised for 60 min. From *day 1 of week 2*, until mating, rats were exercised for 60 min/day at 20 m/min [23,33–36]. Following mating, rats were exercised for 50 min/day at 17 m/min for *week 1 of pregnancy*, 30 min/day at 13 m/min for *week 2 of pregnancy*, and 20 min/day at 11 m/min for *week 3 of pregnancy* [23,24]. Females allocated to the *PregEx* group remained *Sedentary* prior to mating and for the first week of pregnancy then underwent exercise in *weeks 2* and *3 of pregnancy* at the same intensities and durations as the *Exercise* group. Rats were encouraged to run by blowing compressed air near the base of their tail. *Sedentary* rats were placed on a stationary treadmill for the same duration as the exercising rats. The exercise protocol is presented in Fig. 1A.

2.3. Physiological measures

Stress during pregnancy was induced using a series of physiological measures [18,37], which elicits a stress response as evidenced by increased maternal corticosterone concentrations [18,38]. Briefly, *Stress* allocated females underwent indirect calorimetry (E16), tail cuff blood pressure and glucose tolerance testing (E18), and were placed in a metabolic cage for 24-h (E19) [39]. The *Unstressed* females did not go through the stress protocol.

2.4. Post-mortem

At E20, F1 females where anaesthetized with Ketamine (100 mg/kg; Parnell Laboratories Pty Ltd, Alexandria, NSW, Australia) and Xylazil (30 mg/kg; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia), and their uterus was exposed. F2 fetuses were weighed and killed by decapitation and, due to small sample volumes, fetal blood was pooled per litter. Fetal sex was determined by qPCR assessing the presence/absence of the sex-determining region Y (SRY) in DNA extracted from fetal tails [23,40]. Placentae were excised, weighed, and fixed whole in 10% neutral buffered formalin or separated into labyrinth and junctional regions then frozen immediately in liquid nitrogen and stored at −80 °C. Fetal organs known to be influenced by stress (brain and liver) were excised and weighed, then frozen immediately in liquid nitrogen and stored at −80 °C. For placental analyses, one male and one female from each litter were chosen, with each sample representing a single animal (i.e. n = 1). For fetal analysis, pooled fetal plasma and organ weights were analyzed as a whole litter (i.e. n = 1). The dam was then killed by cardiac puncture.

2.5. Placental morphology

Fixed placentae were processed into paraffin blocks, sectioned at 5 μm and stained with haemotoxylin and eosin (n = 3–4 dams/group).

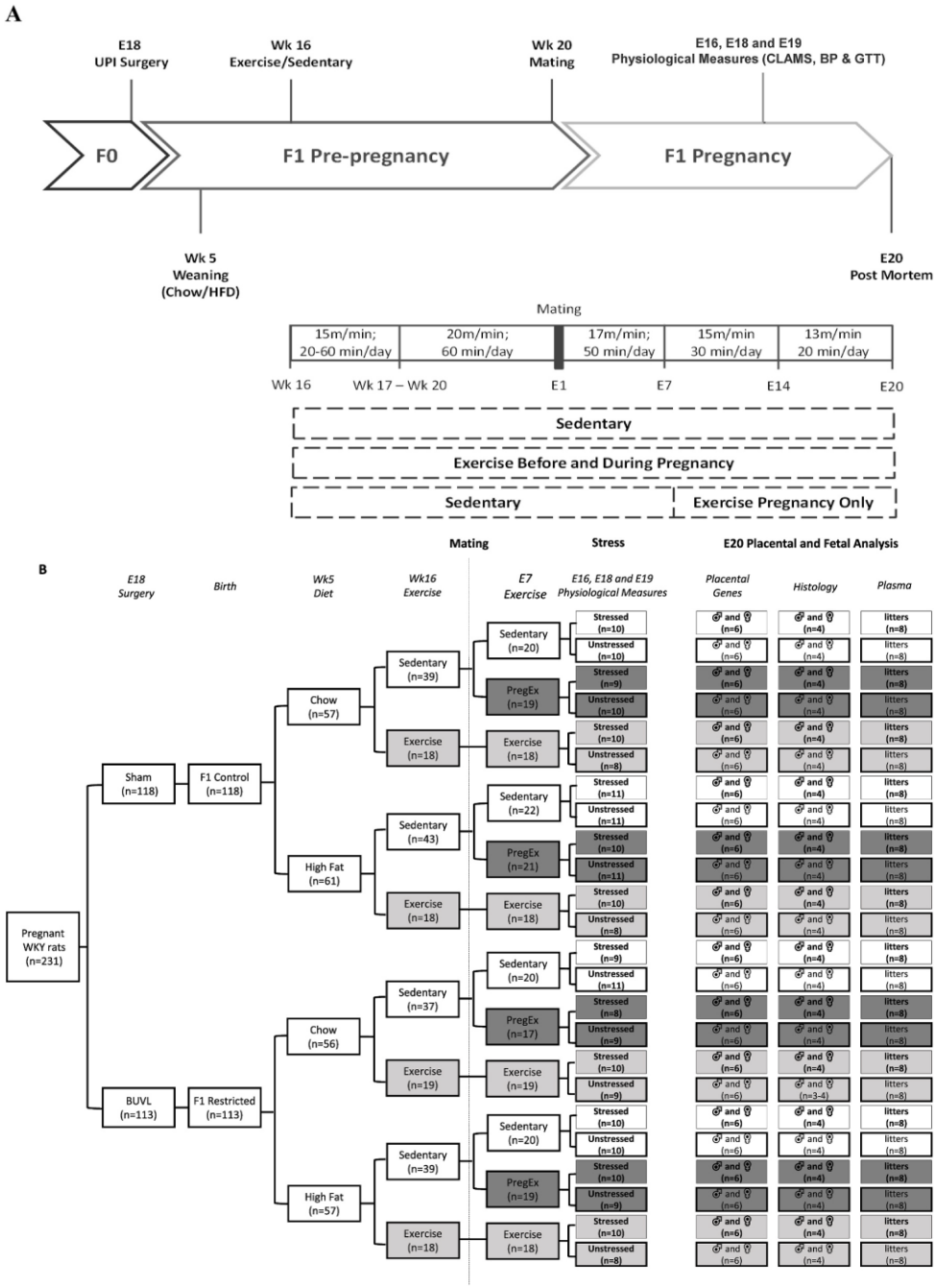

Fig. 1. Flow chat of experimental protocol. (A) Flow chart of the exercise and stress protocols. Rats underwent comprehensive laboratory animal monitoring system (embryonic day 16 (E16)), tail cuff blood pressure and glucose tolerance testing (E18), and 24-h metabolic cage (E20) measurements. HFD - high fat diet; m/min - meters per minute; min/day - minutes per day; UPI- uteroplacental insufficiency; Wk-week. (B) Flow chart of the experimental protocol with sample size per analyses indicating the allocation of female rats between the different Treatment, Diet, Exercise, and Stress groups.

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).

2.6. Placental gene abundance

RNA was extracted from the placental labyrinth [23] and cDNA was generated from 1 μg of RNA using the High Capacity cDNA kit (Life Technologies, Mulgrave, VIC, Australia). PCR primers for stress responsive genes were selected from our previous publications [26,41,42] and were purchased from Life Technologies; O-Linked *N*-Acetylglucosamine Transferase (*Ogt,* Rn00820779_m1; NM_017107.2), Nuclear Receptor Subfamily 3 Group C Member 1 *(Nr3c1,* Rn00561369_m1; NM_012576.2), Nuclear Receptor Subfamily 3 Group C Member 2 *(Nr3c2,* Rn00565562_m1; NM_013131.1), *Hsd11b1* (Rn00567167_m1; NM_017080.2), *Hsd11b2* (Rn04341420_g1; NM_017081.2), and Corticotropin Releasing Hormone Receptor 1 (*Crhr1*, Rn00578611_m1; XM_006247542). All qPCR data were normalized to the geometric mean of two housekeeping genes; TATA box binding protein (*Tbp*, Rn01455646_m1; NM_001004198.1) and β-actin (*Actb*, Rn00667869_m1; NM_031144.3). HotStart DNA Taq Polymerase was activated by heating to 95 °C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative changes in mRNA abundance was quantified using the $2^{-\Delta\Delta CT}$ method and reported in arbitrary units normalized to *Unstressed Sedentary* Chow *Control* male values [23–25]. *Tbp* and *Actb* were not different between Treatments, Diets, Exercises, Stresses, or Sexes.

2.7. Steroid hormone analyses

Fetal plasma concentrations of corticosterone, estradiol, testosterone, 11-deoxycorticosterone, and progesterone were measured using liquid chromatography mass spectrometry [43,44]. The limit of quantification were as follows; corticosterone 0.25 ng/ml, testosterone 5 pg/ml, 11-deoxycorticosterone 0.10 ng/ml, progesterone 0.25 ng/ml, and estradiol 25 pg/ml. Appropriate internal standards were used; corticosterone, testosterone, progesterone, and estradiol-d3 for estradiol. The mean inter-assay CV for the quality control samples across all steroids measured was 9.6%.

2.8. Statistical analysis

Normality testing was performed using the D'Agostino & Pearson and the Shapiro-Wilk tests. Given the study complexity, we initially sought advice from the School of Mathematics and Statistics at the University of Melbourne who agreed with the statistical approach employed to answer our specific research questions. We first performed a two-way ANOVA to identify differences between Treatment and Exercise within each Diet, Stress, and Sex [23–25]. If a main Treatment effect was present, the two-way ANOVA provided the p-value. 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 with Duncan's post-hoc determined Exercise effects in *Control* and *Restricted* groups. As we were only interested in *Exercise* and/or *PregEx* differences compared to *Sedentary*, we will only draw reference to those in text. To determine any differences between Diets, the data was split by Sex, Exercise, and Stress and a two-way ANOVA was conducted to report main Diet effects within each exercise regime. If an interaction was observed, the data was split to identify Diet effects within each exercise regime and Treatment using a Student's unpaired *t*-test. To identify any differences between Stress groups, the data was split by Sex, Exercise, and Diet and a two-way ANOVA was conducted to determine main Stress effects within each exercise regime. If an interaction was observed, the data was split to identify Stress effects within each exercise regime and Treatment using a Student's unpaired *t*-test. To identify any sex-specific differences, a Student's unpaired *t*-test 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. 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 $p < 0.05$.

3. Results

3.1. Maternal growth restriction effects

Plasma corticosterone was reduced in fetuses of *Unstressed* HFD *Restricted* dams (Fig. 3A) as was estradiol in *Stressed* Chow *Restricted* dams (Fig. 4D) compared to normal birth weight (*Control*) counterparts, which was largely driven by the reduction in their respective *Sedentary* (−34%) groups. Plasma estradiol was additionally increased with *Exercise* in *Unstressed* Chow and HFD *Restricted* dams (Fig. 4C). Plasma testosterone concentrations were increased in fetuses from *Unstressed* Chow *Restricted* dams and *Stressed* HFD *Restricted* dams (Fig. 4A and B).

Males: As previously demonstrated [23], *Restriction* increased male placental efficiency in *Unstressed Sedentary* Chow dams and reduced placental efficiency in *Unstressed Exercise* Chow dams (Table 1). Male absolute, but not relative, brain weight was reduced by *Restriction* in *Stressed* HFD dams (Table 1). *Exercise* in *Stressed Restricted* dams reduced *Hsd11b2* and *Nr3c1* in male placentae (Table 2).

Females: As previously demonstrated [23], female fetuses from *Unstressed* Chow *Restricted* dams had increased placental, labyrinth, and junctional zone cross-sectional areas (Table 1) as well as increased absolute, but not relative, liver weight (Table 1). *Stressed Sedentary* HFD *Restricted* dams increased placental efficiency, whereas *PregEx* in *Stressed* HFD *Restricted* dams reduced whole placental and labyrinth zone cross-sectional areas (Table 1). Placental *Nr3c1* was increased in *Unstressed* Chow *Restricted* dams, whereas *Nr3c2* was reduced in *Stressed* Chow *Restricted* dams as well as *Ogt* in *Stressed* HFD *Restricted* dams (Table 2).

3.2. Maternal exercise effects

Exercise reduced fetal plasma estradiol in *Unstressed* Chow *Control* dams and in *Stressed* Chow dams (Fig. 4C and D) compared to *Sedentary*. *Exercise* in *Stressed* HFD *Restricted* dams additionally increased plasma estradiol (Fig. 4D).

Males: As previously demonstrated [23,39], *Exercise* increased male fetal weight in *Unstressed* Chow dams, *Stressed* Chow *Restricted* dams, and in *Stressed* HFD dams (Table 1). *Exercise* in *Unstressed* Chow *Control* dams increased placental efficiency [23], whereas *Exercise* in *Stressed* Chow dams increased placental morphology (whole placental, labyrinth, and junctional zone cross-sectional areas) (Table 1, Fig. 2A). *Exercise* increased absolute, but not relative, brain weight in *Unstressed* HFD *Control* dams as well as in *Stressed* Chow *Restricted* dams (Table 1). *Exercise* in *Stressed* Chow *Control* dams downregulated *Hsd11b2* and *Nr3c1*, whereas *Exercise* in *Stressed* HFD *Control* dams downregulated *Nr3c2* (Table 2). *Exercise* in *Stressed* Chow dams additionally reduced *Crhr1* (Table 2).

Females: As previously demonstrated [23,39], *Exercise* increased female fetal weight in *Unstressed* Chow dams, *Stressed* Chow dams, and *Stressed* HFD dams (Table 1). Importantly, *Exercise* in *Unstressed* HFD dams reduced placental weight and increased placental efficiency (Table 1) [23]. Absolute, but not relative, liver weight was increased with *Exercise* in *Unstressed* Chow dams (Table 1). Whereas the fetal brain-to-liver weight ratio was reduced in *Stressed Exercise* Chow dams

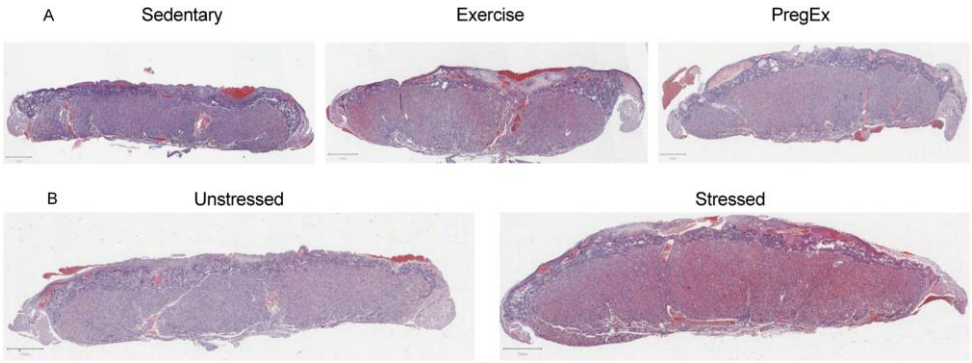

Fig. 2. Placental histology images. Representative whole placental images demonstrating morphological changes. A) *PregEx* and *Exercise* induced changes in male placental morphology in *Stressed* Chow dams compared to *Sedentary*. B) *Stressed* induced changes in placental morphology in *PregEx* Chow dams compared to *Unstressed*.

(Table 1). *Exercise* in *Stressed* Chow dams reduced placental *Hsd11b2*, *Crhr1*, and *Ogt* (Table 2). Whereas *Exercise* in *Unstressed* HFD dams decreased *Crhr1*, and *Exercise* in *Stressed* HFD dams increased *Ogt* (Table 2).

3.3. Maternal PregEx effects

PregEx increased plasma fetal 11-deoxycorticosterone in *Unstressed* HFD dams (Fig. 3C) and increased testosterone in *Unstressed* Chow and HFD dams (Fig. 4A) compared to *Sedentary*. Additionally, fetal plasma testosterone concentrations were reduced with *PregEx* in *Stressed* Chow *Restricted* dams (Fig. 4B).

Males: Despite no changes in fetal or placental weights, *PregEx* in *Unstressed* Chow *Control* dams increased male placental efficiency (Table 1), as previously reported [23]. *PregEx* in *Unstressed* Chow dams increased whole placental and labyrinth cross-sectional areas, whereas *Unstressed* HFD dams increased junctional zone cross-sectional area (Table 1) [23]. *PregEx* in *Stressed* dams, on the other hand, increased whole placental, labyrinth, and junctional zone cross-sectional areas in both Chow and HFD dams (Table 1, Fig. 2A). Relative brain weight was increased in *Unstressed* Chow *Control* dams with *PregEx*, whereas absolute brain weight was increased with *PregEx* in *Stressed* HFD dams (Table 1). Placental stress responsive genes were primarily downregulated by *PregEx* in males. Specifically, *PregEx* in *Unstressed* Chow dams reduced *Nr3c2* (Fig. 3E), *Hsd11b2* (Fig. 3G), and *Chrh1* (Table 2). However, *PregEx* reduced *Hsd11b2* in *Stressed* Chow dams, reduced *Nr3c1* in *Stressed* Chow *Control* dams, reduced *Nr3c2* in *Stressed* HFD *Control* dams, and reduced *Crhr1* in *Stressed* Chow and *Stressed* HFD dams (Table 2).

Females: As previously demonstrated [23], *PregEx* in *Unstressed* Chow and HFD dams reduced placental weight and increased placental efficiency in female fetuses (Table 1). *PregEx* increased placental and labyrinth zone cross-sectional areas in *Unstressed* HFD dams [23], *Stressed* Chow dams, and *Stressed* HFD *Control* dams (Table 1). However, *PregEx* in *Stressed* Chow dams decreased female brain-to-liver weight ratio (Table 1). *PregEx* downregulated placental *Nr3c2* in *Unstressed* HFD dams (Fig. 3F), reduced *Hsd11b2* in *Unstressed* Chow dams (Fig. 3H), and downregulated *Crhr1* in *Unstressed* HFD dams (Table 2). Whereas, in *Stressed* dams, *PregEx* reduced placental *Hsd11b2* and *Crhr1* in *Stressed* Chow dams, and increased *Ogt* in *Stressed* HFD dams (Table 2).

3.4. Maternal stress effects

Stress in *Sedentary* HFD dams increased fetal corticosterone (Fig. 3B; $p = 0.002$) and reduced progesterone (Fig. 4F; $p = 0.045$) concentrations compared to *Unstressed* counterparts. Fetal estradiol concentrations were reduced in *Stressed Exercise* Chow *Restricted* dams ($p = 0.017$) as well as in *Stressed* HFD *PregEx* dams ($p = 0.049$; Fig. 4D).

Males: *Stress* reduced male fetal weight in *PregEx* HFD dams ($p = 0.047$) and reduced placental weight in *Sedentary* Chow and HFD dams ($p < 0.024$) (Table 1), whereas placental efficiency was increased in *Stressed Exercise* Chow *Restricted* dams ($p = 0.006$) (Table 1). *Stress* conversely increased placental cross-sectional area in *PregEx* Chow dams ($p = 0.041$), increased labyrinth cross-sectional area in *PregEx* Chow and HFD dams ($p < 0.049$), and increased junctional zone cross-sectional area in *PregEx* Chow dams ($p = 0.011$) (Table 1, Fig. 2). Absolute brain weight was reduced in *Exercise* Chow *Control* dams ($p = 0.037$), whereas relative brain weight was increased in *PregEx* HFD dams ($p = 0.027$) (Table 1). Absolute liver weight was reduced in *Stressed Exercise* Chow dams ($p = 0.039$), *Stressed PregEx* Chow *Restricted* dams ($p = 0.050$), and *Stressed PregEx* HFD dams ($p = 0.020$); whereas relative liver weight was reduced in *Stressed Exercise* Chow dams ($p = 0.010$) (Table 1). The fetal brain-to-liver weight ratio was similarly decreased in *Stressed PregEx* HFD dams ($p = 0.004$) (Table 1). *Stress* in *Exercise* dams downregulated male placental *Nr3c2* and *Ogt* in Chow and HFD dams (Table 2; $p < 0.030$).

Females: *Stress* increased placental weight in *PregEx* Chow dams ($p = 0.032$), which was decreased in *Sedentary* HFD dams ($p = 0.002$) despite increased placental efficiency (Table 1; $p = 0.006$). Placental and labyrinth zone cross-sectional areas were increased in *PregEx* HFD *Control* dams (Table 1; $p < 0.016$), whereas they were reduced in *PregEx* HFD *Restricted* dams (Table 1; $p = 0.024$). Placental cross-sectional area was additionally reduced in *Stressed Exercise* Chow dams ($p = 0.043$), and labyrinth cross-sectional area was additionally reduced in *Stressed Exercise* Chow *Restricted* dams ($p = 0.045$) (Table 1). Fetal absolute and relative liver weights were reduced by *Stress* in *Exercise* Chow dams (Table 1; $p = 0.042$), whereas the brain-to-liver weight ratio was increased in fetuses from both *Stressed Sedentary* and *Exercise* Chow dams (Table 1; $p = 0.041$). *Stress* in *Exercise* Chow and *Sedentary* HFD dams reduced female placental *Nr3c2* and *Ogt* (Table 2; $p < 0.021$).

