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Defining the links between paternal diet, metabolic health, and reproductive fitness in mice

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Impact of COVID-19

The 2020-2021 COVID-19 pandemic had a significant impact on global economy, travel, and public health. This impact unfortunately extended to include this study. Due to the pandemic lockdown, there was an inability to conduct lab-based research or have face to face meetings with my supervisors for a period of over 10 months. Travel restrictions were also a hindrance to my ability to attend conferences, both domestic and international during that time. Throughout the pandemic, restricted access to university facilities such as the research laboratory or the animal unit caused significant delays to both animal work and lab work. In that period, I was unable to work on any of the experiments I had designed prior to the pandemic and therefore was unable to generate any data related to these experiments. To mitigate the impact of COVID-19 and the lockdown on my research, I had to pivot in order for things to fit within the limited time I had left in my PhD and took a number of steps to achieve that:

- Had weekly meetings with my supervisor on Teams discussing thesis plans and experimental data.
- Analysed new aspects of the data I had already generated from earlier experiments.
- Designed new experiments / redesigned old experiments to fit within the time in the lab I would have once COVID-19 restrictions are lifted.
- Attended virtual conferences and communicated my research through online oral and poster presentations.

DECLARATION

I hereby declare that this thesis is the result of my own work and that it has not been submitted elsewhere for any other degree or award. It includes nothing which is the outcome of work done in collaboration unless otherwise declared. It is not substantially the same as any work that has already been submitted before for any degree or other qualification except as declared. The work presented herein is my own work and where other sources of information have been used, they have been duly acknowledged.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BSA	Bovine serum albumin
CAD	Coronary artery disease
CD	Control diet
C57BL/6	C57 black 6; an inbred strain of laboratory mouse
DAPI	4′,6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DDX4	DEAD-Box Helicase 4
DEGs	Differentially expressed genes
dNTP	Deoxyribonucleotide triphosphate
DPX	Dibutylphthalate polystyrene xylene
DOHaD	Developmental Origins of Health and Disease
dUTP	Deoxyuridine Triphosphate
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acid(s)
FDR	False detection rate
FFA	Free fatty acids

HCL	Hydrochloric acid
H&E	Hematoxylin & eosin
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IMS	Industrial methylated spirit
LN2	Liquid nitrogen
LPD	Low protein diet
MAPK	Mitogen-activated protein kinase
MDL	Methyl donor-supplemented low protein diet
MDWD	Methyl donor-supplemented Western Diet
mTOR	Mammalian target of rapamycin
NGS	Normal goat serum
PCR	Polymerase chain reaction
PE	Paired end
PLZF	Promyelocytic leukemia zinc finger
PBS	Phosphate buffered solution
PBST	Phosphate buffered solution + Triton-X
ROUT	Robust Regression and Outlier Removal
RT	Room temperature
SCs	Spermatogenic cells

SEM	Standard error of the mean
ss-cDNA	Single-strand cDNA
SSCs	Spermatogonial stem cells
SOX-9	SRY-Box Transcription Factor 9
TdT	Terminal deoxynucleotidyl transferase
TG	Triglyceride(s)
TNF	Tumor necrosis factor
WD	Western Diet

LIST OF PUBLICATIONS

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NATIONAL AND INTERNATIONAL CONFERENCES

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ABSTRACT

Dietary intake is a crucial lifestyle factor that plays a significant role in influencing an individual's health and wellbeing. It is well established that maternal reproductive fitness and subsequent embryo development and offspring health are modified by the mother's diet and physiology. There is strong evidence in the literature linking maternal suboptimal diet and disrupted reproductive function and offspring development. However, the role of the father and his dietary habits remained relatively less understood, and only came into the focus of animal model and human studies in recent decades. In recent years, there has been growing evidence that poor paternal diet adversely impacts sperm quality, consequently impacting on embryonic development and offspring health. In order to better understand the impact of suboptimal diet on the male testicular environment as well as metabolic and physiological health, we fed C57BL/6 male mice either a control normal protein diet (18 % casein; CD), isocaloric low protein diet (9 % casein; LPD), a low protein diet supplemented with methyl donors (betaine, choline chloride, folic acid, methionine, Vitamin B_{12}; MDL), a high fat, high sugar Western diet (21.4 % anhydrous milk fat, 50 % carbohydrate; WD), or a Western diet supplemented with methyl donors (MDWD) for a period of at least 8 weeks. The addition of methyl donors served to test the hypothesis that their supplementation to a sub-optimal diet could potentially have ameliorative effects on gene expression and epigenetic profiles. The males were then mated with females fed a standard chow diet. Females at day 17 of pregnancy and males were culled at the end of the study period. Testes were collected from the males and processed for either morphological assessment (histology) or global gene expression profile

(microarray) analysis. Liver tissue and serum were collected for metabolic assays, and DNA from stool samples was isolated and 16S sequenced for microbiome analysis. While MDL males were significantly lighter than CD upon culling, no other significant differences were observed in total body weight. However, both high fat diet groups WD and MDWD exhibited significantly greater gonadal adiposity coupled with increased liver cholesterol and a relative decrease in carcass weight. While there were no significant differences observed in mean total testicular tubule area, total perimeter, lumen area, or epithelial area between the five groups, abnormalities such as seminiferous tubule epithelial loss, tubule vacuolisation and cell separation from the basement membrane were observed significantly more frequently in testes exposed to WD and MDWD compared to the other groups. Analysis of seminiferous tubule cell populations via immunohistochemical staining revealed no change in Sertoli and germ cell numbers between the groups. However, both LPD and WD displayed a decrease in spermatogonial stem cell numbers. Array analysis of global testicular RNA revealed the upregulation and downregulation of genes involved in embryonic lethality in LPD and MDL, respectively. In both high fat groups, genes involved in abnormal cell and mitochondrial function in addition to regulatory genes of cell survival and differentiation were downregulated. While no significant differences were observed in phylogenetic diversity or species evenness between the groups when examining the gut microbiome through bacterial DNA sequencing, MDWD exhibited an increased percentage distribution of the phylum Proteobacteria compared to the other groups. While paternal diet did not seem to have an impact on maternal gestational weight, it was revealed that both low protein diet groups LPD and

MDL resulted in lighter fetuses compared to CD and WD, with an increased fetal to placental weight ratio observed in WD. Taken together, these data provide further insight into testicular cytoarchitecture and global gene expression patterns in response to suboptimal paternal diet with and without key vitamin and mineral supplementation. Moreover, here the adverse effects of a high fat diet are observed without inducing obesity, suggesting an increase in adiposity at the expense of muscle mass potentially through perturbed lipid metabolism mechanisms characterised by changes in liver cholesterol and free fatty acids. Further, supplementation of either low protein or high fat diets with key methyl donors leads to different physiological and metabolic outcomes, which suggests that outcomes resultant from this form of supplementation are unique to the state of nutrition to which they are supplemented.

CHAPTER ONE: INTRODUCTION

1.1 The impact of lifestyle on reproductive fitness

An individual's lifestyle not only shapes their physiology but can also have an impact on their reproductive health. Studies have demonstrated that a number of lifestyle factors, such as smoking, alcohol consumption, drug use, and dietary intake can affect an individual's reproductive fitness. One of the most extensively investigated factors that can affect reproductive wellbeing is obesity, which is increasingly prevalent (Ng et al., 2014) and has been shown to adversely affect an individual's reproductive health through impairing sperm DNA integrity, promoting oxidative stress and reductions in the levels of androgens (Ozcan Dag and Dilbaz, 2015; Palmer et al., 2012; Raad et al., 2017; Rando and Simmons, 2015). A sedentary lifestyle, lacking the necessary physical activity and a well-balanced diet, is potentially causative of poor reproductive outcomes (Ozcan Dag and Dilbaz, 2015). The impact of lifestyle and nutrition on maternal reproductive health is a well-established area of research in reproductive medicine, as it has been shown to have effects on both the mother and the offspring (Moser et al., 2017; Reijnders et al., 2019). Maternal obesity has been characterised as a risk factor for adverse pregnancy outcomes such as stillbirth, preeclampsia, and hypertensive disorders as well as impaired fetal development (Fuchs et al., 2017). Furthermore, a number of studies in humans (Bunt et al., 2005; Landon et al., 2014; Tam et al., 2017) and rodents (Guo and Jen, 1995; Samuelsson et al., 2008; Yan et al., 2010) reveal that maternal obesity or diabetes during pregnancy is linked to offspring metabolic disorders resulting in the significant upregulation of adipogenesis in

the offspring. Similarly, maternal obesity has been positively correlated with increased rates of obesity in children (Ogden et al., 2006).

Maternal protein intake during pregnancy has also been shown to have an impact on offspring development in a rat model. Carlin et al. (2019) observed an increase in adiposity with a decreased expression of the liver insulin receptor and insulin substrate receptor 1 in rat offspring of dams fed a high protein (HP) diet during gestation. Maternal high, and low, protein diets have also been shown to have an adverse effect on the hypothalamus and liver transcriptome as well as metabolic homeostasis in adult mouse offspring (Martin et al., 2018). However, much remains uncertain with regard to the mechanisms underlying the influence of external factors and individual lifestyle on the development of disease and disease risk factors in their offspring.

1.2 Impact of maternal nutrition

The role that nutrition plays in shaping and influencing one's wellbeing is a significant branch of scientific investigation, particularly in recent decades. The world, in its ever-developing state, has facilitated and often given rise to different diets of varying characteristics. Whether it is overnutrition, with calorie-dense diets saturated with high fat and high sugar content, or undernutrition, with caloric restriction or a lack of certain macronutrients, or a deficiency in key vitamins and minerals, significant changes in dietary intake have been known to play an important role in influencing an individual's health, development, and overall wellbeing. Maternal overnutrition animal models have been used to study the extent to which particularly high fat diet feeding modifies and disrupts adult offspring development. A study in Wistar rats fed a high fat

diet from weaning, or from pregnancy and throughout lactation, showed that offspring from these dams were significantly lighter at birth in addition to presenting with hypoinsulinemia and hypoleptinemia at birth, only to be significantly more obese in adulthood compared to the control group (Howie et al., 2008). This highlights the sensitivity of embryonic and fetal development and how susceptible offspring development is to external factors such as maternal gestational dietary intake (Kind et al., 2006; Lucas et al., 2009; Fleming et al., 2018).

Maternal undernutrition before and during gestation has also been shown to affect fetal growth as well as program offspring to have increased predisposition to some non-communicable diseases such as cardiovascular disease (Black et al., 2008). One of the most notable cases of the effect of parental undernutrition on offspring development is the Dutch famine of 1944-1945, where individuals exposed *in utero* to famine for a period of up to 6 months during pregnancy were associated with increased levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and reduced levels of high-density lipoprotein (HDL) later in life (Lumey et al., 2009; Roseboom et al., 2000; Roseboom et al., 2006). Furthermore, prenatal studies with regard to the Dutch famine began with Stein et al. (1972), who later showed that general reductions in postpartum maternal weight, offspring birth weight, and placental weight were a result of prenatal exposure to the famine. More recently, Veenendaal et al. (2013) found that children (F2) of fathers (F1) who were prenatally exposed to the Dutch famine presented with increased body weights and BMIs compared to children of unexposed F1 fathers, with no such effect being observed in F2 offspring of prenatally exposed F1 mothers (Veenendaal et al., 2013).

Data from large birth registries and human cohort studies of pregnant women and their offspring exposed to malnutrition through famines later gave support to the Fetal Origins of Adult Disease (FOAD) theory, a concept proposed by Dr. David Barker. In the late 1980s, Barker and colleagues studied birth records in a population of women and men born in Hertfordshire, United Kingdom between the years 1911 and 1930 and found that birth weight inversely correlated with an increased risk of mortality due to coronary artery disease (CAD) by 75 years of age (Barker et al., 1989; Barker et al., 1991). The determinants of low birth weight were shown to be more influenced by prenatal environment than genetic variation. In a meta-analysis analysing 43 determinants of low birth weight from "895 published papers in the English and French literature from 1970-1984", Kramer (1987) found that the most important factors were external rather than genetic. Such factors included cigarette smoking, pre-pregnancy weight, and nutrition. However, the relationship between nutrition and complications of CAD have been shown to not be a strictly inversely proportional one, but a U-shaped relationship, indicating that both over and undernutrition are linked to disease (Gluckman et al., 2008). This type of relationship has also been suggested to be the case with regard to the association between birth weight and childhood blood pressure and risk of cardiovascular disease, indicating that both low and high birth weight are potential risk factors (Knop et al., 2018; Lai et al., 2019). Different studies in both humans and animal models consequently emerged to further characterise the effect of maternal over- or undernutrition on reproductive and offspring health (Aiken et al., 2016; Connor et al., 2012; Dunford et al., 2014; Jacobs et

al., 2014; Sellayah et al., 2014; Sinclair et al., 2018; Watkins et al., 2011; Watkins et al., 2015; Wu et al., 2004).

1.3 Role of Paternal Nutrition

Unhealthy diet consumption has been on the rise worldwide, particularly in Western countries (Kopp, 2019). However, the increasing prevalence of poor-quality diets is also of significant concern in developing countries, especially in more recent decades (Müller and Krawinkel, 2005). For example, low to middle-income families in countries in sub-Saharan Africa and southeast Asia have a significantly increased reliance upon processed foods and sugar-sweetened beverages, leading to rapidly increasing rates of obesity and its associated risk factors (Popkin et al., 2012). Several investigative studies into the effects of the consumption of such diets have associated it with the development of a number of different diseases and disorders in addition to obesity, such as type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), hypertension, and metabolic syndrome (Ludwig et al., 2001; Nseir et al., 2010; von Frankenberg et al., 2017). Recent reports suggest that one in four people worldwide have metabolic syndrome (O'Neill and O'Driscoll, 2015; Saklayen, 2018), often contributed to by means of an unhealthy lifestyle characterised chiefly by sub-optimal diet consumption. Metabolic syndrome, or MetS, is defined as a cluster of metabolic diseases and abnormalities that includes insulin resistance, obesity, hypertension, and is associated with increased risk of the development of cardiovascular disease and type 2 diabetes (Rochlani et al., 2017). Metabolic syndrome has been associated with a decline in testosterone levels in men (Lotti et al., 2013), in addition to lower sperm concentration, increased levels of sperm DNA fragmentation, and reduced sperm viability (Leisegang et al., 2014).

There is strong evidence indicating that obesity induced by high fat diet feeding reduces male mating and fertilisation success rates, thereby impairing fecundity, with these effects being exacerbated over time the longer the exposure to high fat diet feeding is (Crean and Senior, 2019). High fat diet feeding has also been linked to insulin resistance, dyslipidaemia, and systemic oxidative stress in mice (Funes et al., 2019), with results mimicked in human studies (Anderson et al., 2009; Furukawa et al., 2004). Furthermore, diet-induced obesity is associated with subfertility characterised by reduced sperm motility, reduced testosterone, and significantly lower pregnancy rates in C57BL/6 mice (Fan et al., 2015). These observations made by Fan and colleagues were accompanied by dramatic disruptions to the blood-testis barrier (BTB) and severe seminiferous tubule epithelial atrophy, with significant reductions in tight junction proteins (Fan et al., 2015). A number of systematic reviews and meta-analyses in recent years have focused on the role of male obesity and increased BMI on reproductive outcomes, sperm viability, and general physiological and metabolic health (Campbell et al., 2010; Crean and Senior, 2019), citing an increased likelihood of infertility, abnormal sperm morphology, and reduced rates of live birth per assisted reproduction technology (ART) cycle in obese men (Campbell et al., 2010). In a 2019 study on a high fat diet-fed male mouse model, Gómez-Elías and colleagues induced a MetS-like condition by implementing a 30 % high fat diet regimen for 19 weeks. The authors observed increased glucose intolerance, hypercholesterolemia, and hyperglycemia in the high fat diet (HFD)-fed mice, but found no impact on fertility, as no significant differences were reported in the mice's *in vitro* fertilisation (IVF) rates (Gómez-Elías et al., 2019). The authors specify the importance of an individual's metabolic status, as they

observed no impairment of fertility in a high reproductive performance mouse model without any preexisting impairments to reproductive wellbeing (Gómez-Elías et al., 2019).

Investigations into dietary models also included both over and undernutrition in different forms including excess nutritional or caloric intake, or caloric restriction or nutrient deprivation. This has not only aided in exploring the impact of suboptimal nutrition on male physiology but also the characterisation of the role of certain macronutrients in an individual's diet, such as protein, carbohydrate, fibre, and fat. In addition to their overconsumption, the restriction of said macronutrients is also a subject of interest. One of the main macronutrients that has been at the forefront of investigative studies is dietary protein, both in high and low quantities. Studies in both animal models and humans have aimed to investigate the role of over and undernutrition with protein in addition to how protein interacts with an individual's caloric intake, which led to the proposal of the "protein leverage" hypothesis, which postulates that when high protein diets are fed, the total caloric intake (and therefore the intake of carbohydrates and fats) is reduced in order to avoid protein excess, and vice versa (Gosby et al., 2014; Raubenheimer et al., 2014; Simpson and Raubenheimer, 2005). While the prolonged consumption of high protein diets has been associated with hepatic and renal dysfunction and precipitated progression of coronary artery disease in humans (Delimaris, 2013), the restriction of dietary protein has been associated with the extension of lifespan and the improvement of cardiometabolic health (Kitada et al., 2019). In fact, and following the "protein leverage" hypothesis logic, low protein, highly processed foods have been linked to a higher energy intake in an obesogenic manner, citing

significant global health implications (Grech et al., 2022). In animal models, studies investigating the impact of low protein diets have been consistently emerging particularly over the past two decades. In most cases, studies report adverse effects of low protein diets on metabolic and reproductive health. In a recent systematic review and meta-analysis of animal clinical trials in rats, the consumption of low protein diets was shown to cause significant body weight reduction in addition to reductions in organ weights such as testis, epididymis, and seminal vesicles (Ajuogu et al., 2020). Furthermore, the meta-analysis revealed that low protein diet consumption has endocrinological implications in rats, as it caused significant reductions in serum testosterone and follicle-stimulating hormone (FSH) (Ajuogu et al., 2020). Additionally, paternal low protein diet intake can also affect the offspring, as it is linked to stalled embryo development (Carone et al., 2010) and the programming of perturbed offspring cardiometabolic health characterised by glucose intolerance and vascular dysfunction (Watkins and Sinclair, 2014) in mouse models. Moreover, paternal consumption of a low protein diet also impacts the maturing sperm by impairing its transfer RNA (tRNA) content, a population of small RNAs found to act as key regulators of gene expression (Sharma et al., 2016). Varying degrees of protein restriction have been shown to have different effects on energy intake and expenditure in rodent models. In a study in obesity-prone rats following a protein restriction dietary regimen, the protein-free and very low protein diet groups displayed fatty liver and decreased lean mass and total body weight, while the moderately low protein diet group displayed weight gain and increased adiposity (Pezeshki et al., 2016). This highlights the sensitive nature of dietary protein intake and the divergent effects its varying levels of restriction can have

on animal physiology. In a mouse model, a very low protein (1 %) model led to a decrease in food intake as well as reduced body weight, with fatty acid and amino acid metabolism also being altered by the intake of less protein content (Wu et al., 2021). This decrease in energy intake following very low protein diet consumption is contradictory to the logic of the "protein leverage" hypothesis, which posits that a very low level of protein would induce an increase in energy intake. Wu and colleagues observed that low protein intake also led to the inhibition of hypothalamic mechanistic target of rapamycin (mTOR) signalling, which they showed was crucial for the lack of hunger response in their model (Wu et al., 2021). The mTOR pathway is a key pathway involved in food intake regulation (Cota et al., 2006) and its expression has been shown to be susceptible to regulation by amino acids (Beugnet et al., 2003; Carroll et al., 2016). Whether this mechanism is chiefly responsible for the decrease in energy intake following a very low protein diet regimen still requires elucidation, however. Controlled protein diet consumption may theoretically be a desirable method of inducing weight loss and caloric restriction. However, it is worth noting that evidence in the literature points to the varying impacts protein diet has in different levels of consumption (Pezeshki et al., 2016). Therefore, further research into the mechanisms underlying the effects observed is warranted in order to better understand and characterise the impact of sub-optimal protein diets on physiological, metabolic, and reproductive health and wellbeing.

While a wide range of studies have investigated the impacts of different diets such as calorie-dense high fat, high sugar, or high/low protein diets on human and animal physiology, other studies have focused on supplementation with specific dietary factors or micronutrients as potential means to reverse or

alleviate the effects of sub-optimal diets. Indeed, supplementation with different vitamins, minerals, and amino acids has been linked with positive physiological and metabolic outcomes. For instance, fish oil supplementation in a high fat diet rodent model has been shown to improve intestinal health through alterations to the microbiome in addition to the attenuation of metabolic dysfunction (Monk et al., 2019). Supplementation of L-Carnitine in Silverfish species has been shown to improve cholesterol transport and promote lipid hydrolysis (Chen et al., 2022). Furthermore, supplementation has also been used in studies assessing the impact on offspring development affected by either paternal or maternal dietary intake. On the paternal side, low protein diet (LPD) feeding in a mouse model resulted in increased fetal weight, while supplementing LPD with methyl donors such as betaine, choline, folate, and vitamin B_{12} has been shown to normalise the weights of fetuses of low protein diet-fed male mice (Morgan et al., 2021) potentially through influencing fetal and placental gene expression patterns that were initially altered by LPD consumption as observed by Watkins et al. (2017). The exact mechanisms underlying this normalisation by methyl donor supplementation is unclear, however, yet the altered gene expression profiles and placental nutrient supply remain a potential cause. In a 2016 study on dietary restriction and supplementation in a mouse model, McPherson et al. (2016) observed that dietary restriction in adult male mice not only resulted in increased sperm oxidative DNA damage, but also in offspring with reduced postnatal weight and increased adiposity. This study also showed that supplementing food with vitamins and antioxidants not only normalised the expression of a number of pancreatic genes in female offspring, but also reduced sperm reactive oxygen species (ROS) in founder males, normalised post-natal

growth and adiposity in both male and female offspring, and to an extent normalised sperm DNA methylation patterns (McPherson et al., 2016). The group proposes potential mechanisms underlying this paternal programming of offspring health and development, such as epigenetic changes to sperm and the constitution of seminal fluid (McPherson et al., 2016). In fact, supplementation of sub-optimal diets with such vitamins, minerals, and crucial amino acids is thought to contribute to the alleviation of the effects caused by said diets on a physiological, metabolic, and even inter and transgenerational level.

