Like father, like daughter:
Can maternal or early life exercise break the cycle of paternal diet-induced metabolic programming in female offspring?

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Abstract

Recent research has suggested that paternal phenotype before conception can have long-lasting metabolic consequences on offspring's health. Little is known, however, about actionable interventions that could stop this loop: unhealthy fathers generating unhealthy offspring that will become parents and so on. We and others have previously demonstrated that both maternal exercise and offspring exercise early in life might be beneficial when offspring's health is threatened due to other insults (*e.g.* maternal undernutrition or placental dysfunction). Therefore, the aim of this research was to investigate if maternal exercise (Chapter 3) or offspring early in life exercise (Chapter 4) could attenuate the negative metabolic consequences in adult rat offspring sired by high-fat fed obese fathers (Chapter 3 and 4).

Male Sprague-Dawley rats were fed high-fat diet (HFD; ~41% energy from fat) or normal chow diet (12% energy from fat) before conception (Chapter 3 and 4). Meanwhile, female breeders performed exercise on a treadmill before (four weeks before pregnancy) and during pregnancy (5 days/week, 60–20 min/day, at ~65–50% $\dot{V}O_{2max}$) or remained sedentary (Chapter 3). Then, a cohort of pups from the sedentary mothers was exercised early in life (from 5 to 9 weeks of age, 5 days/week, 60 min/day, at ~65-75% $\dot{V}O_{2max}$) or remained sedentary (Chapter 4). Two studies were performed based on the following experimental groups:

- Normal diet fathers, sedentary mothers, sedentary offspring (NS, Chapter 3 and 4);
- High-fat diet fathers, sedentary mothers, sedentary offspring (HS, Chapter 3 and 4);
- Normal diet fathers, exercised mothers, sedentary offspring (NE, Chapter 3);
- High-fat diet fathers, exercised mothers, sedentary offspring (HE, Chapter 3);
- Normal diet fathers, sedentary mothers, exercised offspring (NE, Chapter 4);
- High-fat diet fathers, sedentary mothers, exercised offspring (HE, Chapter 4).

In both chapters, we evaluated adolescent (11-12 weeks of age) and adult (23-25 weeks of age) offspring rats. Adolescent and adult offspring underwent intraperitoneal insulin tolerance tests (IPITT, 11 and 23 weeks of age, respectively) and intraperitoneal glucose tolerance tests (IPGTT, 12 and 24 weeks of age, respectively). At 25 weeks of age, *ex vivo* insulin-stimulated glucose uptake and mitochondrial respiration in skeletal muscle as well as pancreatic morphology were completed. Western-blots for key proteins

related to insulin signalling and mitochondrial function provided insights to the underlying molecular mechanisms in the skeletal muscle.

Initially, the phenotype of offspring sired by HFD/obese fathers was characterised. Body weight was lower and retroperitoneal fat was unchanged compared to offspring sired by normal diet fathers (HS vs NS). Adolescent offspring demonstrated significant ($P<0.05$) negative metabolic consequences in glucose tolerance, while adult offspring showed impaired insulin sensitivity (glucose area under the curve during IPITT was 11.5% higher). Basal (40%) and insulin-stimulated glucose uptake (35%) were impaired in isolated epitrochlearis (EPI) but not soleus muscle (HS vs NS). The reason(s) for the lower basal glucose uptake were not clear as GLUT1 protein expression was not altered (25% lower compared to control, but not significant). The lower insulin-stimulated glucose uptake may have related to the observed attenuated increase in phosphorylation of TBC1D4^{Thr642} in response to insulin in offspring of HFD/obese fathers. Paternal HFD/obesity did not change citrate synthase activity (a commonly used quantitative marker for content/volume of mitochondria) in the offspring. Also, mitochondrial respiration in plantaris muscle was not affected, but there was an increase in the reactive oxygen species production during leak respiration through complex I. Nevertheless, paternal HFD/obesity decreased the skeletal muscle protein expression of PHF20 and Tfam (mainly involved mitochondrial transcription factor processes) but had no effect on PGC1α (mitochondrial biogenesis marker) in adult offspring. Paternal diet-induced obesity decreased insulin secretion during the IPGTT (HS vs NS). The lower β-cell mass (31%) and altered islet size distribution might explain, at least in part, this finding.

The first study investigated whether maternal exercise would protect offspring sired by high-fat fed obese fathers. Exercise before and during gestation normalised glucose tolerance in adolescent offspring. In adult rats, however, maternal exercise had no effects on body weight and retroperitoneal fat. Whole body insulin sensitivity was unchanged after exercise training in mothers, however, the lower insulin-stimulated glucose uptake in isolated EPI muscle was normalised. Although maternal exercise increased GLUT1 protein expression, this was not sufficient to normalise the lower basal glucose uptake in adult offspring sired by high-fat fed obese fathers. GLUT4 protein expression as well as insulin-stimulated p-AKT^{Ser473} were higher in offspring born from exercised mothers (NE vs NS). Maternal exercise, however, did not normalise the lower phosphorylation levels of insulin-stimulated TBC1D4^{Thr642} observed in offspring sired by

HFD/obese fathers (HE vs HS). Maternal exercise increased skeletal muscle mitochondrial volume in adult offspring from HFD/obese fathers (HE vs HS), however, it decreased mitochondrial-specific oxidative phosphorylation capacity through complex II and electron transport system capacity through complexes I+II. Maternal exercise attenuated the lower insulin secretion observed during the IPGTT in offspring from HFD/obese fathers. This might be due to the higher number of islets, higher relative islet surface area and normalised β-cell mass (HE vs HS).

The second study investigated whether early life exercise in offspring sired by HFD/obese fathers would protect them in adulthood. Early life exercise normalised glucose tolerance in adolescent offspring sired by high-fat fed obese fathers. Body weight was normalised and retroperitoneal fat was unchanged in adulthood (HE vs HS). Exercise early in life did not change glucose tolerance in adulthood. Whole body insulin sensitivity (IPITT) as well as basal and insulin-stimulated glucose uptake in isolated EPI muscle were normalised after exercise training early in life in offspring (HE vs HS). Offspring exercise early in life increased protein expression of GLUT1 and GLUT4, and normalised the lower phosphorylation of insulin-stimulated TBC1D4^{Thr642} (HE vs HS). Exercise early in life did not change mitochondrial volume, but increased mitochondrial respiration compared to sedentary offspring from HFD/obese fathers (HE vs HS). There were no changes in protein expression related to mitochondrial function (PGC1α, Tfam and PHF20) in the plantaris muscle. Surprisingly, early life exercise did not attenuate the negative effects of paternal HFD/obesity in insulin secretion in adult offspring, and β-cell mass remained low compared to offspring sired by high-fat fed obese fathers (HE vs HS).

In summary, paternal HFD/obesity prejudices insulin sensitivity and insulin secretion in adult offspring. The lower insulin sensitivity after paternal HFD/obesity may have been due, at least in part, to the observed impaired glucose uptake and insulin signalling in isolated skeletal muscle, without affecting mitochondrial respiration. The reduced insulin secretion is supported by the lower β-cell mass. Maternal exercise did not protect offspring's whole body insulin sensitivity, but normalised *ex vivo* insulin-stimulated glucose uptake. Offspring exercise early in life normalised both *in vivo* and *ex vivo* insulin sensitivity. Maternal exercise augmented p-AKT while exercise early in life normalised p-TBC1D4 in skeletal muscle. In offspring sired by HFD/obese fathers, maternal exercise increased mitochondrial volume while offspring exercise improved mitochondrial respiration. The lower insulin secretion in offspring from HFD/obese fathers was attenuated by maternal exercise but not by offspring exercise early in life.

Hence, exercise in different stages of life — before and during gestation or during childhood — may be a plausible intervention able to break the insulin resistance cycle of paternal obesity (associated with high-fat diet) in the next generations. In offspring sired by HFD/obese fathers, maternal exercise might be more beneficial in regards to insulin secretion while the positive effects of offspring exercise early in life are related to skeletal muscle insulin sensitivity.

Declaration

I, *Filippe Falcão Tebas Oliveira*, declare that the PhD thesis entitled '*Like father, like daughter: Can maternal or early life exercise break the cycle of paternal diet-induced metabolic programming in female offspring?'* is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:  Date: 22/03/2017

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List of publications, awards, grants and conferences

Publications (not directly related to this thesis)

- McConell, G.K., Kaur, G., **Falcão-Tebas, F.**, Hong, Y.H., Gatford, K.L. Acute exercise increases insulin sensitivity in adult sheep: a new preclinical model. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, v308. p.500-506, 2015.
- Gatford, K. L., Kaur, G., **Falcão-Tebas, F.,** Wadley, G. D., Wlodek, M. E., Laker, R. C., Ebeling, P. R., McConell, G. K. Exercise as an intervention to improve metabolic outcomes after intrauterine growth restriction. Invited review. American Journal of Physiology: Endocrinology and Metabolism, v.306. p.999-1012, 2014.

Awards

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Grants

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Conference Presentations

- **Falcão-Tebas F.**, Arceri, C., Kerris, J., Marin, E., McConell, G. Can exercise early in life overcome the detrimental effects on insulin sensitivity in rat offspring

of high-fat fed/obese fathers? *Oral presentation*. Victorian Obesity Consortium (VOC), Melbourne, Australia 2016.

- **Falcão-Tebas, F**. Arceri, C. R., Kerris, J. P., Marin, E. C., McConell, G. K.. Fit and fat parents: can maternal exercise overcome the detrimental effects on glucose tolerance in offspring of a high-fat eating dad? *Oral presentation*. European College of Sport Science (ECSS), Vienna, Austria 2016.
- **Falcão-Tebas F.**, Marin, E., Arceri, C., Kuang J., Bishop D., McConell, G.. Like mom, like daughter: maternal exercise improves glucose tolerance, body composition and mitochondrial function in adult female rat offspring. *Oral presentation*. International Biochemistry of Exercise Conference (IBEC). Sao Paulo, Brazil 2015.
- **Falcão-Tebas F.**, Arceri C., Kerris J., Marin E., McConell G. Fit mum, fat dad: can mum exercising during pregnancy overcome the detrimental effects on insulin sensitivity of a high-fat eating dad? *Oral presentation*. Australian Physiological Society (AuPS), Hobart, Australia 2015.
- **Falcão-Tebas F.**, Marin E., Arceri C., Kuang J., Bishop D, McConell G. Exercise only early in life in rats programs a healthier phenotype in adulthood. *Oral and poster presentations*. Development Origins Health and Disease (DOHaD) 9th World Congress. Cape Town, South Africa 2015.
- **Falcão-Tebas F.**, Arceri C, Kuang J, Bishop D, Marin E, McConell GK. Childhood is a critical period for life: Long-term effects of early-life exercise on glucose tolerance and mitochondrial capacity. *Oral presentation*. Australian Diabetes Society (ADS), Adelaide, Australia 2015.
- **Falcão-Tebas, F.**, Marin, E.M., McLennan, E., McConell, G.K. Fit mum, healthy daughter: Exercise training during pregnancy improves offspring glucose homeostasis. *Poster presentation*. Developmental Origins of Health and Disease Society (DOHaD) of Australia and New Zealand. Melbourne, Australia 2015.

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List of abbreviations

AAC	Area above the curve
ADP	Adenosine diphosphate
AEC	Animal Ethics Committee
AKT	AKT serine/threonine kinase, also known as 'Protein kinase B'
AKT2	AKT serine/threonine kinase 2
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AS160	TBC1 domain family member 4, also known as 'Akt substrate of 160 kDa'
ATP	Adenosine triphosphate
AUC	Area under the curve
BioPS	Biopsy Preservation Solution
BMI	Body mass index
BSA	Bovine serum albumin
CI	Mitochondrial complex I
CII	Mitochondrial complex II
CII_E	Electron transport system capacity through complex II
CI_L	Leak respiration through complex I
CI_P	Maximum oxidative phosphorylation capacity through complex I
CO_2	Carbon dioxide
CS	Citrate synthase
d	Day
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and diseases
DPM	Disintegrations per minute
DTNB	5,5-dithiobis-2-nitrobenzoate
DTT	Dithiothreitol
E	Electron transport system capacity
EDL	*Extensor digitorum longus*
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetra acetic acid
EPI	Epitrochlearis

ETS	Electron transport system
F1	First filial generation
F2	Second filial generation
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenyl-hydrazone
FCRs	Flux control ratios
GLUT1	Glucose transporter 1, also known as ‘solute carrier family 2 member 1’
GLUT2	Glucose transporter 2, also known as ‘solute carrier family 2 member 2’
GLUT3	Glucose transporter 3, also known as ‘solute carrier family 2 member 3’
GLUT4	Glucose transporter 4, also known as ‘solute carrier family 2 member 4’
GU	Glucose uptake
H_2O	Water
H_2O_2	Hydrogen peroxide
HE	Offspring born from high-fat diet fathers and exercised mothers (chapter 3) or Exercised offspring born from high-fat diet fathers (chapter 4)
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HFD	High-fat diet
HOMA	Homeostatic model assessment
HRP	Horseradish peroxidase
HRR	High-resolution respirometry
HS	Offspring born from high-fat diet fathers and control mothers
IAR	immediate adaptive response
IGF1R	Insulin-like growth factor 1 receptor
IGF2	Insulin-like growth factor 2
inv-RCR	Inverse respiratory control ratio
IP	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
IR	Insulin resistance
IRS	Insulin-receptor substrate
IRS2	insulin-receptor substrate 2
IUGR	Intrauterine growth restriction
kDa	Kilodalton
kg	Kilogram
L	Leak mitochondria respiration

LBW	Low birth weight
LCR	Leak control ratio
MAPK	Mitogen-activated protein kinases
MES	2-(N-morpholino)ethanesulfonic acid
MHC	Myosin heavy chain
m	meter
min	Minute
MiR05	Mitochondrial Respiration Medium 5
MJ	Megajoule
mL	Millilitre
mM	Millimolar
mmol	Millimole
mRNA	Messenger ribonucleic acid
NADH	Reduced nicotine adenine dinucleotide
NCDs	Noncommunicable diseases
NE	Offspring born from control fathers and exercised mothers (chapter 3) or Exercised offspring born from control fathers (chapter 4)
NS	Offspring born from control fathers and control mothers
OLEFT	Otsuka Long-Evans Tokushima Fatty
OXPHOS	Oxidative phosphorylation
P	Maximum oxidative phosphorylation capacity
PAR	Predictive adaptive response
PCR	Phosphorylation control ratio
PDK1	3-phosphoinositide dependent protein kinase-1
PGC1α	Peroxisome-proliferator-activated receptor-γ co-activator 1α
PHF20	Plant homeodomain finger-containing protein 20
PI-3K	Phosphatidylinositide-3 kinase
PI(3,4,5)P3	Phosphatidylinositol 3,4,5 trisphosphate
PKB	Protein kinase B, also known as AKT
PKC	Protein kinase C
PLANT	Plantaris
PND	Postnatal day
RIA	Radioimmunoassay
RNA	Ribonucleic acid

ROS	Reactive oxygen species
ROX	Residual oxygen consumption
RPM	Revolutions per minute
SCR	Substrate control ratio
SEM	Standard error of the mean
SGA	Small for gestation age
SH2	Src Homology-2
SOL	Soleus
SUIT	Substrate-uncoupler-inhibitor titration
T2DM	Type 2 diabetes mellitus
TA	Tibialis anterior
TBC1D1	TBC1 domain family member 1
TBC1D4	TBC1 domain family member 4
TBS	Tris-Buffered saline
TBSt	Tris-buffered saline with Tween-20
TCA	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
Vd	Volume density
$\dot{V}O_{2max}$	Maximal volume of oxygen uptake (per minute)
WHO	World Health Organization
wk	Week

Chapter 1 – Literature review

1. 1. Introduction

It is well established that the environment during early life can influence individual growth trajectories and ultimately long-term health (Hales and Barker 1992, Gluckman et al. 2010a, Wells 2007b). Classical epidemiological studies reported that alterations in gestation, lactation and childhood can affect the risk of developing cardiometabolic diseases such as type 2 diabetes mellitus (T2DM) in adulthood (Hales et al. 1991, Barker et al. 1989). Experimental data from animal models have generally provided complementary support for this relationship (McMullen and Mostyn 2009). Usually, studies using rodents manipulate intrauterine and/or postnatal environments and investigate the consequences later in life. For example, maternal undernutrition and placental restriction are well established models to induce intrauterine growth restriction (IUGR) and consequently low-birth weight (LBW) followed by health complications in long-term (McMullen and Mostyn 2009).

Recently, the role of fathers in this scenario has drawn attention, and it has been demonstrated that paternal diet-induced obesity can retard embryo development and pregnancy health (Binder et al. 2012). In addition, offspring sired by obese fathers have detrimental physiological traits, including higher visceral fat accumulation, insulin resistance and altered balance of cell types in pancreas (Ng et al. 2010, Fullston et al. 2013). These changes are associated with impaired glucose homeostasis in the offspring (Ng et al. 2010, Fullston et al. 2013). However, little is known about the molecular changes in skeletal muscle related to glucose homeostasis in adult offspring sired by obese fathers fed with high-fat diet (HFD).

Whether lifestyle intervention(s) can attenuate the increased risk of developing metabolic diseases in such circumstances needs further investigation. There is early evidence that either maternal exercise (Carter et al. 2013, Hopkins and Cutfield 2011) or exercise early in life (Gatford et al. 2014, Laker et al. 2011) may play an important role in this regard. Thus, the following review will discuss 1) the theoretical frameworks used in the developmental origins of health and diseases (DOHaD) field, and their contribution to explain the rationale for the subsequent studies presented here; 2) the effects of paternal phenotype on offspring's health and disease status, and; 3) how maternal exercise or

exercise early in life can be implemented to delay or prevent the development of T2DM in offspring born from obese fathers fed HFD before conception.

1. 2. The type 2 diabetes problem

There has been a change in the global burden of disease in the last four decades. What once were considered major causes of global mortality (e.g. tuberculosis, influenza, infectious diseases), are no longer the leading causes of deaths (Jones et al. 2012). Instead, we are observing an alarming increase in the prevalence of noncommunicable diseases that typically strike in older age, such as heart diseases and T2DM (Lozano et al. 2012).

Diabetes mellitus is a group of metabolic diseases, generally defined by a chronic hyperglycaemia caused by defects in insulin sensitivity, insulin secretion or both (American Diabetes 2010, Kerner et al. 2014). It can be classified into different types, with the T2DM being the most common form. In this case, a person may experience difficulties to regulate glucose uptake and its utilization in insulin-dependent tissues such as skeletal muscle and fat, which is then compensated for by hypersecretion of insulin. Defects in insulin secretion will eventually be related to insufficient insulin to overcome the insulin resistance observed in T2DM individuals (Meier and Bonadonna 2013). The function of the islets of Langerhans (site of insulin secretory granules) may be compromised (Forbes and Cooper 2013) as demonstrated by decreased number of insulin-producing cells in the islets in both obese and T2DM individuals (Butler et al. 2003). It is important, thus, to have standard values to measure T2DM worldwide and be able to compare data across different populations.

Despite all the research highlighting the amount of people with diabetes mellitus and its influence in other diseases (Hill et al. 2013), the number of cases worldwide only grows. The estimates are higher than what was predicted 10 years ago (Shaw et al. 2010, Wild et al. 2004). In fact, in 2016 the World Health Organization (WHO) launched a Diabetes report where it was stated that over 0.4 billion people have diabetes, in other words, this corresponds to 1 person in 11 worldwide (WHO 2016b). This might suggest that the current methods and technologies used alone to treat the disease are not enough to counter the incidence of new cases. Even more concerning are the findings showing that both parents' lifestyle can influence offspring's phenotype (Mousseau and Fox 1998, Curley et al. 2011). Obese and sedentary parents are likely to generate offspring with higher risk to develop metabolic diseases, such as diabetes, in adulthood (Hanson and

Gluckman 2014). Accordingly, rather than focus on treating the disease, attention must be given to research investigating if this increased risk is preventable.

Along these lines, although T2DM is largely associated with current lifestyle and genetic predisposition (Hays et al. 2008), a new perspective has been recognised to play a major role in the cause of this disease. It is now well documented that the periods before and during pregnancy as well as early in life are crucial and can potentially increase the risk of T2DM later in life.

1. 3. The developmental origins of health and disease (DOHaD) paradigm

The developmental origins of health and disease (DOHaD) concept is a relatively new field of research (emerged over the last 20 years). It focuses on understanding the physiological and pathophysiological basis of how environmental influences acting during early human development influence the risk of later chronic diseases (Hanson and Gluckman 2014, McMullen and Mostyn 2009, Street et al. 2015). There are 'windows of opportunity' when the organism is more susceptible to environmental cues and able to respond to the stimuli (Hanson and Gluckman 2014) (Figure 1-1). Evidence for the DOHaD had been accumulated since early in the 20th century before the DOHaD concept was even established [reviewed elsewhere (Hanson and Gluckman 2014, McMullen and Mostyn 2009)], but there were various historical reasons that delayed the acceptance of the field, including lack of conceptual framework and its biological mechanisms (Hanson and Gluckman 2014).

It is now known that due to developmental plasticity, a genotype is able to generate different phenotypes. The phenotype is likely to best suit the current environment that the organism is living to survive, ideally, until reproductive age (Barker 2004). Developmental plasticity continues after birth, and some studies suggest that it decreases with age (Hillier et al. 2007, Hanson and Gluckman 2014). Over time, cumulative changes in lifestyle and environment increase the risk to develop chronic diseases, such as T2DM (Figure 1-1) (Hillier et al. 2007).

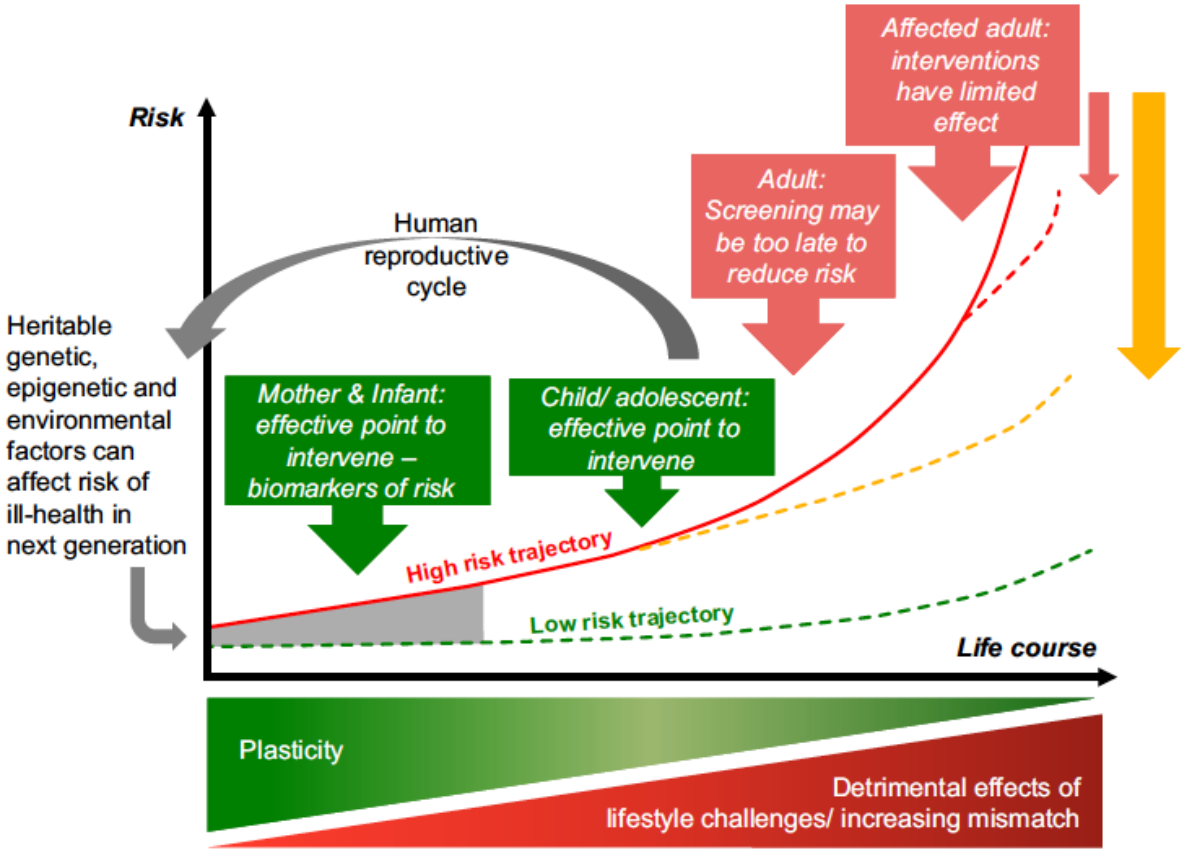

Figure 1-1. Noncommunicable diseases (NCDs) risk from a developmental origin of health and disease (DOHaD) perspective. As plasticity declines and accumulative damage from lifestyle challenges are more likely to happen, the risk of NCDs increases. Interventions early in life might be more beneficial than in adults, and can reduce the risk of NCDs in the next generation (Hanson and Gluckman 2014).

1. 3. 1. Early studies

One of the first studies linking perinatal life to long-term consequences emerged early in the 20th century. In 1911, Hertfordshire, in the United Kingdom, all newborns had their birth weight recorded (Barker et al. 1989, Barker 1999). Afterwards, it was observed in over 10,000 men up to 65 years of age that deaths caused by coronary diseases were inversely proportional to birth weight (Barker et al. 1989). Another key study in the history of the field occurred during World War II (1944–1945). The 'Dutch Famine' was an episode where the Dutch population had approximately a 50% reduction in food supply (~1400 kcal to 670 kcal per day) due to the invasion by Germany (Ravelli et al. 1976). Women also were exposed to limited nourishment during gestation, which resulted in low birth weight babies (Lumey et al. 1995, Lumey 1998). Furthermore, these offspring (19 year old men) exposed to food restriction especially during the first trimester of the

intrauterine life demonstrated higher prevalence of obesity (Ravelli et al. 1976) and coronary diseases (Roseboom et al. 2000). These and further epidemiological studies were important to originate the hypothesis of the foetal origins of adult disease (Hales and Barker 1992). Simply put, it was stated that if the allocation of supplies (e.g. nutrients and oxygen) from the mother to her baby was compromised, her child's health could be at risk later in life.

Animal models have replicated and provided useful insights related to the epidemiological and clinical studies. Under controlled conditions, pre-clinical models showed that both maternal under- and over-nutrition contribute to persistent metabolic alterations in offspring which are associated with an increased risk of obesity (Lillycrop and Burdge 2011). In fact, early studies in rats elucidated the importance of maternal nutrition on foetal-placental growth (Brasel and Winick 1972) and brain development (Roeder and Chow 1972). Protein restricted dams during late pregnancy and lactation had offspring with increased abdominal fat compared to offspring from control dams (Bellinger et al. 2006). Similarly, HFD during pregnancy and lactation causes abdominal obesity, hypertension and higher fasting insulin and glucose levels in 6 months old offspring compared with pups born from control dams (Samuelsson et al. 2008), which is associated with metabolic diseases. Therefore, it is clear that not only genetic and current lifestyle factors (Walley et al. 2006, Mozaffarian et al. 2009), but also environmental cues in early stages of life, contribute to the risk of developing chronic diseases later in life (Gluckman and Hanson 2004b).

1. 3. 2. **Current theoretical framework**

As the DOHaD field expands, insightful frameworks are been proposed to explain the findings from humans and a range of animal models (Gluckman et al. 2010b, Wells 2010, Vaag et al. 2012, Barker et al. 1989). One of the most used approaches suggests that there is an 'adaptive response' by the organism to environmental cues early in life (Hanson and Gluckman 2014). Although some academic discussions were made in regards to the proposed frameworks in the DOHaD field (Wells 2007a, Gluckman et al. 2008), it is important to mention that these frameworks seem to be originated from a common classical biological concept, the developmental plasticity (West-Eberhard 1989), and therefore, might have common parts in some extent.

One of the DOHaD concepts highlight the idea that the foetus might be able to develop an immediate adaptive response (IAR, short-term) and/or and predictive adaptive

response (PAR, long-term) due to the environmental stimulus (Hanson and Gluckman 2014). The IARs are phenotypic responses to a challenge, usually outside the normal range, which promotes immediate survival but may incur longer term disadvantage. An example of IARs includes a reduction in foetal growth in response or impaired placental function to maternal undernutrition. The foetus has to compromise its somatic growth in order to cope with the limited resources provided by an undernourished mother, otherwise the foetus would not survive. Brain, heart and other essential organs might be protected under this circumstance by redistribution of blood flow (Barker 1998), however other tissues will have limited supply of amino acids and glucose via the placenta (Oliver et al. 1993). Ultimately, this will result in diminished levels of placental glucose, insulin and insulin-like growth factor 1 (Barker 1998). These effects are transitory and may or not be associated with long-term consequences (Hanson and Gluckman 2014).

Not all responses made by the developing foetus may promote immediate advantage, supporting survival in short-term. Some responses may foster survival to reproductive age in a predicted postnatal environment, which have been termed PARs. This response confers advantage in the long-term by using developmental cues to predict or forecast later environment (Gluckman et al. 2005). A general PAR example is the coat of a mouse strain (*Microtus pennsylvanicus*) which is thicker at birth if the offspring is born at a time of decreasing day length, thus preparing the offspring in utero for winter (Gluckman and Hanson 2004a). Therefore, the importance of maternal-placental *milieu* has drawn most of the attention in the field. It is proposed that the foetus is able to 'sense' the external environment by signals sent by the mother and adapt its developmental trajectory accordingly to match with what is expected to be observed when delivered (Bateson et al. 2004). If the prediction is successful, no or less concerns will arise based on the PAR. However, if there is a mismatch between the foetal prediction and its later environment, there will be an increased risk to develop chronic diseases, such as diabetes (Hanson and Gluckman 2014).

1. 3. 3. **Phenotypical plasticity**

The theoretical basis for these associations between early stages of life and later outcomes in life involve broad concepts in Biology, such as phenotypic plasticity (Gluckman et al. 2010a). Common in all living organisms, phenotypic or developmental plasticity is defined as "the ability of an organism to react to an environmental input with a change in form, state, movement, or rate of activity" (West-Eberhard 2003). Plasticity

can be modulated by inputs of genes (individual's genome) and environment (phenotype variation). Special attention has been given to the way environmental factors, e.g. nutrition, can play in this process (McMullen and Mostyn 2009, Kappeler and Meaney 2010). Metabolic tissues have the ability to respond strongly to perinatal diets (Wells 2012, Godfrey et al. 2010). For example, in mice, feeding a high-fat diet to mothers before and during pregnancy causes pancreatic dysfunction (Gniuli et al. 2008), altered expression of insulin signalling proteins in skeletal muscle (Buckley et al. 2005) and increased expression of hepatic lipogenic genes (Hartil et al. 2009). Additionally, over-nourishing dams during pregnancy alter their phenotype, having unbalanced eating behaviour and body weight gain (George et al. 2010). Their offspring are more likely to have lower energy expenditure due to reduce muscle mass and function (Rooney and Ozanne 2011), which can potentially impair whole-body fuel utilisation. Indeed, there is growing evidence supporting this idea that plasticity can span generations (Uller 2008).

1. 3. 4. **Parental effects**

Parental effects are persistent influences on progeny phenotype that are derived from parents, apart from nuclear genes (Kappeler and Meaney 2010). One current hypothesis is that paternal effects can modulate offspring gene transcription by epigenetic mechanisms (Kappeler and Meaney 2010). Epigenetic studies non-genetic mitotically heritable states in gene expression potential (Jaenisch and Bird 2003) by marks added onto the DNA that will direct the access to genetic information (Waterland and Michels 2007). This includes DNA methylation, histone modifications and variants, chromatin remodelling and non-coding RNAs [see review (Curley et al. 2011) for further details]. Epidemiological studies have provided important support on this association. In a cohort of 854 subjects it was demonstrated that having both parents obese more than doubles the risk of obesity in 15-17 years old individuals, independent of childhood obesity (Whitaker et al. 1997). In fact, maternal obesity has been associated to some extent with offspring adiposity (Boney et al. 2005, Lake et al. 1997). Although it is difficult to disentangle maternal and paternal effects in human studies, a correlation has also been reported between paternal obese phenotype and hypomethylation of the insulin-like growth factor 2 (IGF2) gene in the umbilical cord (Soubry et al. 2013), lower birth weight (McCowan et al. 2011), and body fat in infant girls (Figueroa-Colon et al. 2000).

In rodents, having both parents eating HFD appears to be more prejudicial for the offspring later in life than having only one (Masuyama et al. 2016, Ornellas et al.

2015, McPherson et al. 2015). Paternal and maternal obesity seems to have addictive negative effects in mice, demonstrating mitochondrial dysfunction and aberrant placental gene expression, which results in foetal and embryo development (McPherson et al. 2015). For example, expression of placental glucose transporter 3 (GLUT3) gene was not different from either maternal or paternal HFD alone; however having both parents obese leads to increased gene expression (McPherson et al. 2015). In C57BL/6 mice, both paternal and maternal HFD affected insulin levels and glucose tolerance of the offspring, however, these effects were more pronounced in pups born from obese mothers compared to obese fathers (Ornellas et al. 2015). On the other hand, hepatic steatosis (fat accumulation in liver cells) was caused by aggravated lipogenesis in pups sired by HFD father compared to HFD mothers (Ornellas et al. 2015). In this context, the contribution of the mother alone has been extensively reviewed elsewhere (Burton and Fowden 2012, Aiken and Ozanne 2014, Sferruzzi-Perri and Camm 2016). Indeed, placental adaptations to maternal nutritional challenges (under- and over-nutrition) include placental weight (Coan et al. 2010) and nutrient transport (Jones et al. 2009). On the other hand, little is known about the contributions of the fathers alone.

There is some interesting evidence showing that paternal diet can influence offspring in both short- and long-terms. In fact, diet-induced paternal obesity impairs placental and foetal weight and foetal morphological development (Binder et al. 2012). Remarkably, paternal low-protein diet altered methylation patterns within their pups in mice, resulting in a dysfunctional lipid metabolism (Carone et al. 2010). In the same year of that publication, another research group found that obese and insulin resistant male rats sire offspring with impaired glucose tolerance and insulin secretion (Ng et al. 2010). Despite nutritional programming, paternal exposure to alcohol, drugs and toxins (Little 1987, Hales and Robaire 2001) as well as paternal age (including very young and old fathers) (Auroux et al. 1998, Lundstrom et al. 2010) may disrupt offspring's development and increase chances of health issues later in life. Interestingly, it appears that paternal genotype also influences offspring by regulating the release of placental hormones into the maternal bloodstream (Haig 1993) and placental growth (Curley et al. 2011). Although these studies suggest a paternal genetic influence on maternal metabolism (Hager and Johnstone 2003, Petry et al. 2007), new evidence imply that fathers can directly influence their offspring epigenetically.

1. 4. Metabolic programming on skeletal muscle and pancreas

This section presents key concepts related to insulin-stimulated glucose uptake and mitochondrial respiration in skeletal muscle as well as pancreatic morphology. Then, each of these topics will be incorporated in a discussion about the long-term metabolic effects of paternal HFD/obesity, maternal exercise and exercise early in life later in this chapter.

1. 4. 1. Insulin-stimulated glucose uptake in skeletal muscle

Skeletal muscle utilises approximately 85% of glucose under euglycemic state, which makes it the primary site for glucose uptake (DeFronzo and Tripathy 2009). Insulin is a potent physiological stimulus that promotes glucose uptake in skeletal muscle. The 'classical' insulin-stimulated glucose uptake pathway involves a series of signals. As this pathway has been extensively studied, the events will be only briefly described: The first committed step involves insulin biding to its receptor at the cell membrane level. This is followed by intracellular activation of the insulin-receptor substrate (IRS, isoforms 1 and 2 are the most common in skeletal muscle), which stimulates a series of kinase cascades. It starts with the phosphatidylinositide-3 kinase (PI-3K, a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit); this contains the Src Homology-2 (SH2) domain which binds to phosphotyrosine motifs and docks with IRS proteins (Backer et al. 1992). Once activated, the role of PI-3K is mainly to convert phosphatidylinositol 4,5 bisphosphate to phosphatidylinositol 3,4,5 trisphosphate ($PI(3,4,5)P_3$) by phosphorylation of these membrane glycolipids. This will result in the recruitment of serine/threonine protein kinases. The first one is the 3-phosphoinositide dependent protein kinase-1 (PDK1), which participates in the downstream activation of protein kinase B (PKB, also known as AKT) and C (PKC). AKT/PKB activation is involved in many other insulin responses (including glycogen synthase activity and mammalian target of rapamycin (mTOR)) (Whiteman et al. 2002), but only its main effects on insulin-stimulated glucose uptake are being discussed in this section. The signal then propagates to activate an AKT substrate of 160 kDa (AS160, more recently being called as TBC1 domain family member 4 – TBC1D4; and TBC1D1) (Middelbeek et al. 2013, Sakamoto and Holman 2008). Next, TBC1D4 activates a Rab GTPase protein (Tan et al. 2012a) which will release glucose transporter (GLUT) 4 and allows it to be translocated to the plasma membrane. Many phosphorylation sites were found to be stimuli-specific, being only phosphorylated via

insulin-stimulation (other sites are only responsive to contraction, or both). For instance, phospho-AKT^{Ser308} is a specific site for insulin only, whereas phospho-AKT^{Ser473} and phospho-TBC1D4^{Thr642} are responsive to both insulin and contraction (exercise) (Treebak et al. 2014). Once GLUT4 is docked to the sarcolemma (through complex interactions between several proteins), glucose can then enter the myocyte across the sarcolemma and T-tubule membranes by facilitated diffusion.

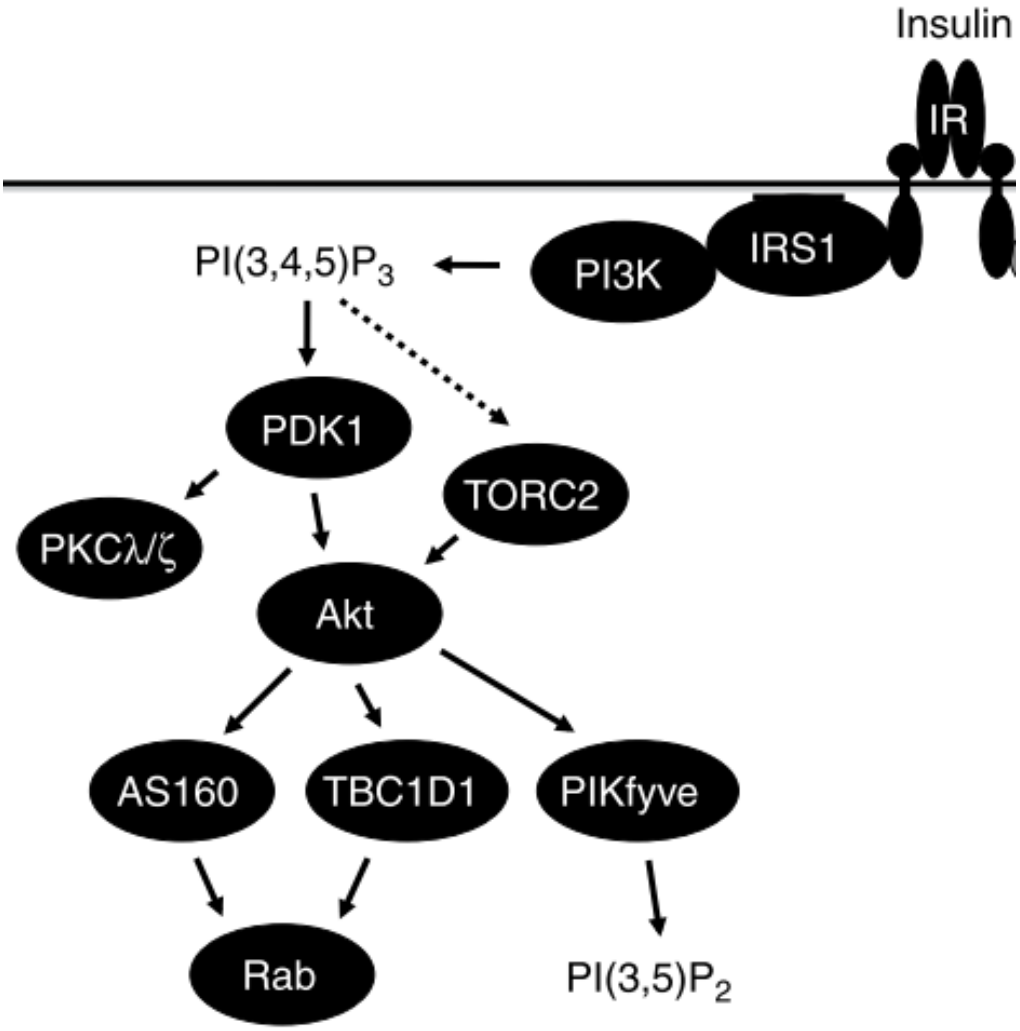

Figure 1-2. Proposed signalling pathway for insulin-stimulated glucose uptake implicated in GLUT4 translocation in skeletal muscle. See text above the detailed explanations for the insulin-stimulated glucose uptake signalling pathway. Adapted from (Leney and Tavare 2009).

1. 4. 2. **Mitochondrial respiration in skeletal muscle**

Mitochondria are the main source of energy production (in form of adenosine triphosphate, ATP) in eukaryotic cells through oxidative phosphorylation (Tzameli

2012). This organelle also plays a central role in cellular function, stress response, cytoprotection, and apoptosis, as well as in reactive radical biology (Duchen 2004). Interestingly, mitochondria is able to change its quantity and/or function in response to a variety of physiological conditions such as diet and exercise (Hancock et al. 2008, Tonkonogi and Sahlin 2002). Alterations in mitochondrial number and/or function have been linked to health problems such as T2DM (Lowell and Shulman 2005, Sivitz and Yorek 2010).

The main function of the mitochondria is to produce energy (ATP), which is made through cellular respiration. It consists of three metabolic processes: glycolysis, tricarboxylic acid cycle (TCA cycle; also known as the Krebs, or citric acid, cycle), and the electron transport system (ETS). The ETS produces ATP through the process of oxidative phosphorylation (OXPHOS), in a sequence of Complexes I–IV located in the mitochondrial inner membrane and arrayed in a supramolecular organization (Lenaz et al. 2006) (Figure 1-3). It is suggested that OXPHOS analysis is very important to increase our knowledge about mitochondrial physiology which can be used to perform functional diagnosis in health and disease (Gnaiger 2014). Developmental programming of mitochondrial function has being demonstrated in several models across different tissues (Warner and Ozanne 2010). In rats, intrauterine growth restriction by placental insufficiency, does not affect state 4 whereas reduces state 3 oxygen consumption in the adult offspring (Selak et al. 2003). Most of the research has focused on maternal effects as the conceptus inherits the mitochondria and its DNA from the maternal line (oocyte) (Cummins 2002). One might then argue that the paternal contribution in terms of mitochondrial programming would be minimal or perhaps irrelevant, but recent research has pointed otherwise.

Some research groups have proposed a link between mitochondrial function and insulin resistance in skeletal muscle (Montgomery and Turner 2015, Lowell and Shulman 2005). A decrease in mitochondrial content/volume, mitochondrial biogenesis, and/or expression of mitochondrial complexes, is likely to lead to decreased substrate oxidation. The Figure 1-3 highlights a proposed mechanism for this link. The diminished electron flow through the ETS can cause ultimately oxidative stress and damage, which can aggravate the decrease in substrate utilisation, leading to lipid accumulation. Lipid intermediates (diacylglycerols and ceramide) are thought to cause inhibition of the insulin signalling pathway, but this is not observed in all studies investigating this link between mitochondrial function and insulin resistance (Holloszy 2013, Martin and McGee 2014,

Fisher-Wellman et al. 2014). Therefore, it is important to investigate whether this relationship between mitochondria and glucose uptake in skeletal muscle is altered by enviromental factors early in life.

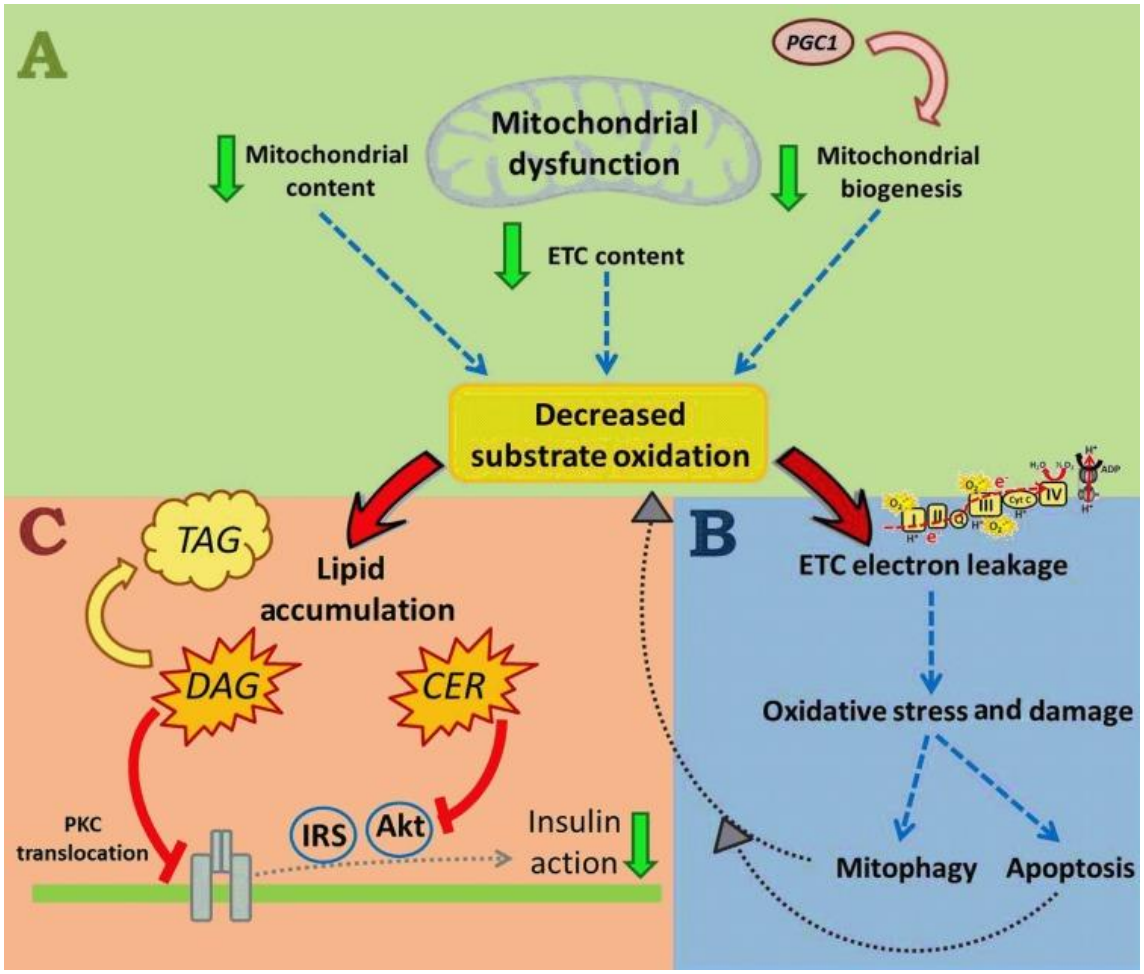

Figure 1-3. A proposed link between mitochondrial dysfunction and insulin resistance. Reduction in substrate oxidation leads to oxidative stress and damage to the mitochondria, which will increased lipid accumulation and impair the insulin signalling pathway. DAG, diacylglycerols. CER, ceramide. (Montgomery and Turner 2015)

1. 4. 3. **Pancreas morphology**

Since the discovery of insulin in 1920s (Bliss 2007) the diabetes research field have focused on the study of this hormone, including the biology of the pancreatic cells responsible for its production. This hormone is produced in the endocrine pancreas (islets of Langerhans), by cells that were first identified by Michael Lane in 1907 using a silver staining technique: “I will hereafter designate the cells in which… the granules are fixed

with the chrome-sublimate fluid as β-cells" (Lane 1907). Nowadays, it is known that pancreatic β-cells are the only physiological source of insulin and are the predominant islet cell type (Figure 1-4); hence, it has essential role for diabetes research. In fact, β-cell mass seems to decrease in T2DM regardless of body weight as demonstrated in pancreatic tissue from 124 autopsies including obese cases with T2DM and nondiabetic subjects, as well as lean subjects with T2DM and nondiabetic cases (Butler et al. 2003) (Figure 1-4). In complement to pancratic morphology, there are many other indices of insulin secretion and β-cell function that can be used *in vivo*. Some are glucose clamp-based indices (e.g. disposition index), other indices are based on glucose tolerance test, such as the insulinogenic index (increment of insulin divided by increment of glucose during first 30 min) and the insulin AUC (area under the curve) divided by glucose AUC (Saisho 2016). Single blood samples are also used to assess β-cell function when determined fasting C-peptide and its ratio to glucose (Saisho 2016).