4. Discussion

Maternal stress increases the risk of having a growth restricted baby, predisposing offspring to adult-onset metabolic and cardiorenal disease, which can be transmitted to subsequent generations through placental programming [17,45]. We recently demonstrated that F2 disease programming may be due to alterations in the placental insulin-like growth factor system [23], nutrient transport pathways [24], and placental angiogenesis [25]. This study follows on from our previous publications and demonstrates, for the first time, that maternal birth weight, diet, timing of exercise initiation, and maternal stress exposure

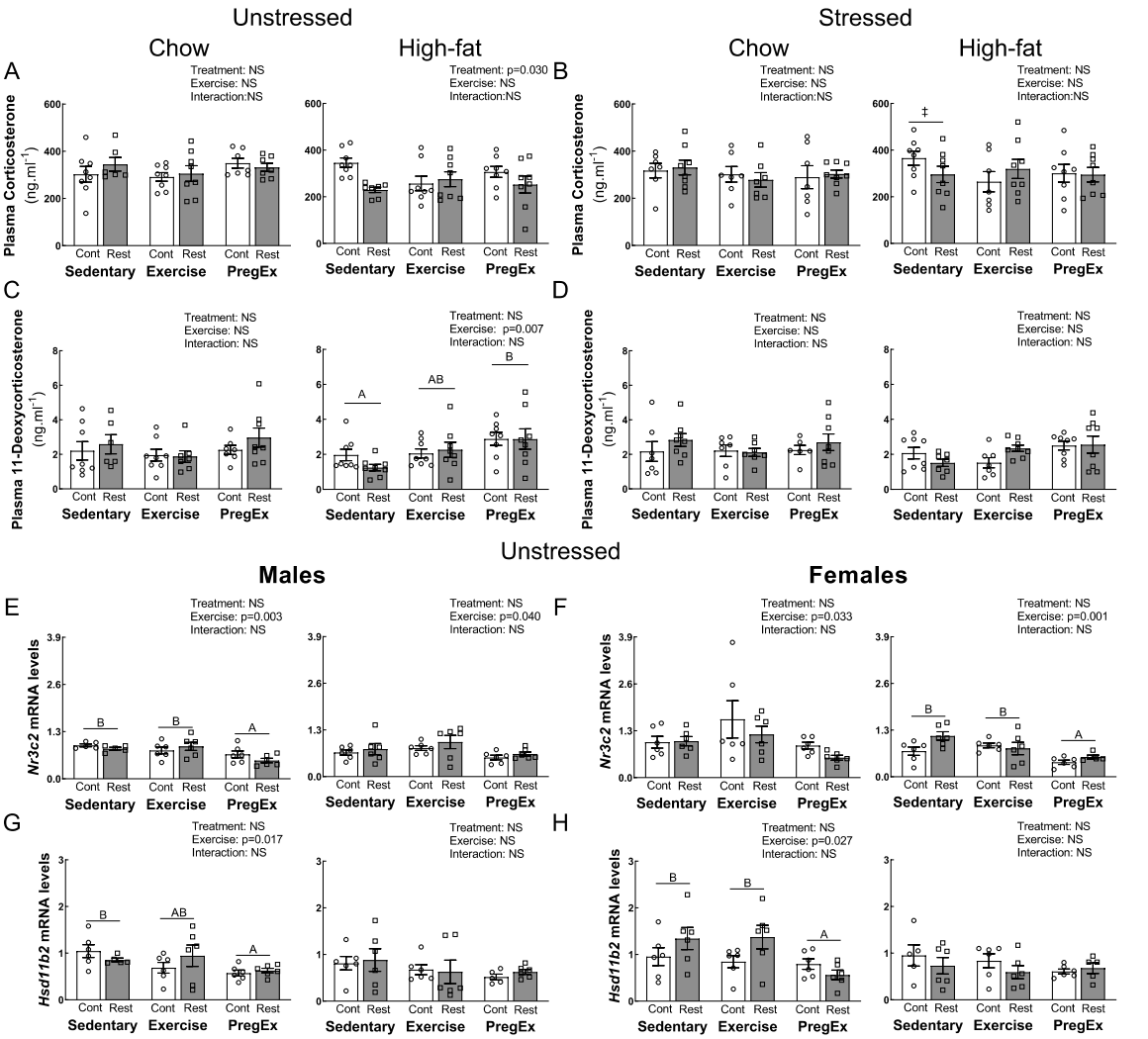

Fig. 3. Fetal plasma glucocorticoids and placental glucocorticoid barrier genes. Pooled fetal plasma corticosterone (A and B) and 11-deoxycorticosterone (C and D) concentrations from *Unstressed* and *Stressed* dams fed a Chow or High-fat diet (n = 8–10 litters in each group). *Nr3c2* (E and F) and *Hsd11b2* (G and H) in male (left) and female (right) associated placentae (n = 6 in each group/sex with n = 1 representing 1 pup from 1 litter) whose mothers were *Control* (Cont; open bars) or *Restricted* (Rest; black bars). Data were analyzed by a series of two-way ANOVA's to identify the differences between *i)* Treatment (maternal birth weight) and Exercise (split by Diet, Stress and Sex), and *ii)* Treatment (maternal birth weight) and Stress (split by Exercise, Diet and Sex). Data presented as mean ± SEM where 'NS' is not significant. ‡$p < 0.05$ *vs. Unstressed* and differences across exercises are denoted by different letters where 'A' is different to 'B' but not to 'AB'.

independently regulate fetoplacental weight outcomes, the placental stress system, and fetal steroid concentrations with sex-specific responses.

4.1. Effect of maternal growth restriction

We have previously demonstrated that maternal growth restriction and stress independently program F2 offspring disease [21], which may be due to alterations in placental nutrient transporter and stress responsive genes [26]. However, as the previous work did not assess sex-specific placental changes or fetal steroids, this was the focus of the present study. Interestingly, in *Restricted* dam's, fetal testosterone was elevated in fetuses from *Unstressed* Chow and *Stressed* HFD dams, with estradiol reduced in fetuses from *Stressed* Chow dams. This may suggest that fetuses of *Restricted* dams are prioritizing testosterone production over its aromatization to estradiol. Indeed, previous studies have demonstrated that IUGR offspring have altered neural and brain development *in utero* and in early postnatal life [46], which increases the risk of altered behavioral outcomes due to reduced testosterone *in utero*. This upregulation in testosterone concentrations in *Unstressed* Chow and *Stressed* HFD dams is, therefore, likely an intrinsic adaptation aimed at protecting/improving neural outcomes and adult behavior in F2 offspring of growth restricted dams; especially in males of *Stressed* HFD dams as absolute brain weight was reduced. Conversely, reduced fetal estradiol concentrations are also associated with detrimental developmental outcomes as it is associated with the imprinting of various behaviors in male rats (sexual, aggression, vocalization, learning, cognition), the development of the fetal neuroendocrine axis, and

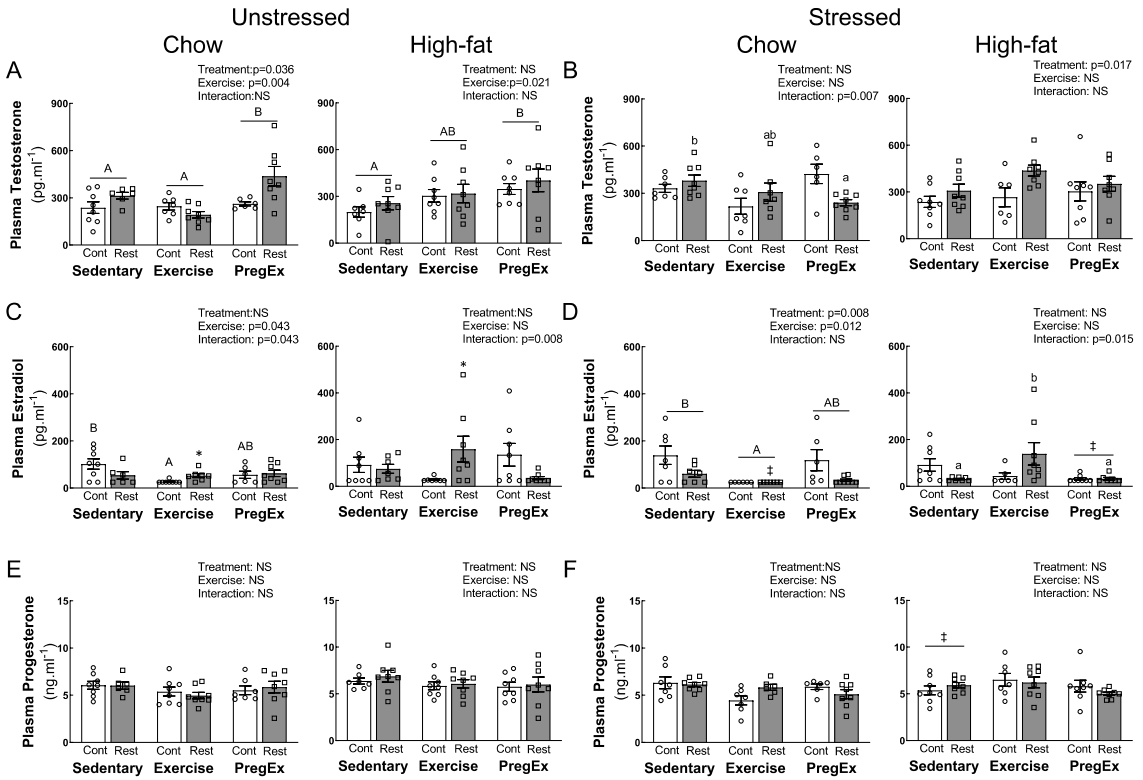

Fig. 4. Fetal plasma steroids. Pooled fetal plasma testosterone (A and B), estradiol (C and D), and progesterone (E and F) concentrations from *Unstressed* and *Stressed* dams fed a Chow (left) or High-fat (right) diet (n = 8–10 litters/group) that were *Control* (Cont; open bars) or *Restricted* (Rest; black bars). Data were analyzed by a series of two-way ANOVA's to identify the differences between *i)* Treatment (maternal birth weight) and Exercise (split by Diet, Stress and Sex), and *ii)* Treatment (maternal birth weight) and Stress (split by Exercise, Diet and Sex). Data presented as mean ± SEM, where 'NS' is not significant. ‡$p < 0.05$ *vs. Unstressed* and differences across exercises are denoted by different letters where 'a/A' is different to 'b/B' but not to 'ab/AB', with lower case letters denoting *Restricted* dams.

estrogen receptor maturation in the brain during late gestation [47–49]. Therefore, these adaptations may be an attempt to improve F2 fetal outcomes, especially in males, in growth restricted mothers; although increased testosterone has the potential to harm the fetus by causing the virilization of female fetuses.

4.2. Effect of maternal stress exposure

To our surprise, only plasma corticosterone was upregulated in fetuses of *Stressed Sedentary* HFD dams, which coincided with reduced progesterone. Although the magnitude of change in progesterone appears minimal, it suggests that either *i)* progesterone is preferentially converted into corticosterone over sex steroids, or *ii)* more maternally derived corticosterone is crossing the placenta. Given the lack of changes in placental gene abundance of the glucocorticoid barrier enzymes, it is likely that the altered plasma steroid concentrations in *Stressed Sedentary* HFD dams are driven by the fetus and not the placenta [50]. Importantly, the upregulated plasma corticosterone may be responsible for the reduced placental weight in males and females as placental morphology was unchanged. In female placentae from *Stressed Sedentary* HFD dams, this reduction in placental weight was accompanied by increased placental efficiency; further suggesting that female fetuses have an ability to adapt to increased stress by altering their placental efficiency to maintain appropriate fetal development, unlike their male counterparts [51]. Therefore, the combination of maternal stress and diet appear to be a driving force in altering fetal steroid concentrations and placental growth in *Stressed Sedentary* dams, which may program offspring disease outcomes.

Maternal stress also modulated the expression of sex steroids depending on the maternal diet and exercise. Specifically, *Stress* in *PregEx* HFD dams reduced plasma estradiol, which plays a key role in placental vascular development [52] and may result in the programming of adult disease [53]. Indeed, placental morphology in females was altered in a manner that was dependent on the maternal birth weight, which suggests that the reduced placental morphology (whole placental and labyrinth zone cross-sectional areas) in *Stressed PregEx* HFD *Restricted* dams may be part of the driving force in the reduced plasma estradiol concentrations. Interestingly, in males fetal and absolute liver weights were reduced in *Stressed PregEx* HFD dams, which resulted in an increased brain-to-liver weight ratio. This suggests that, despite an increased labyrinth zone area, male placentae from *Stressed PregEx* HFD dams are not able to improve fetal outcomes by enhancing placental nutrient transportation to overcome the reduced estradiol concentrations, hence potentially poor placental vascular development. However, future studies are required to investigate this theory as plasma samples were pooled. These sexually dimorphic responses to maternal stress are not surprising as previous studies have demonstrated that female fetuses are sensitive to chronic maternal stress whereas male fetuses are sensitive to acute stress [54,55].

4.3. Exercise effects

Exercise had minimal effects on fetal steroids and placental genes, whereby expression of *Nr3c1* and *Hsd11b2* were decreased in *Stressed*

Table 1
Fetal and placental weight parameters (n = 8–10 litters in each group) and placental histology (n = 3–4 in each group/sex n = 1 representing one pup from one litter) of F2 male and female fetuses from Control and Restricted mothers on a Chow or High-fat diet at E20. Data were analyzed by a series of two-way ANOVA's to identify the differences between *i)* Treatment (maternal birth weight) and Exercise (split by Diet, Stress, and Sex), *ii)* Diet (split by Exercise, Stress, and Sex), and *iii)* Stress (split by Exercise, Diet and Sex). Data presented as mean ± SEM, where NS is not significant. *p < 0.05 *vs. Control*, ‡p < 0.05 *vs. Unstressed*, and differences across exercises are denoted by different letters where 'a/A' is different to 'b/B' but not to 'ab/AB'.

							Two-way ANOVA		
Parameter				Sedentary	Exercise	PregEx	Treatment	Exercise	Interaction
Fetal weight (g)									
Male	Unstressed	Chow	Control	1.796 ± 0.031^{A}	1.941 ± 0.038^{B}	1.814 ± 0.026^{A}	NS	**p = 0.020**	NS
			Restricted	1.870 ± 0.025^{A}	1.888 ± 0.038^{B}	1.876 ± 0.018^{A}			
		HFD	Control	1.843 ± 0.041	1.891 ± 0.045	1.886 ± 0.023	NS	NS	NS
			Restricted	1.862 ± 0.019	1.861 ± 0.077	1.862 ± 0.038			
	Stressed	Chow	Control	1.814 ± 0.024	1.833 ± 0.047	1.829 ± 0.026	NS	**p = 0.013**	**p = 0.047**
			Restricted	1.795 ± 0.017^{a}	1.940 ± 0.024^{b}	1.811 ± 0.022^{a}			
		HFD	Control	1.817 ± 0.017^{A}	1.939 ± 0.041^{B}	1.811 ± 0.039^{A‡}	NS	**p = 0.001**	NS
			Restricted	1.839 ± 0.024^{A}	1.910 ± 0.030^{B}	1.805 ± 0.028^{A‡}			
Female	Unstressed	Chow	Control	1.705 ± 0.045^{A}	1.835 ± 0.015^{B}	1.726 ± 0.022^{A}	NS	**p = 0.003**	NS
			Restricted	1.774 ± 0.018^{A}	1.858 ± 0.044^{B}	1.785 ± 0.032^{A}			
		HFD	Control	1.734 ± 0.023	1.792 ± 0.043	1.782 ± 0.018	NS	NS	NS
			Restricted	1.742 ± 0.035	1.764 ± 0.057	1.753 ± 0.017			
	Stressed	Chow	Control	1.750 ± 0.023^{A}	1.752 ± 0.032^{B}	1.792 ± 0.017^{AB}	NS	**p = 0.048**	NS
			Restricted	1.738 ± 0.027^{A}	1.861 ± 0.029^{B}	1.806 ± 0.031^{AB}			
		HFD	Control	1.728 ± 0.019^{A}	1.829 ± 0.044^{B}	1.764 ± 0.029^{A}	NS	**p = 0.023**	NS
			Restricted	1.794 ± 0.034^{A}	1.840 ± 0.036^{B}	1.734 ± 0.031^{A}			
Placental weight (g)									
Male	Unstressed	Chow	Control	0.357 ± 0.008	0.342 ± 0.005	0.329 ± 0.003	NS	NS	NS
			Restricted	0.339 ± 0.011	0.356 ± 0.008	0.342 ± 0.006			
		HFD	Control	0.345 ± 0.009	0.341 ± 0.008	0.342 ± 0.007	NS	NS	NS
			Restricted	0.349 ± 0.008	0.332 ± 0.010	0.331 ± 0.007			
	Stressed	Chow	Control	0.331 ± 0.008‡	0.337 ± 0.008	0.328 ± 0.006	NS	NS	NS
			Restricted	0.323 ± 0.008‡	0.340 ± 0.006	0.340 ± 0.007			
		HFD	Control	0.329 ± 0.004‡	0.334 ± 0.008^{B}	0.327 ± 0.006	NS	NS	NS
			Restricted	0.330 ± 0.005‡	0.350 ± 0.007	0.330 ± 0.006			
Female	Unstressed	Chow	Control	0.347 ± 0.009^{B}	0.335 ± 0.007^{B}	0.319 ± 0.005^{A}	NS	**p = 0.005**	NS
			Restricted	0.335 ± 0.010^{B}	0.358 ± 0.011^{B}	0.320 ± 0.005^{A}			
		HFD	Control	0.357 ± 0.008^{B}	0.335 ± 0.004^{A}	0.338 ± 0.005^{A}	NS	**p = 0.016**	NS
			Restricted	0.344 ± 0.009^{B}	0.332 ± 0.011^{A}	0.322 ± 0.005^{A}			
	Stressed	Chow	Control	0.331 ± 0.007	0.329 ± 0.005	0.328 ± 0.004‡	NS	NS	NS
			Restricted	0.322 ± 0.007	0.343 ± 0.009	0.334 ± 0.006‡			
		HFD	Control	0.333 ± 0.005‡	0.332 ± 0.004	0.332 ± 0.007	NS	NS	NS
			Restricted	0.322 ± 0.005‡	0.341 ± 0.006	0.330 ± 0.007			
Placental efficiency (g)									
Male	Unstressed	Chow	Control	5.083 ± 0.126^{A}	5.695 ± 0.071^{B}	5.550 ± 0.085^{B}	NS	NS	**p = 0.001**
			Restricted	5.610 ± 0.139*	5.334 ± 0.102*	5.555 ± 0.096			
		HFD	Control	5.389 ± 0.151	5.461 ± 0.061	5.563 ± 0.074	NS	NS	NS
			Restricted	5.389 ± 0.127	5.626 ± 0.162	5.651 ± 0.076			
	Stressed	Chow	Control	5.511 ± 0.100	5.482 ± 0.081	5.621 ± 0.135	NS	NS	NS
			Restricted	5.638 ± 0.179	5.727 ± 0.044‡	5.366 ± 0.066			
		HFD	Control	5.538 ± 0.072	5.848 ± 0.165	5.571 ± 0.147	NS	NS	NS
			Restricted	5.600 ± 0.067	5.480 ± 0.065	5.510 ± 0.109			
Female	Unstressed	Chow	Control	4.964 ± 0.143^{A}	5.513 ± 0.104^{AB}	5.441 ± 0.104^{B}	NS	**p = 0.040**	NS
			Restricted	5.384 ± 0.133^{A}	5.256 ± 0.194^{AB}	5.600 ± 0.115^{B}			
		HFD	Control	4.965 ± 0.159^{A}	5.370 ± 0.108^{B}	5.321 ± 0.075^{B}	NS	**p = 0.008**	NS
			Restricted	5.115 ± 0.157^{A}	5.366 ± 0.129^{B}	5.474 ± 0.053^{B}			
	Stressed	Chow	Control	5.314 ± 0.097	5.353 ± 0.075	5.500 ± 0.084	NS	NS	NS
			Restricted	5.427 ± 0.081	5.487 ± 0.115	5.434 ± 0.127			
		HFD	Control	5.252 ± 0.091‡	5.534 ± 0.100	5.367 ± 0.130	NS	NS	**p = 0.042**
			Restricted	5.608 ± 0.112*‡	5.382 ± 0.070	5.311 ± 0.108			
Placental cross-sectional area									
Male	Unstressed	Chow	Control	11.435 ± 0.780^{A}	14.443 ± 0.471^{A}	15.693 ± 1.982^{B}	NS	**p = 0.019**	NS
			Restricted	13.155 ± 1.375^{A}	12.114 ± 1.233^{A}	17.208 ± 1.743^{B}			
		HFD	Control	14.109 ± 1.088	12.472 ± 1.121	14.417 ± 0.956	NS	NS	NS
			Restricted	14.519 ± 2.506	10.880 ± 0.790	16.708 ± 1.856			
	Stressed	Chow	Control	10.104 ± 0.833^{A}	15.429 ± 0.849^{B}	19.466 ± 0.943^{C‡}	NS	**p = 0.0001**	NS
			Restricted	11.750 ± 1.309^{A}	15.140 ± 2.011^{B}	20.230 ± 0.957^{C‡}			
		HFD	Control	14.052 ± 0.950^{A}	12.710 ± 1.367^{A}	18.677 ± 0.938^{B}	NS	**p = 0.001**	NS
			Restricted	11.110 ± 0.824^{A}	13.169 ± 1.827^{A}	17.354 ± 1.312^{B}			
Female	Unstressed	Chow	Control	14.175 ± 0.444	13.951 ± 1.014	14.506 ± 1.863	**p = 0.004**	NS	NS
			Restricted	15.240 ± 0.645	18.836 ± 1.670	19.281 ± 1.638			
		HFD	Control	14.477 ± 0.605^{A}	13.685 ± 0.912^{A}	15.210 ± 0.980^{B}	NS	**p = 0.006**	NS
			Restricted	14.558 ± 0.652^{A}	14.325 ± 0.879^{A}	18.269 ± 0.579^{B}			
	Stressed	Chow	Control	13.870 ± 1.865^{A}	13.633 ± 0.603^{A‡}	19.523 ± 0.795^{B}	NS	**p = 0.0001**	NS
			Restricted	14.200 ± 0.650^{A}	14.194 ± 0.771^{A‡}	18.822 ± 0.955^{B}			
		HFD	Control	12.882 ± 0.493^{A}	14.698 ± 0.850^{A}	19.310 ± 0.643^{B‡}	NS	**p = 0.009**	**p = 0.026**