1.3.1 Impact of diet on sperm quality

Compared to maternal nutrition, the impact that sub-optimal paternal diet has on reproductive and embryological outcomes has not yet been fully elucidated and its underlying mechanisms are relatively poorly understood. However, there is increasing evidence in animal models and humans showing that poor paternal diet may contribute to male infertility or a general decline in reproductive fitness (Sharma et al., 2013). Data from a number of animal studies contribute to a growing body of evidence showing that paternal nutritional alteration such as a low protein diet (Watkins et al., 2018), high fat diet (Consitt et al., 2018; Fullston et al., 2015; Larson et al., 2019), as well as a low folate diet (Lambrot et al., 2013) can potentially have an array of adverse effects on male reproductive fitness as well as offspring health. Such paternal alterations in dietary intake program the predisposition of certain diseases in the offspring such as cardiovascular disease (CVD) or type 2 diabetes (Fullston et al., 2012; Triggs and Knell, 2012; Watkins et al., 2018). Such studies have also been a subject of a number of reviews assessing the available evidence on the impact of paternal diet, specifically obesity, on fertility and sperm function (Palmer et al., 2012;

Raad et al., 2017; Rando and Simmons, 2015). Indeed, it has been found that obesity has significant adverse effects on sperm quality and thus it hinders fecundity in both mouse models (Bakos et al., 2011) and humans (La Vignera et al., 2012). Significantly, data from a number of large-scale epidemiological studies (Alshahrani et al., 2016; Nguyen et al., 2007; Sun et al., 2017; Wen-Hao et al., 2015) suggest a negative correlation between body mass index (BMI) and male fertility in terms of semen parameters such as motility, concentration, and morphology. Most notable are altered levels of sex hormones, for example a reduction in the level of testosterone, that in turn adversely affect testicular function, thus impairing male reproductive fitness (Fui et al., 2014; Kelly and Jones, 2015). Indeed, a number of recent studies showed that paternal factors, such as age, dietary intake and lifestyle habits, play a key role in altering sperm quality, semen parameters, and embryogenesis (Fullston et al., 2012; Kaarouch et al., 2018; Triggs and Knell, 2012; Pearson and Ehninger, 2018; Schagdarsurengin and Steger, 2016; Soubry, 2015; Watkins et al., 2018), thus highlighting the role of nutritional intake in influencing and shaping an individual's physiological, metabolic, and reproductive fitness and overall wellbeing

Despite there being evidence citing the adverse effects of conditions like obesity on fertility and fecundity, the evidence available on the influence of different types of healthy and unhealthy diets on sperm and semen parameters is surprisingly not as detailed. Diets containing fruit and vegetables, for their vitamin content, and low-fat dairy products and fish as primary protein sources, have been linked to better quality sperm in adult men undergoing fertility treatment (Vujkovic et al., 2009). Furthermore, healthy and varied diets

comprised of different sources of vitamins, minerals and macronutrients are advisable for better sperm parameters and lower sperm DNA fragmentation (Skoracka et al., 2020). However, there is a gap in our understanding of the mechanisms underlying the influence of dietary intake on male reproductive fitness. There is a need for further longitudinal studies aimed at shedding more light onto the roles of different macro and micronutrients on sperm, as data in the literature is not fully conclusive.

1.3.2 Intergenerational and transgenerational impact of paternal diet.

In a study examining the effects of paternal dietary intake on offspring development, Watkins and Sinclair (2014) showed that feeding male mice a low protein diet (LPD) has adverse impacts on adult offspring metabolic health and impairs offspring cardiovascular function. The authors showed that adult offspring from LPD-fed male mice had relatively elevated heart rate, adiposity, and hypertension compared to control group normal protein diet (NPD) mice in addition to vascular dysfunction and impaired glucose tolerance (Watkins and Sinclair, 2014). In another recent study aimed at further characterising the impact of paternal diet on embryonic development and offspring health, Watkins et al. (2017) demonstrated that feeding adult male mice a low protein diet (LPD) alters blastocyst metabolic expression of AMPK (5' AMP-activated protein kinase). Watkins and colleagues suggest that the altered expression is a potential adaptive mechanism in order to preserve embryo viability in response to the LPD (Watkins et al., 2017). In a recent study in a mouse model aimed at determining the impact of paternal LPD on the gene expression in offspring white adipose tissue (WAT), Ly et al. (2019) carried out RNA sequencing analysis on offspring WAT and observed a downregulation of genes involved in

lipogenesis in the LPD group, suggesting a contribution of paternal LPD to offspring metabolic disorder. Furthermore, offspring of LPD-fed male mice present an increased expression of hepatic lipid and cholesterol biosynthesis genes (Carone et al., 2010). It is worth noting that transgenerational effects induced by paternal diets are also reliant on the period during the paternal grandparent's childhood where over or undernutrition exposure takes place (Pembrey et al., 2006). Paternal fasting has also been shown to have an impact on the development of their offspring. Anderson et al. (2006) observed changes in serum glucose, corticosterone concentrations, as well as insulin-like growth factor 1 (IGF1) in offspring of male mice subject to fasting before mating. Ng et al. (2010) found that paternal high fat diet induces dysfunction in pancreatic β cells in addition to glucose intolerance in female offspring in rats. Furthermore, paternal obesity has been linked to an increased risk in breast cancer in female offspring in mice potentially through micro-RNA (miRNA) and other small non-coding RNA populations-mediated pathways (Fontelles et al., 2016). In a 2013 study by Fullston et al. (2013), paternal obesity resulted in metabolic disruptions such as impaired glucose tolerance and insulin resistance in female offspring, with similar changes – albeit less severe – being observed in male offspring. These perturbations were also transmitted to the F2 generation through both maternal and paternal F1 lineages (Fullston et al., 2013). Further studies showed that paternal obesity not only influenced aberrant metabolic phenotypes in F1 and F2 generations, but also caused significant impairment to F1 and F2 offspring reproductive health. In a study examining a HFD-induced obese mouse model without diabetes, Fullston et al. (2012) found that increased sperm ROS and DNA damage was transmitted to F1 male offspring, with a similar effect

being also observed in F2 males from both F1 lineages, in addition to reduced sperm motility (Fullston et al., 2012). The authors speculate that the observed transgenerational effects on reproductive health are likely a result of the impaired metabolic health of the F0 males, which introduced epigenetic alterations to the F0 male sperm (Fullston et al., 2012). Transgenerational effects have also been observed in humans, both paternally mediated and sex specific (Pembrey et al., 2006), and through neonatal exposure to undernutrition as with the case of the 1944-1945 Dutch famine (Painter et al., 2008). The link between poor paternal diet and poor reproductive fitness and subsequent impairment of offspring development has indeed become better understood throughout the last two decades. However, the mechanisms underlying the changes to offspring health and paternal reproductive fitness are still poorly understood. Altered sperm quality in response to factors such as diet suggests an underlying alteration in testicular function accompanied with metabolic and physiological changes.

1.4 The testes

1.4.1 Morphology

The testes are the male's primary reproductive organs, responsible for the production of gametes (spermatozoa) and the secretion of certain androgenic hormones, such as testosterone. They are paired structures that, in many mammalian species are housed inside the scrotum, a sac whose evolutionary purpose is to keep the testes at a certain temperature that is generally lower than the body temperature (3.1° C lower than normal body temperature in humans (Jones and Lopez, 2014)). The most plausible explanation for the difference in

temperature is that for spermatogenesis to properly function, an optimum temperature below the core abdominal temperature is required (Werdelin and Nilsonne, 1999). Testes have an ellipsoid shape surrounded by the tunica albuginea, a dense layer of fibrous tissue, and consist of several lobules, each containing tubular convolutions known as the seminiferous tubules. Extensions of the tunica albuginea form the septa of the testes, which divide the testis into compartments. A transverse cross-section of a testis reveals tens of seminiferous tubules of different sizes and shapes, each at a different stage of the process of spermatogenesis constituting the cycle of the seminiferous epithelium. The stages of the cycle of the seminiferous epithelium would be arranged consecutively along the length of a seminiferous tubule, if the length of the tubule were to be examined at a given point in time (Haschek et al., 2009).

The epithelium that lines the seminiferous tubules contains spermatogenic cells in addition to Sertoli cells which are responsible for the growth, development, and nourishment of germ cells throughout spermatogenesis. Leydig cells occupy the interstitial space between the tubules, and their primary function is to produce testosterone (Matsumoto and Bremner, 2016). As the spermatogenic cells develop through the mitotic and meiotic stages of spermatogenesis, they no longer appear rounded but present characteristics of maturing spermatozoa, such as elongation as characterised during the third stage during which differentiation occurs. The produced spermatozoa pass into the rete testis, where the seminiferous tubules converge and connect to the epididymis through connecting ducts. Spermatozoa are transported to the epididymis for further maturation and eventual storage in the epididymal tail (McGlone and Pond, 2003). The epididymis is a long and tightly coiled duct extending down the

posterior end of the testes, becoming the vas deferens, also known as ductus deferens. The vas deferens connects the epididymis to the urethra through the inguinal canal, where the different accessory glands also terminate in the ejaculatory ducts. The testicular morphology is presented in *Figure 1.1* (Tiwana and Leslie, 2023).

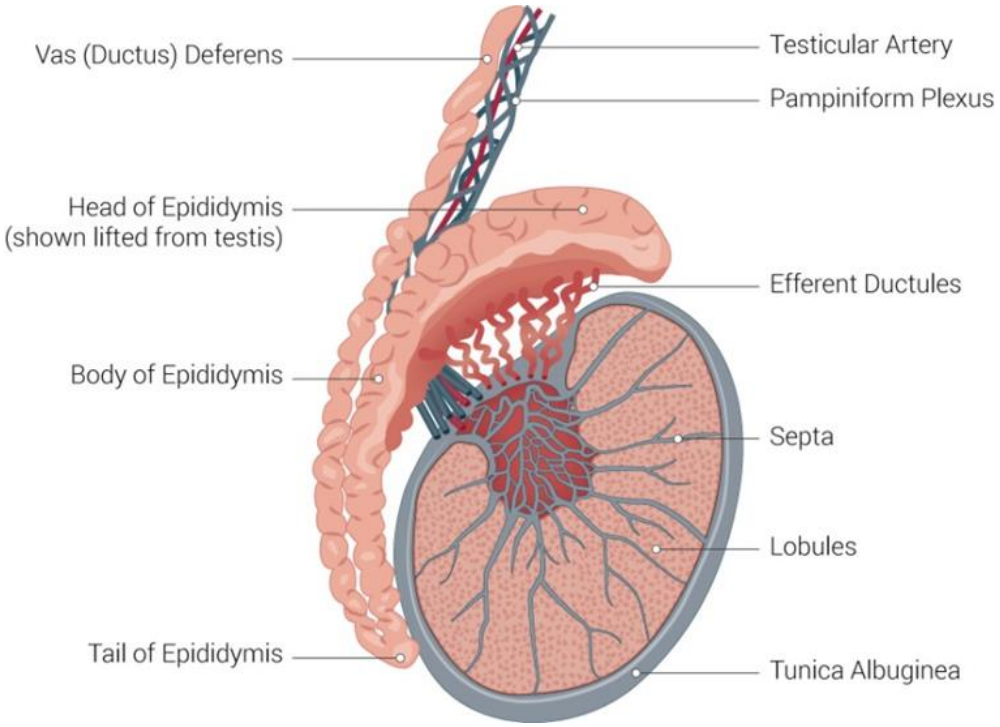

Figure1. 1: Anatomy, Abdomen and Pelvis, Testicle. Contributed Illustration by Beckie Palmer (Tiwana and Leslie, 2023).

1.4.2 Spermatogenesis

The testes are responsible for the production of male gametes, or spermatozoa, through the process of spermatogenesis. This process is an intricate and highly regulated process that allows the production of specialised and differentiated cells – spermatozoa – from undifferentiated spermatogonial stem cells present along the seminiferous tubule basal membrane. Diploid spermatogonia undergo mitotic division in order to replenish the population of stem cells and resulting haploid spermatocytes after the first meiotic division go on to further undergo meiosis resulting in spermatids that finally differentiate to produce spermatozoa (Desjardins and Ewing, 1993; Russell et al., 1993). The duration of this process in humans is reportedly around 74 days (Griswold, 2016), whereas in mice it is estimated to be around 34.5 days (Oakberg, 1956).

Spermatogenesis is broadly divided into 3 main phases; first, the mitotic proliferation of spermatogonia, followed by meiotic reduction division forming haploid spermatids and then the morphological transition to spermatozoa (spermiogenesis), and finally spermiation, which is the process of releasing the mature spermatozoa into the seminiferous tubule lumen (de Kretser et al., 1998). Diploid primary spermatocytes, following their first meiotic division, generate two haploid secondary spermatocytes. They then undergo a second division, producing four round haploid spermatids (Jones and Lopez, 2014). Spermatids then undergo morphological transformation into specialised spermatozoa through the process of spermiogenesis, which also involves nuclear compaction, acrosome formation, and flagellum assembly (O'Donnell, 2014). Cytoplasmic remodelling through phagocytosis by Sertoli cells also occurs and, finally, spermatozoa are released into the lumen during spermiation. Apart from

spermatozoa, spermatogenic cells express the RNA helicase DDX4, a cytoplasmic marker, highlighting a germ-cell specific marker in the human (Castrillon et al., 2000) and mouse model (Fujiwara et al., 1994). The process is demonstrated in *Figure 1.2* by Hunter et al. (2012).

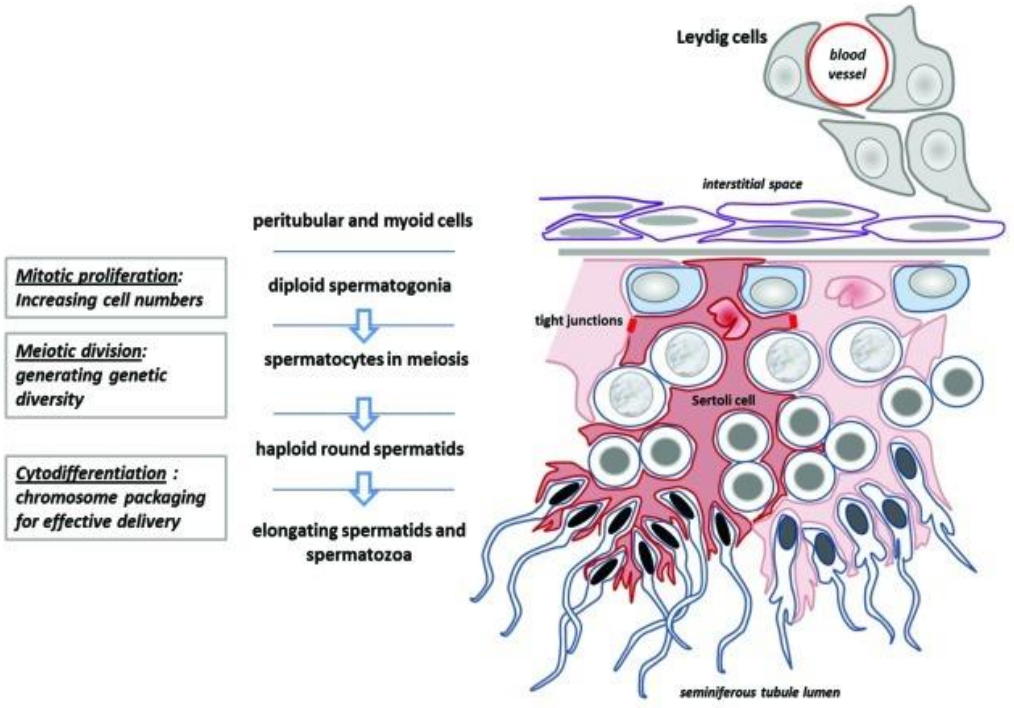

Figure1. 2: Spermatogenesis by Hunter et al. (2012).

1.4.3 The Sertoli cell

All spermatogenic cells are in contact with the Sertoli cells, which not only support the growth and maturation of spermatogenic cells, but also promote cell division. These cells extend from the basement membrane to the lumen of the seminiferous tubules – as demonstrated in *Figure 1.2* by Hunter et al. (2012) – and are stimulated by follicle stimulating hormone (FSH) and testosterone. Sertoli cells secrete inhibin, which inhibits the negative feedback exerted by gonadotropin-releasing hormone (GnRH) and FSH on spermatogenesis. Furthermore, Sertoli cells secrete Androgen Binding Protein (ABP), which stimulates spermatogenesis by binding androgens and retaining them in

seminiferous tubules (França et al., 2016; Ma et al., 2015). Another key hormone secreted by Sertoli cells is the Anti-Müllerian Hormone (AMH) or Müllerian-Inhibiting Hormone (MIH) which inhibits the formation of Müllerian ducts in males upon activation by *SOX-9* gene (Li et al., 2019; Taguchi et al., 1984). High levels of SOX-9 are known to be detected within Sertoli cells of developing and adult testis (da Silva et al., 1996), therefore making it a useful cell-specific marker (Hemendinger et al., 2002). An important feature of Sertoli cells is their role in the maintenance of the blood-testis barrier (BTB) through tight junctions that regulate the tubal environment (Mruk and Cheng, 2015). Mature and viable spermatozoa possess the ability to fertilise oocytes in order to produce a zygote. Through that process, the male passes on half of his genetic and epigenetic material to the offspring.

1.4.4 Leydig cells

Leydig cells play a crucial role in the maintenance of sperm production and spermatogenesis, hormonal regulation, and secondary sexual characteristic in males. Located between seminiferous tubules which contain Sertoli and germ cells, Leydig cells are known to be one of the cell populations in the interstitial compartment of the testis. These cells are the main source of androgens and testosterone in males (Aldamat and Tadi, 2022). There are two distinct populations of Leydig cells: fetal Leydig cells, which originate in the fetus but begin to regress soon after birth, and adult Leydig cells. Fetal Leydig cells are known to produce high levels of testosterone, which is required for the stimulation of male sexual differentiation (Huhtaniemi and Teerds, 2018). On the other hand, adult Leydig cells form from precursor cells during postnatal life and produce testosterone (Chen et al., 2010).

1.5 Overview of epigenetics

Epigenetics, a term first introduced by Conrad Waddington in the early 1940s was defined as the "causal interactions between genes and their products which bring the phenotype into being" (Waddington, 2012). Today's generally accepted definition of epigenetics refers to the study of (mitotically and/or meiotically) heritable changes in gene expression that do not involve a change in the DNA sequence (Dupont et al., 2009). These changes are carried out through the post-translational modification of histones, chromatin remodelling, and RNA-based mechanisms such as non-coding RNAs (Gibney and Nolan, 2010). Epigenetics is influenced by a number of epidemiological factors including diet, infections, and drugs. In fact, a number of alterations to testicular function as well as offspring health are epigenetically mediated.

1.6 Mediators of offspring programming

In addition to direct genetic transmission of alleles by the father to their offspring, thus influencing their genotype and predisposition to disease (Sharp and Lawlor, 2019), a number of paternal impacts on offspring are epigenetically mediated. Several studies demonstrate the impact of paternal low protein (Carone et al., 2010), high fat (de Castro Barbosa et al., 2016), and low folate diets (Lambrot et al., 2013) on sperm epigenetic status. Genome-wide DNA hypomethylation has been reported in sperm of LPD-fed mice, correlating strongly with alterations in 1-carbon metabolism and DNA methylation (Watkins et al., 2018). This finding was observed by the authors through reduced relative expression of key DNA methyltransferases *Dnmt1* and *Dnmt3L* and the altered expression of dihydrofolate reductase (*Dhfr*), methylenetetrahydrofolate reductase (*Mthfr*), and methionine synthase (*Mtr*), all key enzymes in the folate

cycle (Watkins et al., 2018). Reinforcing the important role of paternal epigenetic status, evidence from Grandjean et al. (2015) reports the establishment of Western-like diet-induced offspring phenotypes resulting from the microinjection of either testis or sperm micro-RNA (miRNA) from high fat Western diet-fed male mice into one-cell embryos. Further studies emerged to investigate the role of small non-coding RNAs (sncRNA) such as transfer RNA-derived small RNAs (tsRNA) in sperm. Chen et al. (2016) showed that the injection of sperm tsRNAs derived from HFD-fed male mice into normal zygotes resulted in F1 generations expressing metabolic disorders and altered metabolic pathway gene expression, highlighting tsRNAs as an epigenetic factor and possible mediator of the intergenerational inheritance of metabolic disorders through dietary intake. sncRNAs, a series of RNA species including different RNA subspecies such as tsRNAs, micro RNAs (miRNAs), and ribosomal RNAs (rRNAs) (Dozmorov et al., 2013) have more recently become the focus point of a number of animal model and human studies investigating their role as mediators of transgenerational transmission in paternally acquired metabolic disorders (Zhang et al., 2018). sncRNAs have been identified as important regulators of numerous biological processes including cellular stress response as well as cellular differentiation (Cai et al., 2013; Wang et al., 2020). sncRNAs are able to silence gene expression, and thus have been a key component in therapeutic interventional studies (Galasso et al., 2010). The methylation of non-coding RNA subspecies such as tsRNAs has been suggested to play an essential role in the regulation and stabilisation of paternal phenotypic transmission (Lismer and Kimmins, 2023). In a study highlighting the interaction between DNA methylation mechanisms and RNA populations, Zhang et al. (2018) found

that the inactivation of *Dnmt2*, a tRNA methyltransferase involved in epigenetic transmission (Tuorto et al., 2015), prevents paternally acquired HFD-induced metabolic disorders from being intergenerationally transmitted in male mice through sperm sncRNA-mediated pathways (Zhang et al., 2018).

