Optimising parental fertility and offspring health through diet

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DECLARATION

I hereby declare that this thesis contains my original work completed under the supervision of Dr. Samantha M. Solon-Biet and Dr. Angela J. Crean, unless otherwise acknowledged. It contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

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PRESENTATIONS

The work from this thesis has been presented at the following conferences:

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- Finalist for ECR Award (Top 5 in Basic Science Category)
- Oral Presentation

ANZOS Annual Scientific Meeting 2021
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- Oral Presentation

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- E-Poster and Video Presentation

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- Poster Presentation

Asia-Oceania Conference on Obesity-Malaysian Association for the Study of Obesity (AOCO-MASO) Scientific Conference 2021
Virtual Conference 6 – 8 April 2021.

- Oral presentation

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- Oral presentation

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ABSTRACT

The pre- and peri-natal environment of an individual can significantly influence their development and alter the trajectory of many health outcomes. Various determinants during these stages, including parental disease states, over- or undernutrition, stress, and exposure to toxins, can have life-long consequences for offspring health. Nutrition is often considered as the single most modifiable factor that influences reproduction, health and the intergenerational risks of obesity and metabolic disease. While animal studies have focused primarily on the effects of either under-nutrition (e.g., calorie or protein restriction), or over-feeding of high fat diets, much less is known about the effects of parental macronutrient balance (the proportions of dietary protein, fat, and carbohydrate) in modulating offspring health. In this thesis, I conduct a series of experiments (detailed in data chapters 2-4) to investigate how manipulating macronutrient balance in maternal and paternal diets influence offspring health.

In Chapter 2, I explore the role of maternal diet on offspring health. In many animals, including humans, protein-specific appetite is prioritised over carbohydrate and fat. When confined to protein-poor, imbalanced diets, this can result in the overconsumption of total energy, leading to obesity. The question remains as to how this protein appetite is programmed. Given previous work showing that later-life health can be impacted by nutritional exposure *in utero*, we hypothesised that the programming of protein appetite may also occur at this stage. We examine this hypothesis by manipulating the protein:carbohydrate (P:C) balance of maternal diets and testing offspring food intake, macronutrient targets (points at which the necessary requirements for certain macronutrients are achieved), body composition, various metabolic outcomes and behaviour in early and late life stages. We show that offspring from dams fed high P:C diets throughout gestation and lactation have greater intrinsic protein targets and increased body weights in early life, a result consistent across sexes. We also show that these greater protein targets lead to increased offspring food intake when placed on no-choice diets in adulthood, resulting in an overall increase in body weight and fat mass persisting later in life.

We next investigated the effect of paternal diet on offspring health. The sperm and seminal

fluid from fathers play a crucial role in mediating changes outside of the genome informing their offspring of environmental insults, such as nutritional excess. The “Western” diet is an example of an obesogenic diet used widely in diet research and is usually characterised as high in fat and sugar, although compositions vary greatly between studies. In Chapter 3, we set out to isolate the effects of dietary sugar and fat on male fertility and metabolism by varying the carbohydrate and fat components in paternal diets. We show that although offspring macronutrient selection was not as strongly influenced by paternal diet compared to the maternal manipulations, numerous sex-specific outcomes in the offspring in terms of body composition are present. In Chapter 4, we focus on exploring how varying carbohydrate and fat components in paternal diets can affect anxiety-related behaviour in the studs. Additionally, we investigate how this can influence offspring behaviour in later life. Here, we show behaviour responses that are distinct between male and female offspring, as influenced by paternal dietary manipulations. We also show that paternal influences on offspring health are highly interactive and dependent on other major factors, such as offspring diet and sex.

This work highlights the important implications of early life programming from both maternal and paternal influences on later life outcomes. It could aid in explaining known patterns in the epidemiology of obesity and related cardio-metabolic disorders and will provide fundamental new understanding of the ways in which parental nutrition shapes offspring health.

ABBREVIATIONS

ANOVA Analysis of variance

ALT Alanine Transaminase

AST Aspartate Aminotransferase

BAT Brown Adipose Tissue

cAMP cyclic Adenosine Monophosphate

CD Control Diet

CHOL Cholesterol

CNS Central Nervous System

DNA Deoxyribonucleic Acid

DoHaD Developmental Origins of Health and Disease

EE Energy Expenditure

EPM Elevated Plus Maze

FGF21 Fibroblast Growth Factor 21

GABA Gamma-aminobutyric acid

GTT Glucose Tolerance Test

GWAT Gonadal White Adipose Tissue

HDL High-density Lipoprotein

HF High Fat

HS High Sucrose

HP High Protein

HPA Hypothalamic-pituitary-adrenal

iAUC incremental Area Under the Curve

IL- 1β Interleukin 1β

LDL Low-density Lipoprotein

LP Low Protein

MRI Magnetic Resonance Imaging

NAFLD Non-alcoholic fatty liver disease

P:C Protein:Carbohydrate

PE Protein Energy

PKA Protein Kinase A

OF Open Field

RNA Ribonucleic Acid

ROS Reactive Oxygen Species

RQ Respiratory Quotient

SD Standard Diet

SWAT Subcutaneous White Adipose Tissue

WD Western Diet

CHAPTER 1: General Introduction

1.1 Obesity

1.1.1 Background

Obesity is a global epidemic, with worldwide rates tripling between 1975 to present day. The World Health Organization (WHO) has reported that as of 2016, 39% of people aged 18 years were overweight and over 13% of the world's adult population were classified as obese. Moreover, overnutrition now accounts for a higher number of mortalities than malnutrition (World Health Organization, 2021). Obesity is a multifactorial condition that involves complex interplays between dietary components, lifestyle practices, and various internal (e.g., genomic, epigenomic, and metabolic) factors. It is typically defined by the accumulation of excess adipose tissue within the body, putting an individual at an increased propensity for developing various cardiometabolic comorbidities including type 2 diabetes mellitus, cardiovascular disease, hypertension, various types of cancer, and an increase in early mortality (Lichtenstein, 2015). The high rates of morbidity and mortality both in Australia and worldwide present difficulties not only at an individual level, but also for government and health care systems as they attempt to combat the demands of preventable diseases such as these. Therefore, it is crucial to continually improve our understanding in how this disease is developed, and risk factors that increase an individual's susceptibility to it.

1.1.2 Metabolism, energy homeostasis and obesity

Fundamentally, obesity is a condition that occurs from an energy imbalance, wherein energy intake (through food consumption) exceeds energy expenditure (Romieu et al., 2017). The regulation between these two systems is tightly maintained, and energy homeostasis is achieved through complex interactions between appetite regulatory centres in the central nervous system (CNS) and adipose tissue depots that store energy within the body (Singla et al., 2010). The molecular mechanisms that are responsible for this tight regulation have received a great deal of attention in recent decades (Elmquist et al., 2005). The hypothalamus, located in the CNS, is considered as the major centre that controls hunger and satiety (Woods et al., 1998). Various

neuropeptides such as neuropeptide Y and the melanocortin system (Arora and Anubhuti, 2006, Baldini and Phelan, 2019) and neurotransmitters including serotonin, dopamine, gamma aminobutyric acid (GABA), and oxytocin (Miller, 2019) are involved in both energy intake and expenditure pathways. Other peripheral organs secrete hormones that interact with appetite centres in the hypothalamus. Gut secreted hormones, such as ghrelin and cholecystokinin can also transmit signals to either stimulate appetite or inhibit food intake (Cowley and Grove, 2004, Berthoud, 2007). Adipose tissue functions not only as a storage system of energy for the body, but also an important endocrine organ, secreting factors called adipokines (Halberg et al., 2008). One such hormone that plays an important role in body weight regulation is leptin. It is considered the main regulator of the "brain-gut axis" and acts on receptors in the hypothalamus to signal satiety and promote energy expenditure (Friedman and Halaas, 1998, Zhang et al., 1994). Like leptin, insulin is a key hormone that circulates important signals to the CNS. After being recognised to be proportionate to body adiposity, it was found to regulate energy homeostasis and adiposity between the periphery and the CNS (Porte et al., 2002). Administration of it directly into the brain reduces food intake, while its deficiency results in hyperphagia (Woods et al., 1979, Brüning et al., 2000).

1.2 Nutrition and obesity- the importance of macronutrient balance

1.2.1 The importance of macronutrient balance

It is widely acknowledged that improvements in the nutritional environment are vital for the future management of obesity and its co-morbidities (Simpson et al., 2015). A major finding from past studies is that the balance of macronutrients in the diet has a profound impact on food and energy intake, metabolic health, lifespan, and reproduction (Solon-Biet et al., 2014, Wali et al., 2021b, Solon-Biet et al., 2015). By observing diverse animal species, ranging from insects to mammals, important evidence has been uncovered supporting the existence of independent appetite systems for protein, carbohydrate, and fat (Figure 1.1A,B). Moreover, their interaction and regulation have been associated as a major proponent to the dietary causes of obesity (Dussutour and Simpson, 2009, Shariatmadari and Forbes, 1993, Simpson and Raubenheimer, 2012).

1.2.2 Protein leverage

In many animals, including humans, protein energy (PE) is often more highly prioritized than non-protein energy (carbohydrates and fats; nPE), when confined to diets of fixed macronutrient composition (Sørensen et al., 2008, Gosby et al., 2014, Gosby et al., 2011). This phenomenon, called protein leverage, describes the theory that when macronutrient compositions are imbalanced within the diet, the drive to fulfill and maintain a pre-established protein target takes precedence, thus altering feeding behaviour with various consequences for body weight, composition, and overall metabolism (Simpson and Raubenheimer, 2005; Figure 1.1). In protein replete environments, individuals eat less food and under consume calories as a function of being able to reach their protein targets early. However, in protein-poor environments overconsumption of fats and carbohydrates occurs in an effort to reach this protected protein target. The increased consumption of diets higher in carbohydrate and/or fat, (thereby diluting available protein) ultimately leads to an increased overall energy intake (Figure 1.1C). Hence, it has been proposed that this protein prioritisation may play a key role in the obesity epidemic, and this has been dubbed the protein leverage hypothesis (Raubenheimer and Simpson, 2019).

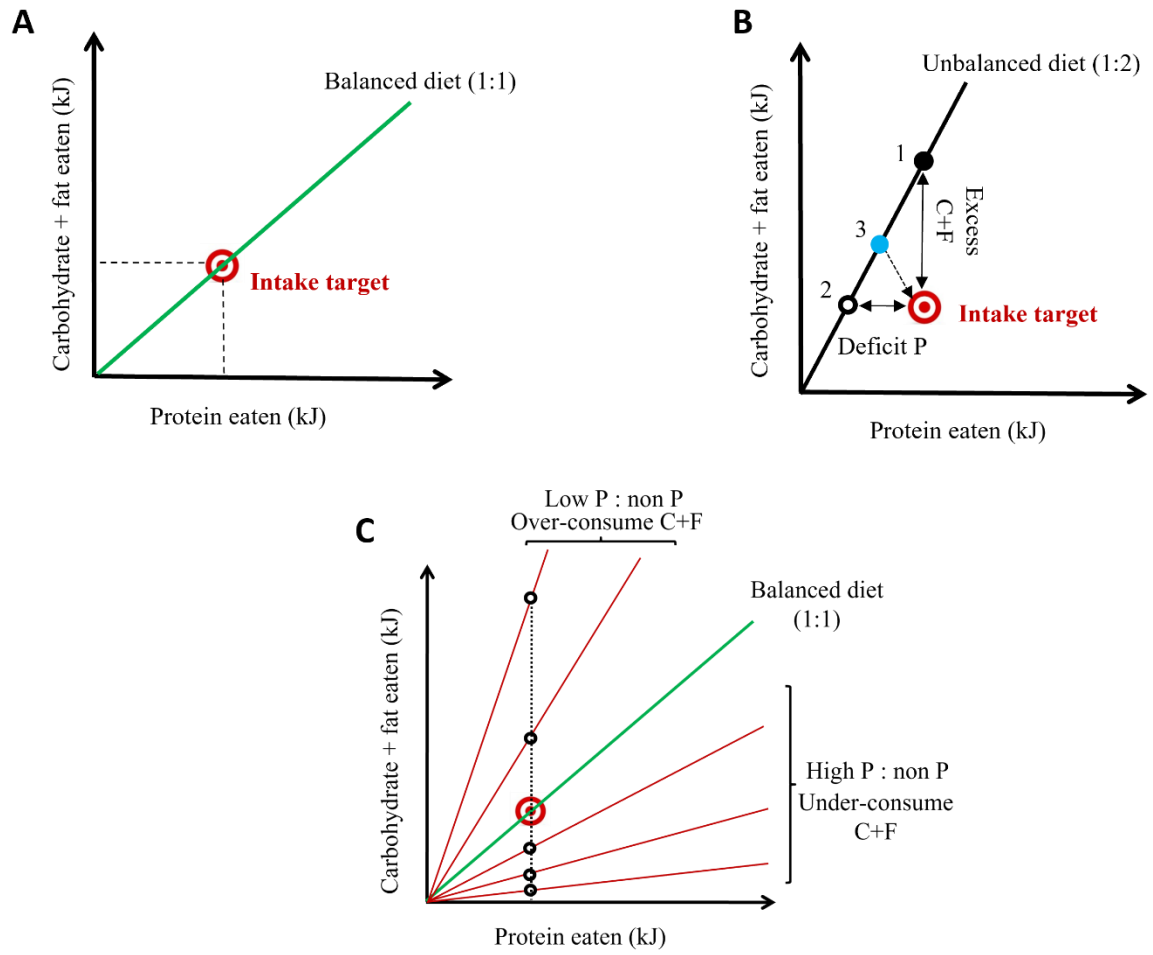

Figure 1.1 Representation of nutrient targets and protein leverage within a nutrient space.

(A) An intake target within a two-dimensional nutrient space, indicating the point at which the ratios of protein (P) *vs* carbohydrate and fat energy (C+F; non-protein) are optimal for maintaining energy balance. A balanced diet (shown as a green line) is that trajectory that runs from the origin to intersect the intake target. (B) An example of an unbalanced diet, in this case with a P:(C+F) ratio of 1:2. An animal is shown to have 3 options here. Eating until they reach point 1 will allow them to achieve intake target of P, but will result in consuming an excess of C+F. At point 2, they will have reached their target for C+F, but undereaten P. Animals can also eat until they maintain total energy intake (point 3) but will not reach target for either P or C+F. (C) Intake arrays when confined to unbalanced diets of varying P:(C+F) ratios. This describes the rule of compromise in which reaching the intake target of P is prioritised over that of C+F when a balanced diet is not available. An extreme example of complete protein leverage is shown here, where it follows a perfectly vertical pattern. The result is either over- or under-consumption of C+F, depending on the amount of protein within the diet.

1.2.3 Protein leverage and how we have eaten over time

There is evidence showing that the environments we developed in overtime were typically more abundant in protein compared to non-protein sources (Konner and Eaton, 2010, Cordain et al., 2000). Because of this, it is understandable that regulatory systems would be developed to ascertain carbohydrate and fat rich foods as highly palatable and allow for a high tolerance for their over-ingestion through storage as fat. Protein, on the other hand must be regulated more firmly in case of a protein deficit, such as in the instance of food shortages. There are very limited ways to store protein in the body, and the ingestion of toxically high levels of protein is prevented by its satiating effect in contrast to the other two macronutrients (Soenen and Westerterp-Plantenga, 2008).

From the 1980s onwards, the rapid invention of modern food manufacturing techniques has brought forth a wide range of ultra-processed products (Monteiro et al., 2013, Martínez Steele et al., 2018). Driven strongly by global corporations and a socio-cultural shift towards cheap, easily accessible, and highly palatable goods, this has coincided closely with a period of dramatic increases in obesity, diabetes and other common non-communicable diseases (Fardet, 2018). Being extremely rich in processed carbohydrates and fats, and typically poor in protein, this group of foods take advantage of protein leverage by promoting overconsumption of nPE in a protein-dilute environment. Moreover, the addition of artificial savoury flavours usually found in PE-rich foods works to detract the individual from complementing their diet with additional protein rich sources and ultimately lead to the further intake of nPE (Simpson and Raubenheimer, 2014).

The protein leverage hypothesis successfully integrates the consequences of regulatory feeding strategies and the nutritional environments in which we have existed in over time, and continue to exist in. However, it does not entirely explain why obesity has increased at such an alarming rate in the past couple of decades, and how it is differentially distributed across and within population groups. It is likely that obesity is caused by complex interactions between several factors including diet, genetics, level of physical activity, and the direct environment. Although several obesity-associated genetic polymorphisms have been identified (Farooqi et al., 2003, Kilpeläinen et al., 2011), genetics only account for a portion of an individual's predisposition to inherit the condition (Bell et al., 2005, Locke et al., 2015, Goldstein, 2009). The incredibly

rapid shift of populations towards a more obese phenotype over the past several generations suggests that it cannot be explained by genetics alone and that environmental factors also play a major role in driving its development. Although much focus has been dedicated in investigating the role of traditional adult risk factors, such as unbalanced diets and low levels of physical activity, a growing understanding is forming regarding the importance of nutrition during the peri-conceptional and early childhood period in determining an individual's susceptibility of developing later life disease (Barker, 2004, Friedman, 2018, Vickers, 2011).

1.3 Developmental programming

1.3.1 Background

Through the expansion of the concepts of "developmental programming" and the "Developmental Origins of Health and Disease (DoHaD)", we have come to better understand the link of *in utero* environment and early development of major disorders and diseases (Barker, 2004, Gluckman et al., 2008b). In animals, including humans, it has been shown that modifications to parental environments around the time of conception and *in utero* can produce lifelong changes in the physiology and metabolism of the offspring. We are now aware that in humans, the first 1000 days of life (starting from conception up to early childhood) is critical in determining a host of health outcomes (Barker, 2012). The adaptations an individual may develop during this window of organogenesis and rapid growth are believed to have immediate implications on overall cardio-metabolic health, as well as long-term implications in the development of disease in later life (Lane et al., 2014).

A well-researched example of this is from the Dutch Famine Birth Cohort of 1944-1945. Exposure to famine at gestation during this period has been shown to lead to a poorer health prognosis in adulthood versus control cohorts born either before or after the famine (De Rooij et al., 2022). This has been reported across a wide range of outcomes, including greater risk of adult obesity, decreased glucose tolerance and insulin regulation, increased plasma lipid levels, and the earlier onset of coronary heart disease and adult mortality (Ravelli et al., 1999, De Rooij et al., 2006a, De Rooij et al., 2006b, Roseboom et al., 2000). Additionally, neurological disorders were also found to be increased in famine exposure in early gestation (de Rooij et al.,

2010).

1.3.2 Thrifty phenotype hypothesis and birth weight

Similarly, the *thrifty phenotype hypothesis* is often used to exemplify how poor foetal and early post-natal nutrition often results in low birthweight and an increased risk of cardiometabolic disease in later life (Hales and Barker, 1992, Barker, 1990, Hales and Barker, 2001). Subsequent research that has stemmed from this continued to explore how the rapid catch-up growth that follows low infant weight is closely associated with permanent deleterious effects on glucose-insulin metabolism, increased fat deposition and therefore greater susceptibility to developing obesity and type two diabetes (Eriksson et al., 2003, Fernandez-Twinn et al., 2005), particularly when challenged postnatally with a hyper-nutritional diet in adulthood (Vickers et al., 2000).

Emerging evidence has proposed that parental overnutrition during conception and pregnancy and increased birth weight can also put an individual at a higher risk of developing metabolic diseases (Armitage et al., 2004). Most studies achieve overnutrition in pregnancy using high fat models and this has been shown to result in fat cell hypertrophy, and increased body weight and visceral fat depots in adulthood, stemming from higher energy intakes (Taylor et al., 2005, Samuelsson et al., 2008b). Additionally, several sex-dependent effects were reported in these studies, where changes were observed in either males or females only (Khan et al., 2004, Sun et al., 2012). Thus, the relationship between human birth weight and the increased susceptibility for developing adult obesity and related metabolic disorders is described to follow a "U-shaped" curve, with heightened risk from both parental under and over nutrition (Curhan et al., 1996, Ong, 2006).

1.3.3 Mismatched pre- and post-natal environments

More recently, it has been proposed that the degree of mismatch between pre- and post-natal environment can also be a major determinant of later life disease (Gluckman et al., 2005, Cleal et al., 2007). It is explained that the more dissimilar the two are, the less prepared the offspring would be to respond to it, putting them at a greater risk of developing cardio-metabolic diseases, such as obesity, in the long term. For example, if the infant is introduced to an energy deficit in the intrauterine environment, they may be predisposed to become more energy thrifty,

but become susceptible to developing obesity and other related diseases particularly when presented with an energy surplus in later life (Barker et al., 1993, Ravelli et al., 1976).

1.4 Paternal effects

1.4.1 Background

While the role of maternal influence on offspring health has been widely examined, the significance of paternal effects often does not receive the same level of attention. It is now becoming increasingly evident that paternal exposures can also impact offspring health outcomes, and any insults or stressors fathers may face prior to and at the time of conception can increase their offspring's susceptibility to developing deleterious health outcomes (Sharma and Rando, 2014, McPherson et al., 2014, Godschalk et al., 2017). Moreover, 'paternal effects' can be an important source of change in offspring phenotype and overall health, and it is therefore important to identify and distinguish them from other sources of variation (Crean et al., 2013, Wolf and Wade, 2009). Paternal effects are influences derived from the paternal line that modify offspring phenotype but does not reflect variation in offspring genotype (Crean and Bonduriansky, 2014). Precedent work suggests that non-genetic changes coming from fathers can also have transgenerational consequences (Carone et al., 2010, Pembrey et al., 2014).

1.4.2 Early work in the field of paternal effects

In humans, the most well-studied evidence for male line effects of nutrient availability on future generations comes from the Overkalix cohorts of northern Sweden (Pembrey et al., 2006, Bygren et al., 2001). From records taken from these groups, researchers identified that paternal and grand paternal food availability during the slow growth period prior puberty are linked to cardiovascular risks in subsequent generations (Bygren et al., 2001). Not only does it exemplify the notion that food scarcity can have deleterious impacts on subsequent generation's mortality, it also brings forth the proposition that a sex specific, male-line transgenerational response system exists in humans (Pembrey et al., 2006). An increasing number of rodent studies have also demonstrated that changes in paternal environment, more specifically paternal nutrition, lead to further changes in offspring metabolism and behaviour. Common dietary models employed in these studies range from energy poor diets, such as caloric restriction and low-

protein diet (Watkins and Sinclair, 2014, Govic et al., 2016), to diets of nutrient excess such as high-fat or ‘‘Western’’ diets (Ng et al., 2014b, Crisóstomo et al., 2021).

1.4.3 Mechanisms behind paternal programming

While the transference of paternal life experiences to offspring outcomes have been demonstrated in various epidemiological studies, the molecular mechanisms underlying these intergenerational transmissions remain elusive. Epigenetic changes in sperm are widely considered to be a transmitter of paternal experience across generations through DNA methylation, post-translational histone modifications, and changes to long and small non-coding RNAs (Donkin and Barrès, 2018, Carone et al., 2010, Ng et al., 2014). During embryonic development, DNA methylation is involved in an array of functions, including the regulation of gene expression, transposon silencing, X chromosome inactivation and genomic imprinting (Lister et al., 2009, Jaenisch, 1997). Moreover, lifestyle exposures such as exercise, diet and alcohol consumption have been shown to change DNA methylation profiles in individuals (Denham et al., 2015, Radford et al., 2014, Ouko et al., 2009). DNA in sperm is packaged distinctly to somatic cells. Here, histones are replaced by protamines bound to DNA which allows a denser packaging structure that serves a protection of extracellular stressors. This replacement process removes most of the information carried by the previous histones, however, it may suggest that those that remain may be essential in early developmental stages (Donkin and Barrès, 2018). Small RNAs (sRNAs) have been suggested to epigenetically alter gene expression either by interaction with the translation machinery and/or by inducing degradation of their complementary mRNA targets. A type of sRNA, called transfer RNA (tRNA), has been found in sperm and implicated in the alteration of gene expression and metabolic outcomes in offspring (Chen et al., 2016, Sharma et al., 2016).

1.5 Dietary effects on behaviour

Environmental factors, including nutrition, have been shown to have a significant impact in the behaviour of an individual (Pyndt Jørgensen et al., 2014, Bellisle, 2004). Aside from immediate diet, studies showing that the peri-conceptional and early life nutritional environment can impact behaviour are increasing (Rabasa et al., 2016, Peleg-Raibstein et al., 2012, Braun and Champagne, 2014). Both maternal and paternal obesogenic diets (such as high

fat, or Western) are often considered to increase the risk of developing behavioural conditions such as stress and anxiety. The mechanisms involved in this are varied and include the reduction of dopamine transmission (Vucetic et al., 2012, Naef et al., 2011), dysregulation of the serotogenic system (Sullivan et al., 2010), and altered glucocorticoid production (Sasaki et al., 2014). Inflammatory pathways have also been implicated to have a potential link in the development of these conditions through developmental programming (Bolton and Bilbo, 2014).

1.6 Scope of thesis

It is probable that the programming of obesity occurs through modification of several pathways during the stage of development. The targeted programmed pathways are likely to include those involving the regulation of appetite and energy expenditure, through altered tissue metabolism and propensity for physical activity (Lemes et al., 2018, Chang et al., 2008, Rajia et al., 2010, Gali Ramamoorthy et al., 2015). An unanswered question is whether developmental programming might target not only the capacity for total energy intake, but also the regulation of specific targets for macronutrients such as protein. Although some studies have manipulated macronutrient balances in their design, it can often be confounded by the diets' energy density. Many studies still place a greater focus on the effects of either parental undernutrition or overnutrition and there are still gaps in what we know about the effects of parental dietary macronutrient balance in modulating offspring health. Another key unanswered question is *if* or *how* any early phenotypic variations influenced by paternal nutrition that are established in early life can be sustained in later life. This thesis aims to explore these questions. To isolate maternal and paternal impacts successfully, and avoid any confounding effects from the opposite parent, we have chosen to perform our diet manipulations on each parent separately. We seek to address parent-specific aims using distinct macronutrient compositions detailed further below.

1.6.1 Maternal line aims

Through the maternal line, we explore whether protein leverage can be programmed by maternal protein intake and whether susceptibility to obesity of individuals from high protein diet fed mothers occurs via the mismatch of pre- and post-natal nutritional environments.

Specific aims are:

1. To test if offspring nutrient selection is altered by maternal diet through developmental programming
2. To investigate if maternal protein:carbohydrate (P:C) intake influences offspring susceptibility to obesity in early life
3. To examine how strongly maternal impacts on offspring metabolism and behaviour can persist long-term

1.6.2 Paternal line aims

As explained above, many paternal effects studies have looked at diet-induced obesity using high fat or Western diets. Some confounders in these models include the total energy content of these obesogenic diets (which are often more energy dense than control diets), and that they are also often low in protein, thus exacerbating established protein leverage effects. Examining the effects of specific macronutrients in parental diet is an avenue that can be explored further. In recent years, several studies have comprehensively demonstrated the effects of low protein diets on offspring health and development (Morgan et al., 2021, Watkins and Sinclair, 2014). Since common obesogenic diets often contain high levels of fat and carbohydrate, we wanted to focus on the specific consequences that an increase of fat, or carbohydrates (and varying the source of carbohydrates from starch to sucrose) in the parental diet may have on offspring outcomes. Therefore, we address these questions through the following aims:

1. To isolate the effects of dietary sugar and fat on male fertility and metabolism
2. To test whether offspring nutrient selection is altered by paternal diet
3. To investigate if paternal sugar/fat intake influences offspring susceptibility to obesity and other cardiometabolic disorders
4. To examine if paternal sugar/fat intake influences offspring anxiety-related behaviour

CHAPTER 2: Maternal macronutrient intake effects on offspring nutrient targets, metabolism, and behaviour

2.1 Introduction

Many animals ranging from insects to mammals, have been shown to possess nutrient-specific appetite systems (Shariatmadari and Forbes, 1993, Münch et al., 2020). In various species, protein intake is more highly prioritized than non-protein energy intake (carbohydrates and fats) when animals are confined to imbalanced diets of fixed macronutrient composition, a phenomenon called *protein leverage* (Simpson and Raubenheimer, 2005, Sørensen et al., 2008, Gosby et al., 2014, Gosby et al., 2011). Protein leverage is pronounced in some primates (Felton et al., 2009, Uwimbabazi et al., 2021) and has been proposed to have played a key role in the human obesity epidemic by driving excess energy intake in modern food environments in which protein has been diluted by highly processed fats and carbohydrates (Simpson and Raubenheimer, 2005, Raubenheimer and Simpson, 2019, Hill and Morrison, 2019, Hall, 2019).

The hormone, fibroblast growth factor 21 (FGF21), is an important signal in the regulation of protein intake and macronutrient selection (Wu et al., 2022, Hill et al., 2020, Flippo and Potthoff, 2021). In mice, Fgf21 is elevated in circulation under low protein feeding and acts via the brain to stimulate selection of higher protein intake under choice feeding conditions, and increased food intake when animals are confined to low protein diets (Solon-Biet et al., 2016, Hill et al., 2019, Laeger et al., 2014). The leveraging of excess energy intake on a low-percent protein diet will be exacerbated when protein requirements ("protein target") increase (Simpson and Raubenheimer, 2005). Reasons for increased dietary protein requirements could include higher demands during lean growth and reproduction; decreased protein efficiency with menopause, senescence, or insulin resistance; physiological adaptation to a higher protein diet or anabolic regime, and genetic adaptation to ancestral diets that are high in protein (Simpson and Raubenheimer, 2005, Raubenheimer et al., 2022).

Additionally, it has been suggested that the protein target could be influenced during early development and even prenatally via the uterine environment or preconception (Raubenheimer et al., 2022). The elevated risk of later obesity associated with formula vs. breast-fed infants, for example, has been linked to the protein content of infant formula being higher than that of breast milk (Huang et al., 2018, Raubenheimer et al., 2015, Totzauer et al., 2018). That the protein target of offspring might be influenced by maternal diet *in utero* is not implausible, given that other features of appetite control and energy metabolism are known to be affected by maternal undernutrition or overnutrition (Lemes et al., 2018, Chang et al., 2008, Rajia et al., 2010, Gali Ramamoorthy et al., 2015). However, the effects of the mother's macronutrient balance in modulating offspring behaviour and health remain unclear. Findings from animal models are often conflicting and studies have demonstrated that both restricted and excess maternal protein intakes during pregnancy can delay intrauterine growth, lower birthweight and influence offspring susceptibility to obesity (Blumfield, 2015).

In the present study, we hypothesized that offspring from mothers fed a high-protein diet during pregnancy and lactation would select a higher protein intake than offspring from mothers on a low-protein diet and, consequently, be more susceptible to hyperphagia, obesity and metabolic dysregulation on a fixed diet low in PE and high in nPE. We tested these predictions in mice and showed that a high protein maternal diet increased self-selected protein targets in offspring from an early age. Higher protein targets in young mice were associated with increased food intake and increased susceptibility to obesity. Our results provide new understanding of the ways in which maternal nutrition shapes offspring behaviour and health and illustrate a possible mechanism for transgenerational accumulation of risks of obesity and metabolic disease in human populations.

2.2 Methods

2.2.1 Ethical approval

Animal protocols were approved by Sydney University's Animal Ethics Committee (Protocol number 2018/1384) to ensure they were in line with the NSW Animal Research Act 1985 and the Australian code for the care and use of animals for scientific purposes 8th edition (2013).

2.2.2 Animals and husbandry

2.2.2.1 General husbandry and cage allocations

Mice (C57BL6/JArc) were sourced from Animal Resources Centre (Perth, WA) and housed at the Charles Perkins Centre, University of Sydney at an ambient temperature of 22°C and with constant access to water. Thirty dams, housed 3-4 per cage, were started on diets at 12 weeks of age and remained on diets for 4 weeks. Outside of the mating period, dam body weights and food intake were measured once a week. Thirty studs arrived at 4 weeks of age and were housed individually outside of the mating period to prevent fighting. Body weights were measured weekly.

2.2.2.2 Breeding protocol

At 16 weeks of age, dams were moved to breeding cages where they were paired with one stud and mated for 1 week. To confirm successful insemination, the stage of oestrous cycle and presence of vaginal plugs were recorded. Once a plug had been observed, the mating pairs were left undisturbed for the remainder of the week-long mating period. During gestation, dams were housed individually where they were fed 1 of 2 diets (detailed below). Body weights were measured twice weekly to detect pregnancies. . To prevent infant cannibalism, litter body weights were not measured until three days post birth. Litters were continually monitored until they were weaned at 3 weeks of age, where the sex of the offspring was recorded.

Since offspring remained housed with their mothers from birth until weaning, they were exposed to either LP or HP diet for 3 weeks, initially via the mother's milk and then through

direct consumption as they transitioned to eating solid food. Upon weaning, male and female offspring from both groups were subjected to a food choice experiment where they were given simultaneous access to LP and HP diets.

2.2.2.3 Offspring choice experiment (3-8 weeks, 40-42 weeks)

Upon weaning at 3 weeks of age, 64 male and 64 female offspring were randomly chosen from the pool of dams (ca. 2 males and 2 females per dam), yielding n=32/sex/maternal diet group. From 3-8 weeks of age, the pups were housed individually. Body weights were measured weekly. To test whether offspring protein targets were impacted in utero and in early life, a food choice experiment was performed. The pups were allowed to self-select between two foods, one low- and the other high-protein, as outlined below. A second round of choice testing was conducted at 40 weeks of age to examine whether protein targets that may have been programmed in early life are retained in later life. The same diets were used for the late life choice experiment.

2.2.2.4 Offspring metabolic testing (8-46 weeks)

Upon completion of the choice experiment at 8 weeks of age, offspring were housed with another mouse of the same sex and diet treatment for the remainder of the study, during which they were restricted to either a Standard or "Western" diet (SD or WD; detailed below). Food intake and body weights were measured once a week for each cage and the average grams of food eaten per day per animal was calculated. Additional metabolic phenotyping, including body composition using EchoMRI 900 (EchoMRI, TX, USA), oral glucose tolerance tests and fasting insulin levels, were measured at 16 and 46 weeks of age (n=13-16/sex/diet).

2.2.3 Experimental Diets

Experimental diets were manufactured in dry, pelleted form by Specialty Feeds Australia. All were based on modification of the standard rodent AIN-93G formulation. A more detailed breakdown of diet compositions can be found in Table 2.1.

2.2.3.1 Dam and offspring choice diets

Dams were fed one of two foods with different protein: carbohydrate - one with a lower P:C ratio (LP; 10% Protein) and another with a higher P:C ratio (HP, 35% Protein) - from 4 weeks prior to mating (females were mated at approximately 15–16-weeks-old) and remained on these diets throughout gestation and lactation.

- **P10 (Low Protein)**

  Containing 10% protein, 70% carbohydrate and 20% fat, yielding net metabolisable energy content of 14.7 kJ/g.

- **P35 (High Protein)**

  With 35% protein, 45% carbohydrate and 20% fat and a net metabolisable energy content of 14.7 kJ/g kJ/g.

2.2.3.2 Adult offspring no-choice diets

- **Standard diet (AIN-93G)**

  19% protein, 63% carbohydrate and 18% fat, with a net metabolisable energy content of 14.4 kJ/g.

- **"Western" diet (Low protein, high fat, high sucrose)**

  10% protein, 50% carbohydrate, and 40% fat, with a net metabolisable energy content of 17.3 kJ/g.

Table 2.1 Breakdown of compositions for diets used in study.

	Maternal and Offspring Choice		***Offspring No-Choice***	
Diet	**Low Protein (P10)**	**High Protein (P35)**	**Standard Diet**	**Western Diet**
NME (kJ/g)	14.7	14.7	14.4	17.3
% Protein energy	10	35	19	10
% Carbohydrate energy	70	45	63	50
% Fat energy	20	20	18	40
Other (g/kg)				
Casein	106	383	200	126
Soy oil	80.3	80.3	70	29
Wheat Starch	458	286	404	254
Sucrose	113.1	71	100	231
Dextrinised Starch	150	94	132	100
Cellulose	49.2	43	50	56

2.2.4 Body composition

Body weights were measured weekly for all mice, unless otherwise specified. Body composition (fat mass and lean mass) was assessed using an EchoMRI 900 (Houston, TX, USA) at 16 weeks and 46 weeks of age in offspring (n=13-16/sex/group). Organ weights were measured at cull including liver, gonadal and subcutaneous white adipose tissue, brown adipose tissue, and quadriceps and are further detailed below.

2.2.5 Tissue collection

At 12 months of age for dams and 46 weeks of age for male and female offspring, mice were anaesthetised using a 1:1 mix of ketamine and xylazine, administered through an intraperitoneal injection. Animals were humanely euthanised and tissues were harvested for analysis. Tissues were snap frozen in liquid nitrogen and stored at -80°C. Blood was collected via cardiac puncture and stored on ice. Organ weights were measured at cull including liver (entire organ) and quadriceps muscle. Multiple fat depots were identified (Bagchi and MacDougald, 2019) and collected, including gonadal white adipose tissue (fat pad surrounding testes or ovaries), subcutaneous white adipose tissue (posterior section made up of dorsolumbar, inguinal and gluteal depots), and brown adipose tissue (interscapular). Serum was collected and stored at -80°C until further analysis.

2.2.6 Glucose metabolism

Glucose tolerance tests were performed on all offspring at 16 and 46 weeks of age. Mice were fasted 4 h prior to testing. Basal blood samples were obtained by tail tipping and blood glucose measured using a clinical glucometer (Accu-Chek Performa, Roche Diagnostics Australia Pty Ltd). Glucose (2g kg-1 lean mass) was then administered via oral gavage. Blood was collected at baseline, 15, 30, 45, 60 and 90 min from the original tail wound and serial tail tipping was not required. The incremental area under the curve (iAUC) was calculated. The iAUC indicates the time taken to clear a bolus dose of glucose from the bloodstream (n= 13-16/sex/diet).

2.2.7 Metabolic hormones and serum biochemistry

To determine the long-term metabolic effects of both maternal and adult diets and their

interaction, levels of fibroblast growth factor 21 (FGF21), markers of liver function, ALT and AST, blood cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels were measured in the serum of male and female offspring at 46 weeks. Blood was collected from tail-tipping at a basal timepoint after 4 h of fasting from 16- and 46-week-old mice for the analysis of insulin levels using an enzyme-linked immunosorbent assay (ELISA) following manufacturer's instructions (Crystal Chem IL) (n=13-16/sex/diet). At 46 weeks, FGF21 was measured in serum collected at 46 weeks using the mouse/rat FGF21 ELISA kit as per the manufacturer's protocols (n=7-8, Crystal Chem IL). Blood cholesterol, triglycerides, liver function tests (alanine transaminase, ALT and aspartate amino transferase, AST) were performed on serum collected at 46-week-old mice (n=13-16/sex/diet).

2.2.8 Metabolic cage phenotyping

To determine whole-animal metabolic rate, substrate utilisation and activity, 4-6 male and female mice per treatment group were housed individually and assessed by indirect calorimetry in a Promethion high-definition behavioural and continuous respirometry system for mice (Sable Systems International, NV, USA) at 45 weeks of age. Oxygen consumption (VO2) and carbon dioxide production (VCO2) were measured over 48 h, following an 8-h acclimation period, with mice maintained at 22°C under a 12:12 hour light: dark cycle. Energy expenditure is presented as kcal/h/g lean mass. Mice were not given access to a running wheel.

2.2.9 Behavioural testing

2.2.9.1 Elevated plus maze

Behaviour of offspring was assessed at 30 weeks in an elevated plus maze, consisting of 2 closed arms (30x5 cm), 2 open arms (30x5 cm), and a central zone (5x5 cm) elevated 50 cm above the ground. Light intensity in the open arms of the maze was 700-750 lx, and in the closed arms was 175-225 lx. For consistency, each mouse was placed into the centre of the maze facing the left open arm. Mice were exposed to the elevated plus maze for a total of 5 min and the apparatus was cleaned thoroughly between animals using ethanol. The following

variables were derived: time spent in the open and closed arms of the maze; entries into the arms, and the total distance travelled. An entry was defined as crossing the dividing line between an arm and the centre platform with all four feet.

2.2.10 Statistical analysis

Data are presented as means ± SEM and statistical significance determined when $P < 0.05$. Relevant t-tests were conducted using GraphPad Prism (v9.0.2; GraphPad Software Inc., La Jolla, CA, USA). Post hoc analysis was performed using Tukey's multiple comparisons test unless otherwise stated in the relevant figure legends. Offspring data was analysed using mixed effect models in R (RStudio version 1.4.1717; Vienna, Austria) with lme4 (Bates et al., 2015) and LmerTest (Kuznetsova et al., 2017) packages. Dam diet, Pup diet, Sex, and their interactions were included as fixed effects. Litter size at weaning and body weight were initially included as covariates in all models and then removed if shown to not have a significant effect. Offspring data were analysed with both sexes included in the model to measure main effect of sex but were separated into males and females in figures to allow better visualization of trends in each sex. All other statistical parameters are included in the figure legends.

2.3 Results

2.3.1 Maternal diet influenced the protein intake target of offspring

Dams were given one of two diets - one with a lower P:C ratio (LP; 10% Protein) and another with a higher P:C ratio (HP, 35% Protein) - from 4 weeks prior to mating, and remained on these diets throughout gestation and lactation (Figure 2.1A). Body weights for both diet groups were similar throughout the pre-mating and early gestation periods (Figure 2.1B). HP dams were heavier than the LP group from the end of gestation ($p = 0.0185$) to the first 2 weeks of lactation ($p = 0.0008$ and $p = 0.0439$, respectively), but body weights of the two groups converged by the end of lactation. Litter weights were measured at 3 days, and again at 21 days after birth where an average weight per pup was calculated based on number of pups in the litter. Average pup weights/litter were not significantly different between LP and HP diets at 3 days of age (Figure 2.1C). At 21 days of age, HP pups weighed more than LP groups (Figure 2.1D; $p = 0.0298$). There were no significant differences found in the average number of pups, and ratios of male to female offspring in the litters of both maternal diet groups (Figure 2.1E).

Upon weaning, male and female offspring from both groups were subjected to a food choice experiment where they were given simultaneous access to LP and HP diets (Figure 2.1F). During the macronutrient selection experiment, pups from HP mothers consistently ingested a higher intake of protein than did offspring from LP dams (85 kJ vs 74kJ across the 5-week period) for both females (Figure 2.1G; $p= 0.0469$) and males (Figure 2.1H; $p=0.0313$), whereas their intake of carbohydrate did not differ. These differences in protein selection were mirrored in body weight and composition for both female and male offspring, measured at 8 weeks of age upon completion of the choice experiment. Here, we detected increased body weight (Figure 2.1F; Table 2.2), lean mass (Figure 2.1G; Table 2.2) and fat mass (Figure 2.1H; Table 2.2) in HP compared to LP pups.

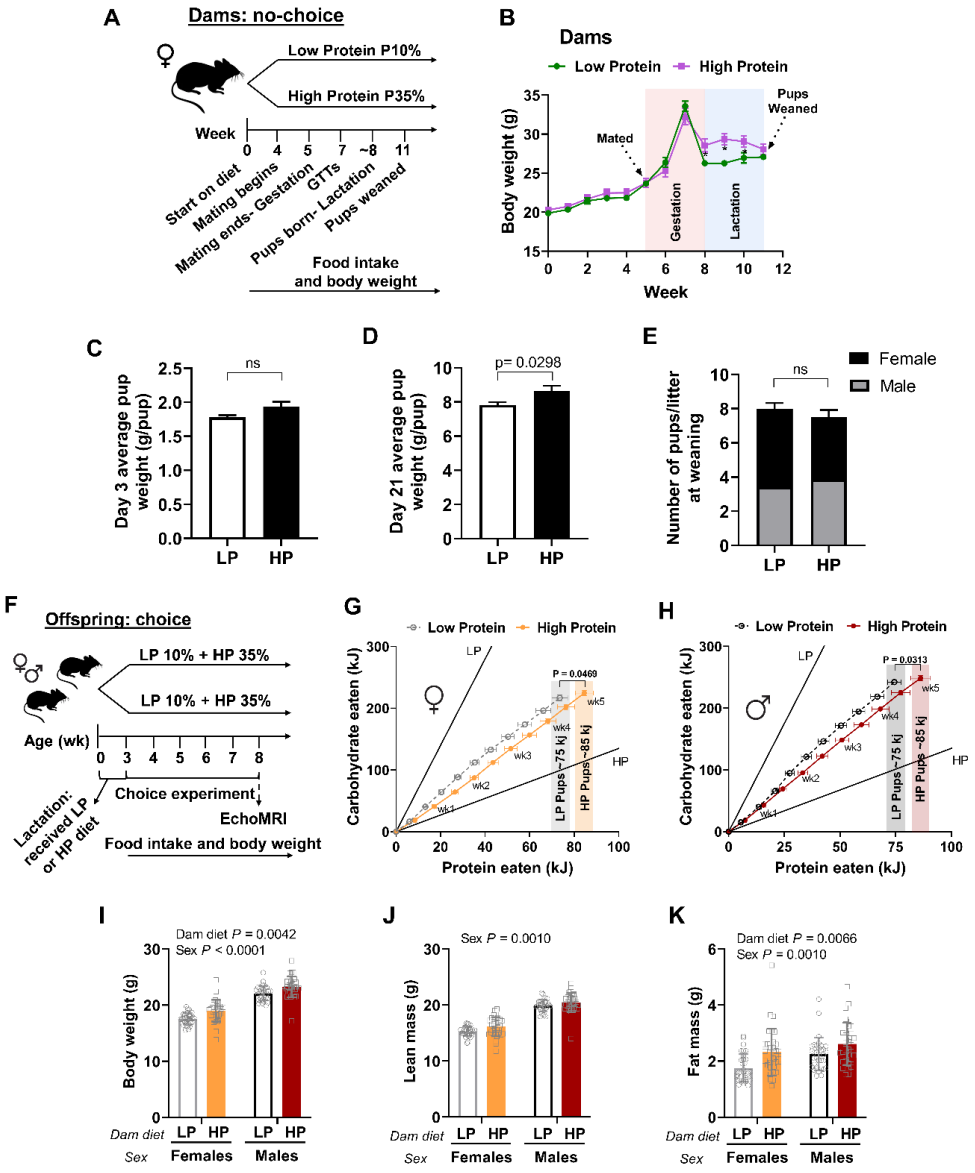

Figure 2.1 Maternal protein intake influences protein targets in offspring.