(*continued on next page*)

Table 1 (*continued*)

Parameter				Sedentary	Exercise	PregEx	Two-way ANOVA		
							Treatment	Exercise	Interaction
			Restricted	15.103 ± 1.085	14.473 ± 1.795	15.428 ± 0.735*‡			
Labyrinth zone cross-sectional area									
Male	Unstressed	Chow	Control	8.399 ± 0.728^{A}	11.322 ± 0.451^{A}	12.038 ± 1.581^{B}	NS	**p = 0.014**	NS
			Restricted	9.905 ± 1.103^{A}	8.879 ± 0.720^{A}	13.563 ± 1.399^{B}			
		HFD	Control	10.676 ± 0.489	9.582 ± 0.970	10.325 ± 0.707	NS	NS	NS
			Restricted	11.124 ± 1.945	8.173 ± 0.703	12.003 ± 1.421			
	Stressed	Chow	Control	8.204 ± 0.969^{A}	11.133 ± 0.669^{B}	15.440 ± 0.680^{C‡}	NS	**p = 0.0001**	NS
			Restricted	9.744 ± 0.907^{A}	11.606 ± 1.607^{B}	15.423 ± 0.899^{C‡}			
		HFD	Control	10.799 ± 0.704^{A}	9.701 ± 1.026^{A}	13.915 ± 0.538^{B‡}	NS	**p = 0.001**	NS
			Restricted	8.544 ± 0.655^{A}	9.626 ± 1.432^{A}	13.065 ± 1.270^{B‡}			
Female	Unstressed	Chow	Control	10.407 ± 0.472	11.048 ± 0.719	11.521 ± 1.621	**p = 0.008**	NS	NS
			Restricted	11.764 ± 0.481	14.038 ± 1.321	14.605 ± 0.969			
		HFD	Control	10.710 ± 0.528^{A}	10.519 ± 0.560^{A}	11.329 ± 0.632^{B}	NS	**p = 0.004**	NS
			Restricted	11.009 ± 0.494^{A}	10.142 ± 0.931^{A}	13.985 ± 0.417^{B}			
	Stressed	Chow	Control	10.494 ± 1.434^{A}	10.584 ± 0.536^{A}	15.128 ± 0.528^{B}	NS	**p = 0.0001**	NS
			Restricted	10.538 ± 0.711^{A}	9.856 ± 0.426^{A‡}	14.636 ± 1.012^{B}			
		HFD	Control	9.765 ± 0.199^{A}	10.767 ± 0.544^{A}	14.772 ± 0.519^{B‡}	NS	**p = 0.006**	**p = 0.017**
			Restricted	11.303 ± 0.891	10.894 ± 1.401	11.500 ± 0.450*‡			
Junctional zone cross-sectional area									
Male	Unstressed	Chow	Control	2.638 ± 0.233	3.216 ± 0.211	3.578 ± 0.317	NS	NS	NS
			Restricted	3.210 ± 0.398	3.213 ± 0.477	3.296 ± 0.400			
		HFD	Control	3.196 ± 0.550^{A}	3.197 ± 0.273^{A}	4.097 ± 0.342^{B}	NS	**p = 0.010**	NS
			Restricted	3.405 ± 0.596^{A}	2.649 ± 0.171^{A}	4.651 ± 0.496^{B}			
	Stressed	Chow	Control	2.420 ± 0.241^{A}	3.297 ± 0.221^{B}	4.122 ± 0.324^{C‡}	NS	**p = 0.0001**	NS
			Restricted	2.699 ± 0.323^{A}	3.458 ± 0.487^{B}	4.624 ± 0.152^{C‡}			
		HFD	Control	3.171 ± 0.284^{A}	3.033 ± 0.376^{A}	4.862 ± 0.407^{B}	NS	**p = 0.001**	NS
			Restricted	2.541 ± 0.236^{A}	3.504 ± 0.444^{A}	3.992 ± 0.303^{B}			
Female	Unstressed	Chow	Control	3.703 ± 0.172	3.023 ± 0.254	3.377 ± 0.396	**p = 0.037**	NS	NS
			Restricted	3.220 ± 0.181	4.811 ± 0.602	4.677 ± 0.828			
		HFD	Control	3.970 ± 0.178	3.610 ± 0.113	3.930 ± 0.498	NS	NS	NS
			Restricted	3.587 ± 0.228	3.921 ± 0.341	4.278 ± 0.363			
	Stressed	Chow	Control	3.984 ± 0.434	3.176 ± 0.219	4.300 ± 0.300	NS	**p = 0.011**	NS
			Restricted	3.527 ± 0.377	3.180 ± 0.206	4.193 ± 0.263			
		HFD	Control	3.118 ± 0.324	4.118 ± 0.567	4.581 ± 0.282	NS	NS	NS
			Restricted	3.902 ± 0.404	3.736 ± 0.551	4.011 ± 0.389			
Absolute brain weight (g)									
Male	Unstressed	Chow	Control	0.121 ± 0.002	0.128 ± 0.002	0.122 ± 0.002	NS	**p = 0.045**	NS
			Restricted	0.124 ± 0.002	0.124 ± 0.002	0.122 ± 0.001			
		HFD	Control	0.117 ± 0.002^{A}	0.126 ± 0.002^{B}	0.123 ± 0.002^{B}	NS	NS	**p = 0.015**
			Restricted	0.122 ± 0.002	0.119 ± 0.003	0.120 ± 0.002			
	Stressed	Chow	Control	0.122 ± 0.002	0.120 ± 0.003‡	0.123 ± 0.001	NS	NS	**p = 0.006**
			Restricted	0.122 ± 0.002^{a}	0.129 ± 0.001^{b}	0.117 ± 0.002^{a}			
		HFD	Control	0.121 ± 0.002	0.127 ± 0.002	0.124 ± 0.001	**p = 0.034**	NS	NS
			Restricted	0.118 ± 0.003	0.121 ± 0.002	0.121 ± 0.002			
Female	Unstressed	Chow	Control	0.116 ± 0.002	0.121 ± 0.002	0.120 ± 0.001	NS	NS	NS
			Restricted	0.121 ± 0.002	0.123 ± 0.002	0.117 ± 0.002			
		HFD	Control	0.117 ± 0.002	0.124 ± 0.003	0.120 ± 0.002	NS	NS	NS
			Restricted	0.118 ± 0.002	0.119 ± 0.002	0.119 ± 0.002			
	Stressed	Chow	Control	0.116 ± 0.003	0.118 ± 0.003	0.119 ± 0.003	NS	NS	NS
			Restricted	0.122 ± 0.002	0.125 ± 0.001	0.120 ± 0.004			
		HFD	Control	0.118 ± 0.001	0.121 ± 0.001	0.121 ± 0.003	NS	NS	NS
			Restricted	0.121 ± 0.002	0.121 ± 0.002	0.119 ± 0.002			
Relative brain weight (%BW)									
Male	Unstressed	Chow	Control	6.683 ± 0.155	6.613 ± 0.096	6.746 ± 0.049	NS	NS	NS
			Restricted	6.645 ± 0.105	6.601 ± 0.085	6.511 ± 0.117			
		HFD	Control	6.545 ± 0.152	6.556 ± 0.147	6.547 ± 0.106	NS	NS	NS
			Restricted	6.607 ± 0.105	6.546 ± 0.247	6.471 ± 0.140			
	Stressed	Chow	Control	6.734 ± 0.124	6.572 ± 0.145	6.666 ± 0.097	NS	NS	NS
			Restricted	6.802 ± 0.108	6.645 ± 0.086	6.514 ± 0.160			
		HFD	Control	6.582 ± 0.085^{A}	6.557 ± 0.088^{A}	6.856 ± 0.114^{B‡}	NS	**p = 0.047**	NS
			Restricted	6.434 ± 0.148^{A}	6.520 ± 0.152^{A}	6.721 ± 0.125^{B‡}			
Female	Unstressed	Chow	Control	6.845 ± 0.089	6.617 ± 0.094	6.980 ± 0.076	NS	NS	NS
			Restricted	6.713 ± 0.122	6.641 ± 0.084	6.600 ± 0.123			
		HFD	Control	6.688 ± 0.084	6.822 ± 0.182	6.752 ± 0.082	NS	NS	NS
			Restricted	6.805 ± 0.174	6.870 ± 0.215	6.774 ± 0.123			
	Stressed	Chow	Control	6.832 ± 0.057	6.965 ± 0.222	6.679 ± 0.133	NS	NS	NS
			Restricted	7.012 ± 0.108	6.735 ± 0.131	6.664 ± 0.262			
		HFD	Control	6.829 ± 0.094	6.618 ± 0.136	6.791 ± 0.133	NS	NS	NS
			Restricted	6.745 ± 0.073	6.527 ± 0.159	6.883 ± 0.124			
Absolute liver weight (g)									
Male	Unstressed	Chow	Control	0.124 ± 0.004	0.136 ± 0.004	0.120 ± 0.007	NS	NS	NS
			Restricted	0.130 ± 0.004	0.134 ± 0.003	0.140 ± 0.005			
		HFD	Control	0.127 ± 0.004	0.137 ± 0.004	0.135 ± 0.003	NS	NS	NS

(*continued on next page*)

Table 1 (*continued*)

Parameter				Sedentary	Exercise	PregEx	Two-way ANOVA Treatment	Exercise	Interaction
			Restricted	0.132 ± 0.002	0.130 ± 0.008	0.133 ± 0.003			
	Stressed	Chow	Control	0.121 ± 0.002	0.122 ± 0.006[‡]	0.125 ± 0.003	NS	NS	NS
			Restricted	0.122 ± 0.003	0.130 ± 0.003	0.123 ± 0.003[‡]			
		HFD	Control	0.127 ± 0.003	0.136 ± 0.003	0.129 ± 0.004[‡]	NS	NS	NS
			Restricted	0.130 ± 0.003	0.133 ± 0.005	0.123 ± 0.003[‡]			
Female	Unstressed	Chow	Control	0.120 ± 0.005[A]	0.132 ± 0.002[B]	0.119 ± 0.005[A]	**p = 0.037**	**p = 0.034**	NS
			Restricted	0.126 ± 0.003[A]	0.135 ± 0.004[B]	0.131 ± 0.003[A]			
		HFD	Control	0.127 ± 0.003	0.132 ± 0.005	0.127 ± 0.002	NS	NS	NS
			Restricted	0.126 ± 0.002	0.121 ± 0.008	0.126 ± 0.004			
	Stressed	Chow	Control	0.116 ± 0.005	0.116 ± 0.005[‡]	0.125 ± 0.002	NS	NS	NS
			Restricted	0.118 ± 0.004	0.132 ± 0.004	0.126 ± 0.002			
		HFD	Control	0.124 ± 0.002	0.133 ± 0.004	0.132 ± 0.005	NS	NS	NS
			Restricted	0.128 ± 0.003	0.134 ± 0.002	0.124 ± 0.003			
Relative liver weight (%BW)									
Male	Unstressed	Chow	Control	6.770 ± 0.178	7.004 ± 0.101	6.627 ± 0.373	NS	NS	NS
			Restricted	6.923 ± 0.159	7.094 ± 0.120	7.491 ± 0.297			
		HFD	Control	7.057 ± 0.094	7.084 ± 0.101	7.129 ± 0.137	NS	NS	NS
			Restricted	7.063 ± 0.056	6.965 ± 0.187	7.140 ± 0.058			
	Stressed	Chow	Control	6.683 ± 0.103	6.621 ± 0.208[‡]	6.720 ± 0.112	NS	NS	NS
			Restricted	6.764 ± 0.149	6.693 ± 0.115[‡]	6.789 ± 0.107			
		HFD	Control	6.906 ± 0.094	7.003 ± 0.116	7.088 ± 0.089	NS	NS	NS
			Restricted	7.059 ± 0.112	7.056 ± 0.143	6.796 ± 0.079			
Female	Unstressed	Chow	Control	7.028 ± 0.152	7.184 ± 0.093	6.890 ± 0.326	NS	NS	NS
			Restricted	6.965 ± 0.131	7.255 ± 0.073	7.310 ± 0.101			
		HFD	Control	7.231 ± 0.114	7.195 ± 0.117	7.127 ± 0.124	NS	NS	NS
			Restricted	7.205 ± 0.138	6.750 ± 0.324	7.160 ± 0.214			
	Stressed	Chow	Control	6.745 ± 0.133	6.777 ± 0.158[‡]	6.988 ± 0.087	NS	NS	NS
			Restricted	6.759 ± 0.151	7.103 ± 0.155[‡]	6.972 ± 0.063			
		HFD	Control	7.196 ± 0.076	7.191 ± 0.088	7.400 ± 0.131	NS	NS	NS
			Restricted	7.135 ± 0.118	7.160 ± 0.104	7.093 ± 0.097			
Brain-to-liver weight ratio									
Male	Unstressed	Chow	Control	0.986 ± 0.028	0.942 ± 0.024	1.072 ± 0.112	NS	NS	NS
			Restricted	0.962 ± 0.026	0.927 ± 0.016	0.877 ± 0.030			
		HFD	Control	0.928 ± 0.027	0.922 ± 0.024	0.918 ± 0.022	NS	NS	NS
			Restricted	0.930 ± 0.018	0.954 ± 0.056	0.901 ± 0.018			
	Stressed	Chow	Control	1.004 ± 0.021	0.996 ± 0.045	0.991 ± 0.022	NS	NS	NS
			Restricted	1.002 ± 0.016	0.994 ± 0.025	0.955 ± 0.029			
		HFD	Control	0.952 ± 0.011	0.936 ± 0.021	0.967 ± 0.026[‡]	NS	NS	NS
			Restricted	0.908 ± 0.023	0.927 ± 0.037	0.983 ± 0.019[‡]			
Female	Unstressed	Chow	Control	0.977 ± 0.030	0.920 ± 0.012	1.037 ± 0.068	NS	NS	NS
			Restricted	0.971 ± 0.020	0.911 ± 0.015	0.902 ± 0.016			
		HFD	Control	0.925 ± 0.016	0.949 ± 0.039	0.944 ± 0.017	NS	NS	NS
			Restricted	0.937 ± 0.018	1.052 ± 0.088	0.955 ± 0.041			
	Stressed	Chow	Control	1.018 ± 0.028[B‡]	0.985 ± 0.030[A‡]	0.955 ± 0.026[A]	NS	**p = 0.029**	NS
			Restricted	1.046 ± 0.031[B‡]	0.952 ± 0.031[A‡]	0.954 ± 0.034[A]			
		HFD	Control	0.946 ± 0.015	0.925 ± 0.021	0.930 ± 0.026	NS	NS	NS
			Restricted	0.943 ± 0.017	0.914 ± 0.031	0.964 ± 0.019			

Exercise Chow *Control* dams. This suggests an inability of the placenta to cope with the increased maternal metabolic demand of exercise in addition to maternal stress in normal birth weight dams, thus reducing the glucocorticoid barrier. Interestingly, *Exercise* had little effect on the fetal steroid milieu; suggesting that *Exercise* likely elicits an earlier adaptive mechanism to allow the fetal and placental systems to regulate appropriate steroid concentrations for optimal fetal growth and development.

To our knowledge, this is the only study that characterizes the placental and fetal endocrine milieu in response to maternal growth restriction, exercise, diet, and stress. A study by Rauramo and colleagues [56] in humans suggests that acute exercise results in a short-term increase in maternal estriol (major estrogen during human pregnancy), but not cortisol, concentrations, which is surprising as exercise has also been shown to influence glucocorticoid and oxidative stress responses [57,58]. These maternal changes are linked to alterations in placental blood flow and increased placental steroid production [56]. Although changes in maternal glucocorticoids were not characterized in the present study, we demonstrate that *Exercise* attenuates fetal glucocorticoid concentrations, consistent with Rauramo et al. (1982), which is likely due to a maternal-to-fetal mechanism designed to protect fetal growth outcomes. Supporting this postulation, we report increased fetal weights in *Unstressed Exercise* Chow dams as well as in *Stressed Exercise* Chow and *Stressed Exercise* HFD dams. Additionally, placental morphology (whole placental, junctional zone and labyrinth cross-sectional areas) was increased in males from *Stressed Exercise* Chow dams; an adaptation that is likely to enhance nutrient transportation capacity in this group.

4.4. PregEx effects

We recently demonstrated that *PregEx* is a key driver of placental gene and protein expression, which is likely due to a late placental adaptation to the increased maternal metabolic demand that threatens fetal energy supplies [23–25]. We report that *PregEx* in *Unstressed* Chow

Table 2
Labyrinth gene abundance for F2 male and female fetuses from Control and Restricted dams on a Chow or a High-fat diet (HFD) at E20 (n = 6 in each group/sex with n = 1 representing 1 pup from 1 litter). Data were analyzed by a series of two-way ANOVA's to identify the differences between *i)* Treatment (maternal birth weight) and Exercise (split by Diet, Stress and Sex) and *ii)* Treatment (maternal birth weight) and Stress (split by Exercise, Diet and Sex). Data presented as mean ± SEM, where NS is not significant. ‡p < 0.05 *vs. Unstressed* and differences across exercises are denoted by different letters where 'a/A' is different to 'b/B' but not to 'ab/AB'.