More recently, emerging evidence suggests the implication of dietary intake in altering sperm sncRNA profiles in humans. In a recent study investigating the effect of a 2-week diet intervention on human sperm sncRNA profiles, Nätt et al. (2019) notably reported an upregulation of a subpopulation of mitochondrial tsRNAs and rRNAs as well as a specific tsRNA derived from nuclear tsRNA after one week of a high sugar dietary regimen. Additionally, the authors also reported the increase of a specific type of nuclear tsRNA derived from the T-loop of genomic tsRNAs which they named nitRNA, in response to the dietary intervention. Furthermore, the authors observed an increase in sperm motility after the 2-week period of dietary intervention and attributed that to an increase in mitochondrial tsRNAs and nuclear nitRNAs, highlighting that sncRNA populations in human sperm are also sensitive to dietary changes (Nätt et al., 2019). In the scope of assisted reproduction technology (ART) and fertility treatment, it has been suggested that sncRNAs in biological fluids such as semen could be used as an effective biomarker for fertility and to benefit ART interventions (Zhu et al., 2021). However, before this promising idea becomes a reality, much further investigation is required with regard to the role of sncRNAs and their interaction with external factors and the downstream implications of the changes they undergo. Furthermore, while animal studies are emerging at a more consistent rate in recent years, extensive research is yet to be done in the

realm of human studies when investigating sncRNAs and their role as a marker for paternally mediated epigenetic transmission.

1.7 The role of seminal plasma

Seminal plasma is fundamentally a combination of secretions from the seminal vesicles and the accessory glands i.e., the prostate and the bulbourethral glands. The seminal vesicles are absent in carnivores, marsupials, and monotremes (McGraw et al., 2014), whereas the prostate is present in all mammals with minor structural variations between species (Marx and Karenberg, 2008). The bulbourethral (or Cowper's) glands are present in most species of placental mammals but are absent in dogs, whales, and dolphins. Seminal plasma plays a critical role in providing the optimal viscosity and pH for sperm to survive and be able to maintain motility, but it also provides spermatozoa with an essential medium of fructose and ions for optimal function (Cheng and Ko, 2019). Seminal plasma contains water, serving as the vehicle for spermatozoa into the female reproductive tract, in addition to mucus secreted by the bulbourethral gland and buffers secreted by both the prostate and bulbourethral glands that serve to neutralise the acidic environment of the female reproductive tract (Jones and Lopez, 2014). Seminal plasma also plays a key role in providing metabolic support to spermatozoa, regulating sperm capacitation, and providing a protective medium for sperm against immune cells in the uterus (Rodriguez-Martinez et al., 2011; Szczykutowicz et al., 2019). Additionally, seminal plasma also contains components such as proinflammatory cytokines that potentially stimulate uterine gene expression as possible means of priming the uterine lining for implantation (Robertson et al., 2017).

Epigenetic transmission in sperm through DNA methylation, non-coding RNAs, and chromatin modification is widely believed to be the primary mediating mechanism in epigenetic inheritance (Day et al., 2016). However, the role of seminal plasma, until recent years, has been relatively overlooked compared to the role of sperm in programming offspring development. In addition to its role in male reproductive fitness, seminal plasma plays a role in post-fertilisation fetal growth and subsequently offspring development (Morgan and Watkins, 2019). Disruptions to blastocyst development characterised by reduced rates of cleavage to two-cell stage, significant reductions to blastocyst number, developmental delay or arrest, and morphological abnormalities with distorted offspring cardiometabolic status were observed as a result of the removal of seminal plasma at the time of conception in mice (Bromfield et al., 2014). Paternal diet and its impact on seminal plasma in turn play a significant role in modifying the female reproductive tract, consequently influencing offspring development. Watkins et al. (2017) observed changes to the uterine environment at early gestation (d3.5) in response to semen from male mice fed a low protein diet (LPD), with significant alterations to uterine gene expression profiles. Blastocysts from the same female mice had significantly supressed expression of genes involved in the AMPK pathway. AMPK activation stimulates glucose uptake as well as fatty acid oxidation (Mihaylova and Shaw, 2011). Glucose uptake is positively correlated with embryonic development and post-implantation survival in both human and mouse embryos (Gardner et al., 2011). However, slower rates of both amino acid and glucose metabolism have been linked to increased developmental potential in human embryos (Baumann et al., 2007). Therefore, Watkins et al. (2017) suggest that the suppression of AMPK

pathway genes may be an adaptive mechanism in response to LPD in order to preserve embryo viability. In another study, to determine whether seminal plasma has a direct effect on offspring health, Watkins et al. (2018) combined artificial insemination with vasectomised male mice mating, thus generating offspring from LPD sperm and control NPD seminal plasma, and vice versa. LPD sperm and/or seminal plasma resulted in heavier offspring displaying glucose intolerance and liver gene expression profiles symptomatic of non-alcoholic fatty liver disease (NAFLD). The greatest changes in hepatic gene expression were observed in offspring from NPD sperm/LPD seminal plasma and vice versa. Therefore, it is suggested that while genetic/epigenetic transmission is sperm-mediated, seminal plasma plays a critical role in modifying maternal uterine environment, and subsequently impacting offspring development. It is proposed that a mismatch between the two results in impaired offspring development, whereas if both sperm and seminal plasma are derived from males fed the same diet, post-fertilisation development occurs normally (Watkins et al., 2018).

The impairment of male reproductive health due to lifestyle factors, however, has been shown to be reversible in a number of cases, as discussed in different studies and reviews. Corrective measures include weight loss (Cabler et al., 2010; Kahn and Brannigan, 2017), maintenance of a balanced diet supplemented with different vitamins and antioxidants (Ahmadi et al., 2016), and cessation of smoking and alcohol intake (Gude, 2012; Harlev et al., 2015; Oyeyipo et al., 2011; Sansone et al., 2018). It is therefore of interest and importance to investigate the interaction between sperm and seminal plasma in programming both the maternal reproductive tract and embryonic/offspring development.

Furthermore, it is crucial to identify key mediators in the programming of sperm epigenetic status and the underlying mechanisms taking place, which ultimately result in alterations to offspring phenotype and developmental health.

1.8 The microbiome and dietary intake

The mammalian microbiome is comprised of anywhere between 10 to 100 trillion microorganisms and it functions in a symbiotic relationship with its host (Turnbaugh et al., 2007). Until recently, our perception of microbes has been chiefly centred on their role in pathogenic processes. However, it is now widely accepted that our body's microbiota plays an essential role in many developmental, physiological, metabolic, and psychological aspects of our daily life. Our bodies play hosts to a number of different and diverse bacterial populations including our skin, gut and oral microbiomes. Thanks to modern advances in sequencing capabilities, the gap in our understanding with regard to the association between our microbiota and complex conditions such as inflammatory bowel disease (Frank et al., 2007) and even obesity (Ley et al., 2006) is being addressed. The human gut microbiome is chiefly populated by the phyla Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, Fusobacteria, and Verrucomicrobia (Rinninella et al., 2019), with Firmicutes and Bacteroidetes making up the vast majority of microbiota in the gut (Arumugam et al., 2011). It has been shown that external factors such as the host's dietary intake can impact these microbial populations (Leeming et al., 2019). An increased dietary diversity, particularly that of plant foods rich in vitamins and dietary fibre, has been shown to improve diversity of microbial populations in the gut (Heiman and Greenway, 2016). On the other hand, reductions in gut microbial diversity have been associated with the exclusion of

crucial nutrients in certain diets such as a gluten-free diet or diets low in fermentable carbs (low-FODMAP diets) (Reddel et al., 2019). Furthermore, low-fibre and low-diversity diets such as Western diets, for example, have been observed to influence the composition of the gut microbiome and promote certain health risk factors such as metabolic diseases and obesity. While the effects of certain dietary patterns on the gut microbiome have been established, the mechanisms governing them remain largely unclear. Some studies speculate certain mechanisms may be behind alterations in microbial composition and metabolic disease in cases of Western diet, for instance. It is thought that the increased population of endotoxin-producing bacteria may be a potential candidate responsible for metabolic endotoxemia (Leeming et al., 2019). While the role of certain macronutrients such as fat or protein is the subject of investigation in a number of studies on dietary intake and the gut microbiome, recent evidence in mouse studies has shown that the reduction in fibre plays a greater role in influencing gut microbiota than dietary fat content (Morrison et al., 2020). Designing the appropriate diet model with the appropriate control groups is both challenging and necessary in the attempt to investigate the role of a sole macro or micronutrient on the gut microbiome and any metabolic, reproductive, or physiological implications resulting from it. A macronutrient that remains relatively under-investigated with regard to its influence on gut microbiota populations is dietary protein. Emerging evidence suggests that dietary protein may play an important role in influencing microbiota compositions, thereby impacting its host. However, data from controlled diet studies in humans is relatively inconclusive, with some studies reporting no alterations in microbiota compositions when overweight study participants

consumed protein supplements (Beaumont et al., 2017), and others reporting changes after protein supplement consumption by athletes (Moreno-Pérez et al., 2018). In rodent studies, increased consumption of protein has been observed to increase the total microbial population while decreasing microbial diversity (Holmes et al., 2017; Kim et al., 2016; Reese et al., 2018). In a review of the association between dietary protein and gut microbiota composition, Zhao et al. (2019) concluded that an appropriate ratio between carbohydrate and protein content or even a low protein diet is preferred over a diet very high in protein, as an increase in undigested protein levels may contribute to the increase in pathogenic microorganisms which are associated with metabolic diseases. The impact of dietary consumption on the gut microbiome leads to subsequent alterations to the host's physiology. In a recent study, Ding et al. (2020) found that the transplantation of fecal microbes from male mice fed a high fat diet to normal diet-fed mice significantly increased the genera *Bacteroides* and *Prevotella* in the control mice's intestinal tract, which resulted in endotoxemia and local inflammation in addition to an increase in pro-inflammatory cytokines in the epididymis (Ding et al., 2020). Furthermore, the authors revealed that a strong negative correlation exists between the increase in genera *Bacteroides* and *Prevotella* and sperm motility (Ding et al., 2020). After carrying out sequencing on testicular RNA, the authors discovered significant downregulation of gamete meiosis and testicular mitochondrial function genes in the microbial transplantation group (Ding et al., 2020). Taken together, these findings suggest a role of high fat diet feeding on gut bacterial dysbiosis, leading to an impairment of spermatogenesis and testicular function.

The relationship that exists between our microbiota and reproductive fitness and wellbeing has gained momentum in recent years with the discussion regarding the sterility of the intra-uterine environment (Stinson et al., 2019). Gaining a better insight into the parental influence between the maternal reproductive tract and the fetus is important for developing new biomarkers for gestational wellbeing and long-term health. Initial studies into the seminal microbiome focused on the detection of pathogenic bacterial species, using comparatively simple techniques such as microscopy and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Earlier studies reported negative associations between the levels of Anaerococcus and semen quality (Hou et al., 2013). In a different study, semen samples identified as 'normal' within a clinical setting were populated predominantly with Lactobacillus, while 'low quality' samples were predominantly populated by Prevotella (Weng et al., 2014). One influence of bacteria on male reproduction stems from the toxic effects of inflammatory cytokines or reactive oxygen species (ROS) they produce within the male reproductive tract (Alahmar, 2019). In addition, bacteria may bind directly to the sperm, influencing motility or even inducing apoptosis (Calogero et al., 2017). Prebiotics supplementation in both human and animal models has been observed to influence seminal plasma composition and sperm quality. In a mouse model, supplementation with Lactobacillus rhamnosus PB01 (DSM 14870) improved sperm kinetics (Dardmeh et al., 2017). Interestingly, the authors in this study observed increased levels of testosterone and higher velocity and motility sperm in supplemented obese males then non-supplemented obese males (Dardmeh et al., 2017). Increased sperm motility and reduced sperm DNA fragmentation were also reported following

supplementation of Lactobacillus rhamnosus CECT8361 and Bifidobacterium longum CECT7347 in men with asthenozoospermia (Valcarce et al., 2017).

Not only can the seminal microbiome influence male reproductive health, but unprotected sexual intercourse can result in the exchange of microbes between partners, suggesting that each partner's reproductive microbiota can affect that of the other. Factors such as frequency of sexual intercourse and number of partners can all be related to the vaginal microbiota and incidences of bacterial vaginosis (Brotman et al., 2010; Plummer et al., 2018). Therefore, it is conceivable that the male's metabolic status at the time of conception could influence his seminal microbiome, which in turn can influence the female reproductive microbiota. As the female reproductive microbiota is directly related to that of the neonate (Mueller et al., 2015), this offers a novel mode of paternal programming of offspring metabolic health. However, such direct demonstration of seminal microbiota paternal programming has yet to be reported.

The intricate interaction between paternal and maternal factors with external and environmental factors such as dietary intake in addition to the interaction with each other has been at the centre of research questions for a number of years. The increasing number of studies offering robust evidence both in animal models and humans has been instrumental in addressing how parental factors can influence and program offspring development, whether through epigenetic programming or nutritional availability through the placenta, or through the microbiome. *Figure 1.3* summarises and highlights the interconnectedness of these influences by visualising both paternal and maternal effects that ultimately

lead to altered offspring development. *Figure 1.3* also highlights the different elements of the current study as discussed in Chapters 3, 4 and 5.

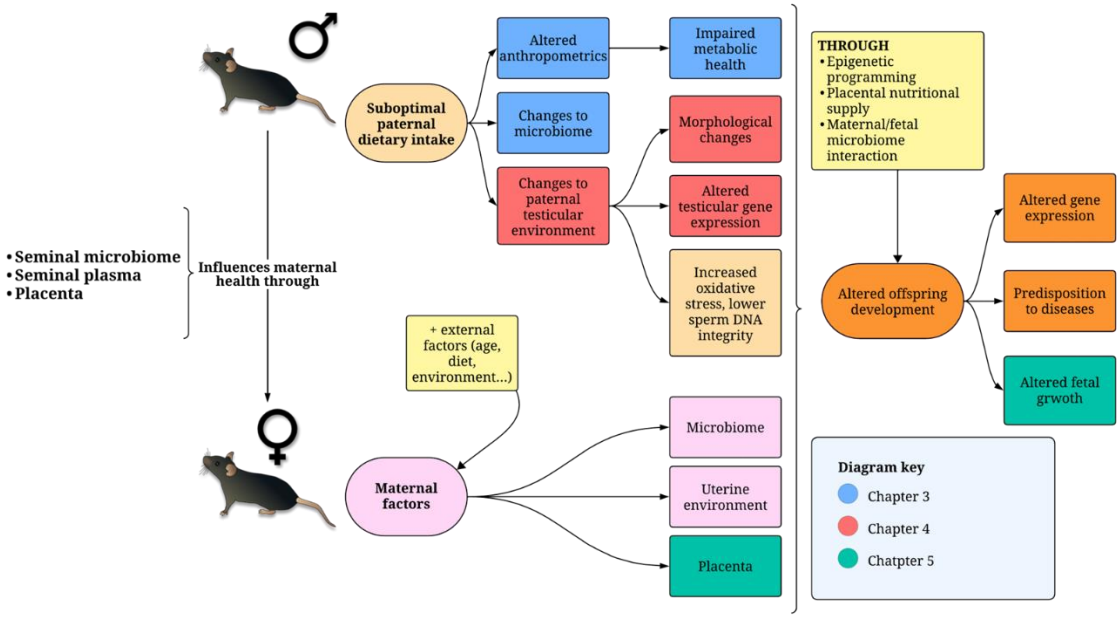

Figure1. 3: Summarised visual representation of paternal and maternal factors influencing offspring development as they are discussed in the current study.

Figure represents a visual guide on the interactions between both paternal and maternal factors discussed in addition to their relation to offspring development. As per the diagram key, colour-coded cells are discussed in their respective results chapters. Blue cells in the diagram are the subject of Chapter 3, red cells are the subject of Chapter 4, and green cells are the subject of Chapter 5.

1.9 RESEARCH OBJECTIVES

Over or undernutrition has been proven to play a significant role in shaping male reproductive fitness and subsequently offspring health. With regard to reproductive fitness, this study hypothesises that it is conceivable that behind any alteration in sperm quality, there are changes affecting the testicular environment itself. Furthermore, it is also possible that behind changes in the sperm or gut microbiome level there lie several other changes to an individual's physiology, highlighting the intricacies and interactions between different aspects of one's health. The mechanisms linking sub-optimal paternal diet and impaired testicular function are, however, not fully elucidated. Studies have shown that paternal diet influences embryogenesis as well as adult offspring development (Watkins et al., 2017; Watkins et al., 2018). Sub-optimal diet also influences sperm and seminal plasma, the latter of which has a significant impact on the maternal periconception environment, allowing uterine remodelling and embryonic implantation (Morgan and Watkins, 2020). This study also hypothesises that sub-optimal dietary intake could impair metabolic and reproductive fitness, and that supplementing those diets with methyl donors could potentially lessen the extent of damage that sub-optimal diets inflict. Therefore, we have used a C57BL/6 mouse model to help further our understanding of how sub-optimal diet can impact an individual's physiological, metabolic, and reproductive fitness through different feeding regimens including a low protein diet and a high fat, high sugar Western Diet. Additionally, we supplemented each of the two diets with key vitamins and minerals to help determine the impact of sub-optimal diet supplementation. Therefore, this study aims to:

- Characterise the impact of sub-optimal diet on adult male (paternal) growth profiles, adiposity, and gross organ morphology.
- Investigate the impact of sub-optimal diet on adult male gross testicular morphology and total gene expression.
- Study the effect of sub-optimal paternal diet on maternal gestational anthropometrics and fetal growth.
- Determine the role of key vitamin and nutrient supplementation, particularly supplementation of methyl donors, and its effectiveness when supplemented to low protein and high fat high sugar diets.

CHAPTER TWO: METHODOLOGY

2.1 Animal treatments

All animal procedures were carried out in accordance with the United Kingdom Home Office Animal (Scientific Procedures) Act 1986 (ASPA) regulations and approval from the Animal Welfare and Ethical Review Board (AWERB) at the University of Nottingham. Four batches of virgin 8-week-old male C57BL/6 mice (n=40/batch) and 5 to 9-week-old female C57BL/6 mice (Charles River) were maintained at the Bio Support Unit at the University of Nottingham on a 12-hour light: 12-hour dark light cycle over the course of the study. The batches were labelled from 1 to 4. Each batch of male mice contained an n number of 40, with 8 males per group. Over the course of 36 months in this study, four batches of males were maintained and underwent the same treatment. Male mice were randomly divided into five different groups (n=8/group in each batch). Each group was allocated either a control normal protein diet (CD; 18 % casein), an isocaloric low protein diet (LPD; 9 % casein), a methyl donor supplemented low protein diet (MDL; LPD with the addition of 5 g/kg choline chloride, 15 g/kg betaine, 7.5 g/kg methionine, 15 mg/kg folic acid, and 1.5 mg/kg vitamin B_{12}, a high fat, high sugar Western diet (WD; 21.4 % anhydrous milk fat, 50 % carbohydrate), or a methyl donor supplemented Western diet (MDWD; WD with the addition of 5 g/kg choline chloride, 15 g/kg betaine, 7.5 g/kg methionine, 15 mg/kg folic acid, and 1.5 mg/kg vitamin B_{12} (Special Dietary Services Ltd, UK) for a period of at least 7-8 weeks, ensuring that the duration on the diets covers all stages of spermatogenesis and spermiogenesis. The composition of all the diets fed to the stud males are listed in *Appendix A1*. All female mice had access to standard rodent chow (Teklad Global 18 % Protein Rodent Diet, Sterilizable,

2018S) and water *ad libitum*. The male mice were group housed for 12 weeks on average before being individually housed for weight recording and mating. Throughout the study, all males were individually weighed on a weekly basis by being taken out of their respective cages and placed onto a scale. Weight progression tracking was maintained throughout the study until time of sacrifice.

2.2 Mating

Virgin 8–9-week-old C57BL/6 females were housed singly overnight with either CD, LPD, MDL, WD, or MDWD stud males for mating. Vaginal plugs were checked the next day, and the presence of a vaginal plug was taken as a sign of successful mating. Females positive for plugs were maintained on regular chow *ad libitum* until late gestation (day 17, term being day 19) when they would be culled via cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986.

2.3 Tissue harvesting

Females were dissected after being weighed and having the uterus removed, weighed, and placed in a phosphate buffer solution (PBS) dish over ice. Blood was collected in 1.5 ml collection tubes (Eppendorf) and kept on ice until it was later centrifuged at 10,000 g for 10 minutes in an accuSpin Micro 17R centrifuge (Fisher Scientific) at 4º C. The serum was then pipetted out into a fresh 1.5 ml collection tube (Eppendorf) and stored at -80º C. Maternal liver, heart, and kidneys were collected and weighed. The apex of each of the maternal hearts was cut off with a transverse cut with a scalpel blade, and that section of the heart was fixed in 10 % formalin for future use. The remaining section was snap frozen in liquid nitrogen (LN2) before being stored at -80º C in a 2 ml nuclease-

free tube (Eppendorf). Portions of liver tissue from different lobes were also fixed in 10 % formalin (Sigma-Aldrich®, Poole, UK) for later histological analysis while the rest was snap frozen. One kidney (left) was fixed in 10 % formalin for histological use while the other (right) was snap frozen and was stored at -80º C in a 2 ml DNase/RNase-free tube (Eppendorf).

After the collection of maternal tissue, the uterus was cut open and the concepti were removed into labelled dishes. Fetuses were labelled based on their position on either uterine horn i.e *L1*, *L2*, *R1*, *R2* with *L* being the left horn and *R* representing the right horn. The numbers represent the position of the fetus in reference to the ovary i.e *L1* is the fetus closest to the ovary on the left horn, whereas *R1* is the closest fetus to the ovary on the right horn. Each conceptus was weighed before dissection. This weight included the weight of the fetus, yolk sac, and placenta. The yolk sac was then carefully cut open and the fetus was removed. Each component of the conceptus (fetus, yolk sac, placenta) was removed and weighed individually. Placentas were either snap frozen whole, fixed in 10 % formalin, or separated into the junctional or labyrinth zones to be collected individually in 0.5 ml nuclease-free tubes (Eppendorf) and snap frozen before being stored at -80º C for later use. Fetuses were also either fixed whole in 10 % formalin for histological work or dissected and snap frozen. Fetal heads were cut off and weighed, in addition to the weighing of hearts, kidneys, and livers. After being weighed, fetal hearts, kidneys, and livers were snap frozen before being stored at -80º C.