(A) Timeline of dam diet regimes and treatments (B) Dam body weights during pre-mating, gestation, and lactation periods (n = 15/diet). Average pup weights in litter for Day 3 (C) and Day 21 (D). (E) Number of pups per litter at Day 21 separated indicating both males and females. (F) Timeline of offspring choice experiment and treatments from birth to 8 weeks of age. Food intake during offspring choice experiment broken down into protein and carbohydrate components for (G) female and (H) male pups (n = 32/sex/maternal diet) (I) body weight (J) lean mass and (K) fat mass measurements of offspring at 8 weeks of age (n = 32/sex/maternal diet). For figures G and H, solid black lines indicate intake trajectories of an

animal within a nutrient space if they were to eat LP or HP diets exclusively. For comparisons between LP and HP groups in Figures B, C, D, G, and H, an unpaired t-test (two tailed, with Welch's correction) was used to determine significance between treatment groups at the indicated time points. For all other bar graphs, an ANOVA was used. All bars indicate means ± SEM. Significant p-values for main effects are provided on the relevant figures.

Table 2.2 Model outputs of 8-week timepoint results for the main effects of dam diet, sex, and dam diet:sex interaction on body weight and composition, accounting for litter size. Dam was included as a random variable in all models. Relevant post-hoc tests are indicated on each figure. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	Factor	Df	Sum Sq	F value	Pr(>F)	Sig
2.1I	**Body weight**	Dam diet	1	18.73	9.9012	0.004198	**
		Sex	1	600.59	317.5499	<2.2E-16	***
		Litter	1	0.94	0.4959	0.487264	NS
		Dam diet: Sex	1	0.38	0.2009	0.654955	NS
2.1J	**Fat mass**	Dam diet	1	3.7482	8.7068	0.0065864	**
		Sex	1	4.9411	11.4778	0.0009946	***
		Litter	1	0.0572	0.1330	0.7178765	NS
		Dam diet: Sex	1	0.3476	0.8074	0.3709677	NS
2.1K	**Lean mass**	Dam diet	1	2.72	2.1120	0.1581	NS
		Sex	1	632.81	491.3801	<2E-16	***
		Litter	1	0.00	0.0003	0.9864	NS
		Dam diet: Sex	1	0.30	0.2295	0.6329	NS

2.3.2 Higher protein targets in young mice were associated with increased food intake and susceptibility to obesity on a fixed adult diet

The next question was whether the protein target defined in early life had consequences for food intake, body composition and metabolic outcomes when pups were restricted to a fixed diet in adulthood. After completing the macronutrient selection experiment, offspring from both HP and LP dams were provided with either a Standard (SD) or Western diet (WD) and various outcomes were measured over time (Figure 2.2A). Based on analysis of cumulative intake at 40 weeks, both male and female mice confined to WD had elevated food intake, irrespective of maternal diet (Figure 2.2B,C). Mice on SD, however, showed divergence in food intake, with pups born from mothers on HP consuming more food than offspring of dams on LP ($p = 0.0019$ for females; $p = 0.0493$ for males).

We found that at 16 weeks of age, both maternal and adult diets impacted various body composition measures for both sexes. Pups from HP dams had greater body weight, fat mass and lean mass indicated by a significant main dam effect (Figure 2.2F-K; Table 2.3) This effect was exacerbated by offspring feeding on WD as an adult. This trend, paired with an overall greater body weight of male vs female offspring, led to a pup diet versus sex interaction. Although WD-fed mice had higher body weights compared to SD mice, the effects of maternal diet were more prominent in the latter (Figure 2.2D-G; Table 2.3). A significant three-way Dam diet: Pup diet: Sex interaction was detected in the iAUC values (a measure of glucose tolerance) of offspring (Figure 2.2L-O; Table 2.3). Here, we observed that a combination of a maternal HP diet and a Western adult diet led to the greatest iAUC values, particularly evident in male pups (Figure 2.2O; Table 2.3). For basal glucose and fasting insulin levels at 16 weeks of age, a significant effect of maternal diet was not detected. However, we observed an interactive pup diet:sex effect in both outcomes at 16 weeks (Figure 2.3; Table 2.3).

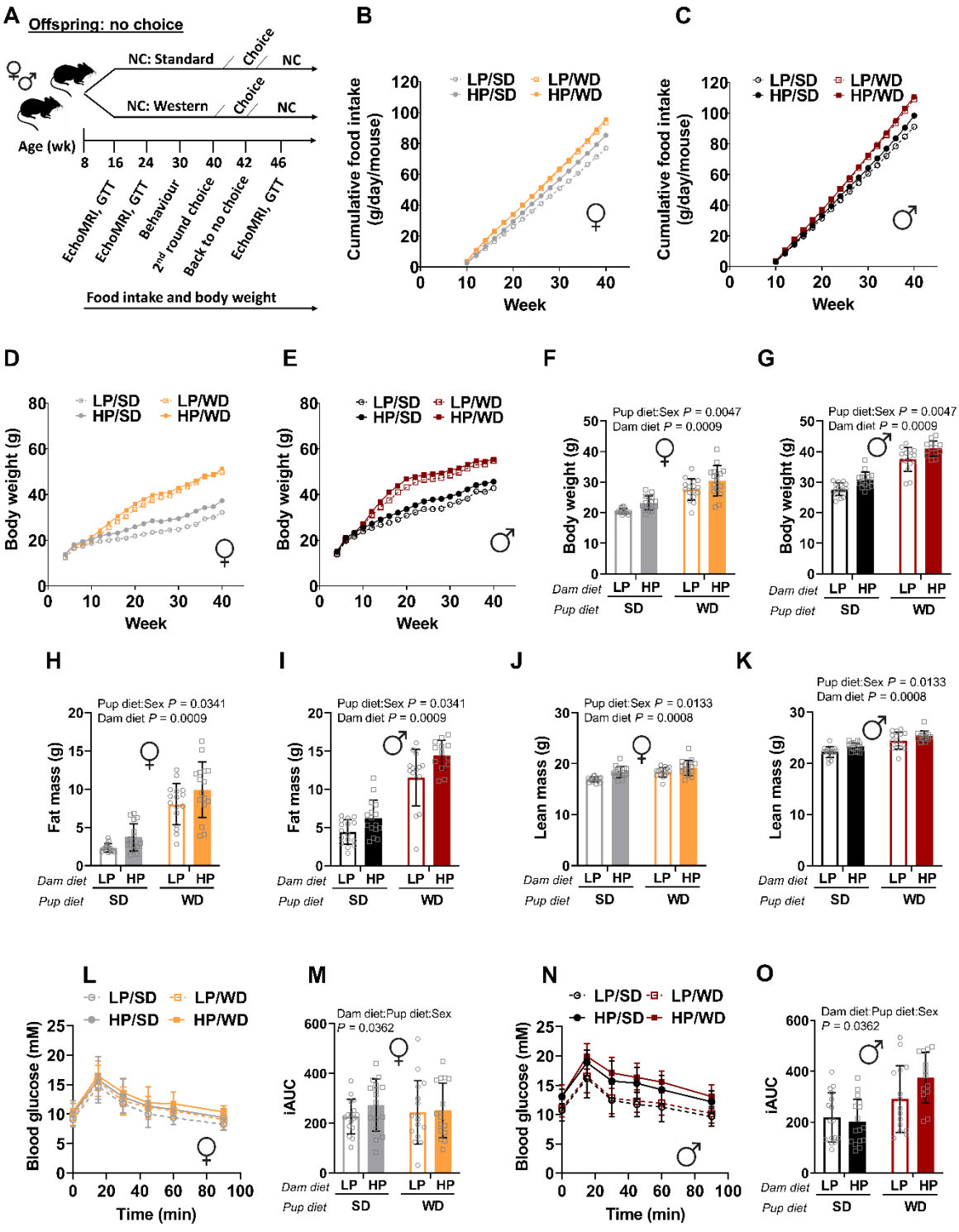

Figure 2.2 Higher protein targets in young mice increase food intake and susceptibility to obesity.

(A)Female and male C57BL/6Arc mice were fed a standard or western diet from 8 weeks of

age and subjected to metabolic phenotyping. No-choice food intake was measured once weekly from 10-40 weeks of age for (B) females and (C) males (n=8 cages/sex/treatment group). Body weights from 3-40 weeks for (D) female and (E) male offspring. Body weight at 16 weeks of age for (F) female and (G) male offspring. Fat mass at 16 weeks of age for (H) female and (I) male offspring. Lean mass at 16 weeks of age for (J) female and (K) male offspring. Blood glucose levels and iAUC levels from an oGTT for (L-M) female and (N-O) male offspring (n = 15-18 animals/sex/group). All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

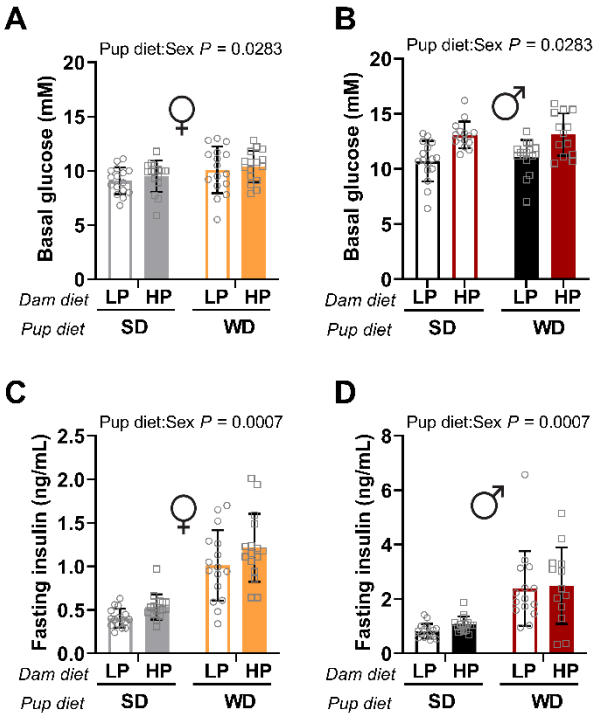

Figure 2.3 Basal glucose and fasting insulin levels were not affected by maternal diet at 16 weeks

16-week-old measurements of basal glucose for (A) female and (B) male offspring and fasting insulin for (C) female and (D) male offspring recorded after 4 hrs of fasting.

Table 2.3 Model outputs of 16-week timepoint results for the main effects of dam diet, pup diet, sex, and their interactions on metabolic traits. Dam was included as a random variable in all models. Relevant post-hoc tests are indicated on each figure. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	Factor	Df	Sum Sq	F value	Pr(>F)	Sig
2.2F,G	**Body weight**	Dam diet	1	109.98	14.6626	0.0009009	***
		Pup diet	1	1328.24	177.0881	<2.2E-16	***
		Sex	1	2322.48	309.6459	<2.2E-16	***
		Dam diet: Pup diet	1	0.00	0.0001	0.9932585	NS
		Dam diet: Sex	1	0.53	0.0703	0.7915062	NS
		Pup diet: Sex	1	62.76	8.3680	0.0047409	**
		Dam diet: Pup diet: Sex	1	0.01	0.0007	0.9787262	NS
2.2H,I	**Fat mass**	Dam diet	1	52.69	10.3125	0.003883	**
		Pup diet	1	854.82	167.3122	<2.2E-16	***
		Sex	1	307.31	60.1480	9.975E-12	***
		Dam diet: Pup diet	1	0.64	0.1245	0.725915	NS
		Dam diet: Sex	1	2.52	0.4932	0.484239	NS
		Pup diet: Sex	1	23.62	4.6238	0.034059	*
		Dam	1	0.13	0.0261	0.872090	NS

		diet: Pup diet: Sex					
2.2J,K	**Lean mass**	Dam diet	1	16.14	14.7332	0.0008151	***
		Pup diet	1	55.69	50.8262	7.812E-09	***
		Sex	1	941.46	859.3128	<2.2E-16	***
		Dam diet: Pup diet	1	0.62	0.5696	0.4544654	NS
		Dam diet: Sex	1	0.35	0.3166	0.5749548	NS
		Pup diet: Sex	1	6.96	6.3527	0.0133622	*
		Dam diet: Pup diet: Sex	1	0.36	0.3259	0.5694286	NS
2.2M, O	**iAUC**	Dam diet	1	11206	1.2505	0.2751040	NS
		Pup diet	1	51149	5.7080	0.0210733	*
		Sex	1	15026	1.6768	0.1985102	NS
		Dam diet: Pup diet	1	3598	0.4015	0.5294750	NS
		Dam diet: Sex	1	970	0.1083	0.7428497	NS
		Pup diet: Sex	1	115258	12.8621	0.0005321	***
		Dam diet: Pup diet: Sex	1	40441	4.5129	0.0362367	*
2.3A,B	**Basal glucose**	Dam diet	1	2.104	0.8602	0.36278	NS
		Pup diet	1	64.407	26.3371	7.372E-06	***
		Sex	1	150.881	61.6981	4.891E-12	***

		Dam diet: Pup diet	1	0.420	0.1715	0.68091	NS
		Dam diet: Sex	1	0.354	0.1449	0.70427	NS
		Pup diet: Sex	1	12.114	4.9537	0.02831	*
		Dam diet: Pup diet: Sex	1	0.158	0.0648	0.79967	NS
2.3C,D	**Fasting insulin**	Dam diet	1	0.5004	1.1404	0.2964475	NS
		Pup diet	1	23.6273	53.8437	4.768E-09	***
		Sex	1	24.8772	56.6919	2.696E-11	***
		Dam diet: Pup diet	1	0.0236	0.0539	0.8176203	NS
		Dam diet: Sex	1	0.0001	0.0002	0.9886042	NS
		Pup diet: Sex	1	5.3713	12.2405	0.0007081	***
		Dam diet: Pup diet: Sex	1	0.1678	0.3824	0.5377801	NS

2.3.3 Higher protein targets were retained in later life, but current diet had a stronger effect on long-term body composition and metabolic outcomes

To test if the difference in protein target determined by maternal diet in early life persisted in later life, another choice experiment was performed at 40 weeks of age. There was a main effect of maternal diet for both females (Figure 2.4A) and males (Figure 2.4B), with WD-fed offspring from HP dams (HW) having increased protein intakes compared to all other groups. At 46 weeks of age, dam diet effects persisted in body composition results of offspring (Table 2.4). Mice from HP dams had greater body weight (Figure 2.4C,D), lean mass (Figure 2.4E,F), and fat mass (Figure 2.4G,H) in comparison to offspring from LP dams, although the main effect of maternal diet in offspring fat mass was only found to be significant after considering body weight differences (Table 2.5). WD-fed female and male pups had increased body weight, lean mass and fat mass compared to SD-fed offspring regardless of maternal diet (Figure 2.4C-H; Table 2.4). At this later time point, maternal diets, pup diets and sex did not have a significant effect on overall glucose tolerance of the offspring (Figure 2.4I-L; Table 2.4).

Additionally, we found a significant three-way interactive effect on pup liver mass (Table 2.4). In female pups, WD-fed mice had increased liver mass compared to SD fed groups, and within those groups, pups from HP dams had increased liver mass compared to LP counterparts (Figure 2.5A; Table 2.4). In male offspring, high protein pups fed SD in adulthood displayed greater liver masses but instead had decreased liver mass when fed WD in adulthood (Figure 2.5B; Table 2.4). A similar trend was observed in brown adipose tissue (BAT; Figure 2.5G,H), but only main effects of pup diet and sex were found (Table 2.4). We also observed a significant pup diet:sex interaction (Table 2.4) for gonadal white adipose tissue (GWAT; Figure 2.5C,D) and subcutaneous WAT (SWAT; Figure 2.5E,F) masses. WD fed mice had greater WAT masses compared to SD fed mice overall, but this effect from pup diet groups was more distinct in females (Figure 2.5C-F).

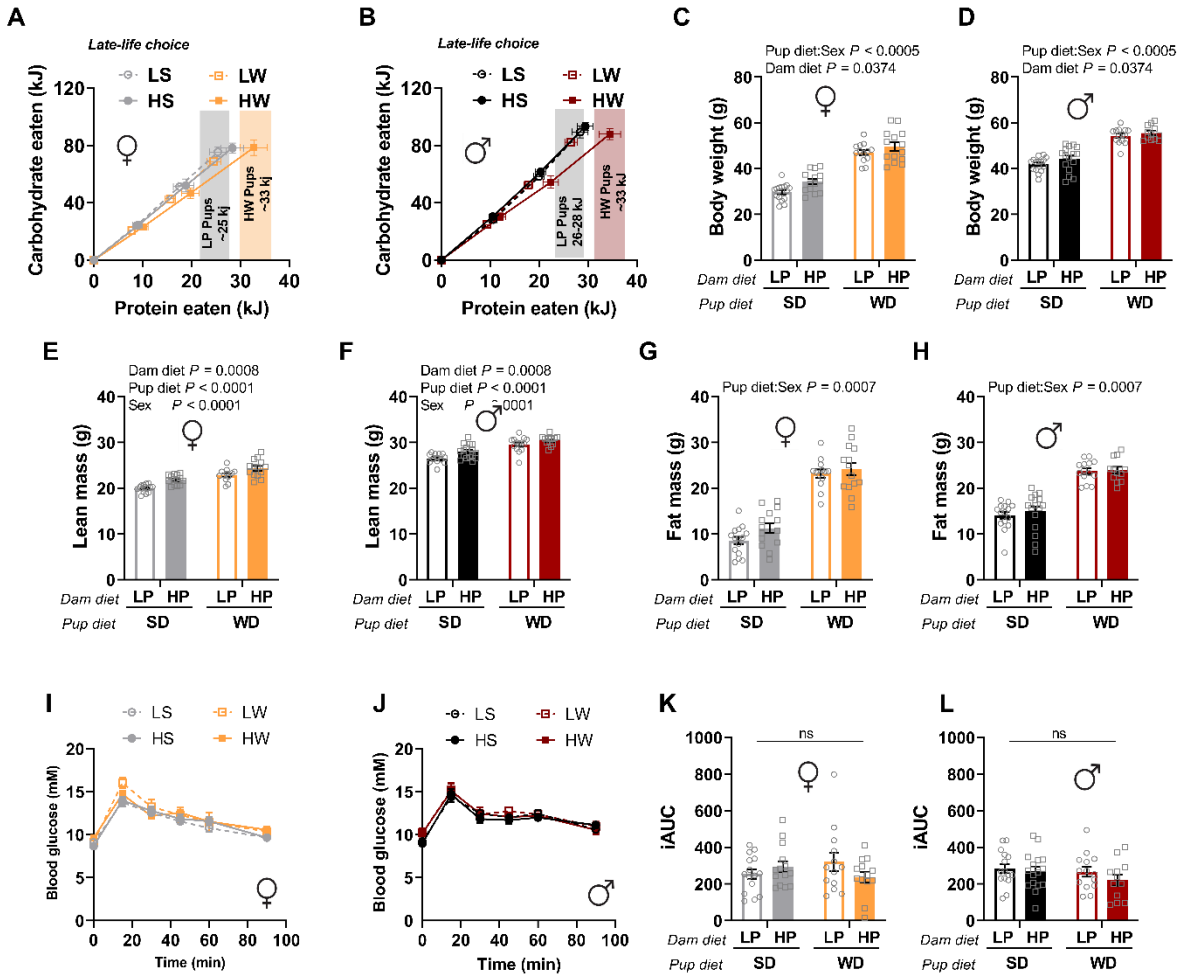

Figure 2.4 High protein targets are retained in later life but adult diets have stronger effects on long-term body composition and metabolic outcomes at 12 months.

Choice experiment food intake from 40-42 weeks of age for (A) female and (B) male offspring. Body weight at 46 weeks for (C) female and (D) male offspring. Lean mass at 46 weeks for (E) female and (F) male offspring. Fat mass at 46 weeks for (G) female and (H) male offspring. Blood glucose levels and iAUC levels from an oGTT for (I-J) female and (K-L) male offspring (n = 15-18 animals/sex/group). All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

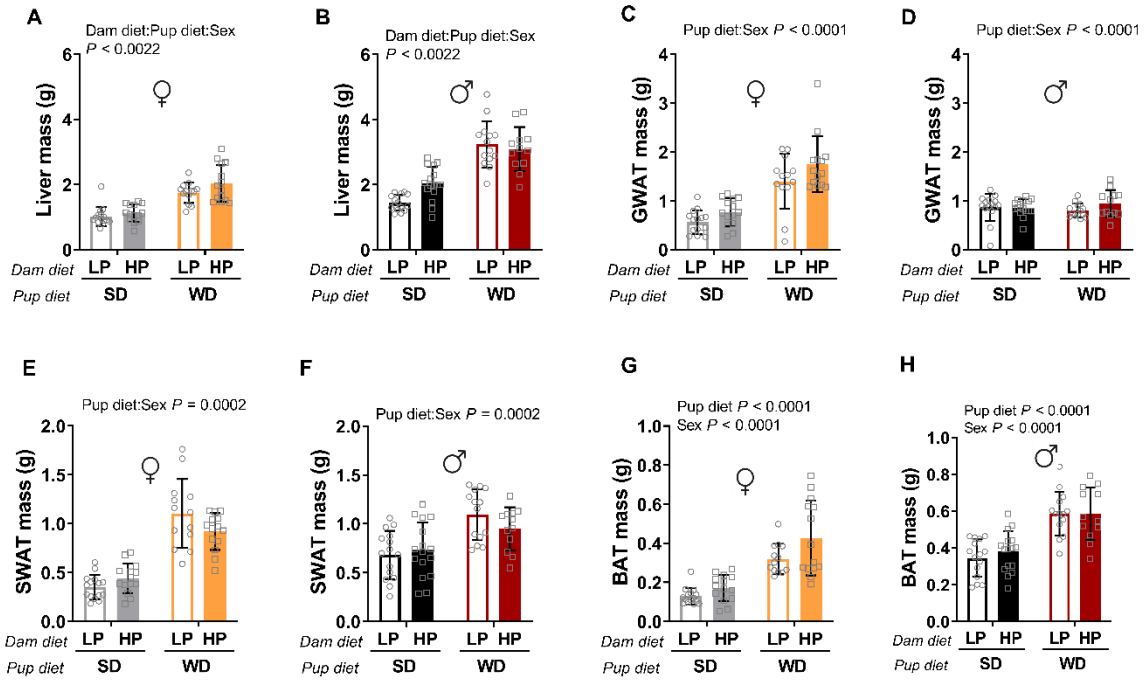

Figure 2.5 Tissue weights of female and male offspring collected at 46 weeks old.

Liver mass for (A) males and (B) females, Gonadal white adipose tissue (GWAT) mass for (C) males and (D) females, Subcutaneous white adipose tissue (SWAT) mass for (E) males and (F) females, Brown adipose tissue (BAT) for (G) males and (H) females. n = 13-16 animals/sex/group. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

Table 2.4 Model outputs of 46-week timepoint results for the main effects of dam diet, pup diet, sex, and their interactions on metabolic traits. Dam was included as a random variable in all analyses. Relevant post-hoc tests are indicated on each figure. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	Factor	Df	Sum Sq	F value	Pr(>F)	Sig. level
2.4C,D	**Body weight**	Dam diet	1	80.02	4.8992	0.037405	*
		Pup diet	1	3103.63	190.0199	<2.2E-16	***
		Sex	1	2009.84	123.0528	<2.2E-16	***
		Dam diet: Pup diet	1	8.08	0.4946	0.485845	NS
		Dam diet: Sex	1	34.77	2.1285	0.148200	NS
		Pup diet: Sex	1	138.96	8.5078	0.004511	**
		Dam diet: Pup diet: Sex	1	0.01	0.0009	0.976537	NS
2.4E,F	**Lean mass**	Dam diet	1	20.37	14.6934	0.0008962	***
		Pup diet	1	113.37	81.7599	1.773E-11	***
		Sex	1	1061.39	765.4411	<2.2E-16	***
		Dam diet: Pup diet	1	0.51	0.3666	0.5480914	NS
		Dam diet: Sex	1	2.72	1.9622	0.1648815	NS
		Pup diet: Sex	1	0.00	0.0013	0.9715934	NS
		Dam diet: Pup diet: Sex	1	0.33	0.2380	0.6268783	NS
2.4G,H	**Fat mass**	Dam diet	1	16.84	1.5674	0.2234559	NS
		Pup diet	1	2212.61	205.9312	<2.2E-16	***
		Sex	1	133.06	12.3842	0.0006893	***
		Dam diet: Pup diet	1	5.52	0.5142	0.4774929	NS
		Dam diet: Sex	1	19.66	1.8299	0.1796260	NS
		Pup diet: Sex	1	133.74	12.4478	0.0006723	***
		Dam diet: Pup diet: Sex	1	0.44	0.0407	0.8405966	NS
2.4K,L	**iAUC**	Dam diet	1	13427.9	1.0857	0.3090	NS

		Pup diet	1	5780.5	0.4674	0.4988	NS
		Sex	1	5454.4	0.4410	0.5083	NS
		Dam diet: Pup diet	1	28542.2	2.3077	0.1379	NS
		Dam diet: Sex	1	4.9	0.0004	0.9842	NS
		Pup diet: Sex	1	8941.8	0.7230	0.3975	NS
		Dam diet: Pup diet: Sex	1	19506.2	1.5771	0.2125	NS
2.5A,B	**Liver weight**	Dam diet	1	0.3873	2.1962	0.1527703	NS
		Pup diet	1	17.7034	100.3817	8.799E-13	***
		Sex	1	23.3087	132.1648	<2.2E-16	***
		Dam diet: Pup diet	1	0.2179	1.2355	0.2725512	NS
		Dam diet: Sex	1	0.0441	0.2501	0.6183294	NS
		Pup diet: Sex	1	2.7716	15.7153	0.0001549	***
		Dam diet: Pup diet: Sex	1	1.7534	9.9421	0.0022463	**
2.5C,D	**GWAT weight**	Dam diet	1	0.7945	6.7120	0.0157136	*
		Pup diet	1	5.7894	48.9116	3.197E-08	***
		Sex	1	1.7502	14.7864	0.0002159	***
		Dam diet: Pup diet	1	0.1353	1.1428	0.2921216	NS
		Dam diet: Sex	1	0.3233	2.7314	0.1016430	NS
		Pup diet: Sex	1	5.6574	47.7962	5.767E-10	***
		Dam diet: Pup diet: Sex	1	0.0000	0.0000	0.9984090	NS
2.5E,F	**SWAT weight**	Dam diet	1	0.02486	0.7065	0.4099714	NS
		Pup diet	1	1.67177	47.4999	5.463E-09	***
		Sex	1	0.77139	21.9175	1.121E-05	***
		Dam diet: Pup diet	1	0.07409	2.1050	0.1524505	NS
		Dam diet: Sex	1	0.00258	0.0734	0.7870719	NS
		Pup diet: Sex	1	0.53514	15.2050	0.0001959	***
		Dam diet: Pup diet: Sex	1	0.00037	0.0104	0.9191090	NS
2.5G,H	**BAT**	Dam diet	1	0.02389	2.1806	0.1537	NS

	weight	Pup diet	1	0.79694	72.7358	1.56E-10	***
		Sex	1	1.14961	104.9241	<2.2E-16	***
		Dam diet: Pup diet	1	0.00008	0.0076	0.9311	NS
		Dam diet: Sex	1	0.02736	2.4973	0.1176	NS
		Pup diet: Sex	1	0.00055	0.0502	0.8232	NS
		Dam diet: Pup diet: Sex	1	0.01620	1.4782	0.2273	NS

Table 2.5 Model outputs of 46-week timepoint results for the main effects of dam diet, pup diet, sex, and their interactions on metabolic traits, accounting for differences in body weight. Dam was included as a random variable in all analyses. Relevant post-hoc tests are indicated on each figure. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Outcome	Factor	Df	Sum Sq	F value	Pr(>F)	Sig. level
Lean mass	Dam diet	1	8.535	12.1512	0.001761	**
	Pup diet	1	0.272	0.3877	0.535121	NS
	Sex	1	249.366	355.0203	<2.2E-16	***
	Body Weight	1	78.445	111.6820	<2.2E-16	***
	Dam diet: Pup diet	1	0.037	0.0530	0.819083	NS
	Dam diet: Sex	1	0.144	0.2044	0.652290	NS
	Pup diet: Sex	1	5.757	8.1957	0.005229	**
	Dam diet: Pup diet: Sex	1	0.257	0.3656	0.547002	NS
Fat mass	Dam diet	1	10.75	13.4993	0.001005	**
	Pup diet	1	2.39	2.9990	0.086853	NS
	Sex	1	253.35	318.1027	<2.2E-16	***
	Body Weight	1	1067.33	1340.1110	<2.2E-16	***
	Dam diet: Pup diet	1	0.00	0.0000	0.994812	NS
	Dam diet: Sex	1	0.00	0.0024	0.960739	NS
	Pup diet: Sex	1	5.70	7.1521	0.008876	**
	Dam diet: Pup diet: Sex	1	0.44	0.5557	0.457989	NS
iAUC	Dam diet	1	5669	0.4493	0.50912	NS
	Pup diet	1	10046	0.7961	0.37506	NS
	Sex	1	4423	0.3505	0.55513	NS
	Body Weight	1	28264	2.2397	0.13774	NS
	Dam diet: Pup diet	1	39867	3.1591	0.08523	NS
	Dam diet: Sex	1	653	0.0518	0.82053	NS
	Pup diet: Sex	1	18371	1.4558	0.23073	NS
	Dam diet: Pup diet: Sex	1	18739	1.4849	0.22626	NS
	Dam diet	1	0.0103	0.0919	0.7644376	NS

Liver weight	Pup diet	1	0.0257	0.2279	0.6344278	NS
	Sex	1	1.1333	10.0626	0.0020037	**
	Body Weight	1	10.8676	96.4964	3.791E-16	***
	Dam diet: Pup diet	1	0.2484	2.2058	0.1473879	NS
	Dam diet: Sex	1	0.0902	0.8011	0.3731147	NS
	Pup diet: Sex	1	6.2276	55.2969	5.949E-11	***
	Dam diet: Pup diet: Sex	1	1.4448	12.8289	0.0005629	***
GWAT weight	Dam diet	1	0.0789	0.9411	0.33423	NS
	Pup diet	1	0.1430	1.7062	0.19436	NS
	Sex	1	5.3565	63.9016	1.899E-12	***
	Body Weight	1	3.7723	45.0023	1.054E-09	***
	Dam diet: Pup diet	1	0.3063	3.6538	0.05869	NS
	Dam diet: Sex	1	0.1347	1.6069	0.20775	NS
	Pup diet: Sex	1	3.3195	39.6003	7.509E-09	***
	Dam diet: Pup diet: Sex	1	0.0013	0.0156	0.90083	NS
SWAT weight	Dam diet	1	0.07739	3.4203	0.07766	NS
	Pup diet	1	0.00002	0.0010	0.97430	NS
	Sex	1	0.04657	2.0580	0.15504	NS
	Body Weight	1	1.12057	49.5223	3.038E-10	***
	Dam diet: Pup diet	1	0.03783	1.6720	0.20046	NS
	Dam diet: Sex	1	0.01632	0.7210	0.39830	NS
	Pup diet: Sex	1	0.14383	6.3566	0.01359	*
	Dam diet: Pup diet: Sex	1	0.00146	0.0646	0.80000	NS
BAT weight	Dam diet	1	0.00000	0.0006	0.980552	NS
	Pup diet	1	0.00033	0.0493	0.824802	NS
	Sex	1	0.06067	8.9982	0.003436	**
	Body Weight	1	0.45388	67.3176	7.055E-13	***
	Dam diet: Pup diet	1	0.00362	0.5366	0.467958	NS
	Dam diet: Sex	1	0.00544	0.8067	0.371464	NS
	Pup diet: Sex	1	0.04075	6.0437	0.015852	*
	Dam diet: Pup diet: Sex	1	0.01639	2.4305	0.122604	NS

2.3.4 Interactions between maternal diet and adult diet were observed in serum biochemistry of offspring

An interaction between dam diets and sex was found in Fgf21 blood serum levels. Fgf21 levels of female offspring from HP dams were slightly greater (Figure 2.6A; Table 2.6), whereas Fgf21 levels of male offspring (Figure 2.6B; Table 2.6) decreased with HP maternal diets at 46 weeks of age. WD in adulthood elevated Fgf21 levels in both sexes, indicated by a main pup diet effect (Table 2.6). A three-way dam diet:pup diet:sex interaction influenced levels of ALT (Figure 2.6C,D), blood cholesterol (Figure 2.6G,H) and LDL (Figure 2.6K,L; Table 2.6). A similar trend was present for all three measures. In females, HP pups had higher levels than LP pups, with a WD adult diet exacerbating this effect. In males, levels increased in offspring from HP mothers when fed SD in adulthood but decreased compared to maternal LP groups when fed WD as an adult (Figure 2.6D, H, J, L; Table 2.6). No differences were found in AST levels of female and male offspring (Figure Figure 2.6E, F; Table 2.6), while an interactive effect of dam and pup diets with a main effect of sex was found for HDL values.

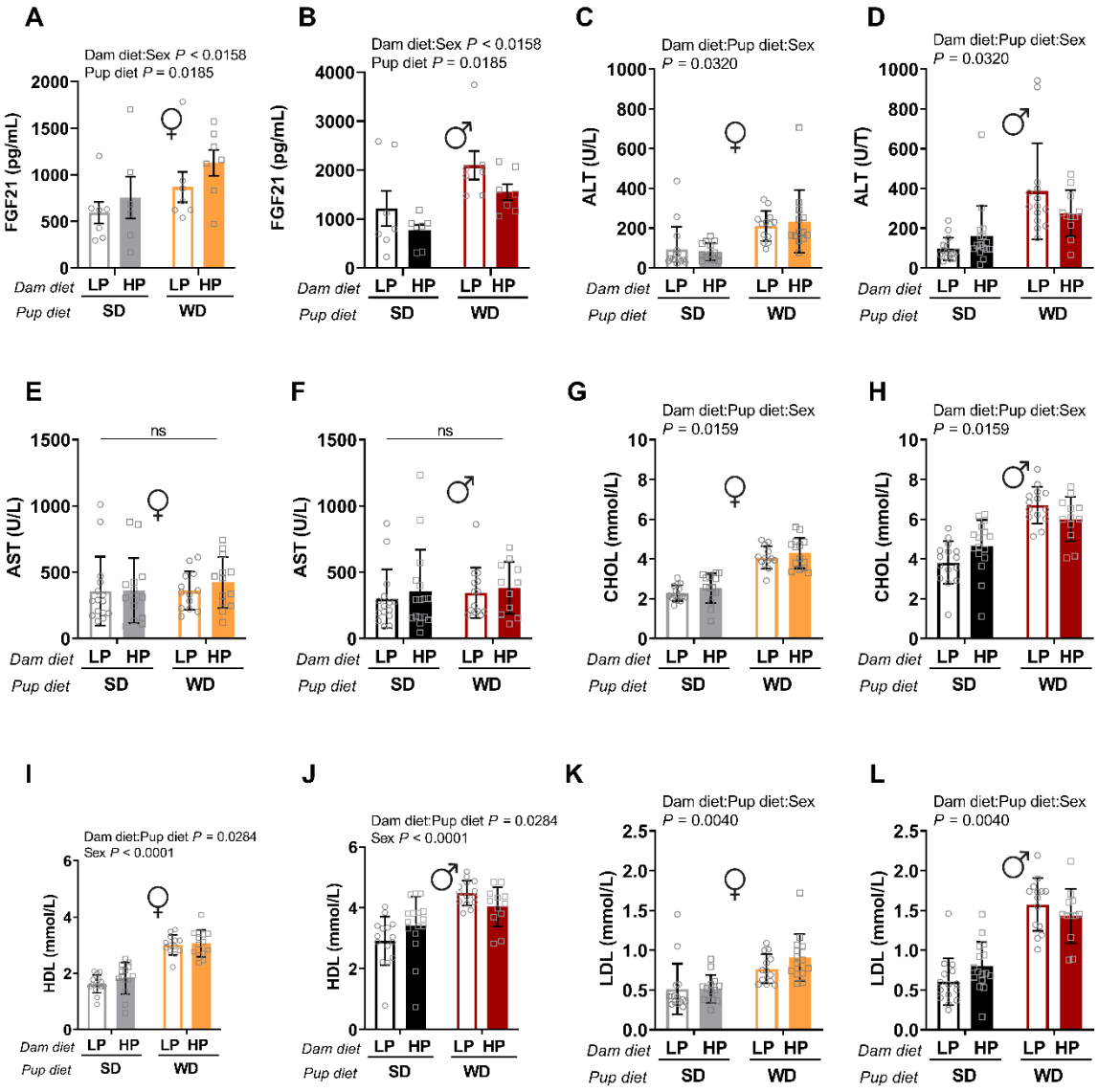

Figure 2.6 Interactions between maternal diet and adult diet were observed in serum biochemistry of male and female offspring.

FGF21 levels for (A) female and (B) male offspring (n = 7 animals/sex/group). Liver function tests as indicated by blood serum ALT levels for (D) females and (D) male offspring, AST levels for (E) female and (F) male offspring. Serum cholesterol for (G) female and (H) male offspring, HDL for (I) female and (J) male offspring, LDL for (K) female and (L) male offspring (n = 15-18 animals/sex/group). All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

Table 2.6 Model outputs of 46-week timepoint results for the main effects of dam diet, pup diet, sex, and their interactions on serum biochemistry. Dam was included as a random variable in all analyses. Relevant post-hoc tests are indicated on each figure. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	Factor	Df	Sum Sq	F value	Pr(>F)	Sig. level
2.6A,B	FGF21	Dam diet	1	10585	0.0211	0.88511	NS
		Pup diet	1	2985452	5.9517	0.01845	*
		Sex	1	2886556	5.7546	0.02038	*
		Dam diet: Pup diet	1	188986	0.3768	0.54224	NS
		Dam diet: Sex	1	3142594	6.2650	0.01577	*
		Pup diet: Sex	1	1934790	3.8572	0.05534	NS
		Dam diet: Pup diet: Sex	1	68396	0.1364	0.71356	NS
2.6C,D	ALT	Dam diet	1	2878	0.1934	0.663761	NS
		Pup diet	1	433834	29.1510	2.404E-06	***
		Sex	1	106995	7.1894	0.008752	**
		Dam diet: Pup diet	1	27089	1.8202	0.184031	NS
		Dam diet: Sex	1	15007	1.0084	0.318031	NS
		Pup diet: Sex	1	30934	2.0786	0.152958	NS
		Dam diet: Pup diet: Sex	1	70720	4.7520	0.031956	*
2.6E,F	AST	Dam diet	1	15660	0.3957	0.5352	NS
		Pup diet	1	3503	0.0885	0.7674	NS
		Sex	1	64979	1.6418	0.2035	NS
		Dam diet: Pup diet	1	4292	0.1084	0.7434	NS

		Dam diet: Sex	1	415	0.0105	0.9187	NS
		Pup diet: Sex	1	1688	0.0427	0.8369	NS
		Dam diet: Pup diet: Sex	1	1504	0.0380	0.8459	NS
2.6G, H	**Cholesterol**	Dam diet	1	0.314	0.4199	0.52347	NS
		Pup diet	1	74.891	100.1318	3.889E-12	***
		Sex	1	109.722	146.7027	<2.2E-16	***
		Dam diet: Pup diet	1	2.724	3.6427	0.06399	NS
		Dam diet: Sex	1	0.342	0.4575	0.50054	NS
		Pup diet: Sex	1	0.802	1.0718	0.30337	NS
		Dam diet: Pup diet: Sex	1	4.521	6.0452	0.01590	*
2.6I,J	**HDL**	Dam diet	1	0.169	0.4597	0.50489	NS
		Pup diet	1	38.247	103.9636	1.193E-11	***
		Sex	1	49.239	133.8433	<2.2E-16	***
		Dam diet: Pup diet	1	1.936	5.2615	0.02841	*
		Dam diet: Sex	1	0.067	0.1809	0.67159	NS
		Pup diet: Sex	1	0.358	0.9720	0.32682	NS
		Dam diet: Pup diet: Sex	1	1.147	3.1166	0.08087	NS
2.6K,L	**LDL**	Dam diet	1	0.0104	0.1774	0.677385	NS
		Pup diet	1	3.9701	67.8045	6.694E-11	***
		Sex	1	4.8166	82.2615	3.607E-14	***

		Dam diet: Pup diet	1	0.0552	0.9422	0.336343	NS
		Dam diet: Sex	1	0.0602	1.0280	0.313477	NS
		Pup diet: Sex	1	1.5460	26.4033	1.709E-06	***
		Dam diet: Pup diet: Sex	1	0.5111	8.7296	0.004038	**

2.3.5 Maternal diet influenced anxiety-related behaviour responses of offspring at 30 weeks of age

Here, we found that maternal diet had a significant effect on the exploratory behaviour of offspring. A decrease in number of entries (Figure 2.7A,B; Table 2.7) and total duration spent in the open arms of the arena (Figure 2.7C,D; Table 2.7) and increase in duration spent in closed arms (Figure 2.7E,F; Table 2.7) was observed in offspring from HP compared with LP dams. A longer duration in the closed arm region considered a marker of anxiety-related behaviour. The entries and time spent in the open arms (anti-anxiety region) were also reduced in offspring fed WD as adults compared with SD counterparts shown as a main pup diet effect (Figure 2.7A-D; Table 2.7). Total distance travelled in the maze was greater in WD than in SD mice and this effect was slightly greater in magnitude in females than males (Figure 2.7G,H; Table 2.7).

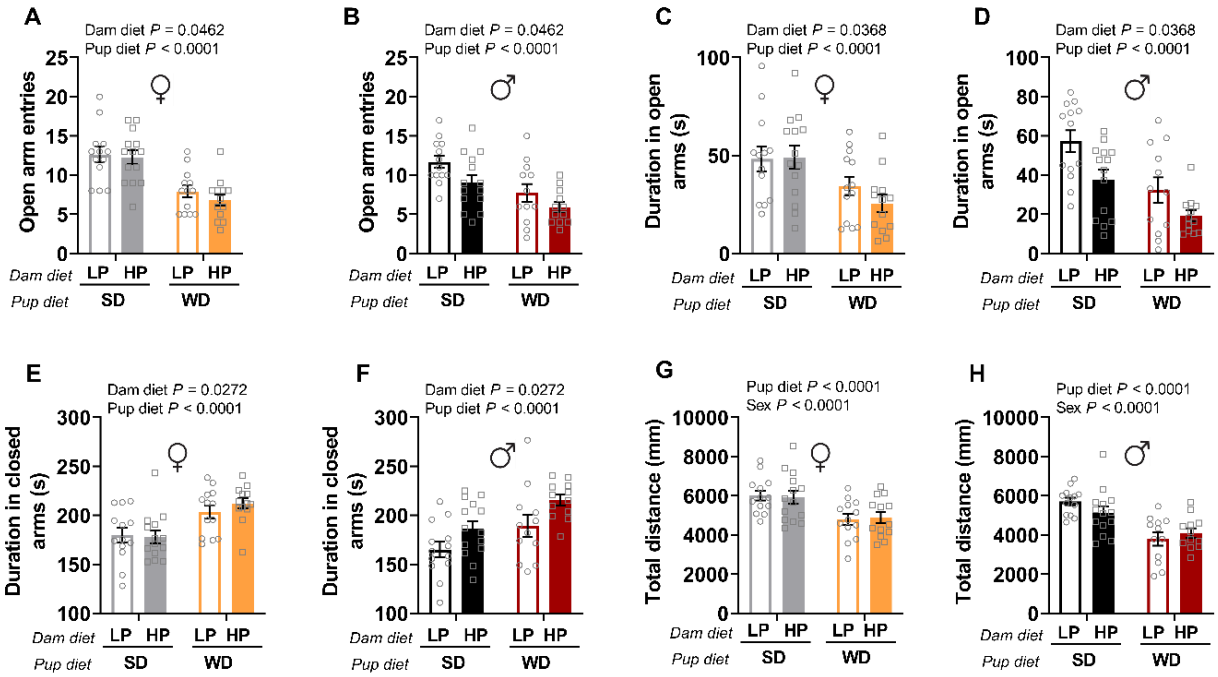

Figure 2.7 Maternal diet influenced anxiety-related behaviour responses of offspring at 30 weeks of age

Using an elevated plus maze, several parameters were measured including number of open arm entries for (A) female and (B) male offspring, duration spent in open arms for (C) female and (D) male offspring, duration spent in closed arms for (E) female and (F) male offspring and total distance travelled around the maze in a five-minute period for (G) female and (H) male offspring (n= 12 animals/sex/group) at 30 weeks of age. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

Table 2.7 Model outputs of 30-week timepoint results for the main effects of dam diet, pup diet, sex, and their interactions on behaviour in an elevated plus maze. Dam was included as a random variable in all analyses. Relevant post-hoc tests are indicated on each figure. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	Factor	Df	Sum Sq	F value	Pr(>F)	Sig. level
2.7A, B	Open arm entries	Dam diet	1	43.33	4.5849	0.04624	*
		Pup diet	1	414.27	43.8381	4.361E-07	***
		Sex	1	26.70	2.8259	0.09619	NS
		Dam diet: Pup diet	1	0.14	0.0149	0.90363	NS
		Dam diet: Sex	1	4.89	0.5179	0.47358	NS
		Pup diet: Sex	1	26.95	2.8517	0.09470	NS
		Dam diet: Pup diet: Sex	1	14.11	1.4930	0.22490	NS
-	Closed arm entries	Dam diet	1	0.668	0.0529	0.8205601	NS
		Pup diet	1	178.808	14.1420	0.0006812	***
		Sex	1	33.057	2.6145	0.1095184	NS
		Dam diet: Pup diet	1	74.003	5.8529	0.0213988	*
		Dam diet: Sex	1	4.362	0.3450	0.5584941	NS
		Pup diet: Sex	1	3.311	0.2619	0.6101238	NS
		Dam diet: Pup diet: Sex	1	22.915	1.8123	0.1816967	NS

2.7C, D	**Duration in open arms**	Dam diet	1	1697.7	4.9720	0.03675	*
		Pup diet	1	8159.1	23.8953	2.858E-05	***
		Sex	1	93.6	0.2740	0.60191	NS
		Dam diet: Pup diet	1	17.7	0.0518	0.82139	NS
		Dam diet: Sex	1	630.8	1.8474	0.17745	NS
		Pup diet: Sex	1	2.5	0.0074	0.93183	NS
		Dam diet: Pup diet: Sex	1	678.1	1.9860	0.16216	NS
2.7E,F	**Duration in closed arms**	Dam diet	1	3992.9	5.7510	0.02718	*
		Pup diet	1	18123.2	26.1029	2.292E-05	***
		Sex	1	745.1	1.0731	0.30294	NS
		Dam diet: Pup diet	1	423.5	0.6100	0.44162	NS
		Dam diet: Sex	1	1386.7	1.9972	0.16095	NS
		Pup diet: Sex	1	337.9	0.4867	0.48717	NS
		Dam diet: Pup diet: Sex	1	185.1	0.2666	0.60685	NS
2.7G, H	**Total distance travelled**	Dam diet	1	1486	0.0020	0.96466	NS
		Pup diet	1	17957313	24.2363	1.199E-05	***
		Sex	1	12556941	16.9476	8.910E-05	***
		Dam diet: Pup diet	1	2213135	2.9870	0.09082	NS
		Dam diet: Sex	1	39820	0.0537	0.81723	NS
		Pup diet: Sex	1	543187	0.7331	0.39427	NS
		Dam diet: Pup diet: Sex	1	893535	1.2060	0.27521	NS

2.3.6 Adult Western Diets led to increased energy expenditure in female mice at 45 weeks.

No differences in energy expenditure in either male or female offspring were detected between maternal diet groups (Figure 2.8A-F) at 45 weeks. In female mice, however, WD was associated with increased energy expenditure for both day (Figure 2.8B) and night periods (Figure 2.8). In males, no significant differences were found in energy expenditure (Figure 2.8,F). No significant effects of diet were found in either sex for respiratory quotient.