							Two-way ANOVA		
Gene				Sedentary	Exercise	PregEx	Treatment	Exercise	Interaction
Hsd11b1									
Male	Unstressed	Chow	Control	1.25 ± 0.30	1.17 ± 0.33	1.09 ± 0.04	NS	NS	NS
			Restricted	1.63 ± 0.30	1.70 ± 0.23	1.20 ± 0.10			
		HFD	Control	1.18 ± 0.23	1.00 ± 0.03	1.42 ± 0.40	NS	NS	NS
			Restricted	0.93 ± 0.09	1.21 ± 0.28	0.98 ± 0.07			
	Stressed	Chow	Control	1.90 ± 0.19	1.46 ± 0.15	1.39 ± 0.18	NS	NS	NS
			Restricted	1.55 ± 0.09	1.34 ± 0.13	0.95 ± 0.26			
		HFD	Control	1.35 ± 0.11	1.24 ± 0.12	1.08 ± 0.09	NS	NS	NS
			Restricted	1.30 ± 0.21	1.42 ± 0.15	0.93 ± 0.06			
Female	Unstressed	Chow	Control	1.17 ± 0.49	3.45 ± 1.24	1.69 ± 0.37	NS	NS	NS
			Restricted	2.67 ± 0.20	2.65 ± 0.33	1.84 ± 0.20			
		HFD	Control	1.83 ± 0.15	1.65 ± 0.10	1.30 ± 0.14	NS	NS	NS
			Restricted	1.58 ± 0.34	1.58 ± 0.14	1.44 ± 0.22			
	Stressed	Chow	Control	1.78 ± 0.26	2.20 ± 0.32	1.74 ± 0.21	NS	NS	NS
			Restricted	1.81 ± 0.37	1.91 ± 0.30	1.78 ± 0.33			
		HFD	Control	1.57 ± 0.24	2.18 ± 0.36	1.68 ± 0.23	NS	NS	NS
			Restricted	1.39 ± 0.20	2.01 ± 0.22	1.49 ± 0.14			
Hsd11b2									
Male	Stressed	Chow	Control	1.39 ± 0.20^{B}	0.37 ± 0.09^{A}	0.60 ± 0.02^{A}	NS	NS	**p = 0.005**
			Restricted	1.15 ± 0.07^{b}	$1.02 \pm 0.19^{b*}$	0.61 ± 0.11^{a}			
		HFD	Control	0.75 ± 0.21	0.55 ± 0.12	0.56 ± 0.05	NS	NS	NS
			Restricted	0.90 ± 0.13	0.71 ± 0.14	0.84 ± 0.13			
Female	Stressed	Chow	Control	1.35 ± 0.22^{B}	0.67 ± 0.09^{A}	0.71 ± 0.09^{A}	NS	**p = 0.011**	NS
			Restricted	1.10 ± 0.29^{B}	0.92 ± 0.15^{A}	0.71 ± 0.06^{A}			
		HFD	Control	0.90 ± 0.20	0.90 ± 0.26	0.73 ± 0.09	NS	NS	NS
			Restricted	0.96 ± 0.12	0.86 ± 0.18	0.52 ± 0.15			
Nr3c1									
Male	Unstressed	Chow	Control	1.05 ± 0.14	0.79 ± 0.04	0.84 ± 0.08	NS	NS	NS
			Restricted	1.08 ± 0.08	0.95 ± 0.25	1.00 ± 0.14			
		HFD	Control	0.97 ± 0.19	0.95 ± 0.09	1.14 ± 0.26	NS	NS	NS
			Restricted	0.82 ± 0.20	0.74 ± 0.33	0.83 ± 0.04			
	Stressed	Chow	Control	1.75 ± 0.39^{B}	0.49 ± 0.15^{A}	0.78 ± 0.03^{A}	NS	**p = 0.005**	**p = 0.008**
			Restricted	1.18 ± 0.05	$1.27 \pm 0.14^{*}$	0.85 ± 0.16			
		HFD	Control	0.99 ± 0.25	0.77 ± 0.21	1.01 ± 0.07	NS	NS	NS
			Restricted	0.96 ± 0.10	0.82 ± 0.14	0.87 ± 0.03			
Female	Unstressed	Chow	Control	1.03 ± 0.05	0.79 ± 0.14	0.97 ± 0.17	**p = 0.021**	NS	NS
			Restricted	1.52 ± 0.24	1.32 ± 0.26	1.01 ± 0.22			
		HFD	Control	1.31 ± 0.08	0.94 ± 0.19	1.07 ± 0.09	NS	NS	NS
			Restricted	0.77 ± 0.20	0.82 ± 0.21	0.90 ± 0.14			
	Stressed	Chow	Control	1.15 ± 0.27	0.91 ± 0.15	1.04 ± 0.11	NS	NS	NS
			Restricted	0.96 ± 0.26	1.06 ± 0.09	0.90 ± 0.12			
		HFD	Control	0.92 ± 0.19	0.84 ± 0.23	1.11 ± 0.13	NS	NS	NS
			Restricted	0.99 ± 0.10	1.07 ± 0.21	0.69 ± 0.24			
Nr3c2									
Male	Stressed	Chow	Control	1.35 ± 0.26	$0.53 \pm 0.08^{‡}$	0.67 ± 0.08	**p = 0.001**	NS	NS
			Restricted	0.91 ± 0.12	$0.73 \pm 0.07^{‡}$	0.57 ± 0.11			
		HFD	Control	0.82 ± 0.07^{B}	$0.51 \pm 0.06^{A‡}$	0.63 ± 0.07^{A}	**p = 0.014**	NS	**p = 0.034**
			Restricted	0.66 ± 0.09	$0.75 \pm 0.11^{‡}$	0.41 ± 0.07			
Female	Stressed	Chow	Control	1.36 ± 0.17	$0.65 \pm 0.13^{‡}$	0.71 ± 0.10	**p = 0.0001**	NS	NS
			Restricted	1.08 ± 0.21	$0.63 \pm 0.08^{‡}$	0.46 ± 0.06			
		HFD	Control	$0.73 \pm 0.07^{‡}$	0.79 ± 0.16	0.68 ± 0.07	NS	NS	NS
			Restricted	$0.65 \pm 0.05^{‡}$	0.67 ± 0.10	0.60 ± 0.06			
Crhr1									
Male	Unstressed	Chow	Control	1.08 ± 0.18^{B}	0.85 ± 0.14^{AB}	0.79 ± 0.20^{A}	NS	**p = 0.048**	NS
			Restricted	1.38 ± 0.09^{B}	1.50 ± 0.39^{AB}	0.66 ± 0.13^{A}			
		HFD	Control	0.62 ± 0.07	0.59 ± 0.06	0.62 ± 0.14	NS	NS	NS
			Restricted	0.74 ± 0.08	1.34 ± 0.41	0.65 ± 0.13			
	Stressed	Chow	Control	1.31 ± 0.30^{B}	0.77 ± 0.18^{A}	0.48 ± 0.07A	NS	**p = 0.007**	NS
			Restricted	1.06 ± 0.20^{B}	0.65 ± 0.19^{A}	0.61 ± 0.10^{A}			
		HFD	Control	1.19 ± 0.21^{B}	0.50 ± 0.11^{AB}	0.47 ± 0.07^{A}	NS	**p = 0.043**	NS
			Restricted	0.82 ± 0.11^{B}	0.87 ± 0.20^{AB}	0.75 ± 0.18^{A}			
Female	Unstressed	Chow	Control	1.21 ± 0.24	1.40 ± 0.29	0.90 ± 0.05	NS	NS	NS
			Restricted	1.67 ± 0.35	1.50 ± 0.67	0.98 ± 0.33			
		HFD	Control	1.54 ± 0.30^{B}	1.13 ± 0.16^{A}	0.82 ± 0.10^{A}	NS	**p = 0.0001**	NS
			Restricted	1.55 ± 0.14^{B}	0.87 ± 0.19^{A}	0.54 ± 0.05^{A}			
	Stressed	Chow	Control	1.82 ± 0.46^{B}	0.65 ± 0.10^{A}	0.77 ± 0.19^{A}	NS	**p = 0.003**	NS
			Restricted	1.18 ± 0.25^{B}	0.77 ± 0.06^{A}	0.60 ± 0.11^{A}			
		HFD	Control	1.17 ± 0.40	0.66 ± 0.15	0.84 ± 0.11	NS	NS	NS
			Restricted	0.88 ± 0.18	1.05 ± 0.28	0.87 ± 0.10			
Ogt									

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Table 2 (*continued*)

Gene				Sedentary	Exercise	PregEx	Two-way ANOVA Treatment	Exercise	Interaction
Male	Unstressed	Chow	Control	1.04 ± 0.13	0.98 ± 0.11	0.90 ± 0.05	NS	NS	NS
			Restricted	1.01 ± 0.07	1.25 ± 0.15	1.12 ± 0.10			
		HFD	Control	0.95 ± 0.06	0.76 ± 0.03	0.96 ± 0.06	NS	NS	NS
			Restricted	0.92 ± 0.06	1.04 ± 0.10	1.17 ± 0.06			
	Stressed	Chow	Control	1.31 ± 0.30	0.84 ± 0.10‡	0.93 ± 0.07	NS	NS	NS
			Restricted	1.01 ± 0.09	0.91 ± 0.06‡	1.04 ± 0.13			
		HFD	Control	0.87 ± 0.03	0.81 ± 0.07‡	0.99 ± 0.08	NS	NS	NS
			Restricted	0.85 ± 0.09	0.91 ± 0.04‡	0.84 ± 0.10			
Female	Unstressed	Chow	Control	1.36 ± 0.15	2.29 ± 0.69	1.34 ± 0.23	NS	NS	NS
			Restricted	1.62 ± 0.19	1.70 ± 0.11	1.69 ± 0.14			
		HFD	Control	1.25 ± 0.09	1.23 ± 0.08	1.24 ± 0.10	NS	NS	NS
			Restricted	1.53 ± 0.23	1.03 ± 0.15	0.45 ± 0.17			
	Stressed	Chow	Control	1.50 ± 0.29^{B}	1.19 ± 0.09^{A‡}	1.28 ± 0.13^{AB}	NS	**p = 0.046**	NS
			Restricted	1.82 ± 0.32^{B}	1.07 ± 0.13^{A‡}	1.52 ± 0.08^{AB}			
		HFD	Control	1.15 ± 0.11^{A‡}	1.41 ± 0.13^{B}	1.44 ± 0.14^{B}	**p = 0.029**	**p = 0.014**	NS
			Restricted	0.91 ± 0.05^{A‡}	1.21 ± 0.10^{B}	1.27 ± 0.10^{B}			

dams reduces *Hsd11b2* in both sexes and reduces *Nr3c2* in males, without changes in *Hsd11b1* or fetal corticosterone and 11-deoxycorticosterone concentrations, which suggests that the fetus is likely modulating glucocorticoid concentrations during late gestation following *PregEx*. Similarly to models of hypoxia in mice [41], the reduction in *Hsd11b2* and *Nr3c2*, at least in male fetuses, is likely due to *PregEx* inducing alterations to maternal oxygen and/or substrate availability rather than inducing a stress response. Thus, it is possible that any glucocorticoid barrier alterations may have manifested in response to reduced oxygen supply around mid-gestation when exercise initiation occurred. The reduction in *Nr3c2* in males and *Hsd11b2* in the absence of corticosterone changes in fetuses of *Unstressed* Chow dams may, therefore, represent mid- and/or late-gestation adaptations to maintain normal fetal growth.

Interestingly, fetal 11-deoxycorticosterone concentrations were upregulated in *Unstressed* HFD dams in the absence of alterations in the glucocorticoid barrier, which suggests that maternally derived corticosterone metabolism may be upregulated that may be due to increased placental HSD11β2 activity; although this association was not investigated. This is supported by the observed increase in junctional zone cross-sectional area in male placentae from *Unstressed* HFD dams, which is likely aimed to improve the placental endocrine response to maternal metabolic stress and the acute stress effect of *PregEx*. This, nevertheless, suggests that high-fat feeding acts as a "second-hit" in dams that exercise only during pregnancy, which occurred independently to alterations in fetal steroids. Although *PregEx* in *Stressed* dams did not alter the fetal endocrine milieu or fetoplacental weight, it resulted in profound increases in placental morphology (whole placental, labyrinth, and/or junctional zone cross-sectional areas) in *Stressed* Chow and *Stressed* HFD dams, which is likely an adaptive mechanism to increase fetal nutrient supply in the presence of a sudden maternal metabolic demand.

4.5. Strengths, limitations, and conclusions

A strength of the study is that it allows the direct comparison of the impact F1 maternal growth restriction and maternal diet have on the fetal stress responsive system in F2 male and female fetuses, thus improving our understanding of the impact these factors have on the transgenerational programming of disease. However, it should be noted that this research question required the generation of a large number of experimental groups (24 maternal groups) and, as such, the sample sizes used throughout the study limits the power of the analysis to be able to statistically compare how these parameters (treatment, exercise, diet, stress, and fetal sex) in combination influence the fetal stress system (i.e. five-way ANOVA), which requires additional studies. Instead our analysis prioritized each of the major effects in isolation.

Despite our attempts to characterize sex-specific responses on the placental stress-system and fetal steroids, several limitations remain. Firstly, as previous studies have demonstrated that maternal steroid concentrations are associated with fetal concentrations and term birth weight [59], it is unknown whether the fetal concentrations reported are due to maternal-to-fetal transportation or fetoplacental synthesis. Secondly, due to the low sample volume available, pooling of samples precludes the investigation of fetal sex-specific steroid responses, although certain inferences can be made. For example, male fetuses have higher concentrations of testosterone during late gestation than females [60]. Thirdly, the status of enzymes associated with placental steroid synthesis are important in understanding the role and extent to which the placenta and fetus are responsible for the conversion of steroid anabolites, which can help elucidate whether the placenta and/or fetus is responsible for fetal steroid concentrations. Fourthly, as only placental labyrinth zone genes were quantified we are not getting the full picture these factors have on the placenta as a whole especially considering the junctional zone is the endocrine region of the rat placenta, which would likely be differentially impacted by these factors. Lastly, as the present study did not investigate long-term F2 offspring health we need to be cautious linking the potential impact of these fetal steroid and placental gene changes on long-term health, which require investigation in future independent studies.

In conclusion, the current study demonstrates sex-dependent fetal steroidogenic responses during a challenging *in utero* environment. Furthermore, maternal *Stress* and *PregEx* appear to play important roles in regulating fetal health through independent placental mechanisms, which highlights the need for future studies to characterize the postnatal outcomes.

Declaration of interest

The authors declare no conflicts of interest.

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Author contribution statement

M.E.W, J.S.M.C, J.F·B, K.M.M, M.H·V and C.M.R. 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. M.H.V and C.M.R. analyzed 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 Placenta.

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Chapter 7 General Discussion

7.1 Overview

It was originally believed in the late sixteenth century and early eighteenth century that fetal growth and development were influenced by what the mother ate, touched, and experienced during pregnancy, which is also known as maternal impression (Shildrick, 2000). This notion changed in the late 1800's to highlight that the environment before and during pregnancy influences the developing fetus. Indeed, in the late 1980's David Barker and colleagues demonstrated a clear link between birth weight and the development of non-communicable adult diseases that ultimately lead to the Developmental Origins of Health and Disease hypothesis, which has been since replicated in numerous populations (Barker & Osmond, 1988; Barker *et al.*, 1989a; Barker *et al.*, 1989b; Barker *et al.*, 1990; Barker, 1991, 1992; Sayer *et al.*, 2004; Syddall *et al.*, 2005; Dennison *et al.*, 2007). More recent studies have focused on animal models which have allowed us to begin to understand the mechanism underpinning this disease programming. Such research has demonstrated that placental growth, development, and function plays a key role in mediating this programmed disease. For example, UPI is the main cause of fetal growth restriction in the Western world and, due to poor placental development, fetal nutrient delivery and blood supply is compromised, which impairs fetal growth and development and, ultimately, programs adult sex-specific diseases.

Placental pathways that are intrinsic to normal fetal growth and development include the IGF-system, nutrient transportation, stress pathways, and placental vascularisation, which numerous studies have demonstrated are altered in pregnancies complicated by fetal growth restriction (Jansson & Powell, 2000, 2006, 2007). Specifically, the IGF and nutrient transportation systems are linked to deregulated fetoplacental growth in pregnancies complicated by fetal growth restriction (Jansson & Powell, 2000; Bell & Ehrhardt, 2002; Sibley *et al.*, 2005; Fowden *et al.*, 2006b; Jansson & Powell, 2006; Jones *et al.*, 2007; Roos *et al.*, 2009; Lager & Powell, 2012; Brett *et al.*, 2014; Rosario *et al.*, 2015; Dimasuay *et al.*, 2016; Rosario *et al.*, 2016a; Sferruzzi-Perri *et al.*, 2017). In addition, dysfunction in the placental stress pathway can also compromise fetal growth by increasing fetal glucocorticoid concentrations and also, independently, regulates the nutrient transportation system (Cuffe *et al.*, 2014; Fowden & Forhead, 2015). However, very few studies have demonstrated whether these placental alterations are similarly present in F2 placentae of mothers who were born growth restricted. Nevertheless we, and others, have demonstrated that F2 offspring of growth restricted mothers

develop sex-specific diseases in adulthood (Master *et al.*, 2015; Cheong *et al.*, 2016a; Cheong *et al.*, 2016b; Anevska *et al.*, 2018). As the placenta is a sexually dimorphic organ it likely plays a key role in the sex-specific programming of fetal growth restriction as well as the transgenerational transmission of disease, which highlights the necessity to characterise placental adaptations in both male and female associated placentae.

In addition to pregnancy complications due to poor placental function, maternal environmental factors (including diet and physical activity) can independently and/or collectively deregulate fetoplacental growth and development; likely resulting in additive negative fetoplacental outcomes or "second-hits" in women that were born growth restricted (Entringer *et al.*, 2012; Gallo *et al.*, 2012a). Our laboratory has, indeed, previously demonstrated that a "second-hit" is required to unmask disease in F1 growth restricted females, whereby metabolic disease only develops during pregnancy (Gallo *et al.*, 2012c; Mahizir *et al.*, 2020); a finding that is consistent in growth restricted humans (Seghieri *et al.*, 2002). Therefore, it is likely that if these F1 growth restricted females are challenged with other adverse events, such as a sedentary lifestyle and/or a high-fat diet, it may further independently or collectively impair placental function, further compromising fetoplacental growth and development. As exercise has been shown to result in beneficial fetal and placental outcomes (Clapp, 2006; May *et al.*, 2010), it is possible that maternal exercise may oppose the negative impact maternal growth restriction, a sedentary lifestyle and/or consumption of a high-fat diet have on fetoplacental outcomes. However, it is likely that these benefits are dependent on the timing and intensity of the exercise whereby exercise will likely have greater maternal and fetal outcomes if it is commenced prior to and throughout pregnancy as opposed to during pregnancy only after placentation has completed.

Therefore, the overall aim of this thesis was to investigate the sex-specific effect F1 maternal growth restriction has on F2 fetoplacental growth and development. Furthermore, I characterised whether these changes were exacerbated by maternal high-fat feeding and if maternal exercise ameliorated and/or prevented any adverse placental outcomes. Specifically, this thesis examined the impact the placental IGF-system (Chapter 3), nutrient transportation (Chapter 4), vasculogenesis and angiogenesis (Chapter 5), and stress (Chapter 6) pathways have on F2 fetoplacental growth.

7.2 Aims and Major Thesis Findings

Specific aims and major findings for each experimental chapter are summarised as follows:

Chapter 3 Maternal exercise in rats upregulates the placental insulin-like growth factor system with diet- and sex-specific responses: minimal effects in mothers born growth restricted

The overall aim of Chapter 3 was to determine changes in the placental labyrinth IGF-system of F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to identify if maternal high-fat feeding exacerbated any changes in the F2 placental IGF-system within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

The major findings for Chapter 3 were that:

- the IGF-system was altered in F2 placentae *Restricted* dams, with maternal diet-, exercise-, and fetal sex-specific responses;
- F1 *Restriction* in Chow-fed dams altered F2 placental morphology only in females, but did not alter F2 male or female fetal weight;
- *Exercise* in *Control* Chow-fed dams increased fetal weight and plasma IGF1 concentrations compared to *Sedentary* dams;
- *PregEx* altered F2 placental morphology and increased placental IGF1 and IGF1R expression, which may in part be modulated by reduced *Let 7f-1* miRNA abundance.

This study was the first to demonstrate that F1 maternal growth restriction differentially dysregulates the F2 placental IGF-system with the responses being dependent on the maternal diet, timing of exercise initiation, and fetal sex. These changes are likely in attempt to improve F2 fetoplacental growth in an adverse *in utero* environment. Structural changes in the F2 placental labyrinth and/or junctional zone are likely aimed at improving fetal growth and development through increased placental nutrient transport efficiency. However, female associated placentae appear to be better at withstanding pregnancy challenges compared to male fetuses given that the placental morphological adaptations were absent in males. Importantly, *PregEx* resulted in profound changes in placental morphology and the placental IGF-system, which may be aimed at improving and/or maintaining a healthy fetal and birth weight trajectory.

Chapter 4 Exercise initiated during pregnancy in rats born growth restricted alters placental mTOR and nutrient transporter expression

The overall aim of Chapter 4 was to determine changes in placental labyrinth nutrient transporter expression in F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to identify if maternal high-fat feeding exacerbated any alterations within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

The major findings of Chapter 4 were that:

- F1 *Restriction* and *Exercise* resulted in minimal changes to the F2 placental nutrient transporter system;
- *PregEx* reduced nutrient transporter abundance in a maternal diet- and sex-specific manner. Specifically, irrespective of maternal diet, *PregEx* downregulated male and female mTOR protein expression, and mTOR activation was increased only in male associated placentae.
- *PregEx* in High-fat fed dams increased placental glycogen cell cross-sectional area in male associated placentae, which was independent of alterations in glucose transporter expression.

This was the first study to demonstrate that F2 placental mTOR and nutrient transporter expression are differentially affected by maternal growth restriction, exercise, and diet; changes of which were dependent on fetal sex. As maternal growth restriction resulted in minimal changes in the F2 placental nutrient transportation system it indicates that alternate pathways, such as placental vascular remodelling and/or fetoplacental steroid regulation, may result in the transgenerational transmission of disease in F2 offspring. It is important to note that the upregulation of p-mTOR protein expression in F2 male associated placentae may be an adaptive mechanism to improve fetal outcomes, despite the absence of changes in nutrient transporters. *PregEx* had profound effects on F2 placental nutrient transporter expression, particularly by decreasing mTOR protein expression and reducing nutrient transporter gene expression; a mechanism potentially aimed at reducing fetal overgrowth.

Chapter 5 Maternal exercise and growth restriction in rats alters placental angiogenic factors and blood space area in a sex-specific manner

The overall aim of Chapter 5 was to determine changes in placental labyrinth angiogenesis in F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to characterize if these outcomes are exacerbated by maternal high-fat feeding within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

The major findings of Chapter 5 were that:

- F1 *Restriction* altered F2 placental vascularization in a diet- and sex-specific manner, which was independent of changes in VEGFA expression;
- Maternal high-fat feeding resulted in minimal alterations in regulators of placental vasculogenesis and/or angiogenesis, and did not alter placental blood spaces;
- *Exercise* had minimal effects on placental regulators of vasculogenesis and angiogenesis, but downregulated *miRNA27a* abundance in female associated placentae;
- *PregEx* increased the expression of placental regulators of vasculogenesis but reduced placental blood spaces in male associated placentae likely due to altered VEGFA and PLGF expression.

This study demonstrated for the first time that maternal growth restriction and high-fat feeding independently modulate the expression of placental vasculogenesis and/or angiogenesis markers, which is dependent on the timing of exercise initiation. Specifically, *PregEx* modulated multiple vasculogenesis and/or angiogenesis regulators and altered placental vasculature formation (blood space area), which may reduce the surface area available for placental nutrient exchange; hence potentially compromising fetal growth and development.

Chapter 6 Maternal exercise alters rat fetoplacental stress response: minimal effects of maternal growth restriction and high-fat feeding

The overall aim of Chapter 6 was to determine changes in the placental labyrinth glucocorticoid barrier in F2 fetuses whose mothers were born growth restricted and the period of exercise initiation (prior to and/or during pregnancy) that is most beneficial in preventing these outcomes. I also aimed to identify if maternal high-fat feeding exacerbated any alterations within each exercise group. I lastly determined if there were any sex-specific differences within each experimental group.

The major findings of Chapter 6 were that:

- F1 maternal growth restriction altered F2 fetal plasma steroid concentrations (reduced corticosterone and increased testosterone) and increased placental *Nr3c1* gene abundance only in females;
- *Exercise* reduced fetal plasma estradiol concentrations and decreased *Crhr1* gene abundance only in females of high-fat fed dams;
- *PregEx* increased fetal steroid concentrations (11-deoxycorticosterone and testosterone) and downregulated placental stress responsive (*Nr3c2* and *Crhr1*) and glucocorticoid barrier (*Hsd11b2*) genes.

This study demonstrated for the first time that maternal birth weight and high-fat feeding independently regulates the placental stress system and fetal steroid concentrations in a sex-dependent manner. Most importantly, this data further adds to the evidence generated from Chapters 3-5 that *PregEx* is an important regulator of fetoplacental growth and development, which may be associated with the programming of adult disease.