At the end of each study, the adult stud male mice were culled via cervical dislocation. After being weighed, the studs were dissected, and organs were weighed and collected. Similar to maternal hearts, the stud male heart was cut,

and the apex was fixed in formalin while the rest of the heart was snap frozen. Samples of the different liver lobes were also fixed in formalin while the remaining sections were snap frozen. For the testes and kidneys, one of each was either fixed in formalin for histological analysis (left) or snap frozen (right) before being stored at -80º C in a 2 ml DNase/RNase-free collection tube. Gonadal fat was collected and weighed from each of the males before being snap frozen. Inguinal, peri-renal, and interscapular fat pads were also collected from males in batch 4. Each of these fat pads was weighed before being collected in 2 ml tubes and snap frozen for later storage at -80º C. The collected seminal vesicles were cut at the base to allow for the collection of seminal fluid. The fluid inside was squeezed out of the vesicles using a pair of sterile forceps and collected in a 1.5 ml collection tube containing 250 µl PBS. The tube was then vigorously mixed to homogenise its contents before being weighed. The seminal vesicles were then weighed before being snap frozen. The seminal fluid was temporarily stored on ice for later processing. The blood was collected in 1.5 ml collection tubes and stored on ice before being centrifuged at 4° C and a speed of 10,000 g for 10 minutes. Serum was pipetted into new collection tubes and stored at -80° C for later use. Epidydimal sperm were collected using the swim-up method in M2 culture media (Sigma-Aldrich®), where sperm were left to swim up for 30 minutes in a 37º C incubator (Kendro BB 6220; Kendro Laboratory Products, Germany) after being pipetted out of epididymal tissue shredded under a dissection microscope. In batch 4, one epididymis per male was used to collect sperm, whereas the caudal region of the other epididymis was fixed in 10 % formalin and processed for histological analysis. For batch 4 males, stool pellets were also collected from the lower gut and were placed in a

2 ml DNase/RNase-free collection tubes and snap frozen before being stored at -80º C for later use. The experimental design is summarised in *Figure 2.1*. Fixed tissues were left in formalin overnight at room temperature (RT) before being washed three times in PBS and stored in 5 ml bijous containing 70 % ethanol.

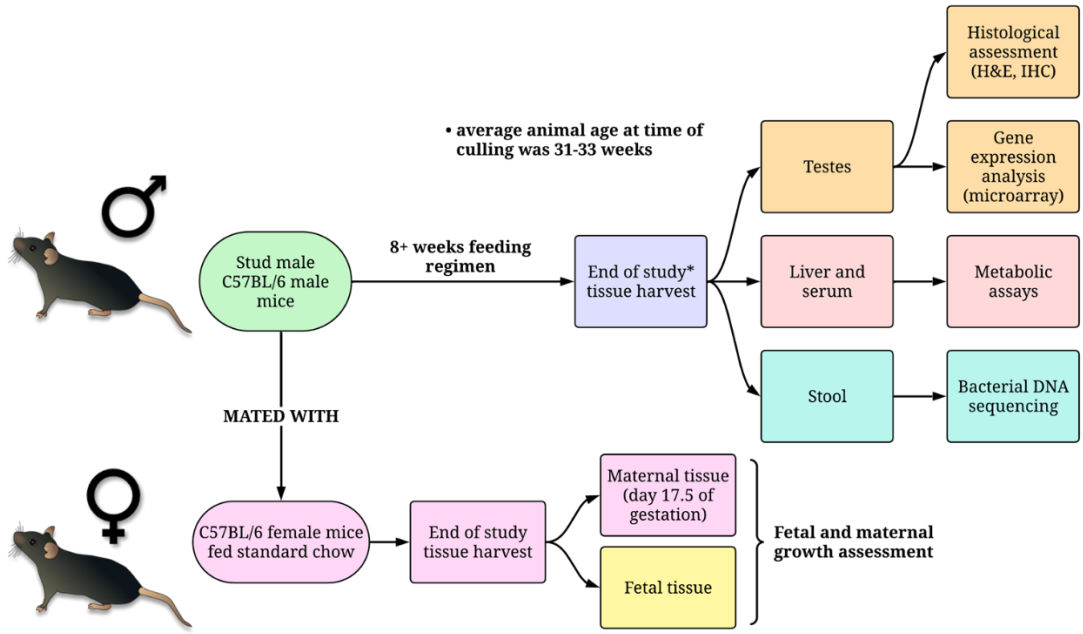

Figure 2.1: Summarised study experimental design. Adult C57BL/6 male mice fed the different experimental diets were mated with females fed a standard chow diet. At the end of the study, animals were culled, and tissue was harvested for either processing and histological analysis or DNA/RNA extraction or metabolite level assessment.

2.4 Histology

After being processed into paraffin blocks on a Shandon Excelsior automated tissue processor (Thermo Fisher), the testes and epididymis tissues (n=8/ group) were cut into 5 µm sections using a Leitz 1512 rotary microtome (Leica) and floated for 30-60 seconds at 40º C in an Electrothermal MH8515X1 Paraffin Section Mounting Bath (Bibby Scientific) containing distilled water. The sections were then mounted on SuperFrost Plus adhesion slides (Menzel-Glaser, Brauncshweig, Germany) before being incubated overnight at 45° C in a SLS OVE1210 30 L general purpose oven (Scientific Laboratory Supplies). Three sets of testis sections were generated for each sample, with each being used to assess a separate feature. One set was stained with hematoxylin and eosin (H&E) for the assessment of gross testicular morphology (see *section 2.4.1* for a detailed description of staining procedure), and two sets were prepared for staining by immunohistochemistry (IHC) (see *section 2.4.2* for full staining protocol details). From each embedded epididymis, sections were taken to be used to assess overall tissue morphology by H&E staining. The sections were floated out in the water bath for 60 seconds and then were left to dry on an Electrothermal MH6616 slide drying bench (Bibby Scientific, UK) at 40º C for 30 minutes before being incubated overnight at 45º C.

2.4.1 Hematoxylin & Eosin staining

For morphological assessment, the slides were stained with hematoxylin and eosin (H&E). The staining protocol was optimised for the type of tissue being used. The sections were de-waxed in xylene (Fisher Scientific) then rehydrated in decreasing concentrations of ethanol (100 %, 95 %, 70 %) (Honeywell). The slides were then stained with Haematoxylin (Sigma-Aldrich®) for 50 seconds

before being washed with running tap water for 3 minutes, followed by a 5-second dip in acid alcohol (1 % hydrochloric acid (HCL) in 70 % industrial methylated spirit (IMS) (Honeywell)) before being washed again in running tap water for 3 minutes. Following that, the slides were then stained with 0.1 % eosin (Acros Organics) for 2 minutes before being washed with tap water for 2 minutes. After staining, the slides were dehydrated with ethanol before being cleared with xylene, rendering the tissue transparent and suitable for microscopy. Dibutylphthalate Polystyrene Xylene (DPX) fixative (Sigma-Aldrich®) was used in mounting cover slips over the stained slides. The slides were left to dry overnight in a fume hood. The staining protocol is summarised in *Table 2.1*.

Procedure	***Solution***	***Time***
De-wax	Xylene I	3 minutes
	Xylene II	3 minutes
Rehydrate	100 % alcohol (IMS) I	3 minutes
	95 % alcohol (IMS) II	3 minutes
	70 % alcohol (IMS)	3 minutes
	Distilled water	3 minutes
H&E staining	Harris' Haematoxylin	50 seconds
	Running tap water	3 minutes
	Acid alcohol (1 % HCL in 70 % IMS)	5 seconds
	Scott's Tap Water	90 seconds
	Running tap water	3 minutes
	0.1% Eosin	2 minutes
	Running tap water	2 minutes
Dehydrate	95 % alcohol (IMS) III	2 minutes
	100 % alcohol (IMS) IV	2 minutes
Clear	Xylene III	2 minutes
	Xylene IV	3 minutes
Mount	Cover slip with DPX mounting medium	

Table 2.1: H&E staining protocol used for testis and epididymis sections.

2.4.2 Immunohistochemistry staining

A set of 5 µm testis sections were dewaxed using xylene as described in *Table 2.1* and rehydrated into PBS. Sections were then equilibrated at room temperature in 10 mM citrate buffer, which was diluted from a 0.1 M stock solution made from tri-sodium citrate (dihydrate) (Sigma-Aldrich®) and distilled water. The pH was measured and adjusted to 6.0 on a pH 212 Microprocessor pH meter (Hanna Instruments). Slide equilibration was carried out at room temperature for a duration of 5 to 10 minutes in an antigen retrieval chamber. After buffer equilibration, the chamber was microwaved four times for 5 minutes each on the 'high' setting. A volume of 50 ml of distilled water was added to the chamber between each microwave run, increasing the total volume in the chamber by 50 ml with every heating step to account for evaporation and water loss. The slides were then allowed to cool for at least 20 minutes at room temperature before being equilibrated in PBS for 5 minutes. After buffer equilibration, the slides were then kept hydrated in a PBS + 0.1 % TritonX-100 (Sigma® Life Science) (PBST) solution. A peroxidase-antiperoxidase (PAP) pen was used to isolate sections on the slides to allow for the pipetting of solutions without loss or cross-contamination. For each batch of immunostaining, a set of three controls was added to ensure experiment validity and the robustness of the results. Negative controls contained neither primary nor secondary antibodies and instead had 5 % Bovine Serum Albumin (BSA; Sigma® A7906) and 10 % normal goat serum (NGS; Sigma® G9023) in a PBST solution. The other two controls were the 'no primary control' which only had secondary antibodies applied to it, and a 'no secondary control' which only had primary antibodies applied. To avoid inconsistency and to reduce error and bias, the sets of samples

were run in two batches, where each batch included 4 samples from each diet group (n=4/group per batch, totalling n=8/group). Each batch had its own set of controls. Both biological and technical replicates were included in this experiment, as each sample within a batch was also run in duplicate on an individual slide.

To prevent non-specific binding of antibodies or other reagents to the tissue, 50 to 100 µl of a prepared blocking solution made up of 5 % BSA and 20 % NGS in a volume of PBST was pipetted onto each tissue for the duration of 1 hour at room temperature in a humidified chamber. After a 1-minute rinse in PBS, the appropriate volume of an anti-DDX4 antibody (mouse anti- DDX4/MVH (Vasa), ab27591 (Abcam®), 1:100) diluted in 5 % BSA and 10 % normal goat serum was added onto each section. The testis sections were dually stained for DDX4 and SOX-9 (i.e an antibody solution containing both primary antibodies was pipetted onto the samples before incubation). The staining for SOX-9 was carried out in a similar fashion to that of DDX4. Rabbit anti-SOX-9 Antibody (ab5535, Chemicon®) was diluted to a concentration of 1:500 in a 5 % BSA and 10 % NGS solution before being added onto the isolated sections. The slides were then incubated overnight at 4º C in a humid chamber. The 'no primary control' had the same solution as the rest of the samples, with the exception of primary antibodies for day 1 before overnight incubation. However, this control had the secondary antibodies applied for day 2 of the staining procedure. Inversely, the 'no secondary control' contained no secondary antibodies, but primary antibodies were pipetted onto control sections on day 1 of the protocol, and only 5 % BSA, 10 % NGS solution on day 2 after overnight incubation.

However, one limitation to this experiment's controls was the absence of a non-specific IgG control to further add an element of accuracy and control.

On day 2, the slides were washed twice in PBST with each wash lasting 5 minutes. As detailed in *section 2.4.*2, the secondary antibody Alexa Fluor® 568 nm (RED) Goat anti-mouse IgG1 (A-21124, Invitrogen™) for the anti-DDX4 primary antibody was diluted to a 1:200 concentration in a solution of 5 % BSA and 10 % NGS. In addition, a polyclonal goat anti-rabbit biotinylated antibody (E0432, DakoCytomation) for the anti-SOX-9 antibody was added at a concentration of 1:200 in a solution of 5 % BSA and 10 % NGS. The slides were incubated at room temperature for 1 hour, protected from light. After incubation, the slides were washed twice in PBST for 5 minutes each. The secondary antibody Alexa Fluor 488 nm (GREEN) Streptavidin conjugate (Invitrogen™, S-32354) was applied to the slides with SOX-9 at a concentration of 1:200 for 30 minutes at room temperature before undergoing two more 5-minute PBST washes. Finally, the slides were mounted with Antifade Mounting Media with DAPI (Vectashield®) before being covered with glass coverslips and sealed with transparent nail polish. The slides were then allowed to dry and were refrigerated overnight before use. In order to prolong the slides' shelf life, the slides were frozen at -20° C after use.

For the detection of PLZF in the testis, the staining had to be run separately and not dually with SOX-9 in order to avoid antibody cross-reactivity, since both primary antibodies were raised in the same species (rabbit). A different set of testis sections was used with a rabbit polyclonal Anti-PLZF antibody - N-terminal (Abcam®, ab189849) as a primary antibody at a concentration of 1:3000 in 5 % BSA and 10 % NGS. After overnight incubation at 4º C, an Alexa

Fluor® 568 nm goat anti-rabbit IgG Heavy & Light chains (Abcam®, ab175471) secondary antibody was used at a concentration of 1:200. The staining protocol was carried out for SOX-9 and PLZF as it was for DDX4. The staining of PLZF was carried out in two batches, with each batch having its own set of 3 controls as previously described.

2.4.3 Imaging

Tissue sections were blinded using masking tape and were assigned random numbers to minimise bias before imaging and analysis. Imaging for H&E sections was conducted on a Leica DMRB microscope, which enabled the scanning of an entire slide to create high resolution images at 20x magnification. Image analysis was carried out using the open-source image processing package Fiji. For each testis sample, an average of 60 seminiferous tubules cross-sections were analysed. Samples with a total number of tubules lower than 50-60 were entirely counted. Each sample was divided into quadrants where tubules were randomly counted and measured. This was done systematically across all samples to maintain consistency. Regular-shaped tubules (i.e round, non-elongated) were analysed for total tubule area (µm²), total perimeter (µm), lumen area (µm²), epithelial area (µm²), and finally the lumen percentage (%). The area of the epithelium was calculated by the subtraction of the lumen area from the total tubular area. Furthermore, the lumen percentage was worked out from the ratio of lumen area over the total area ((lumen area/total area)*100). *Figure 2.2* represents an example image highlighting the measured parameters. After imaging and measurement using Fiji, the slides were then unblinded and the data was collated for statistical analysis.

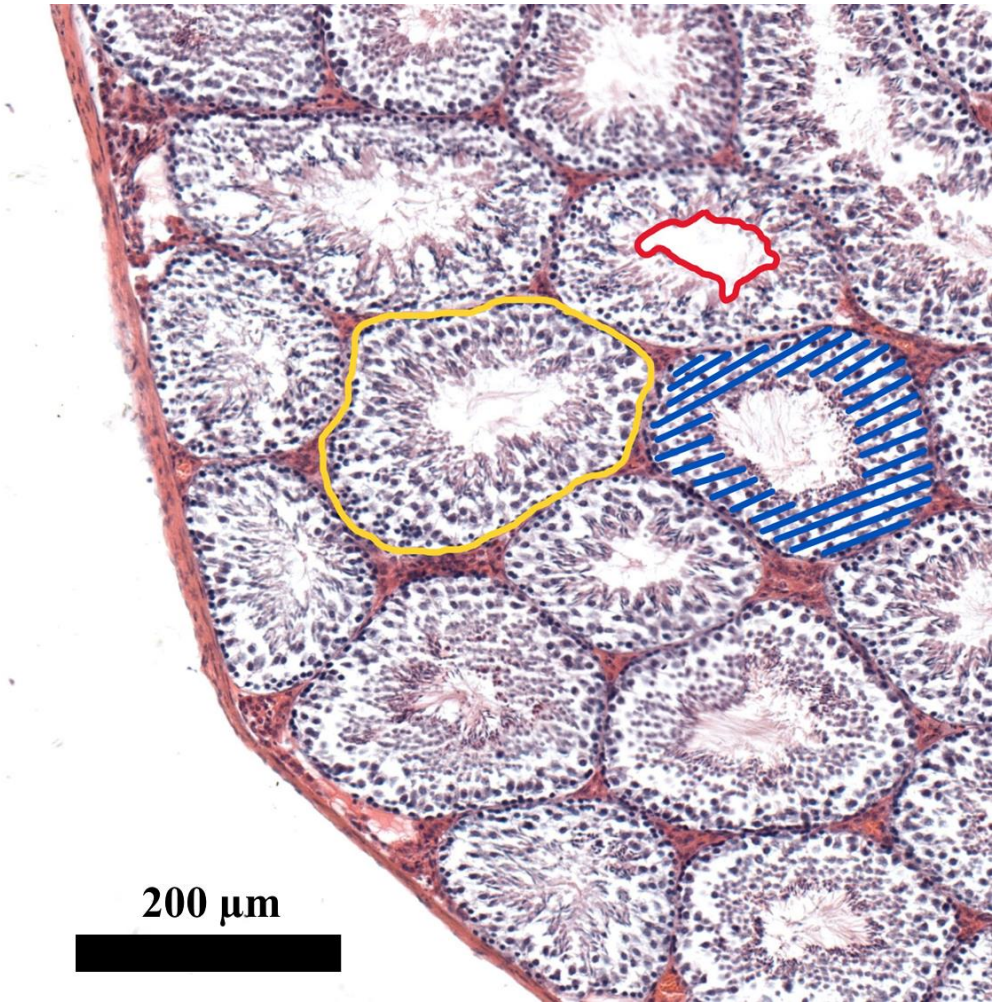

Figure 2.2: Representative H&E-stained image of a testis tissue section highlighting parameters measured: total tubule area (yellow), lumen area (red), and epithelial area (blue).

For the analysis of testicular SOX-9, DDX4 and PLZF expression, sections were viewed and imaged using a Nikon Eclipse 90i fluorescent microscope with the mercury-fiber illuminator Nikon Intensilight C-HGFI and the Hamamatsu ORCA-ER Digital camera Model C4742-80. Images were taken at 10x magnification, capturing an average of about 5-6 tubules per field. Closeups were taken at 20x magnification, capturing about 2-3 tubules per field. Image

viewing and exposure was captured using Volocity® software. Images were captured on three channels, both separately and merged with their respective exposure settings: DAPI (Blue; 200 ms), FITC (Green; 220 ms), and TxRed (Red; 150 ms). To retain consistency, all images of all samples were taken using the above exposure settings.

Grid imaging was carried out, where the stage was moved following a pattern to ensure images of the entirety of each tissue were taken. Landmark tubules and artifacts were used to mark cut-off points in a microscopic field and to avoid imaging the same tubules. In case of duplicate tubules across multiple images, the tubules were analysed once and excluded from subsequent analysis. Images from each grid were generated and saved for analysis. The image analysis was done using Fiji. Seminiferous tubule area was averaged per field for IHC analysis to maintain consistency in quantification. In addition to tubule areas, the number of fluorescent cells per tubule was counted using the software's cell counter as well as the colour threshold and particle counting features, with the exception of SOX-9 positive cells and PLZF positive cells, which were counted manually. Total numbers of SOX-9 positive cells, DDX4-positive cells and PLZF-positive cells were counted and analysed for cell distribution across tubules, tubule size, as well as positive cells in relation to each other. Representative images are showcased in *Figures 2.3* and *2.4*.

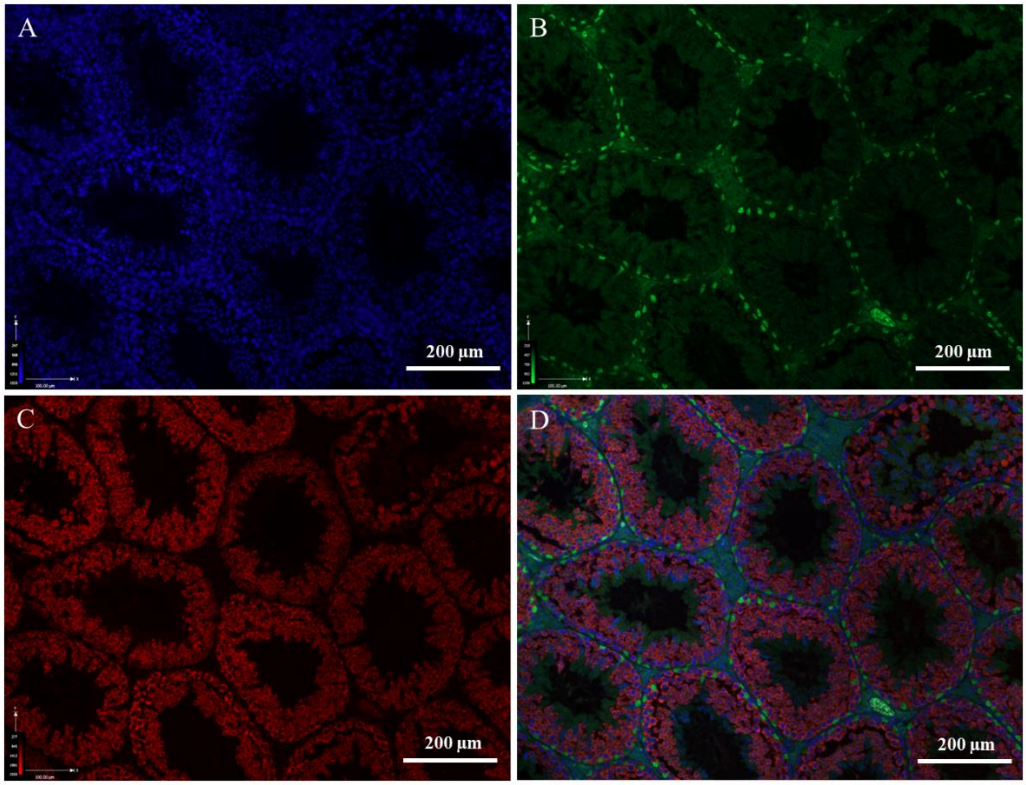

Figure 2.3: Representative images of DDX4/SOX-9 dual staining with a DAPI counterstain with their respective channel. Images represent DAPI-positive nuclear staining (DAPI (blue); A), positive staining for Sertoli cells with anti-SOX-9 (FITC (green); B), positive staining of spermatogenic cells with anti-DDX4 (TxRed (red); C), and a merged image of all three channels (D).