The developmental programming of β-cells have been reported in the literature and some mechanisms have been proposed (Simmons 2007). The β-cell mass is not altered in IUGR rats at 2 weeks of age, however, by ~12 weeks of age, the IUGR group had lower β-cell mass (Stoffers et al. 2003). Genome-wide profiling in islets of IUGR rats identified over one thousand loci with altered DNA methylation (Thompson et al. 2010). Furthermore, IUGR rats show progressive decline in β-cell mass, with a complete absence of *Pdx1* (a transcription factor notably involved in pancreas development and β-cell differentiation) levels in adult rats along with glucose intolerance, which are both prevented by administration of a pancreatic β-cell trophic factor (exendin-4) during neonatal period (Stoffers et al. 2003). This highlights the plasticity of this tissue during early periods of life, and suggests that other stimuli may be effective in positively modulating β-cell phenotype. Therefore, it is important to investigate whether paternal HFD/obesity can negatively influence the islet and β-cell profile in adult offspring, and if maternal or early life exercise can protect against these effects.

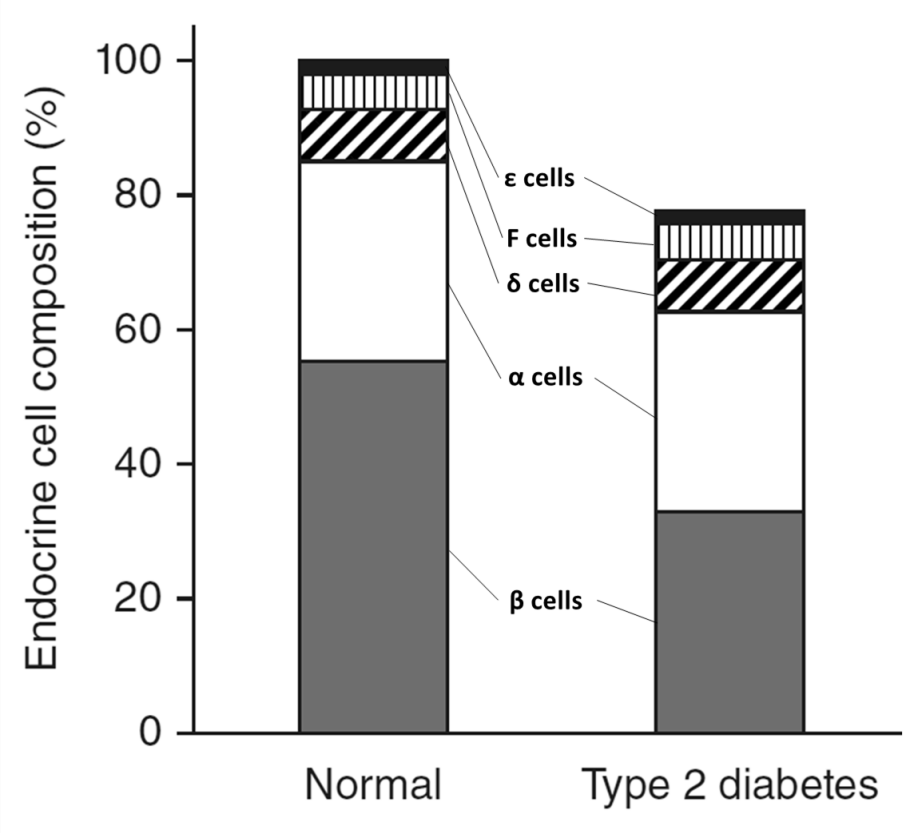

Figure 1-4. Human islet endocrine cell composition in normal (nondiabetic) subjects and those with type 2 diabetes mellitus. Cell types are indicated next to the bars. Adapted from (DeFronzo et al. 2015).

1. 5. Paternal obesity as a risk factor for offspring

According to the WHO, overweight and obesity are an abnormal or excessive fat accumulation likely to prejudice health (WHO 2013). Adults with a body mass index (BMI, kg/m^2) between 25.0 and 29.9 are classified as overweight, and individuals with values equal or above 30 are considered obese (WHO 2013). In 2014, around 39% of adult men aged 18 and over were overweight and 11% of the global male population were obese (WHO 2016a). As paternal phenotype can influence offspring's health, this data should be viewed with even more cautiousness.

The health status of the father at conception can affect the health of subsequent children. In fact, paternal smoking (Lee et al. 2009) and advanced age (van Balkom et al. 2012) have been associated with increased rates of leukaemia and autism, respectively, in subsequent offspring. Paternal BMI has been positively associated with young offspring BMI (5 up to 11 years old) (Li et al. 2009, Danielzik et al. 2002). Indeed, other studies have also found that paternal BMI seems to be a better predictor of childhood metabolic traits than maternal BMI (Figueroa-Colon et al. 2000). In a large cohort, birth

weight of children with mixed-ethnic parentage fell in between that of children with two European or two Indian parents, which can demonstrate that father and mothers contribute to ethnic differences in foetal growth (Wells et al. 2013). A cross-sectional study with over 3000 children aged 5-7 years found that obese fathers are more likely to father an obese child (Danielzik et al. 2002). Sharing the same environment postnatally though makes it rather difficult to separate genetic, epigenetic and environment contributions. Nevertheless, there is evidence that paternal obesity is significantly associated with lower methylation levels in genes important in normal growth and development (namely mesoderm-specific transcript, paternally expressed gene 3 and neuronatin) in the umbilical cord blood leukocytes of 92 newborns (Soubry et al. 2015). Additionally, hypomethylation at the IGF2 differentially methylated regions was associated with paternal obesity, which might suggest that father's phenotype may disrupt the genomic imprinting in germ cells (Soubry et al. 2013).

The effects of paternal HFD/obesity on offspring body weight, from the newborn to the adult rodent, is not clear. Some research groups found no alteration in litter size or birth weight (Masuyama et al. 2016, Ng et al. 2010), while other found lower birth weight (de Castro Barbosa et al. 2016). Recent findings suggest that HFD consumption in male rodents affect fertility and sperm quality (Palmer et al. 2012). In addition, newborn Sprague–Dawley rat offspring sired by father fed HFD (~43% energy from fat for 12 weeks before mating) had lower β-cell mass (de Castro Barbosa et al. 2016). Despite discrepant results in birth weight, studies in rodents sired by HFD fathers show discrepancy in body weight too, with some presenting lower body weight (Chowdhury et al. 2016), no difference (Ng et al. 2010, Ornellas et al. 2015), or higher body weight (Masuyama et al. 2016, Fullston et al. 2013) in adult offspring. Sex-specific differences might also play a role in this matter as early evidence showing male offspring had higher body weight while adult female's body weight did not change (Fullston et al. 2012). Nevertheless, the final outcome of paternal HFD seems to generally be associated with metabolic complications later in life.

Fathers consuming HFD sired F1 mice offspring with increased adiposity and insulin resistance, and more alarming, it also affected offspring metabolism in the F2 generation (Fullston et al. 2013, de Castro Barbosa et al. 2016). Fat mass was also increased at 16 weeks of age compared to pups born from control dads (Masuyama et al. 2016). The same research group also showed significant elevations in the homeostasis model assessment of insulin resistance (HOMA-IR), total triglyceride and leptin levels

while adiponectin levels were decreased in offspring of HFD fathers compared with controls at 24 weeks of age (Masuyama et al. 2016). When male mice were fed with HFD and mated with females fed standard chow, their F1 offspring later in life (from 8 to 39 weeks of age) were heavier, with increase in adiposity, serum triglycerides and reduced glucose tolerance and insulin sensitivity (Fullston et al. 2013). Conversely, there is also evidence reporting lower fat mass in adult offspring sired by HFD fathers (Chowdhury et al. 2016). Another study using rats showed no differences in body weight or fat mass at 12 weeks of age, but offspring sired by high-fat eating dads were still glucose intolerant in adulthood (Ng et al. 2010), with similar results in mice model of paternal HFD (Ornellas et al. 2015). These metabolic impairments in offspring appeared to be due to reduced pancreatic islet cell size, and differentially expressed islet genes related to insulin and glucose metabolism, mitogen-activated protein kinases (MAPK) and calcium signalling, as well as cell cycle (Ng et al. 2010). It has been suggested that transgenerational transmission might occur through epigenetic modifications to the sperm via changes to mRNA and non-coding RNAs, methylation or acetylation content (Rando and Simmons 2015, Fullston et al. 2012) (Figure 1-5). Recently, two independent experiments proposed sperm transfer RNA–derived small RNAs as a candidate to mediate paternal epigenetic factors that drives long-term metabolic consequences in offspring (Chen et al. 2016, Sharma et al. 2016).

As presented above, paternal HFD can alter offspring phenotype and increase the risk to metabolic diseases, such as T2DM. Female Sprague-Dawley rats sired from high-fat eating dads had higher levels of glucose during a glucose tolerance test. This was observed at 6 weeks of age and deteriorated at 12 weeks of age (Ng et al. 2010). As insulin levels were also high during the glucose tolerance tests in both ages, more attention was given to the pancreas of these offspring. Also the same study found no differences in the insulin tolerance test (measured at 0, 30, 60, 90, and 120 min) at 11 weeks of age (Ng et al. 2010). Other research groups, however, showed that female offspring (F1) sired from male mice fed a HFD had high glucose AUC during glucose tolerance tests at 8, 14, 18, 26 and 39 weeks of age (de Castro Barbosa et al. 2016, Park et al. 2015). Insulin AUC (indicating insulin sensitivity) was lower in these offspring at 16, 26, and 39 weeks of age. In fact, similar findings were reported in adult female offspring of HFD fed fathers, showing glucose intolerance, while male offspring was not affected (de Castro Barbosa et al. 2016). Although insulin tolerance test correlates well with insulin sensitivity, it does not differentiate between peripheral and hepatic insulin resistance (Monzillo and Hamdy

2003). It is worth to note that no difference was found in F1 adult offspring of father fed HFD at 12 weeks of age in the glucose area under the curve, but when offspring born from obese dads were also exposed to HFD, latent metabolic defects were reported (higher fasting insulin, hepatic triacylglyceride and diacylglyceride, and impaired glucose area under the curve) (Cropley et al. 2016). To date, no study has investigated whether insulin-stimulated glucose uptake in skeletal muscle is impaired in offspring sired by high-fat eating dads.

In terms of mitochondrial changes, a paternal obesity model using a combined method of *in vitro* fertilisation and 10 weeks of HFD in C57BL/6 mice, found significant changes in resultant embryo mitochondrial activity and kinetic development. The mitochondrial membrane potential of embryos was reduced, affecting embryo development and pregnancy (Binder et al. 2012). To the best of our knowledge, there are no studies investigating the long-term consequences on mitochondrial function in offspring sired by fathers fed HFD.

Paternal HFD has been shown to cause alterations in pancreas morphology and islets DNA methylation, which were associated with impaired insulin secretion in young adult female offspring (13 weeks of age) (Ng et al. 2010). Lower insulin levels were found at 15 min post glucose load during the glucose tolerance test, which might suggest that the β-cells of the offspring were not functioning normally. Paternal HFD contributed to an overall reduction in total islet area within female offspring (-23%). The percentage of small islets (0-5,000 μm^2) was also lower while the percentage of large islets (>10,000 μm^2) was higher compared to control offspring (Ng et al. 2010). A trend ($p=0.09$) for lower total β-cell area in offspring sired by high-fat eating dads was also reported. Mitochondria and oxidative stress may play pivotal roles in altering key cellular events within islets in female offspring sired by HFD fathers (Ng et al. 2014b). Over 640 islets genes were significantly different upon examining the pancreatic tissues in female Sprague-Dawley offspring rats sired by father on HFD compared to control fathers (Ng et al. 2010).

If nothing changes, a 'never-ending obesity cycle' might take place, where obese fathers of today would generate offspring with higher risk to develop diabetes and, likely, obesity tomorrow, and so on (Kappeler and Meaney 2010). It has been proposed that intervention during 'windows of opportunity' (Figure 1-1) might be an effective way to counter these negative programming effects. Various studies indicated that epigenetic processes are potentially reversible in animal models, by manipulation of diet or

endocrine methods (Vickers et al. 2005, Gluckman et al. 2007, Waterland et al. 2008, Street et al. 2015). This may open up a space for other lifestyle interventions to be tested.

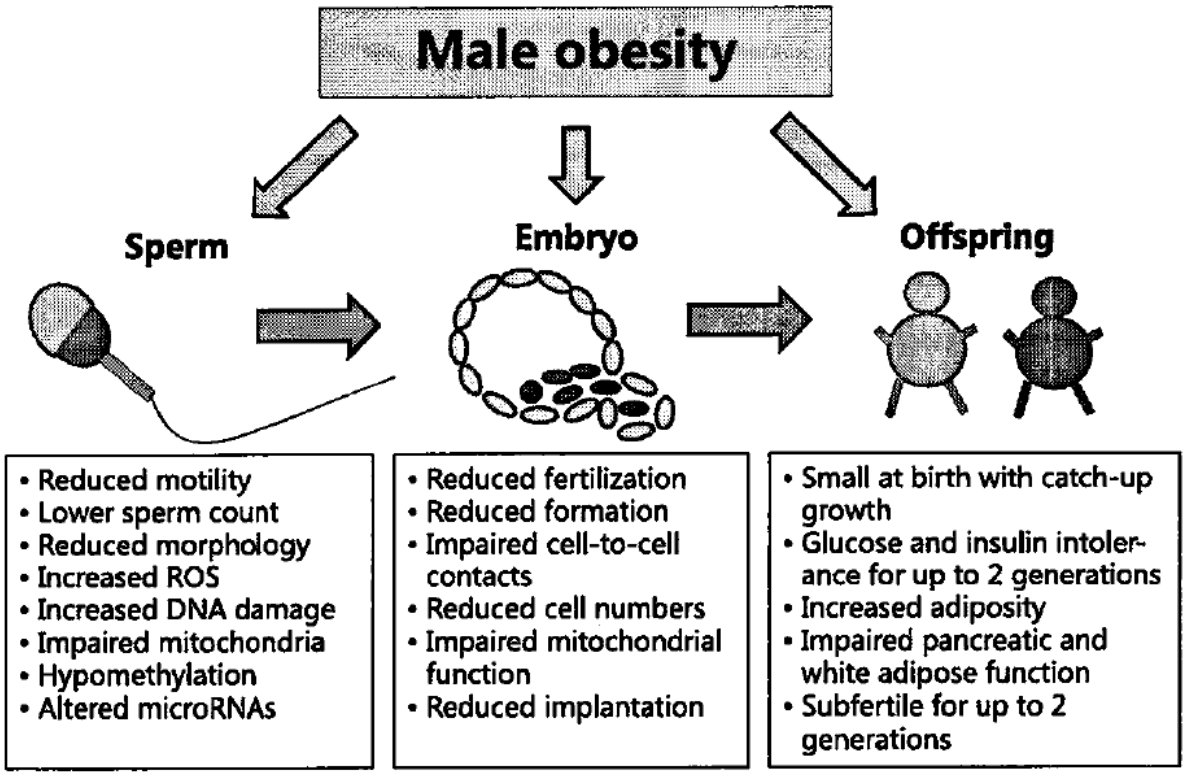

Figure 1-5. Proposed effects of paternal obesity on sperm, embryo development and offspring's health. Male phenotype before conception can influence the offspring at different levels (McPherson et al. 2014). Note: Small birth weight and increased adiposity are controversial findings, with some research groups reporting different outcomes as discussed above.

1. 6. Maternal exercise and long-term effects on offspring

Exercise is known to be a potent regulator of a range of physiological processes and metabolic pathways. During pregnancy, exercise is recommended by several organisations if there is no medical complication during gestation (Artal and O'Toole 2003, Zavorsky and Longo 2011, Artal 2016, Szymanski and Satin 2012). However, little research has been done in terms of the effects of maternal exercise on the offspring. Taking into consideration the early life stages of life is susceptible to environmental cues as discussed above (Figure 1-1), it is possible that exercise during the 'window of opportunity' might influence the phenotype later in life. In fact, we (Falcao-Tebas et al. 2012a, Falcao-Tebas et al. 2012b, Fidalgo et al. 2012, Leandro et al. 2012b) and other

groups (Laker et al. 2014a, Stanford et al. 2015, Carter et al. 2013) have proposed that physical activity before and during pregnancy can act as an environmental factor able to induce long-term effects on the phenotype plasticity of the offspring.

Maternal effects are expected to play an essential role on offspring's phenotype since the foetus experiences direct interaction with the mother through the placenta (Power and Schulkin 2013). There is a redistribution of blood flow to peripheral tissues (working muscles and skin), an increase in core temperature and reduced maternal substrate levels for a short time. Several systematic reviews and meta-analyses, however, have shown that regular exercise does not promote early pregnancy loss, stillbirth, or neonatal death, and is likely to increase the chance for normal delivery, lower incidence of gestational diabetes mellitus and hypertensive disorders among healthy pregnant women (Kramer and McDonald 2006, Poyatos-Leon et al. 2015, Lokey et al. 1991, Di Mascio et al. 2016). The short-term effects of maternal exercise on offspring birth weight and body composition in humans were summarised recently (Hopkins and Cutfield 2011) (Figure 1-6), showing that intensity, volume and timing when maternal exercise is initiated may be a critical component to understand its effects on offspring's phenotype.

It is not clear whether maternal exercise is beneficial to the offspring, as studies in humans are somewhat controversial, showing decrease or increase in birth weight, with or without alteration in lean and fat masses (Figure 1-6). Most of the research in humans only investigates the short-term effects of exercise on placental development, newborn body composition or early childhood (Hopkins and Cutfield 2011). Clinical trials of maternal exercise showed improvements in foetal-placental oxygenation and nutrient supply, as well as advanced neurobehavioral maturation in offspring (Melzer et al. 2010, Clapp Iii 2003, Clapp et al. 2002). Animal studies show similar discrepancy in terms of the outcomes of maternal exercise. In fact, studies have shown negative, neutral and positive effects of exercise during pregnancy. Adult offspring (7-months old) of Wistar rats that performed exercise (55% of maximal aerobic speed) until delivery had lower glucose-simulated insulin secretion, along with reduced protein expression in insulin pathway activation in skeletal muscle and whole body glucose intolerance (Quiclet et al. 2016). Maternal physical activity (running wheel) before and during pregnancy did not change the percentage of fat and lean masses in at 3 weeks of age compared to offspring born from sedentary mothers (Kelly et al. 2015). However, other research groups have found substantial improvement in young and adult offspring phenotype due to maternal

physical activity alone compared to their respective sedentary controls (Carter et al. 2012, Carter et al. 2013, Raipuria et al. 2015).

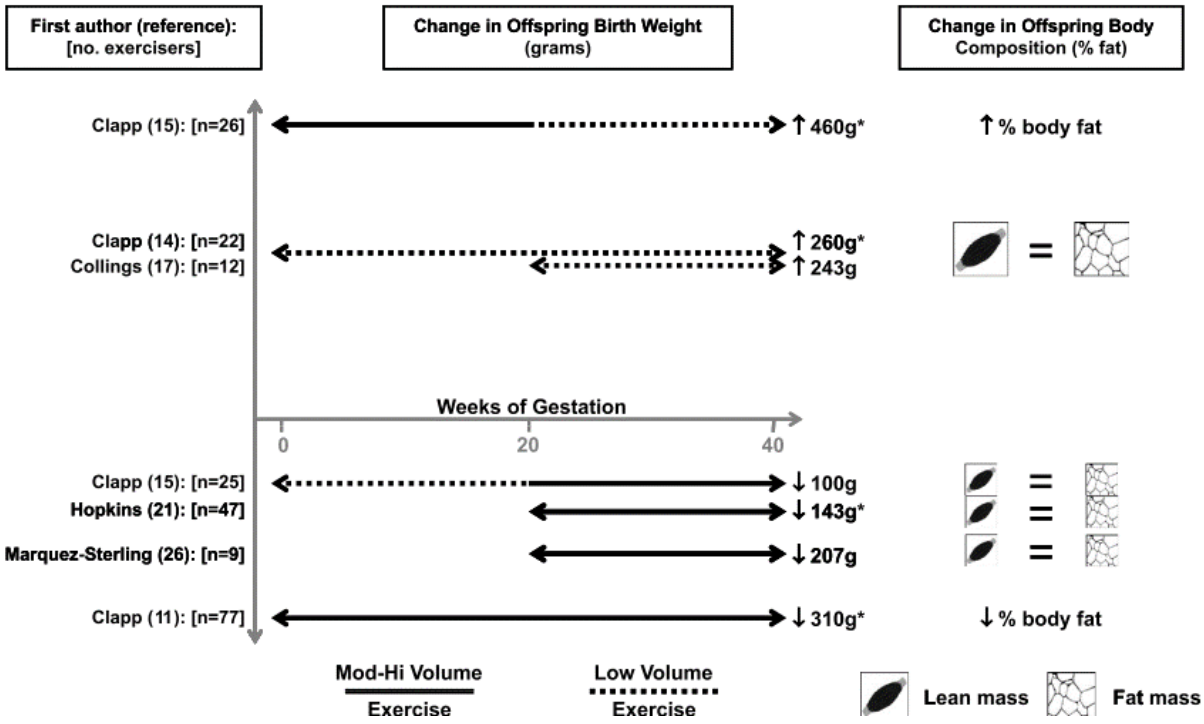

Figure 1-6. Maternal timing- and volume-dependent exercise effects on birth weight and body composition. This review summarised several studies in humans (first authors on the left side, followed by the number of subjects in each study) that used low volume (dotted lines) of exercise throughout gestation and/or started at moderate to high (solid lines) volume of exercise and reduced to low volume in the second half of pregnancy seems to increase birth weight compared to control offspring from sedentary mothers. On the other hand, maintaining a moderate to high volume of exercise (solid lines) during gestation leads to reduced birth weight (middle of the figure). Data about body composition, including lean and fat masses were also presented for their respective studies (right side). Lean mass (skeletal muscle figure), fat mass (adipocytes), and changes in offspring body composition were not significant if lean mass were equal fat mass (=). *Significant change in offspring birth weight according to the original research ($P < 0.05$). (Hopkins and Cutfield 2011).

In rodents, voluntary physical activity or exercise training positively modulates foetal growth and postnatal body weight, biochemical markers and glucose homeostasis (Carter et al. 2012, Rosa et al. 2011, Carter et al. 2013). However, like in humans, these induced-changes in phenotypes may be dependent on the physical fitness of the mother, the time point in the pregnancy when the exercise is carried out and the frequency, duration, and intensity of the exercise (Clapp et al. 2002, Clapp Iii 2003). We have previously demonstrated that exercise training before (5 d/week and 60 min/d, at 65% of VO_{2max}) and during (5 d/week and 20min/d, at 40% of VO_{2max}) pregnancy leads to

positive changes in the growth patterns, nervous system maturation and biochemical markers in adult offspring (up to 21 weeks of age) (Falcao-Tebas et al. 2012a, Falcao-Tebas et al. 2012b, Fidalgo et al. 2012, Leandro et al. 2012b). Physical activity/exercise before and during gestation is able to positively modify the health status of the offspring later in life in healthy (Carter et al. 2012, Carter et al. 2013) and obese mothers (Laker et al. 2014a). However, if exercise is performed in high volume and intensity, especially towards the end of gestation, this may decrease birth weight (Hopkins and Cutfield 2011). One study suggests that these effects are clearer when maternal physical activity is performed before and during pregnancy compared with before or during gestation only (Stanford et al. 2015). Although both female and male offspring are benefited by maternal physical activity/exercise, a sex-dependent response is observed. In rodents, the positive effects on glucose tolerance were reported with no changes in body composition in female offspring (Carter et al. 2012, Carter et al. 2013, Raipuria et al. 2015), while in male offspring both improvements in body composition and insulin sensitivity and secretion are noted (Laker et al. 2014a, Raipuria et al. 2015, Rosa et al. 2013, Stanford et al. 2015, Vega et al. 2015, Falcao-Tebas et al. 2012b, Fidalgo et al. 2013).

In terms of maternal exercise, previous studies have shown that exercise during pregnancy alone improve offspring glucose tolerance test (Carter et al. 2012, Stanford et al. 2015, Vega et al. 2015, Carter et al. 2013) or might have no effects (Rosa et al. 2013, Fidalgo et al. 2013, Laker et al. 2014a). Despite the heterogeneity of the methods in the studies, maternal exercise is generally associated with improvements in offspring's phenotype later in life (across different rodent models and physical activity protocols), which leads to increases of insulin sensitivity. There are evidence in rodents that maternal physical activity (running wheel) can increase RNAm of GLUT4 in young animals (Raipuria et al. 2015) and improve hyperinsulinemic-euglycemic clamp, glucose uptake in isolated skeletal muscle (but not heart) and fat tissues in adulthood (Carter et al. 2012, Carter et al. 2013). Although some studies have reported the protective effects of maternal exercise when associated with other stimuli (for instance, maternal undernutrition or maternal HFD during gestation) (Falcão-Tebas et al. 2012, Falcao-Tebas et al. 2012b, Fidalgo et al. 2013, Laker et al. 2014b), little is known about its effects on offspring sired by fathers fed with HFD before conception.

Only a few studies using maternal physical activity/exercise evaluated mitochondrial parameters in offspring. Swimming exercise before and during pregnancy increased antioxidants (superoxide dismutase, catalase, and glutathione peroxidase) and

increments mitochondrial mass and membrane potential in the different regions of the pups' brain (Marcelino et al. 2013). In terms of skeletal muscle, maternal physical activity (running wheel) increased mRNA expression of PGC1α in both male and female offspring 3 weeks postnatally. However, these effects were lower or not observed in offspring born from high-fat eating mothers who performed physical activity, demonstrating a sex-dependent response (Raipuria et al. 2015). In adult mice though, similar effects were not found as maternal exercise alone did not change expression of PGC1α in skeletal muscle. However, it is important to mention that maternal physical activity before and during pregnancy completely prevented the low expression of PGC1α in the adult offspring caused by maternal HFD (Laker et al. 2014a).

There are no studies investigating pancreatic morphology or β-cell function in offspring born from physically active/exercise dams. By isolating islets and performing *in vitro* insulin secretion experiments, we have previously showed that exercise training before and during gestation increased insulin secretion in the dams in late pregnancy (Leandro et al. 2012c). Whether similar benefits are also found in the offspring later in life is yet to be discovered.

A possible explanation might involve direct changes in maternal-foetal environment, including placental adaptations to exercise, without necessarily involving epigenetic modifications. More studies are required to clarify our understanding about how maternal exercise benefits offspring's health. Nonetheless, whether maternal exercise-induced changes can normalise glucose intolerance in offspring born from HFD fed fathers (Ng et al. 2010), still need to be clarified. Moreover, whilst the gestational period is crucial to the development of the foetus, the early postnatal environment is also important as many organs continue to grow and mature, highlighting further opportunities for lifestyle interventions.

1. 7. Effects of exercise early in life on adulthood

Another potential 'window of opportunity' occurs during childhood, when there is a high capacity to observe phenotype plasticity in response to environmental factors (Figure 1-1). Although there is evidence that epigenomic marks are possibly reversible in adulthood (Weaver et al. 2005), the majority of the studies support the hypothesis that timely intervention early in life might be more effective (Street et al. 2015, Hanson and Gluckman 2014). Current exercise is a lifestyle intervention capable of ameliorating the

prevalence of T2DM. In fact, pre-diabetic patients (with impaired glucose tolerance) who exercise regularly have a significant delay of the onset of the disease or can completely prevent it (Tuomilehto et al. 2001). The timing when this exercise is performed though, appears to be critical as physical activity seems to decline across age between childhood and adulthood (Troiano et al. 2008). Healthy behavioural changes that last for prolonged periods are difficult and not often achieved by imposing better lifestyle habits, including forcing adults to do more exercise and eat healthier foods (Sturm 2002). Therefore, it is important to take action early in life, as children might be more likely to adhere to physical activity programs, and it seems that kids can lose and maintain weight easier than adults (Epstein et al. 1995).

Due to the high potential of plasticity in the early postnatal life (Wells 2007b, Godfrey et al. 2010), perhaps offspring exercise during this period can induce healthy physiological traits related with improved control over glucose homeostasis. A retrospective study with 1436 adults found that physical activity in youth was associated with lower rates of occurrence of T2DM (and hypertension) in adulthood, independent of current physical activity (Fernandes and Zanesco 2010). In addition, an exercise training program (4 days/week for 12 weeks) in post pubertal obese adolescents decreased calculated insulin resistance and decreased visceral and hepatic fat accumulation (van der Heijden et al. 2010). Sedentary low birth weight girls (but not boys) had higher levels of plasma leptin (a circulating hormone mostly produced by the adipose tissue), however, in the other group of low birth weight girls who meet the physical activity recommendations, the leptin levels were normal (Labayen et al. 2013).

In rodents, exercise early in life has been shown to be a potential regulator of later life phenotype in a range of tissues, including gut microbiota, fat, pancreas, skeletal and cardiac muscles (Mika et al. 2015, Gatford et al. 2014, Siebel et al. 2012). Interestingly, starting physical activity early in life (3 weeks of age) resulted in reduced food intake adjusted for lean mass despite higher energy expenditure due to exercise, in a fat mouse predisposed to develop obesity and metabolic syndrome (Wagener et al. 2012). Our model of early-life exercise training (5-9 weeks of age, treadmill running) normalised the decrease in relative islet surface area and β-cell mass and part of these effects were still observed in adulthood in male IUGR Wistar rats (24 weeks of age) (Laker et al. 2011). However, early exercise did not demonstrate a persistent effect on skeletal muscle mitochondrial biogenesis since the increase in markers observed at 9 weeks of age were not observed at 24 weeks of age following cessation of training (Laker

et al. 2012b). Thus, although we have demonstrated that some long-term effects of early exercise were better than exercising in adulthood, and are greater on the pancreas than skeletal muscle (e.g. relative β-cell mass, and islet surface area) (Laker et al. 2011), further studies are required to understand the mechanisms involved. Voluntary post-weaning exercise (3-10 weeks of age) in Sprague-Dawley rats lowered the area under the curve (AUC) during a glucose tolerance test, 5 weeks after cessation of exercise, with similar results observed even when offspring was born from obese dams (Caruso et al. 2013). In fact, only 3 weeks of exercise commenced immediately after weaning is required to provide long-lasting protective effects on genetically predisposed rats to become obese, even after exposure to a high-fat/caloric diet (Patterson et al. 2008). However, to date, there is no data investigating how offspring born from obese fathers respond to early-life exercise training in terms insulin secretion and insulin sensitivity in adulthood.

When applied early in life, exercise may promote long-term effects on glucose tolerance. One of the main conclusions in our recent review (Gatford et al. 2014) was that exercise in healthy population might not be as effective as it is in population with high risk to develop metabolic diseases (e.g. low birth weight individuals), but both have lasting long effects on metabolism. Sprague-Dawley rats were exposed to a running wheel for 3 weeks since the postnatal week 4 and remained sedentary until 14 weeks of age. No differences in body weight, subcutaneous and retroperitoneal fat, plasma leptin, nor glucose or insulin AUC during a glucose tolerance test (Sun et al. 2013). However, when the same exercise model was applied in pups born from mothers that consumed HFD during pregnancy and lactation (which promoted increase in body weight, fat masses, leptin) it normalised subcutaneous and retroperitoneal fat as well as leptin levels (Sun et al. 2013). Otsuka Long-Evans Tokushima Fatty (OLEFT) rats (characterized by insulin resistance, accumulated intra-abdominal fat, dyslipidaemia, and T2DM) that performed exercise early in life (5-19 weeks of age), had lower HOMA-IR, fasting glucose levels compared to the OLEFT sedentary rats (Shindo et al. 2014). Although the benefits of exercise early in life have been reported in different animal models, there is no data available in offspring sired from high-fat eating fathers. Also, it is currently unknown whether early exercise has sustained effects on insulin-stimulated glucose uptake in skeletal muscle and its associated mechanisms in adulthood.

Exercise early in life acutely increases skeletal muscle protein expression of PGC-1α, hydroxyacyl-CoA dehydrogenase β-subunit (β-HAD), and citrate synthase

enzyme activity, as expected (Laker et al. 2012a). In this study, no sustained effects were observed on these skeletal muscle mitochondrial markers in adult offspring (males, 24 weeks of age) following exercise from 5-9 weeks of age (Laker et al. 2012a). However, another animal model used OLEFT rats which performed exercise from 5 to 19 weeks of age in a running wheel and found changes in mitochondrial parameters. At 46 weeks of age, the rats in the early exercise group demonstrated higher RNA expression levels of key regulatory enzymes in the energy-generating metabolic pathway (citrate synthase, succinate dehydrogenase, and phosphofructokinase) compared with control rats (Shindo et al. 2014). It is not clear whether exercise early in life alter mitochondrial function and associated markers, and more studies are required to clarify if permanent effects are observed after exercise cessation early in life.

Exercise early in life has important effects on insulin secretion later in life. The first-phase of insulin secretion during a glucose tolerance test is a whole-body physiological indication of β-cell response. At 24 weeks of age, the first-phase insulin secretion was reduced (~75%, but not significant) in sedentary IUGR offspring compared with sedentary controls, but exercise early in life did not prevent this effect (Laker et al. 2011). Interestingly, in the same model, these Wistar–Kyoto rats exposed to uteroplacental insufficiency, performed exercise early in life (5 days/week, from 5 to 9 weeks) and were examined for any potential improvements in β-cell morphology. Exercised early in life rats had their β-cell mass fully restored, compared with the deficient β-cell mass (~65%) and pancreatic islet surface area of IUGR rats at 24 weeks of age (Laker et al. 2011). In the same study, it was also described that early in life exercise did not affect the gene expression of Pdx1, GLUT2, IRS2, and insulin-like growth factor 1 receptor (IGF1R) in whole pancreas of IUGR rats (Laker et al. 2011). It is important to note that β-cells make up only a small percentage of the pancreas, thus gene/protein expression should be measured in isolated islets whenever possible. Nonetheless, this demonstrates the remarkable long lasting protective effects of exercised early in life to normalise pancreatic islets phenotype later in life, although the mechanisms are yet to be explained.

Studies suggest that being physically active is advantageous in regards to insulin (and leptin) resistance in health population. More importantly is the fact that in people with high risk of developing metabolic diseases, such as individual with low-birth weight (LBW), are able to respond to exercise and obtain similar or even greater benefits (Eriksson et al. 2004, Labayen et al. 2013, Ortega et al. 2011). We published a recent

comprehensive review of how exercise (in both early life and adulthood) has been used as an intervention to improve metabolic health via developmental plasticity (Gatford et al. 2014).

1. 8. Aims and hypotheses

The overall aim of this PhD research was to determine whether the negative metabolic effects in adulthood of offspring sired by HFD/obese fathers could be offset by:

1) Maternal exercise before and during pregnancy (Chapter 3); or,
2) Offspring exercise early in life (Chapter 4).

The general hypothesis was that both maternal exercise and offspring exercise early in life would attenuate the negative metabolic effects of paternal HFD/obesity in adult offspring. Each chapter with original data will present their own specific aims and hypothesis.

Chapter 2 – Methods

2.1 Overview

A schematic design of the experimental studies is shown in Figure 2-1. Male breeders were fed with high-fat diet or normal rat chow diet before conception. Mothers were exercised on a treadmill before and during pregnancy, or remained sedentary (Chapter 3). Thus, four groups were generated based upon paternal and maternal manipulations only:

- Normal diet fathers, sedentary mothers, sedentary offspring (NS, Chapter 3 and 4);
- High-fat diet fathers, sedentary mothers, sedentary offspring (HS, Chapter 3 and 4);
- Normal diet fathers, exercised mothers, sedentary offspring (NE, Chapter 3);
- High-fat diet fathers, exercised mothers, sedentary offspring (HE, Chapter 3);

From sedentary mothers, a cohort of offspring that were either sedentary or exercised early in life was designed (Chapter 4). Therefore, four groups of rat offspring were generated based upon paternal, or their own manipulation:

- Normal diet fathers, sedentary mothers, sedentary offspring (NS, Chapter 3 and 4);
- High-fat diet fathers, sedentary mothers, sedentary offspring (HS, Chapter 3 and 4);
- Normal diet fathers, sedentary mothers, exercised offspring (NE, Chapter 4);
- High-fat diet fathers, sedentary mothers, exercised offspring (HE, Chapter 4).

It is important to mention that Chapter 3 and 4 share the same data for the NS and HS groups, the differences between these chapters are the exercised groups (NE and HE, as outlined above). It was decided to not include the following groups: normal diet dads, exercised mothers, exercised offspring and high-fat diet dads, exercised mothers, exercised offspring as it would have been too many experimental groups to obtain and analyse during the PhD timeline. Also, it would be beyond the scope of the main research questions in this project. In order to replicate and expand previous experiments (Ng et al. 2010), only female offspring were investigated focusing on glucose homeostasis including insulin sensitivity, mitochondrial function and pancreas morphology. In addition, we used the same high-fat diets, and evaluated the offspring at the same age (11

and 12 weeks of age), using similar protocols for insulin and glucose tolerance tests (Ng et al. 2010).

This methods section describes in detail the experimental procedures performed in Chapter 3 and Chapter 4, and appendixes are available with additional information. Each chapter, however, has a brief description of the experimental techniques used to facilitate the reading flow.

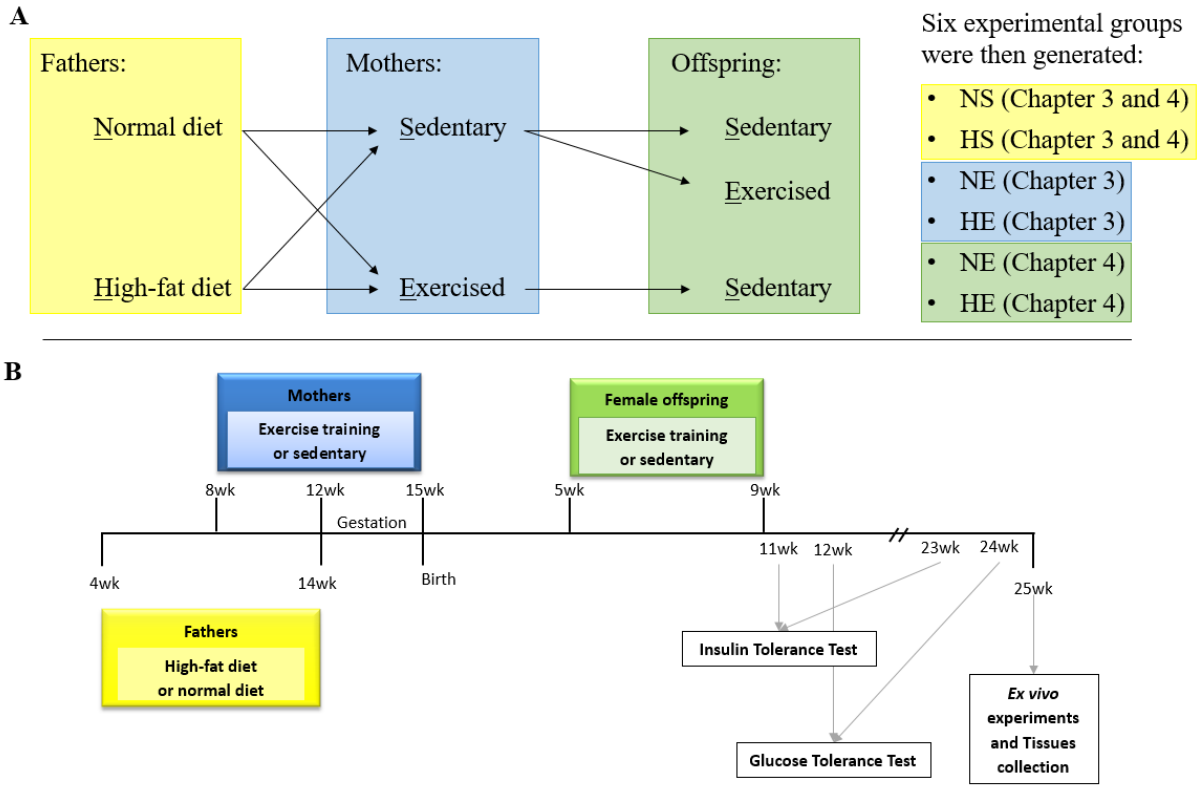

Figure 2-1. Experimental groups and timelines. A. Schematic diagram of breeding performed to produce the six experimental groups. B. Timeline of the experiments, including fathers (yellow), mothers (blue) and offspring (green) interventions and procedures. Normal diet dads, sedentary mothers, sedentary offspring (NS, Chapter 3 and 4); High-fat diet dads, sedentary mothers, sedentary offspring (HS, Chapter 3 and 4); Normal diet dads, exercised mothers, sedentary offspring (NE, Chapter 3); High-fat diet dads, exercised mothers, sedentary offspring (HE, Chapter 3); Normal diet dads, sedentary mothers, exercised offspring (NE, Chapter 4); High-fat diet dads, sedentary mothers, exercised offspring (HE, Chapter 4).

2.2 Animals

All procedures were performed according with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013), after ethics approval from

the Victoria University Animal Ethics Committee (AEC#13/008), Victoria, Australia. Sprague Dawley rats were obtained from the Animal Resources Centre (Western Australia, Australia) and kept in plastic cages at the Footscray Park Campus Animal Facility - Victoria University. Throughout the experiments, all animals were monitored daily by a member of the research team, animal welfare officer or animal technicians. The rats were exposed to a 12 hour light-dark cycle (lights on at 0700 h) and standard environmental conditions of 18-22°C and ~50% relative humidity. All animals had access to food and water *ad libitum*, as well as nesting materials and enrichment items. Male and female breeders were acclimatised to the animal facility and their cages for 5 days with no procedures performed during this period.

2.3 Procedures conducted in parents

2.3.1 Fathers

2.3.1.1 Diets

Twenty-eight male breeders were obtained at 3 weeks of age and equally allocated into control diet or HFD groups randomly. All males rats were mated, and out of the 14 rats in each group, n=9 control diet and n=10 high-fat diet rats mated successfully. Measurement of body weight was conducted weekly. Food consumption was quantified weekly by the difference between the amount of food offered and the amount of food remaining 24 h later, over two consecutive days. Body and food weights were recorded with a 0.01 g accuracy digital scale (ACBplus-600H or AQT-1500, Adam Equipment, U.K.).

Normal diet – Control fathers were fed with a standard control chow diet *ad libitum* throughout the experiments (catalogue name: 'Meat Free Rat and Mouse Diet', with 12.0% energy from fat, Specialty Feeds, Western Australia, Australia).

High-fat diet (HFD) – As per Ng et al. 2010, from 4 to 13 weeks of age, male rats were fed with high-fat diets *ad libitum* (SF01-025 and SF03-020, with 40.7% and 43.0% energy from fat; Specialty Feeds, Western Australia, Australia; detailed diet composition is presented in the Appendix - Diet composition, pages 177-181). Pellets from two different lots of diet were analysed by Agrifood Technology Pty Ltd. (Werribee, Australia) in order to confirm the content of macro and micro nutrients. Diet compositions were similar to that described by the manufacturer.

Table 2-1. Composition of the diets provided to the male breeders from 4 to 14 weeks of age, before mating.

	Control diet	High-fat diets	
		SF01-025	SF03-020
Protein (%)	20.0	19.4	17.0
Fat (%)	4.8	22.3	23.0
Crude fibre (%)	4.8	5.3	4.7
Carbohydrate (%) *	55.3	40.7	49.7
Moisture (%) *	10.1	7.6	3.3
Ash (%) *	5.0	4.7	2.3
Digestible energy (MJ/Kg)	14.0	18.5	20.0

* Determined by private analysis of the diets, not provided by the manufacturer. Both private and manufacturer analyses had very similar outcomes.

2.3.1.2 Mating

At 12 weeks of age, female rats were moved into male cages for breeding. The mating occurred between 0800 h and 1700 h (during the light cycle). In this period, male and female rats were fed with normal diet only. Food intake was measured during the mating period, as rodents are nocturnal, only 5 g of normal diet were consumed while both male and female were in the same cage (independent of father's diet before mating). One male bred with one female rat only.

2.3.1.3 Intraperitoneal Insulin Tolerance Test (IPITT)

In the week following mating was confirmed, male rats were subjected to an IPITT. The test was performed at 1000h, 2 hours after food was removed. Glucose level was determined at 0 minutes in blood samples collected by the tail vein using a portable glucometer (Accu-Chek Performa Nano, Roche Diagnostics, Mannheim, Germany). Then, insulin (0.5 to 1 U/kg based on predicted insulin resistance (Ng et al. 2010); Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was given intraperitoneally (IP) and blood glucose level was measured by a single drop from tail puncture at 20, 40 and 60 min.

2.3.1.4 Intraperitoneal Glucose Tolerance Test (IPGTT)

Three to five days after the IPITT, an IPGTT was performed after an overnight fast (1700 h to 0800 h). Animals were weighed in the morning and the amount of glucose required calculated (Glucose, 50%w/v, Intravenous Infusion, Phebra, New South Wales, Australia; 1g/kg of body weight). Rats were pre-warmed under an incubator at 38ºC for 5 min to facilitate blood flow to their tails. Animals were then gently restrained using a thin tea towel. By using 1 ml heparinised syringe (1000 IU/ml heparin, Pharmacia, New South Wales, Australia) and 25G needle, approximately 0.15 ml of blood was drawn. Glucose was then administered intraperitoneally in the right lower abdominal quadrant. Tail slice was performed by doing a transversal cut approximately 1/5 of the tail (approx. 3 cm from the tip of the tail) for glucose measurement at 15, 30, 60 and 90 min. Glucose levels in the samples were was measured using a hand held glucometer and blood was placed on ice and spun in a centrifuge at 3000 RPM for 10 min at 4ºC for further plasma insulin analysis. Area under the curve (AUC) of blood glucose and plasma insulin was calculated based on the trapezoidal rule (Fidalgo et al. 2013).

2.3.1.5 Post-mortem and tissue collection

Between 3-5 days after the IPGTT, male breeders were anesthetised (60 mg/kg IP; pentobarbitone, Virbac, New South Wales, Australia) and body weight and length, and abdominal circumference were recorded. They were then euthanized by cardiac puncture and had blood collected. Additionally, epididymal, retroperitoneal and subcutaneous fats, as well as liver, kidneys, heart, and spleen were dissected and weighted.

2.3.2 Mothers

2.3.2.1 Exercise before and during pregnancy

Female breeders were obtained at 6 weeks of age and were acclimatised for 5 days to the animal facility. All the rats were acclimatised to the treadmill and then either randomly placed in sedentary or exercise training groups. Measurements of body weight and food intake were conducted weekly prior to gestation and daily during pregnancy and lactation, as described in section 2.3.1.1.

Sedentary group – Sedentary rats were placed in the same room near the treadmill during the same time as the treadmill running was being undertaken for the exercise training group. This was done so that the cage handling and environmental

stresses (e.g. treadmill noise) occurred in both the sedentary and the exercise training groups. Non-exercised females were also removed from their cages to mimic the handling experienced by the exercised group.

Exercise training group – At 7 weeks of age, female breeders performed treadmill acclimatisation. The exercise training was performed in a separate room within the same animal facility. The rats were acclimated over 5 sessions, during 3 consecutive days as per below:

Session		Protocol
1st	(Wednesday) -	1.80 m/min up to 7.50 m/min for 10 minutes
2nd and 3rd	(Thursday) -	1.80 m/min up to 8.40 m/min for 15 minutes
4th and 5th	(Friday) -	1.80 m/min up to 9.96 m/min for 20 minutes

Rats were then randomly assigned into the experimental groups. Trained females were subjected to a protocol of moderate exercise training (treadmill, 5 d/wk, and 60 min/d, estimated at ~ 65-75% $\dot{V}O_{2max}$) from 8 to 12 weeks of age before mating (Amorim et al. 2009). During pregnancy, the intensity and duration of exercise were progressively reduced (5 d/wk, 50–20 min/d, at ~ 65–50% $\dot{V}O_{2max}$) up to 19 days of gestation (Amorim et al. 2009). The training consisted in a warm-up period (5 min), training zone period (20-50 min) and a cool-down period (5 min). Such a protocol has been shown previously to induce protective effects in offspring born from undernourished mothers (Falcão-Tebas et al. 2012, Fidalgo et al. 2013). Table 2-2 and Table 2-3 detail the full protocol adapted from previous studies (Amorim et al. 2009, Bedford et al. 1979). There was no exercise training during lactation. It is also important to mention that no electric shock was used during the exercise training. Only bursts of gas and gentle touch on the animal tail were utilised, especially during the acclimatisation and 1st week of training as these stimuli were barely needed after this period. The burst of air was not used during pregnancy as the rats were already conditioned to run at this stage. The exercise training was performed during the morning between 0700 h and 1000 h.

Table 2-2. Exercise training protocol before pregnancy.

Week No.	Speed (m/min)	Grade (º)	Percentage of $\dot{V}O_{2max}$ #	Duration of each stage (min)	Total time (min)
	4.98	0		5	
1	12.6	0	~ 60	20-40 *	30-50
	4.98	0		5	
	6.66	5.0		5	
2	15.18	5.0	~ 64	50	60
	6.66	5.0		5	
	8.28	10.0		5	
3	17.40	10.0	~ 70	50	60
	8.28	10.0		5	
	8.28	10.0		5	
4	19.26	10.0	~ 76	50	60
	8.28	10.0		5	

* Increasing 5 min per day. # Estimated percentage of $\dot{V}O_2$max based on previous studies (Bedford et al. 1979).

Table 2-3. Exercise training protocol during pregnancy.