7.3 F2 Placental Adaptations in Response to F1 Maternal Growth Restriction

A growing body of experimental evidence suggests that antenatal adjustments in response to inappropriate maternal-to-placental environmental conditions are driven by alterations in placental signalling and activation of growth, nutrient transporter, stress, angiogenic and vasculogenic systems (Robinson & Owens, 1994; Fowden *et al.*, 2008; Klammt *et al.*, 2008; Correa *et al.*, 2012; Gauster *et al.*, 2012; Aiko *et al.*, 2014), which often work together and are intertwined to enhance fetal survival in complicated pregnancies. Epidemiological and experimental models of growth restriction have demonstrated that plasma IGF1 (Burkhardt *et al.*, 2009), labyrinth IGF2, and IGFBP3 (Méio *et al.*, 2009) concentrations are reduced and placental IGFR1 signalling is impaired by up to 33% (Laviola *et al.*, 2005), which is also associated with a reduction in downstream signalling mediators of IGF1R (Klammt *et al.*, 2008). Deregulation of the IGF-system is also a piece of the puzzle to the deregulation of nutrient sensing pathways in F1 growth restricted placentae due to the key role of mTORC1, which is partly regulated through the activation of the IGF-system as well as the influx of placental nutrients, such as amino acids and glucose (Kavitha *et al.*, 2014). It is, therefore, not surprising that the mTOR pathway along with the localization and abundance of nutrient transporters and their ligands are also dysregulated with growth restriction (Roos *et al.*, 2007; Yung *et al.*, 2008). In addition to the IGF and nutrient transportation systems, placental vascularisation via the VEGF family also plays a key role in nutrient transportation by influencing the available surface area for placental nutrient exchange (Arroyo & Winn, 2008); whereby a highly vascularised placenta will have a greater area for nutrient exchange compared to a poorly vascularised placenta. It is also important to acknowledge the important role the placental stress-system plays in maintaining fetal growth, in addition to the IGF-system, where it regulates the exposure of the fetus to physiological glucocorticoid concentrations that are essential for normal fetal growth; however they can compromise fetal growth at excessively high concentrations (Edwards *et al.*, 1993). These changes following growth restriction are attributed to the developmental plasticity of the placentae in challenging metabolic conditions; adaptations of which are likely made to improve birth outcomes. Despite these placental changes following growth restriction being seen as beneficial to fetal survival in the short-term, they have been linked to the development of male-onset adult cardiorenal and metabolic diseases (Anderson *et al.*, 2006).

To my knowledge, the published findings of this thesis are the first to explore the sex-specific impact maternal growth restriction has on the F2 placenta. In contrast to what is observed in F1 placentae, the F2 placental morphology and IGF-system (Chapter 3), nutrient transportation (Chapter 4), vascular development (Chapter 5), and stress enzymes (Chapter 6) are selectively deregulated by maternal growth restriction in a manner that was dependent on the maternal diet. Importantly, F2 male and female associated placentae respond differently to F1 maternal growth restriction, a finding of which is consistently observed following many pregnancy perturbations (Clifton & Murphy, 2004; Brown *et al.*, 2014), including IUGR.

7.3.1 Effects of maternal growth restriction on F2 placental adaptations in chow-fed dams

Chapter 3 demonstrated that F2 male associated placentae from Chow-fed dams had reduced *Igf2r* abundance that, if translated to a similar reduction in IGF2R protein expression, is likely an adaptation aimed at increasing IGF2 ligand activity and/or availability to enhance placental growth. Indeed, a previous study in mice demonstrated that increased IGF2 ligand availability and/or function is associated with increased placental volume and labyrinth zone surface area at term; thus, likely improving placental nutrient delivery (Roberts *et al.*, 2001; Sferruzzi-Perri *et al.*, 2007). Despite no changes in placental nutrient transporter expression (Chapter 4), the increase in p-mTOR (2448), hence mTORC1, suggests that these F2 male associated placentae are programmed for a nutrient deficient environment and the upregulated mTORC1 is likely an adaptation to enhance nutrient transport efficiency. Furthermore, despite no changes in placental region cross-sectional areas (Chapter 3), the reduced blood space cross-sectional area (and consequent increase in labyrinth tissue space) reported in Chapter 5 additionally suggests that the placenta is attempting to enhance nutrient transportation (**Figure 7.1**). This highlights that these F2 male placentae of growth restricted females are programmed to respond in a similar way their F1 mother did *in utero*, which is likely aimed at ensuring appropriate growth and development in defence of an aberrant maternal environment. However, these changes were not observed in F2 female associated placenta, highlighting that they are able to withstand additional pregnancy challenges (**Figure 7.1**). Interestingly, F2 fetal and placental weights were not influenced by F1 maternal growth restriction, which is likely attributed to the lack of changes in IGF ligands (Chapter 3) and stress-responsive (Chapter 6) pathways; a finding that is consistent with previous studies from our laboratory (Gallo *et al.*, 2012c; Briffa *et al.*, 2017). Whilst these placental changes may be beneficial in the short-term, they are likely responsible for the programming of poor cardiometabolic and renal outcomes in adult F2 male offspring and may explain why the F2 females do not develop organ deficits or disease. Ludwig *et al.*

(1996) demonstrated in *Igf2r*$^{-/-}$ mice that despite the fetal overgrowth and increased fetal IGF2 concentrations they present with organomegaly (thickening of the left ventricle without cardiomyocyte hypertrophy and cavity dilation) and are not viable after birth. However, it is important to note that their model was a whole-body *Igfr2* knockout and not specific to the placenta, which is required to clearly delineate the role the placenta plays in this altered organ development. Our laboratory has previously demonstrated that F2 offspring of growth restricted dams have a nephron deficit *in utero* that is resolved by weaning (PN35) (Gallo *et al.*, 2013) and F2 males have left ventricular hypertrophy (Master *et al.*, 2014) and develop cardiometabolic disease (Gallo *et al.*, 2013; Cheong *et al.*, 2016b). Importantly, given the role of IGF2/IGF2R in the development of many organ systems, these F2 male phenotypes may be linked to *i)* increased fetal exposure to IGF2 that may modulate organ set-points and/or development, and/or *ii)* placental-specific programming of deregulated IGF2R in target tissues, such as the heart and pancreas (Casellas *et al.*, 2015); however this association requires further investigation.

7.3.2 Effects of maternal growth restriction on F2 placental adaptations in high-fat fed dams

As discussed previously (**Section 1.4.2**), unlike their male counterparts, F1 females born small only develop glucose intolerance during pregnancy (Gallo *et al.*, 2012b). This highlights that a "second-hit", such as pregnancy or maternal obesity, is required to unmask disease in F1 females, which will likely influence fetal growth and development due to increased maternal and fetal glucose concentrations (Briffa *et al.*, 2017). In line with the theory, this thesis clearly demonstrates that many of the changes reported in High-fat fed *Restricted* dams were not observed in Chow-fed *Restricted* dams. Specifically, maternal high-fat feeding in *Restricted* dams upregulated placental amino acid transporter and *Mtor* gene abundances in male placentae (Chapter 4); a finding of which is consistent with other animal studies (Jones *et al.*, 2009). Interestingly, within these same groups, an increase in the IGF (*Igf2*, *Let-7f* miRNA) and angiogenic (*Vegfa*, *Flt-1*; Chapter 5) systems were also observed. These data suggest that, during late gestation, male associated placentae from high-fat fed *Restricted* dams attempt to adapt to the adverse *in utero* environment by increasing its nutrient transfer capability by *i)* upregulating placental nutrient transporter and growth factor pathways, and/or *ii)* potentially attempting to enhance the placental vascular network to increase area available for nutrient exchange (**Figure 7.1**); although blood space area was not altered. It is also probable that the epigenetic regulation of gene expression pathways may be implicated by the activity of post transcriptional regulators, such as *Let-7f* miRNA, which can be linked to the reduced *Igf1*

abundance. The reduction in *Igf1* abundance indicates that a poor maternal diet acts as a further insult to the already detrimental programmed effects of F1 maternal growth restriction. Therefore, alterations in epigenetic pathways associated with fetal growth and development may program worse cardiometabolic dysfunction and obesity in F2 male offspring of growth restricted mothers.

Similarly, to the F2 males, F2 females also had more over changes in their growth restricted mother consumed a high-fat diet. Specifically, F2 female associated placentae of High-fat *Restricted* dams had increases in the IGF (*Igf2*; Chapter 3), nutrient transportation (*Glut3, Mtor*, *Snat1*, *Snat3*; Chapter 4), and angiogenic (*Vegfa*, *Flt-1*; Chapter 5) systems with a reduction in *Snat4* (Chapter 4) and no changes in stress-responsive genes (Chapter 6). Similarly, to their male counterparts, these data suggest that the late gestation female placentae from high-fat fed *Restricted* dams increases nutrient transport capacity in order to match the increased nutrient availability and, thus, potentially enhancing fetal growth and development (**Figure 7.1**). However, whether the changes in the IGF-system, nutrient transport and angiogenic pathways is detrimental to metabolic health in F2 females from high-fat fed growth restricted mothers is yet to be elucidated.

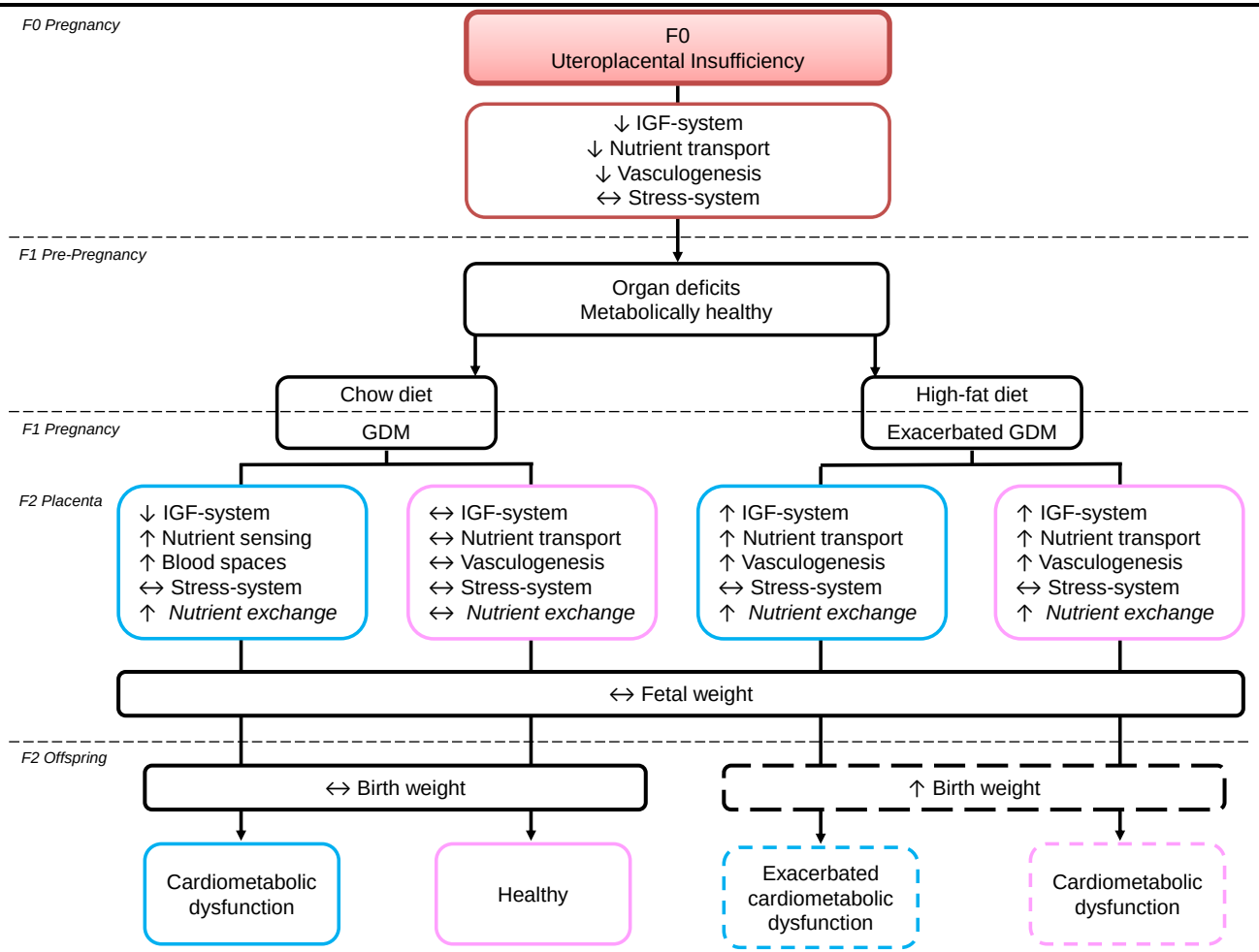

Figure 7. 1 The effects of F1 maternal growth restriction on F2 fetoplacental outcomes.

Maternal growth restriction in first generation (F1) Chow-fed dams deregulated the F2 placental insulin-like growth factor (IGF), nutrient transporter, and vascularisation systems in males, without changes in the placental stress-system or fetal weight at embryonic day (E) 20, which may program the male-onset F2 cardiometabolic disease. Maternal high-fat diet upregulated the IGF, nutrient transporter, and vascularisation systems, which may result in the exacerbation and emergence of F2 cardiometabolic dysfunction in male and female offspring, respectively. Blue outlined boxes denote male data and pink outlines boxes denote female data. Solid boxes = reported effects, dashed boxes = hypothesised links. GDM – F1 gestational diabetes mellitus characterised by Mahizir *et al.* (2020).

7.4 Effects of Maternal High-fat Feeding on F2 Placental Adaptations

7.4.1 Effects of high-fat feeding on F2 placental adaptations

Maternal overweight and obesity are typically associated with fetal macrosomia (Ovesen *et al.*, 2011), which is likely, in part, due to placental alterations in nutrient partitioning, nutrient and metabolic signalling (increased IGF2 and mTORC1 signalling), and morphology (labyrinth surface area reduced by 25%) (Sferruzzi-Perri *et al.*, 2013). In this thesis, it is reported that maternal high-fat feeding did not alter fetal weight (Chapter 3); a finding of which is not too surprising as previous human and animal studies have demonstrated that maternal obesity does not always result in fetal macrosomia (Radulescu *et al.*, 2013). Surprisingly, maternal high-fat feeding led to very few changes in gene and/or protein expression (Chapters 3, 4, and 6; **Figure 7.2**), which may be due to predictive adaptive programming within the high-fat diet model used in this thesis as maternal body weight, thus adiposity, was increased (12%) prior to mating. Although predictive adaptative programming is usually discussed in the context of reduced nutrient availability, Sasson and colleagues suggest this is highly likely to also occur in conditions of excess nutrient availability, such as maternal obesity (Sasson *et al.*, 2015). Specifically, the pregestational exposure of oocytes to a high-fat diet may play an implicit role in programming adaptation mechanisms of the oocyte, making them well prepared for the antenatal stress of fetal life in an *in utero* high-fat environment. Thus, the resultant conceptus and placentae most likely adapted to the maternal environment of nutrient excess. Similarly, to other models of fetal programming these adaptations, although advantageous for fetal survival *in utero*, are likely to result in unfavourable outcomes in adult life, predisposing these offspring to adult onset cardiorenal and metabolic dysfunction (Gallo *et al.*, 2012c).

Despite the limited changes due to maternal high-fat feeding in other placental pathways investigated (Catalano & Ehrenberg, 2006), Chapter 5 reports that the placental angiogenesis and/or vasculogenesis system is impacted in a sex-specific manner. Specifically, maternal high-fat feeding increased VEGFA and PLGF in male associated placentae, which may be in attempt to enhance placental vascularisation and increase nutrient transportation to optimize birth weight (**Figure 7.2**). Whereas, in female associated placentae, VEGFA and FLT-1 expression were reduced (Chapter 5). It is likely that female associated placentae adapted to increased maternal nutrition during early placentation to allow appropriate nutrient transfer during important developmental stages, with placental angiogenic and vasculogenic factor expression reducing with advancing gestation to either reduce fetal overgrowth or because they have reached their optimal adaptive capacity (**Figure 7.2**). These changes in female associated

placentae in angiogenesis and/or vascularisation are likely an indication of increased markers of oxidative stress associated with maternal obesity, as hypoxia is a signal for VEGF production. Specifically, placental hypoxia and altered placental vascularisation have been linked with obesity in humans (Salvolini *et al.*, 2019) and rodents (Stuart *et al.*, 2018), with reduced neonatal survival (Wang *et al.*, 2007; Hayes *et al.*, 2012; Ho & Fong, 2015; Sargent *et al.*, 2015; Chau *et al.*, 2017). In preeclamptic pregnancies both placental and circulating VEGFA and PLGF are downregulated (Cho *et al.*, 2003; Rana *et al.*, 2014), thus the observation in female associated placenta could also be an indicator of aberrant placental vascularisation with advancing gestation in high-fat fed mothers.

7.4.2 Impact of high-fat feeding on F2 placental adaptations in F1 growth restricted mothers

As growth restricted individuals are at an increased predisposition to developing obesity, it is likely that obese growth restricted females are at an even greater risk of a complicated pregnancy (including more severe GDM); a finding of which is consistent with a recent paper from our laboratory (Mahizir *et al.*, 2020). Importantly, it is likely that maternal obesity in growth restricted women is further detrimental to F2 fetoplacental outcomes due to increased nutrient availability. To my surprise, while numerous changes in the IGF-system, nutrient transportation, and angiogenesis systems were reported in High-fat fed *Restricted* dams that were not present in chow-fed counterparts (**Section 7.3.2**), maternal high-fat feeding did not exacerbate any changes in *Restricted* dams. This suggests that maternal high-fat feeding elicits specific changes in F2 fetuses, which is not additive to the initial insult of the perceived poor *in utero* environment of growth restricted mothers. Specifically, it is likely that predictive adaptive programming prepared the oocytes for the stress of fetal life in an *in utero* high-fat environment in mothers who are predisposed to poor metabolic health in pregnancy. If the high-fat feeding insult occurred during pregnancy it is, therefore, likely more overt changes in F2 fetoplacental outcomes would have been observed in growth restricted mothers.

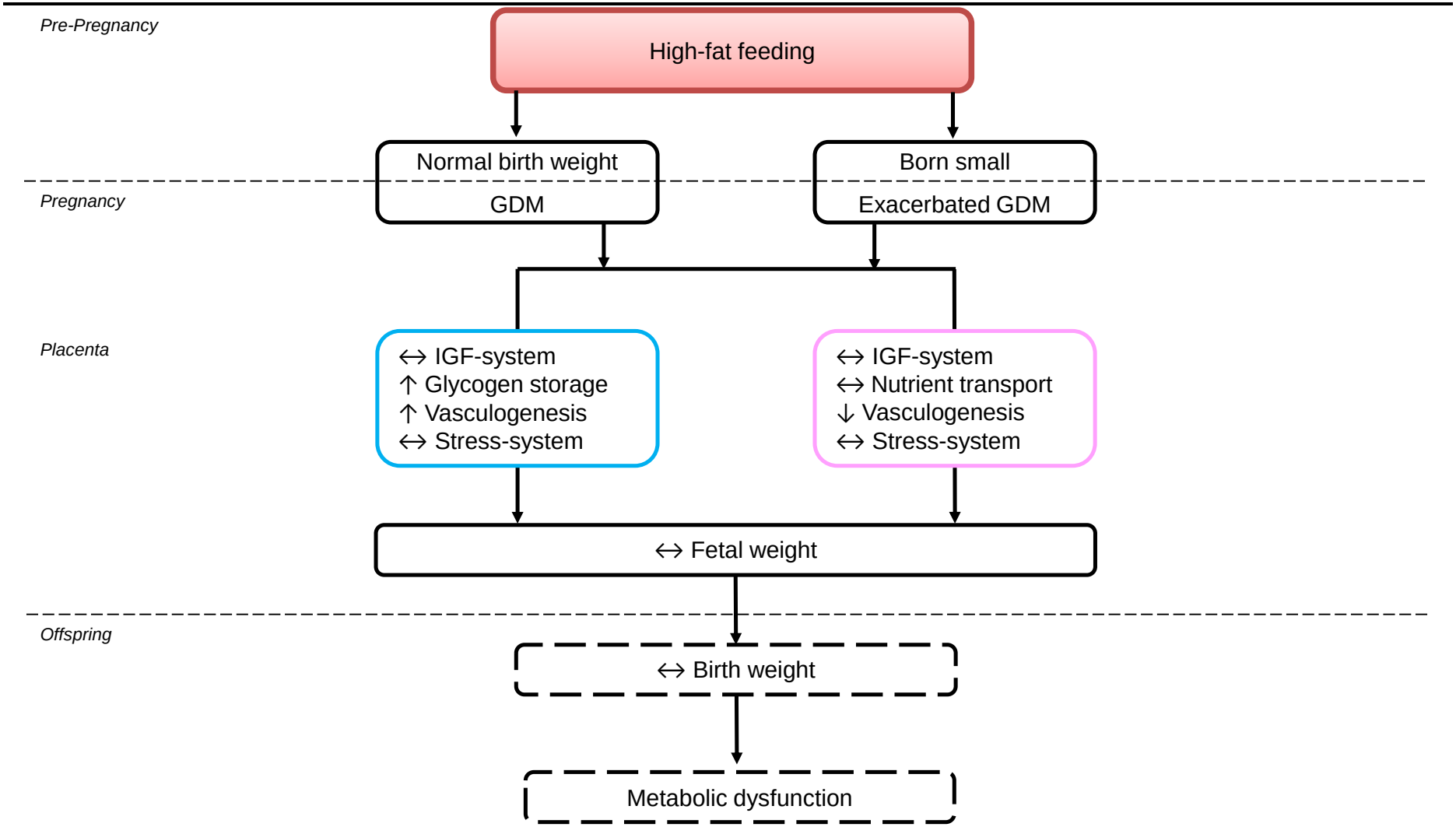

Figure 7. 2 The effects of maternal high-fat feeding on fetoplacental outcomes.