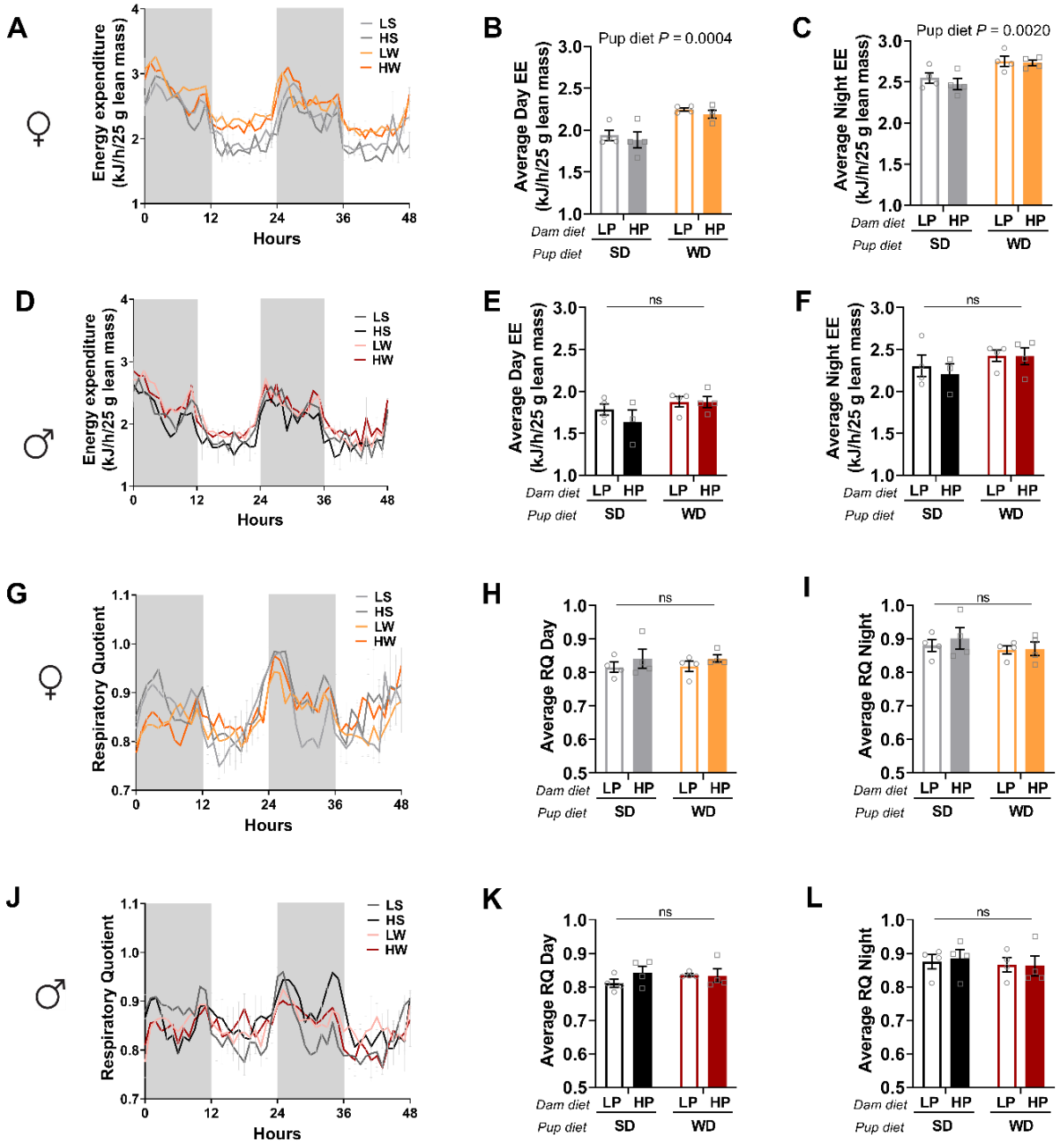

Figure 2.8 Adult Western Diets led to increased energy expenditure in female mice at 45 weeks.

Energy expenditure was measured and normalised to 25 g lean mass at 45 weeks of age for (A) female offspring. Average values were calculated for (B) day and (C) night periods (n = 4 animals/sex/group). Energy expenditure was measured and normalised to 25 g lean mass 45 weeks of age for (D) male offspring. Average values were calculated for (E) day and (F) night periods (n = 3-4 animals/sex/group). Respiratory quotient was measured at 45 weeks of age for

(G) female offspring. Average values were calculated for (H) day and (I) night periods (n = 4 animals/sex/group). Respiratory quotient was measured at 45 weeks of age for (J) male offspring. Average values were calculated for (K) day and (L) night periods (n = 3-4 animals/sex/group).

2.4 Discussion

We explored how maternal P:C diet composition influences offspring nutrient targets and related outcomes such as food selection and intake, body composition, behaviour, and metabolic status. We found that HP maternal diets increased protein targets of offspring. When offspring were subsequently confined to a SD, higher protein targets resulted in an increase in food intake over time. Increased offspring protein targets also increased susceptibility to obesity, at 16 weeks of age. Although maternal protein intake had long-term implications on offspring metabolism and behaviour, these effects were increasingly superseded the longer they were exposed to the adult environments.

We utilised a food choice experiment to investigate whether a higher proportion of protein in the mother's diet during pre-conception, gestation and lactation could lead to higher protein targets being programmed in the offspring. We found that both female and male pups from HP fed mothers consumed a higher amount of protein compared to their LP counterparts, indicating the need to satisfy higher protein targets. Higher consumption of protein may be a consequence of a compensatory decrease in protein efficiency (Raubenheimer et al., 2015). This has likely been programmed in the offspring to counteract excess protein levels of the maternal diet *in utero* and during lactation, presumably through the upregulation of hepatic gluconeogenesis (Veldhorst, Westerterp-Plantenga & Westerterp, 2009).

The implications for higher protein targets established this early on in life are immense. As explained by protein leverage, when placed in environments with low protein composition, many animals will continue to eat (even with the compromise of overconsuming carbohydrates and fats) to reach their protein targets. An elevated protein target from as early as weaning means that HP offspring are even more so predisposed to hyperphagia in these protein poor environments than LP pups, potentially leading to obesity. We observed that the increased consumption of PE also led to higher body weight, lean mass and fat mass in HP pups at 8 weeks of age. Several cases have reported that an increased intake of infant formula in early life, which is typically higher in protein than breast milk, has been associated to an increased

susceptibility of obesity in later life (Huang et al., 2018; Totzauer et al., 2018). In humans, higher intakes of total and animal protein during infancy were associated with higher BMI in childhood and adolescence (Stokes et al., 2021). Other parental diet-driven mechanisms that have been proposed to regulate appetite regulation in offspring include mTORC1 signalling, leptin and ghrelin pathways and changes to the neuronal networks throughout development (Bouret, 2010, Bouret et al., 2004, Morris and Chen, 2009). In protein deficient maternal diets, reduced mTORC1 signaling activity in blastocysts mediated through depleted BCAA and insulin availability is considered to be a crucial proponent in the induction of adverse developmental programming (Fleming et al., 2021, Velazquez et al., 2018). On the other hand, ghrelin blockade in neonatal mice resulted in enhanced hypothalamic neural projections and long-term metabolic effects, including increased body weight, visceral fat, and blood glucose levels and decreased leptin sensitivity (Steculorum et al., 2015).

It is increasingly accepted that foetal exposure to an adverse intrauterine environment such as poor nutrition, infections, toxicity, stress or metabolite and hormonal perturbations, can trigger adaptations to counteract them (Heindel and Vandenberg, 2015). These adaptations may include resetting metabolic and endocrine systems and the downregulation of growth signals to match the intrauterine environment (Mandy and Nyirenda, 2018). The immediate aim of these responses is to maximise its immediate chance for survival, but also its future viability should it encounter a similar hostile environment in later life (predictive adaptive responses) (Gluckman et al., 2008a). While these changes are necessary for short term survival, they may be maladaptive in later life, particularly if the individual is confined to a post-natal environment that is the reverse of what they were exposed to *in utero*.

Therefore, we went on to further investigate whether the effects of maternal diet established in the early life of offspring would remain after they are introduced to new diets in later life. Offspring were provided a fixed diet of either a standard or western diet composition following the initial choice experiment. Given WD is low in protein, it was initially hypothesised that a higher P target should lead to a greater difference in food intake. However, we found that all WD fed groups were eating maximally, independent of maternal diet. Food palatability and

texture may play a role in driving increased consumption for these groups. On the other hand, the distinct food intake trajectories programmed by the maternal diet were much more prominent in the Standard diet group. We observed that even when switched to the same adult diet and remaining on it for an 8-week period, pups from HP fed mothers had elevated food intake, suggesting a higher intake target compared to LP pups. This trend continued long term and was still present when analysed at 32 weeks after the diet switch. At 16 weeks of age (8 weeks after switching to fixed adult diets) we also found that offspring from HP dams were heavier and fatter than those from LP dams, an effect that was exacerbated by offspring feeding on WD. Glucose tolerance was shown to be most greatly impaired from a combination of a HP maternal diet and WD in adulthood.

In humans, similar patterns can be seen when observing the feeding patterns of oceanic and hunter-gatherer populations, such as the Inuit. For many years, these groups predominantly had access to marine-based, protein rich diets and as such, are predicted to possess higher protein targets (Kuhnlein et al., 2004, Ulijaszek, 2003). However, based on the concept of protein leverage, they are also much more susceptible to overconsumption of energy in order to reach these elevated protein targets, especially when transitioned to energy-rich, protein-poor diets (Young, 2007). In recent years, these communities have seen a shift from their traditional diets to more carbohydrate rich, cereal-based food types and then to western-style diets following the development of settled agriculture, and other food processing advancements (Kuhnlein et al., 2004).

It is probable then, that the high incidence of obesity among these populations, as also observed in our study, could be attributed to exacerbation of the protein leverage effect. As animals continue to reach developmentally programmed elevated protein targets in a newer, protein-poor environment, it leads to hyperphagia and an increasingly obese phenotype. Additionally, these oceanic populations have also been reported to have enlarged livers and have increased urine output, which could be possible signs of high rates of hepatic gluconeogenesis and adapted glomerular filtration rates (Cordain et al., 2000, Ho et al., 1972). Although WD pups had larger liver masses in general, we found that male pups from HP mothers who were then

fed SD in adulthood displayed greater liver masses than LP pups on the same fixed adult diet. Exactly why the distinction was greater in SD, rather than WD groups is still unclear. As earlier discussed with food intake and body weight outcomes, it is possible that the high palatability of WD (rich in both sucrose and fat) is driving these pups to consume to maximum capacity. This could allow them to reach an increased level of food consumption independent of earlier protein targets set by maternal diet, and thus results in increased body weights and tissue mass. This suggests that the effect of maternal diet is context dependent and although parental diets can place their offspring on certain trajectories, current diets also have a large effect on overall phenotype.

To test if the difference in protein target determined by maternal diet in early life persisted in later life, another choice experiment was performed at 40 weeks of age. At this timepoint, we observed a main effect of maternal diet for both sexes, suggesting that protein targets programmed in early life can endure into late life. We continued to see the trend observed at the 16-week timepoint, where WD-fed offspring from HP dams (HW) had the highest protein intakes compared to all other groups. However, when we tested how this might have resulted into a continued obese phenotype in the offspring, we instead found that maternal diet effects were increasingly overridden by the adult diet environment over time. WD-fed female and male pups had increased body weight, lean mass and fat mass compared to SD-fed offspring overall. Dam diet still had prolonged impacts on body weights and lean mass, and offspring from HP dams were shown to have greater fat mass in comparison to offspring from LP dams at 46 weeks when body weight was accounted for in the model. There were also no maternal diet-driven significant differences found in the glucose tolerance at this later timepoint.

While maternal diet effects were found to be less pronounced in terms of fat mass and other major metabolic indicators such as glucose at 46 weeks of age, other maternal diet-driven metabolic markers in later life were found and were also highly variable between males and females. In serum Fgf21, we found that males tracked dietary protein as expected, where low protein pups from both adult diets displayed greater Fgf21 levels than their HP equivalents. This follows known patterns of FGF21 as a mediator of protein leverage and could explain

patterns of food intake under no-choice conditions (Hill et al., 2020, Wu et al., 2022). Contrastingly, trends in female offspring remain unclear, with a significant dam diet:sex interaction found for this outcome. This sex-dependent nature of FGF21 has been similarly observed in some studies (Larson et al., 2017, Makarova et al., 2020). Female mice have been shown to have lower sensitivity to the metabolic improvements observed following dietary protein dilution shown by decreased circulating levels of FGF21 (Larson et al., 2017). Additionally, the increased Fgf21 levels in WD compared to SD fed mice may be attributed to the lower protein content of this diet.

More sex specific effects were observed when looking at levels of circulating ALT, cholesterol, HDLs and LDLs. There were interactive effects between maternal and adult diet for these markers in male offspring. The same trend was present for all four measures, with levels increased in offspring from HP mothers when fed SD in adulthood but decreased compared to maternal LP groups when fed WD as an adult. In female offspring, adult diet was the main driver for differences in levels of circulating ALT, cholesterol, HDL and LDL. In past studies, sexually dimorphic responses to early life programming have been typically depicted through settings of famine or energy excess (Schulz, 2010, Dearden et al., 2018). Individuals who encountered periods of undernutrition *in utero* have been shown to develop a range of disease phenotypes as adults, with many of the phenotypes varying depending on sex and what period of gestation the exposure to the famine occurred (Ravelli et al., 1999, Stein et al., 2007). Meanwhile, energy-rich diets (such as high fat diets) have also been used to demonstrate sex-specific responses for a multitude of outcomes including DNA methylation, gene expression, hypertension, adiposity and insulin resistance (Gallou-Kabani et al., 2010, Khan et al., 2003, Nivoit et al., 2009).

In several studies, i*n utero* and early life exposure to maternal diets have been shown to impact the behaviour of their offspring. Similar to typical studies of maternal effects on metabolism, the results from behaviour-focused studies are also often described as consequences of under and overnutrition. Early developmental exposure of dams to chronic LP diets has been shown to reduce food intake and energy expenditure, and increased anxiety like behaviour in an EPM

(Crossland et al., 2017). Maternal low protein diets confined to the preimplantation period in mice has also been reported to result in short term memory deficit and adverse programming of brain development for alterations in brain neuron proportions (Gould et al., 2018). High-fat-diet maternal diets, on the other hand, were shown to increase neuro-inflammation, caused by an increased expression of pro-inflammatory cytokines, such as interleukin- 1β (IL- 1β) (Bilbo and Tsang, 2010, Almeida-Suhett et al., 2016) and the increased expression of several genes involved in inflammation via the glucocorticoid signalling pathway (Sasaki et al., 2014). In this study, we found that an increase in protein in otherwise isocaloric maternal diets led to significant effects in the anxiety-like behaviour of offspring. We observed a significant dam effect in the number of entries and total duration spent in the open arms of the EPM arena, and an increase of time spent in the anxiety associated closed arm region in HP pups. Interestingly, when corticosterone levels were measured in the offspring, a main effect of maternal diet was seen primarily in female offspring, where HP females had increased corticosterone levels. Male offspring were only affected by adult diets. As proposed previously (da Silva et al., 2021), age might be a factor that should be accounted for in these results. EPM tests were conducted at 30 weeks of age, while corticosterone levels were measured at 46 weeks of age.

Our study has provided valuable insights into the influence of maternal diet on offspring health outcomes. However, there are several limitations that should be acknowledged. First, while maternal diet is an important factor in offspring health, it is not the only factor. Paternal influences, including environmental exposures, may also play a role in determining offspring health outcomes. Future studies should consider the contribution of both maternal and paternal factors to better understand the mechanisms underlying offspring health outcomes. Second, we did not distinguish the effects of intrauterine and immediate post-natal environments on offspring. Milk may act as a conduit for maternal diet influences on offspring, but we did not measure the composition of milk in this study. Third, we used a single inbred mouse strain and future studies should consider a range of genetic backgrounds.

We have shown that a higher proportion of protein in the maternal diet from preconception, gestation and lactation leads to higher protein targets for their offspring. Not only does this

have early life consequences on both male and female offspring, it also continues to influence aspects of health in later life, including metabolism and behaviour. Future research in this field should further delve into the mechanisms involved in the programming and long-term persistence of these maternally driven phenotypes. Such work would offer novel discoveries linking the intricate interaction of maternal nutrition, early-life programming and offspring nutritional targets on long-lasting offspring consequences that can be translatable to humans in the future.

CHAPTER 3: The impact of paternal carbohydrate and fat intake on obesity outcomes and offspring metabolism

3.1 Introduction

Obesity has many important interactions with a variety of biological processes. For example, its implications in reproduction, fertility, and programming of offspring health, are widely studied, although much of the work in this field is concentrated towards maternal outcomes (Barker, 2012, Gluckman et al., 2010, Samuelsson et al., 2008a, Ware et al., 2015). In recent years, the impact of the paternal environment and reproductive outcomes on offspring health are becoming more widely acknowledged (Sharp and Lawlor, 2019, Watson, 2016). However, there are still gaps in our understanding of how specific macronutrients, and their balance within the diet, can affect male fertility and offspring health. Hence, there is an urgent need for targeted studies assessing dietary influences on male reproduction and paternally mediated programming.

Multiple studies have demonstrated that paternal diet can influence offspring health outcomes, including adiposity, metabolism, and gene expression (reviewed in Dimofski et al., 2021, Billah et al., 2022). Growing understanding in this field is that the mechanisms of non-genetic transmission via the paternal line occur through both the sperm (Champroux et al., 2018) and non-sperm components, such as seminal plasma (Watkins et al., 2018). Mounting evidence suggests that paternal overnutrition can have negative impacts on conventional sperm parameters including sperm concentration, motility, viability, and morphology (Binder et al., 2015b, Palmer et al., 2012, Raad et al., 2017). Overnutrition and obesity is also associated with increased reactive oxygen species (ROS) production and DNA damage in sperm (Pearce et al., 2019, Bakos et al., 2011). Such increased DNA damage has been linked with adverse consequences such as poor fertilisation and embryo development, and higher risks of miscarriage (Lewis and Simon, 2010, Binder et al., 2012), but also long-term offspring health outcomes such as adiposity and reproductive parameters (Burke et al., 2001, Oshio et al., 2020). Given the continued rise of global obesity, the number of fathers with obesity planning to

conceive is also growing (Ng et al., 2014a). Because the environmental and metabolic states of the fathers at pre-conception can have long-term impacts on their offspring, it is possible that detrimental impacts of obesity on fertility and offspring health will continue to increase in years to come. The diet of a male during this period has been shown to influence the metabolic and reproductive health of his offspring (Ng et al., 2010, Watkins and Sinclair, 2014, Chowdhury et al., 2016) with some studies showing that such 'programmed' effects can even be transmissible to further generations (Chambers et al., 2016, Fullston et al., 2012).

Many studies have examined the association between obesity and male infertility; however, there is often much variation in outcomes across studies, and between male subjects (Katib, 2015, Palmer et al., 2012). Hence, the next step is to investigate potential causes of variation, one of which may be dietary composition (Dimofski et al., 2021). Nutritional studies have shown that it is not just the amount of energy consumed that is crucial, but also the quality and type of nutrients that the energy is derived from (Solon-Biet et al., 2014). Most obesogenic models used in paternal obesity studies use diets with elevated concentrations of fat, typically referred to as "high fat" (Crean and Senior, 2019, Terashima et al., 2015, Deal et al., 2020). The fact that fat is an energy dense macronutrient, typically yielding double the amount of energy per gram of food compared to carbohydrates and protein (Rolls, 2017), partially explains why a high fat diet is often found to result in increased adiposity. Increasing the percentage of fat within the diet not only raises the energy density of food, but also improves palatability while exerting weak satiety-signalling properties (Blundell and MacDiarmid, 1997, Westerterp, 2006), altogether leading to increased food intake.

The role of other dietary components, such as carbohydrates, on male fertility and offspring outcomes is still largely unclear. Some studies that have employed a high sugar diet have demonstrated reduced sperm motility and alteration of semen quality in rodent (Medaglia et al., 2022) and human models (Chiu et al., 2014). Others have reported enhanced adiposity and induced hyperphagia from high sugar diets of differing sources (Peris-Sampedro et al., 2019). Although complex carbohydrates have been shown to offer multiple advantages in terms of overall health (Saris et al., 2000), there are limited published literature explaining their role in the preservation of male reproduction and offspring phenotype. Hence, this is something we

have chosen to explore further in this study.

A semi-purified combination of both a high fat and high carbohydrate dietary composition is a commonly used obesogenic diet, often termed a "Western" or "cafeteria" diet (Bodden et al., 2022, Grandjean et al., 2015). A common issue encountered when analysing results in this field is that the specific contents of Western experimental diets are extremely variable between studies and very rarely properly defined (as reviewed in Pini et al., 2021). Some factors that are changed often, and to different extents, include the source and proportion of fat and sugar, as well as the total energy content of the diet (César et al., 2022, Sertorio et al., 2022). Therefore, it can lead to increased variability in the findings of these studies, and it still remains unclear whether the effects of western (and other "unhealthy") diets on male reproduction result from individual differences in fat, carbohydrate, or total energy content (Suliga and Głuszek, 2019, Pini et al., 2021). Moreover, the way offspring outcomes are affected by these paternal dietary differences could differ depending on the immediate environment of the offspring as well. For example, some studies have shown instances where paternal effects are only revealed when offspring are subjected to stressors in their lifespan (Korgan et al., 2022).

Along with paternal influences, and offspring environments, another factor that could interact to produce certain phenotypes is the sex of the individual. Sex differences occur in most non-communicable diseases, including obesity and metabolic syndrome (Ballestri et al., 2017, Curley and Mashoodh, 2010, Hasegawa et al., 2020), yet the potential role of non-genetic paternal effects in mediating this (and vice versa) is still understudied. A few instances of paternal effects have been shown to vary depending on the offspring's sex in complex ways (Binder et al., 2015a). Studies show that paternal influences can have opposing effects on the same trait in sons compared to daughters (Short et al., 2016), only be present in either sex (Ng et al., 2010), or affect them in different magnitudes (Jazwiec et al., 2022). Not accounting for these sex-specific patterns by pooling both male and female offspring together would potentially mask the extent of paternal influences, therefore, in this research, we opted to investigate paternal effects in both male and female offspring.

Here, we set out to isolate the effects of dietary fat and sugar, without the confounder of energy density, on male fertility and metabolism. Additionally, we wanted to ascertain whether offspring food intake and macronutrient selection was altered by paternal diet, which to our knowledge has not been explored in depth before. Additionally, we aimed to investigate if paternal sugar/fat intake influences offspring susceptibility to obesity and other cardiometabolic disorders in early and later life timepoints. We found that high fat diets in male studs led to the most obese phenotype. We observed that increasing the percentage of sucrose versus starch in the paternal diet had little influence on body weight, fat mass, or fasting insulin levels. In contrast, increasing the percentage of dietary fat (while keeping energy density constant) resulted in increased body weight, fat mass, and fasting insulin levels. No effects were found on mating success and litter sizes as an effect of diet.

Meanwhile, in the offspring, sex specific interactions were observed in a variety of health outcomes. Stud diet driven differences in body composition were clearer in male offspring. Here, we showed that male pups from high fat fed fathers were heavier at 10 weeks of age, but this was mainly attributed to greater lean mass. On the other hand, pups from Control-diet fed studs had greater fat mass. By 20 weeks of age, significant impacts of paternal diet were no longer observed, and the effects of direct environment were strengthened. This work highlights the complex interactions between nutrition, reproduction, and overall health. It provides fundamental new understanding of the ways in which diet and nutrition shape the health of fathers and their offspring, taking the first step towards developing pre-conception dietary guidelines to improve male reproductive outcomes.

3.2 Methods

3.2.1 Ethical approval

All animal protocols were approved by Sydney University's Animal Ethics Committee (Protocol number 2019/1609) to ensure they were in line with the NSW Animal Research Act 1985 and the Australian code for the care and use of animals for scientific purposes 8th edition (2013).

3.2.2 Animals and husbandry

3.2.2.1 General husbandry and cage allocations

All animals in this study were housed in cages at the Charles Perkins Centre, University of Sydney with an ambient temperature of 22°C and constant access to food and water.

Fifty-four C57Bl/6-JArc 3–4-week-old studs were purchased from Animal Resources Centre (Perth, WA) in two cohorts, and housed 3 per cage until mating. They were started on experimental diets immediately upon arrival. This was done so the fathers were exposed to treatments during adolescence, and over multiple rounds of spermatogenesis. They then remained on their assigned diet until culling. Body weights and food intake were measured weekly, and health checks performed at least twice per week. At 18 weeks of age, studs were separated into individual home cages in preparation for mating.

Thirty-six 16-week-old female C57Bl/6-JArc mice that had successfully raised a litter of pups (proven fertile) were purchased from Animal Resources Centre (Perth, WA) and housed individually in pre-allocated mating cages. Dams were allowed to acclimatise to the facility for two weeks prior to mating and exposed to bedding of their assigned mate to prime them for breeding.

3.2.2.2 Breeding protocol

For each breeding cycle, studs were paired with their allocated female overnight for four consecutive nights (n=12 mating pairs per diet) and returned to their home cages during the

day to minimise access to dam food. Although this protocol meant that studs had access to brown chow during mating, it was considered more important that dams were never exposed to experimental diets. To confirm successful copulation, the stage of oestrous cycle and presence of vaginal plugs was recorded. During gestation, dams were housed in individual cages and body weights were measured twice weekly to detect pregnancies. The date and number of any offspring was recorded. To prevent infant cannibalism, litter body weights were not measured until three days post birth. Litters were continually monitored until they were weaned at 3 weeks of age, where the sex of the offspring was recorded.

3.2.3 Offspring experiments

3.2.3.1 Offspring food choice experiment

To test whether offspring intake targets were affected by paternal dietary manipulations, a food choice experiment was performed for a subgroup of the offspring (n=8-10/sex/paternal diet group). Straight after weaning, male and female pups were single-housed and allowed to self-select between two diets (Control and Western) outlined in the offspring experimental diet section above. Food intake was measured twice weekly to determine whether pups were self-selecting more of one type of food, or the total quantity of food consumed. Body weight was measured weekly. The animals were sacrificed at 12 weeks of age and various tissues were collected.

3.2.3.2 Offspring no choice experiment

Upon weaning at 3 weeks of age, 2 male and 2 female offspring (numbers permitting) were randomly chosen from each stud. From weaning (3 weeks of age), these pups were group-housed in cages of 2-3 animals and restricted to either a Standard or Western diet (detailed above), yielding n=8-10/sex/ diet group. Food intake and body weights were measured once a week, and additional metabolic phenotyping, including body composition oral glucose tolerance tests and fasting insulin levels were measured at 10 and 20 weeks (see below). Body composition was assessed via EchoMRI analyser (Houston, TX, USA), where lean mass and fat mass values were recorded. The animals were culled at 21 weeks of age.

3.2.4 Experimental Diets

The experimental diets used in this study were manufactured in dry, pelleted form by Specialty Feeds Australia. Full breakdowns of diet compositions are provided below (Table 3.1).

Table 3.1 Breakdown of compositions for diets used in study.

	Paternal diets			***Offspring No-Choice***	
Diet	**Chow/High Starch**	**High Sucrose**	**High Fat**	**Standard Diet**	**Western Diet**
NME (kJ/g)	14.4	14.7	14.7	14.4	17.3
% Protein energy	18.5	18.5	18.5	18.5	10
% Carbohydrate energy	64	64	42.3	64	50
% Fat energy	17.5	17.5	39.2	17.5	40
Other (g/kg)					
Casein	200	200	200	200	126
Soy oil	70	70	157	70	29
Wheat Starch	404	304	269	404	254
Sucrose	100	300	67	100	231
Dextrinised Starch	132	32	88	132	100
Cellulose	50	50	176	50	56

3.2.4.1 Stud diets

The studs were fed 1 of 3 diets with varying sources of carbohydrate and differing fat: carbohydrate concentrations. Stud diets were matched for metabolisable energy content (i.e. isocaloric) as much as was practically possible through the addition of non-digestible cellulose. The source of protein was casein, fat was soybean oil and carbohydrates were a mixture of sucrose, wheat starch and dextrinised starch.

- **Control**

The control diet (CD; SF18-025, Specialty Feeds, Glen Forrest, WA, Australia) was derived from a standard rodent AIN-93G formulation, with 18.5% protein, 64% carbohydrate and 17.5% fat making up the total calorie content of 14.4 kJ/g. The carbohydrate component consisted of 84.3% starch and 15.7% sucrose.

- **High Sucrose**

The high sucrose diet (HSucrose; SF19-202, Specialty Feeds, Glen Forrest, WA, Australia) was derived from a standard rodent AIN-93G formulation, with 18.5% protein, 64% carbohydrate and 17.5% fat making up the total calorie content of 14.7 kJ/g. The carbohydrate component consisted of 52.8% starch and 47.2% sucrose.

- **High Fat**

The high fat diet (HFat; SF19-203, Specialty Feeds, Glen Forrest, WA, Australia) was derived from a standard rodent AIN-93G formulation, with 18.5% protein, 42.3% carbohydrate and 39.2% fat making up the total calorie content of 14.7 kJ/g. The carbohydrate component consisted of 84.2% starch and 15.8% sucrose.

3.2.4.2 Offspring diets

- **Control**

The control diet fed to pups was the same as the control diet fed to studs (CD; SF18-025, Specialty Feeds, Glen Forrest, WA, Australia).

- **Western**

A high calorie, high fat, and high sucrose “Western” diet (WD; SF18-050, Specialty Feeds, Glen Forrest, WA, Australia) was used as an obesogenic diet for pups in this study, as it was

not logistically feasible to complete a fully crossed 3 paternal by 3 offspring diet design. The Western diet designed for this study was derived from a standard rodent AIN-93G, with 10% Protein, 50% Carbohydrate, and 40% Fat, making up the total calorie content of 17.3 kJ/g. The fat component consisted of 84.7% lard and 15.3% soybean oil. The carbohydrate component consisted of 60.5% starch and 39.5% sucrose.

3.2.4.3 Dam diet

Dams in this study were fed a non-purified brown chow diet.

3.2.5 Body composition

Body weights were measured weekly for all mice, unless specified. Body composition (fat mass and lean mass) was assessed using an EchoMRI 900 (EchoMRI, TX, USA) at 18 weeks of age in studs (n=12/sex/group) and 10 weeks and 20 weeks of age in offspring (n=8-10/sex/group). Organ weights were measured at cull including liver, gonadal and subcutaneous white adipose tissue, brown adipose tissue, and quadriceps.

3.2.6 Tissue collection

After the birth of a successful litter or third round of mating for studs, and 21 weeks of age for male and female offspring, mice were anaesthetised using a 1:1 mix of ketamine and xylazine, administered through an intraperitoneal injection. Animals were humanely euthanised and tissues were harvested for analysis. Tissues were snap frozen in liquid nitrogen and stored at -80°C. Blood was collected via cardiac puncture and stored on ice. Organ weights were measured at cull including liver (entire organ), ceacum and quadriceps muscle. Multiple fat depots were identified (Bagchi and MacDougald, 2019) and collected including, gonadal white adipose tissue (fat pad surrounding testes or ovaries), subcutaneous white adipose tissue (posterior section made up of dorsolumbar, inguinal and gluteal depots), and brown adipose tissue (interscapular). Serum was collected and stored at -80°C until further analysis.

3.2.7 Glucose metabolism

Glucose tolerance tests were performed on all studs at 18 weeks and male and female offspring

(n=8-10/sex/group) at 10 and 20 weeks of age. Mice were fasted for 4 hours prior to testing. Basal blood samples were obtained by tail tipping and blood glucose measured using a clinical glucometer (Accu-Chek Performa, Roche Diagnostics Australia Pty Ltd). Glucose (2g kg-1 lean mass) was then administered via oral gavage. Blood was collected at baseline, 15, 30, 45, 60 and 90 min from the original tail wound and serial tail tipping was not required. The incremental area under the curve (iAUC) was calculated. The iAUC indicates the time taken to clear a bolus dose of glucose from the bloodstream.

3.2.8 Insulin

Blood was collected from tail-tipping at a basal timepoint after 4 h of fasting from 18-week-old studs and 10- and 20-week-old pups (no-choice offspring) for the analysis of insulin levels using an enzyme-linked immunosorbent assay (ELISA) following manufacturer's instructions (Crystal Chem IL) (n=8-10/sex/treatment group).

3.2.9 Metabolic cage phenotyping

To determine whole-animal metabolic rate, substrate utilisation and activity, 6-8 male and female mice per treatment group were housed individually and assessed by indirect calorimetry in a Promethion high-definition behavioural and continuous respirometry system for mice (Sable Systems International, NV, USA) around 9-12 weeks of age. Oxygen consumption (VO2) and carbon dioxide production (VCO2) were measured over 48 h, following an 8-h acclimation period, with mice maintained at 22°C under a 12:12 hour light: dark cycle. Energy expenditure is presented as kcal/h/g lean mass. Mice were not given access to a running wheel.

3.2.10 Statistical analysis

Data are presented as means ± SEM and statistical significance determined when $P < 0.05$. Stud data was analysed using ANOVA in GraphPad Prism (v9.0.2; GraphPad Software Inc., La Jolla, CA, USA). Post hoc analysis was performed using Tukey's multiple comparisons test unless otherwise stated in the relevant figure legends. Offspring data was analysed using mixed effect models in R (RStudio version 1.4.1717; Vienna, Austria) with lme4 (Bates et al 2015) and LmerTest (Kuznetsova et al 2017) packages. Stud diet, Pup diet, Sex, and their interactions

were included as fixed effects. Dam was included as a random variable to account for litter effects. Litter size at weaning and body weight were initially included as covariates in all models and then removed if shown to not have a significant effect. Offspring data were analysed with both sexes included in the model to measure main effect of sex but were separated into males and females in figures to allow better visualization of trends in each sex. All other statistical parameters are included in the figure legends.

3.3 Results

3.3.1 Increased dietary fat, but not carbohydrate source, promotes obesity without a decline in mating success in studs.

High fat-fed studs were heavier than both control and high sucrose studs when measured at 18 weeks of age, 14 weeks after commencement on each allocated diet (Figure 3.1A, Table 3.2). The increased body weight observed in high fat studs was attributed to elevated fat mass, which was significantly higher than both control and high sucrose studs (Figure 3.1B, Table 3.2), whereas there was no significant difference in lean mass across groups (Figure 3.1C, Table 3.2). This was also reflected in the tissue weights of the animals measured upon culling, where different fat sources, and seminal vesicles from high fat animals had increased mass compared to the other two groups (Figure 3.2). Total energy intake was not detected to be significant across the three diets (Figure 3.1D) but was positively correlated to body weight (R^2= 0.0988. P=0.0206). Although high fat studs had higher basal glucose than control studs (Figure 3.1E), glucose tolerance was found to be similar across all groups (Table 3.2) as indicated by the incremental area under the curve (Figure 3.1F-G). Fasting insulin results followed the same trend as body weight and fat mass outcomes (Figure 3.1H), where high fat groups showed elevated levels compared to both chow and high sucrose cohorts (Table 3.2). No significant differences were found in the average number of pups per litter and mating success between paternal diet groups (Figure 3.3A-C). An interaction between stud diet and sex was observed in the number of male to female offspring in the litters (Figure 3.3; P= 0.0472).

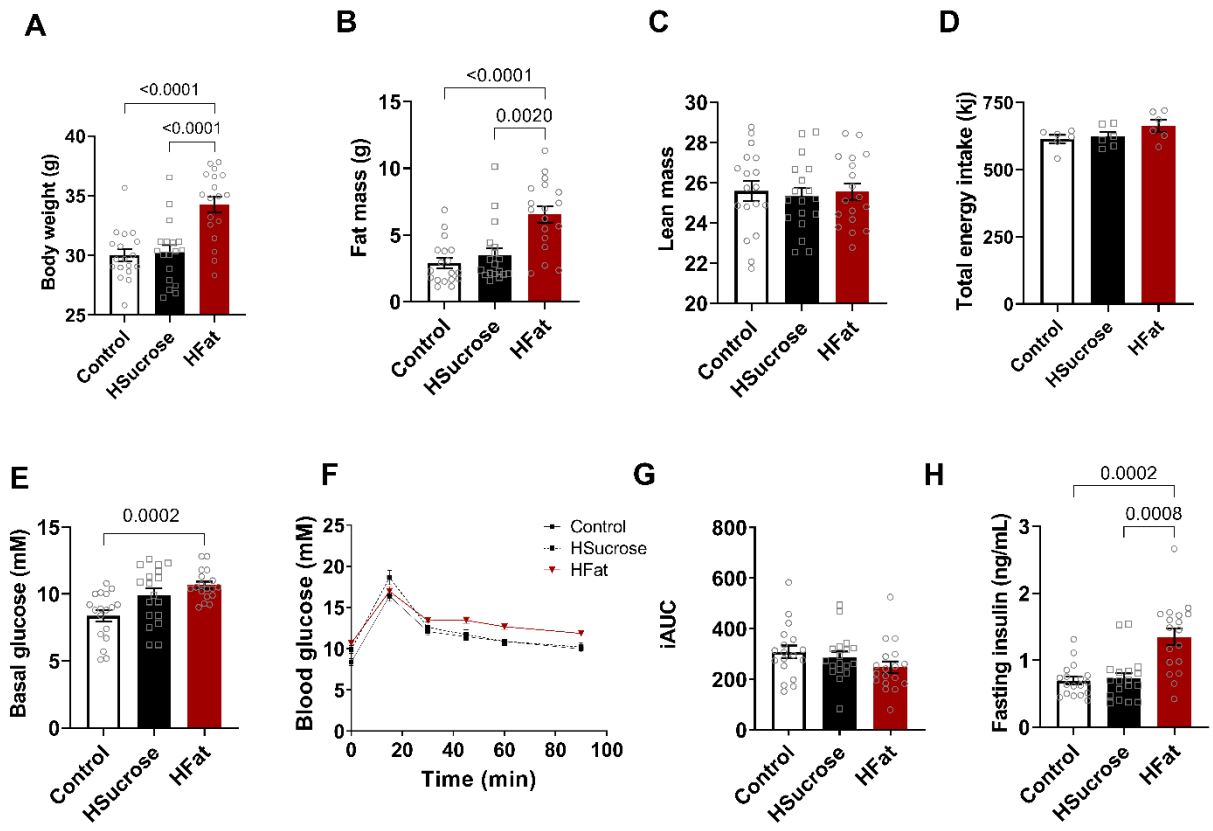

Figure 3.1 Increased dietary fat, not carbohydrate source, promotes obesity without a decline in mating success in studs.

Various outcomes were measured in 18-week old studs, including (A) body weights; (B) fat mass; (C) lean mass; (D) total energy intake from 3-18 weeks of age; (E) basal glucose; (F) glucose tolerance curve from an oGTT as indicated by an (G) incremental area under the curve (iAUC) and (H) fasting insulin. n=18/diet. For all bar graphs, an ANOVA was used for analysis. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

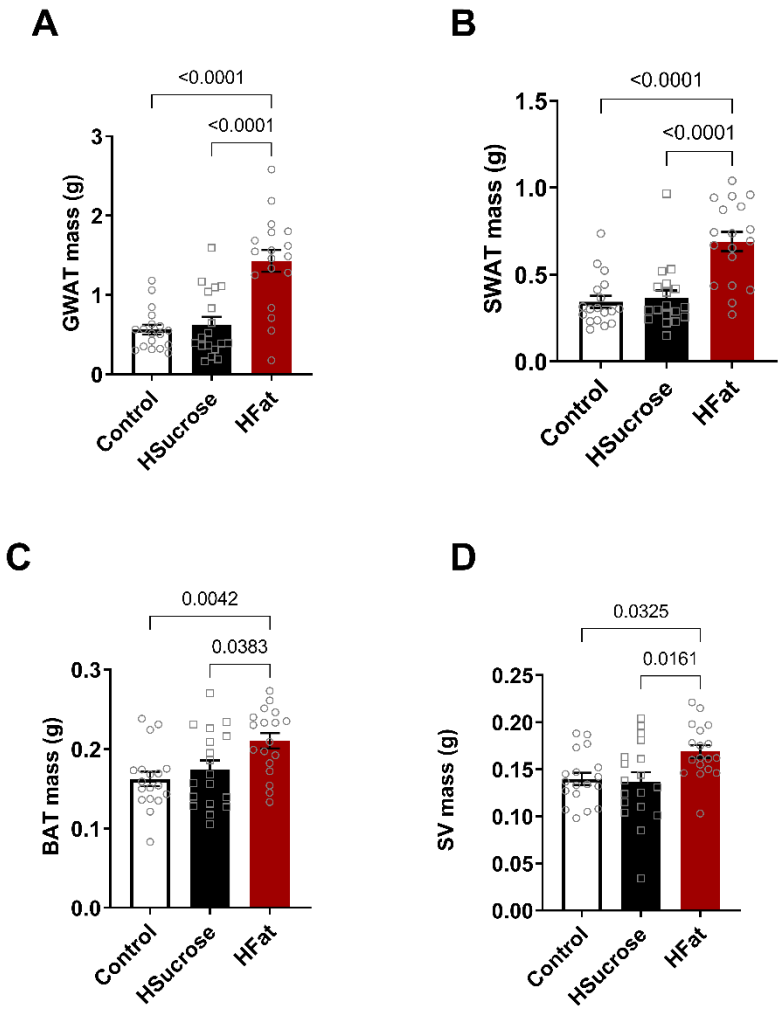

Figure 3.2 Tissue weights of male studs collected at 18 weeks old.