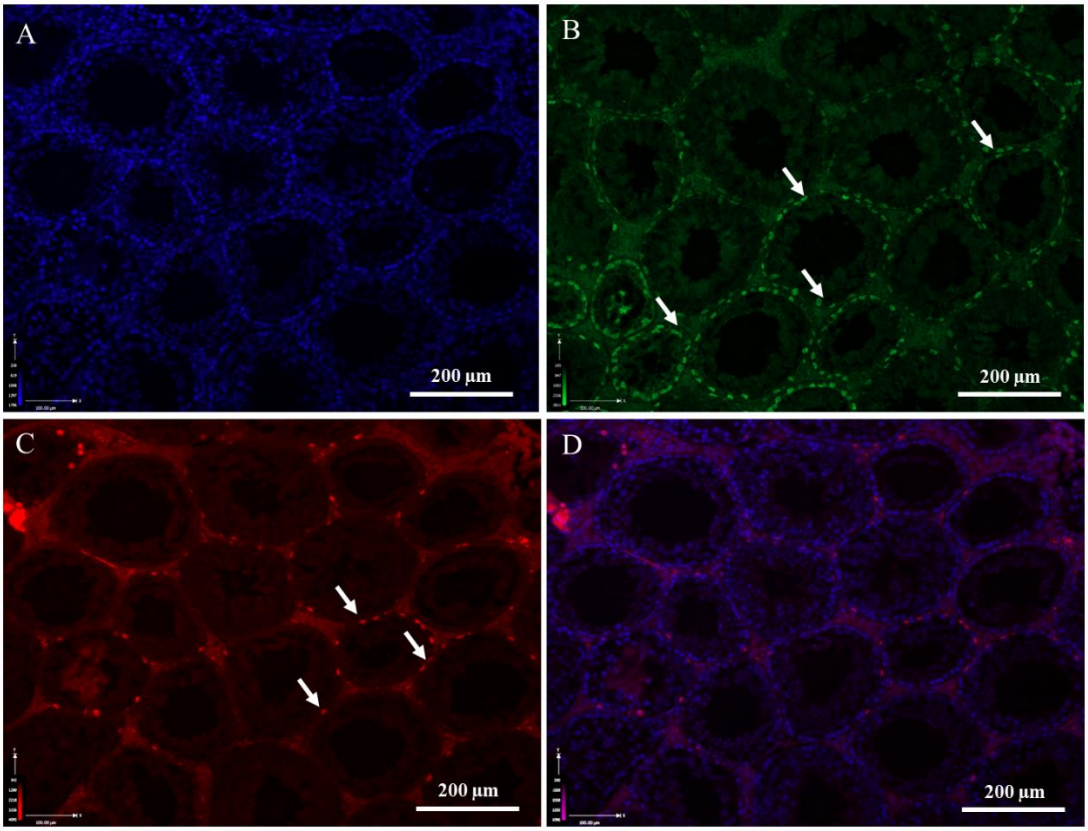

Figure 2.4: Representative images of anti-PLZF/SOX-9 staining with a DAPI counterstain with their respective channel. Images represent DAPI-positive nuclear staining (DAPI (blue); A), positive staining for Sertoli cells with anti-SOX-9 (FITC (green); B), positive staining of spermatogonial stem cells with anti-PLZF (TxRed (red); C), and a merged image of DAPI and PLZF-positive cells (merged; D). Arrows indicate Sertoli cells (B) and PLZF-positive cells (C), respectively.

2.5 RNA extraction

Total RNA was extracted from frozen testicular tissues using the RNeasy® Plus Mini Kit (QIAGEN®) in accordance with the manufacturer's instructions. Testes were cut into the desired weight (15-25 mg) in a mortar over dry ice and using a clean scalpel blade. The samples were transferred into 2 ml Eppendorf collection tubes with the appropriate volume of the Buffer RLT Plus (600 µl for samples ≥ 20 mg, with anti-foaming Reagent DX (0.5 % v/v) and one 5mm stainless steel ball (QIAGEN®) in each tube. The tissues were disrupted at 30 Hz using the QIAGEN TissueLyser for 1 minute. The tubes were then centrifuged for 3 minutes at full speed at room temperature (RT) to sediment any unlysed debris. Each homogenised lysate was transferred to a gDNA Eliminator spin column in a 2 ml collection tube provided by the manufacturer, which was subsequently centrifuged for 30 seconds at ≥ 8000 *g*. After centrifugation, the column was discarded, and 1 volume of 70 % ethanol was added to the flow-through and mixed by pipetting. Up to 700 µl of each sample was transferred to RNeasy spin columns in supplied 2 ml collection tubes. The tubes were centrifuged for 15 seconds at ≥ 8000 *g* at RT. The flow-through was discarded and 700 µl of Buffer RW1 was added into each spin column to be centrifuged for 15 seconds at ≥ 8000 *g* at RT. The flow-through was again discarded and 500 µl of Buffer RPE was added to the spin columns followed by centrifugation for 15 seconds at ≥ 8000 *g*. This step was repeated after discarding the flow-through and the tubes were centrifuged for 2 minutes at ≥ 8000 *g* at RT. After discarding the flow-through, the RNeasy spin columns were placed in new 2 ml collection tubes and were centrifuged at full speed for 1 minute in order to further dry the spin column membranes. The RNeasy spin columns were placed

in new 1.5 ml collection tubes provided by the manufacturer and labelled for each sample. A volume of 30 µl of RNase-free water (QIAGEN®) was added directly to the spin column membrane of each tube, followed by centrifugation for 1 minute at ≥ 8000 *g* at RT to elute the RNA. The flow-through was collected and 1 µl of RNA was used to measure total RNA concentration and purity ratios using the NanoDrop™ Spectrophotometer (Lab Tech). The labelled RNA tubes were then stored at -80° C to be used for cDNA synthesis.

2.6 Microarray

Samples of testes RNA were diluted to 100 ng/µl using the same RNAse-free water used in the elution step in *section 2.5* and made into 20 µl aliquots for microarray preparation. The samples were taken to the Nottingham Arabidopsis Stock Centre (NASC; School of Biosciences, Sutton Bonington Campus), where the RNA was quality control checked. RNA integrity was assessed using microfluidic analysis via an Agilent Bioanalyser 2100 (Agilent), using the RNA Integrity Number (RIN) for evaluation of RNA integrity (Agilent). The average RIN for the RNA samples sent for Microarray analysis was 9.91. GeneChip™ WT PLUS Reagent Kit Manual Target Preparation for GeneChip™ Whole Transcript (WT) Expression Arrays (ThermoFisher Scientific), a reverse transcription priming method, was used to generate a complete transcriptome coverage. First-strand cDNA was synthesised from the extracted RNA using Reverse Transcriptase (RT), followed by a second-strand cDNA synthesis using DNA Polymerase and RNAse H. Complementary RNA (cRNA) was synthesised by in vitro transcription (IVT) of the second-strand cDNA using T7 RNA polymerase. This method is based on the RT-IVT or Eberwine method (Van Gelder et al., 1990). cRNA was then purified by allowing the samples to

incubate with and bind to a volume of Purification Beads (Thermo Fisher) before being placed on a magnetic stand in order to capture the Beads. The supernatant is then discarded carefully. The Purification Beads are then washed twice with an 80 % ethanol wash before being air-dried on a magnetic plate. The cRNA is then eluted using 27 µl of the provided nuclease-free water that is preheated to 65º C. The tubes are then mixed by pipetting before being placed on the magnetic plate to capture the Purification Beads. The supernatant, now containing the purified cRNA is transferred into a provided nuclease-free tube for each sample and placed on ice in preparation for the 2nd cycle single-strand cDNA synthesis by reverse transcription of cRNA. The cRNA template was then hydrolysed using RNase H leaving only a single-strand cDNA (ss-cDNA). In preparation for fragmentation and labelling, the cDNA strand was purified in order to remove salts, enzymes and unincorporated dNTPs using the same method used to purify the cRNA but with the elution in a volume of 30 µl of nuclease-free water. The purified ss-cDNA is fragmented by uracil-DNA glycosylase (UDG; provided) and apurinic/apyrimidinic endonuclease 1 (APE 1; provided) at the unnatural Deoxyuridine Triphosphate (dUTP) residues and breaks the DNA strand. The fragmented cDNA is labelled by terminal deoxynucleotidyl transferase (TdT) using the provided DNA Labelling Reagent that is covalently linked to biotin. After labelling with biotin, the strand is hybridised to DNA probes on Clariom™ S Assay Mouse GeneChip™ (ThermoFisher Scientific).

2.7 Stool DNA extraction

Stool pellets were collected from stud male mice at time of sacrifice by the removal of the lower gut stool pellets. These stool samples were stored in DNase/RNase-free 2 ml tubes (Eppendorf) at -80° C until ready for use. For the

extraction of DNA from the stool pellets, a QIAamp DNA Stool Mini Kit (QIAGEN®) was used in accordance with the manufacturer's instructions. Samples were heated at 70° C in a digital dry bath (Benchmark Scientific) prepared with 1.5 ml inlays in order to heat up the samples. All centrifugation steps were performed at room temperature at approximately 14,000 rpm. Approximately 180-220 mg of sample tool were placed into a 2 ml tube (Eppendorf). Upon the addition of 1 ml of the provided InhibitEX Buffer, the tubes were vortexed continuously until the samples were thoroughly homogenised. The suspension was then heated for 5 minutes at 70° C in a dry bath. For samples that were difficult to lyse, the temperature was increased to 95° C as per the manufacturer's suggestion. The tubes were then vortexed after heating and centrifuged to pellet stool particles. Into new 1.5 ml tubes, 45 µl of the supplied proteinase K was added, followed by 600 µl of supernatant from the centrifuged tube. 600 µl of buffer AL was added to the 1.5 ml tubes and vortexed to form a homogeneous solution. The tubes were then incubated for 10 minutes at 70º C. After incubation, 600 µl of ethanol (96-100 %) was added to the lysate and vortexed to mix. 600 µl of the lysate was transferred to a QIAmp spin column and centrifuged for 1 minute. The step was repeated until all the lysate was centrifuged through the spin column, after which the column was placed in a new 2 ml collection tube. The filtrate was discarded. After that, 500 µl of Buffer AW1 was added to the spin column and the tubes were centrifuged for 1 minute. 500 µl of Buffer AW2 was added and centrifuged for 3 minutes with the filtrate tube being discarded. The spin columns were transferred to new 2 ml collection tubes and were centrifuged for 3 minutes to get rid of the remaining buffer in the spin columns. Finally, 50 µl of buffer ATE was added

directly onto the membrane of each of the tubes to elute the stool DNA. The tubes were left to incubate at room temperature for 1 minute before centrifugation for a duration of 1 minute and assessment of purity and concentration using Nanodrop.

Ethanol precipitation was carried out for samples that recorded sub-standard Nanodrop ratios. A volume of 1 µl carrier Invitrogen™ UltraPure Glycogen (Fisher Scientific) was added to the nucleic acid solution mixed well before a 1:10 volume of 3 M sodium acetate (Sigma-Aldrich®) was added (i.e., 100 µl eluted DNA = 10 µl 3 M sodium acetate). After mixing thoroughly, 2.5 volumes of 100 % ethanol were added. Following that, the tubes were mixed thoroughly and incubated overnight at -80° C.

After overnight incubation, the tubes were centrifuged at maximum speed for 15 minutes at a temperature of 4° C. The supernatant was then carefully removed and discarded. The residual pellet was washed by adding 500 µl of ice cold 70 % ethanol carefully down the side of each tube after which they were centrifuged at maximum speed for 10 minutes at 4° C (or room temperature), before the 70 % ethanol was carefully removed and discarded. This step was repeated a second time before allowing the pellet to air dry for 5 minutes on the bench. A volume of 30-50 µl of elution buffer was added to each tube to dissolve the pellets. The tubes were then mixed, and the purity ratios and concentrations were double-checked using Nanodrop before final storage at -20° C.

2.8 Microbiome DNA sequencing

Extracted stool DNA samples were sent to the sequencing facility DeepSeq at the University of Nottingham where they underwent a 16S sequencing for

bacterial species using the Illumina MiSeq Reagent kit v3 on the Illumina MiSeq Platform (Illumina). A polymerase chain reaction (PCR) was performed to amplify the V3-V4 region of the 16S rDNA gene followed by first stage PCR clean-up, quantification, indexing PCR, further clean-up, and library prep quality control. The 30 samples were then pooled and prepared for sequencing along with a set of positive and negative controls. The samples were then run over a shared 300 paired end (PE) MiSeq run in order to deliver about 60-80,000 PE reads per sample. The sequencing run additionally had a 20 % PhiX library spike-in as an internal quality control. PhiX is a bacteriophage with a balanced genome composition and is often used in Illumina runs as a DNA control and as a way to balance the base composition (Illumina.com, 2023). Data analysis was performed by DeepSeq using the Qiime2 tool, a next-generation microbiome bioinformatics platform (qiime2.org).

2.9 Assays

2.9.1 Cholesterol Quantification

The quantification of total cholesterol in stud male liver tissue was carried out using a Cholesterol Quantification Kit (MAK043, Sigma-Aldrich®) according to the manufacturer's protocol. Samples were run in duplicates in a 96-well plate in addition to standards and blanks. Reagents were brought to room temperature prior to use. A volume of 20 µl of the provided 2 µg/µl Cholesterol standard was diluted with 140 µl of the provided Cholesterol Assay Buffer in order to prepare a 0.25 µg/µl standard solution. Respective volumes of 0, 4, 8, 12, 16, and 20 µl of the standard solution were added to the 96-well plate, thereby generating a 0 (blank), 1, 2, 3, 4, and 5 µg/well standards. The appropriate volume of Cholesterol Assay Buffer was added to bring each of the wells to a 50 µl.

In 2ml Eppendorf tubes, a volume of 200 µl of chloroform (Fisher Scientific): isopropanol (Fisher Scientific): IGEPAL CA-630 (Sigma-Aldrich®) in a ratio of 7:11:0.1 was added to 10 mg of frozen liver tissue. The samples were then lysed in a QIAGEN Tissuelyser II (QIAGEN®) for a duration of 30-60 seconds. The samples were then centrifuged at 13,000 *g* for 10 minutes to allow the removal of insoluble material. The organic phase of each tube was then transferred to a new tube and was left to air dry for 5 minutes at a temperature of 50° C to remove chloroform. The pellet was then discarded, and the samples were placed under a Techne Dri-Block DB-3 sample concentrator for 30 minutes in a fume hood to remove residual organic solvent. The remaining dried lipids were then dissolved with 200 µl of the Cholesterol Assay Buffer and were mixed until homogenous using a vortex mixer. A volume of 30 µl of the sample was brought to a total volume of 50 µl by diluting it with 20 µl of Assay Buffer before use in the assay. The samples were assigned duplicate wells in the plate at random in order to minimise any bias (see *Appendix section A3* for plate map).

Assay Reaction

The reactions were set up in accordance with the manufacturer's instructions, with 50 µl of Reaction Mix being required for each well. The plate was covered and mixed on a horizontal shaker. The plate was then incubated at 37° C for 60 minutes. After incubation, absorbance was measured at 570 nm using an iMark™ microplate reader (Bio-Rad). The value of the Blank (0) wells was then subtracted from the value of each of the other wells to correct for background absorbance. Using the values obtained from the appropriate cholesterol standards, a standard curve was plotted from which the concentration of cholesterol in each sample was calculated.

2.9.2 Free Fatty Acid (FFA) Quantification)

The concentration of Free Fatty Acids (FFA) was determined by a coupled enzyme assay which results in a colorimetric product that is proportional to the amount of FFA present. The Free Fatty Acid Quantitation Kit (MAK044, Sigma-Aldrich®) was used to determine the concentration of FFA in adult male mouse liver samples. The Fatty Acid Probe and Assay Buffer were allowed to come to room temperature prior to use. The provided Acyl-CoA Synthetase (ACS) Reagent and Enzyme Mix were each reconstituted with 220 µl of Fatty Acid Assay Buffer. The vials were mixed well by pipetting and were set aside for later use, protected from light. The Palmitic Acid Standard vial provided was placed in a 90° C bath for 1 minute to redissolve its constituents. Once the standard was clear in colour, it was heated and vortexed a second time before use. A volume of 0, 2, 4, 6, 8, and 10 µl of the standard solution was added (in duplicate) into a 96-well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well standards. The appropriate volume of Assay Buffer was added to each well to bring the total volume to 50 µl.

Adult male mouse liver tissues (weighing 10 mg) in 2 ml collection tubes (Eppendorf) were homogenised in 200 µl of a 1 % Triton X-100 in chloroform solution. The tubes were then centrifuged at 13,000 *g* for 10 minutes to remove any insoluble material. The organic (lower) phases were collected and air dried at 50° C to remove chloroform. The samples were then placed in the Techne Dri-Block DB-3 sample concentrator to remove trace chloroform. The dried lipids were then dissolved in 200 µl of Fatty Acid Assay Buffer by vortex mixing for 5 minutes. A volume of 30 µl of each sample was added into the 96-well plate in duplicates. The final volume was brought to 50 µl by the addition of

Assay Buffer. The samples were assigned duplicate wells in the plate at random in order to minimise any bias (see *Appendix section A3* for plate map).

Assay Reaction

A volume of 2 µl of the provided ACS Reagent was added to each sample and standard well, and the plate was incubated at 37° C for 30 minutes. The Master Reaction Mix was then prepared in accordance with the manufacturer's instructions. A volume of 50 µl of the Master Reaction Mix was added to each of the wells. The plate was then mixed using a horizontal shaker and was incubated for 30 minutes at 37° C with aluminium foil cover to protect from light. The absorbance was then measured at 570 nm. As with the Cholesterol and TG assays, the value obtained for 0 (blank) was subtracted from the other readings to correct for background absorbance. The values obtained from the appropriate palmitic acid standards were used to plot a standard curve form which the concentration of FFA in the samples could be determined.

2.9.3 Triglyceride (TG) Quantification

The Triglyceride Quantification Kit (MAK266, Sigma-Aldrich®) was used to measure the levels of triglycerides in stud male liver tissue samples. In this assay, Triglycerides were converted to free fatty acids (FFA) and glycerol. The glycerol is then oxidised, generating a colorimetric product (measured at 570 nm). Samples were run in duplicates in a 96-well plate in addition to standards and blanks. The Triglyceride Assay Buffer was allowed to come to room temperature prior to the start of the experiment. The triglyceride probe was warmed in a 37° C dry bath for 1-5 minutes to melt the solution prior to use. The supplied Triglyceride Enzyme Mix and Lipase were each reconstituted with 220

µl of the Triglyceride Assay Buffer and mixed well by pipetting. The Triglyceride Standard vial was placed in a 90° C bath for 1 minute to redissolve its contents before vortex mixing for 30 seconds until the cloudy solution becomes clear. The heating step was repeated a second time before the Triglyceride Standard Solution was ready for use as instructed by the manufacturer's guidelines. To prepare the standards, 40 µl of the 1 mM supplied Triglyceride Standard was diluted with 160 µl of Triglyceride Assay buffer to prepare a 0.2 mM standard solution. Respective volumes of 0, 10, 20, 30, 40 and 50 µl of the 0.2 mM solution were added into a 96-well plate, generating a 0 (blank), 2, 4, 6, 8 and 10 nmol/well standards. An appropriate volume of Triglyceride Assay Buffer was added to each well, bringing the total volume to 50 µl.

Each adult male mouse liver tissue weighed 100 mg and was homogenised in 1 ml solution of 5 % Nonidet® P 40 Substitute (nonionic, non-denaturing detergent) (Sigma-Aldrich®) in a QIAGEN Tissuelyser II (QIAGEN®). Each sample tube was then slowly heated to an average temperature of 90° C in a dry bath for 2-5 minutes or until the detergent became cloudy in the solution. The tubes were then cooled to room temperature. The process was repeated a second time to solubilise all triglycerides. The sample tubes were then centrifuged at maximum speed for 2 minutes to remove any insoluble material before being diluted 10-fold with water prior to use in the assay.

A sample volume of 30 µl of was added into duplicate wells in the 96-well plate. The samples were assigned duplicate wells in the plate at random to minimise any bias (see *Appendix section A3* for plate map). The final volume for each sample well was brought to 50 µl by the addition of Triglyceride Assay Buffer.

Sample and standard wells were also treated with 2 µl lipase to convert the triglyceride into FFA and glycerol. The plate was incubated for 20 minutes at room temperature to allow for this step to occur. In addition, the 0 (blank) standard wells, a sample background control was performed by replacing 2 µl of Lipase with 2 µl of Triglyceride Assay Buffer and its value was then subtracted from all readings in accordance with the manufacturer's instructions.

Assay Reaction

The Reaction Mix was set up according to the manufacturer's instructions, with 50 µl of the Master Reaction required per well. A volume of 50 µl of the Master Reaction Mix was added to each sample, standard, and background control well containing the Triglyceride standard. The plate was then put on a horizontal shaker to homogenise the added Reaction Mix and the samples. The plate was then incubated for 45 minutes at room temperature with an added later of aluminium foil to protect from light. After incubation, the absorbance was measured at 570 nm. The value obtained for the 0 (blank) was subtracted from all other readings to correct for background absorbance. Furthermore, the Sample Background Control was subtracted from all sample readings. Using the values obtained from the Triglyceride standards, a standard curve was plotted. Using the values obtained from the standard curve, the concentration of Triglycerides in each sample could be determined.

2.9.4 Glucose Assay

For the quantification of Glucose in adult male mouse serum, the Glucose Colorimetric Detection Kit (EIAGLUC, Thermo Fisher Scientific) was used. Serum samples were diluted to a ratio ≥ 1:15 with the provided Assay Buffer as

recommended by the manufacturer. Standards were prepared by adding a volume of 15 µl Glucose Standard to a tube containing 135 µl Assay Buffer and labelling as 32 mg/dl glucose. A volume of 75 µl Assay Buffer to each of 7 tubes labelled 16, 8, 4, 2, 1, 0.5, and 0 mg/dl glucose, respectively. The standard was then serially diluted and mixed thoroughly between steps.