Week No.	Speed (m/min)	Grade (º)	Percentage of $\dot{V}O_{2max}$ #	Duration of each stage (min)	Total time (min)
	8.28	5.0		5	
1	15.18	5.0	~ 65	40	50
	8.28	5.0		5	
	8.28	5.0		5	
2	12.00	5.0	~ 58	30	40
	8.28	5.0		5	
	4.98	0		5	
3	10.02	0	~ 50	20	30
	4.98	0		5	

Estimated percentage of $\dot{V}O_2$max based on previous studies (Amorim et al. 2009, Bedford et al. 1979).

2.3.2.2 Homeostatic model assessment for insulin resistance (HOMA-IR)

Pregnant rats on day 20 of gestation were fasted for 6 h (from 0800 h to 1400 h) (Cacho et al. 2008). Mothers were pre-warmed under an incubator at 38ºC for 5 min to facilitate tail blood flow. They were then gently restrained using a thin tea towel. By using 1 ml heparinised syringe, approximately 0.15 ml of blood was drawn from the tail. Glucose was immediately measured and blood was placed on ice and centrifuged to collect plasma for insulin analysis. The homeostatic model assessment (HOMA) for insulin resistance (IR) was determined by the following equation: HOMA-IR = (Fasting Plasma Glucose (mmol/l) x Fasting Plasma Insulin (µIU/ml))/2.44, which has been demonstrated to provide an accurate measure of insulin sensitivity in late pregnancy in Sprague-Dawley rats (correlation coefficient between insulin sensitivity index from euglycaemic hyperinsulinaemic clamp and HOMA-IR: r -0.703 and P = 0.0016) (Cacho et al. 2008).

2.3.2.3 Mating

As described previously (session 2.3.1.2), at 12 weeks of age, female rats were mated. Female rats were moved daily into male cages for up to six days after the last pre-pregnancy training section on Friday (4^{th} week training; from 0900 h to 1700 h). During the mating season, from 0700 to 0900 h females continued to run using the same protocol as the last week of exercise training. Once the pregnancy was confirmed by vaginal smear, pregnant rats were housed individually until delivery. The animals that did not mate during this period were killed (60 mg/kg IP; pentobarbitone, Virbac, New South Wales, Australia, followed by cardiac puncture) from the study to avoid discrepancies of pre-gestational training period within the trained group.

2.3.2.4 Post-mortem and tissue collection

After weaning (21 d postnatal), female breeders were anesthetised and body weight and length, and abdominal circumference were recorded. Additionally, retroperitoneal fat, pancreas, liver, kidneys, heart, *extensor digitorum longus* (EDL) and soleus muscles were dissected and weighted.

2.4 Procedures conducted in offspring

2.4.1 Delivery and Postnatal day (PND) 1

From the day 20 of gestation pregnant rats were monitored with a smartphone mobile camera which allowed exact determination of the time that the pups were born. A red filter on the flash was used to record during the dark period (1900 h to 0700 h), and photos were taken in a 1h-interval during the night cycle with the flash light under red filter programmed to trigger for 0.5 second. Postnatal day 1 (PND 1) was considered between 24-36h of the delivery. This study only included litter sizes between 9-15 pups, in order to better control the effects of intrauterine supplies as higher or lower number of pups could potentially under or over nourish the foetuses. At PND 1, litters were adjusted to 12 pups to normalise postnatal nutrition, and when possible six males and six females. Pups in addition to the 12 required were culled by decapitation and blood collected for analysis of glucose levels using a commercial glucometer. Two pups from each breeding pair were used in the study.

2.4.2 Somatic growth

Somatic growth was assessed in terms of body weight and length (tip of the nose to base of the tail), and laterolateral and anteroposterior head axis measurements at PND 1 (24-36h after birth), PND 10 and 21. After PND 21 body weight was determined weekly up to 25 weeks of age when length was measured.

2.4.3 Weaning

At PND 21, all pups were separated from their mothers and fasted for 4h. In order to reduce the number rats used for mating, only two female pups from each breeding pair were randomly included in the study. The other female and male pups that were not included in the study were anesthetised (60 mg/kg ip; pentobarbitone, Virbac, New South Wales, Australia), had muscles (EDL and soleus) dissected and were killed by cardiac puncture. Liver, pancreas, visceral fat, heart, kidney and urine also were collected for further studies. Tissues were weighed and frozen in liquid nitrogen before storing at -80ºC.

2.4.4 Exercise training early in life

At the end of week four of life, all rats performed the treadmill acclimatisation. Rats were then randomly placed into sedentary or exercise training groups. Acclimatisation to the treadmill was the same used for the mothers (2.3.2.1). Trained rats were subjected to a protocol of moderate intensity exercise training (treadmill, 5 d/wk, 60 min/d, at ~65-75% $\dot{V}O_{2max}$) during week 5, 6, 7 and 8 of life. The intensity of the training was estimated based on a protocol originally designed for Sprague-Dawley rats, 74-78 days old (Bedford et al. 1979), and the training protocol was adapted from our previous study (Laker et al. 2011). The training consisted of a warm-up period (5 min), a training zone period (20-50 min) and a cool-down period (5 min) (Table 2-4). The same stimuli used for the maternal exercise (section 2.3.2.1) were used with the offspring early in life. The last week of training was the 9th week of life in female offspring. The rats were then were culled 16 weeks later.

Table 2-4. Protocol of exercise training early in life.

Week No.	Speed (m/min)	Grade (º)	Percentage of $\dot{V}O_{2max}$ #	Duration of each stage (min)	Total time (min)
	4.98	0		5	
1	12.60	0	~ 60	20-40 *	30-50
	4.98	0		5	
	6.66	5.0		5	
2	15.18	5.0	~ 64	50	60
	6.66	5.0		5	
	8.28	10.0		5	
3	17.40	10.0	~ 70	50	60
	8.28	10.0		5	
	8.28	10.0		5	
4	19.26	10.0	~ 76	50	60
	8.28	10.0		5	

** Increasing 5 min per day. # Estimated percentage of $\dot{V}O_2max$ based on previous studies (Bedford et al. 1979).*

2.4.5 Intraperitoneal Insulin Tolerance Test

At 11 and 23 weeks of age, an IPITT was performed as described previously (section 2.3.1.3), except for the insulin concentration that was 0.5 U/kg for the offspring.

2.4.6 Intraperitoneal Glucose Tolerance Test

At 12 and 24 weeks of age, an IPGTT was performed as described previously (section 2.3.1.4).

2.4.7 Post-mortem and tissues collection

At 25 weeks of age, offspring were fasted overnight and anesthetised (60 mg/kg IP; pentobarbitone, Virbac, New South Wales, Australia). The epitrochlearis (EPI) and soleus (SOL) muscles were dissected with the rats still alive, and instantly placed in buffer solution for glucose uptake measurements [(Sequea et al. 2012); described below]. Then, the following muscles were collected: EDL, tibialis anterior (TA), soleus (from the other limb), gastrocnemius, and plantaris (PLANT) with the animal deeply anaesthetised but still alive. Muscles were immediately frozen in liquid nitrogen. Blood was collected by cardiac puncture, and the heart was dissected. The following tissues were then dissected and frozen for future studies: the right lower lobe of the liver; visceral, subcutaneous and brown (dorsal) fat; left and right kidneys; pancreas for morphology (including tail, body and head parts), with a small piece of the body of the pancreas frozen. Sampling and analysing different parts of the pancreas is important as difference among pancreatic areas in regards to distribution of cells may occur (Wang et al. 2013, Trimble et al. 1982). Additionally, right and left tibias and femurs were collected. All tissues described above were collected from one offspring.

2.4.8 Enzyme activity, hormones, plasma metabolites

2.4.8.1 Citrate Synthase activity

Plantaris muscles (2-4 mg) were homogenised at a ratio of 1:400 with the following buffer: 100mM monopotassium phosphate and 100mM dipotassium phosphate, pH 7.5. Homogenates were frozen and thawed three times in liquid nitrogen (to disrupt mitochondrial membrane allowing access of citrate synthase) and spun at 900G for 10min at 4ºC. The citrate synthase (CS; tricarboxylic acid cycle enzyme) assay was adapted to be performed on a 96-wells plate (Srere 1969a, McConell et al. 2015). The CS activity

was measured in triplicate by examining the increase of 5,5-dithiobis-2-nitrobenzoate (DTNB) at a wavelength of 412 nm.

2.4.8.2 Insulin assay

Insulin was analysed with a radioimmunoassay (RIA) kit following the instructions of the manufacturer (SRI-13K RI-13K, Linco Research, St Charles, Missouri, USA). Approximately 100 µL of plasma was used for quantification of insulin.

2.4.9 Ex vivo glucose uptake

2.4.9.1 Experimental procedures

The EPI and SOL muscles were carefully excised from live offspring rats after toe and tail pinch reflex were not observed due to pentobarbital sodium (60 mg/kg IP; Virbac, New South Wales, Australia). Dissection was performed from tendon to tendon, ensuring no muscle damage and stretching. Muscles were quickly split into two strips as described previously (Sharma et al. 2015), and incubated in chambers filled with Krebs Henseleit solution (in mM): 118.5 NaCl, 24.7 $NaHCO_3$, 4.74 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 147.02 $CaCl_2$, 32 mannitol, 7.5% BSA and MilliQ H_2O, with pH 7.4, maintained at 30°C and continuously oxygenated with 95% O_2 and 5% CO_2 (Figure 2-2). During the first 20 min, the muscles were incubated with the Krebs Henseleit solution, in addition to 8 mM glucose (Buffer #1). Then, muscles were transferred to another Krebs Henseleit solution for 30 min, with 4 mM pyruvate and no glucose. This second buffer also contained either 0 or 1.2 nM of insulin (Buffer #2). The last buffer comprised the Krebs Henseleit solution with the addition of 8 mM 2-deoxyglucose (0.75 µCi ml^{-1} 2-[1,2-^{3}H] deoxy-D-glucose), 2 mM mannitol (0.225 µCi ml^{-1} [1-^{14}C]), and 0 or 1.2nM insulin for 10 min (Buffer #3). The 1.2nM of insulin was chosen as it has been considered a physiological concentration of insulin (Sequea et al. 2012). All buffers contained 1.5 ml of final total volume. At the end of the incubation protocol, the muscle pieces were quickly washed in ice-cold Krebs Henseleit solution to stop chemical reactions, rapidly dried in filter paper three times, and frozen in liquid nitrogen for future analyses.

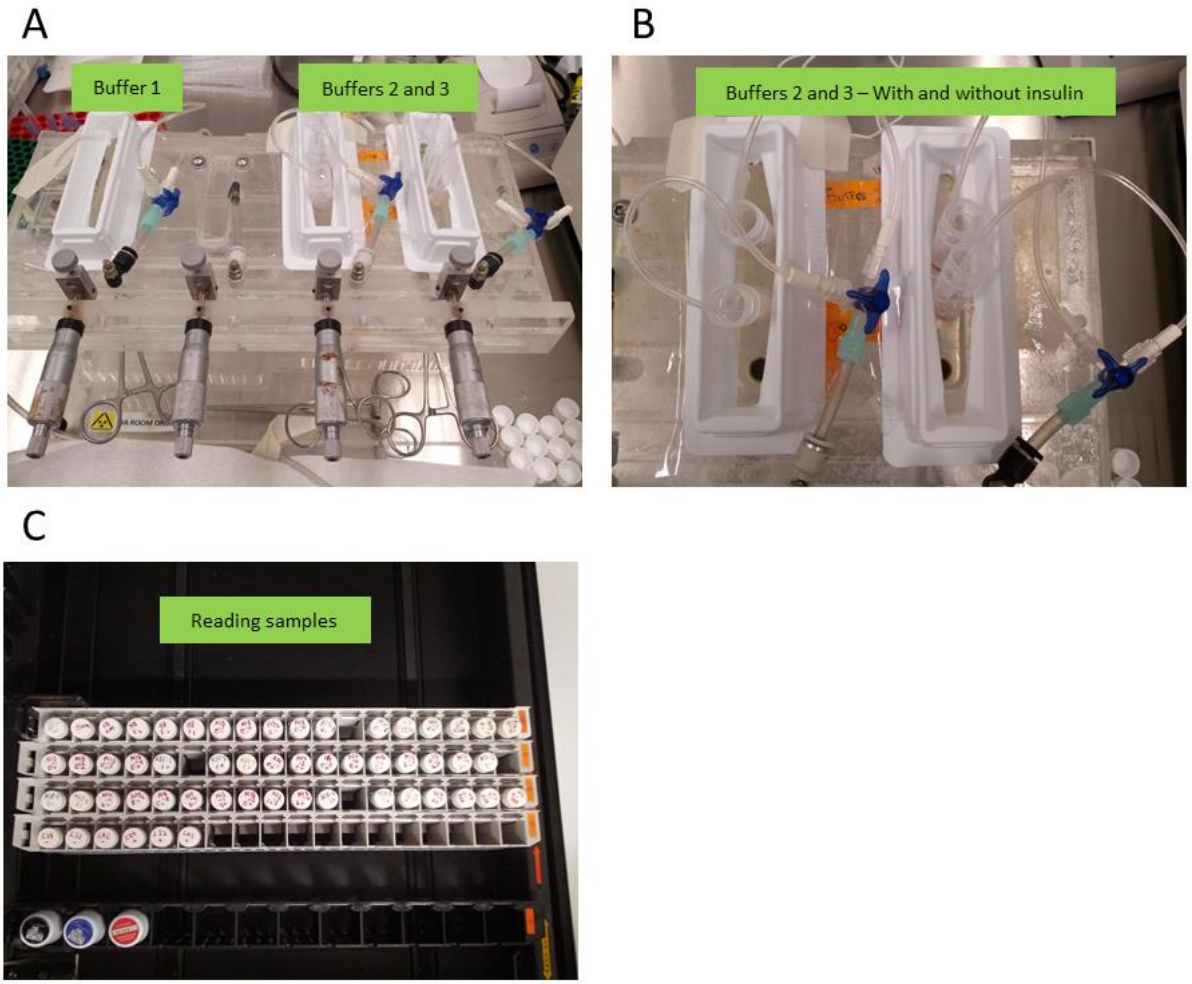

Figure 2-2. Insulin stimulated glucose uptake apparatus. Adapted equipment (A), with baths replaced with 5 ml vials (B) to reduce the amount of buffers used and normal oxygenation tubes substituted by cannulas to ensure proper oxygenation throughout the experiments. Due to limited number of oxygenation exits in this apparatus, buffers 2 and 3 were placed alternatively, using new vials. After the glucose uptake experiments, samples were processed as described below, and read in the scintillation analyser (β counter) (C).

2.4.9.2 Glucose uptake measurements

Approximately 30mg of EPI and 35mg of SOL dried frozen muscles were weighted and 300µl of 1M NaOH were added. The tissues were then kept at 95 °C for 10 min. For neutralisation, 300µl of 1M HCl was added to the solution and vortexed. Then samples were spun for 5 min at 13,000 g at room temperature. Two experimental controls (blank) were prepared in separate glass scintillation vials. The first contained 100µl 1M NaOH and 100µl 1M HCl. The second vial contained 25µl Krebs Henseleit solution collected from each experimental day as previously described (Buffer #3, kept in 4 °C from each experimental day), 87.5µl 1M NaOH and 87.5µl 1M HCl. 200 µl of the homogenate was transferred into glass scintillation vial containing 4 ml of biodegradable

scintillation cocktail (Ultima Gold, PerkinElmer), and mixed thoroughly. Samples sat for at least 1 h at room temperature prior reading on a β-scintillation counter (Liquid Scintillation Analyser, Tri-Carb 2810TR, PerkinElmer, Boston, MA) using dual counts for ^{3}H and ^{14}C protocol with each sample read for 10 minutes (Stephens et al. 2004). The level of radioactivity in disintegrations per minute (DPM) was used for calculations.

2.4.10 Mitochondrial respiration

2.4.10.1 Fibre preparation

After dissection, approximately 10 mg of the medial part of the plantaris muscle was placed in cold Biopsy Preservation Solution (BioPS) containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 50 2-(N-morpholino)ethanesulfonic acid (MES), 15 Na_2-phosphocreatine, 20 imidazole, and 0.5 dithiothreitol (DTT), adjusted to pH 7.1 (Pesta and Gnaiger 2012). Muscle fibres were mechanically separated under a microscope with forceps in a small petri dish on ice (Figure 2-3, B).

The membrane permeabilisation procedure included gentle agitation for 30 min at 4°C in BioPS containing 50 µg/ml saponin, followed by three washes in Mitochondrial Respiration Medium (MiR05). The MiR05 contained (in mM, unless specified) 0.5 EGTA, 3 $MgCl_2$, 60 potassium-lactobionate, 20 taurine, 10 KH_2PO_4, 20 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 110 sucrose and 1 g/L bovine serum albumin (BSA), essentially fatty acid free (pH 7.1) (Pesta and Gnaiger 2012).

2.4.10.2 High-resolution respirometry

High-resolution respirometry (HRR) was performed with the Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria) (Pesta and Gnaiger 2012) combined with the Fluorescence-Sensor Green of the O2k-Fluo LED2-Module for hydrogen peroxide (H_2O_2) measurement (Figure 2-3, A). About 2 mg wet weight of mechanically separated, saponin-permeabilised muscle fibres (Figure 2-3, B) were placed in MiR05, at 37 °C in a closed chamber. Reoxygenation of the medium was made when oxygen concentrations reached 80 µM to avoid potential oxygen diffusion limitation. Using DatLab software (version 5, Oroboros), oxygen concentration (nanomoles per millilitre) was monitored and oxygen flux (picomoles per second per milligram) was measured in duplicate (Figure 2-3, C).

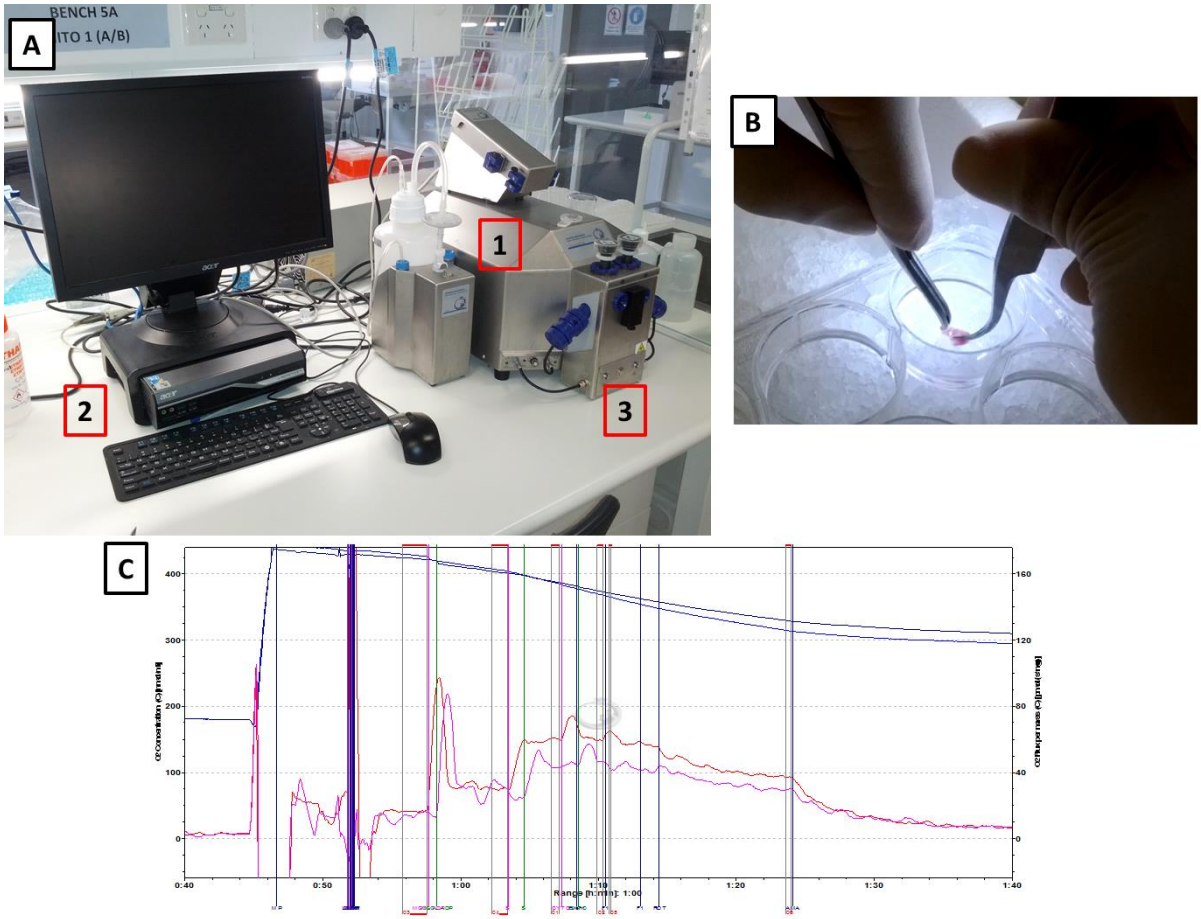

Figure 2-3. Mitochondrial respiration apparatus, muscle fibre separation and respiration data. A, Oxygraph-2k (1), computer system with DataLab software v5.1.0.2 (2), and O2k-Fluo LED2-Module (3). B, mechanical separation of muscle fibres, in preparation for permeabilisation procedure. C, Representative data obtained from DataLab software.

2.4.10.3 Experimental procedure

A mitochondrial substrate–uncoupler–inhibitor titration (SUIT) protocol was used (Pesta and Gnaiger 2012) to investigate respiratory control in a structured order of coupling and substrates states. The SUIT sequence was as follow: Superoxide dismutase (5 U/ml), Amplex® UltraRed (10 µmol) and horseradish peroxidase (1 U/ml) were first added to the chamber to allow H_2O_2 measurements. After stabilisation of the medium, permeabilised muscles were added into the chamber. Approximately 470mmol/ml of O_2 was provided to the medium. Leak respiration (*L*) was measured by adding malate (5 mM) and pyruvate (10 mM), through complex I (CI) (CI_L). Maximum oxidative phosphorylation (oxphos) capacity (*P*) was measured by adding magnesium chloride and adenosine diphosphate (ADP) (5mM) through CI (CI_P), and subsequent addition of succinate (10mM) for measurement of *P* through CI+II combined ($CI+II_P$). Outer

mitochondrial membrane integrity was tested by adding cytochrome *c* (10 µM). At this point, it was possible to estimate whether the experiment was compromised, for instance, due to mitochondrial damage; oxygen flux 10% above the $CI+II_P$ rates were not included in the study. Then, electron transport system (ETS) capacity (*E*) through CI+II ($CI+II_E$) was quantified by adding carbonyl cyanide 4-(trifluoromethoxy) phenyl-hydrazone (FCCP) titrations (0.5 mM) and if an increase in oxygen flux was noticed, another dose of FCCP (0.5mM) was applied. To determine *E* through CII (CII_E), rotenone (1 mM) was added to the media. For quantification and correction of residual oxygen consumption (ROX), antimycin A (5 µM) was added to the experiment. For quantification purposes, only stable portions of real-time fluxes were used, and artifacts induced by additions of chemicals or re-oxygenation were excluded. Representative diagram of the experimental procedure is showed in Figure 2-4.

With the data collected from each stage of the mitochondrial respiration, the following respiratory flux control ratios (FCRs) were calculated (Pesta and Gnaiger 2012). The quotient of CI_L over $CI+II_E$ was considered the leak control ratio (LCR). The quotient of $CI+II_P$ over $CI+II_E$ was considered the phosphorylation control ratio (PCR). While the coupling control ratio is the quotient of CI_L over $CI+II_P$ and is equivalent to the inverse respiratory control ratio (inv-RCR). Lastly, the substrate control ratio (SCR) at constant *P* is the quotient of CI_P over $CI+II_P$.

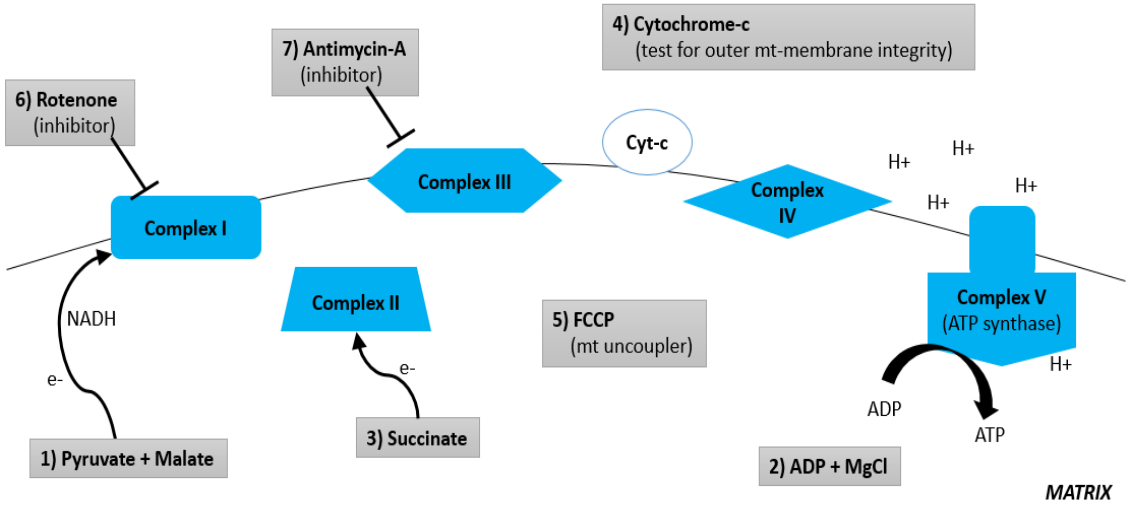

Figure 2-4. Schematic diagram of the electron transport system (ETS) and oxidative phosphorylation (oxphos) in action [Adapted from (Xiong et al. 2009)]. Numbered from 1 to 7 is the sequence of the multiple substrate-uncoupler-inhibitor titrations (SUIT) protocol used in this project. Mitochondrial components are stimulated (→) or inhibited (⊥) by each of the numbered chemical. Cyt C, cytochrome-C. FCCP, Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone. NADH, nicotinamide adenine dinucleotide. e-, electrons. H+ protons.

2.4.11 Pancreas morphology

Pancreases were weighted and stored in 10% Neutral Buffered Formalin at room temperature for up to one week, transferred to 70% ethanol and kept at 4ºC until processed. Five sections per pancreas were immunostained to identify and localize insulin-positive β-cells (n=8-10 per group). Fixed tissue was sent to Anatomical Pathology, Department of Medicine, University of Melbourne (Parkville, Victoria, Australia) to be paraffin embedded, sectioned at 100 µm, stained for insulin using a guinea pig polyclonal anti-porcine insulin antibody [DAKO Corporation (Denmark)] diluted 1:100 and counterstained with haematoxylin. Digital images of microscopic sections were obtained through the Austin Health, Victorian Cancer Biobank Slide Scanning service (Heidelberg, Victoria, Australia). Following standard protocols, whole slide sections were line scanned using an Aperio ScanScope XT (Aperio Technologies, Vista, CA, USA) at 40x magnification at a resolution of 0.5 µm/pixel (Figure 2-5). Digital images were analysed using the Aperio image software (ImageScope version 12.2.2). Slides were code-blinded to remove potential bias. Data analysis was also performed under blind conditions.

Pancreatic islet number was expressed relative to total cross-sectional area (per square millimetre) with islet size arbitrarily classified as small (<5,000 μm^2), medium (5,000 –10,000 μm^2), and large (>10,000 μm^2) (Laker et al. 2011). All five sections were wholly analysed, and relative islet and β-cell volume density (Vd) were quantified by point-counting morphometry (Vd = number of intercepts on islet or insulin-positive cells as a proportion of intercepts on pancreas). Number of islets per millimetre square was obtained by dividing the number of islets by the total pancreas area (mm^2). β-cell mass was determined by multiplying Vd by pancreas weight (g). Additionally, percent of insulin-positive staining cells occupied by β-cells was calculated.

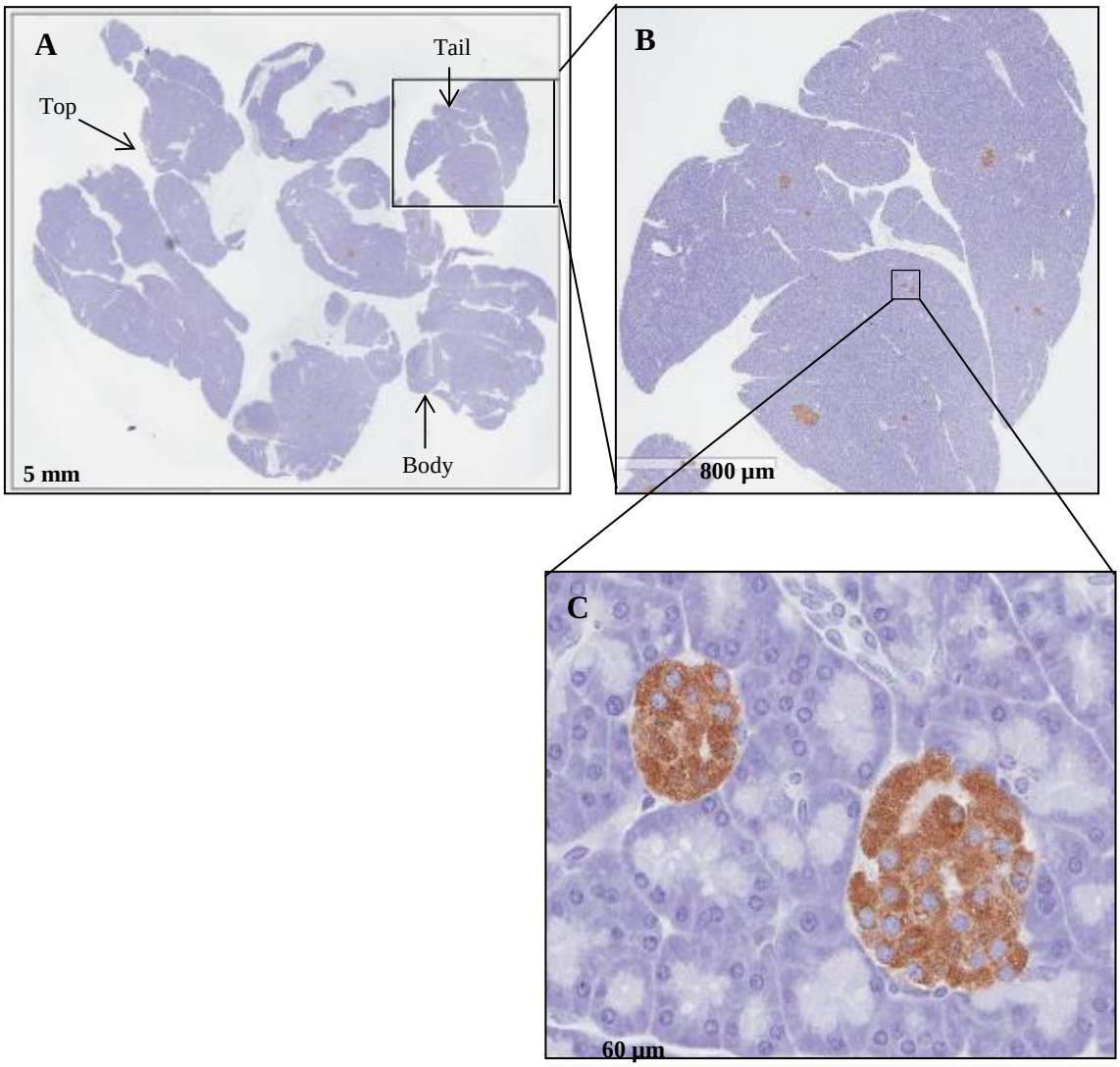

Figure 2-5. Representative image for pancreas morphology. A, Whole pancreas including different sections (top, body and tail of the pancreas). Morphological measurements were performed in at least 5 different sections. B, Tail of the pancreas in an increased magnification. C, Representation of the islets at the amplification level used to perform the analysis.

2.4.12 Western blots

2.4.12.1 Primary Antibodies

GLUT4 (1:2000, abcam, #ab37445), GLUT1 (1:1000, abcam, #ab652), TBC1D4 (1:500, Cell Signaling, #2670), Phospho-AS160 Thr^{642} (1:1000, Cell Signaling, #4288), AKT2 (1:1000, Cell Signaling, #5239), phospho-AKT Thr^{308} (1:1000, Cell Signaling, #9275), phospho-AKT Ser^{473} (1:1000, Cell Signaling, #9271), , Tfam (1:1000, abcam, #ab131607), PGC1α (1:1000, Merck Millipore, #ST1202), and PHF20 (1:500, Cell Signaling, #3934).

2.4.12.2 Protein assay

Protein content of skeletal muscles was measured using RED 660™ Protein Assay with Neutralizer™ (G-Biosciences, A Geno technology, Inc, USA) against standard curves of bovine serum albumin (BSA). 200 µl of Red 660 reagent was added to a well with 10 µl of muscle homogenate, and measured in triplicate at an absorbance of 660 nm after 5 min at room temperature. Concentration was calculated by comparing with a standard curve of known amounts of BSA (0–250 µg).

2.4.12.3 Skeletal muscle preparations

Epitrochlearis (EPI, with and without insulin) and PLANT muscles were homogenised for western blot in the following buffer: 0.125M Tris-HCl, 4% Sodium dodecyl sulphate, 10% glycerol and 10mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 0.1M dithiothreitol. Approximately 3mg of muscle was cut in a -20ºC cryostat. 150µl of buffer was added for each milligram of muscle. The muscle homogenate rocked at room temperature for 1 h.

2.4.12.4 Experimental procedures

Homogenates containing 4 µg of total protein from EPI muscles in basal and insulin-stimulated conditions were analysed for total-TBC1D4, p-$TBC1D4^{Thr642}$, PGC1α, total-AKT, p-AKT Thr^{308}, p-AKT Ser^{373}, GLUT1 and GLUT4 protein contents by Western Blotting. Total protein in muscle samples was separated on 7.5-12% hand-cast criterion TGX Stain-Free gels (Bio-Rad, Hercules, CA, USA) at 60 V for 30 min followed by 120 V for ~35 min with buffer containing 25 mM Tris base, 191.8 mM glycine, 1% sodium dodecyl sulphate. Total proteins in the gel were imaged using Stain Free imager

(ChemiDoc, Bio-Rad). At this stage, the Stain-Free gel was activated (1 min) for detection of myosin heavy chain (MHC), used as an indicator of the amount of protein present due to small sample sizes (Murphy 2011) (Figure 2-6, A). Proteins were wet-transferred to nitrocellulose for 100 min at 100 V in a circulating ice cooled bath using transfer buffer containing 191.8 mM glycine, 25 mM Tris base, and 20% (v/v) methanol. Following transfer, Based on different protein molecular weights, it was possible to obtain data for TBC1D4 (Total or Phospho, 160 kDa), AKT (Total or Phosphos, ~62 kDa), and GLUT1 or GLUT4 (~54 kDa) in the same muscle homogenate by cutting the membranes before primary antibody incubation. Membranes were exposed to primary antibodies overnight at 4°C with constant rocking.

On the following day, appropriate horseradish peroxidase secondary antibodies (Anti-mouse or rabbit IgG, HRP-linked Antibody) were added (1:10000), diluted in 3-5% skim milk powder in Tris-Buffered saline (TBS) with Tween 20 (t) for 60 min. Following washes with TBSt, protein bands were detected after exposures to SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) using a charge-coupled device camera attached to a ChemiDoc (Bio-Rad) and using ImageLab software (Bio-Rad). Densitometry was performed using ImageLab software (Version 5.2.1, Bio-Rad). Each gel contained a ladder (PageRuler™ Plus Prestained Protein Ladder, Thermo Fisher Scientific) which allowed the visualisation of the relative positions of protein molecular mass. Another image was captured under white light before chemiluminescent imaging without moving the membrane and then both images were superimposed (Figure 2-6, B-D).

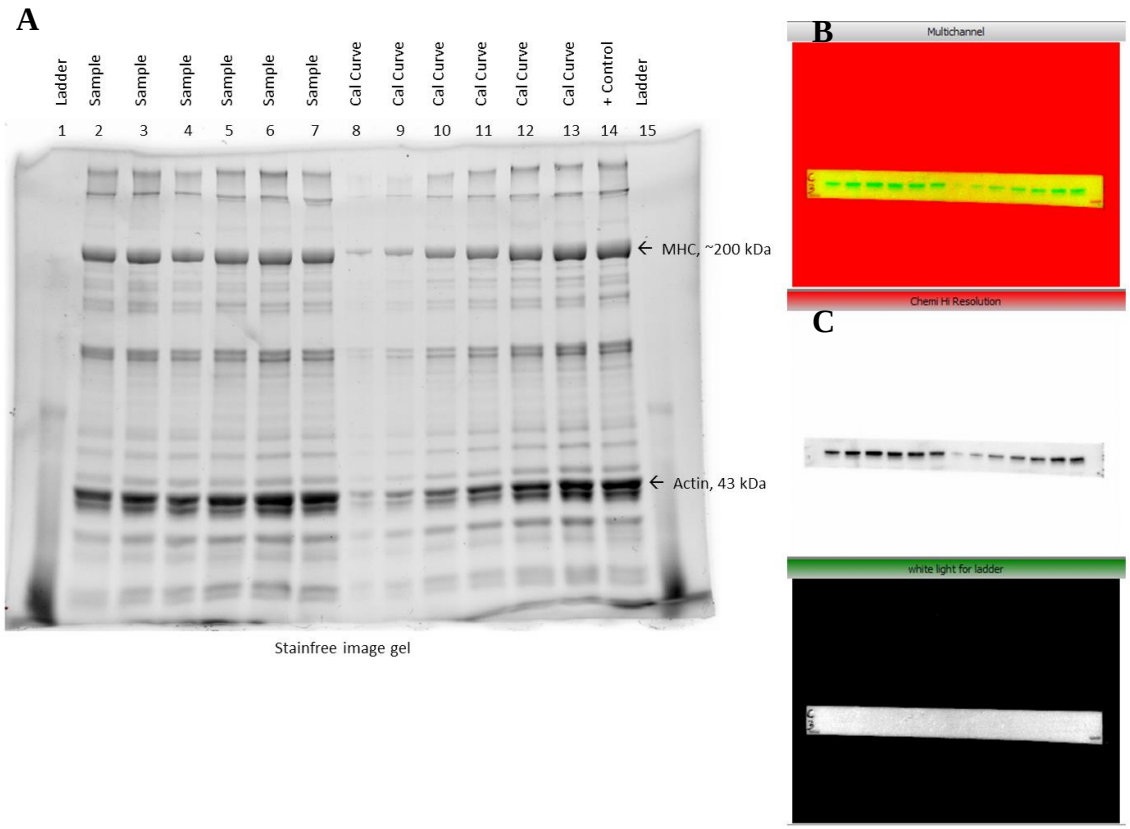

Figure 2-6. Representative gel and blot images. A, Configuration of a standard stain-free gel will the lanes numbered at the top and identification of myosin (MHC) or actin based on molecular weight (kDa) compared against the ladder. B, multichannel image generated after combining (C) images from chemi high-resolution and (D) white light photo for the ladder. Cal curve, calibration curve with pooled samples.

2.5 Statistical analyses

Data are presented as mean ± SEM unless otherwise specified. All data were tested for normality using Shapiro-Wilks test and, if necessary, log-transformed before data analyses. All statistics were run using SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) and/or GraphPad Prism (GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla California USA). Student t test, two or three-way ANOVA with repeated measures were used as appropriate. For ANOVA, 'Paternal diet' and 'Maternal exercise' or 'Offspring exercise' were used as main factors, as well as time points (e.g. during the IPGTT or IPITT) or treatment (e.g. insulin incubation during 2DG uptake). If an interaction was found in a three-way ANOVA, a two-way ANOVA was applied to each time-point/treatment. For two-way ANOVA, if an interaction was found, a post-hoc analysis using the least significant difference (LSD) test was used. The α-level of statistical significance was set *a priori* at $P < 0.05$.

Chapter 3 – Maternal exercise attenuates the reduced insulin secretion in adult female rats sired by paternal diet-induced obesity

3.1 Introduction

The developmental origins of health and disease (DOHaD) paradigm focuses on investigating how environmental cues acting during early development affect the risk of later chronic diseases (Hanson and Gluckman 2014, McMullen and Mostyn 2009, Street et al. 2015). There are 'windows of opportunity' when the organism is more susceptible to environmental signals (Hanson and Gluckman 2014). This ability to respond to external stimuli is named phenotypic plasticity, when a single genotype can have its morphology, physiology and behaviour altered (West-Eberhard 1989). For instance, parental phenotype is able to modify offspring's health, as demonstrated by the increased risk of adult obesity among non-obese children (Whitaker et al. 1997). In large and small animal models, studies have shown that low-protein or high-fat diets during pregnancy are associated with impaired insulin sensitivity and secretion as well as mitochondrial dysfunction in skeletal muscle [reviewed in (Warner and Ozanne 2010, McMullen and Mostyn 2009, Gatford et al. 2014)]. Although much of the research is focusing on maternal effects, the contribution of the fathers in the cause of T2DM has drawn attention in the DOHaD field.

Paternal obesity before mating can negatively influence the phenotype and metabolism of the offspring (Soubry 2015). In fact, a father's body fat was predictive of long-term alterations in total and percentage body fat in their prepubertal daughters (Figueroa-Colon et al. 2000). Although longitudinal studies are still scarce, a prospective epidemiologic cohort observed that obesity in fathers is linked with elevated levels of alanine transaminase in their offspring, independent of body mass index (BMI) (Loomba et al. 2008). Alanine transaminase is argued to be positively correlated with obesity. In rats, Ng *et al*. (2010) have demonstrated that paternal high-fat diet for 10 weeks before conception does not affect body weight and fat mass in female offspring (Ng et al. 2010). In this study, however, during a glucose tolerance test the offspring demonstrated lower insulin area under the curve and higher glucose area under the curve compared to

offspring sired by control diet dads at 12 weeks of age. These findings imply that adolescent offspring (12 weeks of age) developed glucose intolerance in addition to altered pancreatic islets morphology, including reduced total islet area and higher percentage of small islets, as well as disturbed gene expression (Ng et al. 2010). Similar negative effects were reported in mice sired from HFD/obese fathers in terms of glucose metabolism in adulthood (Fullston et al. 2013). None of these studies, however, examined insulin sensitivity in skeletal muscle, which is the main site of insulin-stimulated glucose uptake. Mechanistic studies with a holistic perspective involving insulin sensibility and secretion as well as mitochondrial function are needed. Furthermore, intervention approaches are necessary to break the obesity and T2DM cycle for the next generations.

The involvement of the mothers in this scenario has been directly related to this research field since its origins. Not surprisingly as maternal effects are expected to play an essential role on offspring's phenotype since the foetus experiences direct interaction with the mother through the placenta (Power and Schulkin 2013). This relationship opens up possible ways to positively intervene during pregnancy, such as having a balanced diet as well as a safe environment free of harmful chemicals (Barouki et al. 2012). Another feasible intervention that has been investigated is the maternal physical activity. However, studies in humans in this regard are conflicting with maternal exercise reducing (Clapp and Capeless 1990, Hopkins et al. 2010), having no effect (Marquez-Sterling et al. 2000) or increasing birth weight (Clapp et al. 2000, Clapp et al. 2002). This is thought to depend upon exercise intensity, volume and the time point in the pregnancy when the exercise is performed (Clapp 2003, Clapp et al. 2002). There is, however, very limited number of studies in humans that investigated long-term effects of maternal exercise in the offspring, hence, the need for pre-clinical models in this field. Using voluntary running wheel, research groups have demonstrated that maternal exercise can improve offspring glucose tolerance later in life compared to control/sedentary mothers (Carter et al. 2012, Carter et al. 2013, Stanford et al. 2015).

There are still gaps in the literature related to the combined effects of maternal exercise and paternal HFD/obesity on the offspring. Furthermore, no study has investigated the influence of maternal exercise on offspring mitochondrial function and content. In regards to insulin secretion, only one publication reported that exercise training (treadmill) before and during gestation does not alter pancreatic insulin content, and augments insulin secretory capacity in young rats (3 weeks of age) but tends to reduce

it with aging (~28 weeks of age) (Quiclet et al. 2016). To our knowledge, there is no report on the effects of maternal exercise in offspring's insulin secretion *in vivo*.

Therefore, we will investigate whether paternal diet-induced obesity can impairs insulin sensitivity and the mechanisms associated, as well as its impact on insulin secretion. Another important aim of this chapter is to examine whether maternal exercise before and during gestation can attenuate the negative effects caused by paternal diet-induced obesity and the underlying mechanisms.

3.2 Methods

3.2.1 Ethics

All procedures were performed according with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013), after approval (AEC#13/008) from the Animal Ethics Committee at Victoria University, Melbourne, Australia.

3.2.2 Animals

Sprague Dawley rats were obtained from Animal Research Centre (Perth, Australia) and kept in plastic cages at the animal facility in Footscray Park Campus - Victoria University. The rats were exposed to a 12-hour light-dark cycle (7am lights on) and standard environmental conditions (room temperature at 18-22 °C and ~50% relative humidity). All rats had food and water available *ad libitum*. Cages were supplied with nesting materials and enrichment items. Rats were acclimatised for 3 days with no procedures performed during this period.

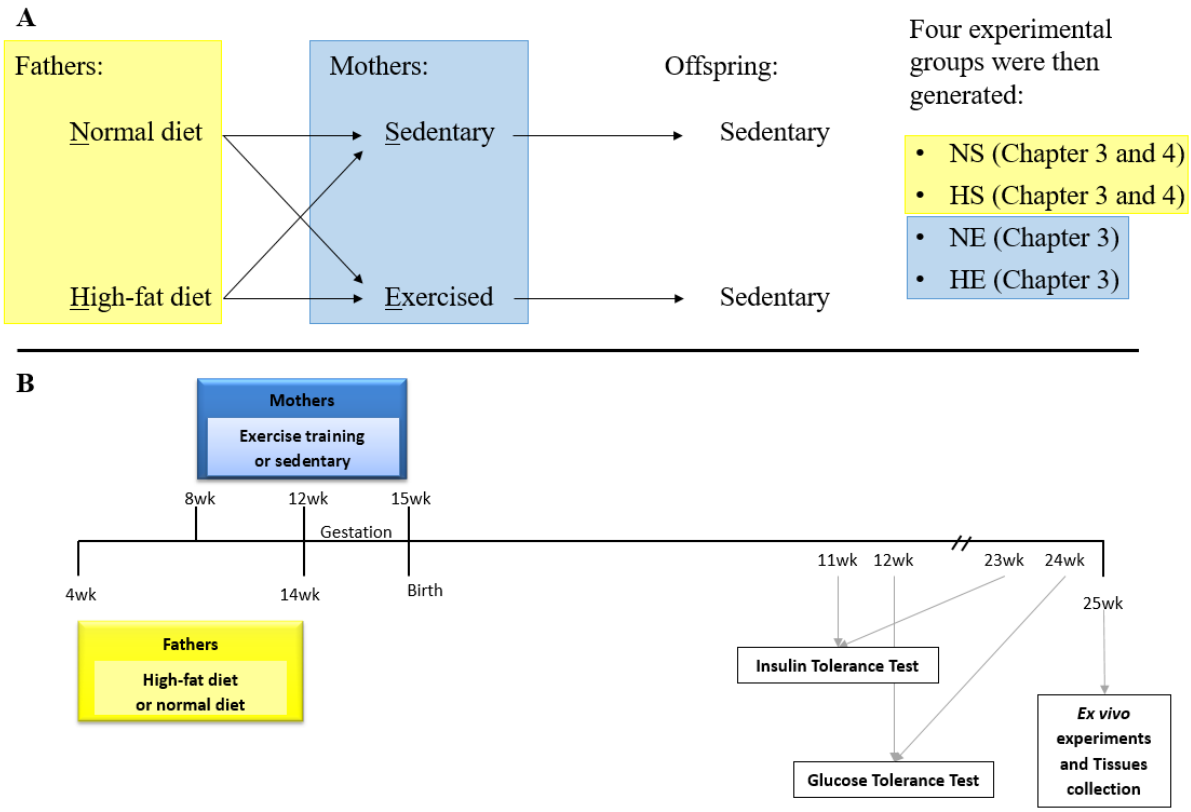

Figure 3-1. Experimental groups and timelines (Chapter 3). A, Experimental groups based on paternal diet (yellow boxes) and maternal exercise (blue boxes) interventions. Male breeders were mated with only one female (1:1). B, Experimental design with timelines for main experiments. NS, pups sired by Normal diet dads and Sedentary mothers. HS, pups sired by High-fat diet dads and Sedentary mothers. NE, pups sired by Normal diet dads and Exercised mothers. HE, pups sired by High-fat diet dads and Exercised mothers.