(A) Gonadal white adipose tissue (GWAT) mass, (B) Subcutaneous white adipose tissue (SWAT) mass, (C) Brown adipose tissue (BAT) (D) Seminal vesicle mass. n = 18 animals/sex/group. For all bar graphs, an ANOVA was used. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

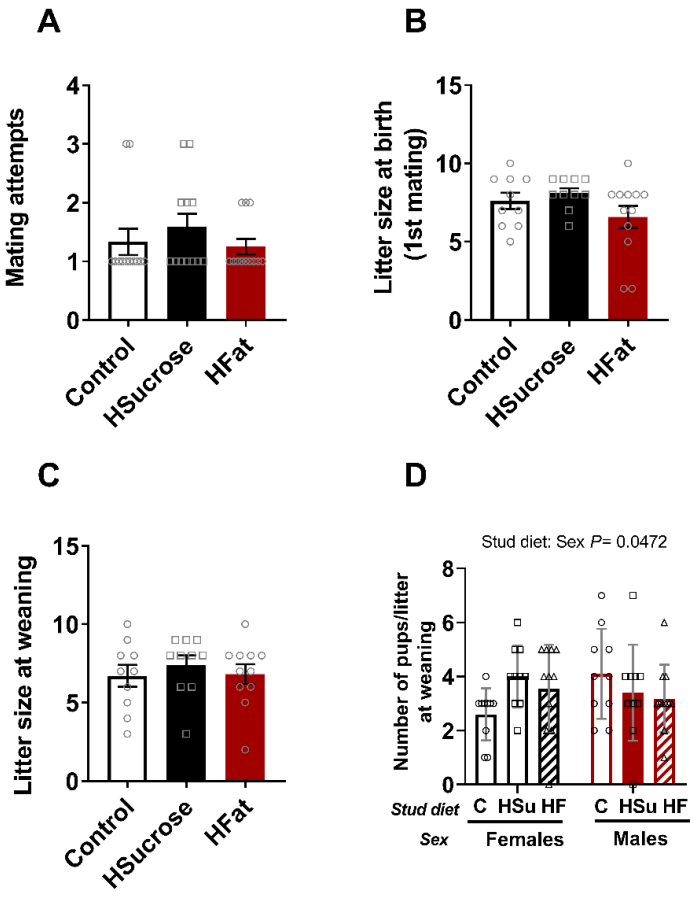

Figure 3.3 Mating results for 18-week-old studs and paired dam.

(A) Mating attempts taken to produce a litter of pups; Average pup weights in litter for Day 3 (B) and Day 21 (C). (D) Number of pups per litter at Day 21 separated by sex indicating both males and females. n = 18 animals/sex/group. For all bar graphs, an ANOVA was used. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

Table 3.2 Statistical summary (one-way ANOVA) of stud metabolic results. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	ANOVA table	Sum.Sq	DF	F (DFn, DFd)	P value	P value
3.1A	**Body weight**	Treatment (between columns)	205.5	2	F (2, 51) = 15.60	P<0.0001	***
		Residual (within columns)	335.9	51			
		Total	541.4	53			
3.1B	**Fat mass**	Treatment (between columns)	137.7	2	F (2, 51) = 14.18	P<0.0001	***
		Residual (within columns)	247.7	51			
		Total	385.4	53			
3.1C	**Lean mass**	Treatment (between columns)	0.723	2	F (2, 51) = 0.1029	P=0.9024	NS
		Residual (within columns)	179.2	51			
		Total	179.9	53			
3.1E	**Basal glucose**	Treatment (between	48.82	2	F (2, 51) = 8.303	P=0.0008	**

		columns)					
		Residual (within columns)	149.9	51			
		Total	198.7	53			
3.1G	**iAUC**	Treatment (between columns)	34408	2	F (2, 51) = 1.729	P=0.1877	NS
		Residual (within columns)	507435	51			
		Total	541843	53			
3.1H	**Fasting insulin**	Treatment (between columns)	4.872	2	F (2, 51) = 15.95	P<0.0001	***
		Residual (within columns)	7.789	51			
		Total	12.66	53			

3.3.2 Offspring macronutrient selection is not influenced by paternal diets

Upon weaning, 1 male and 1 female pup from each stud were subjected to a food choice experiment where they were given simultaneous access to Control and Western diets. Energy intake was measured for each food type, and total intake was also calculated. We found that for both sexes, paternal diet did not influence food selection and the amounts ingested by offspring across all groups were not significantly different (Figure 3.4A-F; Table 3.3). This result was mirrored in body weight and composition for both female and male offspring, measured at 12 weeks of age upon completion of the choice experiment. Here, we detected no significant differences for the body weights, fat mass and lean mass for both sexes (Table 3.3), although variation among individuals was high (Figure 3.4G-L).

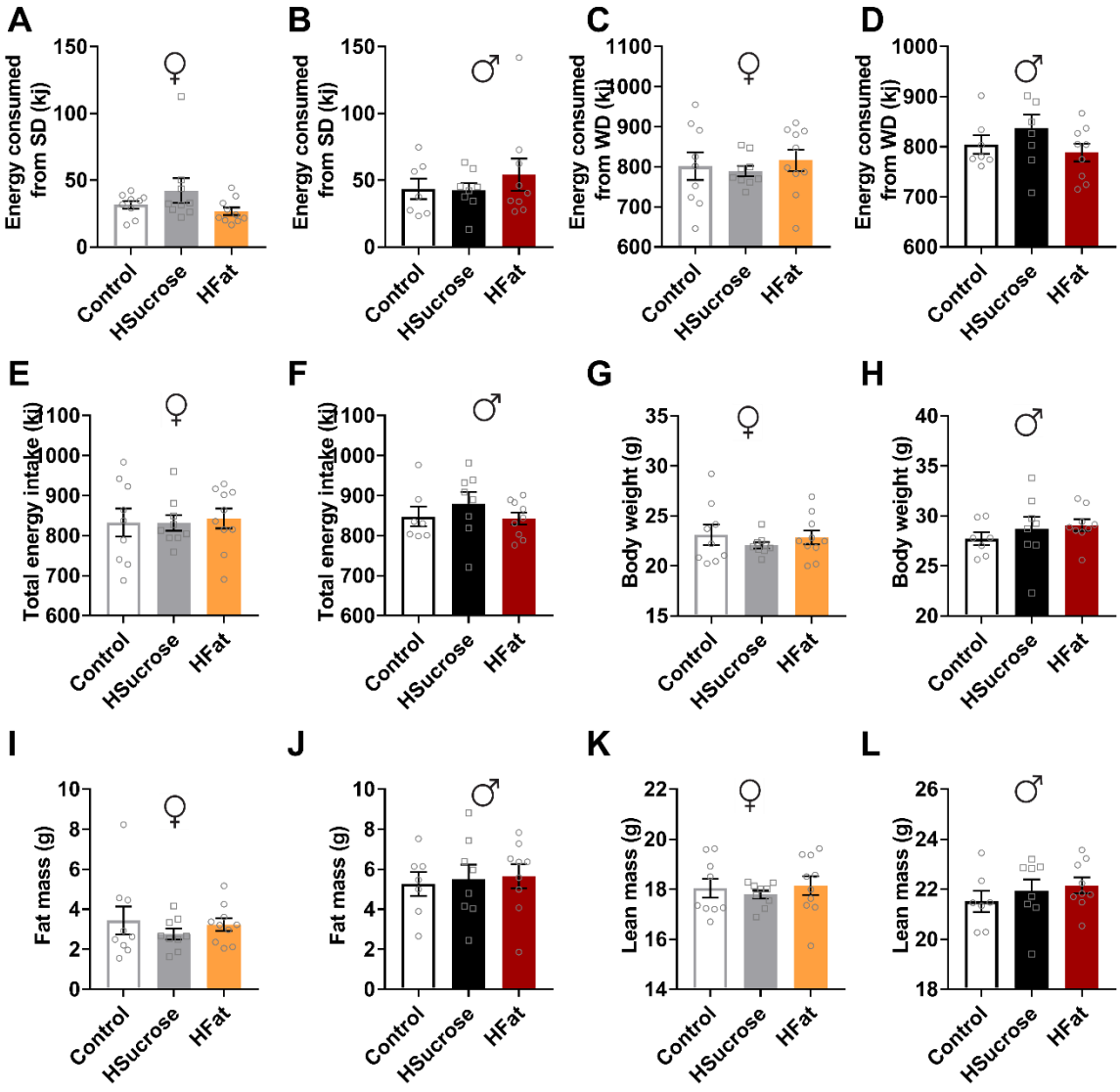

Figure 3.4 Offspring macronutrient selection is not influenced by paternal diets

Three-week old C57BL/6Arc mice given a simultaneous choice between two diets where individual energy intake of control and western diet was measured for (A,C) female and (B,D) male offspring. Total energy intake was also calculated for both (E) female and (F) male pups. Body composition outcomes were measured, including body weight at 12 weeks of age for (F) female and (G) male offspring; fat mass at 12 weeks of age for (H) female and (I) male offspring; lean mass at 12 weeks of age for (J) female and (K) male offspring. (n = 7-10 animals/sex/group). For all bar graphs, an ANOVA was used. All bars indicate means ± SEM.

Table 3.3 Statistical summary (ANOVA) of offspring choice experiment results for the interactive and main effects of pup diet and sex. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	ANOVA table	SS	DF	F (DFn, DFd)	P value	Sig
3.4A,B	**SD consumed (kj)**	Interaction	1663	2	F (2, 46) = 1.719	0.1905	NS
		Stud diet	183	2	F (2, 46) = 0.1892	0.8283	NS
		Sex	2230	1	F (1, 46) = 4.612	0.0370	*
		Residual	22246	46			
3.4C,D	**WD consumed (kj)**	Interaction	12897	2	F (2, 46) = 1.243	0.2980	NS
		Stud diet	1327	2	F (2, 46) = 0.1279	0.8802	NS
		Sex	784.1	1	F (1, 46) = 0.1512	0.6992	NS
		Residual	238600	46			
3.4E,F	**Total food consumed (kj)**	Interaction	5440	2	F (2, 46) = 0.4844	0.6192	NS
		Stud diet	2270	2	F (2, 46) = 0.2021	0.8177	NS
		Sex	5660	1	F (1, 46) = 1.008	0.3207	NS
		Residual	258305	46			
3.4G,H	**Body weight**	Interaction	9.448	2	F (2, 46) = 0.8755	0.4235	NS
		Stud diet	3.825	2	F (2, 46) =	0.7035	NS

					0.3544		
		Sex	435.2	1	F (1, 46) = 80.65	<0.0001	***
		Residual	248.2	46			
3.4I,J	**Fat mass**	Interaction	1.792	2	F (2, 46) = 0.3458	0.7095	NS
		Stud diet	0.8952	2	F (2, 46) = 0.1727	0.8419	NS
		Sex	69.35	1	F (1, 46) = 26.76	<0.0001	***
		Residual	119.2	46			
3.4K,L	**Lean mass**	Interaction	1.038	2	F (2, 46) = 0.4666	P=0.6301	NS
		Stud diet	1.308	2	F (2, 46) = 0.5878	P=0.5597	NS
		Sex	193	1	F (1, 46) = 173.5	P<0.0001	***
		Residual	51.17	46			

3.3.3 Sex specific responses to paternal and offspring diets are observed in body composition and metabolic outcomes of offspring.

A subset of male and female offspring from the three paternal diet groups were subjected to a no-choice allocation of either a Control (CD) or Western diet (WD) and various outcomes were measured over time (Figure 3.5). Total energy intake consumed up to 10 weeks of age was influenced by pup diet and sex, but no significant influence of paternal diet was detected (Table 3.4). We found that at 10 weeks of age, although all WD-fed mice had higher energy intakes compared to CD mice, the magnitude of this effect was more prominent in females (Figure 3.5A; Table 3.4).

Both paternal and offspring diets impacted various body composition measures for both sexes when measured at 10 weeks of age, but to different degrees. When looking at offspring body weights, we found an interactive effect between stud diets and sex, as well as pup diets and sex (Table 3.4). Male offspring of fathers fed high-fat diets were heavier when fed either a control or western diet, whereas paternal diet appeared to have little influence on the weight of female offspring at 10 weeks (Figure 3C,D). The distinction between CD and WD groups was more defined in females, where WD mice were heavier (Figure 3.5C; Table 3.4). Looking at fat mass, we detected a main effect of pup diet and sex, but no effect of stud diet (Figure 3.5E,F; Table 3.4). For lean mass, a stud diet:sex and stud diet:pup diet interaction was observed (Figure 3.5G,H; Table 3.4). As for body weight, effects of paternal diet on lean mass were stronger in male offspring than female offspring. The lean mass of pups from high-fat fathers was greater than control pups regardless of whether they were fed a control or western diet. However, the effect of a paternal high-sugar diet depended on the pup diet, where increased lean mass was observed on a western diet only (Figure 3.5G,H).

No significant effects of stud diet were detected on outcomes of glucose tolerance tests of offspring at 10 weeks of age. No significant differences were observed for the incremental area under the curve (iAUC) levels of the pups, a measure of glucose tolerance (Figure 3.5K,L; Table 3.4). For basal glucose levels, only pup diets yielded a significant effect, where WD fed offspring showed elevated levels for both sexes (Figure 3.5M,N; Table 3.4). In terms of fasting insulin, we detected a main effect of pup diet and sex (Figure 3.5O,P; Table 3.4).

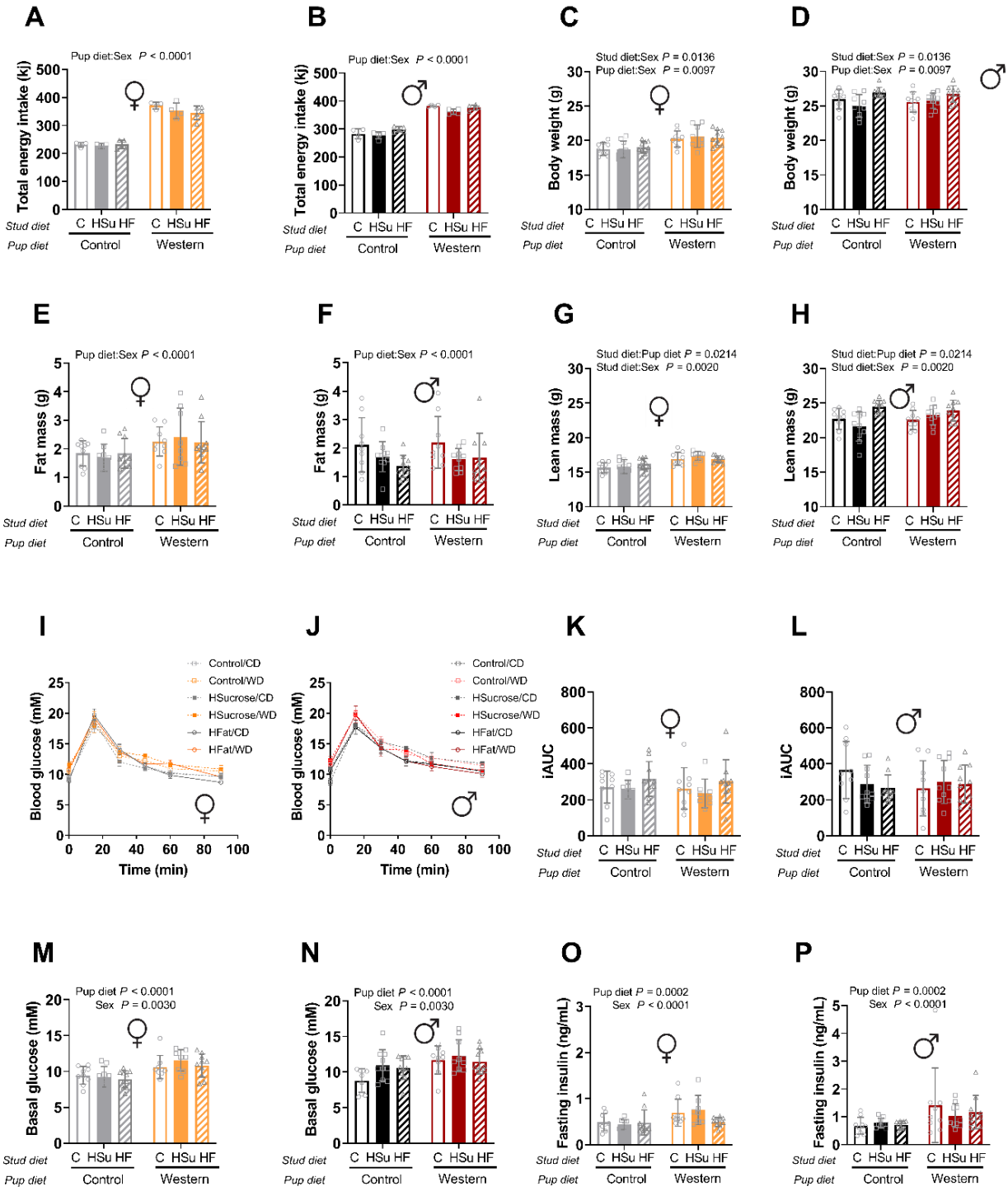

Figure 3.5 Sex specific responses to paternal and offspring diets are observed in body composition and metabolic outcomes of offspring.

Average cumulative food intake was measured up to 10 weeks of age for (A) Females and (B) Males (n=4-5 cages/sex/treatment group). Body weight at 10 weeks of age for (C) female and (D) male offspring. Fat mass at 10 weeks of age for (E) female and (F) male offspring. Lean

mass at 10 weeks of age for (G) female and (H) male offspring. Blood glucose levels and iAUC levels from an oGTT for (I-J) female and (K-L) male offspring (n = 8-10 animals/sex/group). Basal glucose for (M) female and (N) male offspring and fasting insulin for (M) female and (N) male offspring after 4 hrs of fasting. All bars indicate means ± SEM. Significant p-values are provided in a statistical summary table.

Table 3.4 Statistical summary (ANOVA) of 10-week timepoint results (adjusted for litter size when appropriate) for the main effects of sex, stud diet, pup diet; two-way interactions for stud diet:pup diet, stud diet:sex, pup diet:sex; and three-way stud diet:pup diet:sex interaction. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	Factor	Sum.Sq	DF	F.value	Pr..F.	Sig. level
3.5A,B	Total energy intake (kJ)	Stud diet	1266.3240	2	2.8338	0.070639	NS
		Pup diet	146621.6000	1	656.2198	1.98E-26	***
		Sex	17219.6800	1	77.0684	7.18E-11	***
		Stud diet: Pup diet	1356.0090	2	3.0345	0.059297	NS
		Stud diet: Sex	1073.1600	2	2.4015	0.103526	NS
		Pup diet: Sex	4638.4350	1	20.7598	4.81E-05	***
		Stud diet: Pup diet: Sex	17.0866	2	0.0382	0.962521	NS
3.5C,D	Body weight (g)	Stud diet	12.0419	2	3.2982	0.04101	*
		Pup diet	24.5103	1	13.4263	0.000399	***
		Sex	1104.2562	1	604.8904	3.37E-44	***
		Litter	9.6629	1	5.2932	0.023486	*
		Stud diet: Pup diet	7.5972	2	2.0808	0.130203	NS

		Stud diet: Sex	16.3779	2	4.4858	0.013625	*
		Pup diet: Sex	12.6919	1	6.9524	0.009706	**
		Stud diet: Pup diet: Sex	2.6593	2	0.7284	0.485247	NS
3.5E,F	**Fat mass (g)**	Stud diet	1.6067	2	1.9727	0.157726	NS
		Pup diet	2.5993	1	6.3827	0.013537	*
		Sex	2.0347	1	4.9963	0.02822	*
		Stud diet: Pup diet	0.0380	2	0.0467	0.954395	NS
		Stud diet: Sex	2.0085	2	2.4660	0.091428	NS
		Pup diet: Sex	1.1803	1	2.8982	0.09279	NS
		Stud diet: Pup diet: Sex	0.6445	2	0.7913	0.456996	NS
3.5G,H	**Lean mass (g)**	Stud diet	13.0208	2	4.8528	0.016598	*
		Pup diet	16.1692	1	12.0524	0.000853	***
		Sex	1233.5597	1	919.4895	1.07E-44	***
		Litter	6.1660	1	4.5961	0.039421	*
		Stud diet: Pup diet	10.8384	2	4.0394	0.02149	*
		Stud diet: Sex	18.0158	2	6.7145	0.002047	**
		Pup diet: Sex	5.0498	1	3.7641	0.056198	NS

		Stud diet: Pup diet: Sex	3.5025	2	1.3054	0.277275	NS
3.5K,L	**iAUC**	Stud diet	8753.1835	2	0.4066	0.670238	NS
		Pup diet	10947.4690	1	1.0171	0.31645	NS
		Sex	12302.7083	1	1.1430	0.288411	NS
		Stud diet: Pup diet	20603.1720	2	0.9571	0.388654	NS
		Stud diet: Sex	43253.8467	2	2.0093	0.141158	NS
		Pup diet: Sex	362.7026	1	0.0337	0.854869	NS
		Stud diet: Pup diet: Sex	26211.1745	2	1.2176	0.301966	NS
3.5M,N	**Basal glucose (mM)**	Stud diet	7.1046	2	1.4919	0.24186	NS
		Pup diet	82.4793	1	34.6401	9.42E-08	***
		Sex	22.3846	1	9.4012	0.002972	**
		Stud diet: Pup diet	1.8321	2	0.3847	0.681915	NS
		Stud diet: Sex	6.6976	2	1.4064	0.251107	NS
		Pup diet: Sex	0.0257	1	0.0108	0.917459	NS
		Stud diet: Pup diet: Sex	10.8600	2	2.2805	0.109251	NS

3.5O,P	**Fasting insulin (ng/mL)**	Stud diet	0.3648	2	0.8445	0.432807	NS
		Pup diet	3.2230	1	14.9221	0.000199	***
		Sex	4.7135	1	21.8233	9.33E-06	***
		Litter	1.2980	1	6.0098	0.015961	*
		Stud diet: Pup diet	0.2966	2	0.6865	0.505676	NS
		Stud diet: Sex	0.1955	2	0.4525	0.637313	NS
		Pup diet: Sex	0.7232	1	3.3484	0.070251	NS
		Stud diet: Pup diet: Sex	0.4055	2	0.9387	0.394565	NS

3.3.4 Paternal diet effects on offspring body composition and metabolic outcomes are no longer evident at 20 weeks.

By 20 weeks of age, significant effects of paternal diet on offspring body and metabolic traits measured were no longer detected. Analysis of total energy intake at 20 weeks showed similar trends previously found at the earlier 10-week timepoint, with interactive effects of pup diet and sex but no effect of stud diet (Figure 3.6C,D; Table 3.5). Effects of paternal diet on offspring body weight (Figure 3.6E-H) and lean mass (Figure 3.6K,L) appeared to diminish over time, with no significant effect of stud diet detected at 20 weeks of age (Table 3.5). As expected, WD-fed female and male pups had increased body weight, lean mass and fat mass compared to SD-fed offspring (Figure 3.6E-L; Table 3.5). Again, this distinction between SD and WD groups was stronger in females than males, resulting in an interaction between pup diet and sex for body weight, fat mass and lean mass outcomes (Table 3.5). At this later time point, a main effect of sex was detected in the glucose tolerance of the offspring (Figure 3.6M-P; Table 3.5). Similar to the 10-week results, only pup diets led to a significant effect in basal glucose levels, where WD fed offspring showed an increase for both sexes (Figure 3.6Q,R; Table 3.5). In terms of fasting insulin, we detected an interactive effect of pup diet and sex (Figure 3.6S,T; Table 3.5).

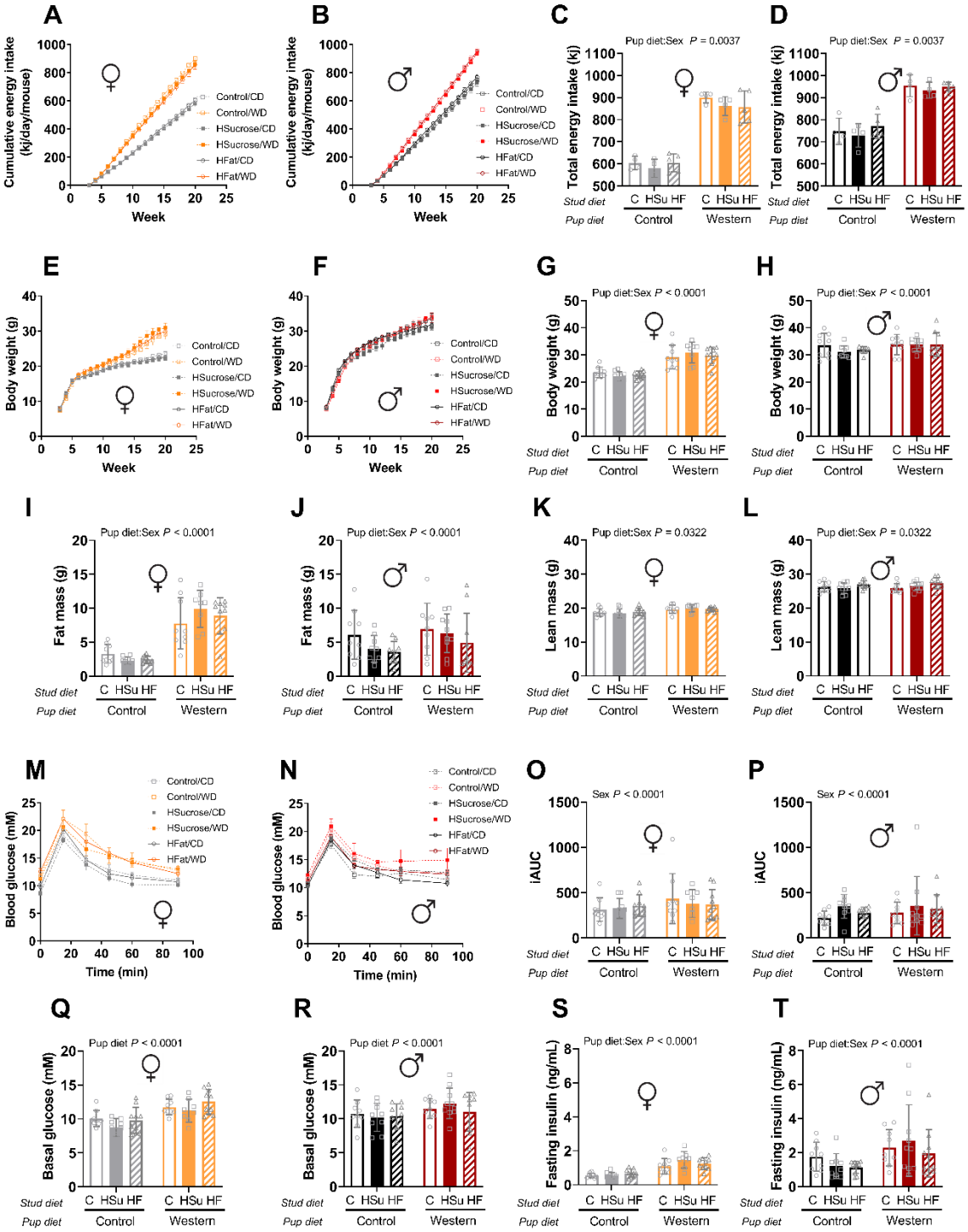

Figure 3.6 Paternal diet effects on offspring body composition and metabolic outcomes are no longer evident at 20 weeks.

Cumulative energy intake over time for (A) female and (B) male offspring, also displayed as total energy intake for (C) female and (D) male offspring. Cumulative body weight trends over

time for (E) female and (F) male offspring, also shown as weight at 20 weeks for (C) female and (D) male offspring. Fat mass at 46 weeks for (I) female and (J) male offspring. Lean mass at 20 weeks for (K) female and (L) male offspring. Blood glucose levels and iAUC levels from an oGTT for (M,O) female and (N,P) male offspring (n = 15-18 animals/sex/group). Basal glucose for (Q) female and (R) male offspring and fasting insulin for (S) female and (T) male offspring after 4 hrs of fasting. (n = 8-10 animals/sex/group). All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

Table 3.5 Statistical summary (ANOVA) of 20-week timepoint results (adjusted for litter size and/or body weight when appropriate) for the main effects of sex, stud diet, pup diet; two-way interactions for stud diet:pup diet, stud diet:sex, pup diet:sex; and three-way stud diet:pup diet:sex interaction. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	Factor	Sum.Sq	DF	F.value	Pr..F.	Sig. level
3.6C, D	Total energy intake (kJ)	Stud diet	5924.167	2	1.364172	0.267226	NS
		Pup diet	720697.3	1	331.9133	5.68E-21	***
		Sex	164211.7	1	75.62685	9.23E-11	***
		Stud diet: Pup diet	3183.03	2	0.732964	0.486824	NS
		Stud diet: Sex	1974.034	2	0.454566	0.637962	NS
		Pup diet: Sex	20614.04	1	9.493686	0.003722	**
		Stud diet: Pup diet: Sex	172.3566	2	0.039689	0.961126	NS
3.6G, H	Body weight (g)	Stud diet	4.710149	2	0.258644	0.773918	NS
		Pup diet	544.8571	1	59.83844	3.13E-11	***
		Sex	1203.885	1	132.2155	1.65E-18	***
		Stud diet: Pup diet	34.97884	2	1.92076	0.153398	NS
		Stud diet: Sex	8.905511	2	0.48902	0.615082	NS
		Pup diet: Sex	209.231	1	22.9786	7.93E-	***

			1		2	06	
		Stud diet: Pup diet: Sex	0.890315	2	0.048889	0.952317	NS
3.6I,J	**Fat mass (g)**	Stud diet	19.76444	2	1.333822	0.279211	NS
		Pup diet	410.5905	1	55.41818	1.09E-10	***
		Sex	5.967651	1	0.805465	0.372183	NS
		Stud diet: Pup diet	23.986	2	1.618718	0.204711	NS
		Stud diet: Sex	30.05732	2	2.028447	0.138314	NS
		Pup diet: Sex	151.2104	1	20.40915	2.22E-05	***
		Stud diet: Pup diet: Sex	3.447309	2	0.232645	0.79299	NS
3.6K, L	**Lean mass (g)**	Stud diet	4.137684	2	1.718379	0.200675	NS
		Pup diet	11.11546	1	9.232493	0.003315	**
		Sex	1415.626	1	1175.818	1.48E-46	***
		Stud diet: Pup diet	1.9453	2	0.807883	0.449818	NS
		Stud diet: Sex	4.597597	2	1.909381	0.155547	NS
		Pup diet: Sex	5.748934	1	4.775061	0.032211	*
		Stud diet: Pup diet: Sex	1.161638	2	0.482428	0.619313	NS
3.6O,	**iAUC**	Stud diet	39285.1	2	0.70499	0.49657	NS

P			5		5	4	
		Pup diet	8.081287	1	0.00029	0.986446	NS
		Sex	223493.1	1	8.021428	0.005601	**
		Body weight 20 wk	112518	1	4.038402	0.047197	*
		Stud diet: Pup diet	43207.57	2	0.775385	0.463303	NS
		Stud diet: Sex	87480.35	2	1.569886	0.213204	NS
		Pup diet: Sex	6827.644	1	0.245052	0.621678	NS
		Stud diet: Pup diet: Sex	12251.37	2	0.219858	0.803024	NS
3.6Q, R	**Basal glucose (mM)**	Stud diet	2.236939	2	0.347418	0.707368	NS
		Pup diet	14.33768	1	4.453555	0.037349	*
		Sex	9.59531	1	2.980486	0.087393	NS
		Body weight 20 wk	33.23493	1	10.3234	0.001773	**
		Stud diet: Pup diet	1.385112	2	0.215121	0.80682	NS
		Stud diet: Sex	16.83508	2	2.614648	0.078243	NS
		Pup diet: Sex	0.11477	1	0.03565	0.850627	NS
		Stud diet: Pup diet: Sex	3.96129	2	0.615226	0.542572	NS
3.6S,T	**Fasting**	Stud diet	0.61670	2	0.68822	0.51083	NS

	insulin (ng/mL)		5		7	2	
		Pup diet	0.005048	1	0.011266	0.915713	NS
		Sex	1.137848	1	2.539621	0.11445	NS
		Body weight 20 wk	29.76641	1	66.43715	1.25E-12	***
		Stud diet: Pup diet	0.444745	2	0.496325	0.610727	NS
		Stud diet: Sex	1.519904	2	1.696174	0.190189	NS
		Pup diet: Sex	9.812086	1	21.90008	1.15E-05	***
		Stud diet: Pup diet: Sex	0.687585	2	0.767328	0.467891	NS

3.3.5 Tissue weights of female and male offspring collected at 21 weeks old.

An interaction between pup diet and sex was detected for the liver mass of the pups where, similar to body composition results the above, the difference between SD and WD groups was more distinct in females than males (Figure 3.7A,B; Table 3.6). The liver mass of male pups remained similar regardless of stud or pup diet. An interaction between stud diet and sex were found in the offspring gonadal white adipose tissue (GWAT; Figure 3.7C,D; Table 3.6) and subcutaneous white adipose tissue (SWAT; Figure 3.7E,F; Table 3.6) masses. Meanwhile, only main effects of pup diet and sex were observed in the brown adipose tissue (BAT; Figure 3.7G,H; Table 3.6) mass. A main effect of stud diet and sex was found for the mass of quadriceps muscles collected (Figure 3.7I,J; Table 3.6). A significant main effect of stud diet, pup diet and sex were all detected in the caecum mass of offspring (Figure 3.7K,L; Table 3.6).

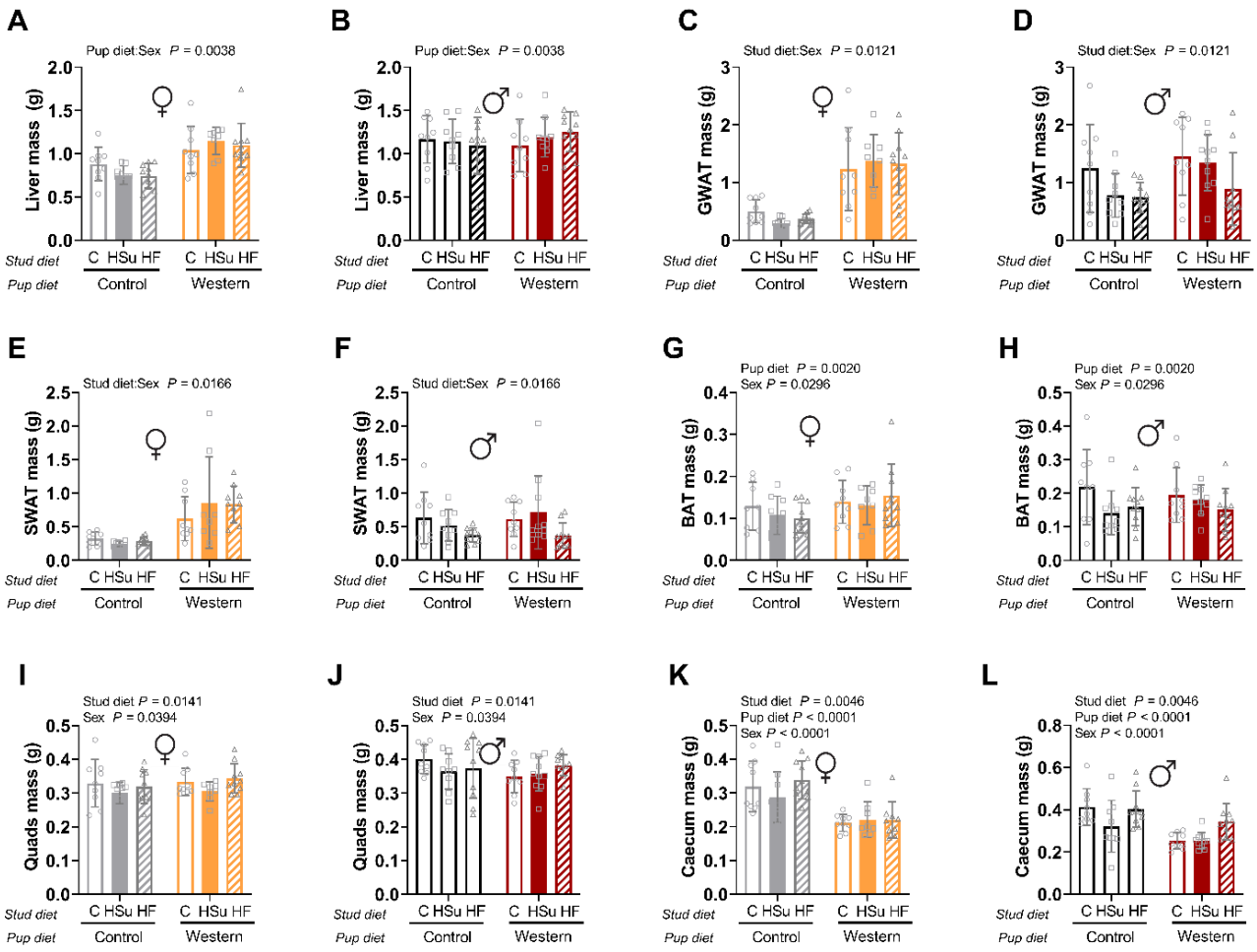

Figure 3.7 Tissue weights of female and male offspring collected at 21 weeks old.

Liver mass for (A) females and (B) males, Gonadal white adipose tissue (GWAT) mass for (C) females and (D) males, Subcutaneous white adipose tissue (SWAT) mass for (E) females and (F) males, Brown adipose tissue (BAT) for (G) females and (H) males. , Quadriceps muscle mass for (I) females and (J) males, Caecum mass for (K) females and (L) males n = 8-10 animals/sex/group. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

Table 3.6 Statistical summary (ANOVA) of tissue collection data at 21 weeks of age (adjustments for body weight shown) for the main effects of sex, stud diet, pup diet; two-way interactions for stud diet:pup diet, stud diet:sex, pup diet:sex; and three-way stud diet:pup diet:sex interaction. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	Factor	Sum.Sq	DF	F.value	Pr..F.	Sig. level
3.7A, B	**Liver mass (g) body weight adjusted**	Stud diet	0.011416	2	0.13587	0.873538	NS
		Pup diet	0.007641	1	0.181898	0.670775	NS
		Sex	0.003995	1	0.095103	0.75847	NS
		Body weight 20 wk	1.082427	1	25.76659	1.81E-06	***
		Stud diet: Pup diet	0.105834	2	1.259662	0.289586	NS
		Stud diet: Sex	0.064007	2	0.761822	0.470296	NS
		Pup diet: Sex	0.024187	1	0.575766	0.450175	NS
		Stud diet: Pup diet: Sex	0.018012	2	0.21438	0.807537	NS
3.7A, B	**Liver mass (g)**	Stud diet	0.001532	2	0.0149	0.985218	NS
		Pup diet	0.860134	1	16.73692	1.04E-04	**
		Sex	1.238307	1	24.0956	4.91E-06	***
		Stud diet: Pup diet	0.226874	2	2.207314	0.116887	NS
		Stud diet: Sex	0.030526	2	0.296994	0.743883	NS
		Pup diet: Sex	0.457961	1	8.911235	0.003813	**
		Stud diet: Pup diet: Sex	0.027277	2	0.265381	0.767623	NS
3.7C, D	**GWAT mass (g)**	Stud diet	0.574351	2	2.744381	0.081093	NS

	body weight adjusted	Pup diet	0.243262	1	2.324726	0.130945	NS
		Sex	2.988776	1	28.56213	6.63E-07	***
		Body weight 20 wk	10.42122	1	99.59002	1.55E-16	***
		Stud diet: Pup diet	0.113747	2	0.543507	0.582935	NS
		Stud diet: Sex	0.979626	2	4.680878	0.012054	*
		Pup diet: Sex	1.07E-06	1	1.03E-05	0.997451	NS
		Stud diet: Pup diet: Sex	0.20938	2	1.000468	0.372555	NS
3.7C, D	**GWAT mass (g)**	Stud diet	0.94086	2	2.206438	0.128038	NS
		Pup diet	10.5548	1	49.50472	6.74E-10	***
		Sex	1.381374	1	6.479	1.29E-02	*
		Stud diet: Pup diet	0.594113	2	1.39327	0.254381	NS
		Stud diet: Sex	1.241004	2	2.910314	0.060341	NS
		Pup diet: Sex	2.613983	1	12.26025	0.000776	***
		Stud diet: Pup diet: Sex	0.144646	2	0.339214	0.713396	NS
3. 7E,F	**SWAT mass (g) body weight adjusted**	Stud diet	0.127303	2	1.079363	0.354153	NS
		Pup diet	0.069039	1	1.170726	0.282379	NS
		Sex	0.990988	1	16.80456	9.31E-05	***
		Body weight 20 wk	1.682804	1	28.53593	6.65E-07	***
		Stud diet: Pup diet	0.084618	2	0.717448	0.491378	NS
		Stud diet: Sex	0.511183	2	4.334162	0.016578	*
		Pup diet: Sex	1.53E-01	1	2.59E+00	0.111853	NS
		Stud diet: Pup diet: Sex	0.074021	2	0.6276	0.536754	NS

3. 7E,F	**SWAT mass (g)**	Stud diet	0.134708	2	0.876004	0.427551	NS
		Pup diet	2.031596	1	26.4228 3	2.07E-06	***
		Sex	0.000501	1	0.006522	9.36E-01	NS
		Stud diet: Pup diet	0.303069	2	1.970847	0.146414	NS
		Stud diet: Sex	0.560341	2	3.643884	0.030784	*
		Pup diet: Sex	1.22755	1	15.96545	0.00015	**
		Stud diet: Pup diet: Sex	0.065579	2	0.426457	0.654402	NS
3.7G, H	**BAT mass (g) body weight adjusted**	Stud diet	0.008847	2	1.940903	0.162116	NS
		Pup diet	0.02318	1	10.17097	0.001987	*
		Sex	0.011133	1	4.884806	2.96E-02	*
		Body weight 20 wk	0.117095	1	51.37983	1.59E-10	***
		Stud diet: Pup diet	0.000656	2	0.143979	0.866143	NS
		Stud diet: Sex	0.005965	2	1.308722	0.276161	NS
		Pup diet: Sex	8.53E-03	1	3.74E+00	0.056663	NS
		Stud diet: Pup diet: Sex	0.009329	2	2.0468	0.136389	NS
3.7G, H	**BAT mass (g)**	Stud diet	0.011395	2	1.707319	0.198877	NS
		Pup diet	0.006655	1	1.994393	1.62E-01	NS
		Sex	0.060165	1	18.02972	5.99E-05	***
		Stud diet: Pup diet	0.007032	2	1.05357	0.353679	NS
		Stud diet: Sex	0.009414	2	1.410543	0.250213	NS
		Pup diet: Sex	0.004728	1	1.416732	0.237681	NS
		Stud diet: Pup diet: Sex	0.007436	2	1.114239	0.333517	NS

3.7I,J	**Caecum mass (g) body weight adjusted**	Stud diet	0.058064	2	5.6878	0.004602	*
		Pup diet	0.135162	1	26.48042	1.37E-06	***
		Sex	0.0738	1	14.45853	2.49E-04	**
		Body weight 20 wk	0.00354	1	0.693637	4.07E-01	NS
		Stud diet: Pup diet	0.023153	2	2.267981	0.108929	NS
		Stud diet: Sex	0.017957	2	1.759076	0.177597	NS
		Pup diet: Sex	4.07E-04	1	7.97E-02	0.778341	NS
		Stud diet: Pup diet: Sex	0.015298	2	1.498541	0.228531	NS
3.7I,J	**Caecum mass (g)**	Stud diet	0.057801	2	5.679662	0.004623	**
		Pup diet	0.256854	1	50.47796	1.88E-10	***
		Sex	0.116657	1	22.92597	5.90E-06	***
		Stud diet: Pup diet	0.020568	2	2.021055	0.137947	NS
		Stud diet: Sex	0.017209	2	1.690966	0.189622	NS
		Pup diet: Sex	3.37E-05	1	0.006615	0.93534	NS
		Stud diet: Pup diet: Sex	0.015263	2	1.499783	0.228205	NS
3.7K, L	**Quad mass (g) body weight adjusted**	Stud diet	0.055433	2	4.889302	0.014977	*
		Pup diet	0.001095	1	0.193124	0.661447	NS
		Sex	0.012825	1	2.262348	1.36E-01	NS
		Body weight 20 wk	0.000324	1	0.057223	8.11E-01	NS
		Stud diet: Pup diet	0.001462	2	0.128923	0.879235	NS
		Stud diet: Sex	0.004696	2	0.414177	0.662365	NS
		Pup diet: Sex	1.56E-02	1	2.75E+00	0.100945	NS

		Stud diet: Pup diet: Sex	0.017174	2	1.514817	0.226584	NS
3.7K, L	**Quad mass (g)**	Stud diet	0.055841	2	4.967204	0.014065	*
		Pup diet	0.000787	1	0.139924	7.09E-01	NS
		Sex	0.024697	1	4.393704	3.94E-02	*
		Stud diet: Pup diet	0.001255	2	0.111658	0.894496	NS
		Stud diet: Sex	0.00483	2	0.429635	0.652295	NS
		Pup diet: Sex	0.017205	1	3.060839	0.084288	NS
		Stud diet: Pup diet: Sex	0.017274	2	1.536592	0.221806	NS

3.3.6 Stud diets had no effect on offspring energy expenditure at 9-12 weeks of age.

Both night-time (Figure 3.8,E; Table 3.7) and daytime energy expenditure was shown to have an interactive effect of both pup diet and sex (Figure 3.8C,F; Table 3.7). Meanwhile, a main pup diet and sex effect was shown for the offspring's respiratory quotient (RQ) at night (Figure 3.8H,K; Table 3.7), while only a main effect of diet was found to be significant during the day (Figure 3.8I,L; Table 3.7). No significant effects of stud diet were found throughout.