Assay Reaction

A 1x HRP solution was prepared by diluting the provided Horseradish Peroxidase (HRP) Concentrate (100x) 1:100 with Assay Buffer. For a full 96-well plate, 30 µl of the concentrate was diluted with 2.97 ml of Assay Buffer, totalling a volume of 3 ml. In addition, a 1x Glucose Oxidase solution was prepared by diluting the provided Glucose Oxidase Concentrate (10x) 1:10 with Assay Buffer. For a full 96-well plate, 275 µl of concentrate with 2.475 ml of Assay Buffer, totalling a volume of 2.75 ml.

Serum samples were diluted 1:20 with Assay Buffer prior to use in the assay. A volume of 20 µl of standards and diluted samples were added in duplicate to the appropriate wells (see *Appendix section A3* for plate map). A volume of 25 µl of 1x HRP solution was then added to each well. The substrate was afterwards added (25 µl) into each well, followed by 25 µl 1x Glucose Oxidase per well. The plate was then incubated for 30 minutes at room temperature.

The plate was then read at 560 nm on the iMark™ microplate reader (Bio-Rad) and the values obtained for the blank wells were subtracted from the rest of the readings and a standard curve was plotted using the values obtained for the glucose standards. The sample concentrations for were determined using the

standard curve. The values obtained for the samples were then multiplied by the appropriate factor to correct for the dilution factor.

2.9.5 Insulin Quantification

The quantification of Insulin was carried out using a Rat/Mouse Insulin ELISA Kit (Millipore®) according to manufacturer's instructions. All reagents were pre-warmed prior to the assay. The provided 10x Wash Buffer was diluted ten-fold with 900 ml deionised water before setting up the ELx50 automatic plate washer (BioTek). Plate strips were assembled in a plate holder and each well was washed 3 times (300 µl Wash buffer per wash). The plate was then decanted and gently tapped on a paper towel without letting the plate dry out. A volume of 10 µl Assay Buffer was added to the Blank and Sample wells followed by 10 µl of the provided Matrix Solution to the Blank, Standard, and Sample wells as instructed in the manufacturer's protocol. A volume of 10 µl Rat Insulin Standards were added in duplicate to each of the standard wells in an ascending concentration gradient. A volume of 10 µl of QC1 and QC2 (provided rat insulin in Quality Control buffer) was added to the appropriate wells followed by 10 µl each of the samples in duplicate to the remaining wells (see *Appendix section A3* for plate map). A volume of 80 µl of the provided Detection Antibody was added to each well and the plate was covered and left to incubate at room temperature for a duration of two hours on a horizontal shaker.

After incubation, the plate sealer was removed, and the contents were decanted from the plate. The plate was then gently tapped on a paper towel. The plate was afterwards washed 3 times (300 µl Wash Buffer per wash), prior to the addition of 100 µl of the provided Enzyme Solution to each well. The plate was covered and left to incubate for 30 minutes at room temperature. After the second

incubation, the contents of the plate were decanted out and the plate was tapped dry on a paper towel before undergoing 6 washes (300 µl Wash Buffer per wash). A volume of 100 µl of the provided Substrate Solution was added to each well. The plate was covered and shaken on a horizontal shaker for a duration of 5 to 20 minutes (until a blue colour fully develops in the standard wells). Once the colour fully developed, 100 µl of the provided Stop Solution was added to each well, immediately stopping the development of the reaction and turning the blue colour yellow after acidification. The plate was then read at 450 nm and 595 nm within 5 minutes and the difference of absorbance units was recorded. The results of the unknown samples were calculated using the plotted standard curve, where the difference in absorbance between 490 nm and 590 nm on the Y-axis and the standard concentrations on the X-axis. The assay would be considered accepted when the QC values fell within the QC Range provided by manufacturer.

2.9.6 TNF-α Quantification

The Mouse TNF alpha SimpleStep ELISA® Kit (ab208348, Abcam®) was used for the quantitative measurement of TNF-α in adult male mouse serum. Following the manufacturer's instructions, all reagents were equilibrated to room temperature prior to use. The reagent volumes stated in the protocol were sufficient for 48 wells. However, the provided reagents in the kit were sufficient for a full plate. Therefore, the volumes were appropriately adjusted for a 96-well plate setup.

Sample diluent 25BS was prepared by diluting 5.5 ml of the provided Sample Diluent NBS with 16.5 ml of the Sample Diluent NS (also provided), totalling a volume of 22 ml (1 in 4). Sample Diluent 10BS was prepared by diluting 2.2 ml

of the provided Sample Diluent NBS with 19.8 ml of the provided Sample Diluent NS (1 in 10), totalling 22 ml of Sample Diluent 10BS. A volume of 200 ml of 1x Wash Buffer PT was prepared by diluting 20 ml of 10x Wash Buffer (provided) with 180 ml of deionised water. Finally, the Antibody Cocktail was prepared by diluting 650 µl each of the 10x Capture and the 10x Detector antibodies (provided) in 5.2 ml of the Antibody Diluent CPI, totalling a volume of 6.5 ml (1 in 10).

For the preparation of 10,000 pg/ml stock standard, TNF-α Standard was reconstituted by adding 1ml of Diluent 25BS. The vial was held at room temperature for 10 minutes and was gently mixed. Eppendorf tubes were labelled 1 to 8, and 240 µl of Diluent 25BS into Tube 1 and 150 µl of Diluent 25BS into the remaining tubes. The Stock standard was used to prepare the dilution series.

Assay Procedure

All reagents were brought to room temperature prior to use. Serum samples were diluted into Diluent 25BS (1:5). A volume of 50 µl of all samples/standards was added to the appropriate wells in duplicate (see *Appendix section A3* for plate map). A volume of 50 µl of Antibody Cocktail was then added into each well. The plate was then sealed and incubated for 1 hour at room temperature on a horizontal shaker. After incubation, the wells were washed with the prepared 1x Wash Buffer PT (3 washes with 350 µl per wash). The plate was then inverted to decant the contents and the tapped gently against a paper towel. A volume of 100 µl of the provided TMB Development Solution was added into each well and the plate was incubated for about 10 minutes on a plate shaker with a cover

of aluminium foil to protect from light. Once a blue colour fully develops without saturation, 100 µl of the provided Stop Solution was added to each well. The plate was shaken for 1 minute before the absorbance was recorded at 450 nm.

2.9.7 Inhibin βA (Inhba)

For the quantitative detection of Inhba in adult male mouse serum, the FineTest® Mouse Inhba (Inhibin beta A chain) ELISA Kit (EM0273, Wuhan Fine Biotech Co.) was used. All reagents were brought to room temperature prior to use. A volume of 30 ml of the provided Concentrated Wash Buffer was diluted with 720 ml of deionised water (1 in 25). For standard preparation, 1ml of Sample Dilution Buffer was added into a Standard vial (provided) (labelled Tube 0) and kept at room temperature for 10 minutes and mixed thoroughly. Seven tubes were labelled respectively as follows: 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank. A volume of 300 µl of Sample Dilution Buffer was added to each tube followed by the addition of 300 µl of the prepared Standard solution (from tube 0) into the first tube. After thorough mixing, 300 µl was transferred from the 1st tube to the 2nd tube. The Standard solution was serially diluted by the transfer of 300 µl from the 2nd tube to the 3rd and so on with thorough mixing between transfers. Sample Dilution Buffer was used for the Blank control.

The required volume of the Biotin-labelled Antibody Working Solution was calculated (100 µl/well x number of wells + 10 % to account for any loss). The Biotin-detection antibody was diluted with Antibody Dilution Buffer at a concentration of 1:100 and was mixed thoroughly. Afterwards, the HRP-Streptavidin Conjugate (SABC) Working Solution was prepared by diluting the SABC with SABC Dilution Buffer at 1:100. The required volume of the working

solution was calculated in accordance with the manufacturer's instructions (100 µl/well x number of wells + 10 % to account for volume loss).

Assay Procedure

The TMB Substrate was equilibrated for 30 minutes at 37° C before being added to the wells. The standards and samples were added to the pre-coated plate in duplicate (see *Appendix section A3* for plate map). A volume of 100 µl of each standard and diluted sample was added into their respective well. The plate was then sealed and incubated at 37° C for a duration of 90 minutes. After incubation, the cover was removed, and the plate content was discarded. The plate was washed twice using prepared Wash Buffer. After washing, 100 µl of Biotin-labelled antibody working solution was pipetted into each of the wells followed by a 60-minute incubation at 37° C. After incubation, the plate was washed 3x with Wash Buffer, and the Wash Buffer was kept in each well for 1-2 minutes each time. Following the 3 washes, 100 µl of the SABC Working Solution was added into each well followed by a 30-minute incubation at 37° C (the plate is covered before every incubation step). The plate was then washed 5 times before 90 µl of TMB Substrate was added into each well. The plate was covered and was incubated for 10-20 minutes at 37° C, making sure the plate is protected from light. Once an apparent colour gradient appeared in the standard wells, the reaction was terminated by the addition of 50 µl of the provided Stop Solution into each well. Due to the acidification, the colour immediately changed to yellow. The plate was then read at 450 nm on the iMark™ microplate reader. The value obtained for the blank was subtracted from each value recorded for the samples and standards. The standard curve was then plotted as the relative value of each standard solution on the Y-axis versus the respective concentration on

the X-axis. The sample concentration was then interpolated from the standard curve.

2.10 Statistics

For the statistical analysis of the data, GraphPad PRISM 9 was used. Outliers in data sets were identified using PRISM's Robust Regression and Outlier Removal (ROUT) method and data was statistically analysed with the outliers excluded from the data sets. The data sets were tested for normality using D'Agostino & Pearson normality test. Normally distributed data were analysed using one-way ANOVAs with Tukey's post hoc test and non-normally distributed data were analysed using non-parametric Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons. Two-way ANOVA was used for the assessment of batch effect on animal weight gain throughout the study period. Area Under the Curve (AUC) analysis was carried out for animal weight progression data. Total gene lists of microarray data sets were generated through Partek Genomics Suite (Partek®). False detection rate (FDR) and fold change thresholding were set to 0.05 and 1.1, respectively. Gene lists were sorted by p value and fold change to determine the top genes of each diet group comparison (CD vs. LPD, CD vs. WD, etc.). Preliminary pathway and enrichment analysis was carried out on microarray data sets using Gene Set Enrichment Analysis (GSEA) functional enrichment analysis tools such as WebGestalt (WEB-based Gene SeT AnaLysis Toolkit) in addition to functional annotation using DAVID (The Database for Annotation, Visualization and Integrated Discovery). Two-way ANOVA was used to analyse array differential gene expression across the different diet groups. A p value ≤ 0.05 was deemed statistically significant for this study.

CHAPTER THREE: THE EFFECT OF SUB-OPTIMAL DIET ON ADULT MALE PHYSIOLOGY AND METABOLISM

3.1 INTRODUCTION

An individual's lifestyle choices and exposure to external or environmental factors can influence their overall physiology and can ultimately have an effect on their metabolic and reproductive fitness. Such external factors include smoking, drug use, exposure to certain chemical compounds such as pesticides, and dietary intake. Nutritional intake and its role in shaping one's physiology have been the subject of multiple studies over the past decades, from investigating undernutrition and dietary restriction (Holden et al., 2019) to overnutrition and supplementation (Pruszyńska-Oszmałek et al., 2021). The prevalence of unbalanced and unhealthy diet consumption has been on the rise worldwide, and particularly in Western countries (Kopp, 2019) but also in developing countries. Furthermore, in a 2019 press release, UNICEF reported that 42 % of school-goer adolescents in low and middle-income countries consume carbonated sugary beverages at least once a day and 46 % consume fast food at least once a week (UNICEF, 2019). This increase in consumption of calorie-dense diets and imbalanced nutritional intake has been correlated with a greater prevalence of obesity. In fact, the prevalence of overweight and obese cases in European citizens has increased as high as 60% in 2022, according to the World health Organisation's (WHO) May 2022 report on the state of the obesity pandemic (Boutari and Mantzoros, 2022). Obesity is known to be the number one risk factor for type 2 diabetes (Barnes, 2011). In addition, it

contributes to several other risk factors for cardiovascular disease (CVD) including hypertension and sleep disorders (Powell-Wiley et al., 2021). Furthermore, not only can obesity have physiological and metabolic implications, but it can also impair reproductive health and subsequent offspring development. Risks of infertility, low conception rates, higher miscarriage rates, and pregnancy complications are increased with obesity and overweight in women (Ozcan Dağ and Dilbaz, 2015). Parental obesity in both human and animal models has also been associated with an increased risk of a number of diseases and disorders in the offspring such as type 2 diabetes, obesity, hypertension, coronary heart disease, and asthma (Godfrey et al., 2016).

Models of undernutrition have also gained attention as it is recognised as a universal public health issue, with increasing prevalence rates becoming of concern, as 462 million adults were reported to be underweight due to malnutrition in recent years (WHO). Studies detailing undernutrition models such as caloric intake restriction have sought to characterise the impact of this state of suboptimal dietary intake on an individual's metabolic and reproductive fitness, and while caloric restriction has been associated with increased lifespan and generally more favourable outcomes for metabolic health (Spadaro et al., 2022), caloric restriction that causes malnutrition impairs reproductive fitness, sperm function, and thereby subsequent offspring health. Studies have shown that low protein diets (with high carbohydrates) have positive outcomes that promote longevity and improve adult cardiometabolic health in both rodent and Drosophila models (Lee et al., 2008; Simpson et al., 2015; Solon-Biet et al., 2014; Solon-Biet et al., 2016). The seemingly contradictory outcomes shown in rodent models in response to protein restriction have been highlighted by Solon-

Biet et al. (2014), who concluded that high protein diets or diets diluted in macronutrients to achieve caloric restriction are not responsible for healthy aging, but low protein diets with compensatory carbohydrates promote longevity (Solon-Biet et al., 2014). It is not currently known if the same concept applies to humans, however.

Another form of dietary alteration comes in the form of supplementation with antioxidants, vitamins, and essential nutrients such as vitamin B_{12}, vitamin A, betaine, choline, methionine, and folate. These are interconnected in a metabolic process known as one-carbon (1C) metabolism, where a number of key metabolic functions are mediated. These include the synthesis of purine and thymidine, methylation, and the metabolism of amino acids such as serine and glycine (Brosnan et al., 2015). The implications of the inhibition of this metabolic pathway, such as the inhibition of folate metabolism, blocks cell proliferation (Ducker and Rabinowitz, 2017). As these nutrients play a crucial role in modifying and regulating metabolic (Cordero et al., 2013), reproductive (Chleilat et al., 2021), and cognitive (McKee and Reyes, 2018) function and wellbeing, it became of importance to further understand and characterise their function and the implications of their supplementation to suboptimal diets. In a rat model fed a high fat and sucrose diet for a period of 8 weeks, Cordero et al. (2013) observed that this high fat diet feeding induced liver steatosis, while supplementing this high fat diet with methyl donors such as folate, choline, betaine, and vitamin B_{12} caused a reduction in hepatic fat development as a form of protective effect (Cordero et al., 2013). Evidence in human studies, on the other hand, is relatively lacking. Two recent systematic reviews and meta-analyses investigated the literature to find evidence on the impact of folate

supplementation on lipid profiles among patients with metabolic diseases (Tabrizi et al., 2017) as well as insulin sensitivity and type 2 diabetes (Lind et al., 2019). Tabrizi and colleagues found no significant impact of folate supplementation on blood pressures and lipid profiles of patients presenting with metabolic diseases (Tabrizi et al., 2017). And while Lind et al. (2019) found that folate supplementation reduces fasting insulin levels compared to placebo, thereby potentially being beneficial for reducing insulin resistance, they found no conclusive evidence with regard to how supplementation impacts the development of type 2 diabetes (Lind et al., 2019). In a randomised double-blinded controlled trial, Grizales et al. (2018) reported that betaine supplementation resulted in reduced fasting glucose levels and intrahepatic triglyceride levels in obese and prediabetic adults (Grizales et al., 2018). However, the authors found no effect of betaine supplementation on HbA1c in addition to no improvement of insulin sensitivity after the intervention period (Grizales et al., 2018). Furthermore, a recent Cochrane review of 15 randomised controlled trials examined the effect of homocysteine-lowering interventions such as vitamin B_6 (pyridoxine), vitamin B_9 (folate), vitamin B_{12} (cobalamin) individually or in combination on cardiovascular disease risk factors including hypertension, elevated total cholesterol, and reduced levels of high-density lipoprotein (HDL) (Martí-Carvajal et al., 2017). The authors found no significant impact of such interventions compared to placebo on the prevention of cardiovascular disease (Martí-Carvajal et al., 2017). In fact, evidence in human studies is relatively less conclusive than that found in animal model studies, and therefore warrants further investigation.

The consumption of sub-optimal diets has been shown to impact metabolic health even on a microbial level by altering key gut microbiota populations. Alterations in macronutrient levels can trigger rapid gut microbiome responses and influence microbiota compositions. Dietary fibre has been shown to be an important determinant of gut microbiota, with fibre interventions increasing fecal Bifidobacterium and Lactobacillus species without affecting alpha diversity in a 2018 systematic review and meta-analysis of 64 studies in human adults (So et al., 2018). Furthermore, diets with low diversity and low fibre such as Western diets have been shown to significantly alter the composition of the gut microbiome and promote a number of health risk factors such as metabolic diseases and obesity. On the other hand, however, a largely unexplored macronutrient with regard to its impact on gut microbiota populations is protein. While more evidence is emerging of the nature of the relationship between certain macronutrients in dietary intake and gut microbiota compositions, more investigation is warranted.

While studies involving different diets such as calorie-dense high fat, high sugar, or high/low protein investigated the effects on human and animal physiology, other studies focused on supplementation as potential means to reverse or alleviate the effects of sub-optimal diets. On the paternal side, methyl-donor supplementation has been shown to normalise the weights of fetuses of low protein diet-fed male mice (Morgan et al., 2020). In fact, supplementation of sub-optimal diets with such vitamins, minerals, and crucial amino acids is thought to contribute to the alleviation of the effects caused by said diets on a physiological, metabolic, and even transgenerational level. However, the full

scope of the mechanisms underlying the changes observed after supplementation is yet to be fully characterised.

3.2 AIMS AND OBJECTIVES

To better understand the effect of sub-optimal diet on the adult male metabolic and reproductive fitness, it is necessary to understand the changes to the physiology being observed throughout the organism's life. The C67BL/6 mouse serves as a versatile model for the study of the influence of sub-optimal diet on different physiological aspects. Therefore, this study hypothesises that sub-optimal diet feeding adversely impacts adult male mouse metabolic fitness that could potentially be balanced by the supplementation of methyl donors. Thus, the aims of this study were to examine the impact of sub-optimal diets with or without methyl-donor supplementation on C57BL/6 adult male mouse:

- Total weight gain trajectory
- Weight gain progression throughout life
- Organ weight to body weight ratios and adiposity
- Serum and liver tissue metabolite levels
- Gut microbiota

3.3 METHODOLOGY

3.3.1 Animals

As detailed in *section* 2, virgin 8-week-old male C57BL/6 mice (n=30/group across 4 batches) were randomly divided into five different groups (n=8 per diet group within each batch). The composition of all the diets fed to the stud males are listed and presented in *Appendix A1*. Livers, kidneys, hearts, testes, seminal vesicles, and gonadal fat were weighed and collected from each animal and snap frozen. In batch 4, the weighing and collection of inguinal, intrascapular, and peri-renal fat pads was included. Stool pellets were collected from the lower gut of the mice and snap frozen.

3.3.2 Serum and liver tissue assays

3.3.2.1 Cholesterol quantification

The quantification of total cholesterol in stud male liver tissue was carried out using a Cholesterol Quantification Kit (MAK043, Sigma-Aldrich®) in accordance with the manufacturer's protocol and as detailed in *section 2.8.1*.

Briefly, total cholesterol was isolated from samples of frozen liver tissue by lysing the tissue before the organic phase of each tube was transferred to a new tube and was left to air dry for 5 minutes at 50° C. The remaining dried lipids were dissolved with 200 µl of the Cholesterol Assay Buffer and were mixed. The samples were analysed in duplicate (see *Appendix A3* for plate map).

Assay Reaction

The reactions were set up according to the manufacturer's instructions, with 50 µl of Reaction Mix being required for each well. See *section 2.8.1* for detailed

steps of the protocol. After the addition of Reaction Mix, the plate was then incubated at 37° C for 60 minutes before the measurement of absorbance at 570 nm.

3.3.2.2 Free Fatty Acid (FFA) Quantification

The concentration of Free Fatty Acids (FFA) was determined using a Free Fatty Acid Quantitation Kit (MAK044, Sigma-Aldrich®) in accordance with the manufacturer's protocols as detailed in *section 2.8.2*. Liver tissues were homogenised before being centrifuged to remove any insoluble material. The organic phases were collected and air dried before the lipids were dissolved in 200 µl of Fatty Acid Assay Buffer by vortex mixing. A volume of 30 µl of each sample was added into the 96-well plate in duplicates (see *Appendix A3* for plate map).

Assay Reaction

See *section 2.8.2* for a detailed description of the procedure. The plate was incubated for 30 minutes at 37° C while being protected from light. The absorbance was then measured at 570 nm and the value of the Blank (0) was subtracted from the other readings to correct for background absorbance.

3.3.2.3 Triglyceride Quantification

A Triglyceride Quantification Kit (MAK266, Sigma-Aldrich®) was used to measure the levels of triglycerides in stud male liver tissue samples (100 mg). The steps of this protocol are detailed in *section 2.8.3*. Samples were homogenised then each sample tube was slowly heated to an average temperature of 90° C in a dry bath for 2-5 minutes. After cooling to room temperature, the samples were centrifuged to remove any insoluble material

before being diluted 10-fold with water prior to use in the assay. A volume of 30 µl was added into duplicate wells in the 96-well plate (see *Appendix A3* for plate map) for each sample. The plate was incubated for 20 minutes at room temperature.