3.2.3 Fathers

After acclimatisation to the animal facility, rats were either fed a control diet or high-fat diet from 4 to 14 weeks of age (section 2.3.1.1; Table 2-1), which was before conception. Control fathers were fed with a standard control chow diet throughout all experiment (Rat and Mouse Cubes, 12.00% energy as fat; Specialty Feeds, Western Australia), while HFD/obese fathers received two high-fat diets (SF01-025 and SF03-020, with 40.7% and 43% energy as fat; Specialty Feeds, Western Australia). Two high-fat diets were offered at the same time (*ad libitum*) in order to replicate Ng *et. al.* (2010). The diet intervention was implemented before mating to cause an obese and diabetic phenotype. This protocol was chosen in order to replicate as close as possible the study published previously by Ng et al. (Ng et al. 2010). Body weight, food intake were measured weekly as described in the previous chapter.

3.2.4 Mothers

Female breeders were obtained at 6 weeks of age and were acclimatised for 3 days to the animal facility and maintained on control diet (same chow diet offered to control fathers). All the rats were acclimatised to the treadmill with 5 sessions of exercise over 3 days (as described in Chapter 2), and then either randomly placed in sedentary or exercise training groups (Table 2-2).

3.2.4.1 Mating

At 12 weeks of age, rats were mated. Female rats were moved daily from 0900 h to 1700 h into male cages (1F:1M) for up to six days after the last pre-pregnancy training session on a Friday (4^{th} week of training). During these days of mating, females continued to run from 0700 h to 0900 h using the same protocol as the last week of exercise training. Once the pregnancy was confirmed by vaginal smear, pregnant rats were housed individually, the exercise group continued performing training on the treadmill with progressive reduction in intensity and volume (Table 2-3) until day 19^{th} of gestation. Male rats remained on HFD and IPGTT was performed as described in Chapter 2.

3.2.4.2 Fasting Plasma Glucose, Fasting Plasma Insulin and Homeostatic model assessment for insulin resistance (HOMA-IR)

Pregnant rats on day 20 of gestation were fasted for 6 h (from 0800 h to 1400 h) (Cacho et al. 2008). Mothers were pre-warmed under an incubator at 38ºC for 5 min to facilitate tail blood flow. Afterwards, the rats were gently restrained using a thin tea towel. By using 1 ml heparinised syringe, approximately 0.15 ml of blood was drawn from the tail vein. Glucose was immediately measured and blood was placed on ice, centrifuged to obtain plasma for insulin analysis later. The homeostatic model assessment (HOMA) for insulin resistance (IR) was determined by the following equation: [HOMA-IR = (Fasting Plasma Glucose x Fasting Plasma Insulin)/2.430]. This has been demonstrated to provide an accurate measure of insulin sensitivity in late pregnancy in Sprague-Dawley rats (correlation coefficient between insulin sensitivity index from isoglycemic hyperinsulinemic clamp and HOMA-IR: r -0.703 and P = 0.0016) (Cacho et al. 2008).

3.2.5 Offspring

3.2.5.1 Body weight and food intake

Body weight and food intake were measured weekly throughout the experiments. Food intake was considered the difference between the amount of food offered and the amount of food remaining 24 h later.

3.2.5.2 Intraperitoneal Insulin Tolerance Test

In the week following mating was confirmed, male rats were subjected to an IPITT. It was performed at 1000 h, 2 hours after food was removed. Blood glucose level was determined at 0 minutes from tail vein by a portable glucometer (Accu-Chek Performa Nano, Roche Diagnostics, Mannheim, Germany). Then insulin (0.5 to 1 U/kg based on predicted insulin resistance (Ng et al. 2010); Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was given intraperitoneally (IP) and blood glucose level was measured by a single drop from tail puncture at 20, 40 and 60 min.

3.2.5.3 Intraperitoneal Glucose Tolerance Test

Glucose tolerance tests were performed after an overnight fast at 12 and 24 weeks of age. After collection of blood sample via tail vein at basal, glucose (1g/kg) was injected IP and further blood samples were obtained at 15, 30, 60 and 90 min and measured using a portable glucometer.

3.2.5.4 Insulin-stimulated glucose uptake in skeletal muscle

With the animal alive and anaesthetised (60 mg/kg IP; pentobarbitone, Virbac, New South Wales, Australia), epitrochlearis (EPI) and soleus (SOL) muscles were dissected. After dissection of skeletal muscles, the rats were killed by cardiac puncture. Both EPI and SOL were split into two strips (Sharma et al. 2015) and incubated in chambers filled with Krebs Henseleit solution (in mM): 118.5 NaCl, 24.7 $NaHCO_3$, 4.74 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 147.02 $CaCl_2$, 32 mannitol, 7.5% BSA and MilliQ H_2O, with pH 7.4, maintained at 30°C and continuously oxygenated with 95% O_2 and 5% CO_2 (Figure 2-2). During the first 20 min, the muscles were incubated with the Krebs Henseleit solution, in addition to 8 mM glucose (Buffer #1). Then, muscles were transferred to another Krebs Henseleit solution for 30 min, with 4 mM pyruvate. This second buffer also contained either 0 nM or 1.2 nM of insulin (Buffer #2) (Sharma et al. 2011). The third incubation was made in the Krebs Henseleit solution with the addition of 8 mM 2-

deoxyglucose (0.75 µCi ml^{-1} 2-[1,2-^{3}H] deoxy-D-glucose), 2 mM mannitol (0.225 µCi ml^{-1} [1-^{14}C]), and 0 nM or 1.2nM insulin for 10 min (Buffer #3). After the last incubation, the reaction stopped by placing muscles on ice-cold Krebs buffer, then, all muscles were dried on filter paper 3 times, and plunged deep in liquid nitrogen.

Approximately 30mg of EPI and 35mg of SOL dried frozen muscles were weighted and homogenised (1M NaOH then 1M HCl). After the neutralisation of the NaOH with HCl, samples were spun for 5 min at 13,000 g at room temperature. Samples sat for at least 1 h at room temperature prior reading on a β-scintillation counter (Liquid Scintillation Analyser, Tri-Carb 2810TR, PerkinElmer, Boston, MA) using dual counts for ^{3}H and ^{14}C protocol with each sample read for 10 minutes (Stephens et al. 2004). The level of radioactivity in disintegrations per minute was used for calculations.

3.2.5.5 Mitochondrial respiration and reactive oxygen species production

In order to obtain enough tissue for quantification of protein expression in the EPI (or SOL) muscle, we opted to use the PLANT muscle for mitochondrial respiration and western blot analyses. Approximately 2 mg of the medial part of the PLANT muscle was placed in cold Biopsy Preservation Solution (BioPS) containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 50 2-(N-morpholino)ethanesulfonic acid (MES), 15 Na_2-phosphocreatine, 20 imidazole, and 0.5 DTT, adjusted to pH 7.1 (Pesta and Gnaiger 2012). Muscle fibres were mechanically separated under a microscope with forceps.

Mitochondrial respiration was performed by high-resolution respirometry with the Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria) (Pesta and Gnaiger 2012) combined with the Fluorescence-Sensor Green of the O2k-Fluo LED2-Module for H_2O_2 measurement. The following substrate–uncoupler–inhibitor titration (SUIT) protocol was used: Superoxide dismutase (5 U/ml), Amplex® UltraRed (10 µmol) and horseradish peroxidase (1 U/ml) were first added to the chamber to allow H_2O_2 measurements, followed by placement of the permeabilised muscles (Pesta and Gnaiger 2012). Then malate (5 mM) and pyruvate (10 mM) were added, followed by magnesium chloride and ADP (5mM) and subsequent addition of succinate (10mM). Cytochrome *c* was used to test for mitochondrial membrane integrity. Then carbonyl cyanide 4-(trifluoromethoxy) phenyl-hydrazone (FCCP) titrations (0.5 mM) were added, and if an increase in oxygen flux was noticed, another dose of FCCP (0.5mM) was applied. This was followed by rotenone (1 mM) and antimycin A (5 µM). Therefore, we measured leak

respiration (*L*) through complex I (CI) (CI_L), Maximum oxidative phosphorylation (oxphos) capacity (*P*) through CI (CI_P), *P* through CI+II combined ($CI+II_P$), electron transport system (ETS) capacity (*E*) through CI+II ($CI+II_E$), *E* through CII (CII_E) and finally residual oxygen consumption (ROX) (Pesta and Gnaiger 2012).

We also measured plantaris skeletal muscle citrate synthase activity, as an indicator of mitochondrial volume (Larsen et al. 2012). Approximately 15 mg of muscle was placed in 300 µL buffer (175 mM KCl and 2 mM EDTA, pH 7.4), mechanically homogenized for 30 s at 30 Hz (TissueLyser II, Qiagen), freeze-thawed three times, then centrifuged at 4°C. Supernatant (5 µL) was loaded in a 96-well plate in duplicate in order to measure citrate synthase activity spectrophotometrically at 412 nm and 37°C [adapted from (Srere 1969b)].

3.2.5.6 Muscle preparation and Western Blots

EPI (both insulin and non-insulin stimulated) and PLANT muscles were homogenised for western blot in the following buffer: 0.125M Tris-HCl, 4% Sodium dodecyl sulphate, 10% glycerol and 10mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 0.1M dithiothreitol. Total protein in muscle samples was separated on 7.5-12% hand-cast criterion TGX Stain-Free gels (Bio-Rad, Hercules, CA, USA) at 60 V for 30 min followed by 120 V for ~35 min with buffer containing 25 mM Tris base, 191.8 mM glycine, 1% sodium dodecyl sulphate. Following the running, the Stain-Free gel was activated (1 min, ChemiDoc, Bio-Rad) for detection of myosin heavy chain (MHC), used as an indicator of the amount of protein present due to small sample sizes (Murphy 2011). Total proteins in the gel were imaged using Stain Free imager (ChemiDoc, Bio-Rad). Proteins were wet-transferred to nitrocellulose for 100 min at 100 V in a circulating ice cooled bath using transfer buffer containing 191.8 mM glycine, 25 mM Tris base, and 20% (v/v) methanol. Membranes were exposed to primary antibodies overnight at 4°C with constant rocking.

On the following day, appropriate horseradish peroxidase secondary antibodies (Anti-mouse or rabbit IgG, HRP-linked Antibody) were added (1:10000), diluted in 3-5% skim milk powder in Tris-Buffered saline (TBS) with Tween 20 (t) for 60 min. Following washes with TBSt, protein bands were detected after exposures to SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) using a charge-coupled device camera attached to a ChemiDoc (Bio-Rad) and using ImageLab software (Bio-Rad). Densitometry was performed using ImageLab software (Version 5.2.1, Bio-

Rad). Each gel contained a ladder (PageRuler™ Plus Prestained Protein Ladder, Thermo Fisher Scientific) allowing visualisation of the relative positions of molecular mass. Protein expression was normalised to total protein using stain free.

3.2.5.7 Pancreas morphology

After the animal was killed by cardiac puncture, the pancreata were dissected, weighted and stored in 10% neutral buffered formalin at room temperature for up to one week, transferred to 70% ethanol and kept at 4ºC until processed. Five parts of the pancreas (including head, body and tail) were randomly chosen, and sectioned with sterile blade to be immunostained to identify and localize insulin-positive β-cells (n=8-10 per group). Fixed tissue was sent to Anatomical Pathology, Department of Medicine, University of Melbourne (Parkville, Victoria, Australia) to be paraffin embedded, sectioned at 100 µm, stained for insulin using a guinea pig polyclonal anti-porcine insulin antibody (DAKO Corporation, Denmark) diluted 1:100 and counterstained with haematoxylin. Digital images of microscopic sections were obtained through the Austin Health, Victorian Cancer Biobank Slide Scanning service (Heidelberg, Victoria, Australia). Following standard protocols, whole slide sections were line scanned using an Aperio ScanScope XT (Aperio Technologies, Vista, CA, USA) at 40x magnification at a resolution of 0.5 µm/pixel. Digital images were analysed using the Aperio image software (ImageScope version 12.2.2). Slides were code-blinded to remove potential bias. Quantification of insulin-positive cells was also performed under blind conditions.

Five random cross-sections from different parts of the pancreas (head, body and tail) were analysed. Relative islet surface area and β-cell area were quantified by point-counting morphometry (number of intercepts on islet or insulin-positive cells as a proportion of intercepts on pancreas or islets). Number of islets per millimeter square was obtained by dividing the number of islets by the total pancreas area (mm^2). β-cell mass was determined by multiplying the relative β-cell area by pancreas weight (g). Additionally, percent of insulin-positive staining cells occupied by β-cells was calculated. Pancreatic islet number was expressed relative to total cross-sectional area (per square millimeter) (Laker et al. 2011) with islet size arbitrarily classified as <5,000 μm^2, 5,000 – 10,000 μm^2, 10,001 – 20,000 μm^2, 20,001 – 50,000 μm^2 and >50,000 μm^2 [adapted from (Ng et al. 2010)].

3.2.6 Statistical analyses

Data are presented as mean ± SEM unless otherwise specified. Statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) and GraphPad Prism (GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla California USA) using student t test, two or three-way ANOVA with repeated measures as appropriate. For ANOVA, 'Paternal diet' and 'Maternal exercise' were used as main factors, as well as time points (e.g. during the IPGTT) or treatment (e.g. insulin incubation during 2DG uptake). If an interaction was found in a three-way ANOVA, a two-way ANOVA was applied each time-point/treatment. For two-way ANOVA, if an interaction was found, a post-hoc analysis using the least significant difference (LSD) test was used. The α-level of statistical significance was set *a priori* at $P < 0.05$.

3.3 Results

3.3.1 Parental phenotype: Effects of paternal high-fat diet and maternal exercise

Table 3-1 shows the negative effects of the high-fat diet from 4 to 14 weeks of age in male rats starting from the same body weight. HFD/obese male breeder rats demonstrated higher body weight (Figure 3-2 A) and cumulative food intake (Figure 3-2 B), which resulted in glucose intolerance (Figure 3-2 C-F).

Table 3-1. Male rats fed with high-fat diets for 10 week demonstrate an obese phenotype.

Parameters	Fathers		
	Control diet (n=10)	High-fat diet (n=9)	*P* value
Initial body weight (g)	106.4 ± 2.2	103.9 ± 1.7	0.399
Final body weight (g)	502.7 ± 11.2	558.6 ± 13.8	0.003
Final body length (cm)	26.3 ± 0.2	27.5 ± 0.1	0.005
Final Ab circumference (cm)	19.6 ± 0.2	22.1 ± 0.2	0.007
Final retroperitoneal fat (g)	7.4 ± 0.3	17.1 ± 0.8	0.000
Final fasting glucose (mmol/l)	6.0 ± 0.2	6.4 ± 0.1	0.125
Final fasting insulin (ng ml^{-1})	0.6 ± 0.0	1.2 ± 0.2	0.007
Final HOMA-IR	0.2 ± 0.0	0.3 ± 0.0	0.006

Ab, abdominal. HOMA-IR, homeostatic model assessment of insulin resistance. HOMA-IR equation was as follows: HOMA-IR = [fasting plasma glucose (mg/dl) x fasting plasma insulin (μU/ml)]/2,430 (Cacho et al. 2008). Values are presented as mean ± SEM.

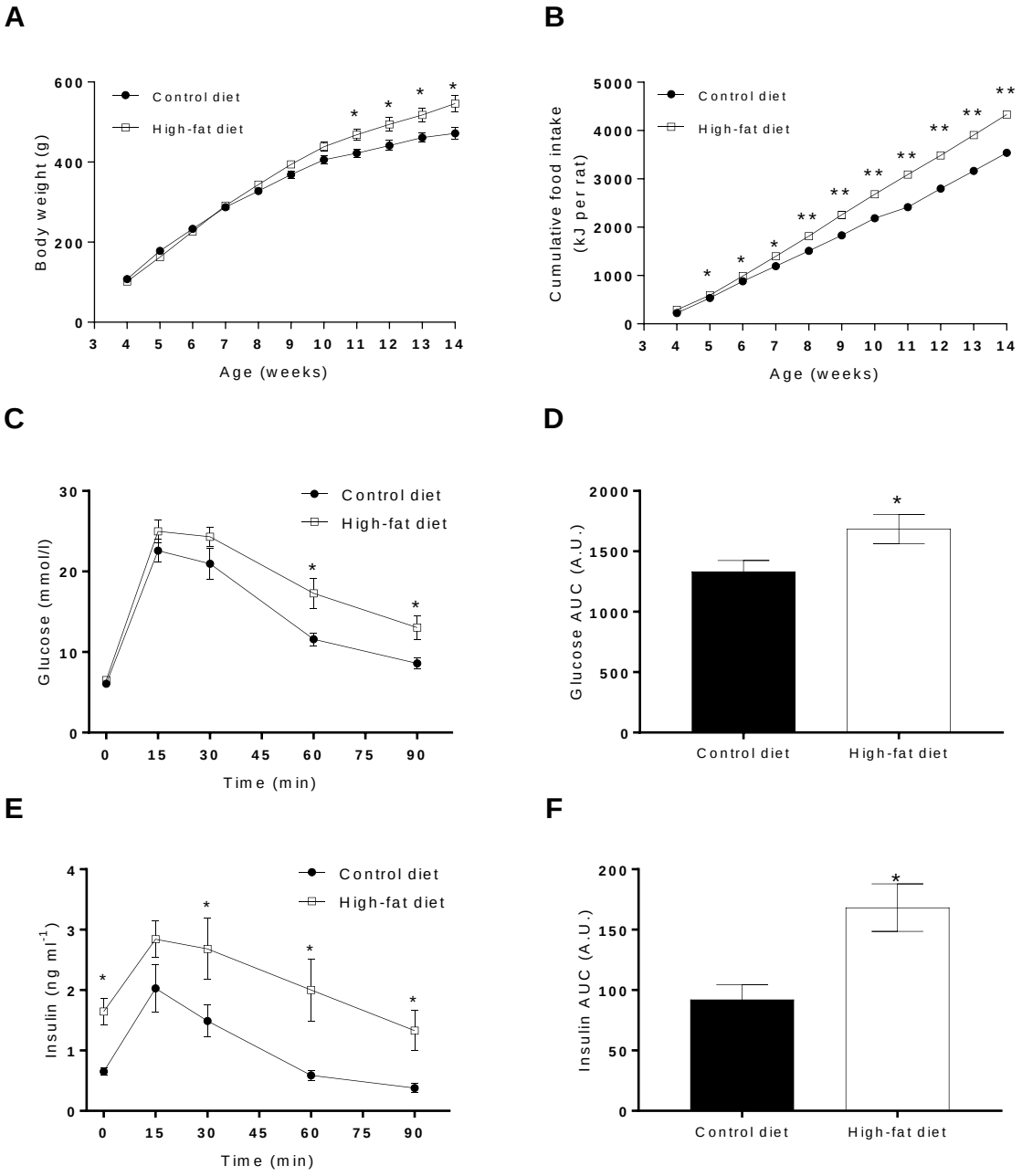

Figure 3-2. Ten weeks of high-fat diet increased body weight, food intake and caused glucose intolerance in male rats (n=9) compared with control diet rats (n=8). Data of body weight (A), cumulative food intake (B) throughout the experiments are presented. Glucose (C) and insulin (E) levels during the glucose tolerance test, and their respective area under the curves (AUC) are presented (D, F). Values are presented as mean ± SEM. * $P < 0.05$ vs fathers on Control diet.

Exercise from 8.5 weeks (including acclimatisation period) until day 19th of gestation did not change body weight (Figure 3-3 A) and food intake in female rats (Figure 3-3 B).

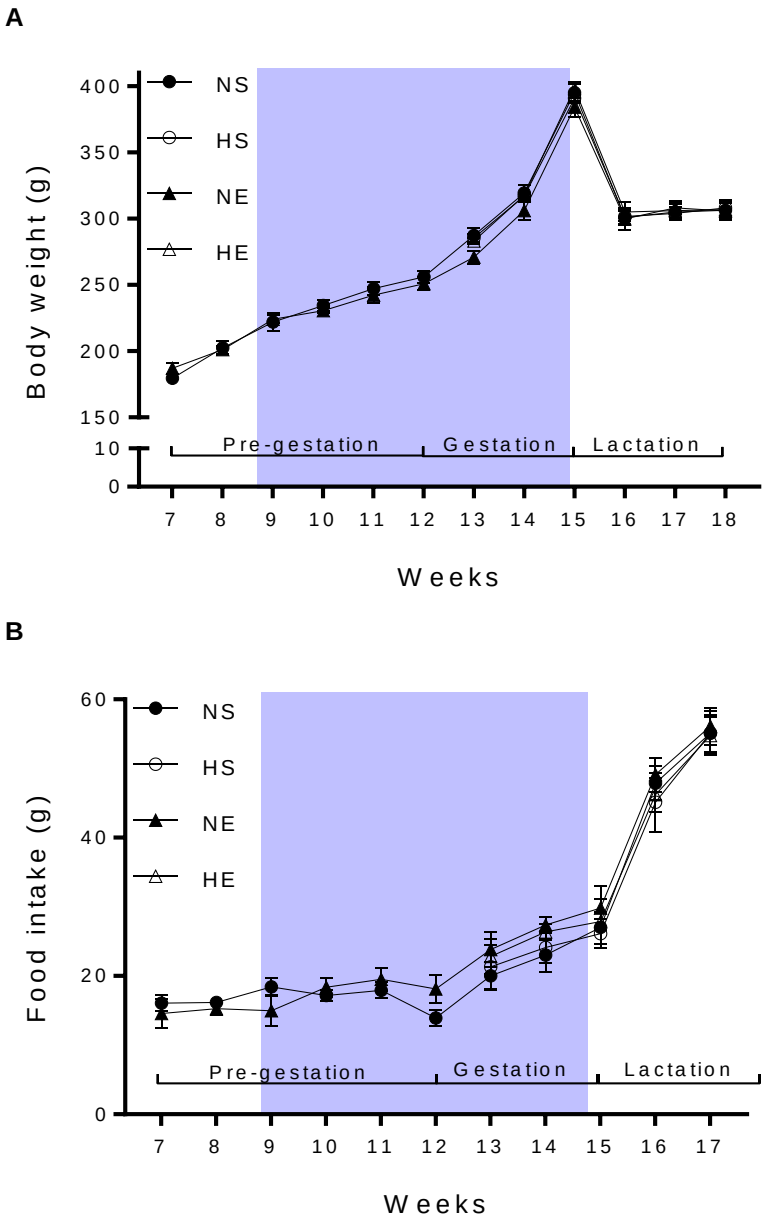

Figure 3-3. Maternal body weight and food intake is not influenced by exercise training. A. Body weight before and during gestation and lactation. B. Food intake per animal, before and during gestation and lactation. Blue rectangles represent the period of exercise training on a treadmill (before gestation and up to day 19 of pregnancy). NS, dams mated with control diet males (n=10); HS, dams mated with high-fat diet males (n=10); NE, exercised dams mated with control diet males (n=9); HE, exercised dams mated with high-fat diet males (n=10). Note that HS and HE groups are displayed after mating (with HFD/obese fathers). Values are presented as mean ± SEM.

3.3.2 No parental effects observed at birth and minor changes on weaning

Paternal high-fat diet did not change the number of male or female rats born, birth weight, or body weight until PND 21 ($P>0.05$) (Table 3-2). The mass of the liver, spleen, heart, kidneys, pancreas and EDL muscle were not affected by paternal high-fat diet at PND 21 ($P>0.05$), while soleus muscle weight was reduced. Maternal exercise also had no effects on number of pups born, birth weight, but a slight reduction in weaning body weight was observed in offspring following paternal HFD/obesity (Table 3-2).

Table 3-2. Paternal high-fat diet and maternal exercise do not alter offspring characteristics from birth (PND1) until weaning (PND21).

Parameters	NS (n=5 litters)	HS (n=5 litters)	NE (n=5 litters)	HE (n=4 litters)
Number of pups born per litter	13.00 ± 1.47	14.80 ± 0.73	12.60 ± 0.68	14.00 ± 0.71
Number of females per litter	5.50 ± 0.50	7.60 ± 1.03	7.20 ± 0.58	8.25 ± 1.31
Number of males per litter	7.50 ± 1.19	7.20 ± 0.73	5.40 ± 0.68	5.75 ± 0.85
PND1 body weight females (g)	7.23 ± 0.11	7.02 ± 0.17	8.09 ± 0.52	6.93 ± 0.53
PND1 body weight of males (g)	7.69 ± 0.31	7.35 ± 0.19	8.55 ± 0.56	6.56 ± 0.46
PND1 blood glucose (mmol/l)	4.67 ± 0.50	5.20 ± 0.25	5.25 ± 0.75	4.90 ± 0.58
Measurements at PND21 (female offspring)				
Body weight (g)	53.94 ± 0.77	52.56 ± 1.00	51.53 ± 0.92	47.36 ± 47.36#
Body length (mm)	128.17 ± 0.76	128.04 ± 1.27	129.57 ± 1.12	125.35 ± 1.68
Liver (g)	2.29 ± 0.06	2.17 ± 0.08	2.25 ± 0.12	2.15 ± 0.23
Spleen (g)	0.24 ± 0.01	0.22 ± 0.03	0.21 ± 0.02	0.20 ± 0.05
Heart (g)	0.28 ± 0.02	0.25 ± 0.02	0.31 ± 0.04	0.25 ± 0.04
EDL (g)	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
Soleus (g)	0.03 ± 0.00	0.02 ± 0.01*	0.03 ± 0.00	0.03 ± 0.00
Kidney (g)	0.31 ± 0.02	0.30 ± 0.02	0.30 ± 0.01	0.27 ± 0.02
Pancreas (g)	0.21 ± 0.01	0.28 ± 0.06	0.22 ± 0.01	0.20 ± 0.01

Blood glucose (mmol/l) ^	11.87 ± 1.31	8.08 ± 1.82	9.03 ± 2.29	8.40 ± 2.07

Sample sizes were considered per litter, not number of individual pups. PND, postnatal day. BMI, body mass index. Values are presented as mean ± SEM (except ranges shown for pups born, number of males, number of females, Mann-Whitney U test was used in these cases). ^, blood glucose collected after a 4h fasting period. NS, offspring sired by control diet fathers and sedentary mothers; HS, offspring sired by high-fat diet fathers and sedentary mothers; NE, offspring sired by control diet fathers and exercised mothers; HE, offspring sired by high-fat diet fathers and exercised mothers. Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$. #, Maternal exercise main effect $P < 0.05$.

3.3.3 Paternal high-fat diet and maternal exercise effects on glucose tolerance in young and adult offspring.

Paternal high-fat diet before mating had no changes in whole body insulin sensitivity (Figure **3-4** A-B), impaired glucose tolerance, increasing the glucose AUC (Figure **3-4** D) in young offspring (12 weeks of age). Insulin levels at 15 and 30 min were higher during the IPGTT, but not significant (P>0.05) (Figure **3-4** E). Maternal exercise did not change whole body insulin sensitivity (Figure **3-4** A-B), but improved glucose tolerance in offspring of a normal diet father (Figure **3-4** C-D). Exercised mothers also attenuated the negative consequences on offspring of having a high-fat eating dad (Figure **3-4** D). When associated with paternal HFD/obesity, maternal exercise decreased the insulin AUC in adolescent female offspring compared to HS group (Figure **3-4** E-F).

At 24 weeks, however, paternal high-fat diet impaired whole body insulin sensitivity in adult rats (Figure 3-5 B). It had no effects on glucose tolerance in adult offspring (Figure 3-5 C-D). Paternal HFD/obesity decreased the insulin levels during the IPGTT at 15 and 30 min, but this was not significant (Figure 3-5 E). In fact, the insulin AUC from 0 to 30 min was significantly lower due to paternal HFD/obesity (Figure 3-5 F). However, the insulin AUC during the IPGTT was significantly lower due to paternal HFD/obesity (Figure 3-5 F). Maternal exercise had no influence on whole body insulin sensitivity (IPITT) in the high-fat father offspring (Figure 3-5 B) and glucose tolerance (Figure 3-5 D), but it attenuated the lower insulin levels during the glucose tolerance test (insulin AUC) (Figure 3-5 F).

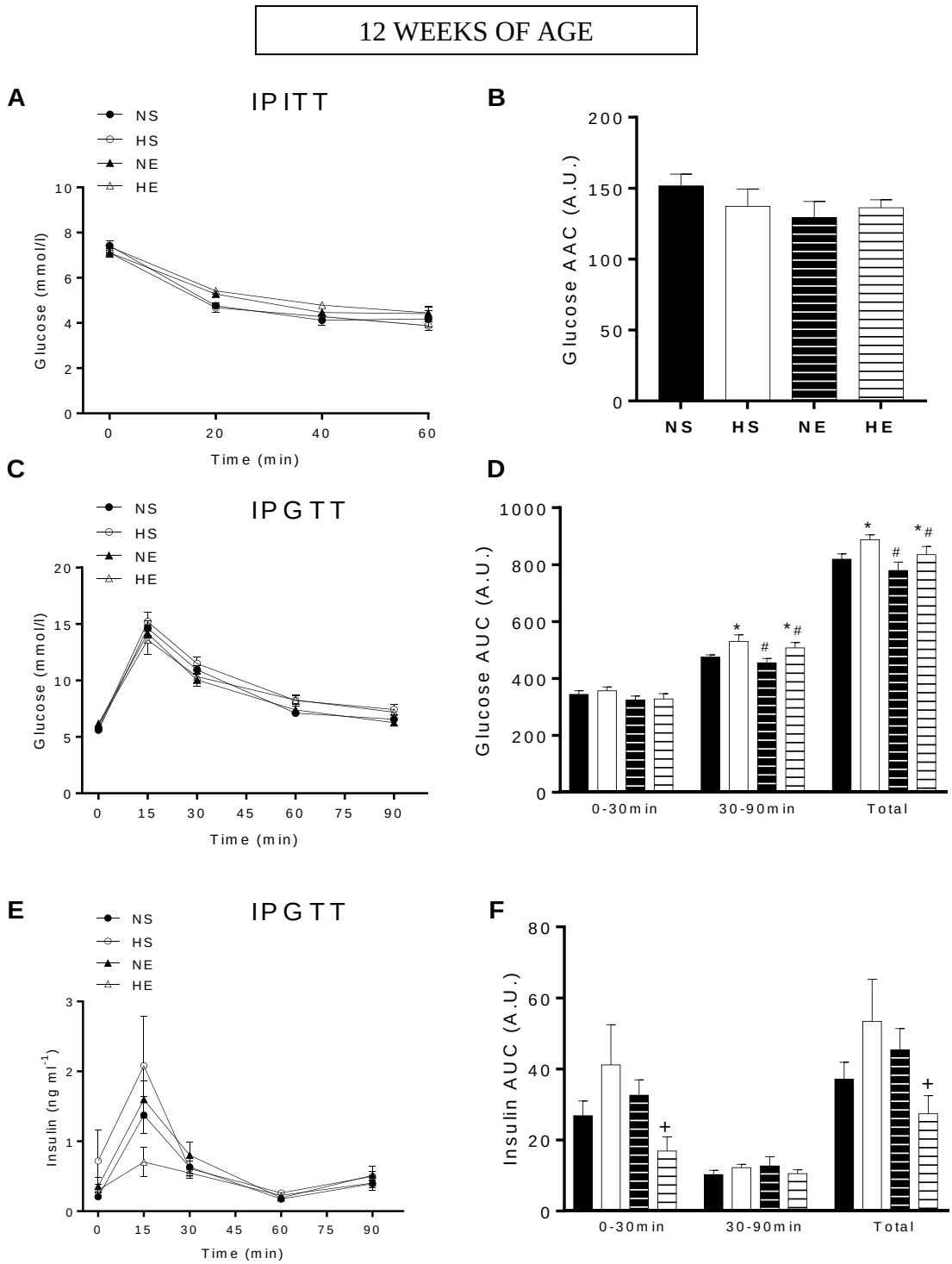

Figure 3-4. Maternal exercise has no effects on whole body insulin sensitivity but protects adolescent (12 weeks of age) female offspring sired by high-fat dads from developing impaired glucose tolerance. Glucose levels during an intraperitoneal insulin tolerance test (IPITT; A) and glucose (C) and insulin (E) levels during an intraperitoneal glucose tolerance test (IPGTT). Area above and under the curve for IPITT (B) and IPGTT (D), respectively. NS, offspring sired by control diet fathers and sedentary mothers (n=10); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=10); NE, offspring sired by control diet fathers and exercised mothers (n=9); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Maternal exercise main effect $P < 0.05$ (NS vs NE; HS vs HE). +, Paternal HFD/obesity vs. Maternal exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

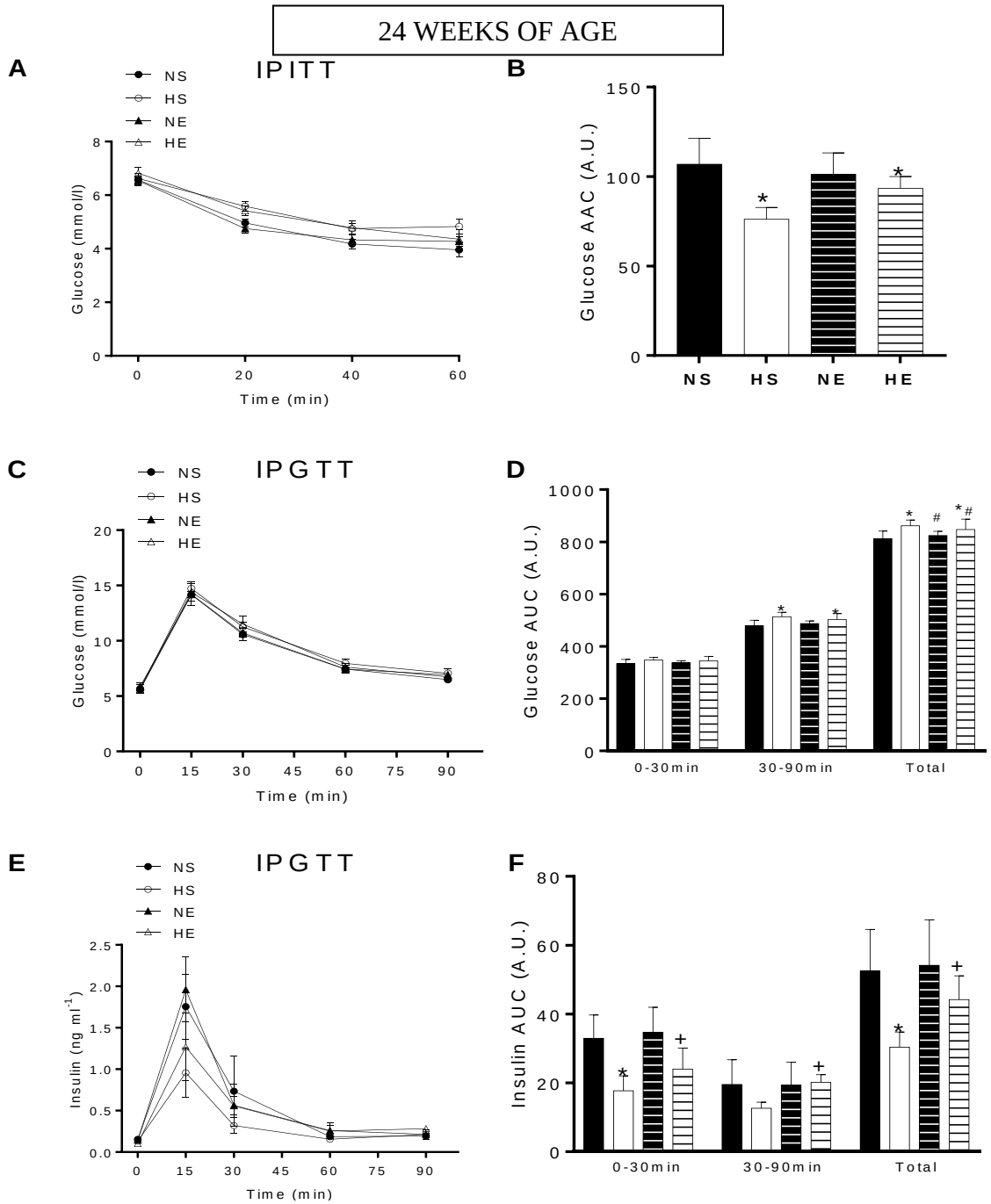

Figure 3-5. Exercise early in life protects older (24 weeks of age) adult female offspring sired by high-fat dads to develop impaired glucose tolerance. Glucose levels during an intraperitoneal insulin tolerance test (IPITT; A) and glucose (C) and insulin (E) levels during an intraperitoneal glucose tolerance test (IPGTT). Area above (AAC) and under (AUC) the curve for IPITT (B) and IPGTT (D, F), respectively. NS, offspring sired by control diet fathers and sedentary mothers (n=10); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=10); NE, offspring sired by control diet fathers and exercised mothers (n=9); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Maternal exercise main effect $P < 0.05$ (NS vs NE; HS vs HE). +, Paternal HFD/obesity vs. Maternal exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

3.3.4 Offspring phenotype at 25 weeks of age

Body weight, SOL and gastrocnemius skeletal muscle weights were lower in offspring sired by HFD/obese fathers (Table **3-3**). Maternal exercise alone decreased to weight of TA muscle, and had no effects on other parameters. Offspring sired by HFD/obese fathers and exercised mothers had normalised soleus and plantaris muscle weights (Table **3-3**).

Table 3-3. Maternal exercise modifies physical characteristics of female offspring sired by high-fat dads at 25 weeks of age.

Parameters	NS n = 10	HS n = 10	NE n = 9	HE n = 10
Body weight (g)	333.47 ± 13.94	299.13 ± 6.98 *	317.23 ± 12.86	315.28 ± 9.55
Length (cm)	24.32 ± 0.25	24.00 ± 0.15	24.61 ± 0.22	24.27 ± 0.15
Abdominal circumference (cm)	18.33 ± 0.45	18.18 ± 0.20	17.90 ± 0.36	17.68 ± 0.33
EDL (g)	0.16 ± 0.07	0.14 ± 0.05	0.16 ± 0.05	0.15 ± 0.01
Soleus (g)	0.15 ± 0.05	0.13 ± 0.06 *	0.15 ± 0.03	0.15 ± 0.03 +
Gastrocnemius (g)	1.75 ± 0.05	1.62 ± 0.02 *	1.73 ± 0.04	1.60 ± 0.02 *
TA (g)	0.67 ± 0.02	0.63 ± 0.01	0.56 ± 0.02 #	0.63 ± 0.01 #
Plantaris (g)	0.33 ± 0.01	0.31 ± 0.07	0.32 ± 0.01	0.33 ± 0.04 +
Liver (g)	8.52 ± 0.31	8.10 ± 0.20	8.75 ± 0.41	9.06 ± 0.42
Pancreas (g)	1.36 ± 0.07	1.20 ± 0.09	1.36 ± 0.07	1.23 ± 0.05
Retroperitoneal fat (g)	6.17 ± 0.35	6.98 ± 0.59	6.43 ± 1.44	6.40 ± 1.50
Kidney right (g)	0.99 ± 0.03	0.91 ± 0.03	0.91 ± 0.03	0.93 ± 0.03
Kidney left (g)	0.98 ± 0.04	0.89 ± 0.02 *	0.92 ± 0.02	0.95 ± 0.03
Heart (g)	1.03 ± 0.03	0.95 ± 0.02	0.98 ± 0.03	1.01 ± 0.03
Fasting glucose (mmol/l)	5.66 ± 0.09	5.63 ± 0.28	5.90 ± 0.35	5.75 ± 0.13

Fasting insulin (ng ml^{-1})	0.15 ± 0.01	0.13 ± 0.02	0.14 ± 0.03	0.11 ± 0.02
HOMA-IR	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00

HOMA-IR, homeostatic model assessment of insulin resistance. Equation was as follows: HOMA-IR = [fasting plasma glucose (mg/dl) x fasting plasma insulin (μU/ml)]/2,430 (Cacho, Sevillano et al. 2008). β-cell function = (Insulin at 0 min / Glucose at 0 min). NS, offspring sired by control diet fathers and sedentary mothers (n=10); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=10); NE, offspring sired by control diet fathers and exercised mothers (n=9); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Maternal exercise main effect $P < 0.05$ (NS vs NE; HS vs HE). +, Paternal HFD/obesity vs. Maternal exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

3.3.5 Offspring skeletal muscle insulin-stimulated glucose uptake

Despite the expected insulin effect, the basal and insulin-stimulated glucose uptake *ex vivo* in soleus muscle was not affected by any treatment (Figure 3-6 A). On the other hand, glucose uptake in EPI muscle of offspring sired by HFD/obese fathers was markedly impaired at basal and insulin-stimulated conditions (Figure 3-6 B). Maternal exercise did not restore basal glucose uptake, but normalised insulin-stimulated glucose uptake in offspring from HFD/obese dads (Figure 3-6 B).

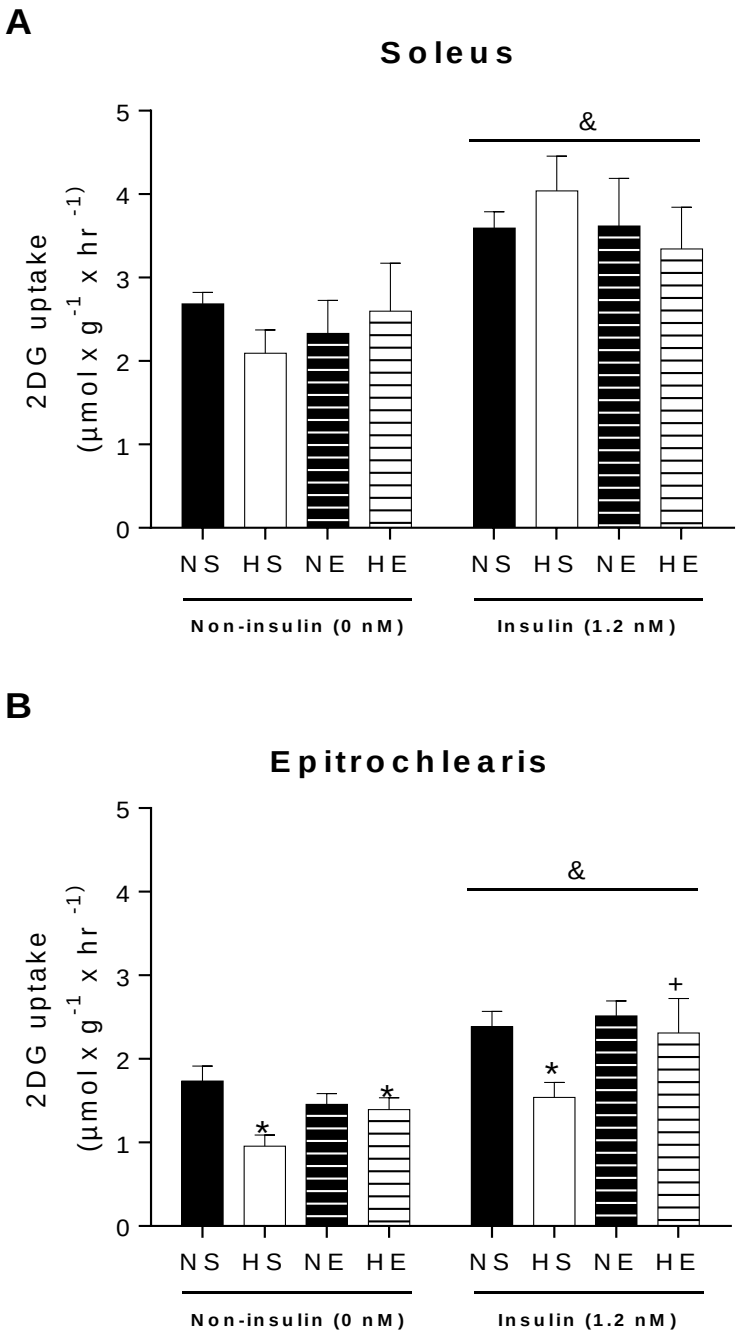

Figure 3-6. Paternal high-fat diet induced obesity before conception impairs basal and insulin-stimulated glucose uptake in a muscle-specific way, while maternal exercise protects insulin-stimulated glucose uptake in adult female offspring (25 weeks of age). NS, offspring sired by control diet fathers and sedentary mothers (n=10); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=10); NE, offspring sired by control diet fathers and exercised mothers (n=9); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM. &, Insulin main effect $P < 0.05$ (0nM vs 1.2nM). * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). +, Paternal HFD/obesity vs. Maternal exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

3.3.6 Offspring skeletal muscle insulin signalling

As glucose uptake was impaired in EPI muscle only, we chose to perform western blots on proteins related to the insulin signalling in this muscle only. In insulin-stimulated condition, paternal HFD/obesity augmented p-AKT^{Se473} (Figure 3-7 C) while p-AKT^{Thr308} did not change (Figure 3-7 B). TBC1D4 phosphorylated protein (Thr642) was also lower in HFD offspring (HS vs NS) (Figure 3-8 B). In terms of glucose transporters, paternal HFD/obesity decreased GLUT1 expression (Figure 3-9 A) and had no significant effect on GLUT4 (Figure 3-9 B).

Maternal exercise increased even more p-AKT^{Se473} in insulin-stimulated conditions (Figure 3-7 C) but was not able to overcome the lower protein expression in HFD offspring in regards to the lower phosphorylation of TBC1D4 (Thr642) (Figure 3-8 B). Exercise before and during pregnancy also increased GLUT1 protein expression (Figure 3-9 A) and GLUT4 (Figure 3-9 B) compared to normal diet fathers. Maternal exercise did not normalise the lower GLUT1 protein expression in offspring sired by HFD/obese fathers (Figure 3-9 A).

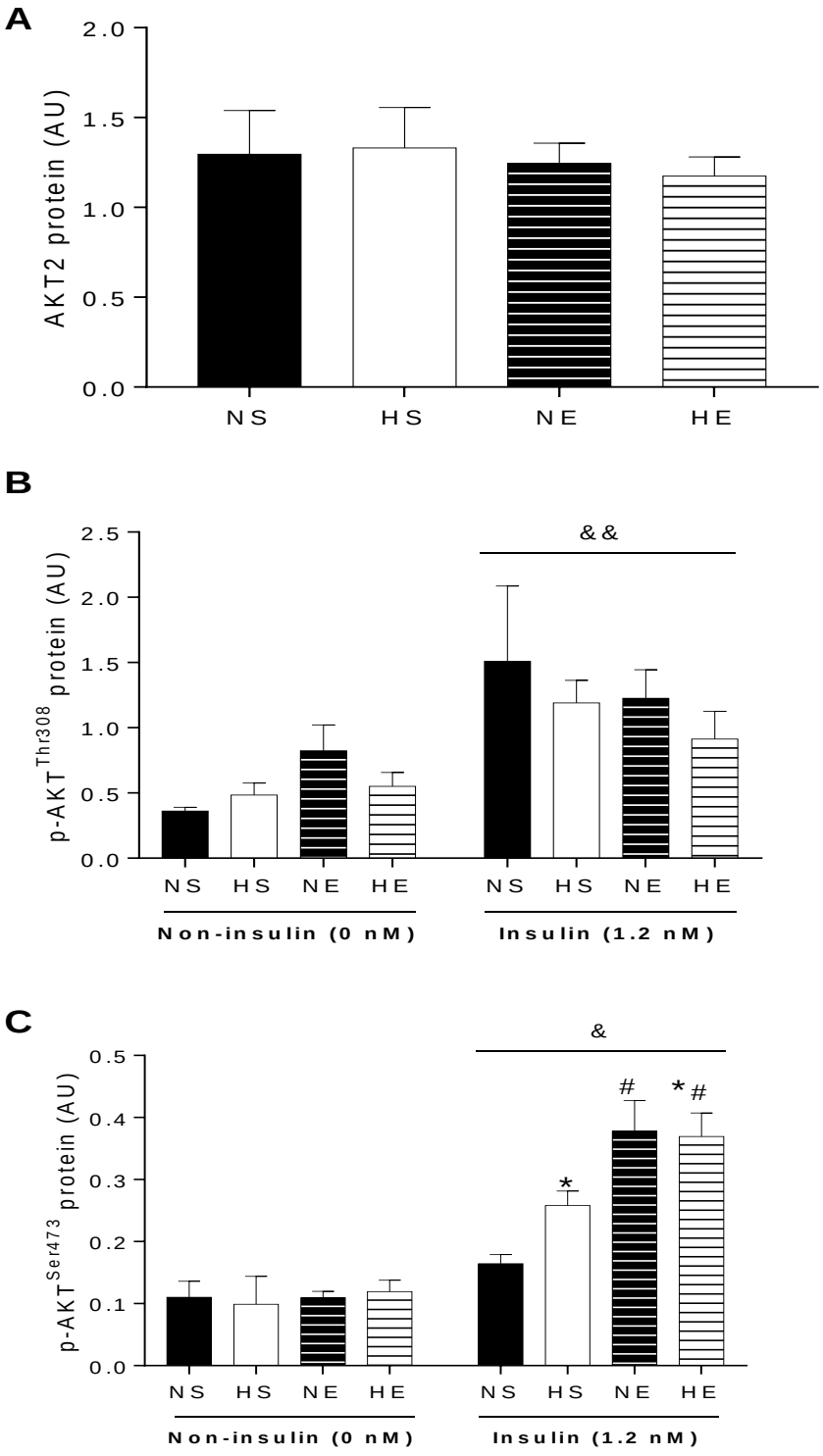

Figure 3-7. Parental effects on proximal insulin signalling in epitrochlearis muscle of adult female offspring. A, total AKT. B, p-AKTThr308. C, p-AKTSer473. NS, offspring sired by control diet fathers and sedentary mothers (n=6); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=6); NE, offspring sired by control diet fathers and exercised mothers (n=6); HE, offspring sired by high-fat diet fathers and exercised mothers (n=6). Values are presented as mean ± SEM. &, && Insulin main effect $P < 0.05$, 0.01 (0nM vs 1.2nM). * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Maternal exercise main effect $P < 0.05$ (NS vs NE; HS vs HE).