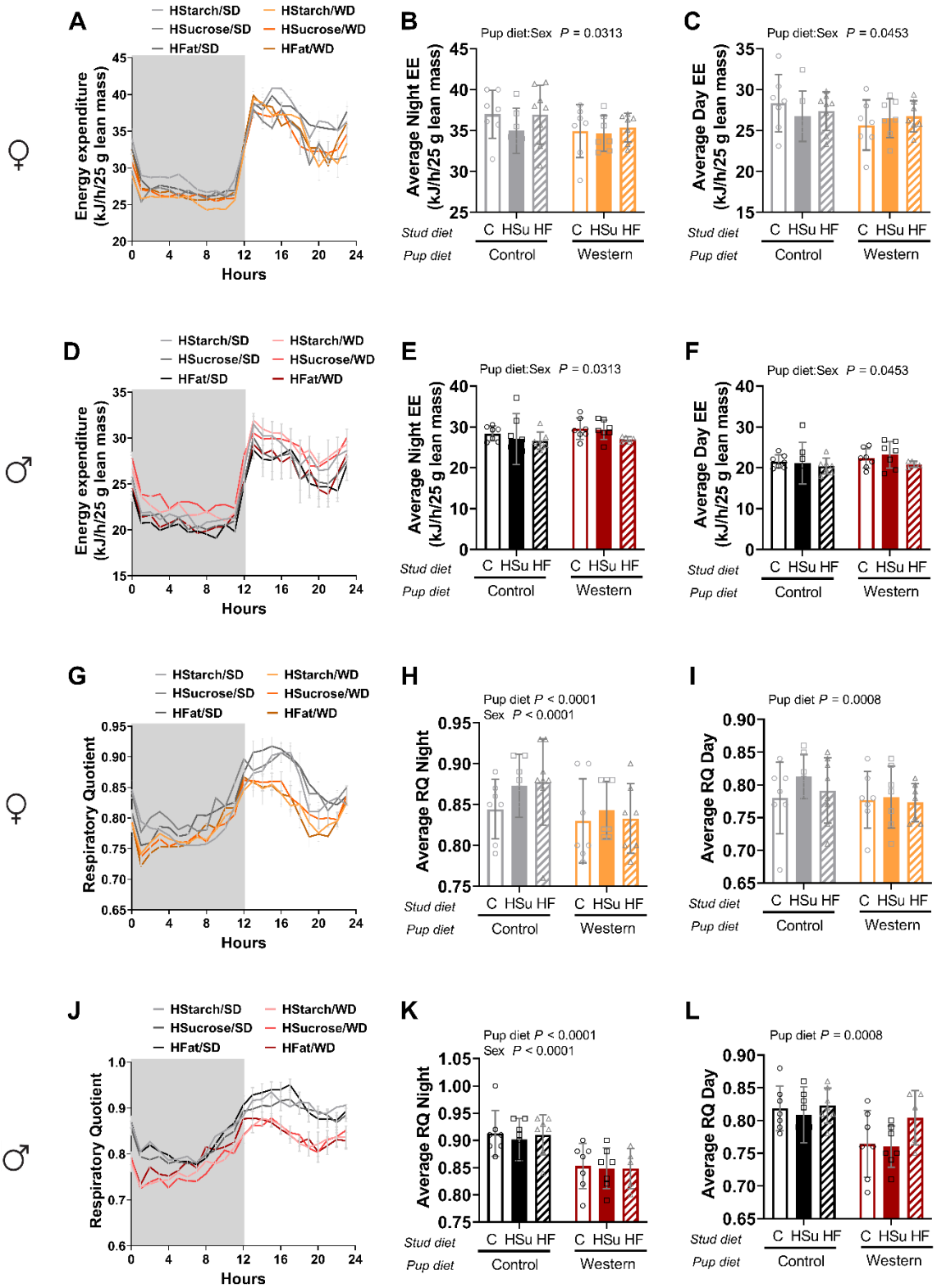

Figure 3.8 Stud diets had no effect on offspring energy expenditure at 9-12 weeks of age.

Energy expenditure was measured and normalised to 25 g lean mass between 9-12 weeks of

age for (A) female offspring. Average values were calculated for (B) night and (C) day periods (n = 6-8 animals/sex/group). Energy expenditure was measured and normalised to 25 g lean mass 45 weeks of age for (D) male offspring. Average values were calculated for (E) night and (F) day periods (n = 6-8 animals/sex/group). Respiratory quotient was measured at 45 weeks of age for (G) female offspring. Average values were calculated for (H) night and (I) day periods (n = 6-8 animals/sex/group). Respiratory quotient was measured at 45 weeks of age for (J) male offspring. Average values were calculated for (K) night and (L) day periods (n = 6-8animals/sex/group).

Table 3.7 Statistical summary (ANOVA) of Promethion metabolic cage system results for the main effects of sex, stud diet, pup diet; two-way interactions for stud diet:pup diet, stud diet:sex, pup diet:sex; and three-way stud diet:pup diet:sex interaction. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	Factor	Sum.Sq	DF	F.value	Pr..F.	Sig. level
3.8B,E	Energy intake night adjusted for lean mass	Stud diet	6.635693	2	0.561127	0.580424	NS
		Pup diet	0.007717	1	0.001305	0.971338	NS
		Sex	1041.365	1	176.1196	2.82E-17	***
		Stud diet: Pup diet	2.604671	2	0.220256	0.803151	NS
		Stud diet: Sex	26.86163	2	2.271471	0.114732	NS
		Pup diet: Sex	29.13026	1	4.926622	0.031328	*
		Stud diet: Pup diet: Sex	3.145184	2	0.265963	0.767619	NS
3.8C,F	Energy intake day adjusted for lean mass	Stud diet	0.587794	2	0.056834	0.944898	NS
		Pup diet	0.005253	1	0.001016	0.974702	NS
		Sex	494.141	1	95.55727	4.02E-13	***
		Stud diet: Pup diet	8.038227	2	0.777219	0.465182	NS
		Stud diet: Sex	11.22342	2	1.085196	0.345791	NS
		Pup diet: Sex	21.78189	1	4.212195	0.045348	*
		Stud diet: Pup diet: Sex	7.796721	2	0.753867	0.475783	NS
3.8H,K	RQ night	Stud diet	0.000847	2	0.244961	0.783394	NS
		Pup diet	0.039681	1	22.96352	8.78E-06	***
		Sex	0.017138	1	9.91789	2.40E-03	**
		Stud diet: Pup diet	0.000943	2	0.272958	0.76192	NS
		Stud diet: Sex	0.003115	2	0.901442	0.410579	NS

		Pup diet: Sex	0.004211	1	2.43675	0.122968	NS
		Stud diet: Pup diet: Sex	0.000777	2	0.224779	0.799259	NS
3.8I,L	**RQ day**	Stud diet	0.001127	2	0.391041	0.681034	NS
		Pup diet	0.01794	1	12.45244	0.000861	***
		Sex	0.001959	1	1.359975	0.248732	NS
		Stud diet: Pup diet	0.001286	2	0.44635	0.642297	NS
		Stud diet: Sex	0.00587	2	2.037349	0.140422	NS
		Pup diet: Sex	0.003171	1	2.201147	0.143602	NS
		Stud diet: Pup diet: Sex	0.002829	2	0.981854	0.381073	NS

3.4 Discussion

In this study, we aimed to characterise how adjusting macronutrient ratios (particularly carbohydrate and fat) while keeping energy and protein content constant, would influence stud and offspring food intake, body composition and metabolism. We observed that increasing the percentage of sucrose in the paternal diet had little influence on body weight, fat mass, or fasting insulin levels. In contrast, high fat diets in male studs led to the most obesogenic effect. Increasing the percentage of dietary fat (while keeping energy density and protein content constant) resulted in increased body weight, fat mass, fasting insulin and glucose levels, with no changes detected in the glucose tolerance of groups. No effects of the diets used in our study were found on mating success, litter size, or offspring sex ratio. However, paternal diet was found to have sex- and pup diet specific effects in 10-week-old pups. Male pups of high-fat fed studs had increased lean mass and body weight when fed either a control or western diet. However, the effects of a high-sugar paternal diet varied depending on pup diet, where increased lean mass was only observed when pups were fed a western diet. These effects of stud diet were no longer evident when the pups were 20 weeks of age.

It is well recognised that obesity, often from overnutrition, is closely associated with the decline of the overall fertility, health, and lifespan of an individual, with an increased risk of passing down deleterious phenotypes to their offspring (Houfflyn et al., 2017, Palmer et al., 2012, Lane et al., 2015). Since fat is a calorie-dense macronutrient, it is often considered as a crucial constituent of obesogenic diets. However, studies that explore the association of high fat diets and development of cardiometabolic disorders often produce contradictory findings (Chowdhury et al., 2014). In animal studies, an obese model is often achieved through an excess of total energy derived from typically palatable, energy rich diets such as "Western" diets that are high in fat and carbohydrate. Interestingly, although the western diet used in our study had an obesogenic effect on female pups, it did not significantly increase the fat mass of males, despite increased energy intake. Aside from total energy intake, the macronutrient balance within diets has been suggested to be an important determinant of metabolic outcomes (Solon-Biet et al., 2014). Hence, a potential reason for the lack of an obesogenic effect of our western diet on males is the low protein content (10%), which may have been limiting for

males. In contrast, stud diets that similarly contained 40% fat, but 18.5% protein caused a significant increase in stud fat mass.

However, unlike what has been shown in past studies (Chowdhury et al., 2016, Chambers et al., 2016, Masuyama et al., 2016), the obesogenic high fat diet of the fathers did not result in an increased risk of lipid accumulation and susceptibility to obesity in their offspring in this study. We observed that male offspring from high fat fed fathers had elevated body weights at 10 weeks, but this was instead attributed to an increased lean mass rather than fat mass, contrasting to previous studies that have reported fat mass changes in offspring (Lecomte et al., 2017). In fact, it was the male pups from chow-fed fathers that were found to have the greatest amounts of fat mass among the three stud diet groups, irrespective of adult diet. It could be possible that the disparity of our findings compared to past studies are due to the paternal diet compositions used. High energy diets have been associated with the declined functioning of the male reproduction and consequently affects the testicular physiology, disrupting its metabolism and bioenergetic capacity (Rato et al., 2014). This has been associated with various mechanisms, such as sperm transfer RNA–derived small RNAs (tsRNAs) exhibiting changes in expression profiles and RNA modifications (Chen et al., 2016). Keeping the energy content constant in all three diets could have maintained a type of paternal influence in the offspring, without leading to a higher susceptibility to obesity and metabolic decline. Similarly, the lack of differences in glucose and insulin metabolism between offspring from the different paternal diet might also be explained by this. Glucose and insulin homeostasis are key outcomes that have been shown to be highly influenced by paternal effects (Stanford et al., 2018, Ng et al., 2014b), but in this study was more strongly driven by pup diets.

In addition to dietary fat, the ratio and source of dietary carbohydrate is also an important predictor of metabolic health (Wali et al., 2021a). Surprisingly, we found that a three-fold increase in dietary sucrose had no significant effects on body composition or metabolic health of studs. This supports recent findings explaining how it is not necessarily a high percentage of certain carbohydrates, but an equal combination of monosaccharides (typically a 50:50 combination of glucose and fructose) that promote deleterious metabolic outcomes (Wali et al.,

2021a). Despite the lack of effects observed in studs, increased sucrose content in the paternal diet led to increased lean mass of pups fed a western diet at 10 weeks of age. The mechanism underlying this effect is unknown as no statistically significant difference in energy intake was detected in the offspring (Figure 3.5A,B). However, interestingly, male pups of high-sucrose studs appear to have a slight preference for western diet (Figure 3.4D).

Effects of paternal diet on food choice and consumption are rarely examined in animal studies. The programming of offspring appetite systems and nutrient selection is an example of an important precursor for future susceptibility to metabolic disease. Most human studies that have explored paternal influence on offspring food selection focus primarily on learned behaviour and behavioural interventions (Klesges et al., 1991, Litchford et al., 2020). However, it is not known whether epigenetic inheritance may play an additional role in paternal effects on feeding behaviour. Here, we employed a choice experiment to test whether paternal diets influence the macronutrient selection of male and female offspring through the programming of innate nutrient targets. We found no statistically significant effects on food preference or intake patterns. Similarly, no significant effect of diet on stud food intake was detected, although intake was correlated with body weight. This lack of an effect on food intake may reflect the consistent protein content of stud diets, in keeping with the concept of *protein leverage*, where the intake of protein is prioritised over carbohydrate and fat (Raubenheimer and Simpson, 2019). It has been proposed that protein targets are defined as early as *in utero* and that the more direct maternal environment, rather than paternal effects, is the main driver of programming this regulation (Raubenheimer et al., 2015). However, feeding assays can lack the sensitivity required to identify subtle differences in energy intake that may result in body fat mass changes over time (Ellacott et al., 2010). Indeed, variation among individuals in our study was high, and significant effects may have been detected with increased replication. Hence, the lack of significant effects found in this study does not mean that paternal diet has no influence on offspring feeding preferences. Effects of paternal diet on feeding behaviour of offspring warrants further investigation, in particular the effects of paternal dietary protein and sugar.

While many of our findings contrast to previous studies, one finding that is commonly observed

in paternal effects studies is sex specific effects, where one sex can display a stronger phenotypic influence from changes in the paternal environment over another in a variety of species (Pembrey et al., 2006, Fullston et al., 2013). These sex-specific results have been attributed to a variety of mechanisms. These include differences in sex chromosomes and hormones (Chen et al., 2012, Gabory et al., 2013), as well as sex-specific alterations in placental gene expression (Binder et al., 2015a, Jazwiec et al., 2022). In this study, we found that paternal effects were primarily evident in male offspring and largely absent in female offspring. While some studies have similarly shown a paternal influence predominantly in male offspring (Pembrey et al., 2006, Sanchez-Garrido et al., 2017), other key studies have demonstrated paternal effects that are more strongly observed in females (Ng et al., 2010). The transmission of phenotypes to subsequent generations can also be sex-specific, e.g., to female descendants (F2 and F3 individuals) via the paternal line (Saavedra-Rodríguez and Feig, 2013, Hellmann et al., 2020b). At 20 weeks of age, sex also had a main effect in the glucose tolerance of the offspring. This is consistent with other studies that have reported sex differences in response to obesogenic diets (Medrikova et al., 2012, De Groef et al., 2022). These include sexually dimorphic responses in serum and tissue metabolomes, gut microbiomes (Hasegawa et al., 2020) and varying propensities in the development of NAFLD and other similar conditions (Ballestri et al., 2017). Altogether, these results draw attention to the potential for sex to influence patterns of paternal effects, but is still a highly understudied area that needs continued attention (Bell and Hellmann, 2019).

By the 20-week timepoint, paternal effects were reduced in both sexes. This resulted to later-life offspring environment effects to become more distinct, and we found that WD-fed female and male pups had increased body weight, lean mass and fat mass compared to SD-fed offspring. Interestingly, this distinction between SD and WD groups remained stronger in females than males, resulting in an interaction between pup diet and sex for body weight, fat mass, lean mass, and fasting insulin outcomes. The reduction of paternal effects as offspring aged is a contrasting trend to what has been reported by other studies (Zalbahar et al., 2017, Cooper et al., 2010), where parental effects were observed from childhood, and persisted up to adulthood. It could be possible that since we allocated offspring to either a CD or WD at 3 weeks of age, an early dietary intervention for the offspring could have made the difference. This suggests that offspring born to parents with obesity may be an important target for

interventions aimed at reducing obesity. Furthermore, the extent and mechanisms by which paternal effects interact with modifiable offspring environmental and lifestyle factors remains to be elucidated.

Altogether, this work highlights the complex interactions between paternal transmission of obesity-associated phenotypes, sex, and offspring metabolism. It brings novel findings regarding paternally influenced food selection, and sex-specific body composition responses to paternal environmental alterations and could provide a good foundation for future research in the field of developmental programming. Further studies are needed to functionally characterise the key organs and timepoints the onset of developmental alterations occur. This will aid in developing future dietary and therapeutic intervention that can be translatable for humans.

CHAPTER 4: Paternal diet effects on offspring anxiety-related behaviour

4.1 Introduction

Obesity is a major public health crisis affecting many countries worldwide. It is connected to a wide range of comorbidities including a higher susceptibility for developing type 2 diabetes (Kahn et al., 2006), cancer (De Pergola and Silvestris, 2013), cardiovascular disease (Powell-Wiley et al., 2021), and a variety of mental illnesses (André et al., 2014, Luppino et al., 2010). Studies have consistently found associations between hypercaloric diets (such as high fat, or Western diets), poor metabolic health, and a decline in cognitive function, and increased risk of anxiety and depressive-like behaviour (Milaneschi et al., 2019, Sharma and Fulton, 2013). It appears that overnutrition, obesity and mental disorders share a strong link, although the underlying mechanisms remain unclear. Obesity and its comorbidities can come with a significant psychosocial burden (Sarwer and Polonsky, 2016). Individuals with extreme obesity have been reported to have a much higher likelihood of experiencing an episode of major depression compared with those of average weight (Onyike et al., 2003, Faith et al., 2002). Inversely, individuals with mental disorders have been shown to have a higher intake of unhealthy foods and reduced physical activity (Rao et al., 2008). This leads to an increased development of obesity compared to individuals without mental illnesses (Holt, 2019). Therefore, obesity can be considered as both the cause and a product of impaired mental health.

Multiple neuro-pathologies have been associated with poor metabolic health and diet-induced obesity (Ogrodnik et al., 2019, André et al., 2014). Rodents fed a high-fat diet (HFD) have been shown to develop anxiety and depressive-like behaviour and metabolic complications (Clark et al., 2022, Dutheil et al., 2016). Meanwhile, studies investigating non-stressed rats that were submitted to a high-sucrose diet have found an increase in anxiety-related phenotypes (Rebolledo-Solleiro et al., 2017). Furthermore, a combination of both high fat and high sucrose components within a diet also appears to exert poorer memory performance and cognitive

decline (Francis and Stevenson, 2013, da Costa Estrela et al., 2015). Obesogenic diets have been shown to drive distinct brain mechanisms that alter behaviour and cognitive function. In mammals, this includes decreased serotonin levels (Veniaminova et al., 2020), altered dopamine transmission (Morris et al., 2015, Naef et al., 2011), suppression of cAMP/PKA signalling in the hypothalamus (Vagena et al., 2019), and increased glucocorticoid receptor expression along with increased stress-induced corticosterone release (Sasaki et al., 2013). There is also a potential link with inflammatory pathways (Dantzer et al., 2008), through the release of inflammatory molecules such as cytokines, that can interact with all these mechanisms (Bolton and Bilbo, 2014).

Although many mental disorders have been shown to have high heritability, and therefore a significant genetic component, many of the anxiety-related loci identified do not seem to fully explain the development of these conditions and phenotypic differences can occur even in the absence of genetically transmitted variation (Cunningham et al., 2021, Alter et al., 2009). It is likely that social and environmental factors also contribute to the risk of developing an anxiety disorder (Brook and Schmidt, 2008). Increasing evidence suggests that the perinatal environment may be particularly crucial in determining long-term consequences on health and disease susceptibility (Dunford and Sangster, 2017, Lane et al., 2015). Though much of the work in this field focusses on maternal influences, there is growing understanding that fathers also play a significant role in affecting offspring phenotypes (Dimofski et al., 2021, Curley et al., 2011). Aside from direct paternal care, epigenetic inheritance has gained traction in recent years as a means of passing down paternally driven phenotypes independent of the genome (Hur et al., 2017, Donkin and Barrès, 2018). Processes included in this form of regulation include DNA methylation, histone modifications, and non-coding RNAs. Furthermore, the influence of paternal environments on offspring may persist across multiple generations, indicating a transgenerational inheritance (Crisóstomo et al., 2021, de Castro Barbosa et al., 2016).

There are several studies that demonstrate how paternal experiences and phenotypes can be passed on through epigenetic mechanisms and other similar processes. Paternal HFD exposure prior to conception can affect offspring metabolic traits, including body composition, glucose intolerance, and adiponectin and leptin gene expression (Masuyama et al., 2016). Socially

defeated fathers in rodent studies have been shown to transmit depressive and anxiogenic behaviours, with more distinct effects observed in male offspring (Dietz et al., 2011). Paternal psychological stress from toxins and other traumatic exposures have been shown to reprogram offspring metabolic outcomes, such as hepatic gluconeogenesis (Wu et al., 2016), memory and cognition (Lupien et al., 2009, Ordyan et al., 2022), and other behavioural outcomes (Dias and Ressler, 2013, Short et al., 2016, Kong et al., 2021). Just as metabolism and behaviour can be linked within a generation, the metabolism and behaviour of male mice may also be linked to their descendants across generations (van Steenwyk et al., 2018).

Although pioneer studies in humans have greatly shaped our understanding of parental influences, they are limited by the low number of successive generations captured, the range of conditions that can be examined and the lack of mechanistic explanations that can be provided. Animal models have been an important tool to overcome these limitations in more reproducible conditions. They have allowed for more focused studies on the mechanisms of inheritance of environmentally induced traits. For example, evidence for effects of paternal stress on subsequent offspring outcomes have primarily stemmed from laboratory rodents (Dietz et al., 2011, Gapp et al., 2014, Rodgers et al., 2013, Bardi et al., 2011). With animal models, we are also able to employ more controlled dietary manipulations and isolate specific drivers of diet-induced obesity and its transmission across generations (Ellacott et al., 2010). An important factor that many studies in this field do not fully explain is whether the anxiogenic effect resulting from diet-induced obesity is driven by the hypercaloric nature of these diets or the specific components (e.g., fat or carbohydrate) *per se*.

In this study, we focus on exploring how varying the carbohydrate and fat proportions within paternal diets (but keeping energy content similar) can affect anxiety-related behaviour in the studs. Additionally, we investigate how this can influence offspring behaviour. To do this, we have employed the elevated plus maze and open field maze- two common tools widely used in research to evaluate anxiety-associated responses, general activity, and locomotion. Both techniques have been widely validated previously and exploit the natural aversion of rodents to exposed fields (Carola et al., 2002, Walf and Frye, 2007, Asano, 1986, Pellow et al., 1985). They are, therefore, based on an anxiety-promoting agent, such as an unprotected, elevated field or an open area, the anxiety level being expressed by the number of entries into, and the

length of time spent in the aversive area. Here, we show anxiety-related responses that are distinct between male and female offspring, as influenced by paternal dietary manipulations.

4.2 Methods

4.2.1 Ethical approval

All animal protocols were approved by Sydney University's Animal Ethics Committee (Protocol number 2019/1609) to ensure they were in line with the NSW Animal Research Act 1985 and the Australian code for the care and use of animals for scientific purposes 8th edition (2013).

4.2.2 Experimental Diets

Table 4.1 Breakdown of compositions for diets used in study.

	Paternal diets			***Offspring No-Choice***	
Diet	**Chow/High Starch**	**High Sucrose**	**High Fat**	**Standard Diet**	**Western Diet**
NME (kJ/g)	14.4	14.7	14.7	14.4	17.3
% Protein energy	18.5	18.5	18.5	18.5	10
% Carbohydrate energy	64	64	42.3	64	50
% Fat energy	17.5	17.5	39.2	17.5	40
Other (g/kg)					
Casein	200	200	200	200	126
Soy oil	70	70	157	70	29
Wheat Starch	404	304	269	404	254
Sucrose	100	300	67	100	231
Dextrinised Starch	132	32	88	132	100
Cellulose	50	50	176	50	56
Choline	1.4	2.1	2.1	1.4	2.1
Folic acid	0.002	0.002	0.002	0.002	0.002
Methionine (% level in diet)	84	83	83	84	63

4.2.2.1 Stud diets

The studs were fed 1 of 3 diets with varying sources of carbohydrate and differing fat: carbohydrate concentrations. Stud diets were matched for metabolisable energy content (i.e., isocaloric) as much as was practically possible through the addition of non-digestible cellulose. The source of protein was casein, fat was soybean oil and carbohydrates were a mixture of sucrose, wheat starch and dextrinised starch.

- **Control**

The control diet (CD; SF18-025, Specialty Feeds, Glen Forrest, WA, Australia) was derived from a standard rodent AIN-93G formulation, with 18.5% protein, 64% carbohydrate and 17.5% fat making up the total calorie content of 14.4 kJ/g. The carbohydrate component consisted of 84.3% starch and 15.7% sucrose.

- **High Sucrose**

The high sucrose diet (HSucrose; SF19-202, Specialty Feeds, Glen Forrest, WA, Australia) was derived from a standard rodent AIN-93G formulation, with 18.5% protein, 64% carbohydrate and 17.5% fat making up the total calorie content of 14.7 kJ/g. The carbohydrate component consisted of 52.8% starch and 47.2% sucrose.

- **High Fat**

The high fat diet (HFat; SF19-203, Specialty Feeds, Glen Forrest, WA, Australia) was derived from a standard rodent AIN-93G formulation, with 18.5% protein, 42.3% carbohydrate and 39.2% fat making up the total calorie content of 14.7 kJ/g. The carbohydrate component consisted of 84.2% starch and 15.8% sucrose.

4.2.2.2 Offspring diets

- **Control**

The control diet fed to pups was the same as the control diet fed to studs (CD; SF18-025, Specialty Feeds, Glen Forrest, WA, Australia).

- **Western**

A high calorie, high fat, and high sucrose “Western” diet (WD; SF18-050, Specialty Feeds, Glen Forrest, WA, Australia) was used as an obesogenic diet for pups in this study, as it was not logistically feasible to complete a fully crossed 3 paternal by 3 offspring diet design. The

Western diet designed for this study was again derived from a standard rodent AIN-93G, with 10% Protein, 50% Carbohydrate, and 40% Fat, making up the total calorie content of 17.3 kJ/g. The fat component consisted of 84.7% lard and 15.3% soybean oil. The carbohydrate component consisted of 60.5% starch and 39.5% sucrose.

4.2.3 Animals and husbandry

4.2.3.1 General husbandry and cage allocations

All animals in this study were housed in cages at the Charles Perkins Centre, University of Sydney with an ambient temperature of 22°C and constant access to water. Studs were sourced from Animal Resources Centre (Perth, WA) and housed individually outside of the mating period to prevent fighting with fellow males (n=18). Studs were divided into 2 cohorts. The first cohort were not mated and were only tested on the elevated plus maze (n=6). The second cohort of males were mated after behaviour testing on both the elevated plus maze and open field test. From the 2nd cohort studs offspring, 2 pups of each sex were restricted to either a Control or Western diet from 3 weeks of age (detailed in chapter 2), yielding n=8-10/sex/ diet group. From up to a week prior to behavioural testing at 15 weeks of age, animals were temporarily moved to the Charles Perkins Centre Animal Behavioural Facility for acclimatisation. Food intake and body weights continued to be measured once a week.

4.2.4 Behavioural analysis

4.2.4.1 Elevated plus maze

The elevated plus maze consisted of 2 closed arms (30 x 5 cm), 2 open arms (30 x 5 cm), and a central zone (5 x 5 cm). The maze was elevated 50 cm above the ground. The light intensity on the open arms of the maze was 700-750 lx, and in the closed arms was 175-225 lx. Each mouse was placed into the centre of the maze facing the left open arm for consistency at the beginning of recording using Logitech webcam software (Logitech; Lausanne, Switzerland). Mice were exposed to the elevated plus maze for a total of 5 min and the apparatus was cleaned thoroughly between animals. The data were analysed using TopScan Image Analysis Software (CleverSys Inc; VA, USA) for the parameters of percentage time spent in the open and closed

arms of the maze and entries into the arms. An entry was defined by crossing the dividing line between an arm and the centre platform with all four feet. Other parameters measured were total distance travelled (in mm), velocity (the animal's average speed when moving in a straight line throughout the maze) , speed (separated into fast or slow speed thresholds; slow speed animal moving at < 20 mm/s, fast speed = animal moving at > 20 mm/s,) and motion (separated into 'fast' or 'slow' detectable motion thresholds defined by the program, where a value of 0 indicates the animal is static; slow motion = animal moving from 0-0.05 , fast motion = animal moving at > 0.05).

4.2.4.2 Open Field Maze

As an additional way to measure anxiety related behaviour and locomotor activity, an open field test was used. The test was performed by placing a mouse into the centre of an open-field arena with a white base (40x40 cm) and black walls bordering the outside (20 cm) and allowing the mouse to explore for 5 min. Bright overhead lighting was approximately 225 lx inside the arenas. Activity in the open-field was quantified by a computer-operated TopScan Image Analysis Software (CleverSys Inc; VA, USA). Total distance (locomotor activity), movement time (in seconds), movement speed (cm/s), and center distance (the distance traveled in the center of the arena) were recorded.

4.2.5 Statistical analysis

Data are presented as means ± SEM and statistical significance determined when $P < 0.05$. Stud data was analysed using ANOVA in GraphPad Prism (v9.0.2; GraphPad Software Inc., La Jolla, CA, USA). Post hoc analysis was performed using Tukey's multiple comparisons test unless otherwise stated in the relevant figure legends. Offspring data was analysed using mixed effect models in R (RStudio version 1.4.1717; Vienna, Austria) with lme4 (Bates et al., 2015) and LmerTest (Kuznetsova et al., 2017) packages. Stud diet, Pup diet, Sex, and their interactions were included as fixed effects. Dam was included as a random variable to account for litter effects. Litter size at weaning and body weight were initially included as covariates in all models and then removed if shown to not have a significant effect. Correlation between parameters was assessed by Spearman rank correlation in RStudio version 1.4.1717. All other statistical parameters are included in the figure legends.

4.3 Results

4.3.1 Stud high fat diet led to increased entries, but not an increase of time spent in the open arms of an elevated plus maze

After 11 weeks on one of 3 diets, an elevated plus maze was used to measure anxiety-related outcomes and movement patterns of 14-week-old studs. A significant effect was observed in the number of entries into the open arms (Figure 4.1A), where males fed a high fat diet had increased entries compared to both control ($p=0.0179$) and high sucrose ($p=0.0195$) groups, indicating increased boldness. Despite this, no significant effects were detected in the number of entries into the closed arms (Figure 4.1B; Table 4.2) and centre zone (Figure 4.1C; Table 4.2). Additionally, no significant differences were found in terms of time spent by the groups in these three regions (Figure 4.1D-F; Table 4.2). There were also no significant effects of stud diet on total distance travelled (Figure 4.1G; Table 4.2), velocity (Figure 4.1H), time travelling in fast or slow speed (Figure 4.1I,J; Table 4.2), and fast and slow motion (Figure 4.1K,L, Table 4.2).

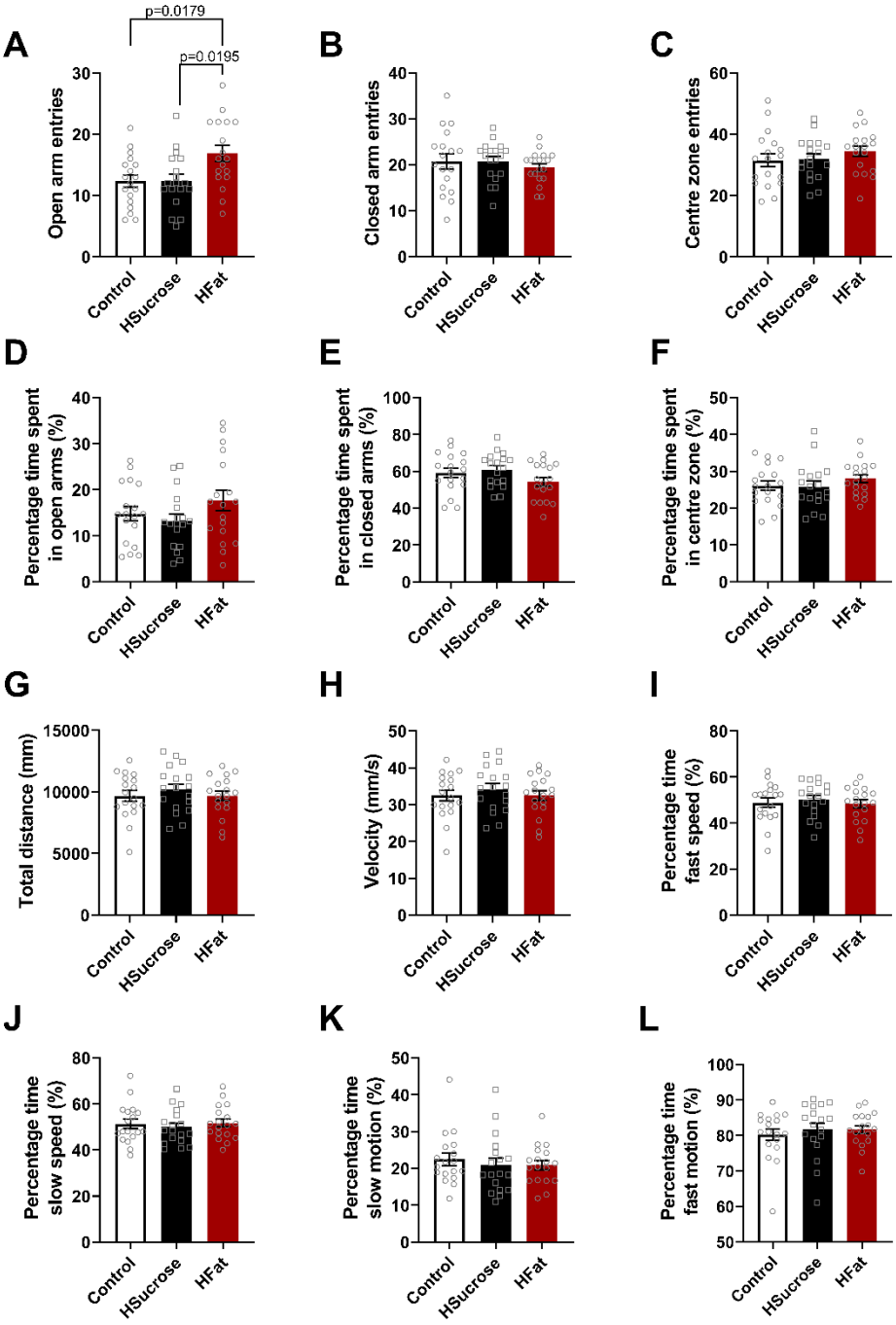

Figure 4.1 Stud high fat diet led to increased entries, but not an increase of time spent in the open arms of an elevated plus maze.

Using an elevated plus maze, several parameters were measured in male studs, including number of entries into (A) open arms (B) closed arms and (C) centre zone. Percentage of time spent in (D) open arms (E) closed arms and (F) centre zone (G) Total distance travelled in

maze (H) Velocity (I) Percentage of time when speed was >20mm/s (fast) (J) Percentage of time when speed was <20mm/s (slow) (K) Percentage of time when motion was >0.0500 (fast) (L) Percentage of time when motion was <0.0500 (slow); (n= 18 animals/group) at 15 weeks of age. For all bar graphs, an ANOVA was used. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

Table 4.2 Statistical summary (one-way ANOVA) of stud elevated plus maze results. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	ANOVA table	Sum.Sq	DF	F (DFn, DFd)	P value	Sig
4.1A	**Open arm entries**	Treatment (between columns)	246	2	F (2, 51) = 5.281	0.0082	**
		Residual (within columns)	1188	51			
		Total	1434	53			
4.1B	**Closed arm entries**	Treatment (between columns)	22.26	2	F (2, 51) = 0.4245	0.6564	NS
		Residual (within columns)	1337	51			
		Total	1359	53			
4.1C	**Centre zone entries**	Treatment (between columns)	87.11	2	F (2, 51) = 0.726	0.4888	NS
		Residual (within columns)	3060	51			
		Total	3147	53			
4.1D	**Percentage time spent in open arms (%)**	Treatment (between columns)	177.8	2	F (2, 51) = 1.571	0.2177	NS
		Residual (within columns)	2886	51			
		Total	3063	53			
4.1E	**Percentage time spent in closed arms (%)**	Treatment (between columns)	408.6	2	F (2, 51) = 2.028	0.142	NS
		Residual (within columns)	5137	51			
		Total	5546	53			
4.1F	**Percentage time spent in centre zone (%)**	Treatment (between columns)	49.4	2	F (2, 51) = 0.8272	0.4430	NS
		Residual (within columns)	1523	51			

		Total	1572	53			
4.1G	**Total distance travelled (mm)**	Treatment (between columns)	3197061	2	F (2, 51) = 0.5054	0.6063	NS
		Residual (within columns)	161317344	51			
		Total	164514405	53			
4.1H	**Velocity (mm/s)**	Treatment (between columns)	35.99	2	F (2, 51) = 0.5052	0.6064	NS
		Residual (within columns)	1816	51			
		Total	1852	53			
4.1I	**Percentage time spent in fast speed (%)**	Treatment (between columns)	31.4	2	F (2, 51) = 0.2541	0.7766	NS
		Residual (within columns)	3151	51			
		Total	3183	53			
4.1J	**Percentage time spent in slow speed (%)**	Treatment (between columns)	30.83	2	F (2, 51) = 0.2489	0.7806	NS
		Residual (within columns)	3159	51			
		Total	3190	53			
4.1K	**Percentage time spent in fast motion (%)**	Treatment (between columns)	25.53	2	F (2, 51) = 0.2915	0.7484	NS
		Residual (within columns)	2233	51			
		Total	2259	53			
4.1L	**Percentage time spent in slow motion (%)**	Treatment (between columns)	29.14	2	F (2, 51) = 0.3039	0.7393	NS
		Residual (within columns)	2445	51			
		Total	2475	53			

4.3.2 Increasing dietary carbohydrates and fat did not affect male mice behaviour in an open field maze.

An open field maze was used to test behaviour and movement patterns of 15-week-old male mice. Similar to the results found in the elevated plus maze, no significant effects were found as a result of dietary fat and carbohydrate changes in a wide range of outcomes measured (Table 4.3). No significant effects were found in the total distance travelled in the maze (Figure 4.2A), velocity (Figure 4.2B), entries into and time spent in either the outer or centre zones (Figure 4.2C-H), time travelling in fast or slow speed (Figure 4.2I,J), and fast and slow motion (Figure 4.2,L).

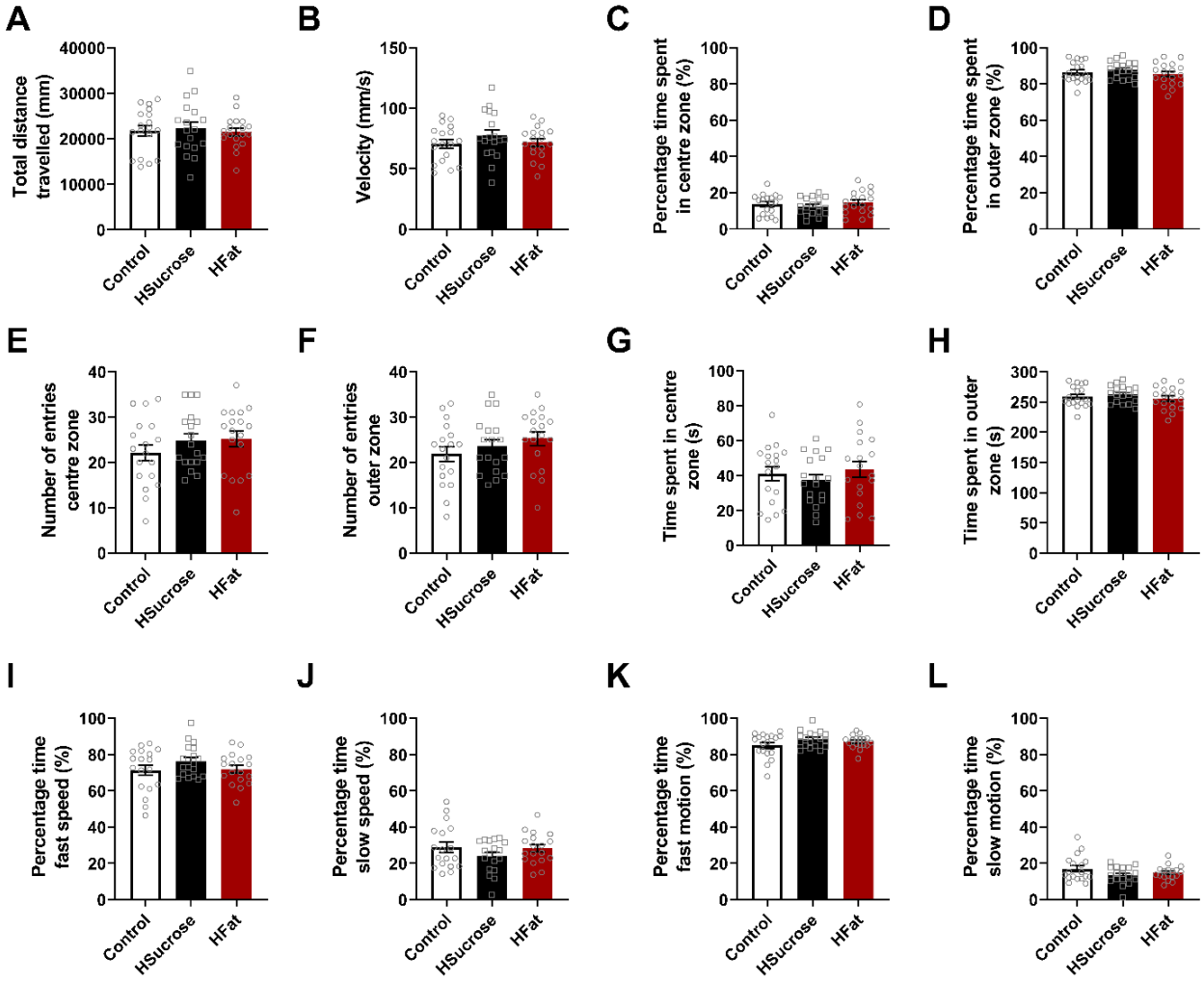

Figure 4.2 Increasing dietary sucrose and fat did not affect male mice behaviour in an open field maze.

Using an open field maze, several parameters were measured in male studs, including (A) Total distance travelled in maze (B) Velocity, Percentage of time spent in (C) centre zone and (D) outer zone. Number of entries into (E) centre zone and (F) outer zone. Duration of time in (G) centre zone and (H) outer zone. (I) Percentage of time when speed was >20mm/s (fast) (J) Percentage of time when speed was <20mm/s (slow) (K) Percentage of time when motion was >0.0500 (fast) (L) Percentage of time when motion was <0.0500 (slow); (n= 12 animals/group) at 15 weeks of age. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

Table 4.3 Statistical summary (one-way ANOVA) of stud open field maze results. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, NS = p>0.05.

Figure	Outcome	ANOVA table	Sum.Sq	DF	F (DFn, DFd)	P value	Sig
4.2A	**Total distance travelled (mm)**	Treatment (between columns)	5867298	2	F (2, 51) = 0.1222	0.8852	NS
		Residual (within columns)	1.224E+09	51			
		Total	1.23E+09	53			
4.2B	**Velocity (mm/s)**	Treatment (between columns)	521.1	2	F (2, 51) = 0.9971	0.376	NS
		Residual (within columns)	13328	51			
		Total	13849	53			
4.2C	**Percentage time spent in centre zone (%)**	Treatment (between columns)	44.07	2	F (2, 51) = 0.7099	0.4965	NS
		Residual (within columns)	1583	51			
		Total	1627	53			
4.2D	**Percentage time spent in outer zone (%)**	Treatment (between columns)	45.47	2	F (2, 51) = 0.7341	0.4849	NS
		Residual (within columns)	1580	51			
		Total	1625	53			
4.2E	**Centre zone entries**	Treatment (between columns)	99.59	2	F (2, 51) = 0.9907	0.3783	NS
		Residual (within columns)	2563	51			
		Total	2663	53			
4.2F	**Outer zone entries**	Treatment (between columns)	106.8	2	F (2, 51) = 1.254	0.2939	NS
		Residual (within columns)	2171	51			
		Total	2277	53			
4.2G	**Time spentin centre zone (s)**	Treatment (between columns)	334.7	2	F (2, 51) = 0.5812	0.5629	NS
		Residual (within columns)	14684	51			
		Total	15018	53			
4.2H	**Time spentin outer zone (s)**	Treatment (between columns)	409	2	F (2, 51) = 0.7329	0.4855	NS
		Residual (within columns)	14231	51			
		Total	14640	53			
4.2I	**Percenta**	Treatment (between	254.5	2	F (2, 51) =	0.28	NS

	ge time spent in fast speed (%)	columns)			1.271	94	
		Residual (within columns)	5109	51			
		Total	5363	53			
4.2J	**Percentage time spent in slow speed (%)**	Treatment (between columns)	255.6	2	F (2, 51) = 1.28	0.2867	NS
		Residual (within columns)	5091	51			
		Total	5347	53			
4.2K	**Percentage time spent in fast motion (%)**	Treatment (between columns)	100.1	2	F (2, 51) = 1.903	0.1596	NS
		Residual (within columns)	1342	51			
		Total	1442	53			
4.2L	**Percentage time spent in slow motion (%)**	Treatment (between columns)	111.4	2	F (2, 51) = 1.912	0.1582	NS
		Residual (within columns)	1486	51			
		Total	1597	53			

4.3.3 Interactions between paternal diet, adult diet and sex were observed in offspring anxiety related responses at 15 weeks of age.

A statistically significant three-way stud diet: pup diet: sex interaction was observed in the amount of time spent in the closed arms (Figure 4.3G,H;Table 4.4). SD females from chow fed studs spent the smallest percentage of time in the open arms, and inversely, the largest percentage of time in the closed arms. In males, pups from high sucrose fathers spent more time in the open arms, and the least in the closed arms, regardless of adult diet. Similar trends were observed in open arm entries, although it did not reach significance (Figure 4.3B; Table 4.4). No significant effects were detected in the number of entries into the closed arms of the maze either (Figure 4.3C,D; Table 4.4). Although a main effect of stud diet was close to significance, it was not detected in the amount of time spent in the open arms (Figure 4.3E,F; Table 4.4). No differences were found between groups for the time spent in the decision-making centre zone (Figure 4.3I,J; Table 4.4).