Maternal high-fat feeding altered placental vasculogenesis without alterations in embryonic day (E) 20 placental or fetal weight outcomes, despite resulting in the emergence and exacerbation of gestational diabetes mellitus (GDM) in normal and growth restricted dams (Mahizir *et al.*, 2020). Blue outlined boxes denote male data and pink outlines boxes denote female data. Solid boxes = reported effects, dashed boxes = hypothesised links. IGF- insulin-like growth factor.

7.5 Effects of Lifestyle Interventions on F2 Placental Adaptations

It is well known that maternal growth restriction and obesity have long-term negative health implications for their children. This highlights the need for simple lifestyle interventions to enhance fetal growth and development, ultimately resulting in healthy mothers and children. Previous research has indeed demonstrated the benefits of a maternal essential nutrient diet on maternal and fetal outcomes (Abu-Saad & Fraser, 2010). However, given that many pregnant women are not likely to comply with dietary modifications it is important to consider the role other simple lifestyle interventions, such as moderate intensity exercise, have on maternal health, which may prevent the multigenerational programming of maternal growth restriction and obesity. While exercise initiation prior to pregnancy is likely to have the most beneficial effects on overall maternal health by reducing the risk of pregnancy complications, such as GDM, it is important to consider that this may not always be practical as ~50% of pregnancies in Australian women are unplanned (Shelton *et al.*, 2018) and pregnancy confirmation is often not known until after the first trimester, once the placenta is already established. This highlights the need to study different onsets of exercise initiation (before and/or during pregnancy) on fetoplacental outcomes.

Despite the known benefits of exercise during pregnancy on maternal health, one of the primary challenges for its effectiveness in today's society is the increasingly sedentary lifestyle and a decreased compliance to physical activity interventions/guidelines, particularly in women of reproductive age. Indeed, analysis from the American National Health and Nutrition Examination Survey (NHANES) in 2007-2016 by Amstrong and colleagues identified that young women are less likely to exercise following high school due to numerous factors such as reduced accessibility, affordability, and increased gender biased societal factors (Armstrong *et al.*, 2018). In Australia, initiatives such as the '*Find your 30*' encourages adults to find practical ways of exercising for at least 30 mins a day with a focus on improving mental, brain, cardiovascular and respiratory health (AIS, 2020); however, there is no mention of the benefits of regular exercise on reproductive health. This is especially surprising as research has well demonstrated that the periconceptional environment is essential for pregnancy progression as well as maternal and fetal outcomes. Specifically, prenatal supplements, such as folic acid, are supported by the world health organisation and the RANZCOG women's health committee to be taken by women of childbearing age to improve the periconceptional environment and reduce the incidence of adverse pregnancy adaptation and/or poor fetal outcomes (WCH, 2015; WHO, 2016). This highlights the need for the public health message to include the importance

and benefits of periconceptional exercise in improving pregnancy and fetal outcomes to increase physical activity/exercise compliance in young women of reproductive age.

7.5.1 Effects of F1 maternal exercise prior to and throughout pregnancy on F2 placental adaptations

Endurance exercise increases metabolic demand as it is associated with increased lean mass, which consequently alters insulin and glucose handling (Richter *et al.*, 1982). However, the impact maternal exercise has on fetal metabolic health as well as nutrient availability is largely unknown. Similarly, to what is observed in humans, Chapter 3 demonstrates that maternal physical activity initiated before and continued during pregnancy (*Exercise*) increased fetal weight (Clapp *et al.*, 2002). There is, however, limited research prior to this thesis that investigated the potential placental mechanism/s associated with this enhanced fetal growth. Interestingly, the increased fetal weight with *Exercise* was only evident in F2 fetuses from Chow-fed dams, which coincided increased fetal plasma IGF1 concentrations (*Control* dams only) as well as IGF ligand expression (Chapter 3; **Figure 7.3**), which is consistent with previous studies in humans (Bauer *et al.*, 1998; Lindsay *et al.*, 2007). This finding is not too surprising as more energy is required to fuel the increased fetal mass and IGF signalling is essential to ensure optimal nutrient availability for fetal growth. Although male associated placentae had coinciding increases in amino acid transporters, the expression of glucose transporters (Chapter 4), angiogenesis markers (Chapter 5) and placental labyrinth zone area (Chapter 3) were unaltered (**Figure 7.3**). This, somewhat unexpected finding, is likely due to placentation occurring in a maternal metabolically demanding environment, which resulted in early angiogenic and/or vasculogenic adaptations driving increased placental efficiency and nutrient transportation. Similarly, to maternal high-fat feeding, these early placental adaptations are likely due predictive adaptive programming as the oocyte was programmed for a metabolically demanding *in utero* environment resulting in significant adaptive changes during placentation to facilitate normal fetoplacental outcomes. Indeed, numerous human and animal studies have identified that the increased fetal weight following maternal exercise is likely beneficial as the children have decreased adiposity with increased lean muscle mass (Clapp & Capeless, 1990) and a reduced risk of chronic disease (May *et al.*, 2010; May *et al.*, 2012; May *et al.*, 2014; Moyer *et al.*, 2016).

While *Exercise* in chow-fed dams increased fetal growth, likely via placental IGF signalling, these changes were not observed in high-fat fed dams. Specifically, *Exercise* did not alter fetal

or placental weight and largely did not alter gene abundance (**Figure 7.3**). This is in contrast to a recent study by Son *et al.* (2019) that reported that maternal *Exercise* in mice improved placental vascularization via increased *Vegfa* and *Vegfr1* abundances and improved mTORC1 signalling (Son *et al.*, 2019). However, the exercise duration used (1 hr.day^{-1}) throughout their study was high compared to the exercise regime used in this thesis that gradually decreased with advancing gestation (50 min.day^{-1} for week 1; 30 min.day^{-1} for week 2; 20 min.day^{-1}) with 2 rest day.wk^{-1} and the exercise prior to pregnancy in the study by Son *et al.* (2019) was purely for training purposed (*i.e.*, 10 m.min^{-1}.day^{-1} for 3 mins). In addition, the study by Son *et al.* (2019) changed the high-fat diets to a lesser amount of energy from fat (60% kcal to 45% kcal) one week prior to mating, which may independently alter fetal outcomes. Given their study utilised a more intensive exercise regime compared to the one implemented in this thesis and the onset occurred from conception, their increased placental vascularisation and, likely, enhance nutrient transportation via mTORC1 is due to the high demanding maternal and *in utero* environments. Whereas the gradual reduction in exercise intensity used in this thesis would require additional late gestational adaptions to facilitate enhanced nutrient transfer for optimal fetal growth and development. The lack of changes in fetal weight with maternal high-fat feeding and minimal gene changes may, however, not be detrimental to offspring outcomes as a previous study has reported improved offspring metabolic health as glucose handling was improved despite the absence of fetal weight differences (Vega *et al.*, 2015).

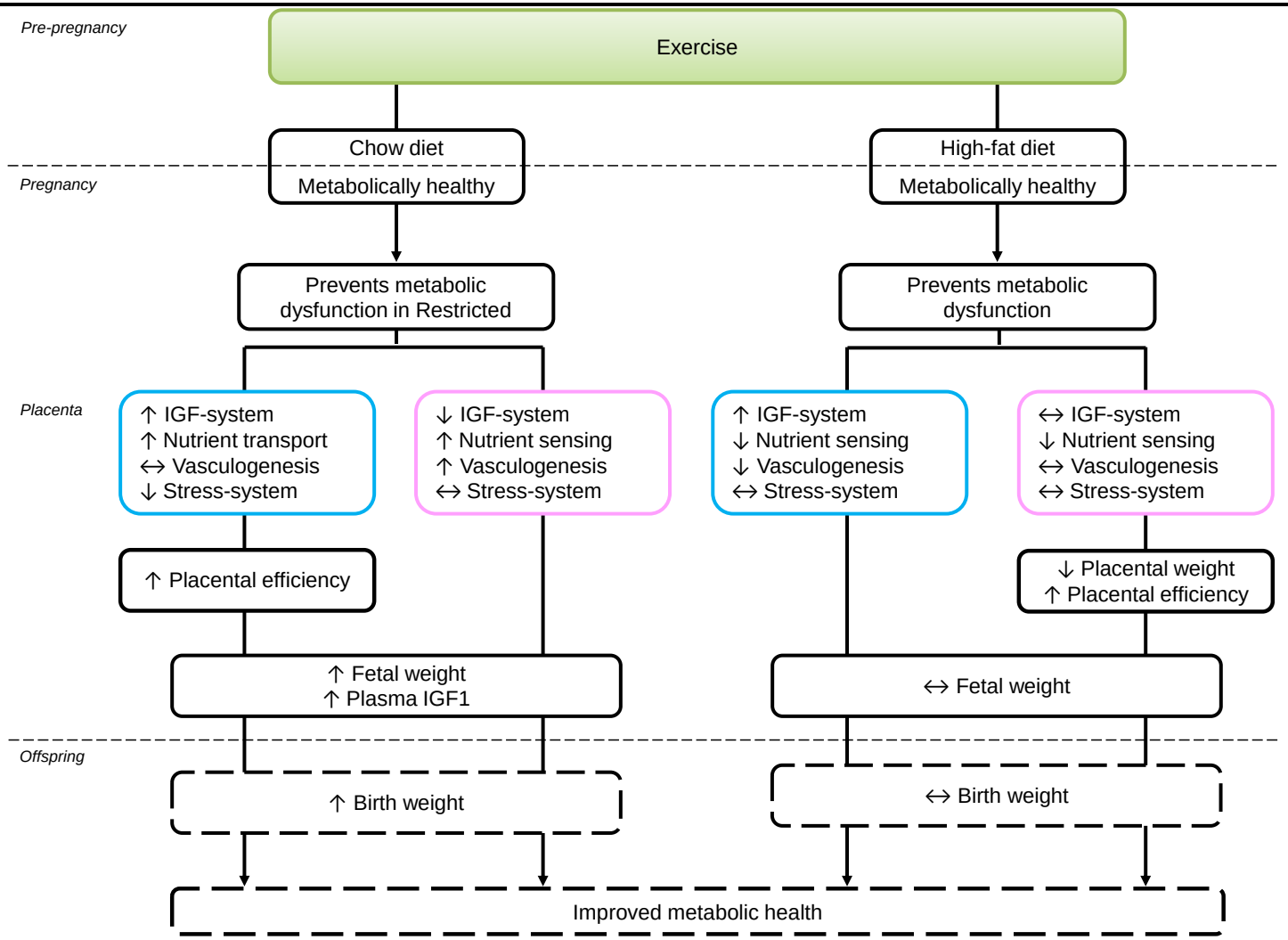

Figure 7. 3 The effects of maternal exercise prior to and throughout pregnancy on fetoplacental outcomes.

Exercise improved maternal metabolic health (Mahizir *et al.*, 2020) and increases placental efficiency in a sex specific manner depending on maternal diet. Importantly, *Exercise* in Chow-fed dams increased placental and fetal IGF1, which may drive the increased fetal weight at embryonic day (E) 20. Blue outlined boxes denote male data and pink outlines boxes denote female data. Solid boxes = reported effects, dashed boxes = hypothesised links. IGF- insulin-like growth factor.

7.5.1.1 Impact of *Exercise* in F1 growth restricted dams on F2 placental adaptations

Our laboratory recently demonstrated the benefits of maternal *Exercise* in F1 *Restricted* dams, where it prevented the development and exacerbation of glucose intolerance (in Chow and High-fat diet dams, respectively) (Mahizir *et al.*, 2020). Given that maternal metabolic health was restored, likely by exerting beneficial effects prior to pregnancy, it is not surprising changes observed were conserved in F2 fetuses of normal and growth restricted mothers. The only change that occurred in F2 placentae of high-fat fed *Restricted* dams that did not occur in normal birth weight counterparts was an increase in *Snat1* and *Snat2* gene abundances. This suggests that the combination of *Exercise* improving the maternal metabolic system in high-fat fed mothers and the pre-programmed adaptations in the F2 placentae results in an upregulated SNAT expression, which is likely aimed at fully utilising the increased nutrient supply and, thus, securing an appropriate F2 fetal weight.

7.5.2 Effects of F1 maternal exercise only during pregnancy on F2 placental adaptations

Previous studies in humans by Clapp and colleagues have clearly demonstrated that pregnancy and fetal programming outcomes are dependent on the timing of exercise initiation as well as exercise type and intensity (Clapp & Rizk, 1992; Clapp & Little, 1995; Clapp & Kiess, 2000; Clapp *et al.*, 2000; Clapp *et al.*, 2002); however, the mechanisms responsible for any programming adaptations are poorly defined. This likely, in part, explains why more profound placental changes were observed following *PregEx*, compared to *Exercise*, as exercise was initiated during a period of exponential placental growth and vascular bed development. Critically, for this thesis, the rodent placenta begins to form during the second week of gestation (E7-14) in a primarily hypoxic environment (Jaffe *et al.*, 1997; Aplin, 2000), whereby uninterrupted placental processes prior to E13.5 would result in appropriate placental growth and development. It is, therefore, possible that when initiating exercise during pregnancy that maternal blood flow during exercise bouts may be preferentially redirected to maternal peripheral organs over the placenta, at least at the start of exercise initiation, resulting in an acute and severe hypoxic environment for trophoblast differentiation and expansion by further reducing placental oxygen perfusion and, potentially, reducing nutrient availability/transportation. This thesis clearly demonstrates that the placenta responds to this hypoxic environment by triggering adaptations to stimulate vasculogenic and/or angiogenic as well as nutrient and growth factor pathways via mTOR regulation, which are likely adaptations to enhance fetal oxygen and nutrient delivery in a high energy demanding maternal environment (**Figure 7.4**). Specifically, Chapter 3 demonstrated that *PregEx* increased

placental efficacy and upregulated IGF1 and IGF1R expression, highlighting that the placenta is attempting to optimise fetal growth and nutrient delivery during sudden shifts in nutrient supply. Although IGF1 is a known regulator of mTOR, the increased placental IGF1 was unable to increase mTOR or MTORC1 expression (Chapter 4), which further suggests nutrient transportation capacity is impaired with *PregEx* (**Figure 7.4**); highlighting that the hypoxic environment and, potentially, low maternal energy is overriding the IGF1-stimulated benefits on mTOR expression. This maternal hypoxic and low energy environment is also causing some degree of placental and fetal stress as placental *Hsd11b2* gene abundance was reduced (Chapter 6), highlighting that there is likely increased corticosterone being transported to the fetus. Thus, it is also not surprising that the MR and *Crhr1* expression is reduced, similar to what Cuffe et al 2014 presented in their hypoxic model (Cuffe *et al.*, 2014). The reduction in receptor gene expression in placental tissue suggests an adaptation to reduce glucocorticoid influence on placental tissue; however, the impact this may have fetal tissue is yet to be elucidate. It is interesting to note, however, that the male and female associated placentae respond differently to this perceived decreased nutrient transfer capacity. Specifically, male associated placentae increase placental and labyrinth cross-sectional areas (Chapter 3) and decrease placental blood space area (inversely associated with tissue spaces; Chapter 5); highlighting that male placenta adapt to this nutrient transportation deficit caused by *PregEx* by increasing the tissue area available (labyrinth size and increasing vascular branching via increased VEGFA) for nutrient transportation to maintain adequate fetal growth in an adverse *in utero* environment (**Figure 7.4**). In females, however, these changes in placental morphology were not observed, which highlights that they have adapted to the adverse *in utero* environment by altering their growth trajectory to allow for normal organ growth and development to occur (**Figure 7.4**), a finding of which is consistent with numerous programming models. Nevertheless, these lack of changes in female associated placentae are in contrast to what is observed in mid-gestation chronic hypoxia (12% oxygen), which results in reduced *Igf2* abundance and labyrinth cross-sectional area only in females (Cuffe *et al.*, 2014); highlighting that *PregEx* likely induces an acute, but not chronic, hypoxic environment. However, as previous studies indicate that aberrant mTOR signalling as well as male fetuses failure to adapt to pregnancy complications facilitates the programming of obesity and cardiometabolic diseases (Fernandez-Twinn *et al.*, 2012) it is possible that *PregEx* may program male onset adult disease, which required further investigation.

While most of the changes in growth-factor (Chapter 3) and nutrient transporters (Chapter 4) were similarly observed in placentae of *PregEx* dams fed a high-fat diet, there were some notable differences especially in morphological outcomes. Specifically, female associated placentae have increased placental and labyrinth zone cross-sectional areas, which was not characterised in male fetuses. This suggests that the female associate placentae are attempting to redirect nutrient resources to promote normal fetal growth and development in a maternal environment that is abundant in nutrients, especially to overcome reduced amino acid transport (**Figure 7.4**). Whereas, in male associated placentae only junctional zone cross-sectional area was increased, which coincided with increased glycogen cell accumulation. This highlights that the male placenta is likely facilitating increased nutrient transportation, despite reduced amino acid transporter abundance (Chapter 4), by increasing placental vascular branching (Chapter 5) and is storing excess maternal nutrients in the form of glycogen (Chapter 4; **Figure 7.4**), which may be an adaptation in case any further *in utero* insults occur that may further compromise fetal growth and development.

7.5.2.1 Impact of *PregEx* in F1 growth restricted dams on F2 placental adaptations

Although *PregEx* resulted in profound alterations in the placental outcomes measured in this thesis, to my surprise the changes were similarly conserved between placentae of normal and small birth weight mothers. This suggests that placentae of F2 fetuses from growth restricted mothers are pre-programmed to adapt to an adverse *in utero* environment similar to that experiences by their F1 *Restricted* dam and that they respond to novel maternal environmental stimuli in the same manner as women with a normal birth weight.

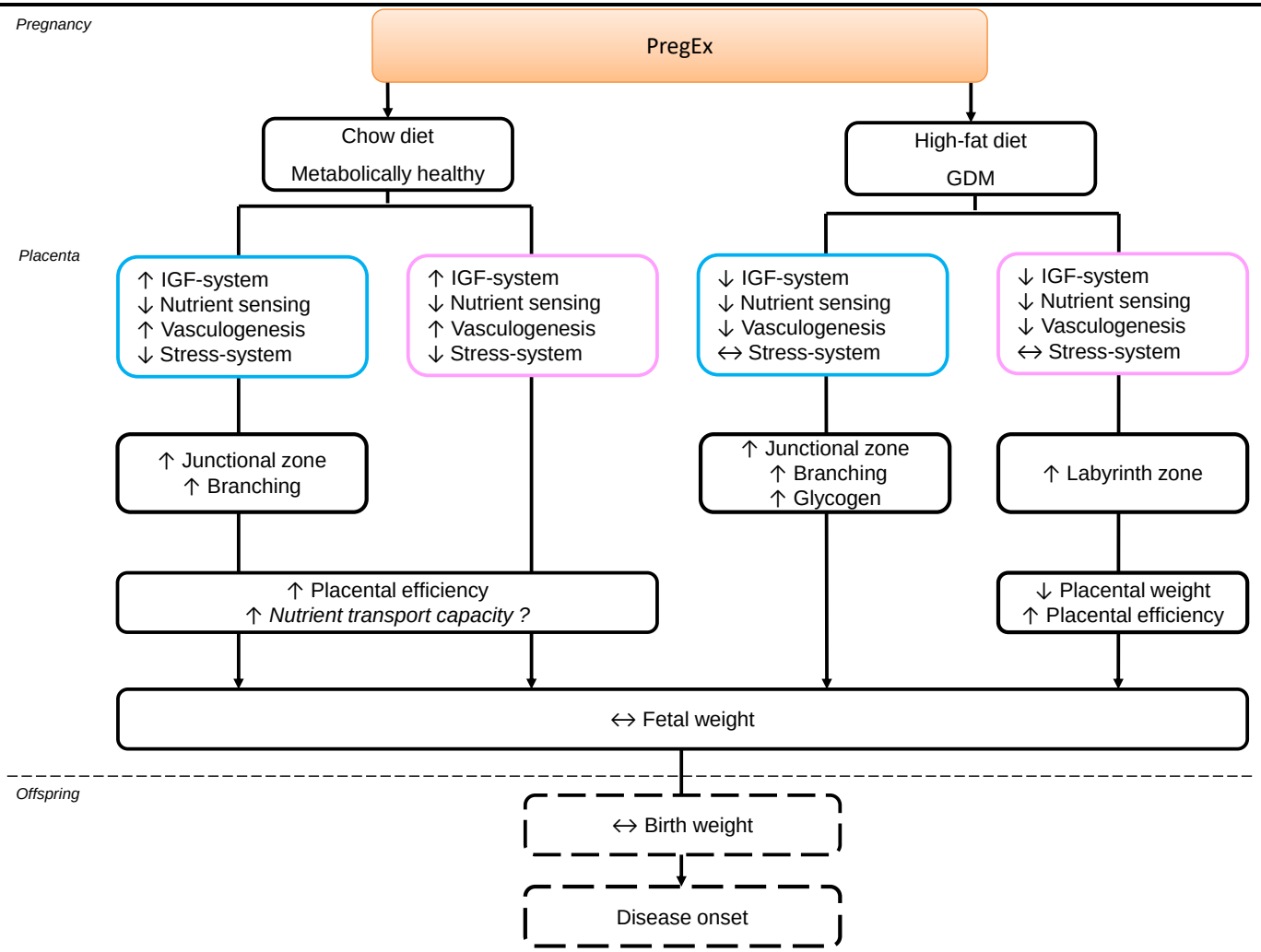

Figure 7. 4 The effects of maternal exercise only during pregnancy on fetoplacental outcomes.