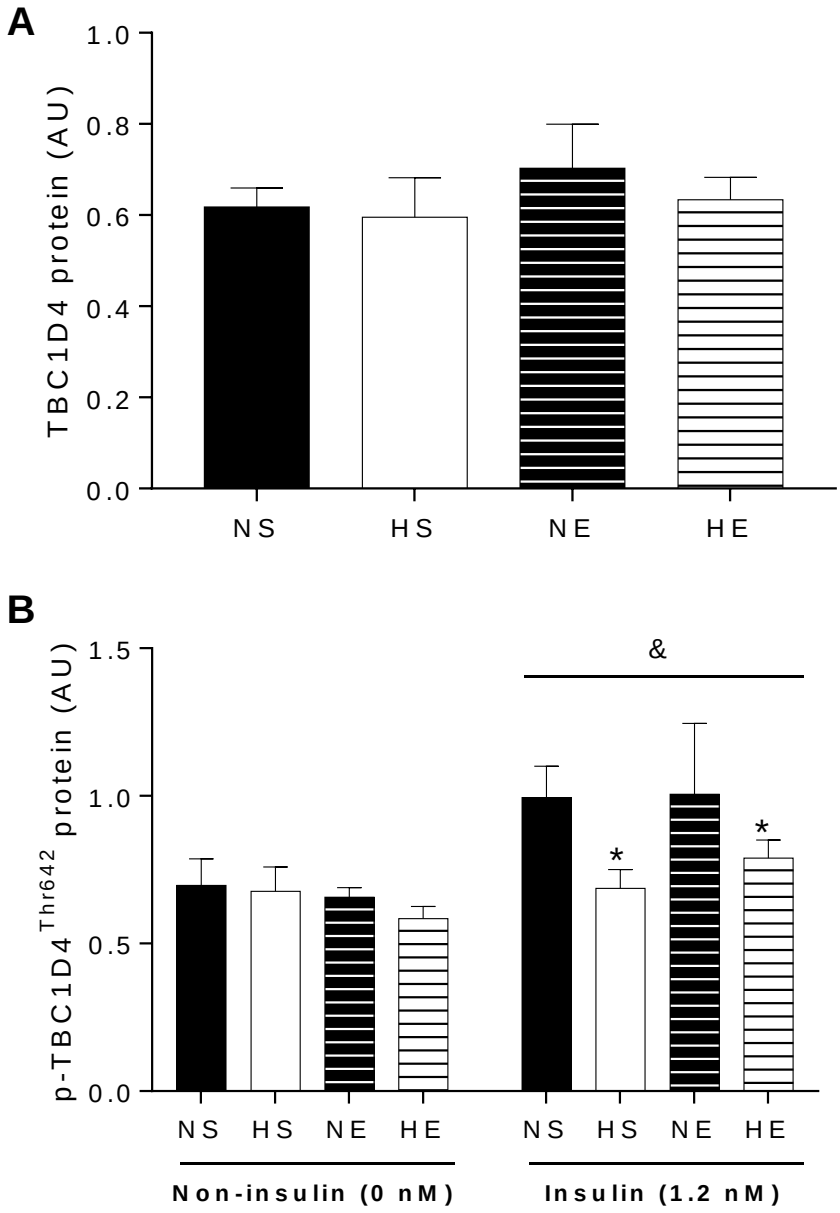

Figure 3-8. Parental effects on distal insulin signalling in epitrochlearis muscle of adult female offspring. A, total TBC1D4. B, p-TBC1D4Thr642. NS, offspring sired by control diet fathers and sedentary mothers (n=6); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=6); NE, offspring sired by control diet fathers and exercised mothers (n=6); HE, offspring sired by high-fat diet fathers and exercised mothers (n=6). Values are presented as mean ± SEM. &, Insulin main effect $P < 0.05$ (0nM vs 1.2nM). * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE).

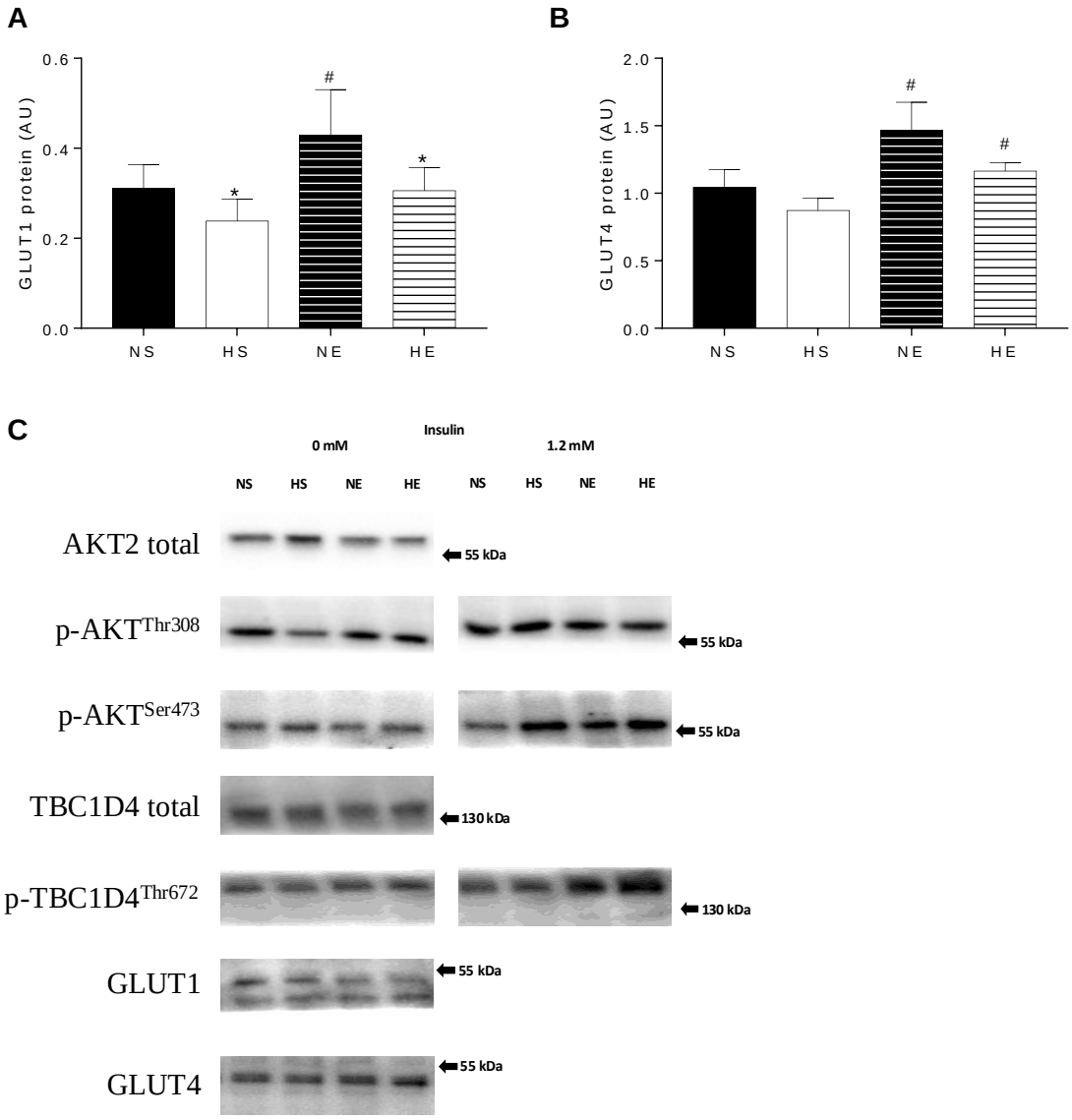

Figure 3-9. Parental effects on GLUT1 and GLUT4 protein expression in epitrochlearis muscle of adult female offspring. A, GLUT1. B, GLUT4. C, representative blots of proteins measured. Representative western blots show the quality and signal obtained with the respective antibodies; because they represent one animal, they do not necessarily represent an exact mean of their experimental group. NS, offspring sired by control diet fathers and sedentary mothers (n=6); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=6); NE, offspring sired by control diet fathers and exercised mothers (n=6); HE, offspring sired by high-fat diet fathers and exercised mothers (n=6). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Maternal exercise main effect $P < 0.05$ (NS vs NE; HS vs HE).

3.3.7 Mitochondrial respiration, flux control ratios and citrate synthase activity

Paternal HFD/obesity did not change citrate synthase activity (Figure **3-10**) and most of the measured mitochondrial respiration components (Figure **3-11** and Figure

3-12), or respiratory control ratios (Figure 3-13). Leak through complex I was lower due to paternal HFD/obesity in mitochondrial respiration (Figure **3-11** A). Maternal exercise increased O_2 flux in both CI_P (Figure 3-12 C) and CII_E (Figure 3-12 C) when compared to sedentary sired by HFD/obese fathers (HE vs HS). Maternal exercise increased CS activity in offspring sired by HFD/obese fathers (Figure **3-10**). In addition, when mitochondrial respiration was normalised by CS activity, maternal exercise decreased $CI+II_P$ (Figure **3-11** F) and CII_E (Figure 3-12 D). Respiratory ratios did not change among treatments (Figure 3-13).

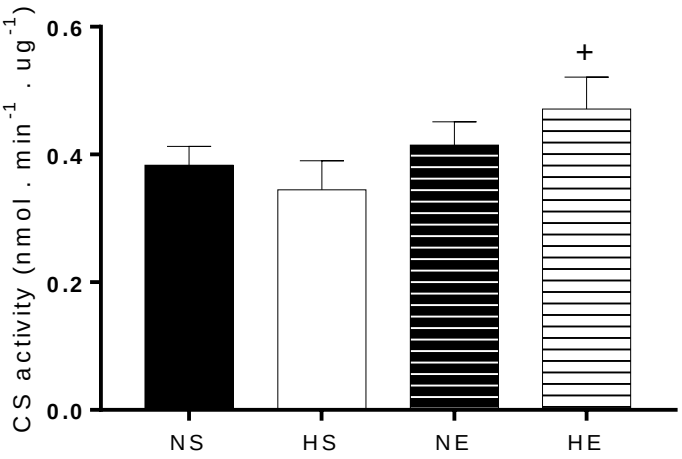

Figure 3-10. Parental effects on citrate synthase activity in adult female offspring. NS, offspring sired by control diet fathers and sedentary mothers (n=9); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=8); NE, offspring sired by control diet fathers and exercised mothers (n=9); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM. +, Paternal HFD/obesity vs. Maternal exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

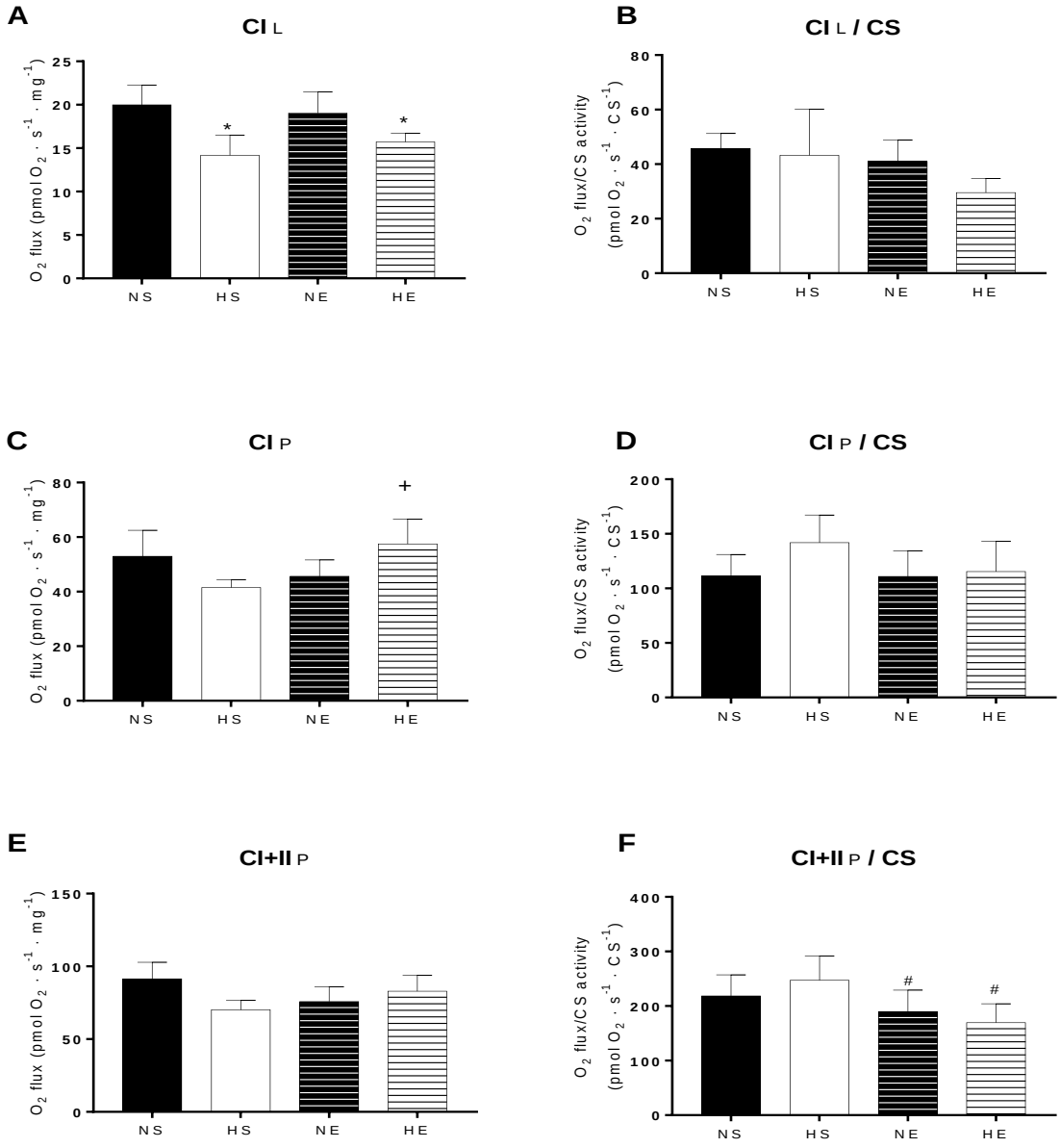

Figure 3-11. Mass-specific mitochondrial respiration in adult offspring sired by control or high-fat eating fathers and sedentary or exercised mothers. A, Leak respiration through complex I (CI_L). B, Maximum oxidative phosphorylation (oxphos) capacity (*P*) through CI (CI_P). C, *P* through complexes I+II ($CI+II_P$). D, electron transport system (ETS) capacity (*E*) through CI+II ($CI+II_E$). E, *E* through complex II (CII_E). NS, offspring sired by control diet fathers and sedentary mothers (n=9); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=8); NE, offspring sired by control diet fathers and exercised mothers (n=9); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Maternal exercise main effect $P < 0.05$ (NS vs NE; HS vs HE). +, Paternal HFD/obesity vs. Maternal exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

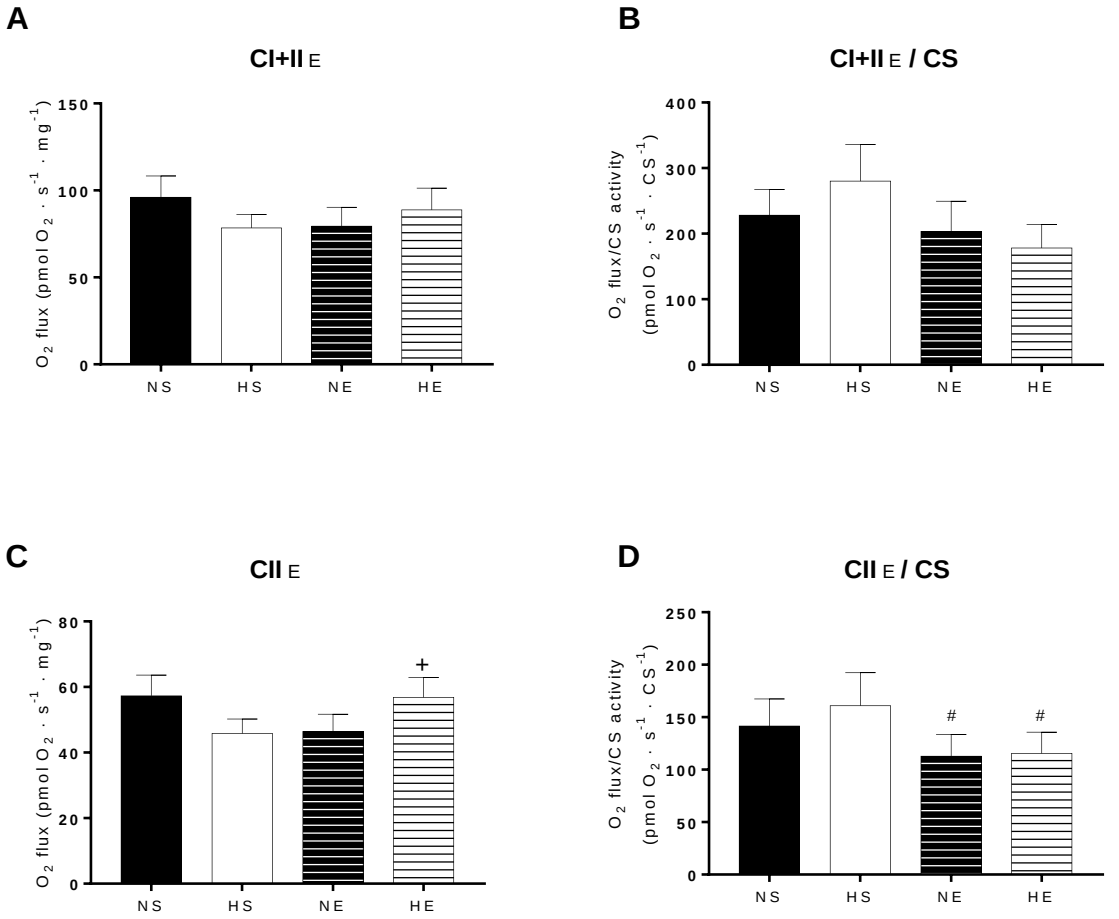

Figure 3-12. Mitochondrial-specific respiration (normalised by citrate synthase activity) in adult offspring sired by control or high-fat eating fathers and sedentary or exercised mothers. Mitochondrial respiration data normalised to citrate synthase (CS) activity. A, Leak respiration through complex I (CIL). B, Maximum oxidative phosphorylation (oxphos) capacity (P) through CI (CIP). C, P through complexes I+II (CI+IIP). D, electron transport system (ETS) capacity (E) through CI+II (CI+IIE). E, E through complex II (CIIE). NS, offspring sired by control diet fathers and sedentary mothers (n=9); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=8); NE, offspring sired by control diet fathers and exercised mothers (n=9); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM. #, Maternal exercise main effect $P < 0.05$ (NS vs NE; HS vs HE). +, Paternal HFD/obesity vs. Maternal exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

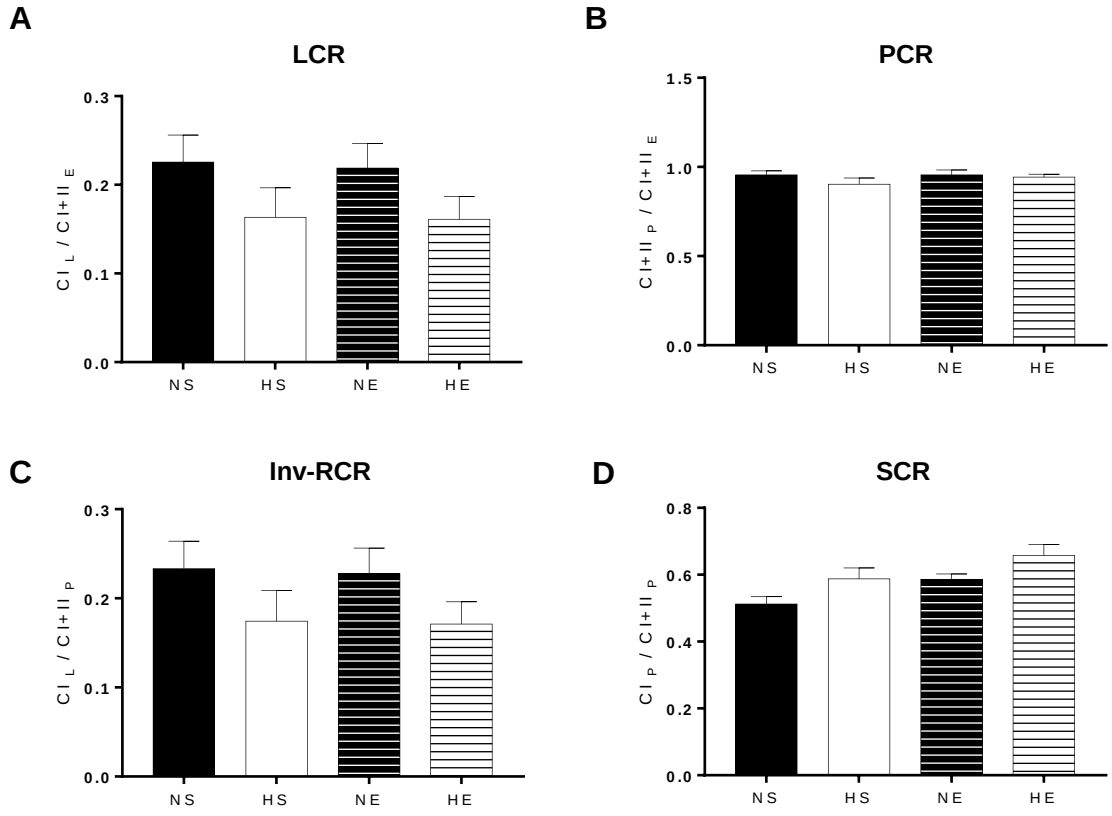

Figure 3-13. Respiratory flux control ratios (FCRs) in adult offspring which performed exercise early in life or not, sired by control or high-fat eating fathers before conception. LCR, leak control ratio (CIL/CI+IIE); PCR, phosphorylation control ratio (CI+IIP/CI+IIE); inv-RCR, inverse of respiratory control ratio (CIL/CI+IIP); SCR, substrate control ratio at constant P (CIP/CI+IIP). FCRs were calculated from mass specific respiration measurements in permeabilised muscle fibres (plantaris muscle) obtained from female rats at 25 weeks of age. NS, offspring sired by control diet fathers and sedentary mothers (n=9); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=8); NE, offspring sired by control diet fathers and exercised mothers (n=9); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM.

3.3.8 Mitochondrial ROS production

Mitochondrial H_2O_2 production was measured simultaneously to the mitochondrial respiration. Paternal HFD/obesity did not affect H_2O_2 emission (Figure 3-14). Maternal exercise, when compared to HS group, decreased H_2O_2 emission on the OXPHOS CI+II stage. Other stages were not affected by maternal exercise (Figure 3-14).

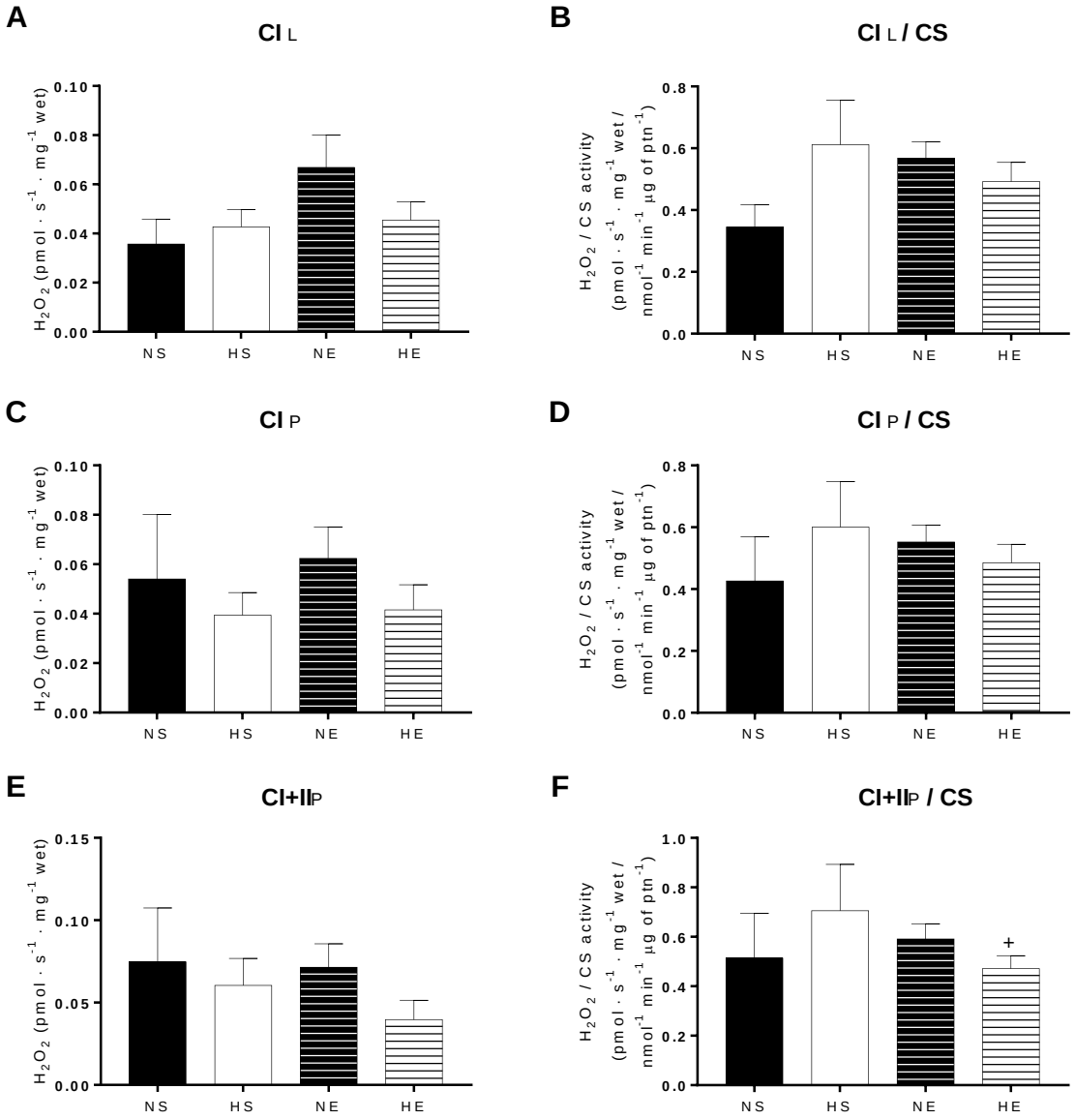

Figure 3-14. H_2O_2 production simultaneously measured during the mitochondrial respiration. A, Hydrogen peroxide (H_2O_2) levels during leak respiration through complex I (CIL). B, H_2O_2 levels during the maximum oxidative phosphorylation capacity through complex I (CIP). C, H_2O_2 levels during the maximum oxidative phosphorylation capacity through complex I and II combined (CI+IIP). B, D and F are these respective values normalised by citrate synthase (CS) activity. NS, offspring sired by control diet fathers and sedentary mothers (n=9); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=8); NE, offspring sired by control diet fathers and exercised mothers (n=9); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM. +, Paternal HFD/obesity vs. Maternal exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

3.3.9 Mitochondrial-related protein expression

HS offspring had lower Tfam and PHF20 protein expression. PGC1α, was not affected by paternal HFD/obesity. Maternal exercise did not prevent any of these lower protein expressions (Figure 3-15).

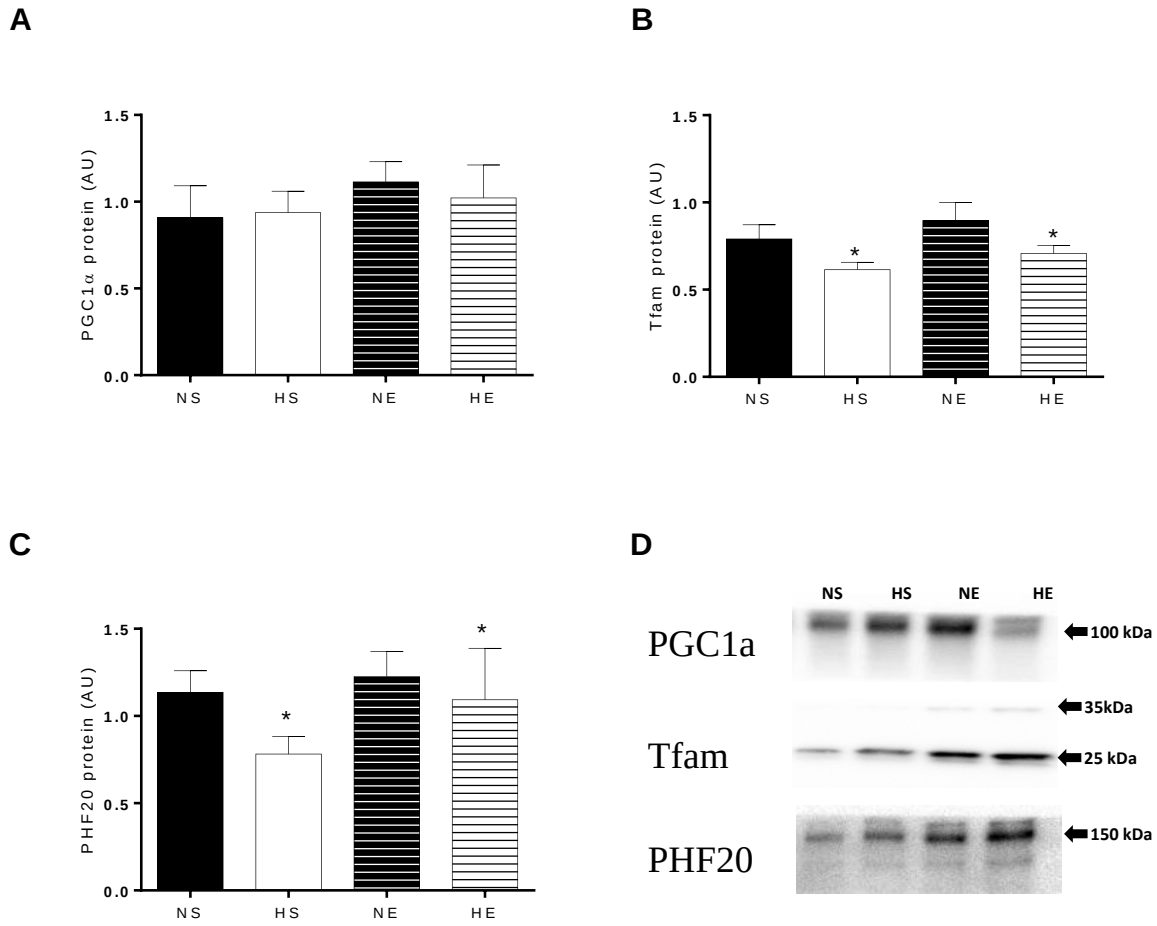

Figure 3-15. Parental effects on protein expression in plantaris muscle in adult female offspring. A, PGC1α. B, TFAM. C, PHF20. D, Representative blots. Representative western blots show the quality and signal obtained with the respective antibodies; because they represent one animal, they do not necessarily represent an exact mean of their experimental group. NS, offspring sired by control diet fathers and sedentary mothers (n=10); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=10); NE, offspring sired by control diet fathers and exercised mothers (n=10); HE, offspring sired by high-fat diet fathers and exercised mothers (n=9). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE).

3.3.10 Pancreas morphology in adult offspring

Relative islet surface area, number of islets, β-cell area and proportion were not affected by paternal HFD/obesity (Figure 3-16 A-B, D-E). However, β-cell mass and the insulinogenic index were lower (Figure 3-16 C), and islet size distribution was altered with a higher number of small islets and a lower number of large and very large islets in HFD offspring (Figure 3-17). The insulinogenic index was lower due to paternal HFD/obesity (Figure 3-16 F). Maternal exercise increased the relative islet surface area, the number of islets, and normalised the lower β-cell mass and insulinogenic index in HS offspring (Figure 3-16). Maternal exercise also prevented the alterations in islet size distribution observed in offspring sired by HFD/obese fathers (Figure 3-17).

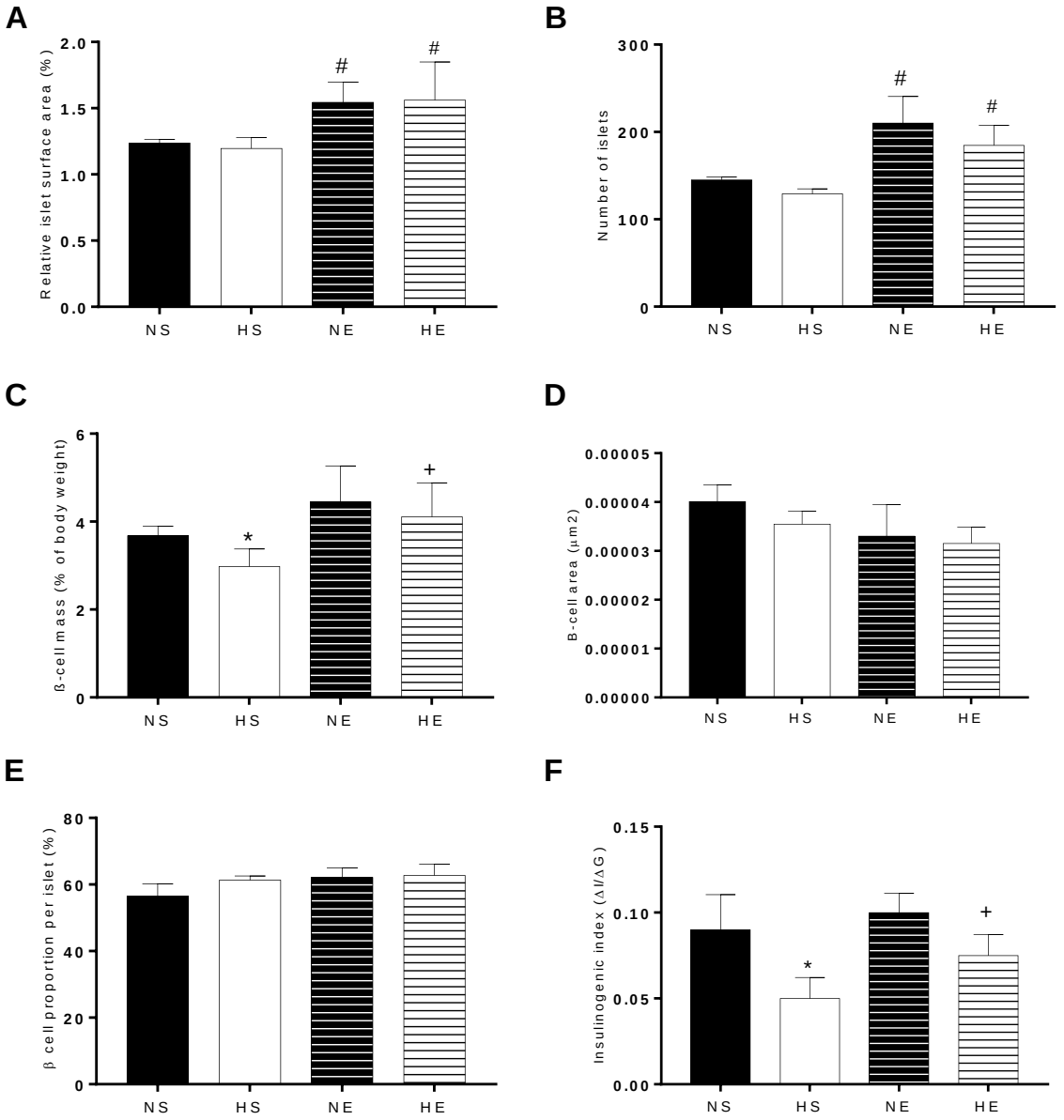

Figure 3-16. Pancreas morphology in adult offspring sired by fathers fed high-fat or control diets and exercised mothers. A, relative islet surface area expressed as a percentage of total pancreas surface area. B, number of islets. C, β-cell mass was calculated as the product of whole pancreas weight before fixation and the ratio of insulin positive/total pancreas cross-sectional area. D, β-cell area. E, β-cell proportion per islet. F. Insulinogenic index, derived from IPGTT at 24 weeks of age with the following formula: Insulinogenic index = AUC insulin (0–30 min) / AUC glucose (0–30 min) (Ng et al. 2010). NS, offspring sired by control diet fathers and sedentary mothers (n=10); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=10); NE, offspring sired by control diet fathers and exercised mothers (n=10); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Maternal exercise main effect $P < 0.05$ (NS vs NE; HS vs HE). +, Paternal HFD/obesity vs. Maternal exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

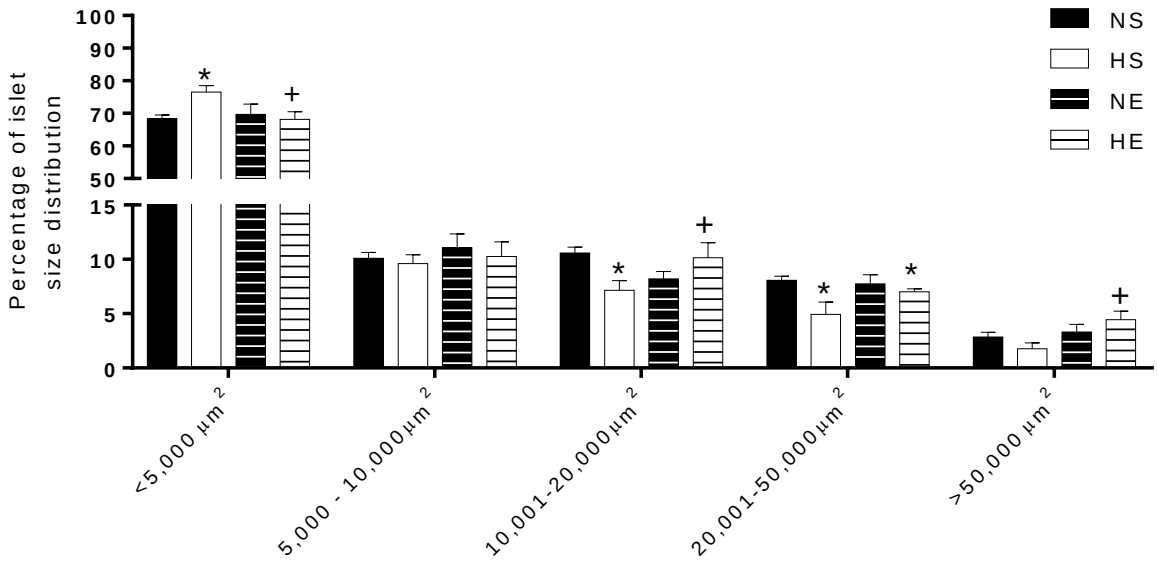

Figure 3-17. Islet distribution. Islets were arbitrarily classified according to their size: <5,000 µm^2, 5,000 – 10,000 µm^2, 10,001 – 20,000 µm^2, 20,001 – 50,000 µm^2 and >50,000 µm^2. NS, offspring sired by control diet fathers and sedentary mothers (n=10); HS, offspring sired by high-fat diet fathers and sedentary mothers (n=10); NE, offspring sired by control diet fathers and exercised mothers (n=10); HE, offspring sired by high-fat diet fathers and exercised mothers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). +, Paternal HFD/obesity vs. Maternal exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

3.4 Discussion

As mentioned before (section 2.1), the data of offspring sired by control diet fathers and HFD/obese fathers are the same in Chapter 3 and 4. To avoid repetition in the discussion sections of these chapters, paternal HFD/obesity will be discussed thoroughly in Chapter 3 and only mentioned in Chapter 4 for the context in comparison to the exercised groups.

The main findings of this study were that adult offspring sired by HFD/obese father had reduced insulin sensitivity due predominantly to effects on skeletal muscle as demonstrated by a reduced insulin tolerance test (*in vivo*) and reduced insulin-stimulated glucose uptake (*ex vivo*). Here, we have also shown that, in offspring sired by HFD/obese fathers, maternal exercise did not normalise the reduced insulin sensitivity *in vivo* or insulin signalling in skeletal muscle. Remarkably, maternal exercise attenuated the lower insulin secretion, which can be partially explained by the higher β-cell mass compared to adult offspring sired by HFD/obese fathers.

3.4.1 Effects of high-fat diet in male breeders

We firstly confirmed that feeding high-fat diet (40-43% of energy as fat) to young male Sprague-Dawley rats for 10 weeks increased body weight, abdominal circumference, retroperitoneal fat, and predisposes the rats to glucose intolerance with adverse impacts in both glucose and insulin levels. This has been demonstrated by other research groups including in different strains and species of rats (Ng et al. 2010, Cropley et al. 2016, Fullston et al. 2013), reassuring that high-fat feeding is a reliable model to induce obesity and insulin resistance in rodents. Previous studies do not indicate if all animals on HFD diet were included in the experiments (Ng et al. 2010, Lecomte et al. 2016, Chowdhury et al. 2016), but it is worth mentioning that we did not exclude any animals, despite some rats not gaining excessive body weight due to 10 weeks of high-fat feeding. Also, it was not our focus of investigation to make a distinction between diet-induced obesity or insulin resistant fathers and its consequences on the offspring. Our model used a HFD that increased body weight, fat mass, and caused glucose intolerance in the fathers. There is, however, evidence supporting that obese, non-diabetic mice generates offspring with disturbed metabolism later in life (Fullston et al. 2013).

3.4.2 Parental effects on offspring characteristics at birth, weaning and growth

Paternal diet-induced obesity before mating did not affect the number of pups born, the ratio between males and females and the birth weight of the litter. Our results corroborate previous findings where no differences in birth weight were found after paternal diet-induced obesity (Masuyama et al. 2016, Ng et al. 2010). Although studies in humans have shown that paternal BMI is associated with offspring BMI in youth (11 years old), and may persist until adulthood (~45 years) (Li et al. 2009), studies in rodents are controversial, demonstrating higher (Fullston et al. 2013, Masuyama et al. 2016) or no difference in body weight of adult offspring after HFD in dads (Ng et al. 2010). Unexpectedly, we found decreased body weight in adult offspring fathered by male rats fed with high-fat diet before conception. This was particularly surprising as we purposely attempted to very closely match the protocol of Ng *et al*, and they found no difference in body weight of their offspring sired by HFD father at 12 weeks of age (Ng et al. 2010). It is possible that if older animals were examined as we did (25 weeks of age) different results could have been observed in the Ng *et al.* study. In fact, recent data from the same laboratory showed that older males sired by HFD/obese fathers have lower body weight

from 8 until 25 weeks of age (Lecomte et al. 2016). This same study also found that adolescent (8 weeks of age) offspring sired by HFD fathers at had lower expression of growth markers in skeletal muscle (Lecomte et al. 2016). It is also important to mention that in the Ng *et al.* manuscript that we closely followed, we were unaware that, in addition to the high-fat pellets, "a small amount of standard laboratory chow was also available in the cage" [p.38, extracted from the doctorate thesis which originated the publication (Ng 2011)]. This was not mentioned in the Ng *et al.* manuscript (Ng et al. 2010) but has been mentioned in recent follow up publications from this group (Lecomte et al. 2016, Chowdhury et al. 2016). Therefore, it is possible their offspring were not lighter as the fathers had HFD and some chow diet while our rats had high-fat only, as described in the methods section.

Both maternal and paternal effects have the capacity to shape offspring's phenotype (Uller 2008). Like us, others found in rats no effects of maternal physical activity (running wheel) on birth weight (Carter et al. 2012, Carter et al. 2013, Stanford et al. 2015). However, exercise intensity and volume, as well as when the exercise starts, can influence neonatal weight [reviewed elsewhere (Hopkins and Cutfield 2011)]. Our protocol of maternal exercise training has started with progressive increase in intensity and volume until gestation, and continuing with progressive decrease in intensity and volume until day 19 of pregnancy. Our results show that maternal exercise training had no effect of birth weight of all groups. Previously, we have reported no changes in birth weight with a similar exercise training protocol (Fidalgo et al. 2012).

We found that adult offspring (25 weeks of age) sired by obese fathers had lower body weight. The soleus, gastrocnemius and plantaris skeletal muscles were also lighter compared to offspring from normal fed fathers. The lower the muscle mass, the lower the energy requirements as demonstrated by the lower food intake in offspring sire by HFD/obese fathers. On the other hand, maternal exercise was able to normalise body and skeletal muscle weights (except gastrocnemius) as well as attenuate the reduced food intake, suggesting that further benefits could be associated with this stimulus *in utero*.

3.4.3 Offspring glucose tolerance and insulin sensitivity *in vivo*

In this study, we showed glucose intolerance (higher glucose AUC) in adolescent female rats (12 weeks of age) sired by obese fathers. Maternal exercise was able to prevent glucose tolerance in the adolescent offspring exposed to paternal diet-induced obesity. To the best of our knowledge, we are the first group showing the combined

effects of maternal exercise and paternal HFD/obesity. Other research groups reported similar benefits due to maternal physical activity alone. For instance, both mice (Carter et al. 2012) and rats (Carter et al. 2013, Stanford et al. 2015) born from dams with access to running wheel before and during gestation had improved glucose tolerance later in life (lower glucose AUC). In offspring born from HFD/obese mothers, maternal exercise is able to normalise glucose tolerance (Stanford et al. 2015). This suggests that maternal exercise can protect the offspring early in life in terms of glucose tolerance, even when the offspring is exposed to other insults, such as maternal undernutrition (Fidalgo et al. 2012), maternal obesity (Stanford et al. 2015), or, as we showed here, paternal diet-induced obesity.

In adulthood, paternal HFD/obesity compromised insulin sensitivity (estimated *in vivo* by IPITT) in the offspring. This finding is in agreement with other research groups that showed that offspring fathered by obese mice had glucose intolerance along with insulin resistance (IPITT) later in lifer (26, and 39 weeks of age) (Fullston et al. 2013). Surprisingly, we found maternal exercise before and during gestation was not able to attenuate the negative effects of paternal diet-induced obesity on insulin sensitivity *in vivo*, as there were no changes in IPITT or IPGTT. In both mice and rats, maternal physical activity (running wheel) before and during gestation has been shown to improve insulin sensitivity in adult offspring compared to control (sedentary, normal chow fed parents) rodents (Carter et al. 2012, Carter et al. 2013, Stanford et al. 2015). Induced changes in offspring phenotype may be dependent on the regime of exercise as well as the physical fitness of the mother, the time point in the pregnancy when the exercise is performed, and the frequency, duration and intensity of the exercise (Clapp 2003, Clapp et al. 2002). Free physical activity in a running wheel results in a higher exercise volume compared with the exercise usually performed on a treadmill. In fact, pregnant mice and rats can run up to 6-8km per day in a running wheel (Carter et al. 2013, Stanford et al. 2015) compared to ~1km achieved in an average session on 1h of exercise training in our protocol on the treadmill. In addition, with our treadmill training, we control the exercise intensity, which is generally not addressed in studies using running wheel models. These differences in the protocol of exercise (intensity, duration and volume) could explain, at least in part, the divergent findings. Further studies should focus on investigating appropriate volume and intensity of exercise required to obtain positive impact on the offspring. Nevertheless, we showed that maternal exercise on a treadmill before and

during pregnancy has effects that are more prominent early in life (adolescent offspring) compared to later in life (adulthood) in terms of insulin sensitivity *in vivo*.

3.4.4 Offspring glucose uptake and insulin signalling in skeletal muscle

The skeletal muscle contributes most of the insulin-stimulated glucose uptake *in vivo* (DeFronzo and Tripathy 2009). Paternal diet-induced obesity did not affect glucose uptake in the predominantly oxidative soleus muscle while the predominantly glycolytic epitrochlearis muscle was markedly compromised. No other studies have investigated muscle-specific glucose uptake in these offspring. We not only demonstrated this negative effect on insulin-stimulated glucose uptake in skeletal muscle, but we also showed that this might be due to lower phosphorylation of TBC1D4.

We have highlighted that this is the first evidence that paternal HFD/obesity reduces both basal and insulin-stimulated skeletal muscle glucose uptake *ex vivo* in adult offspring. In regards to the lower basal glucose uptake, GLUT1 is the main protein related to basal glucose uptake in skeletal muscle, and does not necessarily require insulin for its action (Ebeling et al. 1998). Although paternal diet-induced obesity attended to reduce GLUT1 protein expression in epitrochlearis muscle (25%), this did not reach statistical significance (P=0.185). Therefore, the reasons for the lower basal glucose uptake are not clear. Maternal exercise, on the other hand, increased GLUT1 protein expression, independent of paternal HFD/obesity but basal glucose uptake was not increased.