Assay Reaction

A volume of 50 µl of the Master Reaction Mix was added to each well. After RT incubation, the absorbance was measured at 570 nm. The value obtained for the 0 (blank) was subtracted from all other readings to correct for background absorbance.

3.3.2.4 Glucose Assay

For the quantification of Glucose in adult male mouse serum, the Glucose Colorimetric Detection Kit (EIAGLUC, Thermo Fisher Scientific) was used. Serum samples were diluted to a ratio ≥ *1:15* with Assay Buffer as recommended by the manufacturer. The steps of this protocol are detailed in *section 2.8.4*.

Assay Reaction

As described in *section 2.8.4*, a 1x HRP solution was prepared by diluting the provided Horseradish Peroxidase Concentrate (100x) 1:100 with Assay Buffer. In addition, a 1x Glucose Oxidase solution was prepared by diluting the provided Glucose Oxidase Concentrate (10x) 1:10 with Assay Buffer. Serum samples were diluted 1:20 with Assay Buffer prior to use in the assay. A volume of 20 µl of standards and diluted samples were added in duplicate to the appropriate wells (see *Appendix A3* for plate maps). A volume of 25 µl of 1x HRP solution was then added to each well. The substrate was added (25 µl) into each well, followed by 25 µl 1x Glucose Oxidase per well. After incubation, the plate was

read at 560 nm and the values obtained for the blank wells was subtracted from the rest of the readings.

3.3.2.5 Insulin Quantification

As described in detail in *section 2.8.5*, The quantification of Insulin was carried out using a Rat/Mouse Insulin ELISA Kit (Millipore®) according to manufacturer's instructions. Plate strips were assembled in a plate holder and each well was washed 3 times. A volume of 80 µl of the provided Detection Antibody was added to each well and the plate was covered and left to incubate at room temperature for a duration of two hours.

After incubation, the plate was washed 3 times prior to the addition of 100 µl of Enzyme Solution to each well. The plate was covered and left to incubate for 30 minutes at RT. After the second incubation, the plate was washed again before a volume of 100 µl of Substrate Solution was added to each well. The plate was covered until a blue colour fully developed in the standard wells. 100 µl of Stop Solution was then added to each well. The plate was read at 450 nm and 595 nm within 5 minutes and the difference of absorbance units was recorded.

3.3.2.6 TNF-α Quantification

A Mouse TNF alpha SimpleStep ELISA® Kit (ab208348, Abcam®) was used for the quantitative measurement of TNF-α in adult male mouse serum following the manufacturer's instructions and as described in *section 2.8.6*.

Assay Reaction

Serum samples were diluted into Diluent 25BS (1:5). A volume of 50 µl of all samples/standards was added to the appropriate wells in duplicate (see *Appendix A3* for plate map). A volume of 50 µl of Antibody Cocktail was then added into

each well. The plate was then sealed and incubated for 1 hour at RT. The plate was washed before a volume of 100 µl of the provided TMB Development Solution was added into each well and the plate was incubated for about 10 minutes. Once a blue colour fully developed, 100 µl of Stop Solution was added to each well before the absorbance was recorded at 450 nm.

3.3.2.7 Inhibin βA (Inhba)

For the quantitative detection of Inhba in adult male mouse serum, a FineTest® Mouse Inhba (Inhibin beta A chain) ELISA Kit (EM0273, Wuhan Fine Biotech Co.) was used in accordance with the manufacturer's instructions and as detailed in *section 2.8.7*.

Assay Reaction

The standards and samples were added to the pre-coated plate in duplicate (see *Appendix A3* for plate map). A volume of 100 µl of each standard and diluted sample was added into their respective well. The plate was sealed and incubated at 37° C for 90 minutes. The plate was washed before 100 µl of Biotin-labelled antibody working solution was pipetted into each of the wells followed by a 60-minute incubation at 37° C. The plate was washed before 100 µl of the SABC Working Solution was added into each well followed by a 30-minute incubation. The plate was washed before 90 µl of TMB Substrate was added into each well. The plate was covered and incubated for 10-20 minutes. Once an apparent colour gradient appeared in the standard wells, the reaction was terminated by the addition of 50 µl Stop Solution into each well. The plate was then read at 450 nm.

3.3.3 Stool DNA extraction

As detailed in *section 2.6*, a QIAamp DNA Stool Mini Kit (QIAGEN®) was used to extract DNA from the collected stool pellets. All centrifugation steps were performed at room temperature at approximately 14,000 rpm. Approximately 180-220 mg of mouse stool was thoroughly homogenised with provided buffer before being heated for 5 minutes at 70° C. The tubes were vortexed and centrifuged. Into new 1.5 ml tubes, 45 µl of the provided proteinase k was added, followed by 600 µl of supernatant from the centrifuged tube. Samples were homogenised with buffer solution before being incubated for 10 minutes at 70º C. After incubation, 600 µl of ethanol (96-100 %) was added to the lysate and vortexed. 600 µl of the lysate was added to a QIAmp spin column and centrifuged for 1 minute. The filtrate was discarded. After that, buffers AW1 and AW2 were added with centrifugation after the addition of each of the buffers. Finally, 50 µl of buffer ATE was added directly onto the membrane of each of the tubes to elute the stool DNA. The tubes were left to incubate at RT for 1 minute before centrifugation (1 minute). Ethanol precipitation was carried out for samples that recorded sub-standard Nanodrop ratios as highlighted in *section 2.7*.

3.3.4 Stool DNA sequencing for microbiome analysis

Extracted stool DNA samples were sent to DeepSeq at the University of Nottingham where they underwent a 16S sequencing for bacterial species using the Illumina MiSeq Reagent kit v3 on the Illumina MiSeq Platform (Illumina). A polymerase chain reaction (PCR) was performed to amplify the V3-V4 region of the 16S rDNA gene followed by first stage PCR clean-up, quantification, indexing PCR, further clean-up, and library prep quality control. Data analysis

was performed by DeepSeq using Qiime2, a next-generation microbiome bioinformatics platform (qiime2.org).

3.3.5 Statistics

All data are presented as mean ± standard error of the mean (SEM). The data sets were tested for normality using D'Agostino & Pearson normality test. Normally distributed data were analysed using one-way ANOVAs (with Tukey's post hoc test) and non-normally distributed data were analysed using non-parametric Kruskal-Wallis test (with Dunn's post hoc test) using GraphPad Prism 9. Two-way ANOVA was also carried out to assess batch effect. A p value ≤ 0.05 was deemed statistically significant for this analysis. Area under the curve (AUC) analysis was additionally carried out for weight progression data sets. Outliers in data sets were identified using PRISM's Robust Regression and Outlier Removal (ROUT) method and data was statistically analysed with the outliers excluded from the data sets.

3.4 RESULTS

3.4.1 Total body weight progression

While all batches of animals underwent the same treatments and feeding regimen under the same conditions, it was necessary to identify any significant differences prior to pooling the samples for analysis. In order to check for significant differences across the different batches, batch data was compared at different time points throughout the experiment. No significant difference was observed between the starting weight for all males prior to feeding (*Figure 3.1*). Total body weights of males from each diet group within each batch were compared at Week 1, Week 7, Week 15, and Week 23, respectively. *Figures 3.2, 3.3, 3.4, 3.5* and *3.6* represent the differences in male weights from each group across the four batches used in this study.

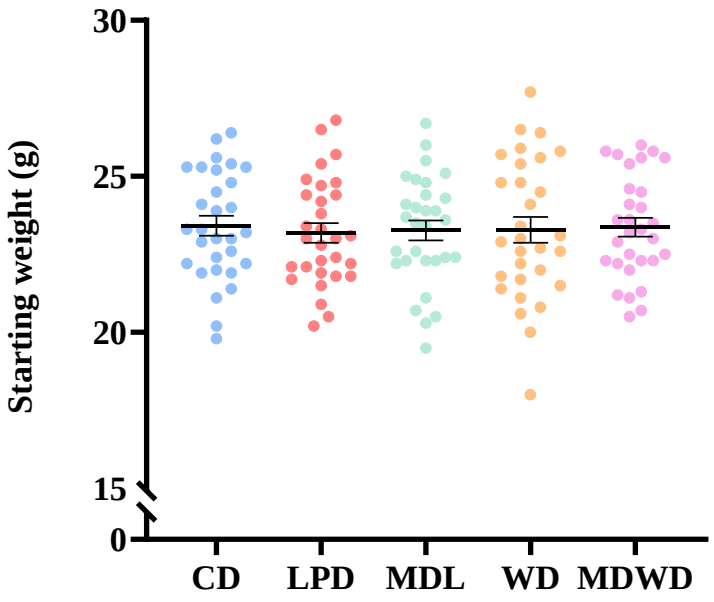

Figure 3. 1: Starting weight of male mice before feeding (n=30/group). Error bars are mean ± SEM. D'Agostino and Pearson normality test was carried out followed by a One-way ANOVA with Tukey's post-hoc test.

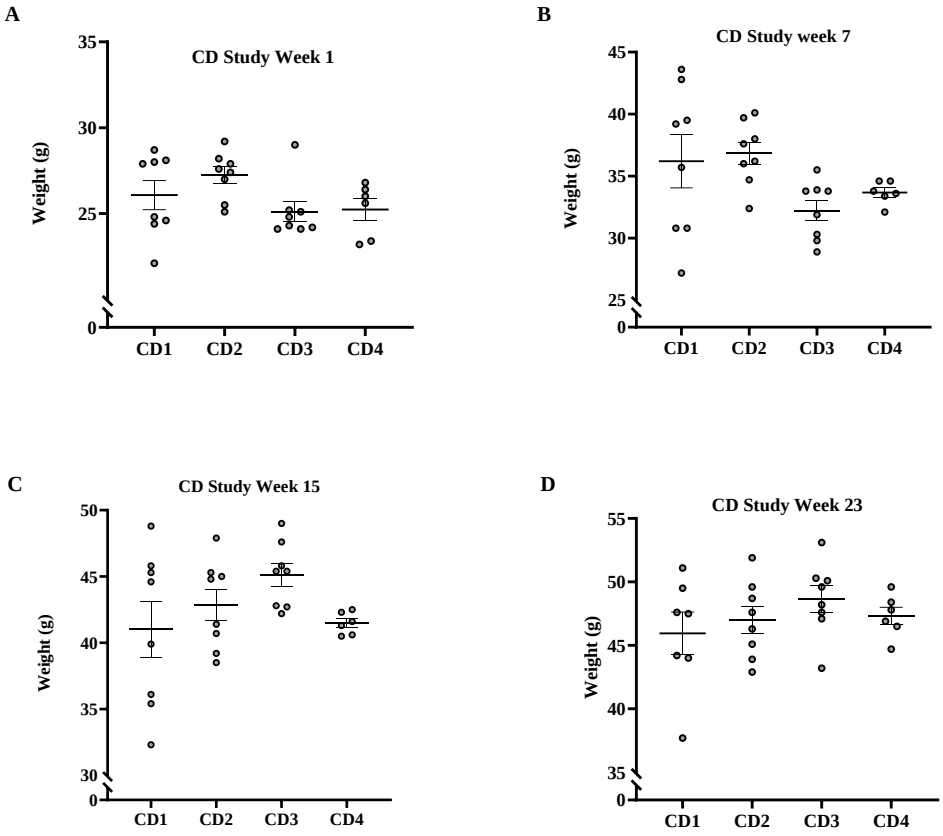

Figure 3. 2: CD batch comparisons (CD1 = Batch 1 CD group, CD2 = Batch 2 CD group, etc.) at different points in time throughout the study. Graphs represent stud male (n=6-8/group/batch) weights at study week 1 (A), week 7 (B), week 15 (C), and week 23 (D). D'Agostino and Pearson normality test was carried out followed by either one-way ANOVA analysis with Tukey's post-hoc test (B, C, D) for the sets that pass the normality test or Kruskal-Wallis analysis (A) for data not normally-distributed. Error bars represent mean ± SEM.

In addition, no significant differences were observed between animal weights in the LPD groups across the batches between week 1 and week 23 of the study (*Figure 3.3*).

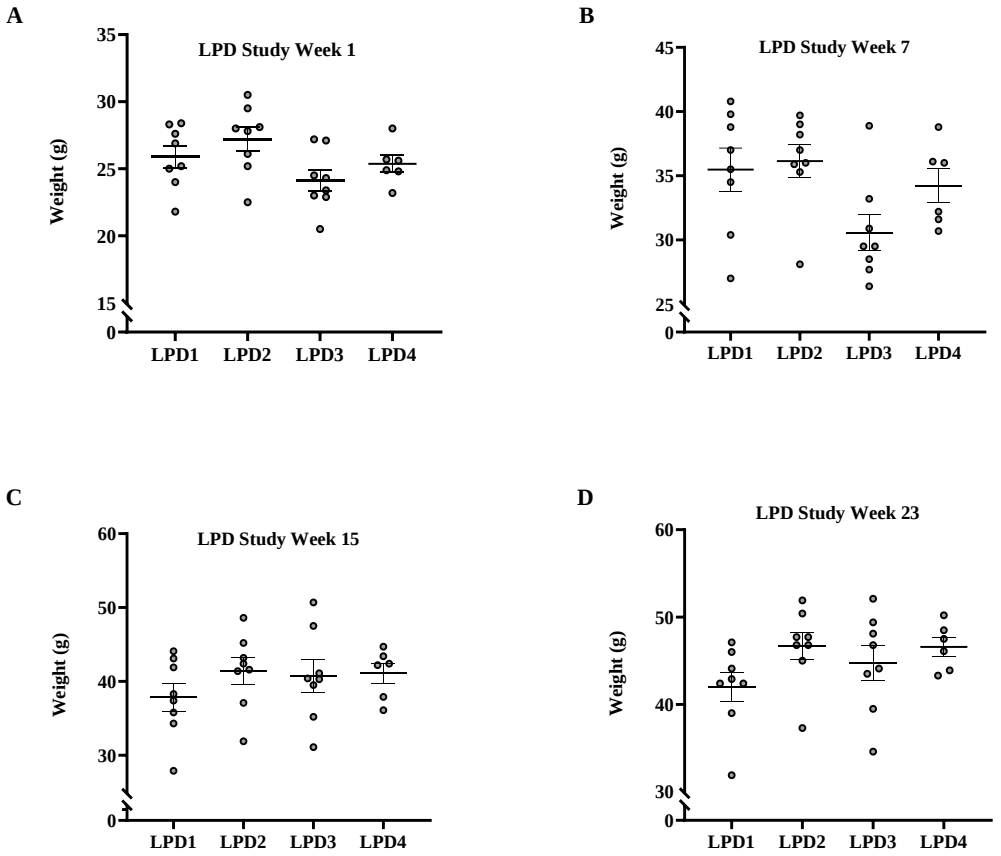

Figure 3. 3: LPD batch comparisons (LPD1 = Batch 1 LPD group, LPD2 = Batch 2 LPD group, etc.) at different points in time throughout the study. Graphs represent stud male weights at study week 1 (A), week 7 (B), week 15 (C), and week 23 (D); n=8/group/batch. D'Agostino and Pearson normality test was carried out followed by either one-way ANOVA analysis with Tukey's post-hoc test (A, C) for the sets that pass the normality test or Kruskal-Wallis analysis (B, D) for data not normally-distributed. Error bars represent mean ± SEM.

At week 1 of the study, significant differences in body weight were observed between the MDL groups of batches 2, 3, and 4. Animals from the MDL batch 2 had significantly greater weight at week 1 of the study than those of batches 3 and 4 (*Figure 3.4A*). Moreover, MDL-fed males from batch 2 had significantly greater weight than those in batch 3 at the 7th week of the study (*Figure 3.4B*). However, as the study progressed through week 15 and week 23, no significant differences in total body weight were observed between MDL males across all four batches (*Figure 3.4C, D*).

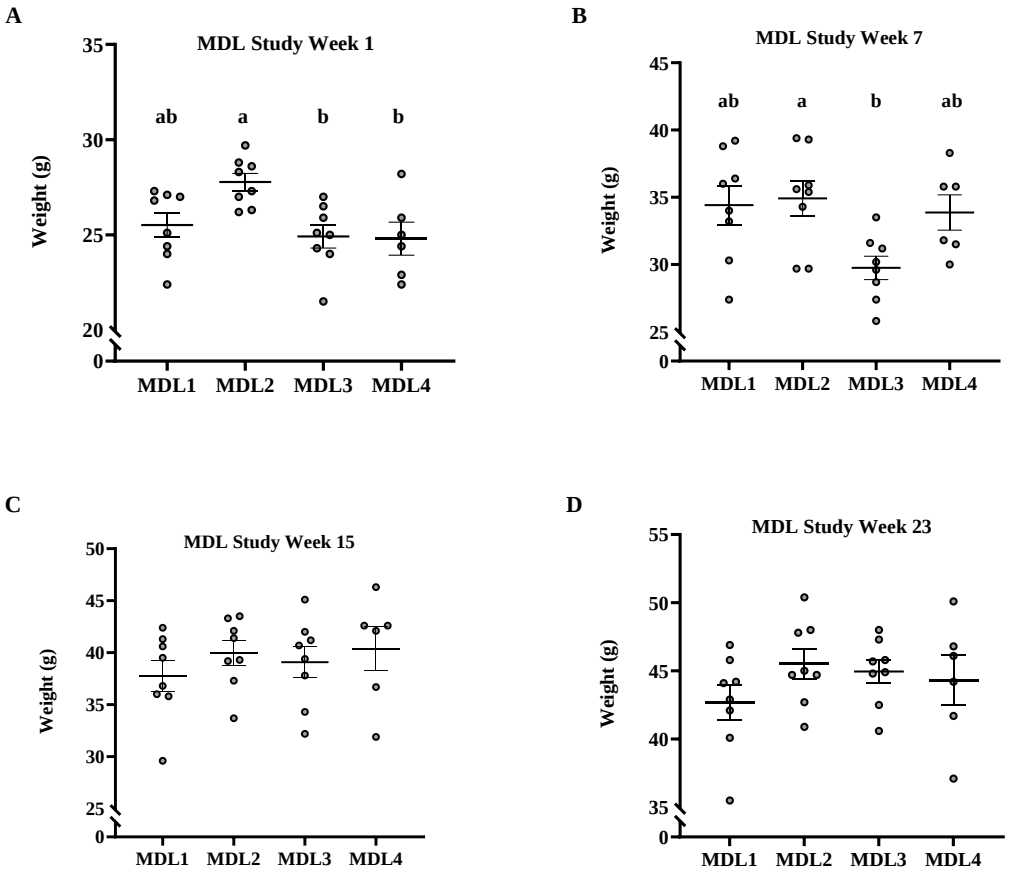

Figure 3. 4: MDL batch comparisons (MDL1 = Batch 1 MDL group, MDL2 = Batch 2 MDL group, etc.) at different points in time throughout the study. Graphs represent stud male weights at study week 1 (A), week 7 (B), week 15 (C), and week 23 (D); n=8/group/batch. D'Agostino and Pearson normality test was carried out followed by one-way ANOVA analysis with Tukey's post-hoc test (A, B, C, D). Graphs represent individual data points ± SEM.

In WD-fed males, significant differences were only observed in week 1 analysis across the different batches. WD-fed males from batch 2 had significantly greater total body weights than those from batch 4 (*Figure 3.5A*). However, no significant differences were observed between the WD groups of all four batches in weeks 7, 15, and 23 (*Figure 3.5B, C, D*).

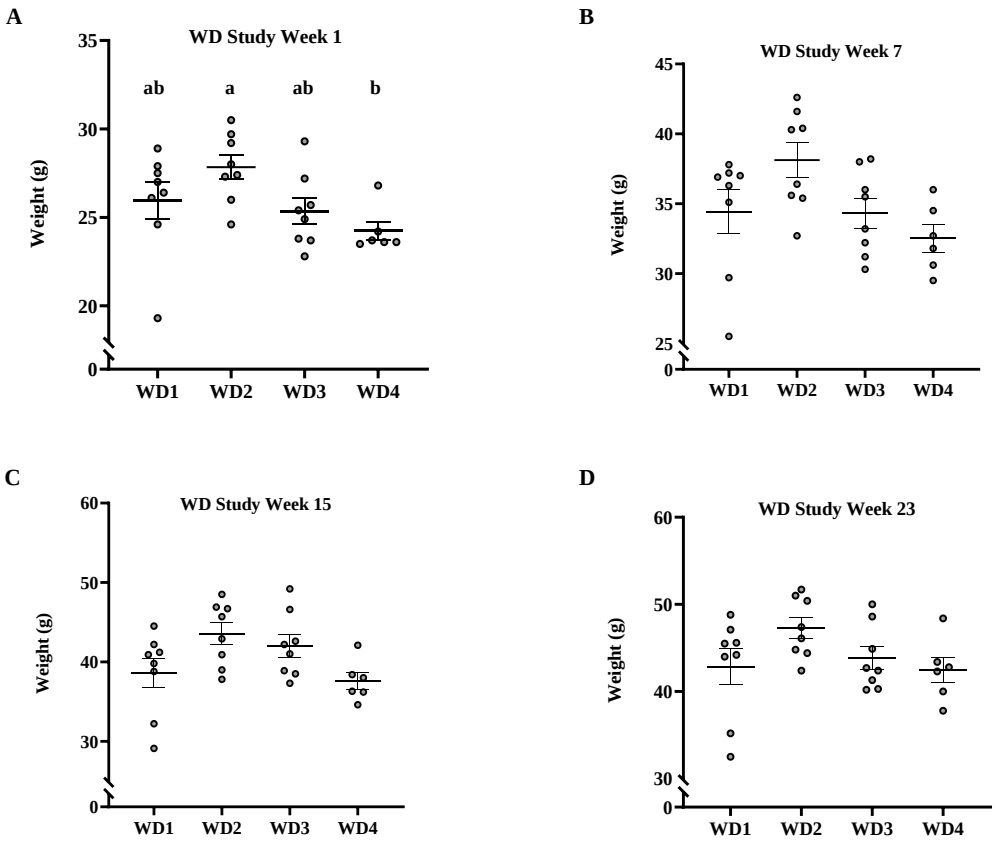

Figure 3. 5: WD batch comparisons (WD1 = Batch 1 WD group, WD2 = Batch 2 WD group, etc.) at different points in time throughout the study. Graphs represent stud male weights at study week 1 (A), week 7 (B), week 15 (C), and week 23 (D); n=8/group/batch. D'Agostino and Pearson normality test was carried out followed by one-way ANOVA analysis with Tukey's post-hoc test (C) for the sets that pass the normality test or Kruskal-Wallis analysis (A, B, D) for data not normally-distributed. Graphs represent individual data points ± SEM.