PregEx dysregulates placental gene and protein expression regardless of maternal birth weight and diet. Importantly, placental vascular remodelling occurs without changes to fetal weight at embryonic day (E) 20. Placentae demonstrate late adaptations to maternal insults introduced from the second week of gestation. Blue outlined boxes denote male data and pink outlines boxes denote female data. Solid boxes = reported effects, dashed boxes = hypothesised links. F1 GDM – gestational diabetes mellitus characterised by Mahizir *et al.* (2020); IGF- insulin-like growth factor.

7.6 Future Directions

The overall aim of this thesis was to identify the impact additional maternal insults have on the F2 placentae of F1 *Restricted* dams. While this thesis clearly demonstrates that *PregEx* results in profound changes in the placental IGF-system, nutrient sensing, and angiogenesis pathways, the impact that these changes have on fetal and long-term postnatal outcomes are yet to be explored. Specifically, the absence of fetal weight differences suggests that the placental changes are either *i)* not adequate to influence changes in fetal weight at E20, and/or *ii)* are remnants of mechanisms from earlier in gestation, such as exercise initiation within the second week of gestation. In order to unravel this question, additional studies are required at different gestational timepoints throughout placentation, late gestation, and birth to identify the impact these maternal insults have on placental growth, development, and function as well as fetal growth and development. However, the large number of experimental groups (12 dam groups) and animals (10 litters/dam group) required, limits the potential to perform these studies and highlights that key targeted studies should be conducted on specific groups to answer the main questions raised by this thesis.

A limitation of the current thesis was the time required to generate the animals required for the study as it is transgenerational in nature (~3 years) as well as the time to process and analyse the placenta in order to explore the various aims of this thesis. As a consequence of this, as well as the lack of EchoMRI availability in the animal facility, I was unable to characterise fetal body and placental composition (lean and fat masses). Indeed, recent studies have demonstrated that maternal exercise improves skeletal glucose handling and insulin sensitivity, which may be associated lean and fat mass at birth (Falcao-Tebas *et al.*, 2020). Given the link between maternal exercise and metabolic health, it would further highlight the benefit of *Exercise* in growth restricted and high-fat fed dams as it restores maternal metabolic health (Mahizir *et al.*, 2020), which may have beneficial effects on short and long-term F2 offspring metabolic health by improving fetal body composition as well as placental outcomes (morphology and gene/protein) and plasma metabolites/hormones. Furthermore, other in-depth fetal analysis via ultrasonographic technology and pulsed-waved colour Doppler sonography may provide invaluable insight of the rate of the growing fetus as well as fetal heart rate and placental/fetal oxygen flow in response to maternal diet, exercise, or stress (Ypsilantis *et al.*, 2009; Szymanski & Satin, 2012; Skow *et al.*, 2019). However, the repeated use of anaesthetic in pregnant dams would compromise maternal and fetal adaptations to pregnancy and may independently influence outcomes.

The absence of significant high-fat diet effects could be attributed to a number of factors including the amount of energy provided by the main energy source within the diet (43% kcal from fat), animal species, as well as the duration of diet exposure. As maternal weight increased by only 12% with high-fat feeding, the dams cannot be considered as obese but overweight, despite 15 weeks of dietary intervention, which is likely why only marginal changes in placental and fetal weight were reported. Indeed, a recent systematic review by Christians *et al*. (2019) demonstrated that the percentage of fat within rodent diets used in obesity modelling is important as this affects fetal weight outcomes, whereby in the majority of studies a 60% fat diet in rats does not affect fetal growth. On the other hand, rats fed a 45% fat diet reduces fetal and birth weight, which is in stark contrast to the findings of this thesis (Chapter 3) where fetal weight was unaltered. As protein and carbohydrate macronutrients as well as micronutrients were matched in the diets used in this thesis it is not surprising that fetal outcomes were not profoundly changed as the ratio of protein and carbohydrate intake is still optimal to influence a relatively healthy growth trajectory. Furthermore, these outcomes where also confounded by species as rats and mice responded differently to the same concentration of fat. This highlights that the choice of diet as well as its impact on fetal outcomes are important considerations to be made when modelling different human conditions. As Western diets usually have a disproportionate amount of carbohydrate and fat content (Zhang *et al.*, 2006)it is, therefore, likely that a Western diet would have been a better option for modelling obesity in Western populations for this thesis. Therefore, future studies should endeavour to consider the ratio macronutrients and not just fat content when studying the effects of diet induced obesity.

Despite the inadequacy of the HFD used in inducing maternal obesity, F2 placental gene and protein expression was differentially deregulated in response to the maternal diet. Previous research has demonstrated that fatty acid metabolism is altered in IUGR placenta (Chassen *et al.*, 2018). Therefore, the analysis of placental lipid transportation via *in vitro* trophoblast experiments lipid distribution (Brown *et al.*, 2016), metabolism (Easton & Regnault, 2020) and accumulation may, in future, provide an important facet of information that may assist in explaining the nutrient transport and fetal outcomes characterised in the current study. However, due to technological constraints lipidomic placental analysis was not able to be performed and due to the method of placental fixation (10% neutral buffered formalin) oil red o staining was unable to be performed as the ethanol processing dissolves most lipid depots. These would have assisted in characterising placental lipid droplet area, a technique for which invaluable data could have been collected. Furthermore *in vitro* analysis of commercial cell

lines or, importantly, primary cells from the animals used in this thesis may also provide invaluable insight in the expression of lipid transporters as well as the metabolic products produced by the isolated cells from *Restricted* and HFD dams in a controlled environment.

The growth factor pathway, in particular, has been a key target of interest in placental research as studies have demonstrated reduced expression of IGF1 and IGF1R in fetal growth restriction (Leger *et al.*, 1996; Crossey *et al.*, 2002; de Vrijer *et al.*, 2006). In this thesis, these two key factors in the IGF-system were downregulated in F2 placentae of maternal F1growth restriction as well as with maternal high-fat feeding. However, in order to fully understand the mechanistic pathways of these growth factors and how they play intrinsic regulatory roles in the propagation of fetal disease programming, via the placenta, required investigation. Studies have already explored the impact knockout and overexpression of this pathway has on the placenta (Constancia *et al.*, 2002; Coan *et al.*, 2008; Sferruzzi-Perri *et al.*, 2011). However, the link between epigenetic regulation, programming, and placental function in response to maternal growth restriction is yet to be explored. Epigenetics alterations including histone modification, DNA methylation and, most importantly, miRNA regulation have been proposed to play important roles in the regulation of placental propagated disease programming. Further exploration of this epigenetic link with the use of F2 placental trophoblast isolation techniques and the concurrent targeting of deregulated and matched miRNA, such as *Let-7f1*, may provide a suitable *ex vivo* landscape to isolate specific pathways that may help clarify the findings from this thesis in response to maternal growth restriction, diet, and exercise.

The rate, volume, and efficiency of placenta nutrient transfer are important outcomes that would provide insight on the metabolic activity of the F2 placenta. Previous studies have already attempted single-perfusion organ baths of the maternal-fetal interface in order to understand the placental transport of drugs and sugars (D'Errico *et al.*, 2019). The placental perfusion method would allow us to measure and assess the F2 placenta transfer, metabolism and alterations that affect maternal-to-fetal nutrient flow during compromised pregnancies. In particular, this would provide robust functional evidence of whether the gene and protein changes characterised in the current thesis actually result in altered transport of glucose, amino acids and/or fatty acids in F2 placentae. This approach would, importantly, highlight any translational changes as a consequence of Exercise or *PregEx* on placental transport.

While this thesis explored the impact maternal growth restriction has on glucose and amino acid nutrient transporter expression, an important factor that was not assessed was mTORC1

signalling due to financial constraints. Analysis of mTORs signalling activity by assessing phosphorylation of its downstream targets, including ribosomal protein S6 (rpS6) , S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Hay & Sonenberg, 2004). These signalling targets are used as functional readouts of MTOR activity and are pro-translational factors involved in the regulation of cell size, proliferation, and nutrient homeostasis through nutrient transporter and receptor upregulation (Hay & Sonenberg, 2004). The characterisation of these well-established downstream regulators of mTOR, could provide more robust intel on the mechanistic regulation of the placental transporter expression in the labyrinth zone in this thesis. mTOR activity is indeed altered in conditions of aberrant nutrient availability, particularly excess nutrient supply as indicated by the increased phosphorylation of rpS6 and 4E-BP1 in placentae from obese pregnant mice (Rosario *et al.*, 2016b). Furthermore, mTOR is a positive regulator of both system A and System L amino acid transporters in the placenta (Rosario *et al.*, 2013). Establishing the status of its downstream targets may have also provided a direct link between mTOR activity and transporter abundance.

The junctional zone of the placenta is the main source of prolactin (PRL) family of hormone/cytokine and placental lactogens (PL)s (Soares, 2004). The deregulation of these hormones is associated with altered placental hormone production such a progesterone in the rodent. Furthermore, evidence indicates that the PRLs modulate maternal pregnancy changes including pancreas biology, immune system as well as brain function (Sorenson & Brelje, 1997; Mann & Bridges, 2001; Yu-Lee, 2002; Shingo *et al.*, 2003). Although the labyrinth is the main site for placental nutrient exchange, the status of the junctional zone associated gene and protein expression was not explored in this thesis due to time and funding limitations. Previous studies report that a maternal obesity inducing diet results in a reduced junctional zone without labyrinth changes (Mark *et al.*, 2012). This reduction in size and weight of this endocrine region is believed to be associated to the fetal growth restriction characterised in the rodent (Mark *et al.*, 2012). Although we do not identify fetal growth restriction with the diet utilized, the characterisation of junctional zone weight, gene and protein expression would provide a clearer picture on the synthesis and secretion of steroid and peptide hormones that may have an impact on maternal adaptations to pregnancy particularly in response to "second-hit" mechanisms such as a maternal HFD in *Restricted* F1 dams. Characterising the junctional zone is likely to also provide the missing link, providing further illumination of the labyrinth nutrient transport changes we characterised as well as growth factor expression. In particular exogenous IGF2 administration has been reported to stimulate growth of the junctional zone

endocrine cells (Van Mieghem *et al.*, 2009) as well as glycogen cells (Esquiliano *et al.*, 2009). Furthermore, Sferruzzi-Perri and team demonstrate that the placental endocrine function and resultant consequences of changed maternal physiology are dependent on *Igf2* (Petry *et al.*, 2010; Sferruzzi-Perri *et al.*, 2011). Thus, it is evident that future studies should characterise junctional zone and labyrinth in unison in order to present the complete placental picture.

As our laboratory has previously reported maternal HFD exacerbated the glucose intolerance in F1 *Restricted* females and revealed glucose intolerance in F1 normal birth weight dams (Mahizir *et al.*, 2020). GDM is associated with increased oxidative stress (Peuchant *et al.*, 2004) and maternal obesity alters oxidant/antioxidant status, specifically resulting in fetal changes in lipid peroxidation, free radical and antioxidant enzymes (Malti *et al.*, 2014). Increased levels of malonyl dialdehyde have been characterised in GDM placenta (Biri *et al.*, 2006). Thus, the characterising of this and other markers of oxidation such as protein carbonyl content, markers of DNA damage such as 8-hydroxy-2′-deoxyguanosine would provide essential information to the extent to which our high-fat feeding may have imparted damage on F2 placenta. This highlights the need to quantify the placental oxidation status as it may provide an additional facet of evidence to the mechanistic processes associated with programming of disease in the F2 generation.

7.6.1 Considerations for clinical applicability

The outcomes of this thesis demonstrate the potential benefits of exercise in complicated rodent pregnancies on placental outcomes. Indeed, the lack of changes in the placental expression of systems of interest, despite increased F2 fetal weight, indicate that *Exercise* may protect the offspring from developing metabolic dysfunction (Cheong *et al.*, 2016b); thus, reducing the transgenerational transmission of non-communicable diseases (NCDs). Previous studies in humans by Clapp and associates indicate that moderate to high intensity exercise during pregnancy is safe for the fetus and has several benefits on maternal health (Hinman *et al.*, 2015). However, there is a lack of rigorous human studies exploring the benefits of maternal exercise on fetoplacental and child health outcomes as well as a lack of consensus on the recommendation of exercise during pregnancy. Given the known benefits of exercise on health in pregnancy and non-pregnancy individual, it is even more concerning that there are limited, if any, studies exploring the benefits of exercise as a periconceptional preventative in women at risk of a complicated pregnancy, such as obese women and individuals born growth restricted. The increased prevalence of NCDs is predicted to impose a great economic burden

on the global economy of an estimated USD $47 trillion by 2030 (Chen *et al.*, 2018). With a cost of such an insurmountable magnitude it is imperative that as many safe and cost-effective measures are taken to reduce the ever-growing burden associated with cardiovascular disease, diabetes, and obesity, particularly in relation to fetal growth restriction. It is, therefore, important that affordable solutions, such as exercise education in adolescence and early adulthood, be presented and prescribed as an effective method to improve maternal and child health outcomes in healthy women as well as those as risk of a complicated pregnancy. Initiatives and programs that present the prescription of exercise of as little as 30 mins per day in young females and males for the benefit of cardiovascular, respiratory, and reproductive health as well as pregnancy outcomes would be of key importance. Such initiatives can be, for example, introduced at a high school level to supplement their basic physical and sexual health education curriculum, which will allow for the importance of exercise to reach beyond just physical health but also reproductive health. The introduction at such an early age is more likely to improve exercise compliance before and during future pregnancies, which will likely result in fewer pregnancy complications as well as healthy mothers and children reducing the burden of NCDs on the Australian economy.

7.7 Concluding Remarks

Findings from this thesis demonstrate that the F2 placenta respond differently to additional maternal insults. Research has well established that IUGR dysregulates placental IGF, nutrient, angiogenesis, and stress pathways that may lead to the programming of sex-specific disease. This thesis described, for the first time, that maternal growth restriction results in similar changes in placental pathways as the observed in IUGR placenta (**Figure 7.5**), suggesting that intergenerational transmission, likely via epigenetic mechanisms, may be responsible for these conserved changes, which may result in the programming of sex-specific F2 disease (Gallo *et al.*, 2012b; Gallo *et al.*, 2013; Cheong *et al.*, 2016b). It is well known that growth restricted individuals are a greater predisposition to develop obesity and that maternal obesity independently influences fetal, placental, and long-term offspring health. Although the F2 placenta responded differently based on the maternal diet, maternal HFD did not exacerbate the placental changes in growth restricted dams. This lack of change is likely due to the HFD used did not induce maternal obesity nor, consequently, an inflammatory environment, which plays a key role in placental outcomes in maternal obesity. The lack of exacerbated changes in high-fat fed growth restricted dams may be due to predictive adaptive programming of the maternal high-fat diet, as it was initiated at weaning (PN35), in the F1 oocyte to modulate fetoplacental growth and development pathways (**Figure 7.5**).

From a clinical perspective, simple lifestyle interventions in women at risk of a complicated pregnancy, such as growth restricted and obese women, are highly desired to improve both maternal and fetal health. This thesis demonstrated that exercise dynamically alters placental nutrient transporters, growth factors, and vasculogenesis regulators in late gestation in a sex-dependent manner. A novel outcome from this thesis is the differential placental outcomes associated with the timing of exercise initiation. Specifically, while this thesis confirmed that exercise initiated before and continued throughout pregnancy increases fetal weight, it resulted in very few placental changes. This lack of change with *Exercise* may be due to the exercise being initiated four weeks prior to pregnancy, which would have resulted in predictive adaptive programming of the oocyte to the high maternal energy demanding environment that required very few changes in placental growth factor, nutrient, and angiogenic pathways to facilitate normal fetal developmental outcomes (**Figure 7.5**). *PregEx*, on the other hand, altered placental but not fetal outcomes and resulted in profound alterations in placental growth factor, nutrient, and angiogenic pathways; changes of which highlight the plasticity of placental mechanisms in adapting to challenging maternal metabolic environments with the aim of

ensuring successful fetal growth (**Figure 7.5**). Importantly, the impact of *Exercise* and *PregEx* were similarly reported in normal and small birth weight dams irrespective of the maternal diet, highlighting indiscriminate benefits on fetoplacental outcomes.

The findings of this thesis have major public health relevance and clinical implications for growth restricted individuals, as the major themes of diet and lifestyle have varying effects on maternal health outcomes, which are known to influence both fetal and placental outcomes. Therefore, it is important to target those at risk of a complicated pregnancy to prevent disease onset and improve overall maternal and fetal health by restoring placental function. Given that growth restriction and obesity are major risk factors for developing GDM and preeclampsia it is imperative to develop easily attainable therapies during pregnancy, such as exercise, to improve overall maternal health as well as short and long-term fetal health by improving placental function to reduce the burden of maternal conditions, diabetes, and cardiovascular disease on the healthcare system; diseases of which collectively cost a reported $21.6 billion AUD ($7.7, $3.5 and $10.5 billion, respectively) in 2015-2016 (AIHW, 2019a).

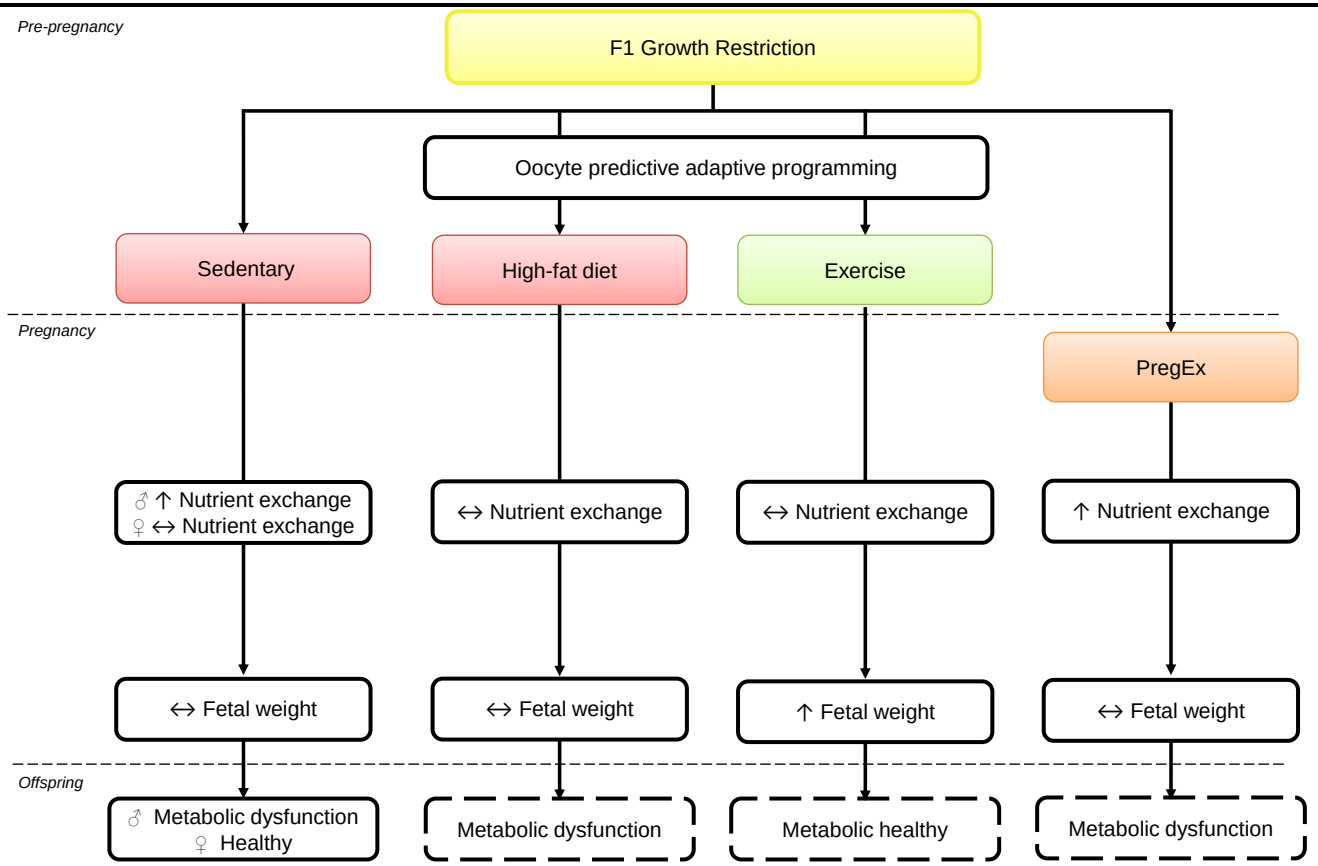

Figure 7. 5 Summary of thesis findings.

Maternal first generation (F1) growth restriction programs altered placental function in anticipation of a depleted maternal nutrient environment. F2 placentae from *Restricted* dams have deregulated gene and protein expression aimed to increase placental nutrient exchange and efficiency, resulting in a normal fetal and birth weight. However, these placental adaptions may be detrimental to postnatal life as F2 males develop metabolic disease as adults. Maternal high-fat feeding did not exacerbate placental dysfunction or alter fetal weight, despite exacerbated maternal metabolic dysfunction. This lack of changes with maternal high-fat feeding may be due to oocyte predictive adaptive programming. Despite very few changes in gene and protein expression, *Exercise* but not *PregEx* increased F2 fetal weight, which may be beneficial to offspring metabolic health; an adaptation that may be due to oocyte predictive adaptive programming. However, *PregEx* resulted in more profound alterations in placental gene and protein expression suggesting that despite the late initiation of exercise the placenta is still plastic allowing changes to occur within itself to optimize fetal growth. However, it is unknown whether these profound changes with *PregEx* would lead to offspring metabolic dysfunction. Solid boxes = reported effects, dashed boxes = hypothesised links.