We also analysed insulin signalling pathway in order provide mechanistic insights to the effects on the insulin-stimulated glucose uptake. Although required for insulin-stimulated glucose disposal into skeletal muscle (Cleasby et al. 2007), AKT2 was not different among the groups in basal condition. In regards to the insulin-stimulated condition, there were no changes in p-AKT^{Thr308} among the groups. In addition, unexpectedly, paternal diet-induced obesity increased p-AKT^{Ser473} in adult offspring, and even higher phosphorylation in this site was observed due to maternal exercise. However, it should be considered that only a small proportion of AKT needs to be phosphorylated (~10%) to obtain maximum increases in AKT activity (Tan et al. 2012b), so the relevance of increases in p-AKT^{Ser473} above this level are not clear. We also investigated p-TBC1D4^{Thr642}, which was compromised in adult offpsring due to paternal diet-induced obesity. Maternal exercise did not change phosphorylation of insulin TBC1D4^{Thr642}. GLUT4 protein expression was not significantly lower in HFD offspring. Maternal exercise increased GLUT4 protein in adult female offspring, independent of paternal

HFD/obesity. Taking together, these results suggest that: 1) Paternal diet-induced obesity comprise insulin sensitivity *in vivo* and *ex vivo*, which may be, at least in part, due to the lower observed insulin-stimulated increases in p-TBC1D4^{Thr642}, and; 2) Maternal exercise does not normalise *in vivo* insulin sensitivity, but it does protect offspring in terms of insulin-stimulated glucose uptake in isolated skeletal muscle, this may be accounted to the increase in p-AKT^{Ser473} and GLUT4 protein content but surprisingly there was no normalising of p-TBC1D4^{Thr642}.

3.4.5 Offspring mitochondrial respiration in skeletal muscle

This study is also the first to investigate whether paternal diet-induced obesity affects skeletal muscle mitochondria in adult rats and if maternal exercise before and during gestation can also play a role in this scenario. This is important as there is evidence that skeletal muscle mitochondrial function can effect insulin sensitivity, although it is unclear whether mitochondrial dysfunction is a cause or consequence of insulin resistance in T2DM (Montgomery and Turner 2015). Mitochondrial respiration (normalised by muscle weight) across all complexes measured tended to be lower (20-30%) in offspring sired by HFD/obese fathers compared with normal diet fathers although only leak through complex I (CI_L) reached statistical significance. Leak oxygen flux is related to heat production, as this is a dissipative component of respiration (Gnaiger 2007). Leak oxygen flux through complex I was lower in offspring sired by HFD/obese fathers which suggests that more resources may be available for biochemical work. Other parameters related to mitochondrial function, such as respiratory ratios, reactive oxygen species production and citrate synthase activity were not affected by paternal HFD/obesity. This indicates that the insulin resistance observed in skeletal muscle of offspring sired by HFD/obese fathers appeared not to have been linked to mitochondrial dysfunction in our cohort of rats.

Maternal exercise before and during pregnancy was able to positively influence mitochondrial respiration when associated with paternal diet-induced obesity. In fact, maternal exercise increased OXPHOS capacity through complex I and ETS capacity through II in offspring sired by paternal HFD/obesity. This suggests an improved respiratory capacity of mitochondria (with saturated levels of ADP) as well as an enhanced respiratory electron transfer system capacity of mitochondria (Gnaiger 2007). Respiratory flux control ratios and mitochondrial ROS (H_2O_2) emission were not different among the experimental groups.

Changes in mitochondrial respiration can also be due to a higher content of mitochondria rather than changes in its capacity itself. In order to test this, we measured citrate synthase activity, an indirect biomarker of mitochondrial volume/content. CS activity in skeletal muscle has been associated with mitochondrial content/volume (Larsen et al. 2012), and it is, therefore, widely used in experimental studies. Paternal diet-induced obesity did not change CS activity, however, maternal exercise increased CS activity in adult offspring sired by HFD/obese fathers. This is the first study that CS activity has been investigated in offspring of either HFD/obese fathers or exercised mothers. When the mitochondrial respiration data were normalised to CS activity, maternal exercise decreased OXPHOS in complexes I+II and ETS in complex II, which suggests that positive effects of maternal exercise on mitochondrial function might be related to increased mitochondrial volume/content rather than the increased function of the each unit of mitochondria. We also observed a decrease in mitochondrial ROS emission in the $CI+II_P$ state when normalised by CS activity. This is important as excess of mitochondrial ROS is considered to be a deleterious factor leading to insulin resistance in skeletal muscle (Houstis et al. 2006, Anderson et al. 2009).

Neither paternal HFD/obesity nor maternal exercise affected the protein expression of PGC1α, one of the main regulators of mitochondrial biogenesis. In young rats, maternal physical activity (running wheel) increased PGC1α gene expression compared offspring born from control/sedentary mothers (Raipuria et al. 2015), but, like us, others have found that this effect does not seem to be sustained until adulthood (Laker et al. 2014a). Interestingly, the mitochondrial transcription factor A (TFAM) and PHF20 protein expression were lower in adult offspring sired by HFD/obese father compared to control. TFAM is an essential transcription factor of mitochondrial biogenesis (Campbell et al. 2012), while PHF20 regulates another important transcription factor, p53 (Cui et al. 2012), which plays a role in mitochondria. The lower expression of these proteins may suggest that the rate of transcription of genetic information might be compromised in adult offspring sired by HFD/obese fathers, however, this may not be directly involved to mitochondrial function. This is rather surprising, as p53 has been implicated in mitochondrial respiration (Matoba et al. 2006). Although maternal exercise tended to increased PHF20 protein expression in adult offspring sired by HFD/obese fathers (36% increased vs HS), no statistical significance was observed (P=0.087). Recent studies have proposed another role for p53, which is related to exercise training-induced adaptations in mitochondrial respiration (Saleem and Hood 2013, Granata et al. 2016). Therefore, it

remains unclear how maternal exercise increased CS activity and GLUT4 protein expression in offspring sired by HFD/obese fathers despite no increase in PGC1α and no alterations in TFAM and PHF20.

Taken together, our results suggest that paternal HFD/obesity reduces insulin sensitivity in the offspring. This is likely to be due to the impaired distal insulin signalling (p-TBC1D4). On the other hand, maternal exercise normalises insulin sensitivity *ex vivo*, but not *in vivo*, in adult offspring of HFD/obese fathers, and may be related to increasing of p-AKT^{Ser473} and GLUT4 protein content. It also increases mitochondrial volume, without changes in mitochondrial biogenesis and transcription factors in offspring sired by HFD/obese fathers. Additional studies are necessary to further tease out the underlying mechanisms.

3.4.6 Offspring insulin secretion *in vivo* and pancreas morphology

We showed that insulin levels tended to be higher in adolescent offspring from HFD/obese fathers. Conversely, Ng *et al*. reported a significant reduction in insulin secretion in adolescent rats due to paternal high-fat diet (Ng et al. 2010). In adulthood, we found no effects of paternal diet-induced obesity on glucose tolerance in offspring but insulin secretion was lower during the glucose tolerance test. The lower insulin secretion *in vivo* in adult offspring of HFD eating obese dads might be partially related to the lower β-cell mass and increased percentage of small pancreatic islets in these rats. β-cell function *in vivo* (estimated using the insulinogenic index) (Singh and Saxena 2010), in offspring was also lower due to paternal HFD/obesity.

We have previously shown in rats that treadmill exercise before and during pregnancy increases glucose-stimulated insulin secretion in isolated islets from mothers (at the 3rd day of lactation) (Leandro et al. 2012b), but whether similar effects would be passed to their offspring still needs further investigation. Here we showed that maternal exercise attenuated the lower insulin secretion observed in adult offspring sired by HFD/obese fathers. A possible link between improved insulin secretion and higher β-cell mass might explain, in part, such effects. In fact, exercise training before and during pregnancy normalised β-cell mass, attenuated the insulinogenic index and *in vivo* insulin secretion (insulin levels during IPGTT). One study showed that maternal exercise on a treadmill before and during pregnancy has no effects on insulin content in young (3 weeks of age) or adult (~28 weeks of age) offspring (Quiclet et al. 2016). In this publication, the researchers also demonstrated that maternal exercise improves insulin secretory capacity

in young but tend to reduce it (P>0.05) in adult rats. The following points can explain such discrepancies, at least in part. First, this study (Quiclet et al. 2016) only used male pups while we investigated just female offspring. Moreover, their exercise protocol is different from ours as they used same treadmill speed and duration of the training session before and throughout gestation (until day 19 of pregnancy) (Quiclet et al. 2016). This is of major concern as human studies have shown that maintaining high volume (60min) of exercise until close to delivery can have negative impact such as reduced birth weight [reviewed in (Hopkins and Cutfield 2011)]. Similar deleterious findings were reported in animal studies that maintained high volume of exercise during gestation, affecting placental and foetal weight (Treadway and Young 1989, Mottola et al. 1993). Furthermore, rats exposed to running wheel naturally diminish the amount of exercise performed, followed by beneficial effects to the offspring development (Santana Muniz et al. 2014).

Maternal exercise also promoted an increase in islet number and relative surface area in offspring sired by HFD/obese fathers. It was not possible, however, to determine whether maternal exercise effects on islet number were due to cell proliferation or differentiation. This could be examined by staining proliferation markers (such as Ki-67, a component of the mitotic chromosome) (Cuylen et al. 2016) and gene expression analysis. Nevertheless, this increase in islet number may also accounts for the attenuation of islet mass and insulin secretion in adult offspring born from exercised mothers and HFD/obese fathers. There were no changes in β-cell area or β-cell proportion per islet. Maternal exercise normalised the percentage of islet size distribution, by normalising islets distribution of small, medium and large sizes (<5.000 μm^2, 10,001 to 20,000 μm^2 and >50.000 μm^2, respectively), which supports the findings of β-cell mass and function, as well as insulin secretion *in vivo* in our experiments.

3.5 Conclusions

Parental phenotype can give us useful insights on offspring's health. In fact, we showed that HFD/obese fathers and exercised mothers can shape their offspring's phenotype later in life. Paternal diet-induced obesity caused negative metabolic consequences in adolescent (12 weeks of age) and adult (24 weeks of age) rats. The lower insulin-stimulated glucose uptake was likely due to the observed decrease in skeletal muscle TBC1D4 phosphorylation rather than mitochondrial function or other

mitochondrial parameters. In addition, the lower insulin secretion might be explained by the lower β-cell mass and higher distribution of smaller islet size in adult offspring sired by HFD/obese dads.

Maternal exercise overcame the lowering of offspring body weight caused by paternal diet-induced obesity. Exercise before and during pregnancy had no effect, however, on whole body insulin sensitivity in adulthood. In isolated muscle, however, positive effects were demonstrated in regards to insulin sensitivity in offspring born from exercised mothers. This may be due to the observed increase in GLUT4 protein and higher insulin-stimulated AKT phosphorylation (Ser 473) in skeletal muscle after maternal exercise and HFD/obese fathers. It also increased mitochondrial volume/content in skeletal muscle compared with sedentary offspring sired by HFD/obese fathers. Our protocol of treadmill exercise before and during pregnancy normalised the lower insulin secretion, which might be due to an increase in β-cell mass, number and area of pancreatic islets in offspring sired by HFD/obese fathers.

Chapter 4 – Offspring exercise early in life can overcome adult skeletal muscle insulin resistance caused by paternal diet-induced obesity

4.1 Introduction

The frightening rise in type 2 diabetes mellitus (T2DM) worldwide can be partially understood by a biological process called phenotypic plasticity (Hanson and Gluckman 2014). This occurs when diverse environmental conditions modify the morphology, physiology and behaviour of a unique genotype (West-Eberhard 1989). The developmental origins of health and disease (DOHaD) paradigm uses this concept to interpret how environmental cues early in life (gestation, lactation and early childhood) impact on long-term disease susceptibility, such as T2DM (McMullen and Mostyn 2009, Wells 2014, Bateson et al. 2004). In humans, obese mothers are more likely to have obese offspring (Gale et al. 2007). Maternal high-fat diet in rodents decreases the expression of mitochondrial transcription factors (such as nuclear respiratory factor-1 and mitochondrial transcription factor A) along with decrease in complexes I and III activities of the mitochondrial electron transport system complex in skeletal muscle in adult offspring (~21 weeks of age) (Pileggi et al. 2016). Another study showed that adult rat offspring from mothers fed high-fat (and high-sucrose) diet did not become obese, but were predisposed to T2DM associated with mitochondrial function impairments in oxidative phosphorylation (OXPHOS) complexes in skeletal muscle (Latouche et al. 2014). Similarly, rodent offspring also develop insulin resistance (Nivoit et al. 2009), hyperphagia and hypertension (Samuelsson et al. 2008) in adulthood. Although much of the research has focussed on maternal effects, the contribution of fathers in the cause of T2DM is drawing increasing attention in the DOHaD field.

Paternal diet-induced obesity before mating can negatively influence the phenotype and metabolism of the offspring (Soubry 2015). In fact, a father's body fat (percentage or total) was predictive of long-term changes in body fat in premenarcheal girls (Figueroa-Colon et al. 2000). Although longitudinal studies are still scarce, a prospective epidemiologic study observed that obesity in fathers before conception is linked with elevated levels of blood alanine transaminase in their offspring (Loomba et al. 2008). This

enzyme is argued to be positively correlated with obesity, and the results of this study were independent of body mass index (BMI) (Loomba et al. 2008), suggesting that paternal obesity might induce obesity in the offspring. In rodents, studies are controversial and have demonstrated that paternal diet-induced obesity before conception decrease, does not affect or increase body weight and fat mass in the offspring, however, metabolic disorders are generally observed (Ng et al. 2010, Fullston et al. 2013, Cropley et al. 2016). Glucose tolerance tests in the offspring at 12 weeks of age demonstrated lower insulin area under the curve and higher glucose area under the curve compared with offspring sired by control diet dads (Ng et al. 2010), indicating lower insulin secretion in the offspring of high-fat/obese fathers. Indeed, these findings were associated with lower total area, altered size distribution and gene expression of pancreatic islets (Ng et al. 2010). Similar findings have confirmed the negative effects of paternal diet-induced obesity on adult mice offspring showing impaired glucose and insulin tolerance (Fullston et al. 2013), suggesting that insulin sensitivity might also be affected. More comprehensive studies on the effects of paternal diet-induced obesity on insulin sensitivity and secretion as well as skeletal muscle mitochondrial function are needed. In addition, intervention approaches are necessary using this model to break the obesity and T2DM cycle for the next generations.

Taking the concept of phenotypic plasticity, it is known that there is an increased opportunity of adaptation from gestation up to infancy, which decreases over time (Hanson and Gluckman 2014) (Figure 1-1). A large cohort of 1436 adults reported that physical activity in youth was associated with lower rates of occurrence of T2DM (and hypertension) in adulthood, independent of current physical activity (Fernandes and Zanesco 2010). Given this, we and others have proposed early life exercise training as a positive stimulus to (re)program the offspring exposed to detrimental insults *in utero*, such as placental restriction (Gatford et al. 2014, Street et al. 2015, Laker et al. 2011, Laker et al. 2012b). The few studies that have been undertaken so far have mostly used uteroplacental blood flow restriction model, which leads pups to be small for gestation age (SGA) and have metabolic consequences in adulthood (Simmons et al. 2001). In rats, exercise training early in life (5-9 weeks of age, treadmill running) normalised the lower relative islet surface area and β-cell mass in a model of SGA rats at 24 weeks of age (Laker et al. 2011). However, early exercise did not have a sustained effect on skeletal muscle mitochondrial biogenesis in adult offspring using this design (Laker et al. 2012b). In obese and diabetes predisposed rats, exercise early in life (7 to 15 weeks of age) fully

protected the development of T2DM at 28 weeks of age (Shima et al. 1996). Similarly, in diet-induced obesity rats that were weaned onto high-fat diet and had access to a running wheel for 3 weeks, exercise prevented the onset of obesity at 13 weeks of age (Patterson et al. 2009). No study has investigated whether exercise early in life can attenuate the consequences of a paternal high-fat diet before conception.

In the previous chapter, we found that paternal diet-induced obesity impaired insulin sensitivity, likely due to the observed decrease in skeletal muscle TBC1D4 phosphorylation rather than mitochondrial function or other mitochondrial parameters. These offspring also had lower insulin secretion, which might be due to lower β-cell mass. As in chapter 3 we focused on the long-term protective effects of maternal exercise before and during pregnancy, in this chapter, we examined whether offspring exercise early in life can attenuate the negative effects caused by paternal diet-induced obesity and the underlying mechanisms.

4.2 Methods

4.2.1 Ethics

All procedures were performed according with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013), after ethics approval (AEC#13/008) from Victoria University, Melbourne, Australia.

4.2.2 Animals

Sprague Dawley rats were obtained with 3.5 weeks of age from Animal Research Centre (Perth, Australia) and kept in plastic cages at the animal facility in Footscray Park Campus - Victoria University. The rats were exposed to a 12 hour light-dark cycle (7am lights on) and standard environmental conditions (room temperature at 18-22 °C and ~50% relative humidity). All rats had food and water available *ad libitum*. Cages were supplied with nesting materials and enrichment items. Rats were acclimatised to the facility for 3 days with no procedures performed during this period. Before conception, male breeders were fed with high-fat or control diet. As demonstrated previously (Ng et al. 2010), female offspring seems to be more affected by paternal HFD than male offspring, therefore, only female rats were used in these experiments.

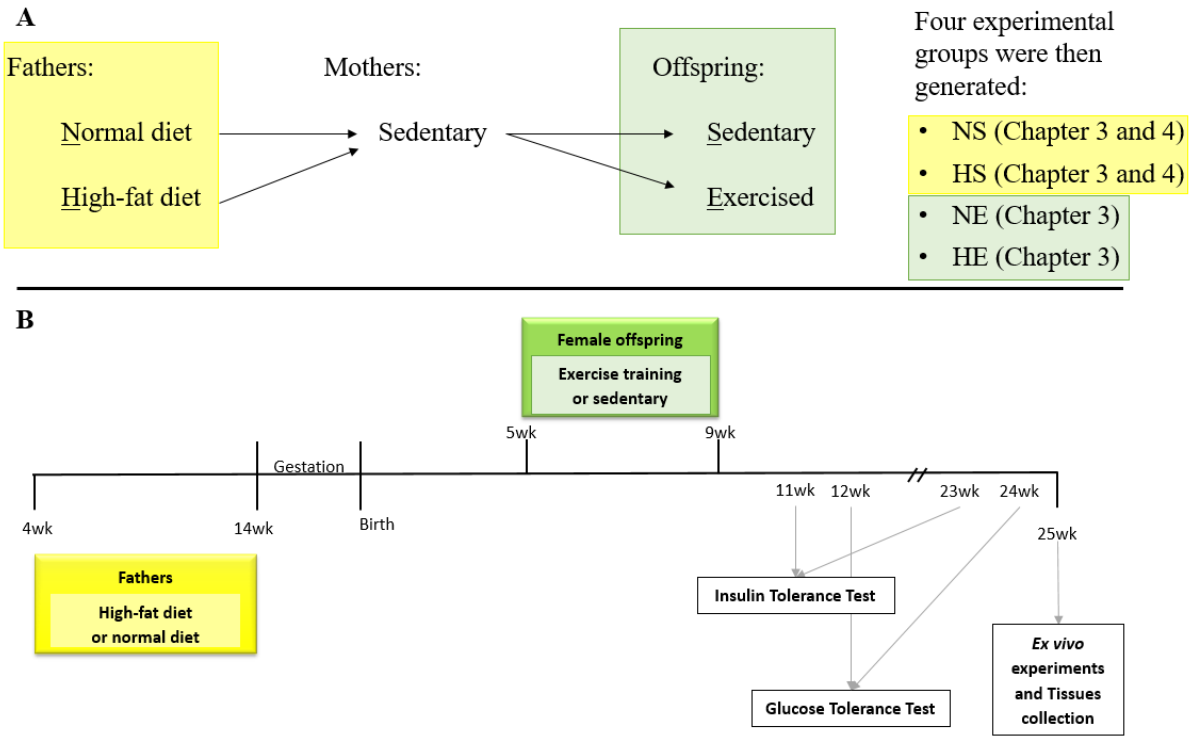

Figure 4-1. Experimental groups and timelines (Chapter 4). A: Experimental groups based on paternal diet and offspring exercise interventions. Male breeders were mated with only one female (1:1). B. Experimental design with timelines for main experiments. NS, pups sired by Normal diet dads that remained Sedentary in life. HS, pups sired by high-fat diet dads which remained Sedentary in life. NE, pups sired by Normal diet dads and Exercised early in life. HE, pups sired by High-fat diet dads and Exercised early in life.

4.2.3 Fathers

After acclimatisation, rats were either fed a control diet or high-fat diet from 4 to 14 weeks of age (section 2.3.1.1; Table 2-1), which was before conception. Control fathers were fed with a standard control chow diet ad libitum throughout the experiment (Rat and Mouse Cubes, 12.00% energy as fat; Specialty Feeds, Western Australia). High-fat fathers were fed high-fat diets (SF01-025 and SF03-020, with 40.7% and 43% energy as fat; Specialty Feeds, Western Australia).

4.2.4 Mating

Female rat breeders were purchased at 6 weeks old, and maintained on control diet until mating period. During the 12th week of age, female rats were placed together with male rats (13 weeks of age). The mating occurred between 8am and 5pm (during the light cycle). During this period male and female rats were fed with control diet only. On average, only 5 grams of chow diet were eaten during this period by each breeding

pair. Once the pregnancy was confirmed by vaginal smear, pregnant dams were housed individually during gestation.

4.2.5 Offspring

This study only included litter sizes between 9-15 pups. Birth weight was considered the postnatal day (PND) 1. At PND21, offspring were weaned and only female pups remained in the study. There were no interventions in the offspring from birth until 4 weeks of age. Body weight and food intake were measured weekly throughout the experiments. Food intake was considered the difference between the amount of food offered and the amount of food remaining 24 h later.

4.2.5.1 Early in life exercise protocol

All offspring rats performed acclimatisation to the treadmill at the end of the 4th week of age. Sedentary rats were placed in the same room near the treadmill during the same time of the exercised group. Trained rats were subjected to a protocol of moderate intensity exercise training (treadmill, 5 d/wk, 60 min/d, at ~65-75% $\dot{V}O_{2max}$) from 5-9 weeks of life (Table 2-4). The intensity of the training was estimated based on a protocol originally designed for Sprague-Dawley rats, 74-78 days old (Bedford et al. 1979), and the training protocol was adapted from our previous study (Laker et al. 2011).

4.2.5.2 Insulin tolerance tests and glucose tolerance tests

Insulin and glucose tolerance tests (ITT and IPGTT, respectively) were performed after an overnight fast in adolescent and adult rats (Figure 4-1). IPITT and IPGTT were performed as described previously (section 2.3.1.3 and 2.3.1.4). After collection of blood sample via tail vein at basal, glucose (1g/kg) was injected intraperitoneally (IP) and further blood samples were obtained from tail at 15, 30, 60 and 90 min and analysed for glucose using a portable glucometer (Accu-Chek Performa Nano, Roche Diagnostics, Mannheim, Germany).

From the IPGTT at 24 weeks of age, the homeostatic model assessment of insulin resistance (HOMA-IR) and the insulinogenic indexes were obtained. The insulinogenic index was provided an estimate of early insulin response to glucose (a measure of β-cell function *in vivo*) (Singh and Saxena 2010), and has been used previously in female Sprague-Dawley rats (Ng et al. 2010).

4.2.5.3 Insulin-stimulated glucose uptake in skeletal muscle

With the animal alive and deeply anaesthetised, epitrochlearis (EPI) and soleus (SOL) muscles were dissected and longitudinally split into two strips (Sharma et al. 2015) and incubated in chambers filled with Krebs Henseleit solution (in mM): 118.5 NaCl, 24.7 $NaHCO_3$, 4.74 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 147.02 $CaCl_2$, 32 mannitol, 7.5% BSA and MilliQ H_2O, with pH 7.4, maintained at 30°C and continuously oxygenated with 95% O_2 and 5% CO_2. Muscles were incubated for 20 min in the Krebs Henseleit solution with 8 mM glucose (Buffer #1). Then, muscles were transferred to another Krebs Henseleit solution for 30 min, with 4 mM pyruvate. This second buffer also contained either 0 nM or 1.2 nM of insulin (Buffer #2), which is considered a physiological concentration of insulin as described previously (Sharma et al. 2011). The third incubation was made in the Krebs Henseleit solution with the addition of 8 mM 2-deoxyglucose, 0.75 µCi ml^{-1} 2-[1,2-^{3}H] deoxy-D-glucose and 0.225 µCi ml^{-1} [1-^{14}C] mannitol, and 0 nM or 1.2nM insulin for 10 min (Buffer #3).

To determine glucose uptake, approximately 30mg of EPI and 35mg of SOL dried frozen muscles were weighted, then 1M HCl was added and neutralised with 1M NaOH. Samples were spun for 5 min at 13,000 g at room temperature and sat for at least 1 h at room temperature prior to reading on a β-scintillation counter (Liquid Scintillation Analyser, Tri-Carb 2810TR, PerkinElmer, Boston, MA) using dual counts for ^{3}H and ^{14}C with each sample read for 10 minutes (Stephens et al. 2004). The level of radioactivity in disintegrations per minute was used for calculations.

4.2.5.4 Skeletal muscle mitochondrial respiration and reactive oxygen species production

Approximately 2 mg of the medial part of the PLANT muscle was placed in cold Biopsy Preservation Solution (BioPS) containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 50 2-(N-morpholino)ethanesulfonic acid (MES), 15 Na_2-phosphocreatine, 20 imidazole, and 0.5 DTT, adjusted to pH 7.1 (Pesta and Gnaiger 2012). Muscle fibres were mechanically separated under a microscope with forceps. Then, permeabilised muscle fibres were used as a mitochondrial preparation by permeabilising the plasma membrane chemically (30 min incubation, 50 µg/ml saponin).

Mitochondrial respiration was performed on muscle fibre bundles by high-resolution respirometry (HRR) with a Oxygraph-2k (O2k, OROBOROS Instruments,

Innsbruck, Austria) (Pesta and Gnaiger 2012) combined with the Fluorescence-Sensor Green of the O2k-Fluo LED2-Module for H_2O_2 measurement. The following substrate–uncoupler–inhibitor titration (SUIT) protocol was used: Superoxide dismutase (5 U/ml), Amplex® UltraRed (10 µmol) and horseradish peroxidase (1 U/ml) were first added to the chamber to allow H_2O_2 measurements, followed by placement of the permeabilised muscles. Then malate (5 mM) and pyruvate (10 mM) were added, followed by magnesium chloride and ADP (5mM) and subsequent addition of succinate (10mM). Cytochrome *c* was used to test for mitochondrial membrane integrity. Then carbonyl cyanide 4-(trifluoromethoxy) phenyl-hydrazone (FCCP) titrations (0.5 mM) were added, and if an increase in oxygen flux was noticed, another dose of FCCP (0.5mM) was applied. This was followed by rotenone (1 mM) and antimycin A (5 µM). Therefore, we measured leak respiration (*L*) through complex I (CI) (CI_L), Maximum oxidative phosphorylation (oxphos) capacity (*P*) through CI (CI_P), *P* through CI+II combined ($CI+II_P$), electron transport system (ETS) capacity (*E*) through CI+II ($CI+II_E$), *E* through CII (CII_E) and finally residual oxygen consumption (ROX) (Pesta and Gnaiger 2012). Experiments were conducted under blinded conditions with coded samples.

4.2.5.5 Western Blots

EPI (both insulin and non-insulin stimulated) and PLANT muscles were homogenised for western blot in the following buffer: 0.125M Tris-HCl, 4% Sodium dodecyl sulphate, 10% glycerol and 10mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 0.1M dithiothreitol. Total protein in muscle samples was determined on 7.5-12% hand-cast criterion TGX Stain-Free gels (Bio-Rad, Hercules, CA, USA) at 60 V for 30 min followed by 120 V for ~35 min with buffer containing 25 mM Tris base, 191.8 mM glycine, 1% sodium dodecyl sulphate. Following the running, the Stain-Free gel was activated (1 min) for detection of myosin heavy chain (MHC), used as an indicator of the amount of protein present due to small amount of protein loaded in each well (Murphy 2011). Total protein in the gel was imaged using Stain Free imager (ChemiDoc, Bio-Rad). Proteins were wet-transferred to nitrocellulose for 100 min at 100 V in a circulating ice cooled bath using transfer buffer containing 191.8 mM glycine, 25 mM Tris base, and 20% (v/v) methanol. Membranes were exposed to primary antibodies overnight at 4°C with constant rocking.

On the following day, appropriate horseradish peroxidase secondary antibodies (Anti-mouse or rabbit IgG, HRP-linked Antibody) were added (1:10000), diluted in 3-

5% skim milk powder in Tris-Buffered saline (TBS) with Tween 20 (t) for 60 min. Following washes with TBSt, membranes were exposed to SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and protein bands were detected using a charge-coupled device camera attached to a ChemiDoc (Bio-Rad) and ImageLab software (Bio-Rad). Densitometry was performed using ImageLab software (Version 5.2.1, Bio-Rad). Each gel contained a ladder (PageRuler™ Plus Prestained Protein Ladder, Thermo Fisher Scientific) allowing visualisation of the relative positions of molecular mass. Experiments were conducted under blinded conditions with coded samples.

4.2.5.6 Pancreas morphology

After dissection of skeletal muscles and cardiac puncture, pancreases were weighted and stored in 10% neutral buffered formalin at room temperature for up to one week, transferred to 70% ethanol and kept at 4ºC until processed. Five sections per pancreas were immunostained to identify and localise insulin-positive β-cells (n=8-10 per group). Fixed tissue was sent to Anatomical Pathology, Department of Medicine, University of Melbourne (Parkville, Victoria, Australia) to be paraffin embedded, sectioned at 100 µm and stained for insulin using a guinea pig polyclonal anti-porcine insulin antibody (DAKO Corporation, Denmark) diluted 1:100 and counterstained with haematoxylin. Digital images of microscopic sections were obtained through the Austin Health, Victorian Cancer Biobank Slide Scanning service (Heidelberg, Victoria, Australia). Following standard protocols, whole slide sections were line scanned using an Aperio ScanScope XT (Aperio Technologies, Vista, CA, USA) at 40x magnification at a resolution of 0.5 µm/pixel. Digital images were analysed using the Aperio image software (ImageScope version 12.2.2). Slides were code-blinded to remove potential bias. Data analysis was also performed under blind conditions.

Five random cross-sections from different parts of the pancreas (head, body and tail) were analysed. Sampling from various parts of the pancreas is important as islet localisation, composition and architecture may vary among species, and within the same species depending on physiological and pathological states (Kharouta et al. 2009). Relative islet surface area and β-cell area were quantified by point-counting morphometry (number of intercepts of insulin-positive cells as a proportion of intercepts on pancreas or islets). Number of islets per millimetre square was obtained by dividing the number of islets by the total pancreas area (mm^2). β-cell mass was determined by multiplying the

relative β-cell area by pancreas weight (g). Pancreatic islet number was expressed relative to total cross-sectional area (per square millimetre) (Laker et al. 2011) with islet size arbitrarily classified as <5,000 μm^2, 5,000–10,000 μm^2, 10,001–20,000 μm^2, 20,001–50,000 μm^2 and >50,000 μm^2.

4.2.6 Statistical analyses

Data are presented as mean ± standard error of the mean (SEM) unless otherwise specified. Statistical analyses were performed with SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) and GraphPad Prism (GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla California USA) using student t test, two or three-way ANOVA with repeated measures as appropriate. For ANOVA 'Paternal diet' and 'Exercise early in life' were used as main factors, as well as time points (e.g. during the IPGTT) or treatment (e.g. insulin incubation during 2DG uptake) as another main factor. If an interaction was found, a two-way ANOVA was applied in cases of three main factors for each time-point. For two-way ANOVA, if an interaction was found, a post-hoc analysis using the least significant difference test was used. The α-level of statistical significance was set a priori at $P < 0.05$.

4.3 Results

Some data from paternal HFD/obesity have been presented in chapter 3 (Table 3-1 and Figure 3-2), consequently these data will not be presented here again.

4.3.1 Paternal high-fat diet does not alter offspring phenotype from birth until weaning

Paternal high-fat diet did not change the number of male or female rats born, birth weight, or body weight from birth until PND 21 (P>0.05) (Table **4-1**). At birth, latero-lateral and antero-posterior head axis in female pups were not influenced by paternal high-fat diet (P>0.05). At PND 21, females pups sired by paternal diet-induced obesity had lower antero-posterior head axis (P=0.04) and tended to have lower mass of the soleus and EDL muscles (P=0.07 and P=0.09, respectively). The weights of the liver, spleen, heart, kidneys and pancreas were not affected by paternal obesity at PND 21 (P>0.05) (Table **4-1**).

Table 4-1. Paternal diet-induced obesity does not alter female offspring characteristics from birth (PND1) until weaning (PND21).

Variables	NS (*n*=5 litters)			HS (*n*=5 litters)			*P* value
Number of pups born per litter	13		(10-16)	15		(12-16)	0.280
Number of females per litter	6		(4-6)	8		(4-10)	0.136
Number of males per litter	7.5		(5-10)	8		(5-9)	0.829
PND1 blood glucose (mmol/l)	4.90	±	0.50	5.25	±	0.25	0.347
PND1 body weight of females (g)	7.25	±	0.11	7.05	±	0.17	0.360
PND1 body weight of males (g)	7.73	±	0.31	7.17	±	0.19	0.359
PND3 body weight (g)	9.36	±	0.34	8.99	±	0.13	0.302
PND6 body weight (g)	14.72	±	0.54	14.36	±	0.26	0.506
PND9 body weight (g)	20.60	±	0.75	18.69	±	0.51	0.054
PND12 body weight (g)	26.48	±	0.83	25.58	±	0.19	0.151
PND15 body weight (g)	33.06	±	0.90	31.50	±	0.34	0.057
PND18 body weight (g)	38.43	±	0.45	37.99	±	0.26	0.373
Measurements at PND21							
Body weight (g)	53.94	±	0.77	52.56	±	1.00	0.272
Body length (mm)	128.2	±	0.7	128.0	±	1.3	0.927
Antero-posterior head axis (mm)	39.3	±	0.3	38.5	±	0.2	0.049
Latero-lateral head axis (mm)	18.1	±	0.2	17.8	±	0.2	0.410
Liver (g)	2.29	±	0.06	2.17	±	0.08	0.265

Spleen (g)	0.24	±	0.01	0.22	±	0.03	0.609
Heart (g)	0.28	±	0.02	0.25	±	0.02	0.302
EDL (g)	0.03	±	0.00	0.02	±	0.01	0.093
Soleus (g)	0.03	±	0.00	0.02	±	0.01	0.078
Kidneys (g)	0.31	±	0.02	0.30	±	0.02	0.761
Pancreas (g)	0.21	±	0.01	0.28	±	0.06	0.273
Blood glucose (mmol/l)^	11.87	±	1.31	8.08	±	1.82	0.177

Sample sizes were considered per litter, not number of individual pups. NS, offspring sired by control (normal, N) diet fathers; HS, offspring sired by high-fat (H) diet fathers. PND, postnatal day. EDL, *extensor digitorum longus*. Values are presented as mean ± SEM (except for number pups born, and number of males and females; median (minimum – maximum) with Mann-Whitney U test was used in those cases). ^, blood glucose collected after a 4h fasting period.

4.3.2 Paternal high-fat diet decreased body weight and food intake as well as reduced glucose tolerance in young and adult offspring, while exercise early in life exerts protective effects.

Offspring sired by HFD/obese fathers had lower body weight and food intake after 12 and 16 weeks of age, respectively (Figure 4-2 A-B). Paternal HFD/obesity before conception did not affect insulin tolerance but it impaired glucose tolerance, increasing the glucose AUC, in adolescent offspring (Figure 4-3 A-F). Exercise early in life increased body weight consistently after week 16 of age and normalised food intake from week 18 until week 24 of age (Figure 4-2 A-B). In adolescent rats, exercise early in life did not alter glucose tolerance, but when offspring sired by high-fat eating dads perform early exercise, the negative consequences were attenuated (Figure 4-3 A-F).

In adult offspring (24 weeks of age), insulin tolerance was impaired by paternal high-fat diet, whereas glucose tolerance was unaffected (Figure 4-4 C-D). Insulin levels during the IPGTT were lower though in offspring sired by HFD/obseity dads (Figure 4-4 E-F). Exercise early in life associated with paternal HFD/obesity did not affect glucose tolerance test (Figure 4-4 C-F), but normalised the insulin tolerance test (Figure 4-4 A-B).

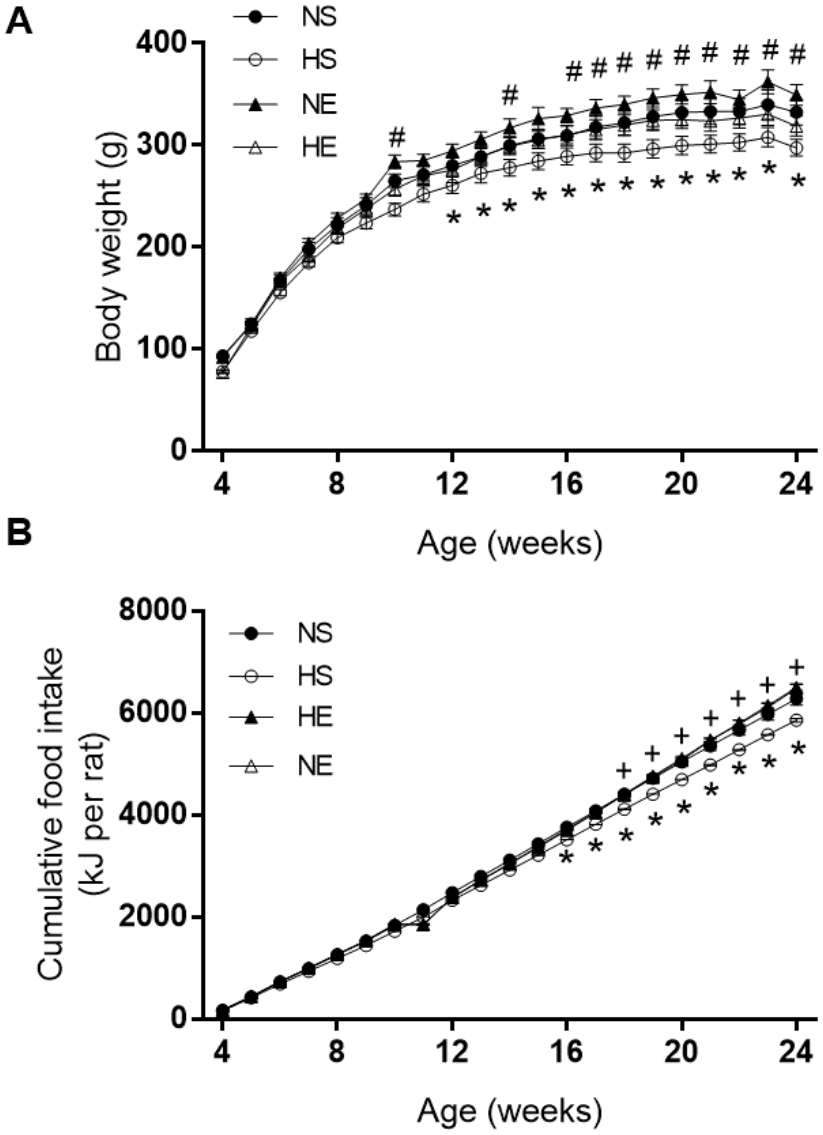

Figure 4-2. Paternal diet-induced obesity reduces body weight (A) and food intake (B) in offspring while exercise early in life attenuate its effects. NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high-fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high-fat diet fathers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity effect $P < 0.05$. #, Exercise early in life effect $P < 0.05$. +, Paternal diet vs. Exercise early in life interaction $P < 0.05$.

12 WEEKS OF AGE

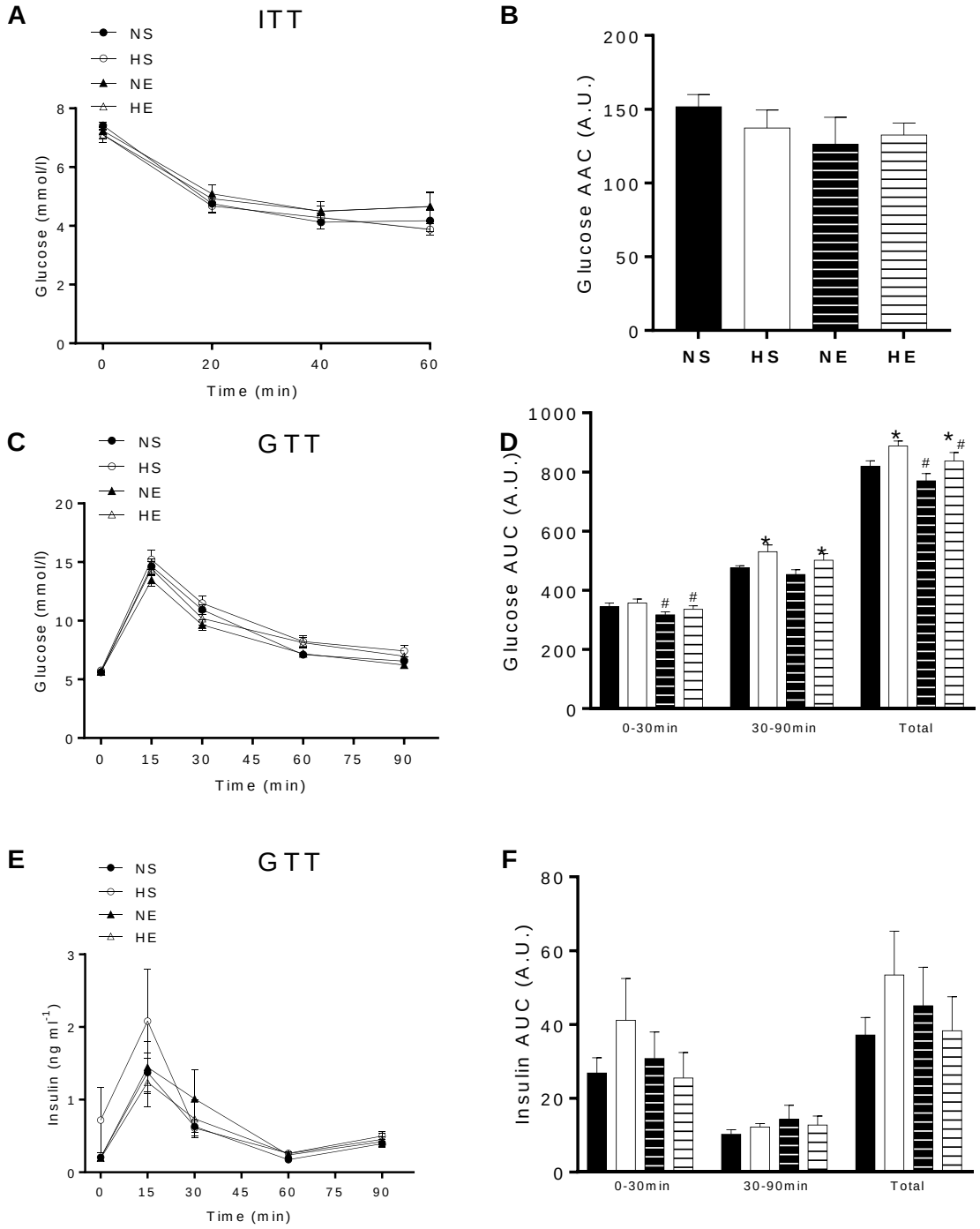

Figure 4-3. Exercise early in life protects young (12 weeks of age) adult female offspring sired by high-fat dads to develop impaired glucose tolerance. Glucose levels (A) and glucose area under the curve (AUC, B) during the IPITT. Glucose (C) and insulin (E) levels during a glucose tolerance test at 12 week of age, and their respective areas under the curves (D, F). NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high-fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high-fat diet fathers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Offspring exercise main effect $P < 0.05$ (NS vs NE; HS vs HE). +, Paternal HFD/obesity vs. Offspring exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

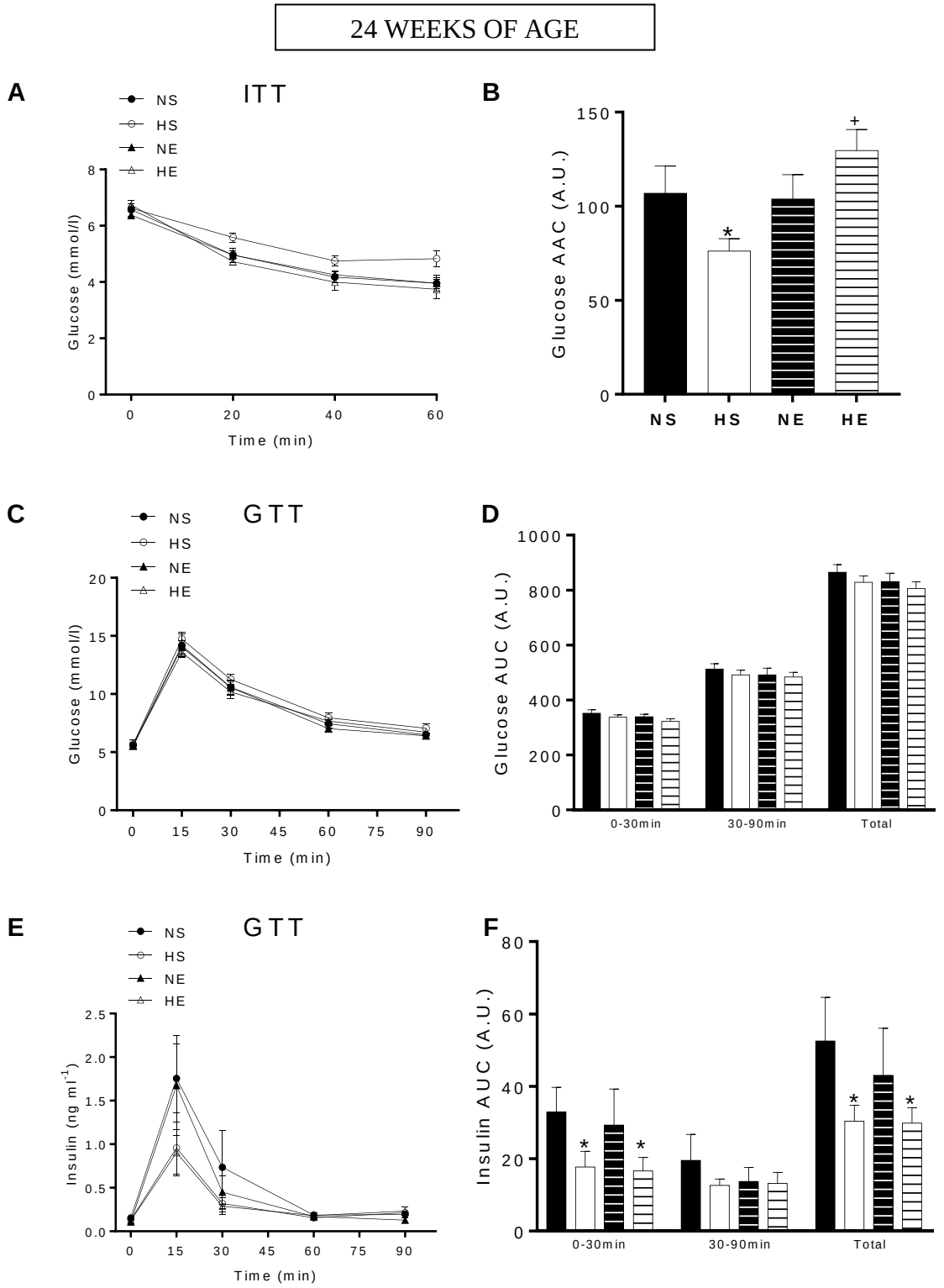

Figure 4-4. Exercise early in life improved insulin tolerance but not glucose tolerance in adult (24 weeks of age) female offspring sired by high-fat dads. Glucose levels (A) and glucose area under the curve (AUC, B) during the IPITT. Glucose (C) and insulin (E) levels during a glucose tolerance test at 24 week of age, and their respective areas under the curves (D, F). NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high-fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high-fat diet fathers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). +, Paternal HFD/obesity vs. Offspring exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

4.3.3 Offspring phenotype at 25 weeks of age (post mortem)

Phenotypical characteristics such as body weight and skeletal muscle weights are affected by paternal high-fat diet (Table **4-1**). The main changes were the lower body weight and BMI due to paternal diet-induced obesity (with no changes in body length), as well as smaller soleus and gastrocnemius muscles (Table **4-1**). Exercise early in life increased body weight and body length, as well as augmented the weight of the soleus, plantaris, tibialis anterior, liver and heart. Exercise early in life also lowered HOMA-IR, mainly due to decreasing fasting insulin (Table **4-1**). When offspring sired by HFD/obese father exercised early in life, their body weight, soleus and plantaris muscle weights were normalised.

Table 4-1. Effects of exercise early in life on adult (25 weeks of age, post mortem) female offspring sired by high-fat dads.