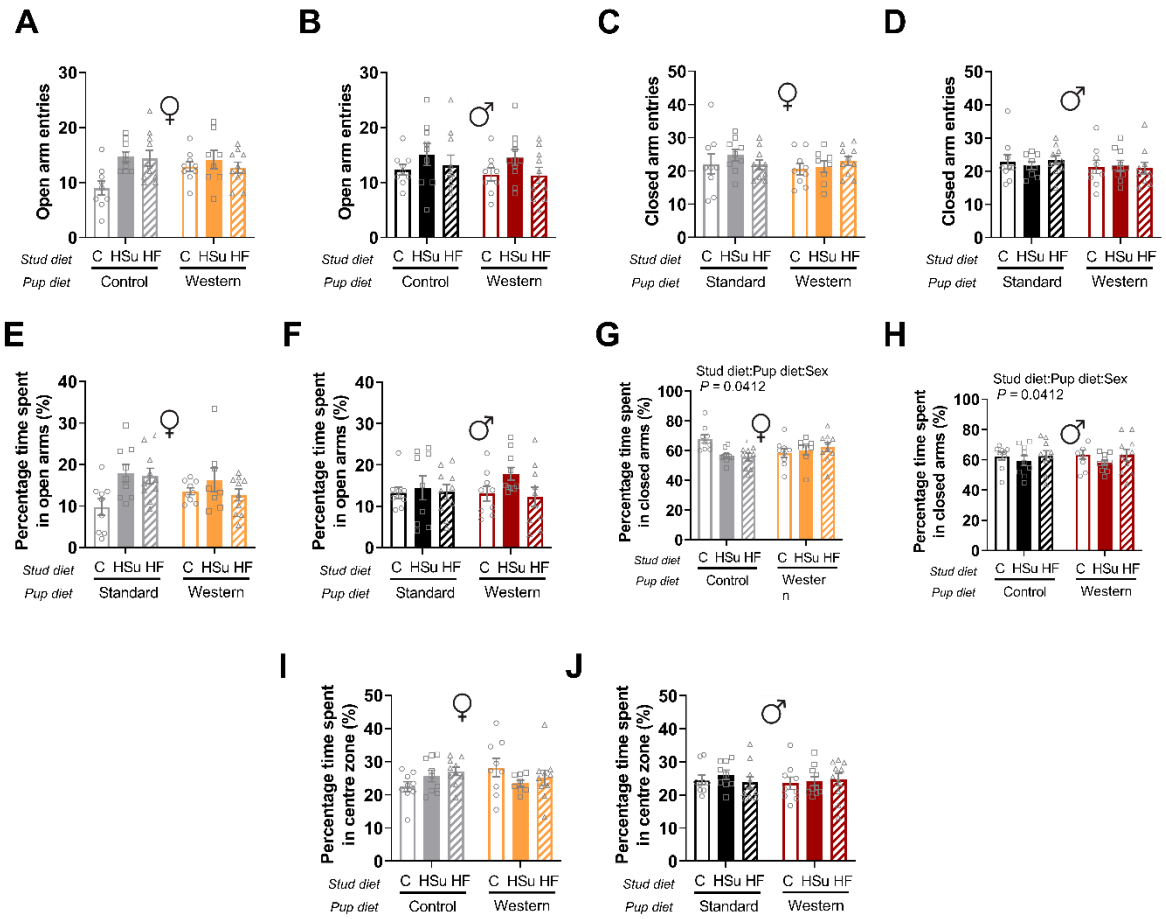

Figure 4.3 Interactions between paternal diet, offspring diet and sex were observed in offspring anxiety related responses at 15 weeks of age.

Using an elevated plus maze, several parameters were measured including number of open arm entries for (A) female and (B) male offspring, number of closed arm entries for (C) female and (D) male offspring, percentage of time spent in open arms for (E) female and (F) male offspring, percentage of time spent in closed arms for (G) female and (H) male offspring and percentage of time spent in centre zone for (I) female and (J) male offspring. (n= 8-10 animals/ sex/group) at 15 weeks of age. For all bar graphs, an ANOVA was used. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

Table 4.4 Statistical summary (ANOVA) of pup elevated plus maze locomotor results for the main effects of sex, stud diet, pup diet; two-way interactions for stud diet:pup diet, stud diet:sex, pup diet:sex; and three-way stud diet:pup diet:sex interaction. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, #p>0.05<0.01, NS = p>0.05.

Figure	Outcome	Factor	Sum.Sq	DF	F.value	Pr..F.	Sig. level
4.3A,B	Open arm entries	Stud diet	87.4155	2	3.2029	0.056795	#
		Pup diet	2.4718	1	0.1811	0.671623	NS
		Sex	0.3066	1	0.0225	0.881258	NS
		Stud diet: Pup diet	42.8101	2	1.5686	0.215132	NS
		Stud diet: Sex	22.3084	2	0.8174	0.445454	NS
		Pup diet: Sex	19.5074	1	1.4295	0.235771	NS
		Stud diet: Pup diet: Sex	33.0126	2	1.2096	0.304316	NS
4.3C,D	Closed arm entries	Stud diet	8.3205	2	0.1961	0.823066	NS
		Pup diet	44.2147	1	2.0838	0.152967	NS
		Sex	6.2912	1	0.2965	0.587662	NS
		Stud diet: Pup diet	5.9599	2	0.1404	0.869199	NS
		Stud diet: Sex	21.3341	2	0.5027	0.606854	NS
		Pup diet: Sex	0.0101	1	0.0005	0.982632	NS
		Stud diet: Pup	55.8168	2	1.3153	0.274632	NS

		diet: Sex					
4.3E,F	**Percentage time in open arms (%)**	Stud diet	148.2249	2	2.7881	0.079249	#
		Pup diet	0.0231	1	0.0009	0.976577	NS
		Sex	8.1362	1	0.3061	0.581714	NS
		Stud diet: Pup diet	119.7655	2	2.2528	0.112153	NS
		Stud diet: Sex	58.1861	2	1.0945	0.339925	NS
		Pup diet: Sex	19.2181	1	0.7230	0.397962	NS
		Stud diet: Pup diet: Sex	112.6041	2	2.1181	0.12764	NS
4.3G,H	**Percentage time in closed arms (%)**	Stud diet	186.1952	2	1.4960	0.240834	NS
		Pup diet	0.1404	1	0.0023	0.962243	NS
		Sex	56.1662	1	0.9025	0.345032	NS
		Stud diet: Pup diet	277.9158	2	2.2329	0.114085	NS
		Stud diet: Sex	80.3465	2	0.6455	0.527152	NS
		Pup diet: Sex	0.6336	1	0.0102	0.919897	NS
		Stud diet: Pup diet: Sex	414.3798	2	3.3293	0.041209	*
4.3I,J	**Percent time in centre zone (%)**	Stud diet	1.2783	2	0.0289	0.971537	NS
		Pup diet	0.0661	1	0.0030	0.956529	NS
		Sex	23.2463	1	1.0513	0.3084	NS
		Stud diet: Pup diet	77.4585	2	1.7515	0.180385	NS

		Stud diet: Sex	27.3026	2	0.6174	0.541995	NS
		Pup diet: Sex	12.4033	1	0.5609	0.456261	NS
		Stud diet: Pup diet: Sex	111.8520	2	2.5292	0.086594	#

4.3.4 Differences in movement were observed in offspring in an elevated plus maze at 15 weeks of age.

Aside from anxiety-related responses, the elevated plus maze was also used to observe movement patterns of the offspring. An interaction between stud diet and pup diet was found in the motion of the offspring (Figure 4.4I-L; Table 4.5). In CD mice, pups from control-fed fathers spent the most time in slow motion. In WD animals, pups from high sucrose fathers spent more time in fast motion than the other groups. Male offspring of high fat fed males spent the largest percentage of time in slow motion. A main pup diet effect was found in the total distance travelled (Figure 4.4A,B; Table 4.5), velocity (Figure 4.4C,D; Table 4.5) and speed of the animals (Figure 4.4E-H; Table 4.5), where those fed a control diet in adulthood were observed to cover larger distances, have increased velocity and spent more time where speed was >20 mm/s when moving around the maze. Removing body weight from the statistical model also led to a statistically significant sex effect being detected in these outcomes.

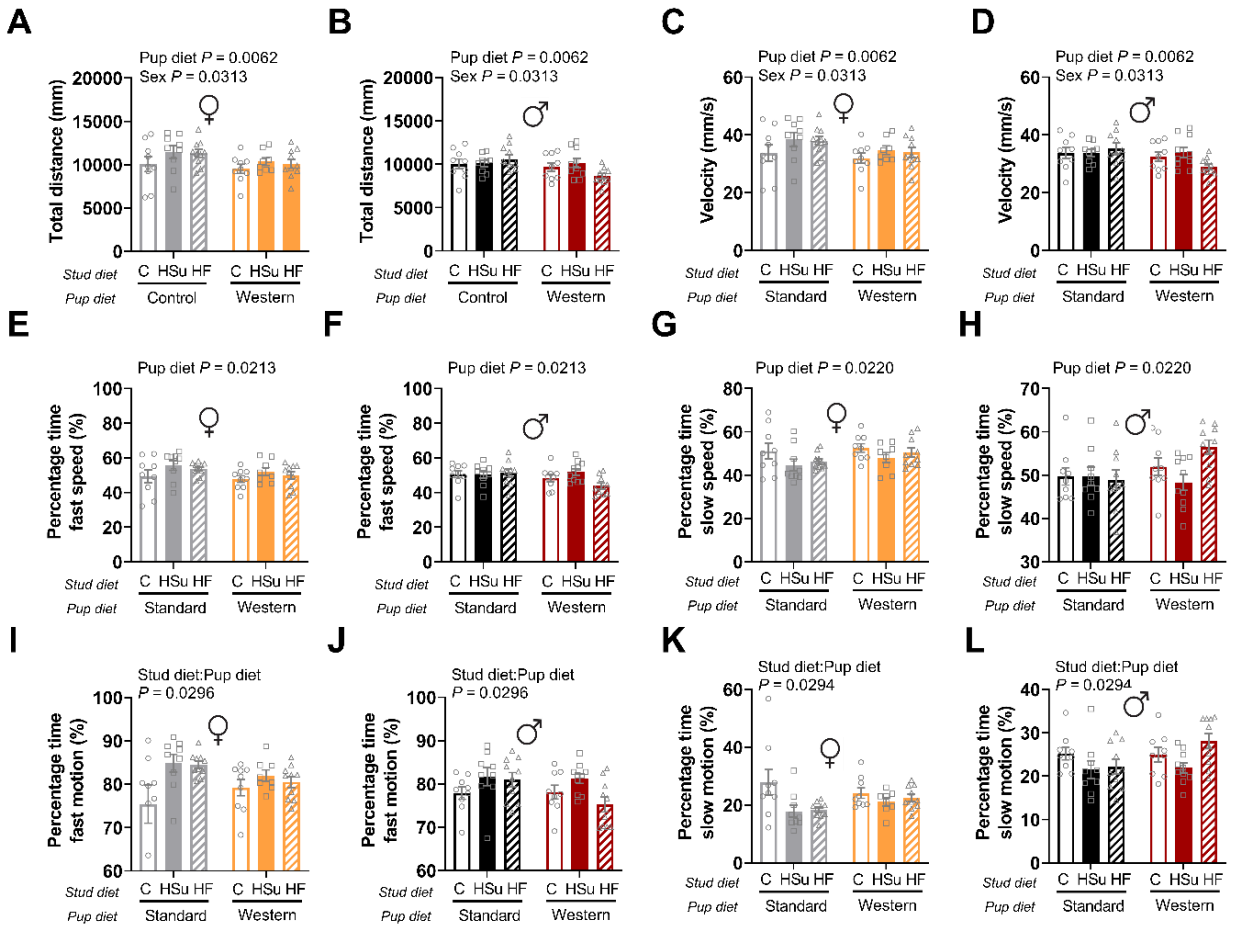

Figure 4.4 Differences in movement were observed in offspring in an elevated plus maze at 15 weeks of age.

Using an elevated plus maze, several parameters were measured including total distanced travelled around the maze for (A) female and (B) male offspring, velocity of movements for (C) female and (D) male offspring, Percentage of time when speed was >20mm/s (fast) for for (E) female and (F) male offspring, Percentage of time when speed was <20mm/s (slow) , for (G) female and (H) male offspring. Percentage of time when motion was >0.0500 (fast) for (I) female and (J) male, Percentage of time when motion was <0.0500 (slow) for (K) female and (L) male offspring, (n= 8-10 animals/ sex/group) at 15 weeks of age. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures

Table 4.5 Statistical summary (ANOVA) of pup elevated plus maze locomotor results for the main effects of sex, stud diet, pup diet; two-way interactions for stud diet:pup diet, stud diet:sex, pup diet:sex; and three-way stud diet:pup diet:sex interaction. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, #p>0.05<0.01, NS = p>0.05.

Figure	Outcome	Factor	Sum.Sq	DF	F.value	Pr..F.	Sig. level
4.4A,B	Total distance travelled	Stud diet	5213281.9880	2	1.1140	0.343062	NS
		Pup diet	18534768.8600	1	7.9210	0.006222	**
		Sex	11257553.4400	1	4.8110	0.03131	*
		Stud diet: Pup diet	7699800.0000	2	1.6453	0.19978	NS
		Stud diet: Sex	7548755.8810	2	1.6130	0.205976	NS
		Pup diet: Sex	401960.0534	1	0.1718	0.679754	NS
		Stud diet: Pup diet: Sex	4399058.1670	2	0.9400	0.395326	NS
4.4C,D	Velocity (mm/s)	Stud diet	58.7153	2	1.1140	0.343044	NS
		Pup diet	208.7863	1	7.9228	0.006216	**
		Sex	126.7362	1	4.8092	0.03134	*
		Stud diet: Pup diet	86.5709	2	1.6425	0.200306	NS
		Stud diet: Sex	84.9402	2	1.6116	0.206255	NS
		Pup diet:	4.5544	1	0.1728	0.678837	NS

		Sex					
		Stud diet: Pup diet: Sex	49.5275	2	0.9397	0.395435	NS
4.4E,F	**Percentage time in fast speed (%)**	Stud diet	165.7847	2	2.0717	0.144775	NS
		Pup diet	221.0382	1	5.5243	0.021304	*
		Sex	115.1775	1	2.8786	0.093744	#
		Stud diet: Pup diet	122.2940	2	1.5282	0.223408	NS
		Stud diet: Sex	131.7071	2	1.6458	0.199461	NS
		Pup diet: Sex	0.6996	1	0.0175	0.895162	NS
		Stud diet: Pup diet: Sex	93.0707	2	1.1630	0.318157	NS
4.4G,H	**Percentage time in slow speed (%)**	Stud diet	180.0777	2	2.2464	0.124461	NS
		Pup diet	219.0297	1	5.4647	0.021991	*
		Sex	116.3405	1	2.9026	0.092406	#
		Stud diet: Pup diet	127.1098	2	1.5857	0.211404	NS
		Stud diet: Sex	136.8168	2	1.7068	0.188143	NS
		Pup diet: Sex	0.3728	1	0.0093	0.923429	NS
		Stud diet: Pup diet: Sex	88.2775	2	1.1012	0.33782	NS
4.4I,J	**Percent time in fast**	Stud diet	273.8651	2	4.4750	0.02008	*
		Pup diet	61.1596	1	1.9987	0.16138	NS
		Sex	99.1485	1	3.2402	0.07565	#

	motion (%)	Stud diet: Pup diet	225.4319	2	3.6836	0.029575	*
		Stud diet: Sex	122.2104	2	1.9970	0.14251	NS
		Pup diet: Sex	6.8245	1	0.2230	0.6381	NS
		Stud diet: Pup diet: Sex	43.2719	2	0.7071	0.496312	NS
4.4K,L	**Percent time in slow motion (%)**	Stud diet	285.2488	2	4.5127	0.019489	*
		Pup diet	71.9882	1	2.2777	0.135253	NS
		Sex	115.0750	1	3.6410	0.059988	#
		Stud diet: Pup diet	233.1850	2	3.6890	0.029429	*
		Stud diet: Sex	148.8449	2	2.3548	0.101515	NS
		Pup diet: Sex	3.8244	1	0.1210	0.72891	NS
		Stud diet: Pup diet: Sex	49.1027	2	0.7768	0.4635	NS

4.3.5 Paternal and adult diet influences on offspring behaviour are not detected in an open field maze at 15 weeks of age.

An open field maze was used to test behaviour and movement patterns of 15-week-old male mice. Contrary to the results found in the elevated plus maze, no significant effects were found as an effect of stud diet, or pup diet in a wide range of outcomes measured (Table 4.6). No significant effects were found in the total distance travelled in the maze (Figure 4.5,B), velocity (Figure 4.5C,D), time spent in either the outer or centre zones (Figure 4.5E-H), time travelling in fast or slow speed Figure 4.5I-L), and fast and slow motion (Figure 4.5M-P).

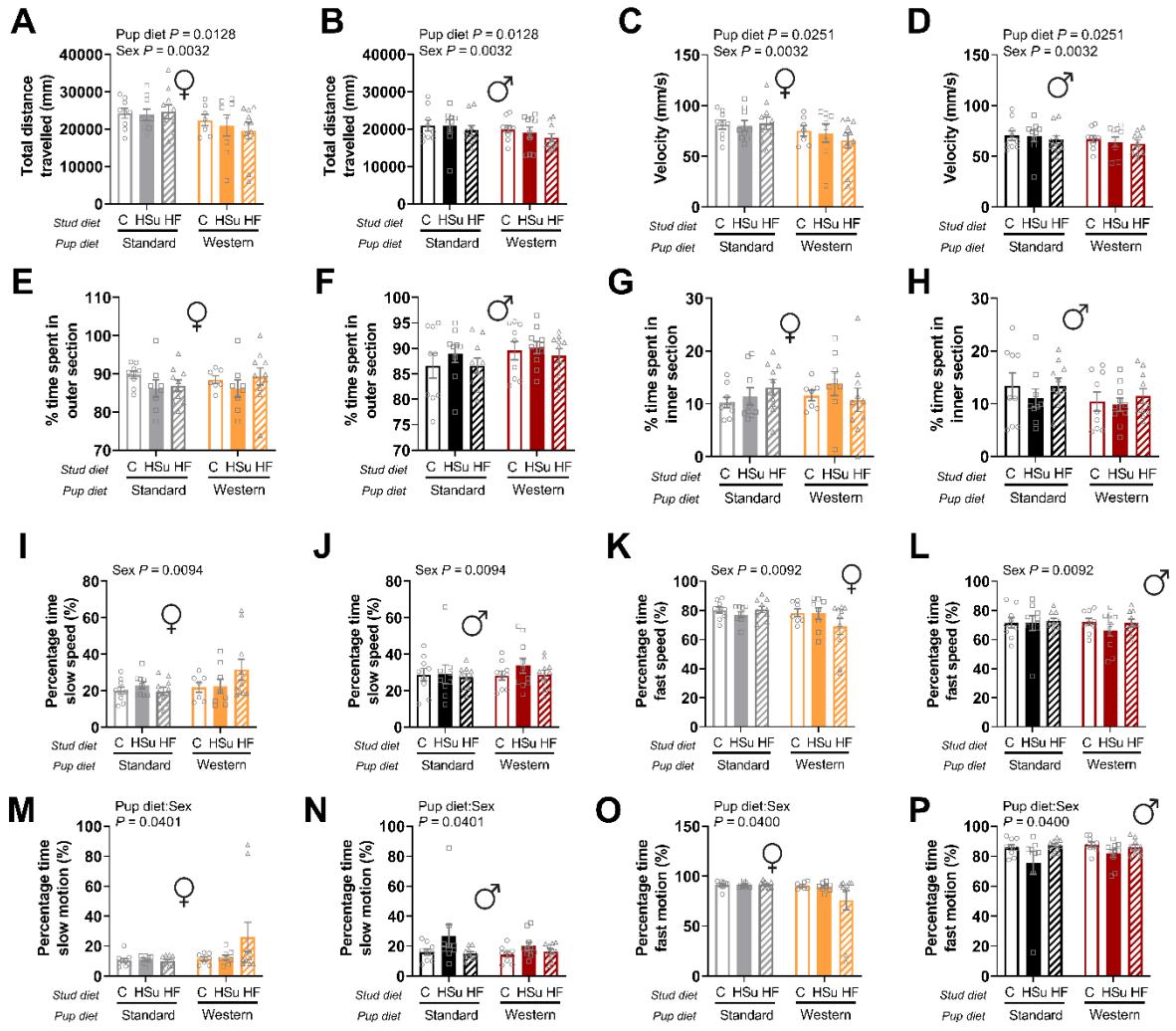

Figure 4.5 Paternal and adult diet influences on offspring behaviour are not detected in an open field maze at 15 weeks of age.

Using an open field maze, several parameters were measured including total distanced travelled around the maze for (A) female and (B) male offspring, velocity of movements for (C) female and (D) male offspring, percentage of time spent in the outer section for (E) female and (F) male offspring, percentage of time spent inner section for (G) female and (H) male offspring, Percentage of time when speed was <20mm/s (slow) for (I) female and (J) male offspring Percentage of time when speed was >20mm/s (fast) for (I) female and (J) male offspring,) Percentage of time when motion was <0.0500 (slow) for (M) female and (N) male offspring, Percentage of time when motion was >0.0500 (fast) for (O) female and (P) male offspring. (n= 8-10 animals/ sex/group) at 15 weeks of age. For all bar graphs, an ANOVA was used. All bars indicate means ± SEM. Significant p-values are provided on the relevant figures.

Table 4.6 Statistical summary (ANOVA) of pup open field maze results for the main effects of sex, stud diet, pup diet; two-way interactions for stud diet:pup diet, stud diet:sex, pup diet:sex; and three-way stud diet:pup diet:sex interaction. Level of significance (Sig) is denoted by ***p≤0.001 ** p≤0.01, *p≤0.05, #p>0.05<0.01, NS = p>0.05.

Figure	Outcome	Factor	Sum.Sq	DF	F.value	Pr..F.	Sig. level
4.5A,B	**Total distance travelled**	Stud diet	41335829.0	2	0.8261	0.440793	NS
		Pup diet	160903157.4	1	6.4311	0.012794	*
		Sex	229184338.5	1	9.1602	0.00316	**
		Stud diet: Pup diet	21397908.1	2	0.4276	0.653269	NS
		Stud diet: Sex	4160987.9	2	0.0832	0.920274	NS
		Pup diet: Sex	18596187.9	1	0.7433	0.390723	NS
		Stud diet: Pup diet: Sex	6847469.7	2	0.1368	0.872275	NS
4.5C,D	**Velocity (mm/s)**	Stud diet	331.2	2	0.6036	0.548847	NS
		Pup diet	1418.6	1	5.1716	0.02514	*
		Sex	2502.1	1	9.1214	0.003222	**
		Stud diet: Pup diet	149.8	2	0.2730	0.761648	NS
		Stud diet: Sex	0.6	2	0.0011	0.998878	NS
		Pup diet: Sex	232.5	1	0.8475	0.359523	NS

		Stud diet: Pup diet: Sex	207.5	2	0.3783	0.68603	NS
4.5E,F	**Percentage time in outer section (%)**	Stud diet	9.7	2	0.2077	0.81405	NS
		Pup diet	20.0	1	0.8535	0.358773	NS
		Sex	1.2	1	0.0513	0.821485	NS
		Stud diet: Pup diet	37.7	2	0.8041	0.451649	NS
		Stud diet: Sex	67.3	2	1.4355	0.244747	NS
		Pup diet: Sex	35.6	1	1.5178	0.222238	NS
		Stud diet: Pup diet: Sex	27.5	2	0.5857	0.559553	NS
4.5G,H	**Percentage time in inner section (%)**	Stud diet	10.5	2	0.2221	0.802631	NS
		Pup diet	19.7	1	0.8336	0.364416	NS
		Sex	1.5	1	0.0621	0.80394	NS
		Stud diet: Pup diet	36.2	2	0.7684	0.467696	NS
		Stud diet: Sex	65.4	2	1.3872	0.256405	NS
		Pup diet: Sex	36.5	1	1.5459	0.218057	NS
		Stud diet: Pup diet: Sex	28.2	2	0.5975	0.553074	NS
4.5I,J	**Percentage time in slow speed (%)**	Stud diet	188.1	2	0.7551	0.472663	NS
		Pup diet	371.4	1	2.9823	0.08733	#
		Sex	873.2	1	7.0108	0.009443	**

		Stud diet: Pup diet	160.4	2	0.6438	0.527495	NS
		Stud diet: Sex	139.5	2	0.5600	0.573035	NS
		Pup diet: Sex	94.1	1	0.7559	0.386733	NS
		Stud diet: Pup diet: Sex	182.1	2	0.7312	0.483947	NS
4.5K,L	**Percentage time in fast speed (%)**	Stud diet	181.1	2	0.7341	0.482569	NS
		Pup diet	372.5	1	3.0196	0.085405	#
		Sex	871.0	1	7.0616	0.009197	**
		Stud diet: Pup diet	152.4	2	0.6180	0.541123	NS
		Stud diet: Sex	130.0	2	0.5271	0.591973	NS
		Pup diet: Sex	87.7	1	0.7109	0.401185	NS
		Stud diet: Pup diet: Sex	185.9	2	0.7535	0.473417	NS
4.5M,N	**Percent time in slow motion (%)**	Stud diet	797.2	2	1.9497	0.147792	NS
		Pup diet	321.9	1	1.5748	0.212499	NS
		Sex	248.0	1	1.2129	0.273449	NS
		Stud diet: Pup diet	414.3	2	1.0134	0.366758	NS
		Stud diet: Sex	452.3	2	1.1063	0.334876	NS
		Pup diet: Sex	884.4	1	4.3264	0.04013	*

						6	
		Stud diet: Pup diet: Sex	258.0	2	0.6310	0.534191	NS
4.5O,P	**Percent time in fast motion (%)**	Stud diet	794.8	2	1.9652	0.145605	NS
		Pup diet	307.1	1	1.5188	0.220747	NS
		Sex	194.7	1	0.9629	0.328863	NS
		Stud diet: Pup diet	388.4	2	0.9604	0.386303	NS
		Stud diet: Sex	448.8	2	1.1098	0.333735	NS
		Pup diet: Sex	876.2	1	4.3329	0.039989	*
		Stud diet: Pup diet: Sex	255.6	2	0.6321	0.533624	NS

4.3.6 Correlations between stud metabolism and offspring behaviour were observed

In chow males, EPM distance and velocity negatively correlated with studs EPM distance and velocity (Figure 4.6). In females, EPM closed bout negatively correlated with stud bouts in OF, and positively correlated with fast motion in EPM. EPM fast motion was negatively correlated with studs closed bout in EPM and positively correlated with stud basal glucose (Figure 4.6).

The distance travelled and velocity in the EPM of WD males were negatively correlated with stud energy intake, time spent in open and fast motion. For these same animals, entries into the closed arms negatively correlated with stud distance travelled and velocity, fast motion, percentage time in the open arms of the EPM, and positively correlated with stud percentage time in the open arms. In WD females, the percentage time spent in open arms negatively correlated with stud insulin and body weight, energy intake and lean mass. The distance travelled and velocity in the EPM of WD females negatively correlated with stud iAUC as well. Their entries into closed arms positively correlated with stud body weight and fat mass, while percentage time in closed arms positively correlated with stud energy intake.

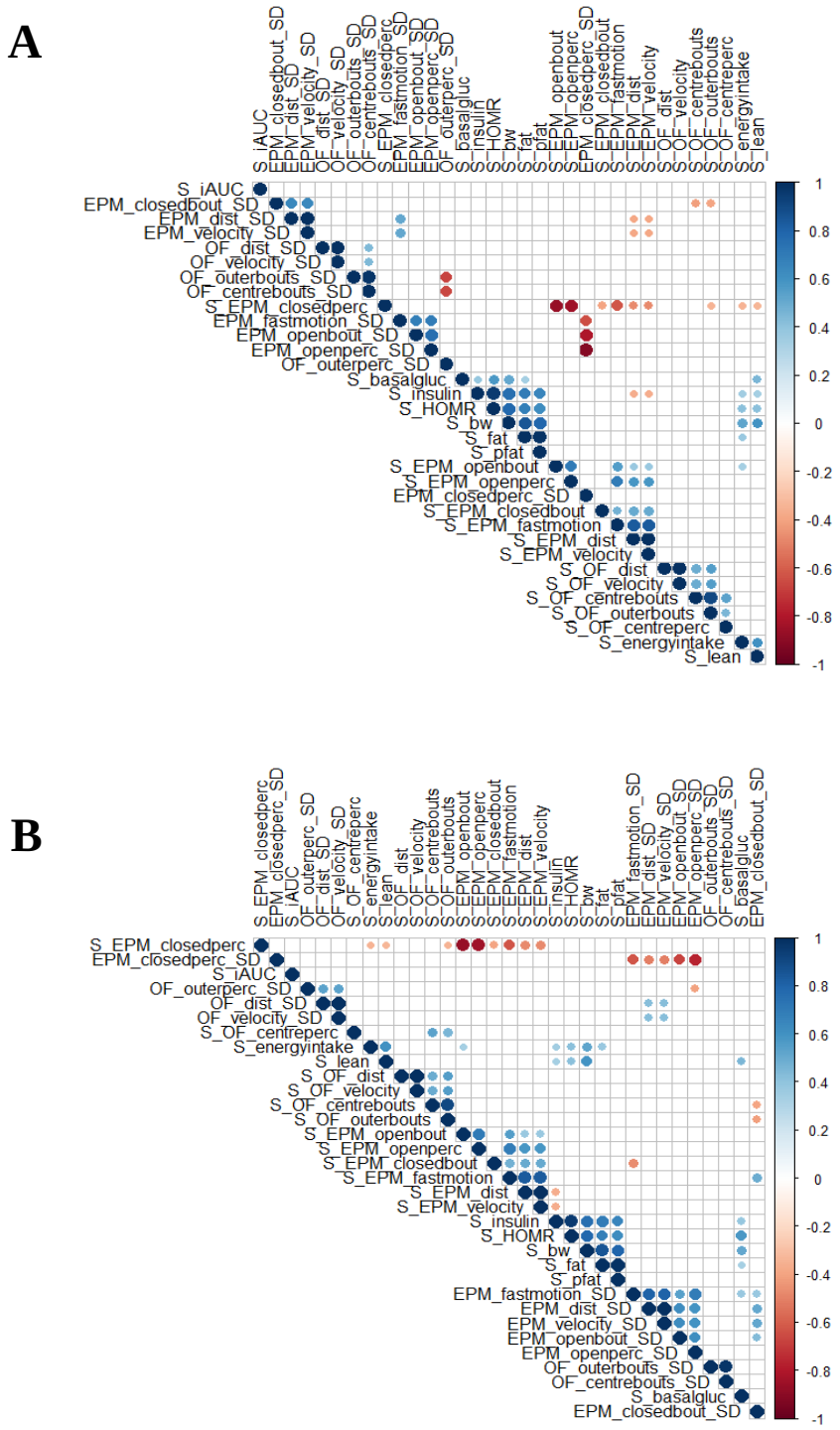

Figure 4.6 Correlogram of stud metabolic outcomes and control-diet fed offspring behavioural parameters

For (A) Male and (B) Female CD fed offspring. Circle size and colour indicate correlation strength (Spearman's rho). Presence of a circle indicates a significant ($p < 0.05$) Spearman correlation.

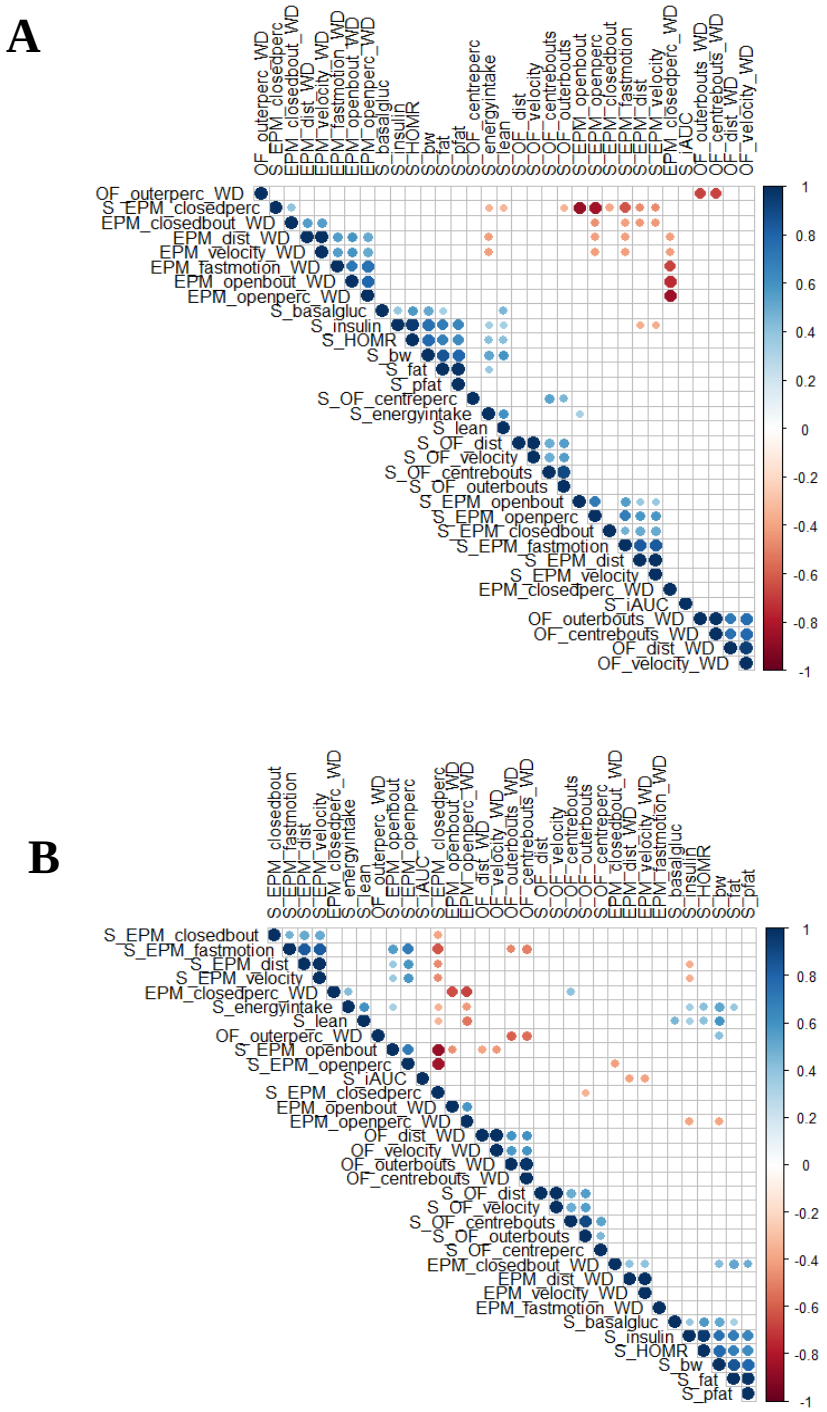

Figure 4.7 Correlogram of stud metabolic outcomes and WD fed offspring behavioural parameters

For (A) Male and (B) Female WD-fed offspring. Circle size and colour indicate correlation strength (Spearman's rho). Presence of a circle indicates a significant ($p < 0.05$) Spearman correlation.

4.4 Discussion

Many animal studies neglect to identify the source and macronutrient content of experimental diets, leading to conflicting and contradictory findings. In this study, we set out to isolate the effects of dietary fat and carbohydrate in paternal diet, without the confounder of energy density. Using two behavioural assays that exploit the natural aversion of rodents to exposed fields - namely, the elevated plus maze and open field maze, we sought to characterise the effect of our dietary manipulation on both paternal and offspring behaviour. Despite finding very minimal effects of diet on stud behaviour, we still observed marked, sex-specific differences in offspring behaviour and locomotion, influenced by both paternal and current diet. This included a three-way stud diet: pup diet: sex interaction, which was observed in the amount of time spent in the closed arms and an interaction between stud diet and pup diet in the motion of the offspring in an EPM. Further correlations found between stud metabolic and behavioural outcomes and offspring behaviour bring forth the idea that paternal effects are highly interactive, depending on factors such as offspring environment and sex, within and across generations.

We initially investigated how changing the percentage of dietary fat and sucrose to starch ratio in the diet of 15-week-old male mice can affect their behaviour. Much of the literature in this field suggests that high fat diets increase the susceptibility of an individual for not only metabolic disorders such as obesity, but also mood disorders such as depression and anxiety (Dutheil et al., 2016, Sharma and Fulton, 2013). On the other hand, increasing the sucrose content of the carbohydrate component within the diet has been shown to both limit activation of the stress system by activating the reward circuits in the brain (Hoebel et al., 2009), but can conversely also promote stress-driven emotional and addictive behaviours and anxiety (Jacques et al., 2019, Witek et al., 2022). Overall, most research find that obesogenic diets are associated with increased anxiety, although there is a large amount of unexplained variation among studies, with some studies reporting opposite trends (Yoshizaki et al., 2020). In this study, we observed that high fat studs had increased open arm entries, which could indicate a predisposition to risk taking behaviour. We found that males fed high fat diets entered open arms more frequently than the control and high sucrose diets, the opposite to most studies that

show rodents on HFDs enter closed arms more, and open arms less often (Sharma and Fulton, 2013, Dutheil et al., 2016). This divergence of these results from the current understanding in literature can be due to a variety of factors such as animal strain (Dutheil et al., 2016) or age they were started on diets (Sharma and Fulton, 2013). These differences could also be attributed to a major experimental consideration that this study has addressed. Much of the findings that link high fat diets to a decline in mental condition are often confounded by their high caloric nature. Contrastingly, we have used diets with similar caloric contents and have instead focused on manipulating the proportions and source of fat and carbohydrates between the three diets. This suggests that it is most likely the energy excess in high fat and Western diets, not increased fat content *per* se, that results in an increased susceptibility to anxiety-like behaviour.

We then chose to perform the same tests on the studs' male and female offspring at the same 15-week timepoint. Interestingly, although we found that HSucrose diets did not affect stud behaviour, their offspring behaved differently to controls. A three-way stud diet: pup diet: sex interaction was observed in the percentage of time spent in the closed arms. It appears that offspring fed WD (high in fat and sucrose) post-weaning were generally spending more time in this zone, indicating an increased anxiety-related response similarly seen in other studies (Gainey et al., 2016, Ogrodnik et al., 2019). Additionally, within the offspring fed control diets, female pups of both HFat and HSucrose studs spent less time in closed arms than those from Control studs, indicating reduced anxiety. Interestingly, the stud control diet in this study has a lower amount of the methyl donor choline (Table 4.1; 1.5g/kg) in comparison to HSucrose and HFat diets (2.1g/kg), This could suggest a link to previous reports, where the levels of methyl donors in the paternal diet led to changes memory-related gene expression, neural functions and anxiety-like behaviour in offspring (Sahara et al., 2019, Ryan et al., 2018). However, further evidence is required to fully elucidate these findings.

An interaction between stud diet and pup diet was found in the motion of the offspring, suggesting a more erratic behaviour in offspring from high sucrose fed fathers and those on WD in adulthood. Though erratic behaviour has previously been reported to have resulted from direct high sucrose diets (Peris-Sampedro et al., 2019), an effect passed on from the paternal line is much less recognised and should be elucidated more.

It is also interesting that influences of paternal diet are reflected in their offspring at all, despite not demonstrating diet-affected changes between the studs themselves. The exact mechanisms that mediate this form of inheritance and phenotypic development should be explored further to distinguish between a multitude of processes, some of which may also be seen in anxiety developed alongside diet-induced obesity. These include epigenetics (Braun and Champagne, 2014, Skinner et al., 2008), hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Rodgers et al., 2013), inflammation (Bolton and Bilbo, 2014), and the alteration of signalling pathways (Vagena et al., 2019, Sasaki et al., 2013). Other studies also suggest that the hippocampus plays a crucial role in influencing performance in behavioural tests and anxiety-related behaviour (Deacon and Rawlins, 2005).

In this study, we found that much of the paternal effects present depended greatly on the offspring's current environment. In later life, paternal effects were not as strong and current environment effects were shown to be more distinct. A main pup diet effect was found in general activity of the animals, where those fed a control diet in adulthood were observed to cover larger distances and have increased velocity and speed when moving around the maze. These results showing the locomotor movements of the animals were also mirrored in the open field maze. In female pups, this could perhaps be attributed to the lesser body weights of SD offspring compared to WD counterparts, allowing the smaller mice to travel more efficiently around the space. Offspring on Western diets displayed a higher tendency towards anxiety-like behaviour; something that is in line with much of the literature in this field (Deal et al., 2020, da Costa Estrela et al., 2015, López-Taboada et al., 2020). Since the western diets used in this portion of the study are indeed hypercaloric (17.3 kJ/g vs 14.7 kJ/g in SD), the idea that anxiety-related behaviour is greater attributed to an increased energy content than specific macronutrient composition is further reiterated.

Sex is also a key factor that defines the consequence of paternal diet impact on their offspring. Aside from the well-recognised sex differences in body weight and morphology (Karastergiou et al., 2012, Palmer and Clegg, 2015), neurophysiological responses (Monroe et al., 2015),and hormonally mediated changes of the inflammatory response (Frye et al., 2000, Zore et al., 2018, Maeng and Milad, 2015), there are also studies that offer evidence regarding the sex-specific nature of parental, and even grand paternal inheritance (Hellmann et al., 2020a, Hellmann et

al., 2020b, Pembrey et al., 2014). Some studies have demonstrated that female offspring and grand offspring, but not males, can exhibit anxiety-like behaviour and social deficits (Mashoodh et al., 2022, Saavedra-Rodríguez and Feig, 2013). However, it was also proposed that in some cases, it is predominantly through males that these effects can be transmitted to subsequent generations, even when they are not displaying the phenotype (Saavedra-Rodríguez and Feig, 2013). This is an interesting comparison to this study, where offspring are shown to display differences in behaviour due to the stud diets, even when we did not detect a significant difference between the studs themselves. It is also noteworthy that in Chapter 3 of this thesis, most of the metabolic stud effects were revealed in male offspring. Other sex-effect papers have previously documented similar findings, where parental effects may be more distinct in one offspring sex over the other (Anway and Skinner, 2006)**.** Past studies also emphasise the differential epigenetic processes in the placenta, noting the abundance of X-linked genes involved and the early unequal gene expression by the sex chromosomes between males and females (Gabory et al., 2013, Champagne, 2013).This then has further consequences for the development of certain traits in offspring.

In this study, it appears as though the specific effects of each stud diet were not very straightforward. It could be due to the high variability of the data, showing near significance in several instances, particularly in the anxiety-related responses from the open field results. The difference in elevated plus maze and open field results could also be explained by different nature of the tests, as discussed in previous papers (Carola et al., 2002). Perhaps, it could also suggest another way by which paternal behaviour influences are facilitated down to their offspring, which may not be as directly causal as seen in maternal studies (Crossland et al., 2017, da Silva et al., 2021), but are highly interactive depending on other key factors such as pup diet and sex. This idea led us to further investigate whether any stud-offspring relationships that were not initially detected as a direct result of our experimental manipulations were still present in an indirect way.

Hence, we explored correlations between stud metabolic and behaviour outcomes and offspring behaviour and found a multitude of significant relationships, where many of the connections were inversely associated. This may reflect a previously observed trend in other paternal effects studies, where a phenotypic change can alternate in presence or direction from one

generation to the next. For example, an increase in effect in paternal generation, decrease or no effect in offspring, then an increase or presence of the effect in grand-offspring generation again. This has been shown in the context of paternal stress regulation through corticosterone (Short et al., 2016), emphasising that environmental manipulations can alter outcomes such as anxiety and depression-related behaviour across multiple generations. A negatively skewed, transgenerational relationship has also been explored in some studies, where paternal effects have been observed to reduce offspring performance in the next generation, especially under highly variable environments (Guillaume et al., 2016). Most of the significant correlations we found in this study stemmed from the WD fed offspring groups, for both sexes. Among these results, we found interesting links between stud metabolism and behaviour and the behaviour of their direct offspring. These correlations continue to demonstrate the complex network of interactions between diet, metabolism, and behaviour across generations.

The findings of this study and correlation results emphasise that it does not seem to be just one direct defining factor that has noticeable consequences on offspring behaviour. Rather, it is the intricate interplay and high correlation of a variety of constituents that is often observed. Although influences of paternal environment are present and detectable in our data, as we have shown, they are interactive and dependent on other major factors, such as offspring diet and sex. Nevertheless, this study provides fundamental new understanding of the ways in which diet and nutrition shape the behaviour of fathers and their offspring, taking the first step towards developing dietary guidelines to improve and optimise mental health conditions such as anxiety and depression.

CHAPTER 5: General Discussion

5.1 Implications of findings

In this thesis, I have employed macronutrient manipulations to parental diets prior to, and during the period of conception, to answer specific and relevant questions in both the maternal and paternal lines of offspring effects. Here, we have shown that varying the ratios of macronutrients, while keeping the energy content constant, has crucial (and in some cases, enduring) impacts on food intake, body composition, metabolism, and behaviour of offspring.

In Chapter 2, we demonstrated that protein leverage occurs from early life and is likely programmed through maternal influences during the stage of gestation and/or lactation. I have shown that an increase of the P:C ratio in maternal diets leads to higher protein targets in the offspring, thus exacerbating the effects of protein leverage and requiring the offspring to consume more food in total to reach these elevated targets. This has large implications when considered through the food landscape of today's society and echoes well recognized evolutionary paradigms of 'mismatch' models. Such examples describe that adaptations prioritising nutritional thriftiness established many generations prior (such as in the hunter-gatherer or Paleolithic era), can be ill-suited when placed in the context of energy-dense modern industrialised diets, resulting in rapidly increasing rates of chronic metabolic disease (Turner and Thompson, 2013, Raubenheimer et al., 2015).