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Appendix 1

Specialty Feeds

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Diet
AIN93G

Standard AIN93G Rodent Diet

A semi-pure diet formulation for laboratory rats and mice based on AIN-93G. This formulation satisfies the nutritional requirements for growth of rats and mice. Some modifications have been made to the original formulation to suit locally available raw materials.

- We have evidence that vitamin losses and other changes to the diet can occur when irradiated at 25KGy. The diet SF09-091 is formulated with extra vitamins to allow for losses during the irradiation process. Please contact us for more information if the diet is to be irradiated.

Calculated data uses information from typical raw material composition. It could be expected that individual batches of diet will vary from this figure. **Diet post treatment by irradiation or auto clave could change these parameters**. We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.

Calculated Nutritional Parameters as Fed	
Protein	19.4%
Total Fat	7.0%
Total Digestible Carbohydrate as defined by FSANZ Standard 1.2.8	56.8%
Crude Fibre	4.7%
AD Fibre	4.7%
Digestible Energy	16.1 MJ / Kg
% Total calculated digestible energy from lipids	16.0%
% Total calculated digestible energy from protein	21.0%
Ash	4.5%

Diet Form and Features

- Semi pure diet. 12 mm diameter pellets.
- Pack size 5 Kg, vacuum packed in oxygen impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

Appendix 1

Ingredients	
Casein (Acid)	200 g/Kg
Sucrose	100 g/Kg
Canola Oil	70 g/Kg
Cellulose	50 g/Kg
Wheat Starch	404 g/Kg
Dextrinised Starch	132 g/Kg
L Methionine	3.0 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
AIN93 Trace Minerals	1.4 g/Kg
Potassium Citrate	2.5 g/Kg
Potassium Dihydrogen Phosphate	6.9 g/Kg
Potassium Sulphate	1.6 g/Kg
Choline Chloride (75%)	2.5 g/Kg
AIN93 Vitamins	10 g/Kg

Calculated Essential Amino Acids as Fed	
Valine	1.26%
Leucine	1.80%
Isoleucine	0.87%
Threonine	0.79%
Methionine	0.84%
Cysteine	0.05%
Lysine	1.49%
Phenylalanine	0.99%
Tyrosine	1.04%
Tryptophan	0.27%
Histidine	0.60%

Calculated Total Minerals as Fed	
Calcium	0.70%
Phosphorous	0.35%
Magnesium	0.06%
Sodium	0.15%
Chloride	0.16%
Potassium	0.40%
Sulphur	0.23%
Iron	49 mg/Kg
Copper	7.0 mg/Kg
Iodine	0.2 mg/Kg
Manganese	19 mg/Kg
Cobalt	No data
Zinc	46 mg/Kg
Molybdenum	0.15 mg/Kg
Selenium	0.3 mg/Kg
Cadmium	No data
Chromium	1.0 mg/Kg
Fluoride	1.0 mg/Kg
Lithium	0.1 mg/Kg
Boron	3.3 mg/Kg
Nickel	0.5 mg/Kg
Vanadium	0.1 mg/Kg

Calculated Total Vitamins as Fed	
Vitamin A (Retinol)	4 000 IU/Kg
Vitamin D (Cholecalciferol)	1 000 IU/Kg
Vitamin E (a Tocopherol acetate)	78 mg/Kg
Vitamin K (Menadione)	1 mg/Kg
Vitamin C (Ascorbic acid)	None added
Vitamin B1 (Thiamine)	6.1 mg/Kg
Vitamin B2 (Riboflavin)	6.3 mg/Kg
Niacin (Nicotinic acid)	30 mg/Kg
Vitamin B6 (Pryridoxine)	7 mg/Kg
Pantothenic Acid	16.5 mg/Kg
Biotin	200 ug/Kg
Folic Acid	2 mg/Kg
Inositol	None added
Vitamin B12 (Cyancobalamin)	103 ug/Kg
Choline	1 470 mg/Kg

Calculated Fatty Acid Composition as Fed	
Myristic Acid 14:0	trace
Palmitic Acid 16:0	0.30%
Stearic Acid 18:0	0.14%
Palmitoleic Acid 16:1	0.02%
Oleic Acid 18:1	3.89%
Gadoleic Acid 20:1	0.07%
Linoleic Acid 18:2 n6	1.51%
a Linolenic Acid 18:3 n3	0.98%
Arachadonic Acid 20:4 n6	No data
EPA 20:5 n3	No data
DHA 22:6 n3	No data
Total n3	0.98%
Total n6	1.51%
Total Mono Unsaturated Fats	3.98%
Total Polyunsaturated Fats	2.50%
Total Saturated Fats	0.50%

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Diet SF03-020

23% Fat, High Simple Carbohydrate 0.19% Cholesterol Semi-Pure Rodent Diet

A semi-pure diet formulation for laboratory rats and mice based on AIN-93G.

- The fat content has been increased to 23%, sucrose content has been increased to improve pellet strength and starch content has been reduced.
- Cholesterol has been added at 0.19%.
- We have evidence that vitamin losses and other changes to the diet can occur during the irradiation process at 25KGy. Please contact us for more information if the diet is to be irradiated.

Calculated Nutritional Parameters

Protein	19.40%
Total Fat	23.00%
Crude Fibre	4.70%
AD Fibre	4.70%
Digestible Energy	20 MJ / Kg
% Total calculated digestible energy from lipids	43.00%
% Total calculated digestible energy from protein	17.00%

Diet Form and Features

- Semi pure diet. 12 mm diameter pellets.
- Pack size 1.5 Kg, vacuum packed in oxygen- impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave. Note, Irradiation can soften pellets.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

Ingredients

Casein (Acid)	200 g/Kg
Sucrose	424g/Kg
Canola Oil	50 g/Kg
Cocoa Butter	50 g/Kg
Hydrogenated Vegetable Oil (Copha)	131 g/Kg
Cellulose	50 g/Kg
Pregelled Wheat Starch	50 g/Kg
DL Methionine	3.0 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
AIN93 Trace Minerals	1.4 g/Kg
Potassium Citrate	2.5 g/Kg
Potassium Dihydrogen Phosphate	6.9 g/Kg
Potassium Sulphate	1.6 g/Kg
Choline Chloride (75%)	2.5 g/Kg
AIN93 Vitamins	10 g/Kg
Cholesterol USP	1.9 g/Kg

Calculated Amino Acids	
Valine	1.26%
Leucine	1.80%
Isoleucine	0.90%
Threonine	0.80%
Methionine	0.80%
Cystine	0.06%
Lysine	1.50%
Phenylanine	1.00%
Tyrosine	1.00%
Tryptophan	0.30%
Histidine	0.60%

Calculated Total Minerals	
Calcium	0.47%
Phosphorous	0.32%
Magnesium	0.09%
Sodium	0.12%
Chloride	0.16%
Potassium	0.40%
Sulphur	0.22%
Iron	73 mg/Kg
Copper	7.1 mg/Kg
Iodine	0.2 mg/Kg
Manganese	19 mg/Kg
Cobalt	No data
Zinc	52 mg/Kg
Molybdenum	0.15 mg/Kg
Selenium	0.3 mg/Kg
Cadmium	No data
Chromium	1.0 mg/Kg
Fluoride	1.0 mg/Kg
Lithium	0.1 mg/Kg
Boron	2.1 mg/Kg
Nickel	0.5 mg/Kg
Vanadium	0.1 mg/Kg

Calculated Total Vitamins	
Vitamin A (Retinol)	4 000 IU/Kg
Vitamin D (Cholecalciferol)	1 000 IU/Kg
Vitamin E (a Tocopherol acetate)	80 mg/Kg
Vitamin K (Menadione)	1 mg/Kg
Vitamin C (Ascorbic acid)	None added
Vitamin B1 (Thiamine)	6.1 mg/Kg
Vitamin B2 (Riboflavin)	6.3 mg/Kg
Niacin (Nicotinic acid)	30 mg/Kg
Vitamin B6 (Pryridoxine)	7.2 mg/Kg
Pantothenic Acid	16.5 mg/Kg
Biotin	200 ug/Kg
Folic Acid	2 mg/Kg
Inositol	None added
Vitamin B12 (Cyancobalamin)	103 ug/Kg
Choline	1 470 mg/Kg

Calculated Fatty Acid Composition	
Saturated fats C12 or Less	6.77%
Myristic Acid 14:0	1.80%
Palmitic Acid 16:0	3.11%
Stearic Acid 18:0	3.05%
Oleic Acid 18:1	5.70%
Gadoleic Acid 20:1	0.07%
Linoleic Acid 18:2 n6	1.50%
a Linolenic Acid 18:3 n3	0.74%
EPA 20:5 n3	No data
DHA 22:6 n3	No data
Total n3	0.74%
Total n6	1.50%
Total Saturated Fats	14.93%
Total Monosaturated Fats	5.89%
Total Polyunsaturated Fat	2.24%
Cholesterol	0.19%

Calculated data uses information from typical raw material composition. It could be expected that individual batches of diet will vary from this figure. **Diet post treatment by irradiation or auto clave could change these parameters**. We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.

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Diet
SF01-028

23% Fat Semi-Pure Rodent Diet
43% of Energy From Fat

A high fat semi-pure modification of AIN93G.

- Fat content has been increased from around 7% in AIN93G to 23%.
- Calculated energy has increased by around 24% over the base diet. 40% of the total calculated energy is from lipids.
- The triglyceride profile has an increased proportion of saturated and mono-unsaturated fatty acids over the standard diet.
- Other nutritional parameters have remained unchanged.
- The high fat content has resulted in a significant reduction in pellet hardness. The pellets must be handled with great care to avoid breakage.

Calculated Nutritional Parameters	
Protein	19.00%
Total Fat	22.60%
Crude Fibre	4.70%
AD Fibre	4.70%
Digestible Energy	19.9 MJ / Kg
% Total calculated digestible energy from lipids	43.00%
% Total calculated digestible energy from protein	17.00%

Diet Form and Features

- Semi pure diet. 12 mm diameter pellets.
- Pack size 1.5 Kg, trays vacuum packed in oxygen impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

Ingredients	
Casein (Acid)	200 g/Kg
Sucrose	388 g/Kg
Canola Oil	48 g/Kg
Cocoa Butter	180 g/Kg
Cellulose	50 g/Kg
Wheat Starch	90 g/Kg
DL Methionine	3.0 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
AIN93 Trace Minerals	1.4 g/Kg
Potassium Citrate	2.5 g/Kg
Potassium Dihydrogen Phosphate	6.9 g/Kg
Potassium Sulphate	1.6 g/Kg
Choline Chloride (75%)	2.5 g/Kg
AIN93 Vitamins	10 g/Kg

Calculated Amino Acids	
Valine	1.10%
Leucine	1.70%
Isoleucine	1.00%
Threonine	0.70%
Methionine	0.70%
Cystine	0.05%
Lysine	1.50%
Phenylanine	0.90%
Tyrosine	1.00%
Histidine	0.60%
Tryptophan	0.10%

Calculated Total Minerals	
Calcium	0.45%
Phosphorous	0.30%
Magnesium	0.09%
Sodium	0.11%
Chloride	0.16%
Potassium	0.40%
Sulphur	0.23%
Iron	70 mg/Kg
Copper	6.8 mg/Kg
Iodine	0.2 mg/Kg
Manganese	18 mg/Kg
Cobalt	No data
Zinc	50 mg/Kg
Molybdenum	0.15 mg/Kg
Selenium	0.3 mg/Kg
Cadmium	No data
Chromium	1.0 mg/Kg
Fluoride	1.0 mg/Kg
Lithium	0.1 mg/Kg
Boron	3.4 mg/Kg
Nickel	0.5 mg/Kg
Vanadium	0.1 mg/Kg

Calculated Total Vitamins	
Vitamin A (Retinol)	4 000 IU/Kg
Vitamin D (Cholecalciferol)	1 000 IU/Kg
Vitamin E (a Tocopherol acetate)	75 mg/Kg
Vitamin K (Menadione)	1 mg/Kg
Vitamin C (Ascorbic acid)	None added
Vitamin B1 (Thiamine)	6.1 mg/Kg
Vitamin B2 (Riboflavin)	6.3 mg/Kg
Niacin (Nicotinic acid)	30 mg/Kg
Vitamin B6 (Pryridoxine)	7 mg/Kg
Pantothenic Acid	16.5 mg/Kg
Biotin	200 ug/Kg
Folic Acid	2 mg/Kg
Inositol	None added
Vitamin B12 (Cyancobalamin)	100 ug/Kg
Choline	1 700 mg/Kg

Calculated Fatty Acid Composition	
Staurated Fats C12:0 and less	0.09%
Myristic Acid 14:0	0.04%
Palmitic Acid 16:0	4.79%
Stearic Acid 18:0	6.55%
Arachidic Acid 20:0	0.21%
Palmitoleic Acid 16:1	0.04%
Oleic Acid 18:1	8.73%
Gadoleic Acid 20:1	0.08%
Linoleic Acid 18:2 n6	1.50%
a Linolenic Acid 18:3 n3	0.55%
Arachadonic Acid 20:4 n6	No data
EPA 20:5 n3	Trace
DHA 22:6 n3	No data
Total n3	0.58%
Total n6	1.50%
Total Mono Unsaturated Fats	8.85%
Total Polyunsaturated Fats	2.09%
Total Saturated Fats	11.80%

Calculated data uses information from typical raw material composition. **Diet post treatment by irradiation or auto clave could change these parameters**. It could be expected that individual batches of diet will vary from this figure. We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.

Appendix 2

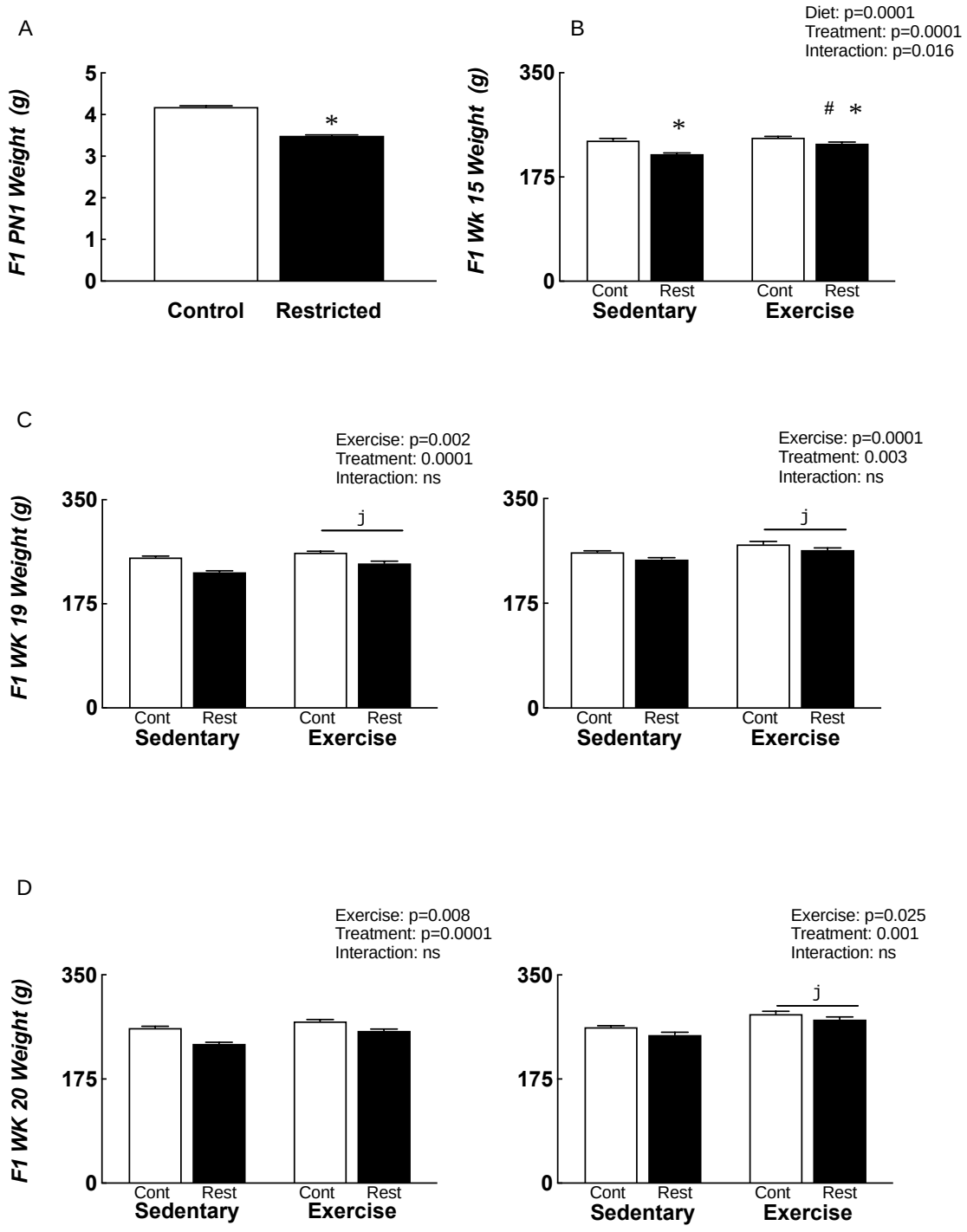

Figure A.1 F1 Female body weights at PN1, week 15, 19 and 20 (mating)

Control (white bars) and Restricted (black bars) females body weights at (A) Postnatal day 1(PN1); (B)15; (C) 19; and (D) 20 weeks of age. All values are expressed as mean ± SEM; 15-39 per group from separate litters. **P*<0.05 vs Control; #*P*<0.05 vs Chow; φ*P*<0.05 vs Sedentary.

Appendix 3

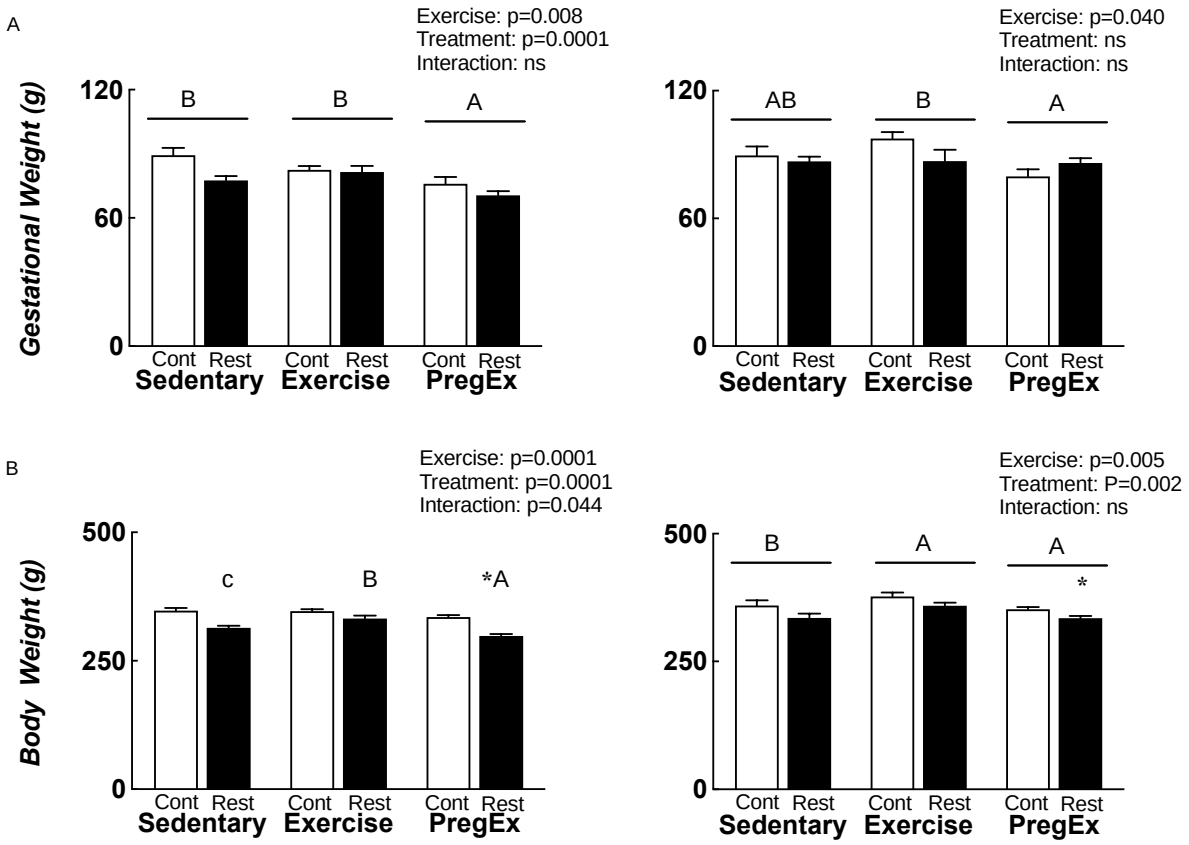

Figure A.2 Gestational weight gain and Body weights at *post-mortem* (E20)

Control (white bars) and Restricted (black bars) gestational weight gain and body weights at *post mortem* (E20). Results from the two-way ANOVA of treatment and exercise separated by diet. (A) Gestational Weight gain and (B) Body weight at PM. All values are expressed as mean ± SEM; 8-12 per group from separate litters. * $P<0.05$ vs Control; 'a' is different to 'b' and 'c' ($P<0.05$).