Parameters	NS n = 10	HS n = 10	NE n = 9	HE n = 10
Body weight (g)	333.5 ± 13.9	299.1 ± 6.9 *	352.8 ± 11.8#	322.0 ± 9.3 *#
Length (cm)	24.30 ± 0.20	24.00 ± 0.10	25.10 ± 0.10 #	24.60 ± 0.20 #
Abdominal circumference (cm)	18.33 ± 0.45	18.18 ± 0.20	19.12 ± 0.69	18.73 ± 0.24
EDL (g)	0.16 ± 0.07	0.14 ± 0.05	0.17 ± 0.03 #	0.16 ± 0.03 #
Soleus (g)	0.15 ± 0.05	0.13 ± 0.06 *	0.16 ± 0.05 #	0.15 ± 0.04 *#
Gastrocnemius (g)	1.75 ± 0.05	1.62 ± 0.02	1.70 ± 0.4	1.72 ± 0.04
Tibialis anterior (g)	0.67 ± 0.02	0.63 ± 0.01	0.69 ± 0.01 #	0.68 ± 0.12 #
Plantaris (g)	0.33 ± 0.01	0.31 ± 0.07 *	0.35 ± 0.01 #	0.33 ± 0.03 *#
Liver (g)	8.52 ± 0.31	8.15 ± 0.28	9.28 ± 0.31 #	9.22 ± 0.46 #
Pancreas (g)	1.36 ± 0.07	1.20 ± 0.09	1.46 ± 0.13	1.27 ± 0.11
Retroperitoneal fat (g)	6.17 ± 0.35	6.98 ± 0.59	7.46 ± 1.05	6.95 ± 0.91
Kidneys (g)^	0.98 ± 0.03	0.90 ± 0.03*	0.98 ± 0.03	1.00 ± 0.04 +
Heart (g)	1.03 ± 0.03	0.95 ± 0.02	1.08 ± 0.03 #	1.04 ± 0.04 #
Fasting glucose (mmol/l)	5.66 ± 0.09	5.63 ± 0.28	5.64 ± 0.12	5.59 ± 0.23
Fasting insulin (ng ml^{-1})	0.15 ± 0.01	0.13 ± 0.02	0.11 ± 0.01 #	0.12 ± 0.01 #
HOMA-IR	0.035 ± 0.006	0.034 ± 0.008	0.026 ± 0.005 #	0.029 ± 0.003 #

EDL, extensor digitorum longus. HOMA-IR, homeostatic model assessment of insulin resistance. Equation was as follows: HOMA-IR = [fasting plasma glucose (mg/dl) x fasting plasma insulin (μU/ml)]/2,430 (Cacho, Sevillano et al. 2008). NS, offspring sired by control diet fathers; HS, offspring sired by high-fat diet fathers; NE, early life exercised offspring sired by control diet fathers; HE, early life exercised offspring sired by high-fat diet fathers. ^, kidneys left and right were combined as there was no statistical difference between them. Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Offspring exercise main effect $P < 0.05$ (NS vs NE; HS vs HE). +, Paternal HFD/obesity vs. Offspring exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

4.3.4 Offspring skeletal muscle insulin-stimulated glucose uptake

Basal and insulin-stimulated glucose uptake *ex vivo* in soleus muscle were not affected by any treatments (Figure 4-5 A). On the other hand, glucose uptake in EPI muscle of offspring sired by HFD/obese fathers was markedly impaired during basal and insulin-stimulated conditions (Figure 4-5 B). Exercise early in life alone did not affect glucose uptake *ex vivo*. Nevertheless, exercise early in life normalised both basal and insulin-stimulated 2DG uptake in rats sired by HFD/obese dads (Figure 4-5 B).

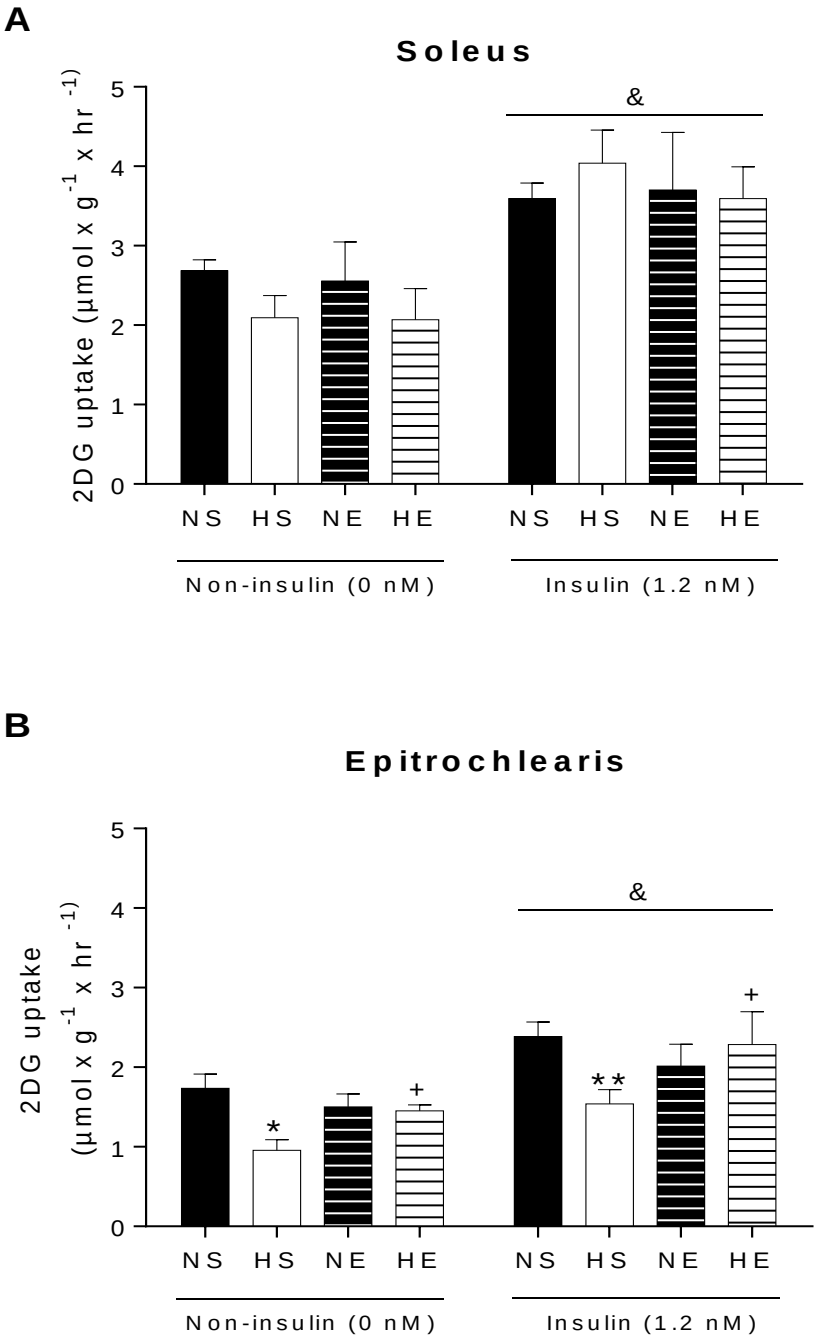

Figure 4-5. Paternal high-fat diet before conception impairs basal and insulin-stimulated glucose uptake in a muscle-specific way, while exercise early in life protects adult female offspring. NS, offspring sired by control diet fathers (n=6); HS, offspring sired by high-fat diet fathers (n=6); NE, early life exercised offspring sired by control diet fathers (n=5); HE, early life exercised offspring sired by high-fat diet fathers (n=5). Values are presented as mean ± SEM. &, Insulin effect $P < 0.05$ (0nM vs 1.2nM). *, ** Paternal HFD/obesity main effect $P < P < 0.05$, 0.01 respectively (NS vs HS; NE vs HE). +, Paternal HFD/obesity vs. Offspring exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

4.3.5 Skeletal muscle insulin signalling and metabolic regulators

As glucose uptake was impaired in EPI muscle but not the SOL muscle, we chose to perform western blots for proteins related to the insulin signalling only in the EPI muscle (Figure 4-6 and Figure 4-7). In basal conditions (0 nM), p-Akt^{Thr308}, p-AKT^{Ser473} as well as p-TBC1D4^{Thr642} were not affected by paternal diet-induced obesity and exercise early in life (Figure 4-6 and Figure 4-7). The only change was observed was an interaction between paternal diet and exercise early in life in p-Akt^{Ser473} in basal condition (Figure 4-6).

In regards to insulin-stimulated condition (1.2 nM), paternal diet-induced obesity increased p-Akt^{Ser473} but decreased p-TBC1D4^{Thr642} compared to normal diet offspring (HS vs NS) (Figure 4-6 and Figure 4-7). There were no differences in p-Akt^{Thr308}. Paternal HFD/obesity tended to decrease GLUT1 expression by 23% but this was not significant (P=0.1077), and did not affect GLUT4 expression (Figure 4-8).

In insulin-stimulated condition, when associated with paternal diet-induced obesity, exercise early in life was able to normalise p-TBC1D4^{Thr642} but there were no effects on p-AKT^{Ser473} and p-AKT^{Thr308}, (HE vs HS) (Figure 4-6 and Figure 4-7). Exercise early in life significantly increased GLUT1 and GLUT4 in adult offspring (NE vs NS) (Figure 4-8).

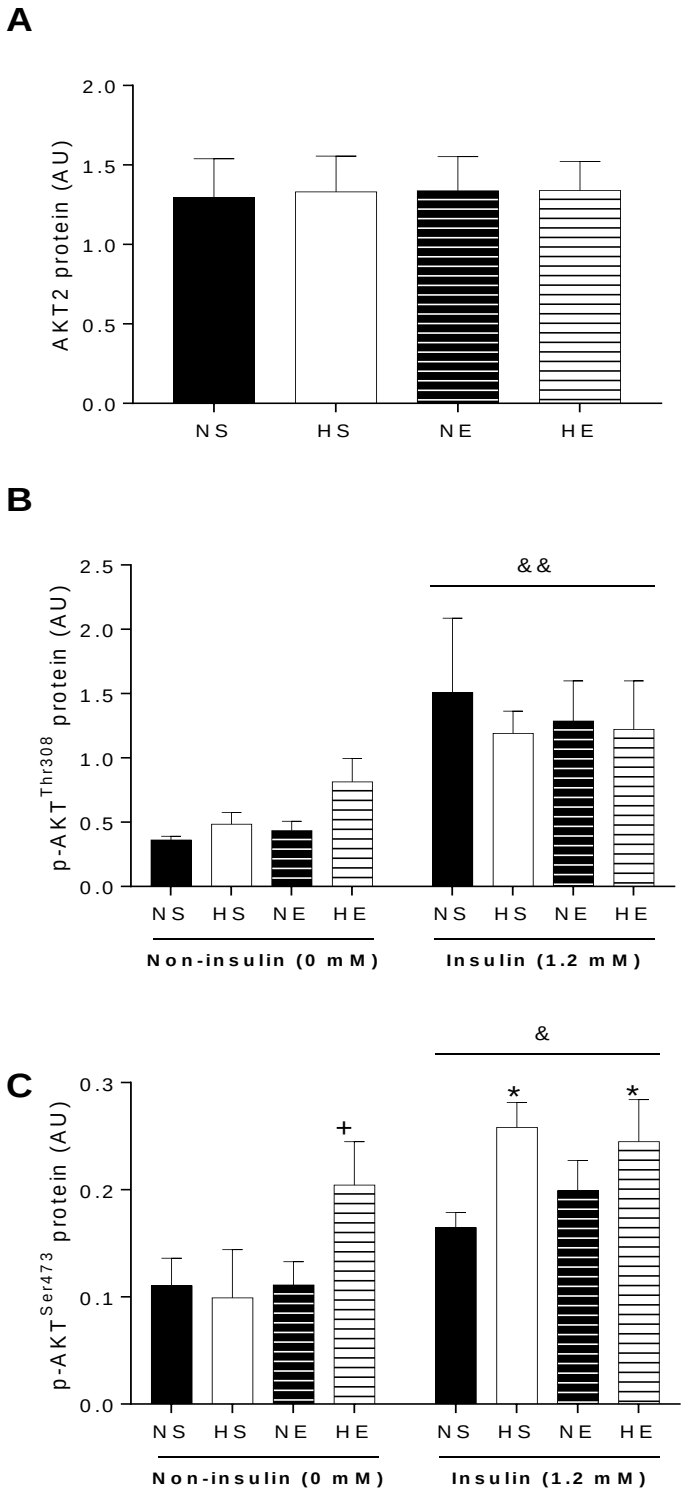

Figure 4-6. Basal and insulin signalling in epitrochlearis muscle of adult female offspring which exercised early in life or not, sired by high-fat eating dads or control diet eating dads. NS, offspring sired by control diet fathers (n=6); HS, offspring sired by high-fat diet fathers (n=6); NE, early life exercised offspring sired by control diet fathers (n=5); HE, early life exercised offspring sired by high-fat diet fathers (n=5). Values are presented as mean ± SEM. &, && Insulin effect $P < 0.05$, 0.01 (0nM vs 1.2nM). * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Offspring exercise main effect $P < 0.05$ (NS vs NE; HS vs HE). +, Paternal HFD/obesity vs. Offspring exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

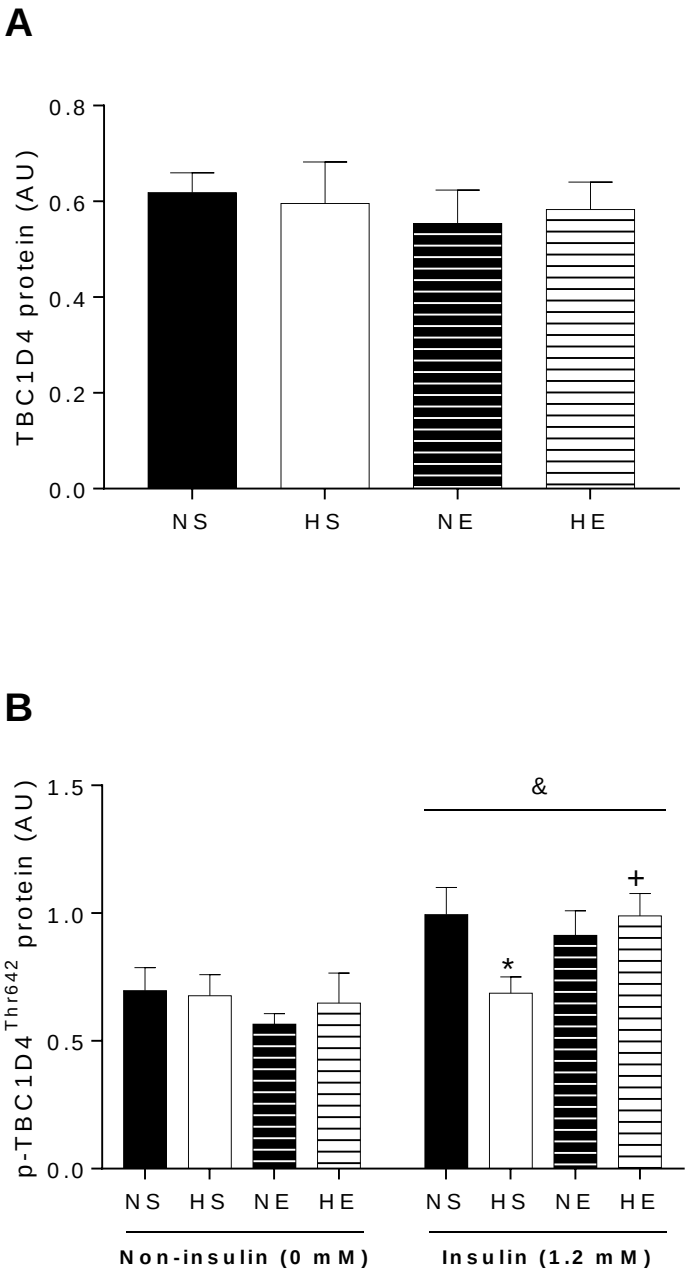

Figure 4-7. Basal and insulin signalling in epitrochlearis muscle of adult female offspring which exercised early in life or not, sired by high-fat eating dads or control diet eating dads. NS, offspring sired by control diet fathers (n=6); HS, offspring sired by high-fat diet fathers (n=6); NE, early life exercised offspring sired by control diet fathers (n=5); HE, early life exercised offspring sired by high-fat diet fathers (n=5). Values are presented as mean ± SEM. &, Insulin effect $P < 0.05$ (0nM vs 1.2nM). * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). +, Paternal HFD/obesity vs. Offspring exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

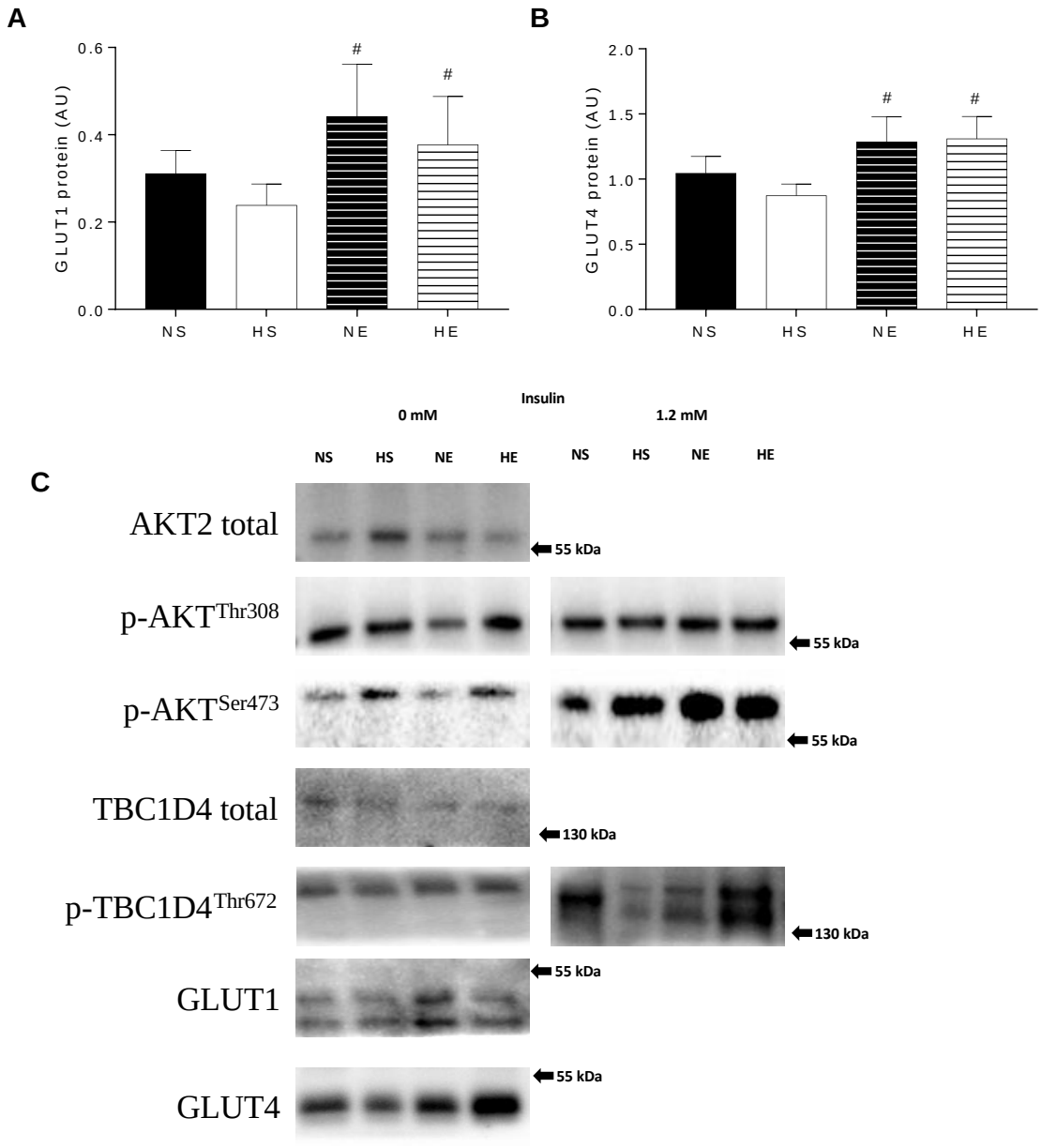

Figure 4-8. Early life exercise increases GLUT1 and GLUT4 in adult offspring. GLUT1 (A) and GLUT4 (B) protein expressions. C. Representative blots. For p-TBC1D4^{Thr672} insulin-stimulated condition (1.2 nM) and GLUT1, two bands were identified and used to measure protein expression. NS, offspring sired by control diet fathers; HS, offspring sired by high-fat diet fathers; NE, early life exercised offspring sired by control diet fathers; HE, early life exercised offspring sired by high-fat diet fathers. Representative western blots show the quality and signal obtained with the respective antibodies; because they represent one animal, they do not necessarily represent an exact mean of their experimental group. Values are presented as mean ± SEM. #, Offspring exercise main effect $P < 0.05$ (NS vs NE; HS vs HE).

4.3.6 Mitochondrial respiration and flux control ratios

Paternal HFD/obesity caused an overall reduction of mitochondrial respiration (20-30% lower throughout all cases) but no statistical significance was reached in any of the stages measured (HS vs NS) (Figure 4-9). Citrate synthase activity (Figure **4-10**) and respiratory control ratios (Figure 4-11) were not affected by paternal HFD/obesity. Exercise early life in offspring born from normal diet fathers only increased CI_L and had no further effects (Figure 4-9 A). When compared to HS offspring, the HE group showed increased mass-specific mitochondrial respiration (muscle weight) (Figure 4-9). CS activity (Figure **4-10**) and the respiratory control ratios (Figure 4-11) did not change. As CS activity was unchanged, no mitochondrial-specific respiration was calculated to avoid further variation in the normalisation process.

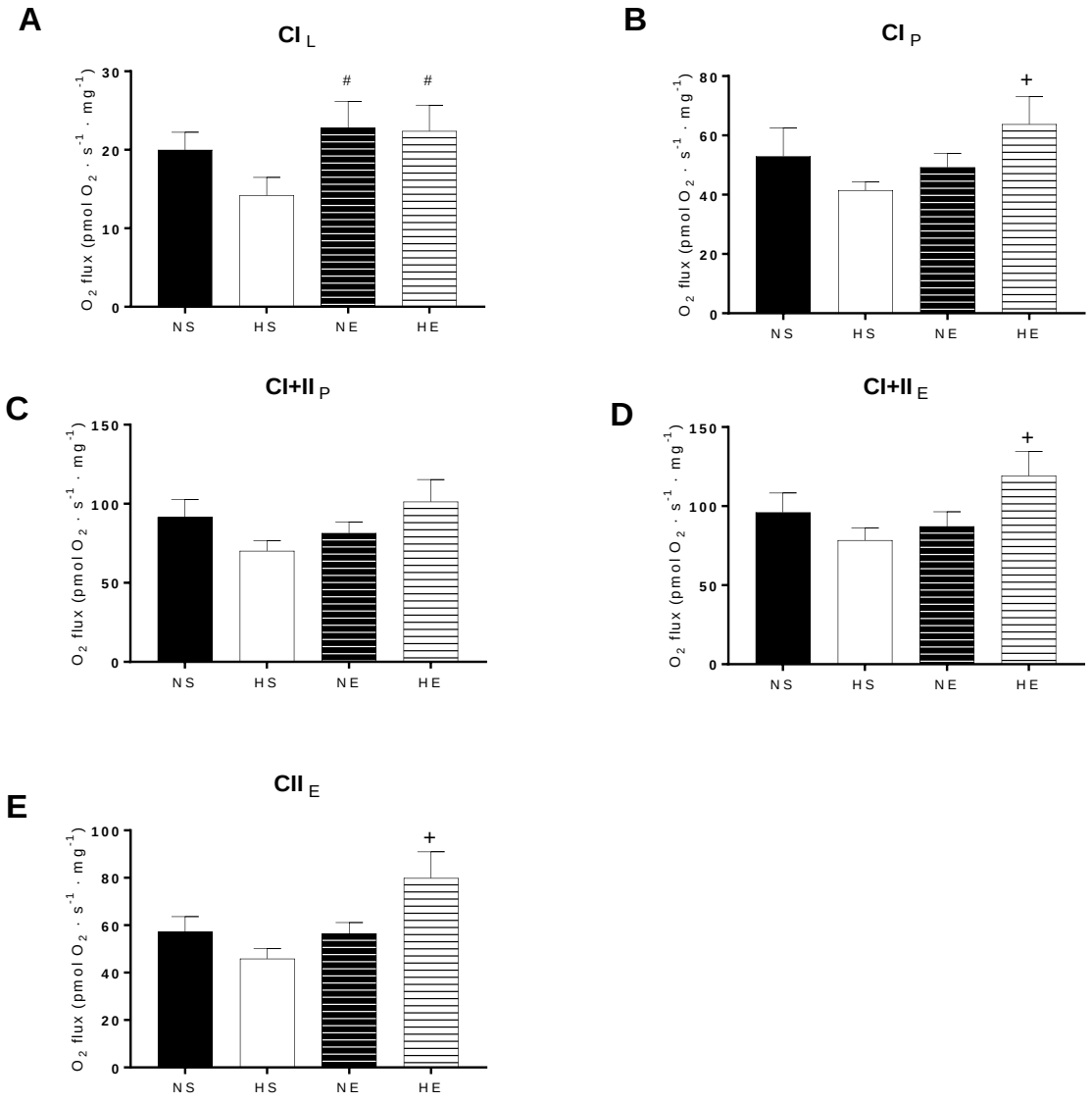

Figure 4-9. Mass-specific mitochondrial respiration in adult offspring which performed exercise early in life or not, sired by control or high-fat eating fathers before conception. NS, offspring sired by control diet fathers (n=9); HS, offspring sired by high-fat diet fathers (n=8); NE, early life exercised offspring sired by control diet fathers (n=7); HE, early life exercised offspring sired by high-fat diet fathers (n=8). Values are presented as mean ± SEM. #, Offspring exercise main effect $P < 0.05$ (NS vs NE; HS vs HE). +, Paternal HFD/obesity vs. Offspring exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

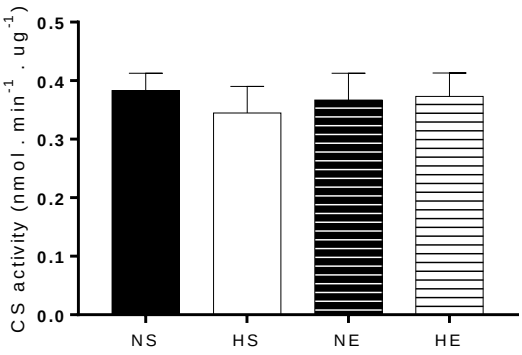

Figure 4-10. Paternal high-fat diet and early in life exercise does not affect citrate synthase activity. NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high-fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high-fat diet fathers (n=10). Values are presented as mean ± SEM.

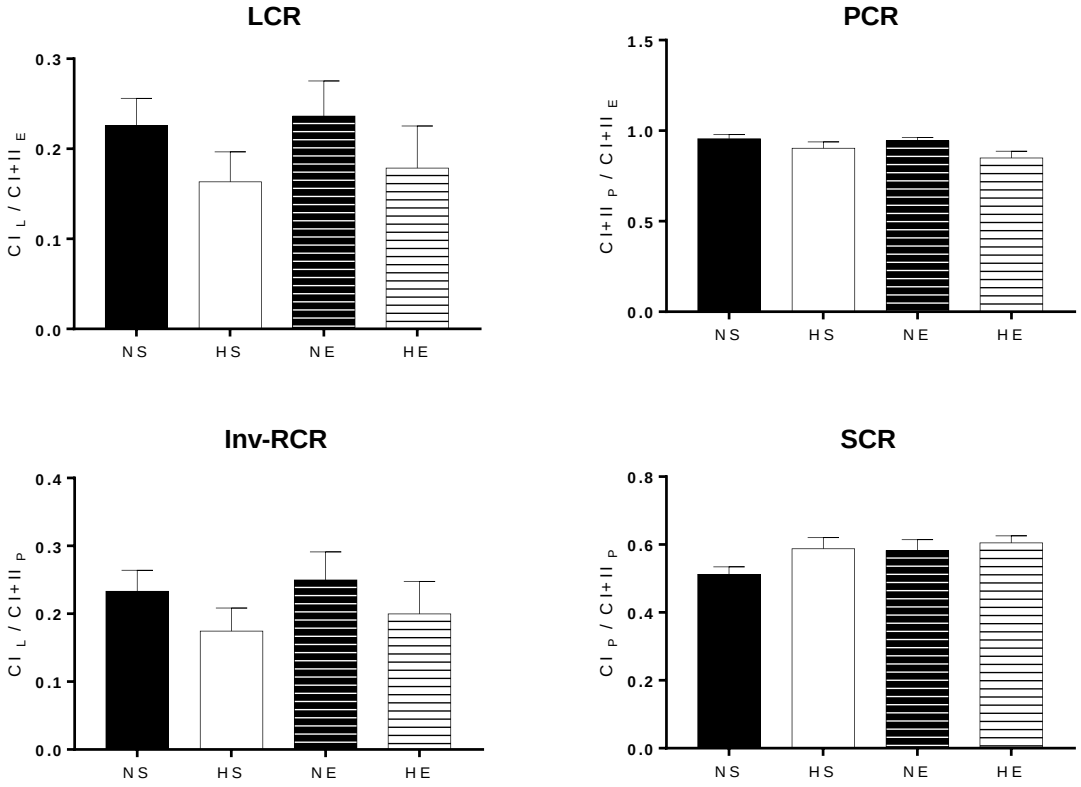

Figure 4-11. Respiratory flux control ratios (FCRs) in adult offspring which performed exercise early in life or not, sired by control or high-fat eating fathers before conception. LCR, leak control ratio (CIL/CI+IIE); PCR, phosphorylation control ratio (CI+IIP/CI+IIE); inv-RCR, inverse of respiratory control ratio (CIL/CI+IIP); SCR, substrate control ratio at constant P (CIP/CI+IIP). FCRs were calculated from mass specific respiration measurements in permeabilised muscle fibres (plantaris muscle) obtained from female rats at 25 weeks of age. NS, offspring sired by control diet fathers (n=9); HS, offspring sired by high-fat diet fathers (n=8); NE, early life exercised offspring sired by control diet fathers (n=7); HE, early life exercised offspring sired by high-fat diet fathers (n=8). Values are presented as mean ± SEM.

4.3.7 Mitochondrial ROS production

We also measured H_2O_2 production simultaneously to the mitochondrial respiration. H_2O_2 production was not altered by either paternal diet-induced obesity or exercise early in life in any of the stages of the respiration (Figure 4-12).

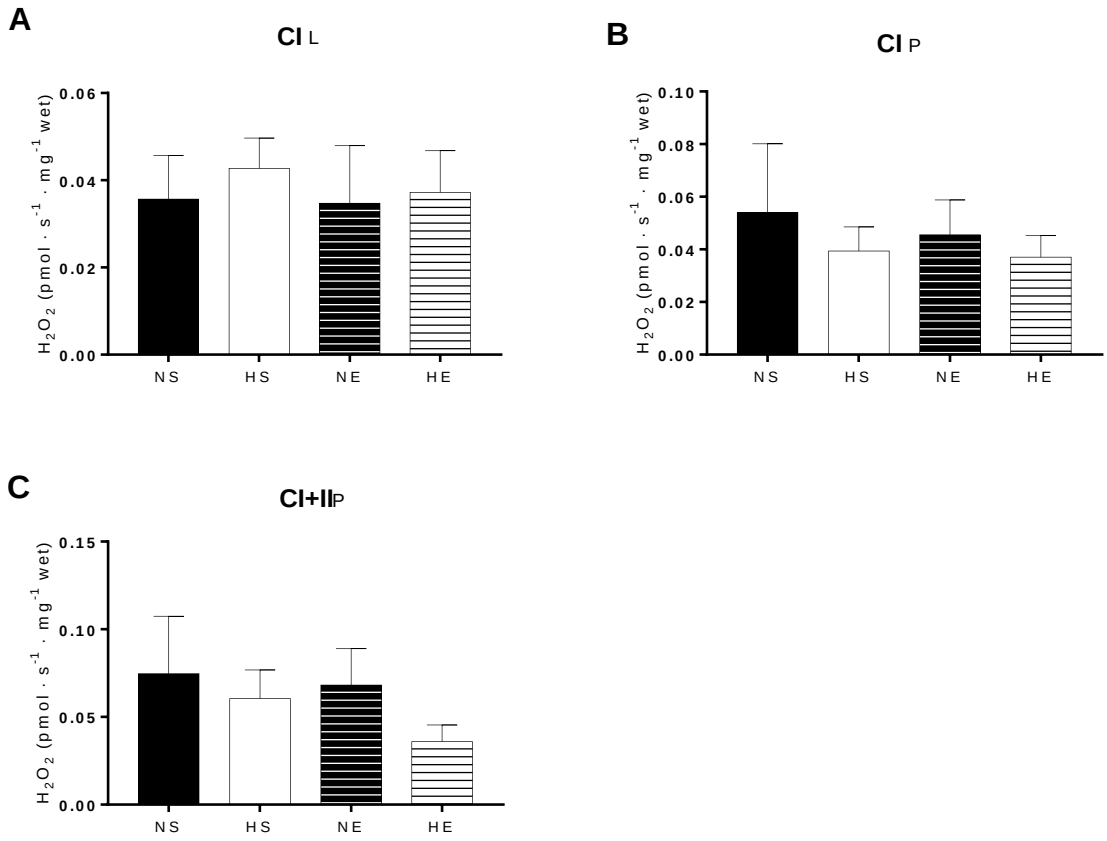

Figure 4-12. H_2O_2 production simultaneously measured during the mitochondrial respiration. A, Hydrogen peroxide (H_2O_2) levels during leak respiration through complex I (CIL). B, H_2O_2 levels during the maximum oxidative phosphorylation capacity through complex I (CIP). C, H_2O_2 levels during the maximum oxidative phosphorylation capacity through complex I and II combined (CI+IIP). B, D and F are these respective values normalised by citrate synthase (CS) activity. NS, offspring sired by control diet fathers (n=9); HS, offspring sired by high-fat diet fathers (n=8); NE, early life exercised offspring sired by control diet fathers (n=7); HE, early life exercised offspring sired by high-fat diet fathers (n=8). Values are presented as mean ± SEM.

4.3.8 Mitochondrial-related protein expression

HS pups tended to have lower Tfam protein expression (P=0.07) and had significantly lower plant homeodomain finger-containing protein 20 (PHF20) protein

expression. PGC1α was not affected by paternal HFD/obesity. Exercise early in life did not affect any of these protein expressions (Figure 4-13).

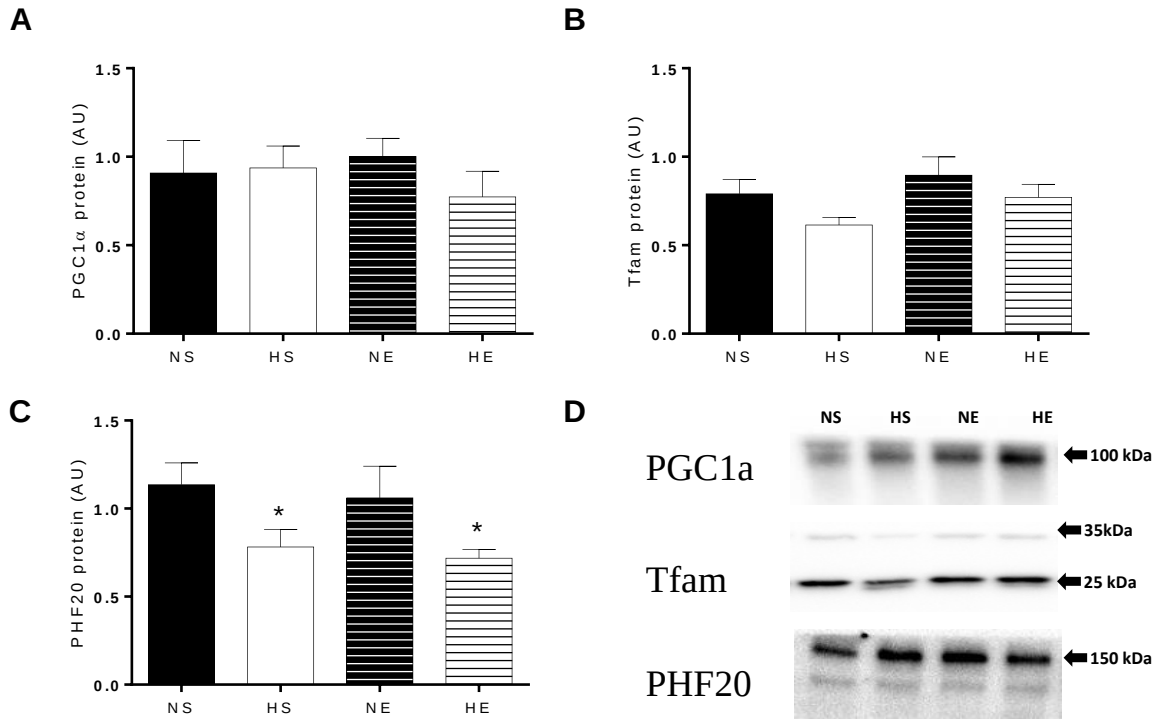

Figure 4-13. Protein expression in plantaris muscle of adult offspring which performed exercise early in life or not, sired by control or high-fat eating fathers before conception. For TFAM and PHF20, two bands were identified and used to measure protein expression. NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high-fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high-fat diet fathers (n=10). Representative western blots show the quality and signal obtained with the respective antibodies; because they represent one animal, they do not necessarily represent an exact mean of their experimental group. Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE).

4.3.9 Pancreas morphology in adult offspring

Relative islet surface area, number of islets, β-cell area and proportion did not change due to paternal HFD/obesity (Figure 4-14). However, β-cell mass was lower (Figure 4-14 C) and islet size distribution was altered with more small size islets and less large and very large islets in HS (Figure 4-15). The percentage of <5,000 μm^2 islets were higher due to paternal HFD/obesity in contrast to the lower 10,000-20,000 and 20,000-

50,000 μm^2 islet sizes. Paternal diet-induced obesity decreased this index in adult offspring (Figure 4-14).

Exercise early in life increased the number of islets in both normal and high-fat dad offspring while β-cell mass and the insulinogenic index remained lower in exercised offspring sired by diet-induced obese fathers (HE vs HS) (Figure 4-14). Exercise early in life normalised the amount of 20,000-50,000 μm^2 islets, but did not change the number of <5,000 μm^2 and 10,000-20,000 μm^2 islets (Figure 4-15). Other islet sizes were not affected by either paternal diet-induced obesity or exercise early in life.

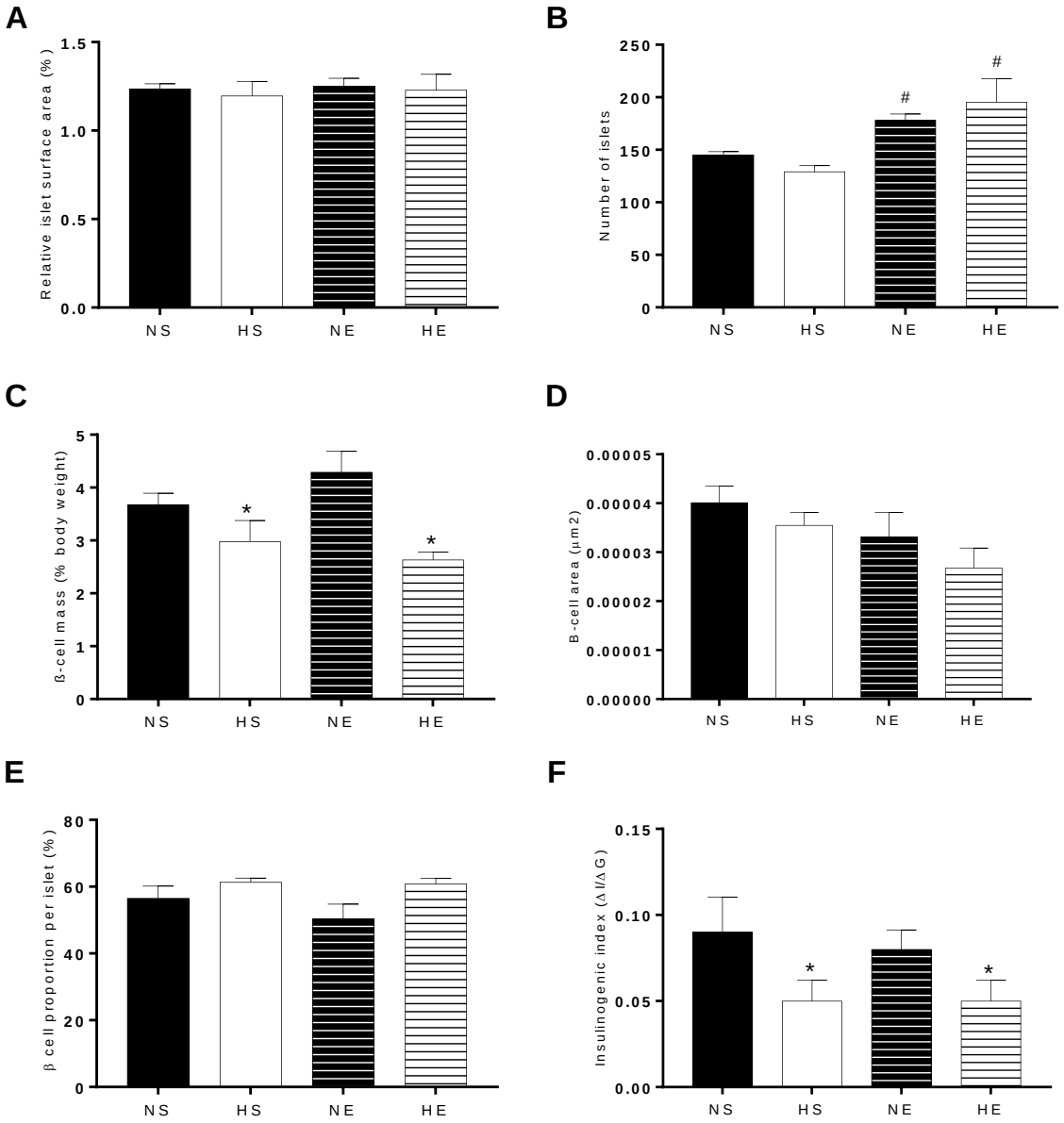

Figure 4-14. Pancreas morphology in adult offspring sired by fathers fed high-fat or control diets which performed or not exercised early in life. A. Relative islet surface area expressed as a percentage of total pancreas surface area. B. Number of islets. C. β-cell mass was calculated as the product of whole pancreas weight before fixation and the ratio of insulin positive/total pancreas cross-sectional area. D. β-cell area. E. β-cell proportion per islet. F. Insulinogenic index, derived from IPGTT at 24 weeks of age with the following formula: Insulinogenic index = AUCinsulin(0–30 min) / AUCglucose(0–30 min) (Ng et al. 2010). NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high-fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high-fat diet fathers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). #, Offspring exercise main effect $P < 0.05$ (NS vs NE; HS vs HE).

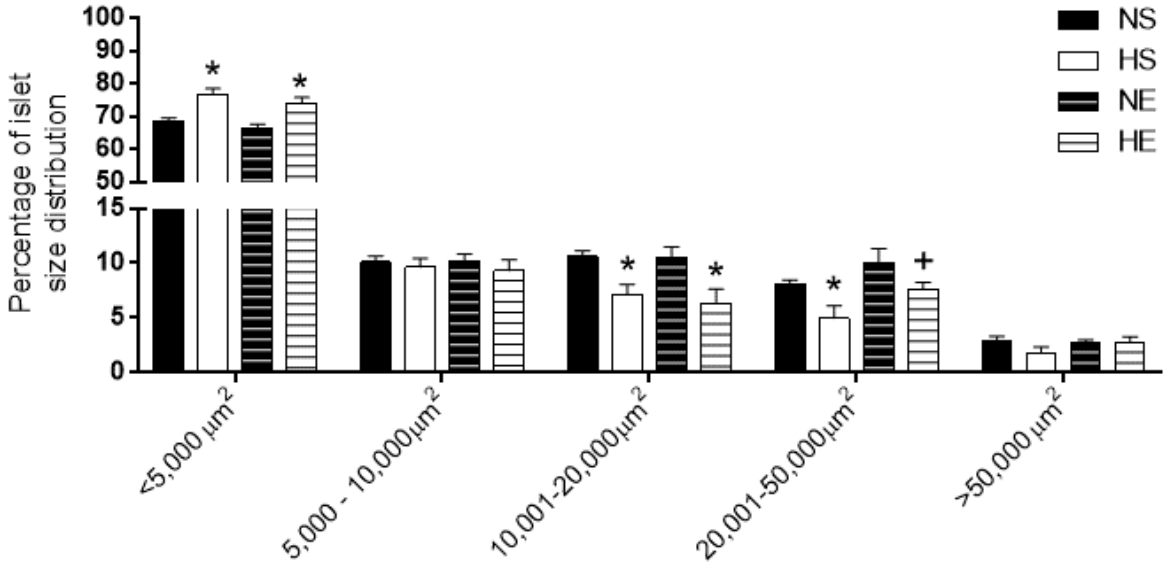

Figure 4-15. Islet distribution. Islets were arbitrarily classified according to their size: <5,000 µm², 5,000 – 10,000 µm², 10,001 – 20,000 µm², 20,001 – 50,000 µm² and >50,000 µm². NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high-fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high-fat diet fathers (n=10). Values are presented as mean ± SEM. * Paternal HFD/obesity main effect $P < 0.05$ (NS vs HS; NE vs HE). +, Paternal HFD/obesity vs. Offspring exercise interaction $P < 0.05$, followed by LSD test (HS vs HE).

4.4 Discussion

As the data of paternal diet-induced obesity has been discussed previously in Chapter 3, this section will rather emphasise the offspring exercise early in life effects. For the first time, we have demonstrated that exercise early in life normalises insulin sensitivity in adult offspring sired by HFD/obese fathers. The benefits of early exercise appeared to be due mainly to positive effects on the skeletal muscle mitochondrial function and insulin-stimulated glucose uptake because reductions in pancreatic β-cell mass in adult offspring sired by HFD/obese fathers were not normalised by early exercise training. These findings differed to the effects of maternal exercise (Chapter 3) after HFD/obese fathers, which did not normalise the reduced insulin sensitivity but attenuated the lower observed β-cell mass and insulin secretion.

4.4.1 Effects of paternal high-fat diet on offspring characteristics at birth, weaning and growth

Paternal diet-induced obesity and maternal exercise training did not affect any of the measured parameters at birth. Similarly, other groups reported no changes in birth weight in rats and mice offspring sired by fathers eating high-fat diet before conception (Masuyama et al. 2016, Ng et al. 2010).

Exercise early in life increased body weight and food intake, which was not associated with increased fat mass but rather an increase in lean mass such as skeletal muscles (*e.g.* soleus, tibialis anterior, plantaris), liver and heart weights. Body weight and body composition were normalised in offspring that were sired by HFD/obese fathers and who exercised early in life. Other exercise models have shown that physical activity in rats (free access to running wheel) from 3-6 weeks of age attenuates the development of obesity in genetically predisposed diet-induced obesity rats in long-term (Patterson et al. 2009). Our results also corroborate findings from prior research showing that free running wheel from 3 to 10 weeks of age promoted positive effects on body weight and adiposity offspring at 15 weeks of age, born from both obese and lean mothers (Caruso et al. 2013).

4.4.2 Offspring glucose tolerance, insulin sensitivity and secretion *in vivo*

We demonstrated impaired glucose tolerance (higher glucose AUC), however, we did not observe a decreased insulin secretion *in vivo* in adolescent rats (12 weeks). This was surprising as female rats at 12 weeks of age sired by HFD/obese father showed impaired glucose tolerance and reduced insulin secretion (Ng et al. 2010). The reason for such a difference is not clear as we intentionally attempted to replicate their experiments (Ng et al. 2010). One of the novel findings in this study is the improvement in glucose tolerance in adolescent rats (12 weeks of age) due to exercise early in life, but no effects on insulin secretion were found. In fact, short-term positive adaptations to exercise are reported in humans and animal models a few days after the last training session (AbouAssi et al. 2015, Leandro et al. 2012a, Laker et al. 2011). The positive effects of 8 months of exercise training in adults (196 subjects, combined aerobic and resistance training) on insulin sensitivity, β-cell function (estimated by the disposition index) and glucose effectiveness (the ability of glucose to cause its own uptake at resting) can still be observed after 2 weeks of exercise training cessation (AbouAssi et al. 2015). In adolescent rats, exercise early in life improves pancreatic β-cell mass (Laker et al. 2011) and skeletal muscle fibre composition (Leandro et al. 2012a), even when exposed to other

negative stimuli (placental restriction or maternal undernutrition, respectively). Similar positive effects were observed in our experiments in terms of body weight and glucose tolerance as both were independent of paternal HFD/obesity, suggesting exercise early in life might be able to protect adolescent offspring in short-term (3 weeks after the last training session).