The way that early nutritional targets can be developmentally programmed is likely complex and multifactorial. We have shown that higher protein targets in offspring can be present as early as the weaning period because of maternal diet, but the exact mechanism by which this occurs still requires further elucidation. There are, however, some clues as to how this happens from past studies. For example, high protein diets have been shown to result in enhanced gluconeogenesis and higher nitrogen excretion rates. This in turn could both potentially reduce protein efficiency and increase the protein target (Veldhorst et al., 2009). The relevant signaling

pathways responsible for its transmission within and across generations, and the critical timeframe during which this, and other health outcomes, is programmed is still vague. It is likely that protein target programming begins as early as *in utero* from direct access to their mother's environment, but it is also possible that such signals can occur in or be strengthened in the lactation period from the mother's milk, for instance (Totzauer et al., 2018, Weber et al., 2014). Again, the identification of this critical timepoint could be an avenue for future work in the field to focus on.

Additionally, we illustrated that maternal effects (such as protein target programming and susceptibility to obesity) can remain over time and can be exacerbated by a high caloric offspring Western diet. We found multiple interactive effects of maternal diets, adult diet, and sex in the serum lipid profiles of offspring (Figure 2.4), highlighting the idea that strength of parental effects over time can be determined by acclimation to current environments as well (Beaman et al., 2016). Another interesting concept to explore is how the ideal diets for different stages in life, and for each individual could be varied. Here, we have shown that an increase of dietary protein in mothers prior to conception can raise offspring protein targets, which can then lead to a high susceptibility to obesity in offspring. However, an offspring low protein diet high in fat, carbohydrate, and total energy content (Western diet) was shown to exacerbate an earlier established obese phenotype. This accentuates the importance of the continual development of precision nutritional techniques (Simpson et al., 2015). This will allow for diet optimization for improved health, fecundity and lifespan at critical windows that might occur at multiple stages throughout life.

In Chapter 3 and 4, we manipulated the carbohydrate and fat components in isocaloric paternal diets and examined how it would vary paternal metabolic state, and as a result alter male and female offspring health outcomes. We observed that changing most of the carbohydrate component in high carbohydrate diets from starch (control) to sucrose led to few metabolic differences in male mice, while high fat diets in male studs led to the most obesogenic effect. Increasing the percentage of dietary fat (while keeping energy density constant) resulted in increased body weight, fat mass, fasting insulin levels and basal glucose, with no changes detected in the glucose tolerance, or anxiety-related behaviour of stud groups.

Meanwhile, in the offspring, paternal diet was found to have sex- and pup diet specific effects which were more distinct at an earlier timepoint (10 weeks vs 20 weeks). Importantly, we showed that paternal effects are highly dependent on other key factors such as pup diet and sex. For example, male pups of high-fat fed studs had increased lean mass and body weight when fed either a control or western diet. However, the effects of a high-sugar paternal diet varied depending on pup diet, where increased lean mass was only observed when pups were fed a western diet. When anxiety-related responses were measured in offspring, interaction between stud diet and pup diet was found in some outcomes, such as the motion of the offspring. Some past studies have shown that the appearance of parental effects can highly depend on personal immediate experience, and vice versa (Donelan and Trussell, 2015). For example, when observing anti-predator behaviour in snails it was found that offspring only integrated parental signals, and resulting outcomes were only revealed, when they were exposed to similar cues in their immediate environment. This suggests that personal, maternal, and paternal cues all interact to shape offspring escape behaviour and activity (Tariel et al., 2020).

5.2 Limitations and Future directions

As shown in this body of work, both maternal and paternal environments can shape a wide range of offspring health outcomes, but experimental studies disentangling how, and to what extent their effects interact together, and jointly with offspring environments, are still rare. In this thesis, we wanted to concentrate on separate questions for the different parental effects, and therefore chose specific dietary manipulations to examine them further. One variation to the experimental design of this study would have been to expose both mothers and fathers to similar diet landscapes. Indeed, there is an increasing appreciation for the study of parent-of-origin models (Curley and Mashoodh, 2010, Emborski and Mikheyev, 2019). Moreover, biparental manipulations are increasingly acknowledged as a more relevant model for developing inclusive and translatable research (reviewed in Champagne, 2020, Bonduriansky et al., 2016). Such models could reveal answers for additional layers to the parental story, but a rigorous and logistically experimental design is extremely crucial. Assessing the relative strength of paternal compared to maternal effects is possible when we expose fathers and mothers independently and then pair them up with control individuals (Rutkowska et al., 2020).

An experimental design that manipulates both maternal and paternal environments simultaneously and assesses resulting offspring outcomes would likely be the most appropriate model to humans. Past studies have employed designs comparing two groups of offspring from biparental exposure vs. non-exposure groups and assess if it has a stronger impact on offspring (Ganiger et al., 2007, Ornellas et al., 2016). The downfall of such a design is that it makes it difficult to account for any interactions or mediated effects between paternal and maternal sides independently. More layers of complexity are added when we then look at interactive effects between maternal and paternal environment and offspring sex and environment, making it very challenging to isolate any driving forces for phenotypic changes that occur (Tariel et al., 2020). Disentangling these interactions may be fundamental to understanding how offspring integrate past and present environmental cues to produce variant phenotypes.

5.3 Conclusion

There remains much work to be able to fully unravel the complex interplay between parental influences, non-genetic changes, inherent offspring variations and current environments. This thesis has shared some novel insight on how nutrient targets and food selection are programmed in offspring, with further consequences to later-life metabolism and behaviour. We have provided a good foundation for future research in the field of developmental programming and obesity preventions, but the specific mechanistic pathways responsible, and the degree of interaction between the many factors involved still require further elucidation. A multidiscipline approach combining reproductive biology, genomics, molecular biology, neuroendocrinology, behavioural ecology, and evolutionary theory needs to be combined and corroborated to create comprehensive models that not only predict the phenomenon of parental effects but also develop a framework for understanding the broader context of when and why these effects may interact.

REFERENCES

ALMEIDA-SUHETT, C., GRAHAM, A., CHEN, Y. & DEUSTER, P. 2016. Behavioral changes in male mice fed a high-fat diet are associated with IL-1β expression in specific brain regions. *Physiology & Behavior,* 169.

ALTER, M. D., GILANI, A. I., CHAMPAGNE, F. A., CURLEY, J. P., TURNER, J. B. & HEN, R. 2009. Paternal transmission of complex phenotypes in inbred mice. *Biol Psychiatry,* 66**,** 1061-6.

ANDRÉ, C., DINEL, A.-L., FERREIRA, G., LAYÉ, S. & CASTANON, N. 2014. Diet-induced obesity progressively alters cognition, anxiety-like behavior and lipopolysaccharide-induced depressive-like behavior: Focus on brain indoleamine 2,3-dioxygenase activation. *Brain, Behavior, and Immunity,* 41**,** 10-21.

ANWAY, M. D. & SKINNER, M. K. 2006. Epigenetic Transgenerational Actions of Endocrine Disruptors. *Endocrinology,* 147**,** s43-s49.

ARMITAGE, J. A., KHAN, I. Y., TAYLOR, P. D., NATHANIELSZ, P. W. & POSTON, L. 2004. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? *The Journal of Physiology,* 561**,** 355-377.

ARORA, S. & ANUBHUTI 2006. Role of neuropeptides in appetite regulation and obesity – A review. *Neuropeptides,* 40**,** 375-401.

ASANO, Y. 1986. Characteristics of open field behavior of Wistar and Sprague-Dawley rats. *Jikken Dobutsu,* 35**,** 505-8.

BAGCHI, D. P. & MACDOUGALD, O. A. 2019. Identification and Dissection of Diverse Mouse Adipose Depots. *J Vis Exp*.

BAKOS, H. W., MITCHELL, M., SETCHELL, B. P. & LANE, M. 2011. The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model. *International Journal of Andrology,* 34**,** 402-410.

BALDINI, G. & PHELAN, K. D. 2019. The melanocortin pathway and control of appetite-progress and therapeutic implications. *J Endocrinol,* 241**,** R1-r33.

BALLESTRI, S., NASCIMBENI, F., BALDELLI, E., MARRAZZO, A., ROMAGNOLI, D. & LONARDO, A. 2017. NAFLD as a Sexual Dimorphic Disease: Role of Gender and Reproductive Status in the Development and Progression of Nonalcoholic Fatty Liver Disease and Inherent Cardiovascular Risk. *Adv Ther,* 34**,** 1291-1326.

BARDI, M., FRANSSEN, C. L., HAMPTON, J. E., SHEA, E. A., FANEAN, A. P. & LAMBERT, K. G. 2011. Paternal experience and stress responses in California mice (Peromyscus californicus). *Comp Med,* 61**,** 20-30.

BARKER, D. J. 1990. The fetal and infant origins of adult disease. *British Medical Journal,* 301**,** 1111-1111.

BARKER, D. J. 2004. The developmental origins of adult disease. *J Am Coll Nutr,* 23**,** 588s-595s.

BARKER, D. J. P. 2012. Developmental origins of chronic disease. *Public Health,* 126**,** 185-189.

BARKER, D. J. P., GODFREY, K. M., GLUCKMAN, P. D., HARDING, J. E., OWENS, J. A. & ROBINSON, J. S. 1993. Fetal nutrition and cardiovascular disease in adult life. *The Lancet,* 341**,** 938-941.

BATES, D., MÄCHLER, M., BOLKER, B. & WALKER, S. 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software,* 67**,** 1 - 48.

BEAMAN, J. E., WHITE, C. R. & SEEBACHER, F. 2016. Evolution of Plasticity: Mechanistic Link between Development and Reversible Acclimation. *Trends in Ecology & Evolution,* 31**,** 237-249.

BELL, A. M. & HELLMANN, J. K. 2019. An Integrative Framework for Understanding the Mechanisms and Multigenerational Consequences of Transgenerational Plasticity. *Annual Review of Ecology, Evolution, and Systematics,* 50**,** 97-118.

BELL, C. G., WALLEY, A. J. & FROGUEL, P. 2005. The genetics of human obesity. *Nature Reviews Genetics,* 6**,** 221+.

BELLISLE, F. 2004. Effects of diet on behaviour and cognition in children. *Br J Nutr,* 92 Suppl 2**,** S227-32.

BERTHOUD, H.-R. 2007. Interactions between the "cognitive" and "metabolic" brain in the control of food intake. *Physiology & Behavior,* 91**,** 486-498.

BILBO, S. D. & TSANG, V. 2010. Enduring consequences of maternal obesity for brain inflammation and behavior of offspring. *The FASEB Journal,* 24**,** 2104-2115.

BILLAH, M. M., KHATIWADA, S., MORRIS, M. J. & MALONEY, C. A. 2022. Effects of paternal overnutrition and interventions on future generations. *International Journal of Obesity,* 46**,** 901-917.

BINDER, N. K., BEARD, S. A., KAITU'U-LINO, T. J., TONG, S., HANNAN, N. J. & GARDNER, D. K. 2015a. Paternal obesity in a rodent model affects placental gene expression in a sex-specific manner. *Reproduction,* 149**,** 435-44.

BINDER, N. K., HANNAN, N. J. & GARDNER, D. K. 2012. Paternal Diet-Induced Obesity Retards Early Mouse Embryo Development, Mitochondrial Activity and Pregnancy Health. *PLOS ONE,* 7**,** e52304.

BINDER, N. K., SHEEDY, J. R., HANNAN, N. J. & GARDNER, D. K. 2015b. Male obesity is associated with changed spermatozoa Cox4i1 mRNA level and altered seminal vesicle fluid composition in a mouse model. *Mol Hum Reprod,* 21**,** 424-34.

BLUMFIELD, M. L. 2015. Update on the role of maternal diet in pregnancy and the programming of infant body composition. *Nutrition Bulletin,* 40**,** 286-290.

BLUNDELL, J. E. & MACDIARMID, J. I. 1997. Fat as a risk factor for overconsumption: satiation, satiety, and patterns of eating. *J Am Diet Assoc,* 97**,** S63-9.

BODDEN, C., PANG, T. Y., FENG, Y., MRIDHA, F., KONG, G., LI, S., WATT, M. J., REICHELT, A. C. & HANNAN, A. J. 2022. Intergenerational effects of a paternal Western diet during adolescence on offspring gut microbiota, stress reactivity, and social behavior. *Faseb j,* 36**,** e21981.

BOLTON, J. L. & BILBO, S. D. 2014. Developmental programming of brain and behavior by perinatal diet: focus on inflammatory mechanisms. *Dialogues Clin Neurosci,* 16**,** 307-20.

BONDURIANSKY, R., RUNAGALL-MCNAULL, A. & CREAN, A. J. 2016. The nutritional geometry of parental effects: maternal and paternal macronutrient consumption and offspring phenotype in a neriid fly. *Functional Ecology,* 30**,** 1675-1686.

BOURET, S. G. 2010. Development of hypothalamic neural networks controlling appetite. *Forum Nutr,* 63**,** 84-93.

BOURET, S. G., DRAPER, S. J. & SIMERLY, R. B. 2004. Trophic action of leptin on hypothalamic neurons that regulate feeding. *Science,* 304**,** 108-10.

BRAUN, K. & CHAMPAGNE, F. A. 2014. Paternal Influences on Offspring Development: Behavioural and Epigenetic Pathways. *Journal of Neuroendocrinology,* 26**,** 697-706.

BROOK, C. A. & SCHMIDT, L. A. 2008. Social anxiety disorder: a review of environmental risk factors. *Neuropsychiatr Dis Treat,* 4**,** 123-43.

BRÜNING, J. C., GAUTAM, D., BURKS, D. J., GILLETTE, J., SCHUBERT, M., ORBAN, P. C., KLEIN, R., KRONE, W., MÜLLER-WIELAND, D. & KAHN, C. R. 2000. Role of brain insulin receptor in control of body weight and reproduction. *Science,* 289**,** 2122-5.

BURKE, V., BEILIN, L. J. & DUNBAR, D. 2001. Family lifestyle and parental body mass index as predictors of body mass index in Australian children: a longitudinal study. *Int*

J Obes Relat Metab Disord, 25**,** 147-57.

BYGREN, L. O., KAATI, G. & EDVINSSON, S. 2001. Longevity Determined by Paternal Ancestors' Nutrition during Their Slow Growth Period. *Acta Biotheoretica,* 49**,** 53-9.

CAROLA, V., D'OLIMPIO, F., BRUNAMONTI, E., MANGIA, F. & RENZI, P. 2002. Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. *Behav Brain Res,* 134**,** 49-57.

CARONE, B. R., FAUQUIER, L., HABIB, N., SHEA, J. M., HART, C. E., LI, R., BOCK, C., LI, C., GU, H., ZAMORE, P. D., MEISSNER, A., WENG, Z., HOFMANN, H. A., FRIEDMAN, N. & RANDO, O. J. 2010. Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell,* 143**,** 1084-1096.

CÉSAR, H., SERTORIO, M. N., DE SOUZA, E. A., JAMAR, G., SANTAMARINA, A., JUCÁ, A., CASAGRANDE, B. P. & PISANI, L. P. 2022. Parental high-fat high-sugar diet programming and hypothalamus adipose tissue axis in male Wistar rats. *Eur J Nutr,* 61**,** 523-537.

CHAMBERS, T. J. G., MORGAN, M. D., HEGER, A. H., SHARPE, R. M. & DRAKE, A. J. 2016. High-fat diet disrupts metabolism in two generations of rats in a parent-of-origin specific manner. *Scientific Reports (Nature Publisher Group),* 6**,** 31857.

CHAMPAGNE, F. A. 2013. Effects of Stress Across Generations: Why Sex Matters. *Biological Psychiatry,* 73**,** 2-4.

CHAMPAGNE, F. A. 2020. Interplay between paternal germline and maternal effects in shaping development: The overlooked importance of behavioural ecology. *Functional Ecology,* 34**,** 401-413.

CHAMPROUX, A., COCQUET, J., HENRY-BERGER, J., DREVET, J. R. & KOCER, A. 2018. A Decade of Exploring the Mammalian Sperm Epigenome: Paternal Epigenetic and Transgenerational Inheritance. *Frontiers in cell and developmental biology,* 6**,** 50-50.

CHANG, G.-Q., GAYSINSKAYA, V., KARATAYEV, O. & LEIBOWITZ, S. F. 2008. Maternal High-Fat Diet and Fetal Programming: Increased Proliferation of Hypothalamic Peptide-Producing Neurons That Increase Risk for Overeating and Obesity. *The Journal of Neuroscience,* 28**,** 12107-12119.

CHEN, Q., YAN, M., CAO, Z., LI, X., ZHANG, Y., SHI, J., FENG, G. H., PENG, H., ZHANG, X., ZHANG, Y., QIAN, J., DUAN, E., ZHAI, Q. & ZHOU, Q. 2016. Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder.

Science, 351**,** 397-400.

CHEN, X., MCCLUSKY, R., CHEN, J., BEAVEN, S. W., TONTONOZ, P., ARNOLD, A. P. & REUE, K. 2012. The Number of X Chromosomes Causes Sex Differences in Adiposity in Mice. *PLOS Genetics,* 8**,** e1002709.

CHIU, Y. H., AFEICHE, M. C., GASKINS, A. J., WILLIAMS, P. L., MENDIOLA, J., JØRGENSEN, N., SWAN, S. H. & CHAVARRO, J. E. 2014. Sugar-sweetened beverage intake in relation to semen quality and reproductive hormone levels in young men. *Hum Reprod,* 29**,** 1575-84.

CHOWDHURY, R., WARNAKULA, S., KUNUTSOR, S., CROWE, F., WARD, H. A., JOHNSON, L., FRANCO, O. H., BUTTERWORTH, A. S., FOROUHI, N. G., THOMPSON, S. G., KHAW, K. T., MOZAFFARIAN, D., DANESH, J. & DI ANGELANTONIO, E. 2014. Association of dietary, circulating, and supplement fatty acids with coronary risk: a systematic review and meta-analysis. *Ann Intern Med,* 160**,** 398-406.

CHOWDHURY, S. S., LECOMTE, V., ERLICH, J. H., MALONEY, C. A. & MORRIS, M. J. 2016. Paternal High Fat Diet in Rats Leads to Renal Accumulation of Lipid and Tubular Changes in Adult Offspring. *Nutrients,* 8**,** 521.

CLARK, T. D., CREAN, A. J. & SENIOR, A. M. 2022. Obesogenic diets induce anxiety in rodents: A systematic review and meta-analysis. *Obesity Reviews,* 23**,** e13399.

CLEAL, J. K., POORE, K. R., BOULLIN, J. P., KHAN, O., CHAU, R., HAMBIDGE, O., TORRENS, C., NEWMAN, J. P., POSTON, L., NOAKES, D. E., HANSON, M. A. & GREEN, L. R. 2007. Mismatched pre- and postnatal nutrition leads to cardiovascular dysfunction and altered renal function in adulthood. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 9529-9533.

COOPER, R., HYPPÖNEN, E., BERRY, D. & POWER, C. 2010. Associations between parental and offspring adiposity up to midlife: the contribution of adult lifestyle factors in the 1958 British Birth Cohort Study. *The American Journal of Clinical Nutrition,* 92**,** 946-953.

CORDAIN, L., MILLER, J. B., EATON, S. B. & MANN, N. 2000. Macronutrient estimations in hunter-gatherer diets. *The American Journal of Clinical Nutrition,* 72**,** 1589-1590.

COWLEY, M. A. & GROVE, K. L. 2004. Ghrelin--satisfying a hunger for the mechanism. *Endocrinology,* 145**,** 2604-6.

CREAN, A. J. & BONDURIANSKY, R. 2014. What is a paternal effect? *Trends in Ecology & Evolution,* 29**,** 554-559.

CREAN, A. J., DWYER, J. M. & MARSHALL, D. J. 2013. Adaptive paternal effects? Experimental evidence that the paternal environment affects offspring performance. *Ecology,* 94**,** 2575-2582.

CREAN, A. J. & SENIOR, A. M. 2019. High-fat diets reduce male reproductive success in animal models: A systematic review and meta-analysis. *Obes Rev,* 20**,** 921-933.

CRISÓSTOMO, L., JARAK, I., RATO, L. P., RAPOSO, J. F., BATTERHAM, R. L., OLIVEIRA, P. F. & ALVES, M. G. 2021. Inheritable testicular metabolic memory of high-fat diet causes transgenerational sperm defects in mice. *Scientific Reports (Nature Publisher Group),* 11.

CROSSLAND, R. F., BALASA, A., RAMAKRISHNAN, R., MAHADEVAN, S. K., FIOROTTO, M. L. & IGNATIA, B. V. D. V. 2017. Chronic Maternal Low-Protein Diet in Mice Affects Anxiety, Night-Time Energy Expenditure and Sleep Patterns, but Not Circadian Rhythm in Male Offspring. *PLoS One,* 12.

CUNNINGHAM, A. M., WALKER, D. M. & NESTLER, E. J. 2021. Paternal transgenerational epigenetic mechanisms mediating stress phenotypes of offspring. *Eur J Neurosci,* 53**,** 271-280.

CURHAN, G. C., CHERTOW, G. M., WILLETT, W. C., SPIEGELMAN, D., COLDITZ, G. A., MANSON, J. E., SPEIZER, F. E. & STAMPFER, M. J. 1996. Birth weight and adult hypertension and obesity in women. *Circulation,* 94**,** 1310-5.

CURLEY, J. P. & MASHOODH, R. 2010. Parent-of-origin and trans-generational germline influences on behavioral development: The interacting roles of mothers, fathers, and grandparents. *Developmental Psychobiology,* 52**,** 312-330.

CURLEY, J. P., MASHOODH, R. & CHAMPAGNE, F. A. 2011. Epigenetics and the origins of paternal effects. *Hormones and Behavior,* 59**,** 306-314.

DA COSTA ESTRELA, D., DA SILVA, W. A. M., GUIMARÃES, A. T. B., DE OLIVEIRA MENDES, B., DA SILVA CASTRO, A. L., DA SILVA TORRES, I. L. & MALAFAIA, G. 2015. Predictive behaviors for anxiety and depression in female Wistar rats subjected to cafeteria diet and stress. *Physiology & Behavior,* 151**,** 252-263.

DA SILVA, L. O., DA SILVA ARAGÃO, R., DUARTE BARROS, M. D. L., NOGUEIRA FERRAZ-PEREIRA, K., LINS PINHEIRO, I. & GALINDO, L. C. M. 2021. Maternal exposure to high-fat diet modifies anxiety-like/depression-like behaviors and compounds of Serotonergic System in offspring: A preclinical systematic review. *Int J Dev Neurosci,* 81**,** 371-385.

DANTZER, R., O'CONNOR, J. C., FREUND, G. G., JOHNSON, R. W. & KELLEY, K. W.

2008. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nature reviews. Neuroscience,* 9**,** 46-56.

DE CASTRO BARBOSA, T., INGERSLEV, L. R., ALM, P. S., VERSTEYHE, S., MASSART, J., RASMUSSEN, M., DONKIN, I., SJÖGREN, R., MUDRY, J. M., VETTERLI, L., GUPTA, S., KROOK, A., ZIERATH, J. R. & BARRÈS, R. 2016. High-fat diet reprograms the epigenome of rat spermatozoa and transgenerationally affects metabolism of the offspring. *Molecular Metabolism,* 5**,** 184-197.

DE GROEF, S., WILMS, T., BALMAND, S., CALEVRO, F. & CALLAERTS, P. 2022. Sexual Dimorphism in Metabolic Responses to Western Diet in Drosophila melanogaster. *Biomolecules,* 12**,** 33.

DE PERGOLA, G. & SILVESTRIS, F. 2013. Obesity as a major risk factor for cancer. *J Obes,* 2013**,** 291546.

DE ROOIJ, S. R., BLEKER, L. S., PAINTER, R. C., RAVELLI, A. C. & ROSEBOOM, T. J. 2022. Lessons learned from 25 Years of Research into Long term Consequences of Prenatal Exposure to the Dutch famine 1944–45: The Dutch famine Birth Cohort. *International Journal of Environmental Health Research,* 32**,** 1432-1446.

DE ROOIJ, S. R., PAINTER, R. C., PHILLIPS, D. I. W., OSMOND, C., MICHELS, R. P. J., GODSLAND, I. F., BOSSUYT, P. M. M., BLEKER, O. P. & ROSEBOOM, T. J. 2006a. Impaired insulin secretion after prenatal exposure to the Dutch famine. *Diabetes Care.*

DE ROOIJ, S. R., PAINTER, R. C., ROSEBOOM, T. J., PHILLIPS, D. I., OSMOND, C., BARKER, D. J., TANCK, M. W., MICHELS, R. P., BOSSUYT, P. M. & BLEKER, O. P. 2006b. Glucose tolerance at age 58 and the decline of glucose tolerance in comparison with age 50 in people prenatally exposed to the Dutch famine. *Diabetologia,* 49**,** 637-43.

DE ROOIJ, S. R., WOUTERS, H., YONKER, J. E., PAINTER, R. C. & ROSEBOOM, T. J. 2010. Prenatal undernutrition and cognitive function in late adulthood. *Proceedings of the National Academy of Sciences,* 107**,** 16881-16886.

DEACON, R. M. J. & RAWLINS, J. N. P. 2005. Hippocampal lesions, species-typical behaviours and anxiety in mice. *Behavioural Brain Research,* 156**,** 241-249.

DEAL, A. W., SESHIE, O., LENZO, A., COOPER, N., OZIMEK, N. & WOODS, L. C. S. 2020. High-fat diet negatively impacts both metabolic and behavioral health in outbred heterogeneous stock rats. *Physiological Genomics,* 52**,** 379-390.

DEARDEN, L., BOURET, S. G. & OZANNE, S. E. 2018. Sex and gender differences in

developmental programming of metabolism. *Molecular metabolism,* 15**,** 8-19.

DENHAM, J., O'BRIEN, B. J., HARVEY, J. T. & CHARCHAR, F. J. 2015. Genome-wide sperm DNA methylation changes after 3 months of exercise training in humans. *Epigenomics,* 7**,** 717-731.

DIAS, B. G. & RESSLER, K. J. 2013. Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nature Neuroscience,* 17**,** 89.

DIETZ, D. M., LAPLANT, Q., WATTS, E. L., HODES, G. E., RUSSO, S. J., FENG, J., OOSTING, R. S., VIALOU, V. & NESTLER, E. J. 2011. Paternal transmission of stress-induced pathologies. *Biol Psychiatry,* 70**,** 408-14.

DIMOFSKI, P., MEYRE, D., DREUMONT, N. & LEININGER-MULLER, B. 2021. Consequences of Paternal Nutrition on Offspring Health and Disease. *Nutrients,* 13**,** 2818.

DONELAN, S. C. & TRUSSELL, G. C. 2015. Parental effects enhance risk tolerance and performance in offspring. *Ecology,* 96**,** 2049-2055.

DONKIN, I. & BARRÈS, R. 2018. Sperm epigenetics and influence of environmental factors. *Molecular metabolism,* 14**,** 1-11.

DUNFORD, A. R. & SANGSTER, J. M. 2017. Maternal and paternal periconceptional nutrition as an indicator of offspring metabolic syndrome risk in later life through epigenetic imprinting: A systematic review. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews,* 11**,** S655-S662.

DUSSUTOUR, A. & SIMPSON, S. J. 2009. Communal Nutrition in Ants. *Current Biology,* 19**,** 740-744.

DUTHEIL, S., OTA, K. T., WOHLEB, E. S., RASMUSSEN, K. & DUMAN, R. S. 2016. High-Fat Diet Induced Anxiety and Anhedonia: Impact on Brain Homeostasis and Inflammation. *Neuropsychopharmacology,* 41**,** 1874-87.

ELLACOTT, K. L. J., MORTON, G. J., WOODS, S. C., TSO, P. & SCHWARTZ, M. W. 2010. Assessment of Feeding Behavior in Laboratory Mice. *Cell Metabolism,* 12**,** 10-17.

ELMQUIST, J. K., COPPARI, R., BALTHASAR, N., ICHINOSE, M. & LOWELL, B. B. 2005. Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. *Journal of Comparative Neurology,* 493**,** 63-71.

EMBORSKI, C. & MIKHEYEV, A. S. 2019. Ancestral diet transgenerationally influences offspring in a parent-of-origin and sex-specific manner. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 374**,** 20180181.

ERIKSSON, J. G., FORSÉN, T., TUOMILEHTO, J., OSMOND, C. & BARKER, D. J. 2003. Early adiposity rebound in childhood and risk of Type 2 diabetes in adult life. *Diabetologia,* 46**,** 190-4.

FAITH, M. S., MATZ, P. E. & JORGE, M. A. 2002. Obesity–depression associations in the population. *Journal of Psychosomatic Research,* 53**,** 935-942.

FARDET, A. 2018. Characterization of the Degree of Food Processing in Relation With Its Health Potential and Effects. *Adv Food Nutr Res,* 85**,** 79-129.

FAROOQI, I. S., KEOGH, J. M., YEO, G. S. H., LANK, E. J., CHEETHAM, T. & O'RAHILLY, S. 2003. Clinical Spectrum of Obesity and Mutations in the Melanocortin 4 Receptor Gene. *New England Journal of Medicine,* 348**,** 1085-1095.

FELTON, A. M., FELTON, A., RAUBENHEIMER, D., SIMPSON, S. J., FOLEY, W. J., WOOD, J. T., WALLIS, I. R. & LINDENMAYER, D. B. 2009. Protein content of diets dictates the daily energy intake of a free-ranging primate. *Behavioral Ecology,* 20**,** 685-690.

FERNANDEZ-TWINN, D. S., WAYMAN, A., EKIZOGLOU, S., MARTIN, M. S., HALES, C. N. & OZANNE, S. E. 2005. Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 288**,** R368-R373.

FLEMING, T. P., SUN, C., DENISENKO, O., CAETANO, L., ALJAHDALI, A., GOULD, J. M. & KHURANA, P. 2021. Environmental Exposures around Conception: Developmental Pathways Leading to Lifetime Disease Risk. *International Journal of Environmental Research and Public Health,* 18**,** 9380.

FLIPPO, K. H. & POTTHOFF, M. J. 2021. Metabolic Messengers: FGF21. *Nature Metabolism,* 3**,** 309-317.

FRANCIS, H. & STEVENSON, R. 2013. The longer-term impacts of Western diet on human cognition and the brain. *Appetite,* 63**,** 119-128.

FRIEDMAN, J. E. 2018. Developmental Programming of Obesity and Diabetes in Mouse, Monkey, and Man in 2018: Where Are We Headed? *Diabetes,* 67**,** 2137-2151.

FRIEDMAN, J. M. & HALAAS, J. L. 1998. Leptin and the regulation of body weight in mammals. *Nature,* 395**,** 763-70.

FRYE, C. A., PETRALIA, S. M. & RHODES, M. E. 2000. Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3α,5α-THP. *Pharmacology Biochemistry and Behavior,* 67**,** 587-596.

FULLSTON, T., PALMER, N. O., OWENS, J. A., MITCHELL, M., BAKOS, H. W. & LANE, M. 2012. Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Human Reproduction,* 27**,** 1391-1400.

FULLSTON, T., TEAGUE, E. M. C. O., PALMER, N. O., DEBLASIO, M. J., MITCHELL, M., CORBETT, M., PRINT, C. G., OWENS, J. A. & LANE, M. 2013. Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *The FASEB Journal,* 27**,** 4226-4243.

GABORY, A., ROSEBOOM, T. J., MOORE, T., MOORE, L. G. & JUNIEN, C. 2013. Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics. *Biol Sex Differ,* 4**,** 5.

GAINEY, S. J., KWAKWA, K. A., BRAY, J. K., PILLOTE, M. M., TIR, V. L., TOWERS, A. E. & FREUND, G. G. 2016. Short-Term High-Fat Diet (HFD) Induced Anxiety-Like Behaviors and Cognitive Impairment Are Improved with Treatment by Glyburide. *Frontiers in Behavioral Neuroscience*.

GALI RAMAMOORTHY, T., BEGUM, G., HARNO, E. & WHITE, A. 2015. Developmental programming of hypothalamic neuronal circuits: impact on energy balance control. *Frontiers in Neuroscience,* 9.

GALLOU-KABANI, C., GABORY, A., TOST, J., KARIMI, M., MAYEUR, S., LESAGE, J., BOUDADI, E., GROSS, M.-S., TAURELLE, J., VIGÉ, A., BRETON, C., REUSENS, B., REMACLE, C., VIEAU, D., EKSTRÖM, T. J., JAIS, J.-P. & JUNIEN, C. 2010. Sex- and Diet-Specific Changes of Imprinted Gene Expression and DNA Methylation in Mouse Placenta under a High-Fat Diet. *PLOS ONE,* 5**,** e14398.

GANIGER, S., MALLESHAPPA, H. N., KRISHNAPPA, H., RAJASHEKHAR, G., RAMAKRISHNA RAO, V. & SULLIVAN, F. 2007. A two generation reproductive toxicity study with curcumin, turmeric yellow, in Wistar rats. *Food Chem Toxicol,* 45**,** 64-9.

GAPP, K., SOLDADO-MAGRANER, S., ALVAREZ-SÁNCHEZ, M., BOHACEK, J., VERNAZ, G., SHU, H., FRANKLIN, T. B., WOLFER, D. & MANSUY, I. M. 2014. Early life stress in fathers improves behavioural flexibility in their offspring. *Nature Communications,* 5**,** 5466.

GLUCKMAN, P. D., HANSON, M. A., BEEDLE, A. S. & SPENCER, H. G. 2008a. Predictive adaptive responses in perspective. *Trends Endocrinol Metab,* 19**,** 109-10; author reply

112.

GLUCKMAN, P. D., HANSON, M. A. & BUKLIJAS, T. 2010. A conceptual framework for the developmental origins of health and disease. *Journal of Developmental Origins of Health and Disease,* 1**,** 6-18.

GLUCKMAN, P. D., HANSON, M. A., COOPER, C. & THORNBURG, K. L. 2008b. Effect of In Utero and Early-Life Conditions on Adult Health and Disease. *New England Journal of Medicine,* 359**,** 61-73.

GLUCKMAN, P. D., HANSON, M. A., SPENCER, H. G. & BATESON, P. 2005. Environmental influences during development and their later consequences for health and disease: implications for the interpretation of empirical studies. *Proceedings. Biological sciences,* 272**,** 671-677.

GODSCHALK, R., REMELS, A., HOOGENDOORN, C., VAN BENTHEM, J., LUIJTEN, M., DUALE, N., BRUNBORG, G., OLSEN, A.-K., BOUWMAN, F. G., MUNNIA, A., PELUSO, M., MARIMAN, E. & VAN SCHOOTEN, F. J. 2017. Paternal Exposure to Environmental Chemical Stress Affects Male Offspring's Hepatic Mitochondria. *Toxicological Sciences,* 162**,** 241-250.

GOLDSTEIN, D. B. 2009. Common Genetic Variation and Human Traits. *New England Journal of Medicine,* 360**,** 1696-1698.

GOSBY, A. K., CONIGRAVE, A. D., LAU, N. S., IGLESIAS, M. A., HALL, R. M., JEBB, S. A., BRAND-MILLER, J., CATERSON, I. D., RAUBENHEIMER, D. & SIMPSON, S. J. 2011. Testing Protein Leverage in Lean Humans: A Randomised Controlled Experimental Study. *PLoS ONE,* 6**,** e25929.

GOSBY, A. K., CONIGRAVE, A. D., RAUBENHEIMER, D. & SIMPSON, S. J. 2014. Protein leverage and energy intake. *Obesity Reviews,* 15**,** 183-191.

GOULD, J., SMITH, P., AIREY, C., MORT, E., AIREY, L., WARRICKER, F., PEARSON-FARR, J., WESTON, E., GOULD, P., SEMMENCE, O., RESTALL, K., WATTS, J., MCHUGH, P., SMITH, S., DEWING, J., FLEMING, T. & WILLAIME-MORAWEK, S. 2018. Mouse maternal protein restriction during preimplantation alone permanently alters brain neuron proportion and adult short-term memory. *Proceedings of the National Academy of Sciences,* 115**,** 201721876.

GOVIC, A., PENMAN, J., TAMMER, A. H. & PAOLINI, A. G. 2016. Paternal calorie restriction prior to conception alters anxiety-like behavior of the adult rat progeny. *Psychoneuroendocrinology,* 64**,** 1-11.

GRANDJEAN, V., FOURRÉ, S., DE ABREU, D. A. F., DERIEPPE, M.-A., REMY, J.-J. &

RASSOULZADEGAN, M. 2015. RNA-mediated paternal heredity of diet-induced obesity and metabolic disorders. *Scientific Reports (Nature Publisher Group),* 5**,** 18193.

GUILLAUME, A. S., MONRO, K. & MARSHALL, D. J. 2016. Transgenerational plasticity and environmental stress: do paternal effects act as a conduit or a buffer? *Functional Ecology,* 30**,** 1175-1184.

HALBERG, N., WERNSTEDT-ASTERHOLM, I. & SCHERER, P. E. 2008. The adipocyte as an endocrine cell. *Endocrinol Metab Clin North Am,* 37**,** 753-68, x-xi.

HALES, C. N. & BARKER, D. J. P. 1992. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia,* 35**,** 595-601.

HALES, C. N. & BARKER, D. J. P. 2001. The thrifty phenotype hypothesis: Type 2 diabetes. *British Medical Bulletin,* 60**,** 5-20.

HALL, K. D. 2019. The Potential Role of Protein Leverage in the US Obesity Epidemic. *Obesity,* 27**,** 1222-1224.

HASEGAWA, Y., CHEN, S.-Y., SHENG, L., JENA, P. K., KALANETRA, K. M., MILLS, D. A., WAN, Y.-J. Y. & SLUPSKY, C. M. 2020. Long-term effects of western diet consumption in male and female mice. *Scientific Reports,* 10**,** 14686.

HEINDEL, J. J. & VANDENBERG, L. N. 2015. Developmental origins of health and disease: a paradigm for understanding disease cause and prevention. *Curr Opin Pediatr,* 27**,** 248-53.

HELLMANN, J. K., BUKHARI, S. A., DENO, J. & BELL, A. M. 2020a. Sex-specific plasticity across generations I: Maternal and paternal effects on sons and daughters. *J Anim Ecol,* 89**,** 2788-2799.

HELLMANN, J. K., CARLSON, E. R. & BELL, A. M. 2020b. Sex-specific plasticity across generations II: Grandpaternal effects are lineage specific and sex specific. *Journal of Animal Ecology,* 89**,** 2800-2812.

HILL, C. M., LAEGER, T., DEHNER, M., ALBARADO, D. C., CLARKE, B., WANDERS, D., BURKE, S. J., COLLIER, J. J., QUALLS-CREEKMORE, E., SOLON-BIET, S. M., SIMPSON, S. J., BERTHOUD, H.-R., MÜNZBERG, H. & MORRISON, C. D. 2019. FGF21 Signals Protein Status to the Brain and Adaptively Regulates Food Choice and Metabolism. *Cell reports,* 27**,** 2934-2947.e3.

HILL, C. M. & MORRISON, C. D. 2019. The Protein Leverage Hypothesis: A 2019 Update for Obesity. *Obesity,* 27**,** 1221-1221.

HILL, C. M., QUALLS-CREEKMORE, E., BERTHOUD, H.-R., SOTO, P., YU, S., MCDOUGAL, D. H., MUNZBERG, H. & MORRISON, C. D. 2020. FGF21 and the

Physiological Regulation of Macronutrient Preference. *Endocrinology,* 161**,** 1c+.

HO, K.-J., MIKKELSON, B., LEWIS, L. A., FELDMAN, S. A. & TAYLOR, C. B. 1972. Alaskan Arctic Eskimo: responses to a customary high fat diet. *The American Journal of Clinical Nutrition,* 25**,** 737-745.

HOEBEL, B. G., AVENA, N. M., BOCARSLY, M. E. & RADA, P. 2009. Natural Addiction: A Behavioral and Circuit Model Based on Sugar Addiction in Rats. *Journal of Addiction Medicine,* 3.

HOLT, R. I. G. 2019. The Management of Obesity in People with Severe Mental Illness: An Unresolved Conundrum. *Psychotherapy and Psychosomatics,* 88**,** 327-332.

HOUFFLYN, S., MATTHYS, C. & SOUBRY, A. 2017. Male Obesity: Epigenetic Origin and Effects in Sperm and Offspring. *Current Molecular Biology Reports,* 3**,** 288-296.

HUANG, J., ZHANG, Z., WU, Y., WANG, Y., WANG, J., ZHOU, L., NI, Z., HAO, L., YANG, N. & YANG, X. 2018. Early feeding of larger volumes of formula milk is associated with greater body weight or overweight in later infancy. *Nutrition Journal,* 17**,** 12.

HUR, S. S. J., CROPLEY, J. E. & SUTER, C. M. 2017. Paternal epigenetic programming: evolving metabolic disease risk. *Journal of Molecular Endocrinology,* 58**,** R159-R168.

JACQUES, A., CHAAYA, N., BEECHER, K., ALI, S. A., BELMER, A. & BARTLETT, S. 2019. The impact of sugar consumption on stress driven, emotional and addictive behaviors. *Neuroscience & Biobehavioral Reviews,* 103**,** 178-199.

JAENISCH, R. 1997. DNA methylation and imprinting: why bother? *Trends in Genetics,* 13**,** 323-329.

JAZWIEC, P. A., PATTERSON, V. S., RIBEIRO, T. A., YEO, E., KENNEDY, K. M., MATHIAS, P. C. F., PETRIK, J. J. & SLOBODA, D. M. 2022. Paternal obesity induces placental hypoxia and sex-specific impairments in placental vascularization and offspring metabolism†. *Biol Reprod,* 107**,** 574-589.

KAHN, S. E., HULL, R. L. & UTZSCHNEIDER, K. M. 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature,* 444**,** 840-6.

KARASTERGIOU, K., SMITH, S. R., GREENBERG, A. S. & FRIED, S. K. 2012. Sex differences in human adipose tissues - The biology of pear shape. *Biology of Sex Differences,* 3**,** 13.

KATIB, A. 2015. Mechanisms linking obesity to male infertility. *Cent European J Urol,* 68**,** 79-85.

KHAN, I., DEKOU, V., HANSON, M., POSTON, L. & TAYLOR, P. 2004. Predictive

adaptive responses to maternal high-fat diet prevent endothelial dysfunction but not hypertension in adult rat offspring. *Circulation,* 110**,** 1097-102.

KHAN, I. Y., TAYLOR, P. D., DEKOU, V., SEED, P. T., LAKASING, L., GRAHAM, D., DOMINICZAK, A. F., HANSON, M. A. & POSTON, L. 2003. Gender-Linked Hypertension in Offspring of Lard-Fed Pregnant Rats. *Hypertension,* 41**,** 168-175.

KILPELÄINEN, T. O., QI, L., BRAGE, S., SHARP, S. J., SONESTEDT, E., DEMERATH, E., AHMAD, T., MORA, S., KAAKINEN, M., SANDHOLT, C. H., HOLZAPFEL, C., AUTENRIETH, C. S., HYPPÖNEN, E., CAUCHI, S., HE, M., KUTALIK, Z., KUMARI, M., STANČÁKOVÁ, A., MEIDTNER, K., BALKAU, B., TAN, J. T., MANGINO, M., TIMPSON, N. J., SONG, Y., ZILLIKENS, M. C., JABLONSKI, K. A., GARCIA, M. E., JOHANSSON, S., BRAGG-GRESHAM, J. L., WU, Y., VAN VLIET-OSTAPTCHOUK, J. V., ONLAND-MORET, N. C., ZIMMERMANN, E., RIVERA, N. V., TANAKA, T., STRINGHAM, H. M., SILBERNAGEL, G., KANONI, S., FEITOSA, M. F., SNITKER, S., RUIZ, J. R., METTER, J., LARRAD, M. T. M., ATALAY, M., HAKANEN, M., AMIN, N., CAVALCANTI-PROENÇA, C., GRØNTVED, A., HALLMANS, G., JANSSON, J.-O., KUUSISTO, J., KÄHÖNEN, M., LUTSEY, P. L., NOLAN, J. J., PALLA, L., PEDERSEN, O., PÉRUSSE, L., RENSTRÖM, F., SCOTT, R. A., SHUNGIN, D., SOVIO, U., TAMMELIN, T. H., RÖNNEMAA, T., LAKKA, T. A., UUSITUPA, M., RIOS, M. S., FERRUCCI, L., BOUCHARD, C., MEIRHAEGHE, A., FU, M., WALKER, M., BORECKI, I. B., DEDOUSSIS, G. V., FRITSCHE, A., OHLSSON, C., BOEHNKE, M., BANDINELLI, S., VAN DUIJN, C. M., EBRAHIM, S., LAWLOR, D. A., GUDNASON, V., HARRIS, T. B., SØRENSEN, T. I. A., MOHLKE, K. L., HOFMAN, A., UITTERLINDEN, A. G., TUOMILEHTO, J., LEHTIMÄKI, T., RAITAKARI, O., ISOMAA, B., NJØLSTAD, P. R., FLOREZ, J. C., LIU, S., NESS, A., SPECTOR, T. D., TAI, E. S., FROGUEL, P., BOEING, H., LAAKSO, M., MARMOT, M., et al. 2011. Physical Activity Attenuates the Influence of FTO Variants on Obesity Risk: A Meta-Analysis of 218,166 Adults and 19,268 Children. *PLOS Medicine,* 8**,** e1001116.