Unlike at 12 weeks, paternal diet-induced obesity did not affect glucose tolerance (glucose AUC, P=0.09) in adult offspring (24 weeks of age) but markedly impaired insulin secretion during the glucose tolerance test. The reduction in insulin secretion was similar to what Ng *et al*. observed at 12 weeks (Ng et al. 2010). Insulin sensitivity (estimated *in vivo* by IPITT) was also compromised due to paternal HFD/obesity at 24 weeks. Similar findings have been reported in mice fathered by obese males which caused glucose intolerance in the offspring at various time points (8, 14, 26 and 39 weeks of age) along with insulin resistance (IPITT) at 16, 26, and 39 weeks of age (Fullston et al. 2013). Exercise early in life in offspring sired by obese fathers fed HFD normalised insulin sensitivity (IPITT) in adulthood. However, there were no changes in glucose tolerance or insulin secretion due to exercise early in life. This suggests that the improvement in insulin sensitivity in response to early life exercise in adult offspring sired by HFD/obese dads was due to effects at the level of the skeletal muscle rather than pancreas. Indeed, as discussed below, there were improvements in skeletal muscle *ex vivo* insulin-stimulated glucose uptake and mitochondrial function. However, further studies are needed using tracer and clamp techniques to investigate if hepatic insulin resistance might play an important role.

4.4.3 Offspring skeletal muscle glucose uptake

The main tissue responsible for *in vivo* insulin-stimulated glucose uptake is the skeletal muscle (DeFronzo and Tripathy 2009). The insulin hormone is a powerful stimulator of glucose uptake in skeletal muscle. There were no effects of paternal diet-induced obesity on offspring glucose uptake in the predominantly oxidative soleus muscle while the predominantly glycolytic epitrochlearis muscle was markedly compromised. Both basal and insulin-stimulated glucose uptake in the epitrochlearis muscle were lower in offspring sired by obese/HFD fed fathers compared to offspring of control diet fathers. To the best of our knowledge, this is the first time that reduced glucose uptake in isolated skeletal muscle has been examined or demonstrated in offspring sired by HFD eating

obese fathers. The lower insulin-stimulated glucose uptake may account, as least in part, for the lower insulin sensitivity (demonstrated by IPITT) in HFD offspring.

Due to compromised glucose uptake in the epitrochlearis in HFD offspring, we chose this muscle to perform further analysis and obtain insights into the underlying mechanisms. Basal glucose uptake in skeletal muscle is thought to be related to GLUT1 protein, an insulin-independent glucose transporter found in the sarcolemmal membrane (Ebeling et al. 1998). Despite a 25% decrease in GLUT1 expression in basal state in offspring sired by HFD/obese fathers, this difference was not significant (P=0.185). Similarly, other research groups have shown a significant (Ciaraldi et al. 2005) or a tendency (Kampmann et al. 2011) for a decrease in GLUT1 expression in skeletal muscle of T2DM patients. Nevertheless, exercise early in life increased the amount of GLUT1 in offspring sired by control and HFD/obese fathers, in support of our basal glucose uptake results. It is important to note that some studies report normal GLUT1 expression in T2DM patients (Pedersen et al. 1990), but these individuals are generally less responsive to insulin in skeletal muscle (Frojdo et al. 2009). Hence, we also investigated the insulin signalling.

Akt is activated by two phosphorylation steps (Scheid and Woodgett 2003), an initial phosphorylation by mammalian target of rapamycin 2 (mTORC2) at the p-Akt^{Ser473} site followed by 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylation of Akt^{Thr308} (Sarbassov et al. 2005). We found no differences between groups in the Akt^{Thr308} phosphorylation site, however, offspring sired by HFD/obese fathers had higher phosphorylation at Akt^{Ser473} with and without early life exercise. It was demonstrated that inhibition of Akt following mTORC2 depletion activates transcription factors (the forkhead box protein O1 and O3a), which promotes cellular survival by controlling the expression of genes related to stress resistance, metabolism and apoptosis (Calnan and Brunet 2008). It is not clear why offspring sired by obese fathers fed HFD had higher phosphorylation at Akt^{Ser473} and exercise early in life had no influence. Regardless, it will be important to assess alternative mechanisms that might increase Akt phosphorylation at Akt^{Ser473} (e.g. activation of a Akt^{Ser473} kinase, inhibition of a Akt^{Ser473} phosphatase, or regulation of the binding of a Akt^{Ser473}-regulatory protein).

An important distal to Akt signal within the insulin pathway is Akt substrate of 160 kDa (AS160, more recently being called TBC1 domain family member 4 – TBC1D4; and the 150 kDa TBC1D1) (Middelbeek et al. 2013, Sakamoto and Holman 2008). An important site of TBC1D4 that is regulated by Akt is the Threonine 642, which appears

to control GLUT4 trafficking in skeletal muscle (Chen et al. 2011). In T2DM patients, this phosphorylation site is reduced during an euglycemic-hyperinsulinemic clamp and likely explains, at least in part, the impaired insulin-stimulated glucose uptake (Middelbeek et al. 2013). Here, we report that offspring sired by HFD/obese fathers had lower TBC1D4 phosphorylation at Thr642 site with insulin, which might be associated with the lower insulin-stimulated glucose uptake in epitrochlearis muscle. The Thr642 phosphorylation site of the TBC1D4 has been reported to be responsive to both insulin and exercise stimuli (Treebak et al. 2014). Although other studies have provided some evidence to support that TBC1D4^{Thr642} phosphorylation increases during clamp, 5h into exercise recovery (healthy men, 25-28 years) (Pehmoller et al. 2012) or 2 days after the last bout of exercise training (Frosig et al. 2007), we have demonstrated for the first time its long-term effects (many weeks after exercise training had ceased). Sixteen weeks after the last exercise session, the positive effects of exercise early in life were still observed in adult offspring sired by obese fathers fed with HFD before conception. Further experiments investigating epigenetic markers in these offspring should provide useful insights to explain these findings.

There was no difference in GLUT4 protein expression that could explain the lower insulin-stimulated glucose uptake in offspring of HFD/obese fathers. Since most of muscle GLUT4 is found within intracellular storage sites, one limitation of this study is that we did not investigate the location of the GLUT4; as shown in T2DM patients, this protein might not be able to translocate to the membrane after insulin stimulation (Ryder et al. 2000). Therefore, the lower insulin-stimulated glucose uptake in isolated muscle presented in our experiments might be explained not by the amount of GLUT4, but rather its positioning within the myocyte (that is, less translocation), and/or perhaps due to the lower insulin-stimulated increase in p-TBC1D4. Exercise early in life, however, increased the protein expression of GLUT4 in adult epitrochlearis muscle independent of paternal diet-induced obesity. One of the known early adaptive responses of muscle to exercise is a rapid increase in GLUT4 protein expression in humans (Greiwe et al. 1999) and rodents (Ren et al. 1994). We suggest that the increase in GLUT4 protein expression as well as the increase in p-TBC1D4 in insulin-stimulated condition may account for the normalisation of insulin-stimulated glucose uptake *ex vivo* in the epitrochlearis muscle.

In order to investigate further mechanisms that might explain the lower glucose uptake in skeletal muscle in these offspring, we also examined the mitochondrial function and key metabolic markers.

4.4.4 Mitochondrial respiration

This is the first study examining whether mitochondrial function in rats is affected by HFD/obesity in fathers before conception and whether exercise early in life influences mitochondrial function in adulthood. Although not significant, the mitochondrial function of all measured complexes tended to be lower (20-30%) in offspring sired by HFD/obese fathers compared with normal diet fathers. Paternal diet-induced obesity also did not affect respiratory ratios, reactive oxygen species production or citrate synthase activity. This suggests that insulin resistance observed in skeletal muscle might not be linked to mitochondrial dysfunction in these animals. There is, in fact, an extensive discussion whether dysfunctional mitochondria is linked to insulin resistance (Szendroedi et al. 2012, Holloszy 2013, Martin and McGee 2014). There were no associations between impaired mitochondrial respiratory capacity and content with peripheral insulin resistance in insulin-sensitive lean and insulin-resistant obese subjects (Fisher-Wellman et al. 2014). On the other hand, others have documented lower mitochondrial content by electron microscopy (Chomentowski et al. 2011) and lower mitochondrial capacity (Larsen et al. 2011) related to insulin resistance in skeletal muscle. Given that HS were insulin resistant but there were no mitochondrial function impairments, it appears that in this situation, insulin sensitivity and mitochondrial function did not relate and that the lower p-TBC1D4 may account for the insulin resistance.

Exercise early in life in NS did not affect mitochondrial respiration, but when offspring sired by HFD/obese fathers performed exercise early in life, their mitochondrial respiration was improved in adulthood. This remarkable effect of early life exercise on mitochondrial function months later in offspring of HFD/obese fathers may have contributed to the normalisation of insulin-stimulated glucose uptake in skeletal muscle discussed above. This is not the first time that the effects of exercise are little unless there was a previous detrimental stimulus. In fact, the positive effects of maternal exercise were only observed in offspring of mothers on a high-fat diet (Laker et al. 2014a) or a low-protein diet (Falcao-Tebas et al. 2012a), but maternal exercise in chow fed mums had no effects on the offspring. Similar effects might apply to exercise early in life, only showing positive effects when an adverse stimulus threatens the offspring.

PGC1α (a major regulator of mitochondrial biogenesis) was not affected by our experimental groups. Similar results were reported by our group using exercise early in life with different rat strain and opposite sex. Exercise early in life was not able to

normalise mitochondrial PGC1α in intrauterine growth restricted adult offspring (Laker et al. 2012b). Interestingly, the mitochondrial transcription factor A (TFAM) tended to be lower due to paternal diet-induced obesity (P=0.07). TFAM is a DNA binding protein essential for transcriptional activation and mitochondrial DNA organization (Campbell et al. 2012), however, there is evidence indicating that muscle-specific TFAM-knockout mice do not show insulin resistance in skeletal muscle (Wredenberg et al. 2006). TFAM protein expression matches the mitochondrial respiration data in this study which was lower overall (but not significant). PHF20 plays a role in the regulation of the tumour suppressor protein p53, a transcription factor controlled by various post-translational modifications (Cui et al. 2012). Recently, PHF20 has been shown to be involved in exercise-training mitochondrial respiration adaptions in healthy humans (Granata et al. 2016), however, our early exercise model did not affect the expression of this protein in adulthood. Offspring sired from HFD/obese fathers presented lower expression of this PHF20 independent of exercise early in life. Although PHF20 and p53 have been demonstrated to be involved in insulin resistance in adipose tissue resistance (Minamino et al. 2009) and liver (Derdak et al. 2011), its effects on skeletal muscle are elusive and needs further investigation.

Taken together, our results suggest that the normalising of insulin sensitivity in adulthood after early exercise in offspring of high-fat fathers may be related to normalising of p-TBC1D4, increases in GLUT4 protein content and mitochondrial function independent of mitochondrial biogenesis and PHF20 protein content. Further studies are required to further tease out the mechanisms involved.

4.4.5 Effects of early life exercise on pancreas morphology

We found that insulin secretion during the glucose tolerance test was lower in adult offspring (24 weeks of age) of HFD eating obese dads, and might be related to the lower β-cell mass and altered islet size distribution in these rats. In fact, others have reported an association between pancreatic β-cell mass and insulin secretion in experiments (Kostromina et al. 2013, Matveyenko and Butler 2008). Our findings in adult rats corroborate the results from Ng *et al*. that demonstrated negative effects of paternal HFD on insulin secretion, affecting pancreas morphology in adolescent rat offspring (12 weeks of age) (Ng et al. 2010).

Some studies suggest about >50% of the secretory capacity of the β-cell is lost at the time of diagnosis of T2DM (Holman 1998), with ~40% deficit in β-cell volume in

lean T2DM individuals (Butler et al. 2003). Maybe this relation is aggravated by paternal diet-induced obesity as we showed that a 31% reduction in β-cell mass is linked to insulin secretion *in vivo*. Experiments testing β-cell secretory capacity and apoptosis markers would help understand if this relationship is changed due to paternal diet-induced obesity. Interestingly, we also found in the offspring exercised early in life an increase in islet number despite no difference in β-cell mass. This may be explained by an increase in other cell types within the islets, such as alpha and delta cells. It would be interesting to investigate in future studies whether exercise early in life might be able to alter pancreatic cell types. As demonstrated by others (Ng et al. 2010), we also found altered islet size distribution in offspring sired by HFD/obese fathers. The increase in the percentage of islets smaller than 5,000 μm^2 was followed by a decrease in islets sizing 10,000 to 50,000 μm^2.

The contribution of islet size to glucose-induced insulin release is controversial. Altered islet size distribution in transgenic mice (signal transducer and activator of transcription 3 knockout) does not change islet secretory function *in vitro* (Kostromina et al. 2013). However, another study pointed out that insulin secretion rates *in vitro* was directly associated to islet size (volume or surface area) (Reaven et al. 1981). It has been proposed that large and small islets difference in secretory capacity may be due altered percentage of β-cells within each islet (Farhat et al. 2013), however, we did not find any differences in the insulin-positive counts among the categorical organisation of cell sizes (data no shown). We also showed that β-cell function *in vivo* (estimated using the insulinogenic index) (Singh and Saxena 2010), is lower due to paternal HFD/obesity, in accordance to previous study (Ng et al. 2010).

Exercise early in life had minimal effects on islet size, only normalising islets from 20,000 to 50,000 μm^2. This supports the findings of impaired β-cell mass and insulin secretion in our experiments. We have previously demonstrated that exercise early in life has beneficial impacts on insulin secretion later in life (Gatford et al. 2014, Laker et al. 2011). Intrauterine growth restricted rats (placental blood flow restriction) is a model known for having deficits in β-cell mass especially with aging (Simmons et al. 2001). Exercise early in life fully restored the large decrease in β-cell mass (~65%) and pancreatic islet surface area in this animal model at 24 weeks of age (Laker et al. 2011). Surprisingly, we did not see any normalising effects of exercise early in life on the reduced β-cell mass in our HE group compared to HS. This suggests that the mechanisms causing decreases in β-cell mass with IUGR are different those that involve the fathers

sperm. It will be important to determine which genes are associated with the lower β-cell mass in the two models. In addition, exercise early in life might have fixed β-cell deficits in SGA model due to sex differences, as Laker *et al*. used males and we used female offspring, or rat strain differences between Wistar-Kyoto and Sprague-Dawley rats (Laker et al. 2011).

4.5 Conclusions

Paternal diet-induced obesity decreases body weight of female offspring after weaning, and negative metabolic consequences were observed in adolescence (12 weeks of age) and adulthood (24 weeks of age). Indeed, whole body insulin sensitivity is impaired as is *ex vivo* insulin-stimulated glucose uptake in the predominantly glycolytic EPI muscle, but not in the predominately oxidative soleus muscle. The observed decrease in skeletal muscle TBC1D4 phosphorylation may contribute to the reduced insulin-stimulated glucose uptake. Mitochondrial function and reactive oxygen species, mitochondrial biogenesis markers and citrate synthase activity were not affected by paternal diet-induced obesity. There was also a reduction in insulin secretion in HS likely due to the observed lower β-cell mass and higher distribution of smaller islet size.

Exercise early in life attenuated the negative effects of paternal diet-induced obesity on body weight and whole body insulin sensitivity in adulthood. It appears that the normalising of the reduced insulin sensitivity with early life exercise was due to effects on skeletal muscle rather than the pancreas. Indeed, exercise early in life increased GLUT4 protein and normalised the reduced skeletal muscle insulin-stimulated TBC1D4 phosphorylation. It also increased skeletal muscle mitochondrial respiration compared to sedentary offspring sired by HFD/obese fathers. Surprisingly, early life exercise did not have any effects on the reduced pancreatic β-cell mass in offspring from HFD/obese fathers, which might explain why exercise had no effects on insulin secretion in adulthood.

Chapter 5 – Conclusions

This thesis investigated whether maternal exercise before and during gestation (Chapter 3) or offspring exercise early in life (Chapter 4) could prevent or attenuate the negative metabolic effects in adulthood of offspring sired by high-fat diet-induced obese fathers. This section summarises the key findings from the studies conducted, argues how the results fit with the current knowledge in the field, discusses limitations and considerations related to the project, and recommends potential directions for future research.

5.1 Key findings

Although the main focus and novelty of this thesis are related to the long-term effects on maternal exercise and offspring exercise early in life, some aspects associated to paternal diet-induced obesity per se and its impact on offspring have not been investigated until now. The novel findings in regards to paternal HFD/obesity repercussions are related to the metabolic outcomes in skeletal muscle (insulin-stimulated glucose uptake and mitochondrial function), as no study has investigated this aspect. In particular no study had examined the effects of high-fat fed obese fathers on skeletal muscle.

5.1.1 Paternal diet-induced obesity effects on the offspring

We found for the first time that adult female rat offspring sired by HFD/obese fathers appear to develop whole body insulin resistance due to compromised skeletal muscle metabolism rather than effect on the pancreas. In fact, the insulin-stimulated glucose uptake in the predominantly fast-twitch EPI muscle (but not soleus, slow-twitch fibres) was reduced, and the lower phosphorylation of TBC1D4^{Thr642} might explain, at least in part, this finding.

Although skeletal muscle mitochondrial function of all measured complexes tended to be lower (20-30%, when normalised by muscle weight), this was not significant. There were also no effects of paternal HFD/obesity on ROS production or mitochondrial volume. Given there was insulin resistance, our data do not support the proposed link by others (Lowell and Shulman 2005, Turner and Heilbronn 2008) that insulin resistance might be related to mitochondrial dysfunction and fit more with others (Holloszy 2013,

Martin and McGee 2014, Fisher-Wellman et al. 2014) that these can be dissociated. Compared to control offspring, female rats sired by HFD/obese fathers had lower PHF20 protein expression, which regulates transcription factor p53. This protein is thought to play a role mitochondrial biogenesis and function in skeletal muscle, but further investigation is required. Our findings, therefore, support the indication that mitochondrial function and biogenesis might not be directly related to insulin resistance in skeletal muscle in these offspring.

The previous study that led to this investigation found that adolescent offspring (12 weeks of age) sired by HFD/obese fathers have reduced β-cell mass and accompanying impaired insulin secretion (Ng et al. 2010). Here, we reported glucose intolerance in adolescent offspring (12 weeks of age), but no effects on insulin secretion at this stage (with only a tendency to be higher). The novel aspect of this project was that we expanded Ng *et al* findings and demonstrated that a similar phenotype is observed in adulthood (24 weeks of age) when insulin secretion was remarkably impaired (Ng et al. 2010). The reduced insulin AUC (0-30min) compared with adult offspring from control fathers, which was likely due to the observed lower β-cell mass. We also determined similar pattern of islet size distribution in adult offspring, showing higher percentage of small islets and lower percentage of medium-large islets.

5.1.2 Long-term effects of maternal exercise

One of the most interesting findings in our study is the absence of effects in offspring born from normal diet fathers and exercised mothers in most of the parameters evaluated. Other research groups investigating glucose tolerance in similar experimental designs (voluntary running wheel before and during pregnancy) have reported clear benefits of maternal exercise to the offspring in adulthood (Carter et al. 2012, Carter et al. 2013). However, like us, others have demonstrated the positive effects of maternal exercise only when offspring were exposed to other risks *in utero*, such as maternal high-fat diet (Laker et al. 2014a). It is not clear why such a discrepancy occurs, but as discussed before (section 3.4.3), we hypothesise that disparate exercise protocols (especially in regards to the volume of exercise) might contribute to the different long-term responses to maternal exercise in the offspring.

On the other hand maternal exercise had remarkable beneficial effects in adult offspring sired by HFD/obese fathers, attenuating or even normalising its detrimental effects. For instance, exercise before and during pregnancy improved glucose tolerance

in adolescent offspring. On the other hand, in adult offspring, maternal exercise was not able to normalise *in vivo* insulin sensitivity although in isolated EPI muscle it prevented the negative effects of having a HFD/obese father on insulin-stimulated glucose uptake. Maternal exercise also increased skeletal muscle insulin-stimulated p-AKT^{Ser473} and GLUT4 protein expression above normal levels, there was however, no effects on p-TBC1D4^{Thr642}. This suggests that the skeletal muscle was normalised by maternal exercise in regards to insulin sensitivity but there may have been defects in other tissues, such as the liver, that were not normalised by maternal exercise.

Given that mitochondrial function and mitochondrial biogenesis were not really affected in offspring sired by HFD/obese fathers, it is perhaps not surprising that maternal exercise did not have much effect on adult offspring in this regard. The increased mitochondrial volume (CS activity) may be of importance. Mitochondrial function per unit of mitochondrial volume was decreased ($CI+II_P$ and CII_E) with non-significant changes in other complexes. Surprisingly, the lower level of key mitochondrial transcription factors observed in HS group, Tfam and PHF20, remained lower in offspring from exercised mothers.

Another important response in the offspring to maternal exercise was observed in insulin secretion. In adolescent rats, insulin secretion was lower in offspring sired by HFD/obese fathers and exercised mothers. In adult rats, however, maternal exercise attenuated the lower insulin secretion observed in offspring sired by HFD/obese fathers. This might be related to the increase in β-cell mass as well as increase in number of islets and islet surface area. We also observed a reduction in the percentage of small islets and an increase in medium to large islets, which might contribute to the improvement in the insulinogenic index and insulin secretion.

The effects described above might involve direct changes in maternal-foetal environment. This can include placental adaptations to exercise (Clapp 2006, Clapp and Dickstein 1984), without necessarily involving epigenetic modifications. But more studies are required to clarify the mechanisms involved in the beneficial effects of maternal exercise on offspring diabetes risk. In fact, others have proposed that reduced circulating factors (*e.g.* lipid profiles and inflammatory cytokines) can increase PGC-1α promoter methylation (Barrès et al.), and this can modulate maternal exercise effects on the offspring skeletal muscle in long-term (Laker et al. 2014a).

5.1.3 Long-term effects of offspring exercise early in life

It appears that the positive effects of exercise early in life on adulthood are only observed when the offspring is at higher risk of developing metabolic disease. In fact, exercise early in life compared to control animals had no effects in any of the parameters evaluated. However, a positive impact of exercise early in life was observed in offspring sired by HFD/obese fathers.

In adolescent offspring of HFD/obese fathers, exercise early in life improved glucose tolerance, but had no effects on whole body insulin sensitivity (IPITT). Importantly, however, in adult offspring, exercise early in life normalised whole body insulin resistance as well as the lower insulin-stimulated glucose uptake in EPI muscle *ex vivo*. This might be explained, at least in part, by the normalisation in p-TBC1D4^{Thr642}. This is a remarkable and important finding that indicate that epigenetic effects must be responsible, especially as no difference was found in adolescent but was in adult rats.

Exercise early in life in offspring sired by HFD/obese fathers also increased mitochondrial respiration above the control situation. These long-term effects of exercise training early life have not been reported before in offspring sired by HFD/obese fathers. There were no alterations on mitochondrial ROS emission, mitochondrial volume, mitochondrial biogenesis marker or transcription factors. This suggests that, in this model, the improvement in insulin sensitivity in skeletal muscle due to exercise early in life might not be related to mitochondrial adaptations in adulthood.

Surprisingly, offspring exercise was not able to improve the lower insulin secretion later in life, with insulin levels during the IPGTT remaining lower in offspring sired by HFD/obese fathers. This may have been because despite the higher number of islets, the β-cell mass was unaffected by offspring exercise and therefore remained lower than controls. The sustained lower β-cells in adults from HFD/obese fathers after early exercise corroborates the results about the insulinogenic index and insulin secretion. Early exercise did not normalise β-cell mass as our lab had previously found that it did after IUGR, in male Wistar rats at 24 weeks of age. Although these are different experimental models, they are the only studies that instigated insults early in life and used exercise early in life as a preventive tool.

5.2 Summarising the effects of exercise to correct the metabolic effects of paternal diet-induced obesity

Figure 5-1 highlights all the main results in this thesis in regards to the effects of high-fat fed obese fathers, maternal exercise and offspring exercise early in life, in adult offspring. These were presented in relation to the control offspring (NE vs NS) or the offspring from HFD/obese fathers (HE vs HS).

Our results suggest that exercise early in life can act as an environmental stimulus able to improve offspring's health in long-term. Like others (Laker et al. 2011), we showed that 5-9 weeks of age in rats seem to be a window of opportunity when a brief targeted intervention protects adult rats in high risk to develop metabolic diseases, such as IUGR rats. Our results are also important for translation, as childhood is a stage in life when government intervention in physical activity levels is feasible and compliance with exercise interventions is better than in adults (Schuler et al. 2013).

A few points should be considered related to the different responses in the offspring of early life exercise compared to maternal exercise. First, offspring exercise might be able to develop a sustained effect from a short (4 weeks) period of exercise training early in life. On the other hand, maternal exercise effects could be due to the influence exercise had in the mothers themselves rather than on the offspring directly, which can improve gestational parameters. In fact, uterine blood flow, placental and birth weights are improved during and/or after exercise during gestation (Clapp 2000, Hall and Kaufmann 1987, de Oliveria Melo et al. 2012, Clapp 2003).

The underlying mechanism of how maternal exercise and offspring exercise may modulate offspring's phenotype later in life is likely to be related to epigenetic adaptations. Maternal and offspring exercise, if considered as environmental factors, would be able to modify cellular and physiological phenotypic traits in the offspring (as we have demonstrated), by switching genes on and off by DNA methylation and/or histone modifications (Handy et al. 2011). Future research examining the effects of maternal exercise and offspring exercise should focus on the role epigenetic changes and the interplay between these factors. Different epigenetic markers, such as microRNAs, acetylation and methylation of histones might be involved to the long-term responses observed in the offspring. Knowledge in this area will highlight the importance of prevention, rather than treatment, and will be crucial to fight the alarming rates of metabolic diseases, especially for the next generations.

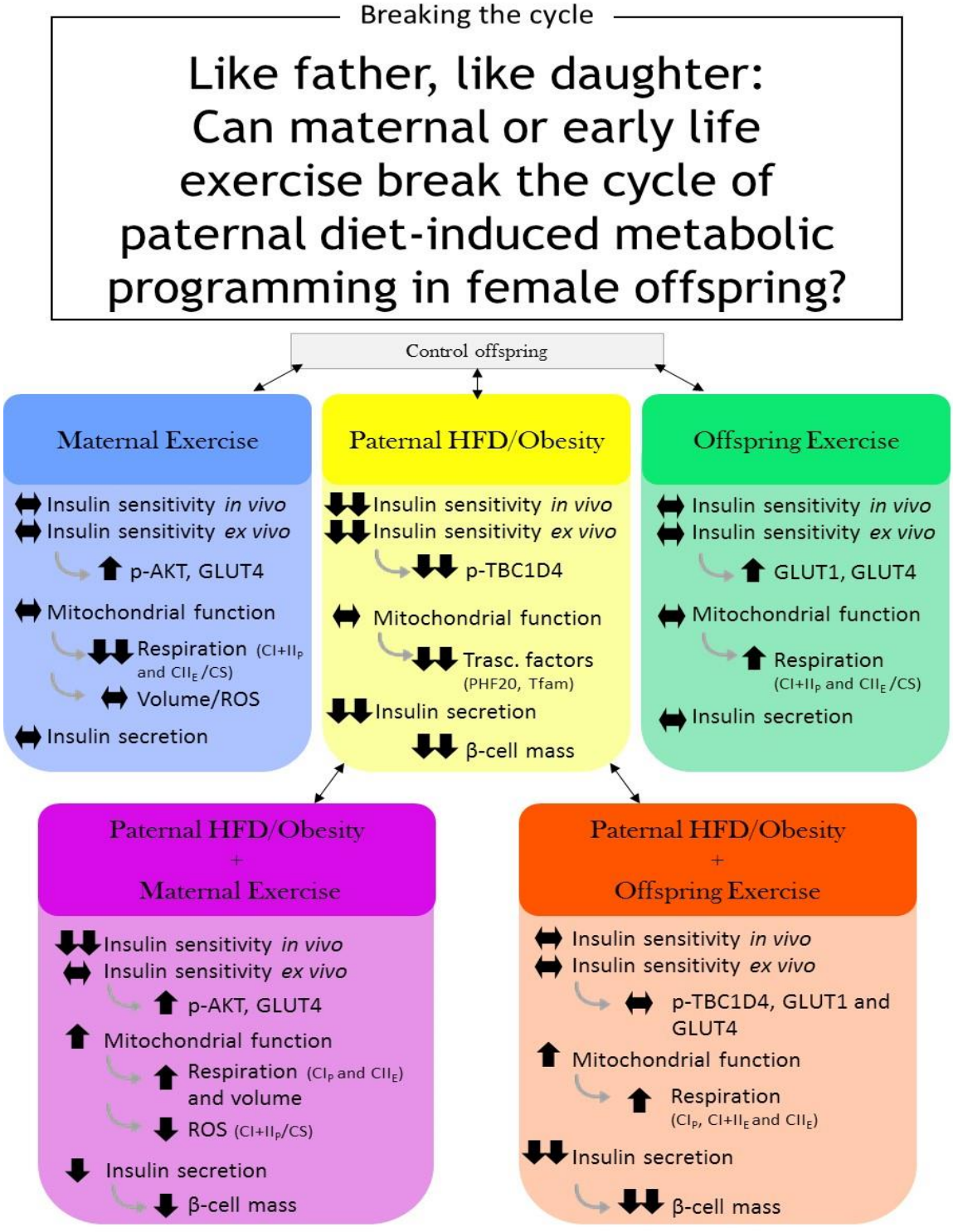

Figure 5-1. Summary of key findings in this thesis. The vivid colours represent the treatment of the parents while the shaded colours represent the effects on their respective adult offspring (25 weeks of age). Arrows indicate a significant difference compared to control animals (blue, yellow and green boxes) or compared to offspring sired by HFD/obese fathers (purple and orange boxes). Two downwards arrows indicate a decrease in a given measurement, while one downwards arrow indicates attenuation of the negative effect. Sidewards arrows indicate normalisation in a given measurement. Upwards arrow indicates improvement in a given measurement.

These results are very important as it is known that once a period of exercise training is ceased the training induced adaptations are lost over weeks to months (Mujika and Padilla 2000, Burstein et al. 1985). However, our findings will cause a paradigm shift as we have now clearly shown that deficits that occur before conception (paternal HFD/obesity) can be reprogrammed and corrected by either maternal exercise or offspring exercise early life.

5.3 Limitations and Considerations

It is important to acknowledge potential shortcomings and limitations of this research. Although it would be interesting to have all possible group combinations, such as exercised offspring of normal diet fathers and exercised mothers or exercised offspring from obese fathers and exercised mothers, it was necessary to limit the number of experimental groups in this research project. It was not our aim to include sedentary and/or exercised offspring born from HFD/obese fathers and exercise mothers in the experimental design. These extra groups would require more time, resources and even more littermate control rats, which could potentially affect the number of experiments we would have been able to perform. A trade-off between number of experimental groups and number of experiments would take place; therefore, we decided to not include these groups in our study. In addition, availability of more animals in the animal facility was another limitation of this study.

Intraperitoneal glucose and insulin tolerance tests are widely used in experimental studies, mostly because they are relatively inexpensive, quick and do not require high technical skills. However, we acknowledge that our studies would had benefited from the use of the euglycaemic hyperinsulinaemic clamp (gold-standard method for assessing of insulin sensitivity). The downside is that the clamp procedure requires (in most cases) anaesthesia of the animal, cannulation and recovery post procedure. As such, clamps are generally performed as end-point experiments and *ex vivo* insulin sensitivity with and without insulin would not have been possible, thus needing to add more animals (with clamp and without) rather than using right and left muscles of the same animal.

Moreover, there are other phosphorylation sites of AKT, TBC1D4 and TBC1D1 involved in the insulin-stimulated glucose uptake in skeletal muscle that could be explored. In fact, this topic has been discussed recently (Cartee 2015), showing the

importance of several phosphorylation sites in insulin- and exercise-stimulated glucose transport. For instance, TBC1D1 has a Thr596 while TBC1D4 has Ser318, Ser341 and Ser704 that are insulin-responsive phosphorylation sites in skeletal muscle (Treebak et al. 2014). Therefore it is possible that we may have missed important potential regulation of insulin signalling at these sites.

It would be interesting to look at the same parameters evaluated at 25 weeks of age (skeletal muscle glucose uptake and mitochondrial respiration, as well as pancreas morphology) in adolescent offspring (~12 weeks of age), to better compare our data with Ng and colleagues (Ng et al. 2010). We do not know whether the changes observed in adult offspring were also present in adolescent rats. Such project would double the amount of animals required to have two cohorts (adolescent and adult offspring). The fact that we did not investigate the pancreas at the same age as Ng *et al*. did (Ng et al. 2010) is a limitation, as we do not know whether our rats had similar changes in that period of life.

Ideally, experiments with isolated islets would had been performed as well. This would provide deeper understanding about the offspring's insulin secretory capacity. With isolated islets, it is possible to examine protein and gene expression relevant to the β-cell function, *e.g.* glucose transporters such as GLUT1 and GLUT3 (humans), and GLUT2 (rodents) (McCulloch et al. 2011), as well as PDX1 (Li et al. 2005). It would also have been possible to do glucose-stimulated insulin secretion and arginine-stimulated insulin secretion which allow us to explore specific pathways in regards to any potential problems.

5.4 Future Directions

The findings from the present studies have identified a number of considerations for future research. Given that having both parents obese is worse than only one parent in embryo and foetal development (McPherson et al. 2015) and metabolic outcomes in adolescent (12 weeks of age) offspring (Ornellas et al. 2015), future experimental models should incorporate obesity in both fathers and mothers, to investigate the long-term consequences in the adult offspring. Moreover, the percentage of reproductive aged couples who are overweight or obese has increased to a point that is the predominant situation in many countries including Australia (Ng et al. 2014a, Scheil 2012).

Although a study examined the difference of applying exercise before, during or before and during pregnancy (Stanford et al. 2015), it would be important to have more studies to examine whether maternal exercise before and during pregnancy is also the optimal period for offspring sired by HFD/obese fathers. Testing in mice as well as other animals (*e.g.* pigs and sheep) would strengthen the translational importance of these findings by replicating similar results in different species, if confirmed.

The insulin tolerance test is a simple, quick and low-cost experiment to evaluate whole body insulin sensitivity, useful in large cohort experiments such as in this project. Future research, however, should perform more complexes analyses including euglycaemic hyperinsulinaemic clamps with tracers, to use a gold-standard measurement of insulin sensitivity and distinguish between insulin resistance in skeletal muscle and liver. It would also be interesting to investigate whether other components of the insulin signalling are related to the results we have reported here. For instance, binding of insulin to its receptor followed by the activation of insulin to its receptor substrates (IRS1 and IRS2) would be of interest.

As we observed positive effects of maternal and offspring exercise on some mitochondrial parameters, it would be interesting to investigate, for instance, metabolites, proteins and genes related to the mitochondrial oxidative phosphorylation (OXPHOS) and electron transport system (ETS). This might help to elucidate some of the positive effects due to maternal and offspring exercise in offspring sired by HFD/obese fathers.

Future studies should consider evaluating pancreatic function *ex vivo*, with isolated islets. Experiments performing glucose-stimulated insulin secretion *in vitro* would provide additional insight into the underlying mechanisms of how paternal HFD/obesity impairs insulin secretion. In addition, isolated islets could be harvested to perform further islet-specific protein and genes expressions (e.g. PDX1 and GLUT2).

Further characterisation of this model, including investigation of leptin, growth hormone, and metabolites such as triglycerides, non-esterified fatty acids and C-peptide would provide useful insights to overall metabolic health of the offspring. If some lipids (*e.g.* ceramides, and diacylglycerol) were measured in the skeletal muscle, we would have been able to provide a more complete discussion about the relationship between mitochondrial function and insulin sensitivity as mentioned previously.

Upcoming research should investigate epigenetic mechanisms related to the effects offspring exercise or maternal exercise later in life. Previous studies have shown that maternal obesity causes hypermethylation of the PGC1α promoter in offspring

skeletal muscle, and maternal physical activity can attenuate this negative effect (Laker et al. 2014a). It would be interesting to explore whether paternal HFD/obesity, exercise early in life and maternal exercise also cause epigenetic modifications to DNA in promoters and/or associated histones in skeletal muscle and pancreas.

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Appendices

Animal ethics approval

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

MEMO

TO	Prof Glenn McConell ISEAL Victoria University	DATE	26th February 2014
FROM	A/Professor Alan Hayes Acting Chair Victoria University AEEC		
SUBJECT	**Ethics Application AEETH 13/008**		

Dear Glenn,

AEEC 13/008 - Effects of maternal or early-life exercise training on insulin secretion and sensitivity in rat female offspring born to fathers fed high-fat diet. (AEEC 13/128)

The AEEC Executive has reviewed your revised application and **approved your project between 26th February 2014 and 26th February 2017.** Attached with this memo is a PDF version of the approved application, this must be provided to all persons named on the project.

Continued approval of the project is conditional upon the following:

- Any variation proposed to the project, and the reasons for that change, must be submitted to the AEEC for approval and must not be implemented until approval is granted. **If a modification is required the AEEC Secretary must be contacted to obtain the word version of the currently approved application.**
- All activities involving the use of animals must occur as specified within the approved application or cited AEEC approved SOPs. Records pertaining to training, numbers of animals used, monitoring and the nature, timing and detail of all experimental activities must be maintained. These records may be inspected at any time by the AEEC, the Animal Welfare Officer or the Bureau of animal Welfare. Records of animal monitoring must be kept in a location accessible to all personnel involved in animal care. Various guidelines have been written to assist investigators to meet their legal obligations in respect to record keeping. These guidelines are available for reference on the VU Animal Ethics website. Annual and Final Reports should be supplied promptly to the Secretary of the AEEC.
- The project must only be conducted in approved premises nominated on the Licence SPPL 77. Use of other premises would constitute a variation and relevant details are to be notified to the AEEC for approval as "field work"

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- The AEEC must be notified in writing of:
  - Any changes to the approved personnel listed on the application the AEEC must approve the addition of a new investigator(s) prior to the investigator commencing work on the project.
  - Any unexpected incidents or complications that result in deaths, euthanasia or pain and suffering for the animals used in the project. Details of the steps taken to deal with adverse incidents must be included in the notification.

- Should the numbers of animals treated exceed that estimated for the first year of the ethics application, the primary investigator should submit a request for a minor amendment to update the numbers accordingly.

On behalf of the Committee, I wish you all the best for the conduct of the project.

If you have any further queries, please do not hesitate to contact me.

Kind Regards,

A/Professor Alan Hayes
Deputy Chair
Victoria University AEEC

Diet composition

Normal diet

Specialty Feeds
3150 Great Eastern Hwy
Glen Forrest
Western Australia 6071
p: +61 8 9298 8111
F: +61 8 9298 8700
Email: info@specialtyfeeds.com

Diet **Meat Free Rat and Mouse Diet**

A fixed formulation diet for Laboratory Rats and Mice fortified with vitamins and minerals to meet the requirements of breeding animals after the diet is autoclaved or irradiated.

- Minor modifications were made to this fixed formulation on 27 Jan 2009. Please contact us for details.
- All nutritional parameters of this diet meet or exceed the NRC guidelines for Rats and Mice.
- The diet has been designed as a general ration for breeding and early growth in all rat and mouse strains. The total fat content has been deliberately kept low at around 5%, to maximise the long term breeding performance of most strains.
- The formulation is designed to be fed ad-lib to rodents of all ages. There is some indication that growth performance in a minority of strains can be improved by increasing dietary energy (fat content). BalbC mice, DA rats and some of the modified strains appear to be most susceptible to this problem. Please contact us if you are concerned about this issue.
- Mammalian meals have been excluded from the diet, however the diet does contain fish meal. We have formulated totally vegetarian diets, and maintained colonies for some time on these diets. Please contact us if you require such a diet.
- The feed is manufactured in a cylindrical form with a diameter of around 12 mm, length is variable from 10 mm to 30 mm. We have found that this form is ideal for overhead hopper feeding, maximising the ease of handling whilst minimising fines formation and the risk of bridging in the feed hopper. Pellet strength has been kept lower than conventional pelletised diets. While this leads to a slight increase in transit and storage damage to the diet (fines generation), we have found that juvenile mice often have a lower feed intake on harder pellets.
- The diet is packed in permeable bags suitable for direct loading into an autoclave. It is recommended that the diet be autoclaved at 120° C for 20 minutes with a post autoclaving vacuum drying cycle. Some clumping of the diet can be expected, but the diet clumps can usually be easily broken. Modifying the drying time to leave some residual moisture in the diet can minimise the clumping. Do not autoclave at 135° C as this will result in significant clumping that will be difficult to break.

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Calculated Nutritional Parameters	
Protein	20.00%
Total Fat	4.80%
Crude Fibre	4.80%
Acid Detergent Fibre	7.60%
Neutral Detergent Fibre	16.40%
Total Carbohydrate	59.40%
Digestible Energy	14.0 MJ / Kg
% Total Calculated Energy From Protein	23.00%
% Total Calculated Energy From Lipids	12.00%

Ingredients

A Fixed formula ration using the following ingredients:
Wheat, barley, Lupins, Soya meal, Fish meal, Mixed vegetable oils, Canola oil, Salt, Calcium carbonate, Dicalcium phosphate, Magnesium oxide, and a Vitamin and trace mineral premix.

Added Vitamins	
Vitamin A (Retinol)	10 000 IU/Kg
Vitamin D (Cholecalciferol)	2 000 IU/Kg
Vitamin E (a Tocopherol acetate)	100 mg/Kg
Vitamin K (Menadione)	20 mg/Kg
Vitamin B1 (Thiamine)	80 mg/Kg
Vitamin B2 (Riboflavin)	30 mg/Kg
Niacin (Nicotinic acid)	100 mg/Kg
Vitamin B6 (Pryridoxine)	25 mg/Kg
Calcium Pantothenate	50 mg/Kg
Biotin	300 ug/Kg
Folic Acid	5.0 mg/Kg
Vitamin B12 (Cyancobalamin)	150 ug/Kg

Diet Form and Features

- Cereal grain base diet. 12 mm diameter pellets.
- Pack size 10 and 20 Kg Bags.
- Diet suitable for irradiation, also suitable for autoclave.
- Lead time 2 weeks

Added Trace Minerals	
Magnesium	100 mg/Kg
Iron	70 mg/Kg
Copper	16 mg/Kg
Iodine	0.5 mg/Kg
Manganese	70 mg/Kg
Zinc	60 mg/Kg
Molybdenum	0.5 mg/Kg
Selenium	0.1 mg/Kg

Calculated Amino Acids	
Valine	0.87%
Leucine	1.40%
Isoleucine	0.80%
Threonine	0.70%
Methionine	0.30%
Cystine	0.30%
Lysine	0.90%
Phenylanine	0.90%
Tyrosine	0.50%
Tryptophan	0.20%
Histidine	0.53%

Calculated Total Minerals	
Calcium	0.80%
Phosphorous	0.70%
Magnesium	0.20%
Sodium	0.18%
Potassium	0.82%
Sulphur	0.20%
Iron	200 mg/Kg
Copper	23 mg/Kg
Iodine	0.5 mg/Kg
Manganese	104 mg/Kg
Cobalt	0.7 mg/Kg
Zinc	90 mg/Kg
Molybdenum	1.2 mg/Kg
Selenium	0.4 mg/Kg
Cadmium	0.05 mg/Kg

Calculated Fatty Acid Composition	
Myristic Acid 14:0	0.03%
Palmitic Acid 16:0	0.50%
Stearic Acid 18:0	0.14%
Palmitoleic Acid 16:1	0.01%
Oleic Acid 18:1	1.90%
Gadoleic Acid 20:1	0.03%
Linoleic Acid 18:2 n6	1.30%
a Linolenic Acid 18:3 n3	0.30%
Arachadonic Acid 20:4 n6	0.01%
EPA 20:5 n3	0.02%
DHA 22:6 n3	0.05%
Total n3	0.37%
Total n6	1.31%
Total Mono Unsaturated Fats	2.00%
Total Polyunsaturated Fats	1.77%
Total Saturated Fats	0.74%

Calculated Total Vitamins	
Vitamin A (Retinol)	10 950 IU/Kg
Vitamin D (Cholecalciferol)	2 000 IU/Kg
Vitamin E (a Tocopherol acetate)	110 mg/Kg
Vitamin K (Menadione)	20 mg/Kg
Vitamin C (Ascorbic acid)	No data
Vitamin B1 (Thiamine)	80 mg/Kg
Vitamin B2 (Riboflavin)	30 mg/Kg
Niacin (Nicotinic acid)	145 mg/Kg
Vitamin B6 (Pryridoxine)	28 mg/Kg
Pantothenic Acid	60 mg/Kg
Biotin	410 ug/Kg
Folic Acid	5 mg/Kg
Inositol	No data
Vitamin B12 (Cyancobalamin)	150 ug/Kg
Choline	1 640 mg/Kg

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.

High-fat diets

Specialty Feeds

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Diet
SF01-025

22% Fat Rat and Mouse Diet

A high fat diet based on our standard rat and mouse diet.

- For general comments see our standard rat and mouse formulation data sheet.
- The total fat content of this diet has been increased to 22%. In our experience this is the maximum fat content that can be included into a standard pelletised diet. Even at this content a significant loss in pellet strength is apparent. Higher fat contents are possible but we have found that the diet form must change.
- The increased fat content has resulted in a 28% increase in calculated energy from 14.3 MJ/Kg to 18.3 MJ/Kg.
- All other nutritional parameters have been kept as close as possible to our standard rat and mouse diet.

Calculated Nutritional Parameters	
Protein	19.40%
Total Fat	22.30%
Crude Fibre	5.30%
Digestible Energy	18.5 MJ / Kg
% Total Calculated Digestible Energy from Protein	17.00%
% total Calculated Digestible Energy From Lipids	44.00%

Added Trace Minerals	
Magnesium	100 mg/Kg
Iron	70 mg/Kg
Copper	16 mg/Kg
Iodine	0.5 mg/Kg
Manganese	70 mg/Kg
Zinc	60 mg/Kg
Molybdenum	0.5 mg/Kg
Selenium	0.1 mg/Kg

Ingredients

A fixed formula ration using the following ingredients.
Wheat, Lupins, Barley, Soya meal, Canola meal, Cocoa butter, Canola oil, Salt, Calcium carbonate, Dicalcium phosphate, Magnesium oxide, and a Vitamin and mineral premix.

Feeding Recommendations

Feed ad-lib to animals of all ages.

Diet Form and Features

- Cereal grain base 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 for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

Added Vitamins	
Vitamin A (Retinol)	6 670 IU/Kg
Vitamin D3 (Cholecalciferol)	1 330 IU/Kg
Vitamin K (Menadione)	13 mg/Kg
Vitamin E (a Tocopherol acetate)	70 mg/Kg
Vitamin B1 (Thiamine)	53 mg/Kg
Vitamin B2 (Riboflavin)	20 mg/Kg
Niacin (Nicotinic acid)	70 mg/Kg
Vitamin B6 (Pyridoxine)	17 mg/Kg
Calcium Pantothenate	33 mg/Kg
Biotin	200 ug/Kg
Folic Acid	3 mg/Kg
Vitamin B12 (Cyanocobalamin)	100 ug/Kg

Calculated Amino Acids	
Valine	0.80%
Leucine	1.40%
Isoleucine	0.80%
Threonine	0.70%
Methionine	0.30%
Cystine	0.30%
Lysine	1.00%
Phenylanine	0.80%
Tyrosine	0.70%
Tryptophan	0.20%
Histidine	0.51%

Calculated Total Minerals	
Calcium	0.78%
Phosphorous	0.67%
Magnesium	0.20%
Sodium	0.18%
Potassium	0.70%
Sulphur	0.17%
Iron	190 mg/Kg
Copper	18 mg/Kg
Iodine	0.3 mg/Kg
Manganese	81 mg/Kg
Cobalt	0.4 mg/Kg
Zinc	70 mg/Kg
Molybdenum	1.4 mg/Kg
Selenium	0.4 mg/Kg
Cadmium	0.03 mg/Kg
Chromium	No data
Boron	3.6 mg/Kg

Calculated Total Vitamins	
Vitamin A (Retinol)	8 070 IU/Kg
Vitamin D (Cholecalciferol)	1 330
Vitamin E (a Tocopherol acetate)	83 mg/Kg
Vitamin K (Menadione)	13 mg/Kg
Vitamin C (Ascorbic acid)	No data
Vitamin B1 (Thiamine)	56 mg/Kg
Vitamin B2 (Riboflavin)	21 mg/Kg
Niacin (Nicotinic acid)	98 mg/Kg
Vitamin B6 (Pryridoxine)	19 mg/Kg
Pantothenic Acid	41 mg/Kg
Biotin	284 ug/Kg
Folic Acid	3.7 mg/Kg
Inositol	No data
Vitamin B12 (Cyancobalamin)	100 ug/Kg
Choline	1 660 mg/Kg

Calculated Fatty Acid Composition	
Saturated Fats C12:0 or less	0.09%
Myristic Acid 14:0	0.06%
Palmitic Acid 16:0	5.20%
Stearic Acid 18:0	6.90%
Arachidic Acid 20:0	0.23%
Palmitoleic Acid 16:1	0.05%
Oleic Acid 18:1	7.50%
Gadoleic Acid 20:1	0.04%
Linoleic Acid 18:2 n6	1.64%
a Linolenic Acid 18:3 n3	0.30%
Arachadonic Acid 20:4 n6	Trace
EPA 20:5 n3	0.02%
DHA 22:6 n3	0.06%
Total n3	0.39%
Total n6	1.64%
Total Mono Unsaturated Fats	7.59%
Total Polyunsaturated Fats	2.04%
Total Saturated Fats	12.60%

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 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%

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