KLESGES, R. C., STEIN, R. J., ECK, L. H., ISBELL, T. R. & KLESGES, L. M. 1991. Parental influence on food selection in young children and its relationships to childhood obesity. *The American Journal of Clinical Nutrition,* 53**,** 859-864.

KONG, Q.-Q., TIAN, X.-D., WANG, J., YUAN, H.-J., NING, S.-F., LUO, M.-J. & TAN, J.-H. 2021. A next-generation sequencing study on mechanisms by which restraint and social instability stresses of male mice alter offspring anxiety-like behavior. *Scientific*

Reports, 11**,** 7952.

KONNER, M. & EATON, S. B. 2010. Paleolithic Nutrition. *Nutrition in Clinical Practice,* 25**,** 594-602.

KORGAN, A. C., FOXX, C. L., HASHMI, H., SAGO, S. A., STAMPER, C. E., HEINZE, J. D., O'LEARY, E., KING, J. L., PERROT, T. S., LOWRY, C. A. & WEAVER, I. C. G. 2022. Effects of paternal high-fat diet and maternal rearing environment on the gut microbiota and behavior. *Scientific Reports (Nature Publisher Group),* 12.

KUHNLEIN, H. V., RECEVEUR, O., SOUEIDA, R. & EGELAND, G. M. 2004. Arctic Indigenous Peoples Experience the Nutrition Transition with Changing Dietary Patterns and Obesity. *The Journal of Nutrition,* 134**,** 1447-1453.

KUZNETSOVA, A., BROCKHOFF, P. B. & CHRISTENSEN, R. H. B. 2017. lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software,* 82**,** 1 - 26.

LAEGER, T., HENAGAN, T. M., ALBARADO, D. C., REDMAN, L. M., BRAY, G. A., NOLAND, R. C., MÜNZBERG, H., HUTSON, S. M., GETTYS, T. W., SCHWARTZ, M. W. & MORRISON, C. D. 2014. FGF21 is an endocrine signal of protein restriction. *The Journal of Clinical Investigation,* 124**,** 3913-3922.

LANE, M., ROBKER, R. L. & ROBERTSON, S. A. 2014. Parenting from before conception. *Science,* 345**,** 756-760.

LANE, M., ZANDER-FOX, D. L., ROBKER, R. L. & MCPHERSON, N. O. 2015. Peri-conception parental obesity, reproductive health, and transgenerational impacts. *Trends in Endocrinology & Metabolism,* 26**,** 84-90.

LARSON, K. R., RUSSO, K. A., FANG, Y., MOHAJERANI, N., GOODSON, M. L. & RYAN, K. K. 2017. Sex Differences in the Hormonal and Metabolic Response to Dietary Protein Dilution. *Endocrinology,* 158**,** 3477-3487.

LECOMTE, V., MALONEY, C. A., WANG, K. W. & MORRIS, M. J. 2017. Effects of paternal obesity on growth and adiposity of male rat offspring. *American Journal of Physiology-Endocrinology and Metabolism,* 312**,** E117-E125.

LEMES, S. F., DE SOUZA, A. C. P., PAYOLLA, T. B., VERSUTTI, M. D., DE FÁTIMA DA SILVA RAMALHO, A., MENDES-DA-SILVA, C., SOUZA, C. M., MILANSKI, M., TORSONI, A. S. & TORSONI, M. A. 2018. Maternal Consumption of High-fat Diet in Mice Alters Hypothalamic Notch Pathway, NPY Cell Population and Food Intake in Offspring. *Neuroscience,* 371**,** 1-15.

LEWIS, S. E. & SIMON, L. 2010. Clinical implications of sperm DNA damage. *Hum Fertil*

(Camb), 13**,** 201-7.

LISTER, R., PELIZZOLA, M., DOWEN, R. H., HAWKINS, R. D., HON, G., TONTI-FILIPPINI, J., NERY, J. R., LEE, L., YE, Z., NGO, Q.-M., EDSALL, L., ANTOSIEWICZ-BOURGET, J., STEWART, R., RUOTTI, V., MILLAR, A. H., THOMSON, J. A., REN, B. & ECKER, J. R. 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature,* 462**,** 315-322.

LITCHFORD, A., SAVOIE ROSKOS, M. R. & WENGREEN, H. 2020. Influence of fathers on the feeding practices and behaviors of children: A systematic review. *Appetite,* 147**,** 104558.

LOCKE, A. E., KAHALI, B., BERNDT, S. I., ET. AL, LIFELINES COHORT STUDY, ADIPOGEN CONSORTIUM, AGEN-BMI WORKING GROUP, CARDIOGRAMPLUSC4D CONSORTIUM, CKDGEN CONSORTIUM, GLGC, ICBP, MAGIC INVESTIGATORS, MUTHER CONSORTIUM, MIGEN CONSORTIUM, PAGE CONSORTIUM, REPROGEN CONSORTIUM, GENIE CONSORTIUM & CONSORTIUM, I. E. 2015. Genetic studies of body mass index yield new insights for obesity biology. *Nature,* 518**,** 197-206.

LÓPEZ-TABOADA, I., GONZÁLEZ-PARDO, H. & CONEJO, N. M. 2020. Western Diet: Implications for Brain Function and Behavior. *Frontiers in psychology,* 11**,** 564413-564413.

LUPIEN, S. J., MCEWEN, B. S., GUNNAR, M. R. & HEIM, C. 2009. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature Reviews Neuroscience,* 10**,** 434+.

LUPPINO, F. S., DE WIT, L. M., BOUVY, P. F., STIJNEN, T., CUIJPERS, P., PENNINX, B. W. J. H. & ZITMAN, F. G. 2010. Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies. *Archives of general psychiatry,* 67**,** 220-229.

MAENG, L. Y. & MILAD, M. R. 2015. Sex differences in anxiety disorders: Interactions between fear, stress, and gonadal hormones. *Horm Behav,* 76**,** 106-17.

MAKAROVA, E. N., YAKOVLEVA, T. V., BALYIBINA, N. Y., BARANOV, K. O., DENISOVA, E. I., DUBININA, A. D., FEOFANOVA, N. A. & BAZHAN, N. M. 2020. Pharmacological effects of fibroblast growth factor 21 are sex-specific in mice with the lethal yellow (A(y)) mutation. *Vavilovskii zhurnal genetiki i selektsii,* 24**,** 200-208.

MANDY, M. & NYIRENDA, M. 2018. Developmental Origins of Health and Disease: the

relevance to developing nations. *International health,* 10**,** 66-70.

MARTÍNEZ STEELE, E., RAUBENHEIMER, D., SIMPSON, S. J., BARALDI, L. G. & MONTEIRO, C. A. 2018. Ultra-processed foods, protein leverage and energy intake in the USA. *Public Health Nutrition,* 21**,** 114-124.

MASHOODH, R., HABRYLO, I. B., GUDSNUK, K. M. & CHAMPAGNE, F. A. 2022. Sex-specific effects of chronic paternal stress on offspring development are partially mediated via mothers. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

MASUYAMA, H., MITSUI, T., EGUCHI, T., TAMADA, S. & HIRAMATSU, Y. 2016. The effects of paternal high-fat diet exposure on offspring metabolism with epigenetic changes in the mouse adiponectin and leptin gene promoters. *American Journal of Physiology-Endocrinology and Metabolism,* 311**,** E236-E245.

MCPHERSON, N. O., FULLSTON, T., AITKEN, R. J. & LANE, M. 2014. Paternal Obesity, Interventions, and Mechanistic Pathways to Impaired Health in Offspring. *Annals of Nutrition and Metabolism,* 64**,** 231-238.

MEDAGLIA, D. S. A., VIEIRA, H. R., SILVEIRA, S. D. S., SIERVO, G., MARCON, M., MATHIAS, P. C. F. & FERNANDES, G. S. A. 2022. High-fructose diet during puberty alters the sperm parameters, testosterone concentration, and histopathology of testes and epididymis in adult Wistar rats. *J Dev Orig Health Dis,* 13**,** 20-27.

MEDRIKOVA, D., JILKOVA, Z. M., BARDOVA, K., JANOVSKA, P., ROSSMEISL, M. & KOPECKY, J. 2012. Sex differences during the course of diet-induced obesity in mice: adipose tissue expandability and glycemic control. *International Journal of Obesity,* 36**,** 262+.

MILANESCHI, Y., SIMMONS, W. K., ELISABETH, F. C. V. R. & PENNINX, B. W. J. H. 2019. Depression and obesity: evidence of shared biological mechanisms. *Molecular Psychiatry,* 24**,** 18-33.

MILLER, G. D. 2019. Appetite Regulation: Hormones, Peptides, and Neurotransmitters and Their Role in Obesity. *Am J Lifestyle Med,* 13**,** 586-601.

MONROE, T. B., GORE, J. C., BRUEHL, S. P., BENNINGFIELD, M. M., DIETRICH, M. S., CHEN, L. M., NEWHOUSE, P., FILLINGIM, R., CHODKOWSKI, B., ATALLA, S., ARRIETA, J., DAMON, S. M., BLACKFORD, J. U. & COWAN, R. L. 2015. Sex differences in psychophysical and neurophysiological responses to pain in older adults: a cross-sectional study. *Biology of Sex Differences,* 6**,** 25.

MONTEIRO, C. A., MOUBARAC, J. C., CANNON, G., NG, S. W. & POPKIN, B. 2013. Ultra-processed products are becoming dominant in the global food system. *Obes Rev,*

14 Suppl 2**,** 21-8.

MORGAN, H. L., ALJUMAH, A., ROUILLON, C. & WATKINS, A. J. 2021. Paternal low protein diet and the supplementation of methyl-donors impact fetal growth and placental development in mice. *Placenta,* 103**,** 124-133.

MORRIS, M. J., BEILHARZ, J. E., MANIAM, J., REICHELT, A. C. & WESTBROOK, R. F. 2015. Why is obesity such a problem in the 21st century? The intersection of palatable food, cues and reward pathways, stress, and cognition. *Neurosci Biobehav Rev,* 58**,** 36-45.

MORRIS, M. J. & CHEN, H. 2009. Established maternal obesity in the rat reprograms hypothalamic appetite regulators and leptin signaling at birth. *Int J Obes (Lond),* 33**,** 115-22.

MÜNCH, D., EZRA-NEVO, G., FRANCISCO, A. P., TASTEKIN, I. & RIBEIRO, C. 2020. Nutrient homeostasis — translating internal states to behavior. *Current Opinion in Neurobiology,* 60**,** 67-75.

NAEF, L., MOQUIN, L., DAL BO, G., GIROS, B., GRATTON, A. & WALKER, C. D. 2011. Maternal high-fat intake alters presynaptic regulation of dopamine in the nucleus accumbens and increases motivation for fat rewards in the offspring. *Neuroscience,* 176**,** 225-36.

NG, M., FLEMING, T., ROBINSON, M., THOMSON, B., GRAETZ, N., MARGONO, C., MULLANY, E. C., BIRYUKOV, S., ABBAFATI, C., ABERA, S. F., ABRAHAM, J. P., ABU-RMEILEH, N. M., ACHOKI, T., ALBUHAIRAN, F. S., ALEMU, Z. A., ALFONSO, R., ALI, M. K., ALI, R., GUZMAN, N. A., AMMAR, W., ANWARI, P., BANERJEE, A., BARQUERA, S., BASU, S., BENNETT, D. A., BHUTTA, Z., BLORE, J., CABRAL, N., NONATO, I. C., CHANG, J. C., CHOWDHURY, R., COURVILLE, K. J., CRIQUI, M. H., CUNDIFF, D. K., DABHADKAR, K. C., DANDONA, L., DAVIS, A., DAYAMA, A., DHARMARATNE, S. D., DING, E. L., DURRANI, A. M., ESTEGHAMATI, A., FARZADFAR, F., FAY, D. F., FEIGIN, V. L., FLAXMAN, A., FOROUZANFAR, M. H., GOTO, A., GREEN, M. A., GUPTA, R., HAFEZI-NEJAD, N., HANKEY, G. J., HAREWOOD, H. C., HAVMOELLER, R., HAY, S., HERNANDEZ, L., HUSSEINI, A., IDRISOV, B. T., IKEDA, N., ISLAMI, F., JAHANGIR, E., JASSAL, S. K., JEE, S. H., JEFFREYS, M., JONAS, J. B., KABAGAMBE, E. K., KHALIFA, S. E., KENGNE, A. P., KHADER, Y. S., KHANG, Y. H., KIM, D., KIMOKOTI, R. W., KINGE, J. M., KOKUBO, Y., KOSEN, S., KWAN, G., LAI, T., LEINSALU, M., LI, Y., LIANG, X., LIU, S., LOGROSCINO,

G., LOTUFO, P. A., LU, Y., MA, J., MAINOO, N. K., MENSAH, G. A., MERRIMAN, T. R., MOKDAD, A. H., MOSCHANDREAS, J., NAGHAVI, M., NAHEED, A., NAND, D., NARAYAN, K. M., NELSON, E. L., NEUHOUSER, M. L., NISAR, M. I., OHKUBO, T., OTI, S. O., PEDROZA, A., et al. 2014a. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet,* 384**,** 766-81.

NG, S.-F., LIN, R. C. Y., MALONEY, C. A., YOUNGSON, N. A., OWENS, J. A. & MORRIS, M. J. 2014b. Paternal high-fat diet consumption induces common changes in the transcriptomes of retroperitoneal adipose and pancreatic islet tissues in female rat offspring. *The FASEB Journal,* 28**,** 1830-1841.

NG, S. F., LIN, R. C., LAYBUTT, D. R., BARRES, R., OWENS, J. A. & MORRIS, M. J. 2010. Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. *Nature,* 467**,** 963-6.

NIVOIT, P., MORENS, C., VAN ASSCHE, F. A., JANSEN, E., POSTON, L., REMACLE, C. & REUSENS, B. 2009. Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. *Diabetologia,* 52**,** 1133-1142.

OGRODNIK, M., ZHU, Y., LANGHI, L. G. P., TCHKONIA, T., KRÜGER, P., FIELDER, E., VICTORELLI, S., RUSWHANDI, R. A., GIORGADZE, N., PIRTSKHALAVA, T., PODGORNI, O., ENIKOLOPOV, G., JOHNSON, K. O., XU, M., INMAN, C., PALMER, A. K., SCHAFER, M., WEIGL, M., IKENO, Y., BURNS, T. C., PASSOS, J. F., VON ZGLINICKI, T., KIRKLAND, J. L. & JURK, D. 2019. Obesity-Induced Cellular Senescence Drives Anxiety and Impairs Neurogenesis. *Cell Metabolism,* 29**,** 1061-1077.e8.

ONG, K. K. 2006. Size at birth, postnatal growth and risk of obesity. *Horm Res,* 65 Suppl 3**,** 65-9.

ONYIKE, C. U., CRUM, R. M., LEE, H. B., LYKETSOS, C. G. & EATON, W. W. 2003. Is obesity associated with major depression? Results from the Third National Health and Nutrition Examination Survey. *Am J Epidemiol,* 158**,** 1139-47.

ORDYAN, N. E., MALYSHEVA, O. V., HOLOVA, G. I., AKULOVA, V. K. & PIVINA, S. G. 2022. Sex-Dependent Effects of Stress in Male Rats on Memory and Expression of the Insulin-Like Growth Factor 2 Receptor Gene in the Brains of Offspring. *Neuroscience and Behavioral Physiology,* 52**,** 242-250.

ORNELLAS, F., SOUZA-MELLO, V., MANDARIM-DE-LACERDA, C. A. & AGUILA, M.

B. 2016. Combined parental obesity augments single-parent obesity effects on hypothalamus inflammation, leptin signaling (JAK/STAT), hyperphagia, and obesity in the adult mice offspring. *Physiology & Behavior,* 153**,** 47-55.

OSHIO, L. T., ANDREAZZI, A. E., LOPES, J. F., SÁ, J. P., BOLOTARI, M., COSTA, V. M. G., GUERRA, M. O. & PETERS, V. M. 2020. A paternal hypercaloric diet affects the metabolism and fertility of F1 and F2 Wistar rat generations. *J Dev Orig Health Dis,* 11**,** 653-663.

OUKO, L. A., SHANTIKUMAR, K., KNEZOVICH, J., HAYCOCK, P., SCHNUGH, D. J. & RAMSAY, M. 2009. Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes: implications for fetal alcohol spectrum disorders. *Alcohol Clin Exp Res,* 33**,** 1615-27.

PALMER, B. F. & CLEGG, D. J. 2015. The sexual dimorphism of obesity. *Molecular and Cellular Endocrinology,* 402**,** 113-119.

PALMER, N. O., BAKOS, H. W., FULLSTON, T. & LANE, M. 2012. Impact of obesity on male fertility, sperm function and molecular composition. *Spermatogenesis,* 2**,** 253-263.

PEARCE, K. L., HILL, A. & TREMELLEN, K. P. 2019. Obesity related metabolic endotoxemia is associated with oxidative stress and impaired sperm DNA integrity. *Basic Clin Androl,* 29**,** 6.

PELEG-RAIBSTEIN, D., LUCA, E. & WOLFRUM, C. 2012. Maternal high-fat diet in mice programs emotional behavior in adulthood. *Behavioural Brain Research,* 233**,** 398-404.

PELLOW, S., CHOPIN, P., FILE, S. E. & BRILEY, M. 1985. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods,* 14**,** 149-67.

PEMBREY, M., SAFFERY, R. & BYGREN, L. O. 2014. Human transgenerational responses to early-life experience: potential impact on development, health and biomedical research. *Journal of Medical Genetics,* 51**,** 563.

PEMBREY, M. E., BYGREN, L. O., KAATI, G., EDVINSSON, S., NORTHSTONE, K., SJÖSTRÖM, M. & GOLDING, J. 2006. Sex-specific, male-line transgenerational responses in humans. *European Journal of Human Genetics : EJHG,* 14**,** 159-66.

PERIS-SAMPEDRO, F., MOUNIB, M., SCHÉLE, E., EDVARDSSON, C. E., STOLTENBORG, I., ADAN, R. A. H. & DICKSON, S. L. 2019. Impact of Free-Choice Diets High in Fat and Different Sugars on Metabolic Outcome and Anxiety-Like Behavior in Rats. *Obesity,* 27**,** 409-419.

PINI, T., RAUBENHEIMER, D., SIMPSON, S. J. & CREAN, A. J. 2021. Obesity and Male Reproduction; Placing the Western Diet in Context. *Frontiers in Endocrinology,* 12.

PORTE, D., JR., BASKIN, D. G. & SCHWARTZ, M. W. 2002. Leptin and insulin action in the central nervous system. *Nutr Rev,* 60**,** S20-9; discussion S68-84, 85-7.

POWELL-WILEY, T. M., POIRIER, P., BURKE, L. E., DESPRÉS, J.-P., GORDON-LARSEN, P., LAVIE, C. J., LEAR, S. A., NDUMELE, C. E., NEELAND, I. J., SANDERS, P. & ST-ONGE, M.-P. 2021. Obesity and Cardiovascular Disease: A Scientific Statement From the American Heart Association. *Circulation,* 143**,** e984-e1010.

PYNDT JØRGENSEN, B., HANSEN, J. T., KRYCH, L., LARSEN, C., KLEIN, A. B., NIELSEN, D. S., JOSEFSEN, K., HANSEN, A. K. & SØRENSEN, D. B. 2014. A Possible Link between Food and Mood: Dietary Impact on Gut Microbiota and Behavior in BALB/c Mice. *PLOS ONE,* 9**,** e103398.

RAAD, G., HAZZOURI, M., BOTTINI, S., TRABUCCHI, M., AZOURY, J. & GRANDJEAN, V. 2017. Paternal obesity: how bad is it for sperm quality and progeny health? *Basic Clin Androl,* 27**,** 20.

RABASA, C., WINSA-JÖRNULF, J., VOGEL, H., BABAEI, C. S., ASKEVIK, K. & DICKSON, S. L. 2016. Behavioral consequences of exposure to a high fat diet during the post-weaning period in rats. *Hormones and Behavior,* 85**,** 56-66.

RADFORD, E. J., ITO, M., SHI, H., CORISH, J. A., YAMAZAWA, K., ISGANAITIS, E., SEISENBERGER, S., HORE, T. A., REIK, W., ERKEK, S., PETERS, A., PATTI, M. E. & FERGUSON-SMITH, A. C. 2014. In utero effects. In utero undernourishment perturbs the adult sperm methylome and intergenerational metabolism. *Science,* 345**,** 1255903.

RAJIA, S., CHEN, H. & MORRIS, M. J. 2010. Maternal Overnutrition Impacts Offspring Adiposity and Brain Appetite Markers-Modulation by Postweaning Diet. *Journal of Neuroendocrinology,* 22**,** 905-914.

RAO, T. S. S., ASHA, M. R., RAMESH, B. N. & RAO, K. S. J. 2008. Understanding nutrition, depression and mental illnesses. *Indian journal of psychiatry,* 50**,** 77-82.

RATO, L., ALVES, M. G., CAVACO, J. E. & OLIVEIRA, P. F. 2014. High-energy diets: a threat for male fertility? *Obesity Reviews,* 15**,** 996-1007.

RAUBENHEIMER, D., MACHOVSKY-CAPUSKA, G. E., GOSBY, A. K. & SIMPSON, S. 2015. Nutritional ecology of obesity: from humans to companion animals. *The British journal of nutrition,* 113 Suppl**,** S26-S39.

RAUBENHEIMER, D., SENIOR, A. M., MIRTH, C., CUI, Z., HOU, R., LE COUTEUR, D. G., SOLON-BIET, S. M., LÉOPOLD, P. & SIMPSON, S. J. 2022. An integrative approach to dietary balance across the life course. *iScience,* 25**,** 104315.

RAUBENHEIMER, D. & SIMPSON, S. J. 2019. Protein Leverage: Theoretical Foundations and Ten Points of Clarification. *Obesity,* 27**,** 1225-1238.

RAVELLI, A. C. J., VAN DER MEULEN, J. H. P., OSMOND, C., BARKER, D. J. P. & BLEKER, O. P. 1999. Obesity at the age of 50 y in men and women exposed to famine prenatally. *The American Journal of Clinical Nutrition,* 70**,** 811-816.

RAVELLI, G.-P., STEIN, Z. A. & SUSSER, M. W. 1976. Obesity in Young Men after Famine Exposure in Utero and Early Infancy. *New England Journal of Medicine,* 295**,** 349-353.

REBOLLEDO-SOLLEIRO, D., ROLDÁN-ROLDÁN, G., DÍAZ, D., VELASCO, M., LARQUÉ, C., RICO-ROSILLO, G., VEGA-ROBLEDO, G. B., ZAMBRANO, E., HIRIART, M. & PÉREZ DE LA MORA, M. 2017. Increased anxiety-like behavior is associated with the metabolic syndrome in non-stressed rats. *PLOS ONE,* 12**,** e0176554.

RODGERS, A. B., MORGAN, C. P., BRONSON, S. L., REVELLO, S. & BALE, T. L. 2013. Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. *J Neurosci,* 33**,** 9003-12.

ROLLS, B. J. 2017. Dietary energy density: Applying behavioural science to weight management. *Nutr Bull,* 42**,** 246-253.

ROMIEU, I., DOSSUS, L., BARQUERA, S., BLOTTIÈRE, H. M., FRANKS, P. W., GUNTER, M., HWALLA, N., HURSTING, S. D., LEITZMANN, M., MARGETTS, B., NISHIDA, C., POTISCHMAN, N., SEIDELL, J., STEPIEN, M., WANG, Y., WESTERTERP, K., WINICHAGOON, P., WISEMAN, M. & WILLETT, W. C. 2017. Energy balance and obesity: what are the main drivers? *Cancer Causes & Control,* 28**,** 247-258.

ROSEBOOM, T. J., VAN DER MEULEN, J. H. P., OSMOND, C., BARKER, D. J. P., RAVELLI, A. C. J. & BLEKER, O. P. 2000. Plasma lipid profiles in adults after prenatal exposure to the Dutch famine. *The American Journal of Clinical Nutrition,* 72**,** 1101-1106.

RUTKOWSKA, J., LAGISZ, M., BONDURIANSKY, R. & NAKAGAWA, S. 2020. Mapping the past, present and future research landscape of paternal effects. *BMC Biology,* 18**,** 183.

RYAN, D. P., HENZEL, K. S., PEARSON, B. L., SIWEK, M. E., PAPAZOGLOU, A., GUO,

L., PAESLER, K., YU, M., MÜLLER, R., XIE, K., SCHRÖDER, S., BECKER, L., GARRETT, L., HÖLTER, S. M., NEFF, F., RÁCZ, I., RATHKOLB, B., ROZMAN, J., EHNINGER, G., KLINGENSPOR, M., KLOPSTOCK, T., WOLF, E., WURST, W., ZIMMER, A., FUCHS, H., GAILUS-DURNER, V., HRABĚ DE ANGELIS, M., SIDIROPOULOU, K., WEIERGRÄBER, M., ZHOU, Y. & EHNINGER, D. 2018. A paternal methyl donor-rich diet altered cognitive and neural functions in offspring mice. *Molecular Psychiatry,* 23**,** 1345-1355.

SAAVEDRA-RODRÍGUEZ, L. & FEIG, L. A. 2013. Chronic social instability induces anxiety and defective social interactions across generations. *Biol Psychiatry,* 73**,** 44-53.

SAHARA, Y., MATSUZAWA, D., ISHII, D., FUCHIDA, T., GOTO, T., SUTOH, C. & SHIMIZU, E. 2019. Paternal methyl donor deficient diets during development affect male offspring behavior and memory-related gene expression in mice. *Developmental Psychobiology,* 61**,** 17-28.

SAMUELSSON, A.-M., MATTHEWS, P. A., ARGENTON, M., CHRISTIE, M. R., MCCONNELL, J. M., JANSEN, E. H. J. M., PIERSMA, A. H., OZANNE, S. E., TWINN, D. F., REMACLE, C., ROWLERSON, A., POSTON, L. & TAYLOR, P. D. 2008a. Diet-Induced Obesity in Female Mice Leads to Offspring Hyperphagia, Adiposity, Hypertension, and Insulin Resistance. *Hypertension,* 51**,** 383-392.

SAMUELSSON, A.-M., MATTHEWS, P. A., ARGENTON, M., CHRISTIE, M. R., MCCONNELL, J. M., JANSEN, E. H. J. M., PIERSMA, A. H., OZANNE, S. E., TWINN, D. F., REMACLE, C., ROWLERSON, A., POSTON, L. & TAYLOR, P. D. 2008b. Diet-Induced Obesity in Female Mice Leads to Offspring Hyperphagia, Adiposity, Hypertension, and Insulin Resistance: A Novel Murine Model of Developmental Programming. *Hypertension (Dallas, Tex. 1979),* 51**,** 383-392.

SANCHEZ-GARRIDO, M. A., RUIZ-PINO, F., VELASCO, I., BARROSO, A., FERNANDOIS, D., HERAS, V., MANFREDI-LOZANO, M., VAZQUEZ, M. J., CASTELLANO, J. M., ROA, J., PINILLA, L. & TENA-SEMPERE, M. 2017. Intergenerational Influence of Paternal Obesity on Metabolic and Reproductive Health Parameters of the Offspring: Male-Preferential Impact and Involvement of Kiss1-Mediated Pathways. *Endocrinology,* 159**,** 1005-1018.

SARIS, W. H. M., ASTRUP, A., PRENTICE, A. M., ZUNFT, H. J. F., FORMIGUERA, X., VENNE, W. P. H. G. V.-V. D., RABEN, A., POPPITT, S. D., SEPPELT, B., JOHNSTON, S., VASILARAS, T. H. & KEOGH, G. F. 2000. Randomized controlled trial of changes in dietary carbohydrate/fat ratio and simple vs complex carbohydrates

on body weight and blood lipids: the CARMEN study. *International Journal of Obesity and Related Disorders,* 24**,** 1310-1318.

SARWER, D. B. & POLONSKY, H. M. 2016. The Psychosocial Burden of Obesity. *Endocrinol Metab Clin North Am,* 45**,** 677-88.

SASAKI, A., DE VEGA, W., SIVANATHAN, S., ST-CYR, S. & MCGOWAN, P. O. 2014. Maternal high-fat diet alters anxiety behavior and glucocorticoid signaling in adolescent offspring. *Neuroscience,* 272**,** 92-101.

SASAKI, A., DE VEGA, W. C., ST-CYR, S., PAN, P. & MCGOWAN, P. O. 2013. Perinatal high fat diet alters glucocorticoid signaling and anxiety behavior in adulthood. *Neuroscience,* 240**,** 1-12.

SCHULZ, L. C. 2010. The Dutch Hunger Winter and the developmental origins of health and disease. *Proceedings of the National Academy of Sciences,* 107**,** 16757-16758.

SERTORIO, M. N., CÉSAR, H., DE SOUZA, E. A., MENNITTI, L. V., SANTAMARINA, A. B., DE SOUZA MESQUITA, L. M., JUCÁ, A., CASAGRANDE, B. P., ESTADELLA, D., AGUIAR, O. & PISANI, L. P. 2022. Parental High-Fat High-Sugar Diet Intake Programming Inflammatory and Oxidative Parameters of Reproductive Health in Male Offspring. *Frontiers in Cell and Developmental Biology,* 10.

SHARIATMADARI, F. & FORBES, J. M. 1993. Growth and food intake responses to diets of different protein contents and a choice between diets containing two concentrations of protein in broiler and layer strains of chicken. *Br Poult Sci,* 34**,** 959-70.

SHARMA, S. & FULTON, S. 2013. Diet-induced obesity promotes depressive-like behaviour that is associated with neural adaptations in brain reward circuitry. *International Journal of Obesity,* 37**,** 382+.

SHARMA, U., CONINE, C. C., SHEA, J. M., BOSKOVIC, A., DERR, A. G., BING, X. Y., BELLEANNEE, C., KUCUKURAL, A., SERRA, R. W., SUN, F., SONG, L., CARONE, B. R., RICCI, E. P., LI, X. Z., FAUQUIER, L., MOORE, M. J., SULLIVAN, R., MELLO, C. C., GARBER, M. & RANDO, O. J. 2016. Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. *Science,* 351**,** 391-396.

SHARMA, U. & RANDO, OLIVER J. 2014. Father-Son Chats: Inheriting Stress through Sperm RNA. *Cell Metabolism,* 19**,** 894-895.

SHARP, G. C. & LAWLOR, D. A. 2019. Paternal impact on the life course development of obesity and type 2 diabetes in the offspring. *Diabetologia,* 62**,** 1802-1810.

SHORT, A. K., FENNELL, K. A., PERREAU, V. M., FOX, A., O'BRYAN, M. K., KIM, J.

H., BREDY, T. W., PANG, T. Y. & HANNAN, A. J. 2016. Elevated paternal glucocorticoid exposure alters the small noncoding RNA profile in sperm and modifies anxiety and depressive phenotypes in the offspring. *Translational Psychiatry,* 6**,** e837-e837.

SIMPSON, S. J., LE COUTEUR, D. G. & RAUBENHEIMER, D. 2015. Putting the Balance Back in Diet. *Cell,* 161**,** 18-23.

SIMPSON, S. J. & RAUBENHEIMER, D. 2005. Obesity: the protein leverage hypothesis. *Obesity Reviews,* 6**,** 133-142.

SIMPSON, S. J. & RAUBENHEIMER, D. 2012. *The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity,* Princeton University Press.

SIMPSON, S. J. & RAUBENHEIMER, D. 2014. Perspective: Tricks of the trade. *Nature,* 508**,** S66-S66.

SINGLA, P., BARDOLOI, A. & PARKASH, A. A. 2010. Metabolic effects of obesity: A review. *World J Diabetes,* 1**,** 76-88.

SKINNER, M. K., ANWAY, M. D., SAVENKOVA, M. I., GORE, A. C. & CREWS, D. 2008. Transgenerational Epigenetic Programming of the Brain Transcriptome and Anxiety Behavior. *PLoS ONE,* 3**,** e3745.

SOENEN, S. & WESTERTERP-PLANTENGA, M. S. 2008. Proteins and satiety: implications for weight management. *Curr Opin Clin Nutr Metab Care,* 11**,** 747-51.

SOLON-BIET, S. M., COGGER, V. C., PULPITEL, T., HEBLINSKI, M., WAHL, D., MCMAHON, A. C., WARREN, A., DURRANT-WHYTE, J., WALTERS, K. A., KRYCER, J. R., PONTON, F., GOKARN, R., WALI, J. A., RUOHONEN, K., CONIGRAVE, A. D., JAMES, D. E., RAUBENHEIMER, D., MORRISON, C. D., LE COUTEUR, D. G. & SIMPSON, S. J. 2016. Defining the Nutritional and Metabolic Context of FGF21 Using the Geometric Framework. *Cell Metab,* 24**,** 555-565.

SOLON-BIET, S. M., MCMAHON, A. C., BALLARD, J. W., RUOHONEN, K., WU, L. E., COGGER, V. C., WARREN, A., HUANG, X., PICHAUD, N., MELVIN, R. G., GOKARN, R., KHALIL, M., TURNER, N., COONEY, G. J., SINCLAIR, D. A., RAUBENHEIMER, D., LE COUTEUR, D. G. & SIMPSON, S. J. 2014. The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. *Cell Metabolism,* 19**,** 418-30.

SOLON-BIET, S. M., WALTERS, K. A., SIMANAINEN, U. K., MCMAHON, A. C., RUOHONEN, K., BALLARD, J. W., RAUBENHEIMER, D., HANDELSMAN, D. J., LE COUTEUR, D. G. & SIMPSON, S. J. 2015. Macronutrient balance, reproductive

function, and lifespan in aging mice. *Proc Natl Acad Sci U S A,* 112**,** 3481-6.

SØRENSEN, A., MAYNTZ, D., RAUBENHEIMER, D. & SIMPSON, S. J. 2008. Protein-leverage in Mice: The Geometry of Macronutrient Balancing and Consequences for Fat Deposition. *Obesity,* 16**,** 566-71.

STANFORD, K. I., RASMUSSEN, M., BAER, L. A., LEHNIG, A. C., ROWLAND, L. A., WHITE, J. D., SO, K., DE SOUSA-COELHO, A. L., HIRSHMAN, M. F., PATTI, M. E., RANDO, O. J. & GOODYEAR, L. J. 2018. Paternal Exercise Improves Glucose Metabolism in Adult Offspring. *Diabetes,* 67**,** 2530-2540.

STECULORUM, S. M., COLLDEN, G., COUPE, B., CROIZIER, S., LOCKIE, S., ANDREWS, Z. B., JAROSCH, F., KLUSSMANN, S. & BOURET, S. G. 2015. Neonatal ghrelin programs development of hypothalamic feeding circuits. *J Clin Invest,* 125**,** 846-58.

STEIN, A. D., KAHN, H. S., RUNDLE, A., ZYBERT, P. A., VAN DER PAL–DE BRUIN, K. & LUMEY, L. 2007. Anthropometric measures in middle age after exposure to famine during gestation: evidence from the Dutch famine. *The American Journal of Clinical Nutrition,* 85**,** 869-876.

SULIGA, E. & GŁUSZEK, S. 2019. The relationship between diet, energy balance and fertility in men. *International Journal for Vitamin and Nutrition Research,* 90**,** 514-526.

SULLIVAN, E. L., GRAYSON, B., TAKAHASHI, D., ROBERTSON, N., MAIER, A., BETHEA, C. L., SMITH, M. S., COLEMAN, K. & GROVE, K. L. 2010. Chronic consumption of a high-fat diet during pregnancy causes perturbations in the serotonergic system and increased anxiety-like behavior in nonhuman primate offspring. *J Neurosci,* 30**,** 3826-30.

SUN, B., PURCELL, R. H., TERRILLION, C. E., YAN, J., MORAN, T. H. & TAMASHIRO, K. L. 2012. Maternal high-fat diet during gestation or suckling differentially affects offspring leptin sensitivity and obesity. *Diabetes,* 61**,** 2833-41.

TARIEL, J., LUQUET, É. & PLÉNET, S. 2020. Interactions Between Maternal, Paternal, Developmental, and Immediate Environmental Effects on Anti-predator Behavior of the Snail Physa acuta. *Frontiers in Ecology and Evolution,* 8.

TAYLOR, P. D., MCCONNELL, J., KHAN, I. Y., HOLEMANS, K., LAWRENCE, K. M., ASARE-ANANE, H., PERSAUD, S. J., JONES, P. M., PETRIE, L., HANSON, M. A. & POSTON, L. 2005. Impaired glucose homeostasis and mitochondrial abnormalities in offspring of rats fed a fat-rich diet in pregnancy. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 288**,** R134-R139.

TERASHIMA, M., BARBOUR, S., REN, J., YU, W., HAN, Y. & MUEGGE, K. 2015. Effect of high fat diet on paternal sperm histone distribution and male offspring liver gene expression. *Epigenetics,* 10**,** 861-871.

TOTZAUER, M., LUQUE, V., ESCRIBANO, J., CLOSA-MONASTEROLO, R., VERDUCI, E., REDIONIGI, A., HOYOS, J., LANGHENDRIES, J. P., GRUSZFELD, D., SOCHA, P., KOLETZKO, B. & GROTE, V. 2018. Effect of Lower Versus Higher Protein Content in Infant Formula Through the First Year on Body Composition from 1 to 6 Years: Follow-Up of a Randomized Clinical Trial. *Obesity (Silver Spring),* 26**,** 1203-1210.

TURNER, B. L. & THOMPSON, A. L. 2013. Beyond the Paleolithic prescription: incorporating diversity and flexibility in the study of human diet evolution. *Nutrition Reviews,* 71**,** 501-510.

ULIJASZEK, S. J. 2003. Trends in body size, diet and food availability in the Cook Islands in the second half of the 20th century. *Economics & Human Biology,* 1**,** 123-137.

UWIMBABAZI, M., RAUBENHEIMER, D., TWEHEYO, M., BASUTA, G. I., CONKLIN-BRITTAIN, N. L., WRANGHAM, R. W. & ROTHMAN, J. M. 2021. Nutritional geometry of female chimpanzees (Pan troglodytes). *American Journal of Primatology,* 83**,** e23269.

VAGENA, E., RYU, J. K., BAEZA-RAJA, B., WALSH, N. M., SYME, C., DAY, J. P., HOUSLAY, M. D. & BAILLIE, G. S. 2019. A high-fat diet promotes depression-like behavior in mice by suppressing hypothalamic PKA signaling. *Translational Psychiatry,* 9**,** 141.

VAN STEENWYK, G., ROSZKOWSKI, M., MANUELLA, F., FRANKLIN, T. B. & MANSUY, I. M. 2018. Transgenerational inheritance of behavioral and metabolic effects of paternal exposure to traumatic stress in early postnatal life: evidence in the 4th generation. *Environmental Epigenetics,* 4.

VELAZQUEZ, M. A., SHETH, B., SMITH, S. J., ECKERT, J. J., OSMOND, C. & FLEMING, T. P. 2018. Insulin and branched-chain amino acid depletion during mouse preimplantation embryo culture programmes body weight gain and raised blood pressure during early postnatal life. *Biochim Biophys Acta Mol Basis Dis,* 1864**,** 590-600.

VELDHORST, M. A. B., WESTERTERP-PLANTENGA, M. S. & WESTERTERP, K. R. 2009. Gluconeogenesis and energy expenditure after a high-protein, carbohydrate-free diet. *The American Journal of Clinical Nutrition,* 90**,** 519-526.

VENIAMINOVA, E., CESPUGLIO, R., CHERNUKHA, I., SCHMITT-BOEHRER, A. G., MOROZOV, S., KALUEFF, A. V., KUZNETSOVA, O., ANTHONY, D. C., LESCH, K.-P. & STREKALOVA, T. 2020. Metabolic, Molecular, and Behavioral Effects of Western Diet in Serotonin Transporter-Deficient Mice: Rescue by Heterozygosity? *Frontiers in neuroscience,* 14**,** 24-24.

VICKERS, M. H. 2011. Developmental programming of the metabolic syndrome - critical windows for intervention. *World journal of diabetes,* 2**,** 137-148.

VICKERS, M. H., BREIER, B. H., CUTFIELD, W. S., HOFMAN, P. L. & GLUCKMAN, P. D. 2000. Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *Am J Physiol Endocrinol Metab,* 279**,** E83-7.

VUCETIC, Z., CARLIN, J. L., TOTOKI, K. & REYES, T. M. 2012. Epigenetic dysregulation of the dopamine system in diet-induced obesity. *Journal of Neurochemistry,* 120**,** 891-898.

WALF, A. A. & FRYE, C. A. 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature protocols,* 2**,** 322-328.

WALI, J. A., MILNER, A. J., LUK, A. W. S., PULPITEL, T. J., DODGSON, T., FACEY, H. J. W., WAHL, D., KEBEDE, M. A., SENIOR, A. M., SULLIVAN, M. A., BRANDON, A. E., YAU, B., LOCKWOOD, G. P., KOAY, Y. C., RIBEIRO, R., SOLON-BIET, S. M., BELL-ANDERSON, K. S., O'SULLIVAN, J. F., MACIA, L., FORBES, J. M., COONEY, G. J., COGGER, V. C., HOLMES, A., RAUBENHEIMER, D., LE COUTEUR, D. G. & SIMPSON, S. J. 2021a. Impact of dietary carbohydrate type and protein–carbohydrate interaction on metabolic health. *Nature Metabolism,* 3**,** 810-828.

WALI, J. A., SOLON-BIET, S. M., FREIRE, T. & BRANDON, A. E. 2021b. Macronutrient Determinants of Obesity, Insulin Resistance and Metabolic Health. *Biology,* 10**,** 336.

WARE, S., VOIGT, J. P. & LANGLEY-EVANS, S. C. 2015. Body composition and behaviour in adult rats are influenced by maternal diet, maternal age and high-fat feeding. *Journal of Nutritional Science,* 4**,** 11.

WATKINS, A. J., DIAS, I., TSURO, H., ALLEN, D., EMES, R. D., MORETON, J., WILSON, R., INGRAM, R. J. M. & SINCLAIR, K. D. 2018. Paternal diet programs offspring health through sperm- and seminal plasma-specific pathways in mice. *Proceedings of the National Academy of Sciences of the United States of America,* 115**,** 10064.

WATKINS, A. J. & SINCLAIR, D. A. 2014. Paternal low protein diet affects adult offspring cardiovascular and metabolic function in mice. *American Journal of Physiology-Heart and Circulatory Physiology,* 306**,** H1444-H1452.

WATSON, ERICA D. 2016. Transferring Fragments of Paternal Metabolism to the Offspring. *Cell Metabolism,* 23**,** 401-402.

WEBER, M., GROTE, V., CLOSA-MONASTEROLO, R., ESCRIBANO, J., LANGHENDRIES, J.-P., DAIN, E., GIOVANNINI, M., VERDUCI, E., GRUSZFELD, D., SOCHA, P. & KOLETZKO, B. 2014. Lower protein content in infant formula reduces BMI and obesity risk at school age: follow-up of a randomized trial. *The American journal of clinical nutrition,* 99**,** 1041-1051.

WESTERTERP, K. R. 2006. Perception, passive overfeeding and energy metabolism. *Physiology & Behavior,* 89**,** 62-65.

WITEK, K., WYDRA, K. & FILIP, M. 2022. A High-Sugar Diet Consumption, Metabolism and Health Impacts with a Focus on the Development of Substance Use Disorder: A Narrative Review. *Nutrients,* 14.

WOLF, J. B. & WADE, M. J. 2009. What are maternal effects (and what are they not)? *Philosophical Transactions of the Royal Society B: Biological Sciences,* 364**,** 1107-1115.

WOODS, S. C., LOTTER, E. C., MCKAY, L. D. & PORTE, D., JR. 1979. Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature,* 282**,** 503-5.

WOODS, S. C., SEELEY, R. J., PORTE, D. & SCHWARTZ, M. W. 1998. Signals That Regulate Food Intake and Energy Homeostasis. *Science,* 280**,** 1378-1383.

WORLD HEALTH ORGANIZATION 2021. Obesity and Overweight Factsheet.

WU, C.-T., CHAFFIN, A. T. & RYAN, K. K. 2022. Fibroblast Growth Factor 21 Facilitates the Homeostatic Control of Feeding Behavior. *Journal of Clinical Medicine,* 11**,** 580.

WU, L., LU, Y., JIAO, Y., LIU, B., LI, S., LI, Y., XING, F., CHEN, D., LIU, X., ZHAO, J., XIONG, X., GU, Y., LU, J., CHEN, X. & LI, X. 2016. Paternal Psychological Stress Reprograms Hepatic Gluconeogenesis in Offspring. *Cell Metabolism,* 23**,** 735-743.

YOSHIZAKI, K., ASAI, M. & HARA, T. 2020. High-Fat Diet Enhances Working Memory in the Y-Maze Test in Male C57BL/6J Mice with Less Anxiety in the Elevated Plus Maze Test. *Nutrients,* 12**,** 2036.

YOUNG, T. K. 2007. Are the circumpolar inuit becoming obese? *American Journal of Human Biology,* 19**,** 181-189.

ZALBAHAR, N., NAJMAN, J., MCINTYRE, H. D. & MAMUN, A. 2017. Parental pre-pregnancy obesity and the risk of offspring weight and body mass index change from childhood to adulthood. *Clinical Obesity,* 7**,** 206-215.

ZHANG, Y., PROENCA, R., MAFFEI, M., BARONE, M., LEOPOLD, L. & FRIEDMAN, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* 372**,** 425-32.

ZORE, T., PALAFOX, M. & REUE, K. 2018. Sex differences in obesity, lipid metabolism, and inflammation—A role for the sex chromosomes? *Molecular Metabolism,* 15**,** 35-44.