MDWD-fed males from batch 2 displayed significantly greater total body weight than those from batch 4 during the first week of the study (*Figure 3.6A*). However, no significant differences in total body weight were observed between MDWD males across the four different batches in weeks 7 (*Figure 3.6B*), week 15 (*Figure 3.6C*), and week 23 (*Figure 3.6D*).

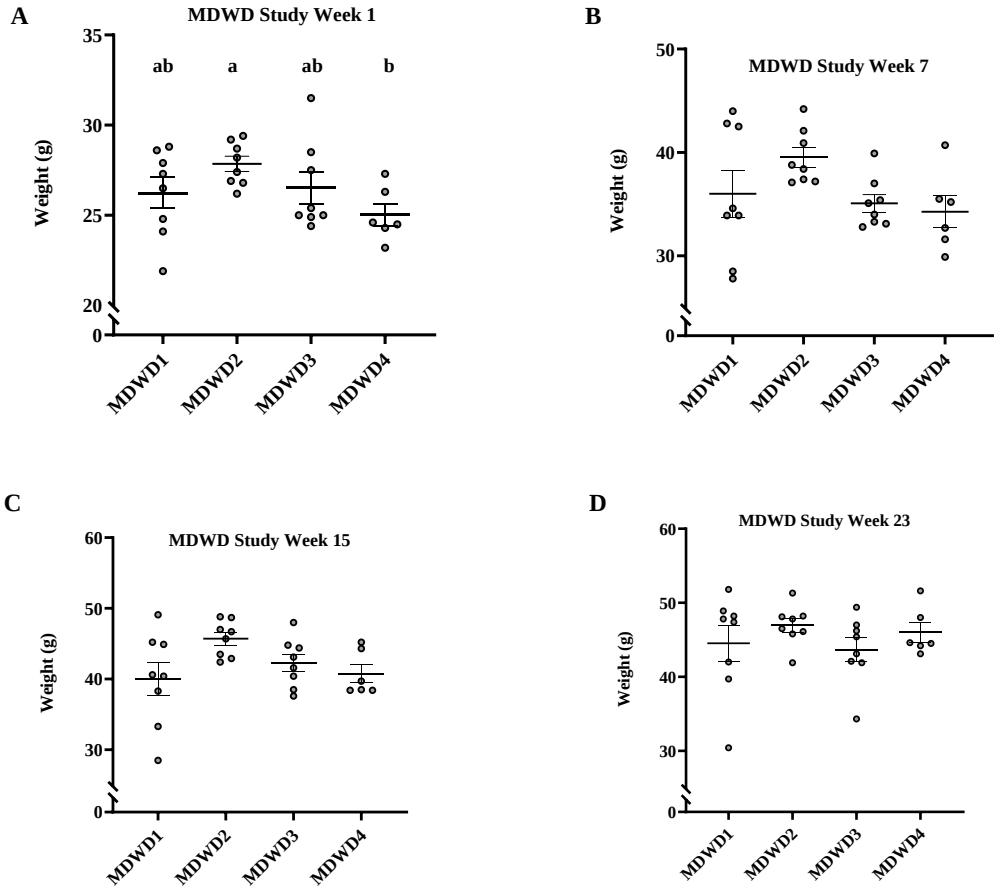

Figure 3. 6: MDWD batch comparisons (MDWD1 = Batch 1 MDWD group, MDWD2 = Batch 2 MDWD group, etc.) at different points in time throughout the study. Graphs represent stud male weights at study week 1 (A), week 7 (B), week 15 (C), and week 23 (D); n=8/group/batch. D'Agostino and Pearson normality test was carried out followed by one-way ANOVA analysis with Tukey's post-hoc test (B) for the sets that pass the normality test or Kruskal-Wallis analysis (A, C, D) for data not normally-distributed. Graphs represent individual data points ± SEM.

Furthermore, no significant difference was observed between the starting individual housing weights for males of all batches across the different diet groups (*Figure 3.7*). Individual housing for males was started at an average mouse age of 16 weeks (average study week 9) after which mating with female

mice started. By the time the male mice were individually housed, all males had spent a minimum of 8 weeks on their respective feeding regimen.

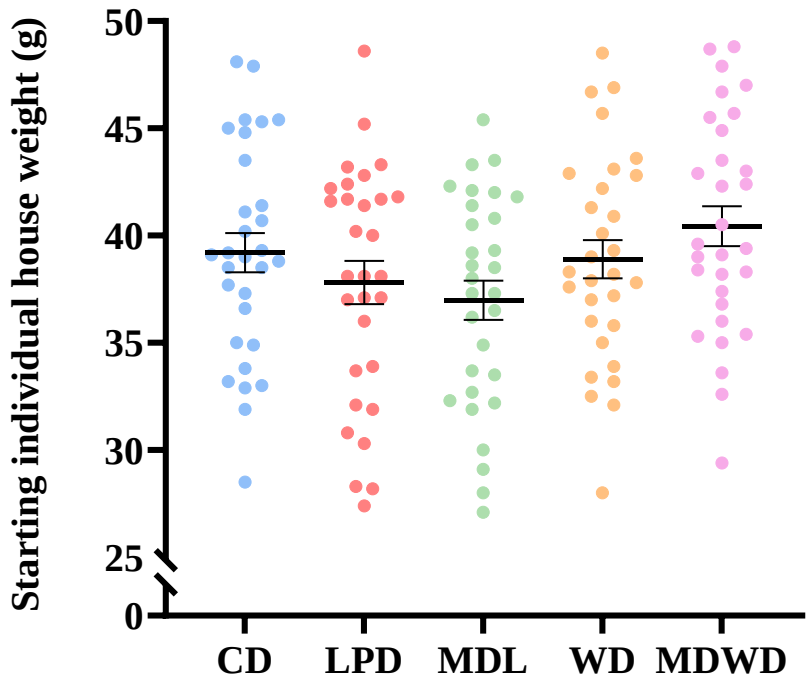

Figure 3.7: Starting individual housing weights of males from all batches. Graph represents average starting weight of males upon individual housing at an average age of 16 weeks (n=30/group) ± SEM. D'Agostino and Pearson normality test was carried out followed by a One-way ANOVA with Tukey's post-hoc test.

Male weight growth trajectory in response to the different diets over the course of a 27-week period is shown in *Figure 3.8*. No significant differences were observed in the weight progression of the animals in each batch over the course of the study period with the exception of Batch 2, in which the MDWD group had a significantly greater area under the curve compared to MDL (*Figure 3.9*).

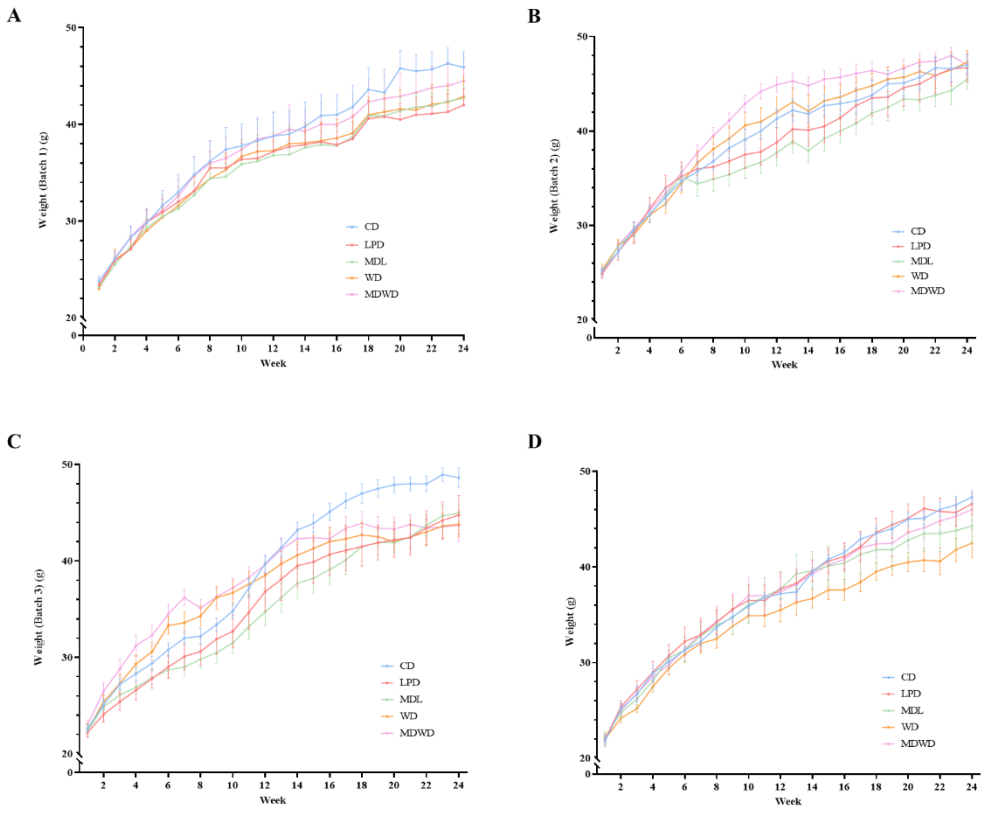

Figure 3.8: Animal weight progression over the study period. The effect of diet on total body weight progression over a 24-week period in batch 1(A), batch 2 (B), batch 3 (C), and batch 4 (D). Individual points represent a mean weight measurement at a given week ± SEM.

Body weight progressions between weeks 7 and 23 of the study were analysed as area under the curve (AUC) analysis, which was performed using GraphPad PRISM, revealing MDL in batches 2 and 3 to have a significantly smaller AUC than MDWD and CD, respectively (*Figure 3.9*).

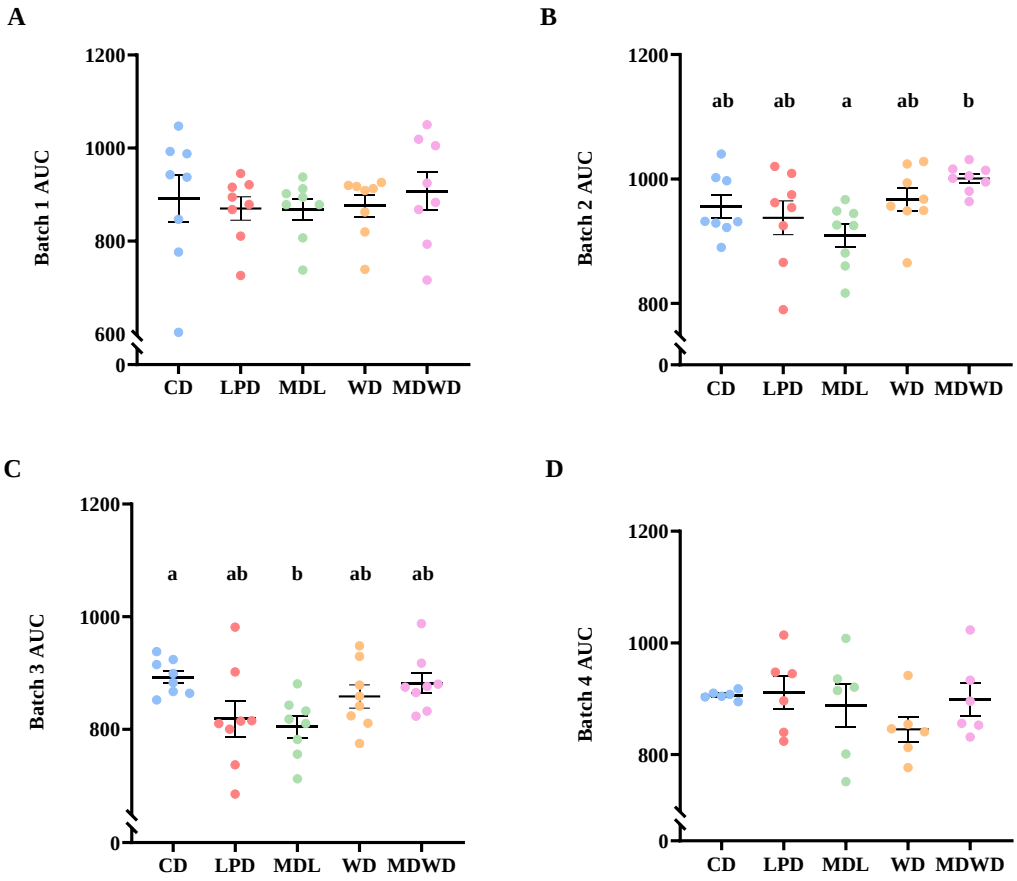

Figure 3. 9: Area under the curve (AUC) analysis of batch weight progression. Graph represents the effect of diet on total body weight progression over a 24-week period in batch 1(A), batch 2 (B), batch 3 (C), and batch 4 (D) through area under the curve (AUC) analysis. Individual points represent a mean area measurement for an individual sample ± SEM (n=8/group). D'Agostino & Pearson normality test was carried out followed by One-way ANOVA analysis and Tukey's post-hoc test for normally distributed data (A) or Kruskal-Wallis and Dunn's post-hoc test for nonparametric data sets (B, C, D).

A two-way ANOVA analysis was also carried out to further define the interactions between the different diet groups and the different batches and to identify the extent of variation within and between the different batches. Batch

2 significantly differed from batches 1, 3, and 4 with regard to animal weight progression (*Figure 3.10*).

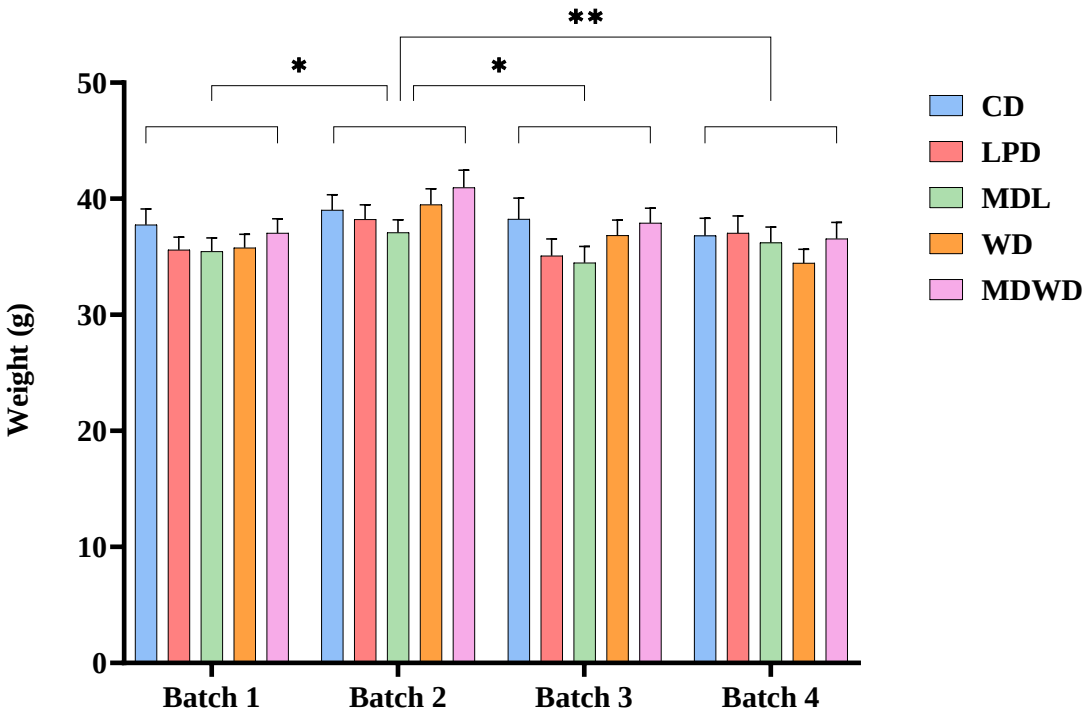

Figure 3. 10: Batch effect on weight progression. Figure shows a two-way ANOVA analysis examining row (batch) effect on weight progression throughout the study period ± SEM. * *p≤0.05; ** p≤0.01*.

No significant differences were observed in the average total body weight across all batches of males at weeks 1 and 7 of the study (*Figure 3.11A, B*). However, MDL males displayed a significantly lower total body weight compared to CD-fed males at week 15 (*Figure 3.11C*) and week 23 (*Figure 3.11D*). Furthermore, WD-fed males were observed to be significantly lighter in total body weight compared to CD males at week 23 (*Figure 3.11D*).

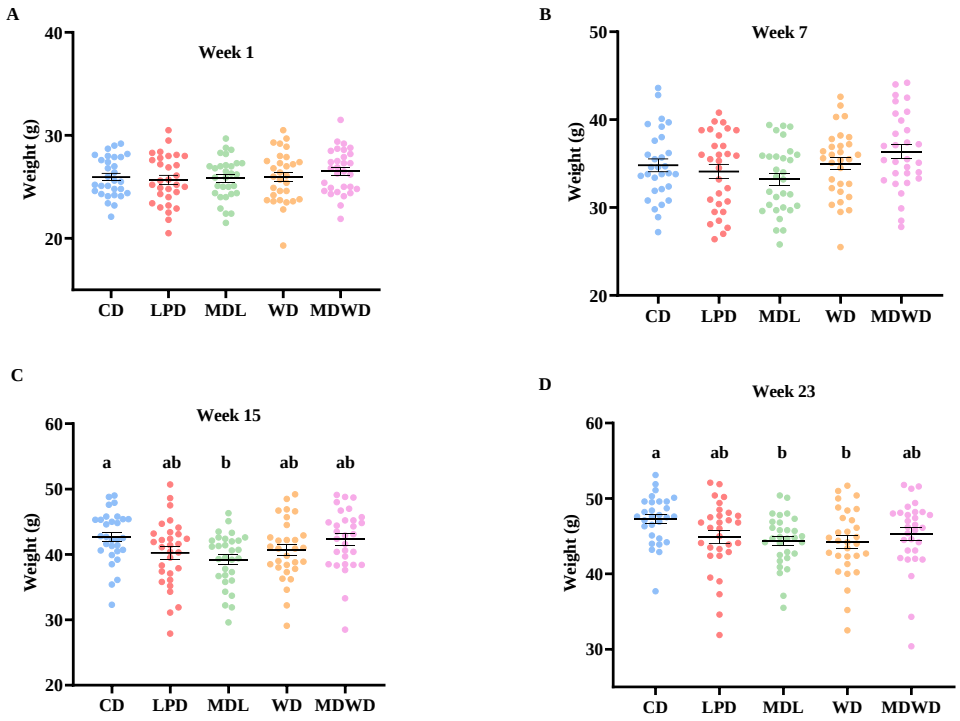

Figure 3.11: Stud male weights at different points in time throughout the study. Graphs represent stud male weights at study week 1 (A), week 7 (B), week 15 (C), and week 23 (D); n=29-30/group. D'Agostino and Pearson normality test was carried out followed by one-way ANOVA analysis with Tukey's post-hoc test (A, C) for the sets that pass the normality test or Kruskal-Wallis analysis (B, D) for data not normally-distributed. Graphs represent individual data points ± SEM.

With regard to total body weight at the time of sacrifice, no statistically significant difference was observed between the CD and the study groups LPD, WD, and MDWD. However, MDL-fed mice had a significantly reduced total body weight compared to CD (*Figure 3.12*).

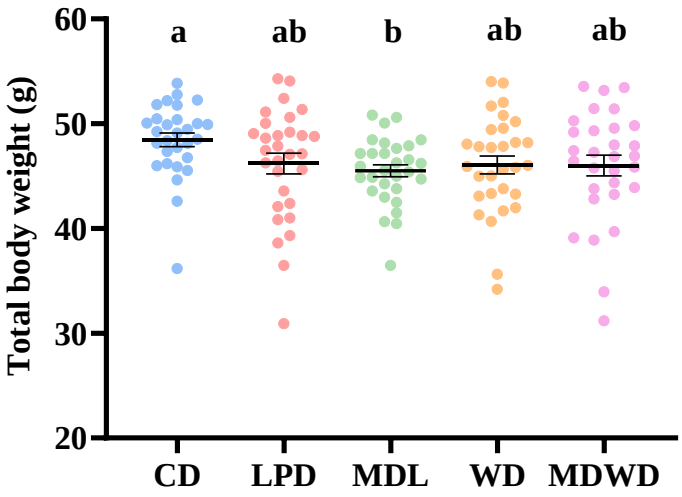

Figure 3. 12: Effect of different diets on total body weight at time of sacrifice. Individual points represent individual males in their respective group (n=30 males per group). The mean is denoted as the horizontal black line ± SEM. Data from 4 batches of males is shown and was analysed using Kruskal-Wallis test with Dunn's post-hoc test. Bars without letters in common are statistically significant ($p \leq 0.05$).

3.4.2 Sub-optimal diet influences organ weight.

Hearts, livers, kidneys, and testes were weighed after animal dissection and before being stored for later use. Analysis of organ weights revealed that MDWD had significantly heavier hearts than CD (*Figure 3.13A*; $p \leq 0.05$)). MDL males had significantly lighter livers compared to WD ($p<0.0001$) and MDWD ($p<0.0005$), whereas the WD group additionally had heavier livers compared to CD ($p<0.01$) and LPD ($p<0.001$) (*Figure 3.13B*). Total kidney weight (both kidneys) was also significantly altered, as MDWD males displayed heavier kidneys compared to CD ($p<0.01$), LPD ($p<0.0001$), and MDL ($p<0.0001$), and WD had significantly greater kidney weight compared to LPD and MDL

(*p<0.0001*) (*Figure 3.13C*). However, no significant difference was observed in total testis weight (both testes) (*Figure 3.13D*).

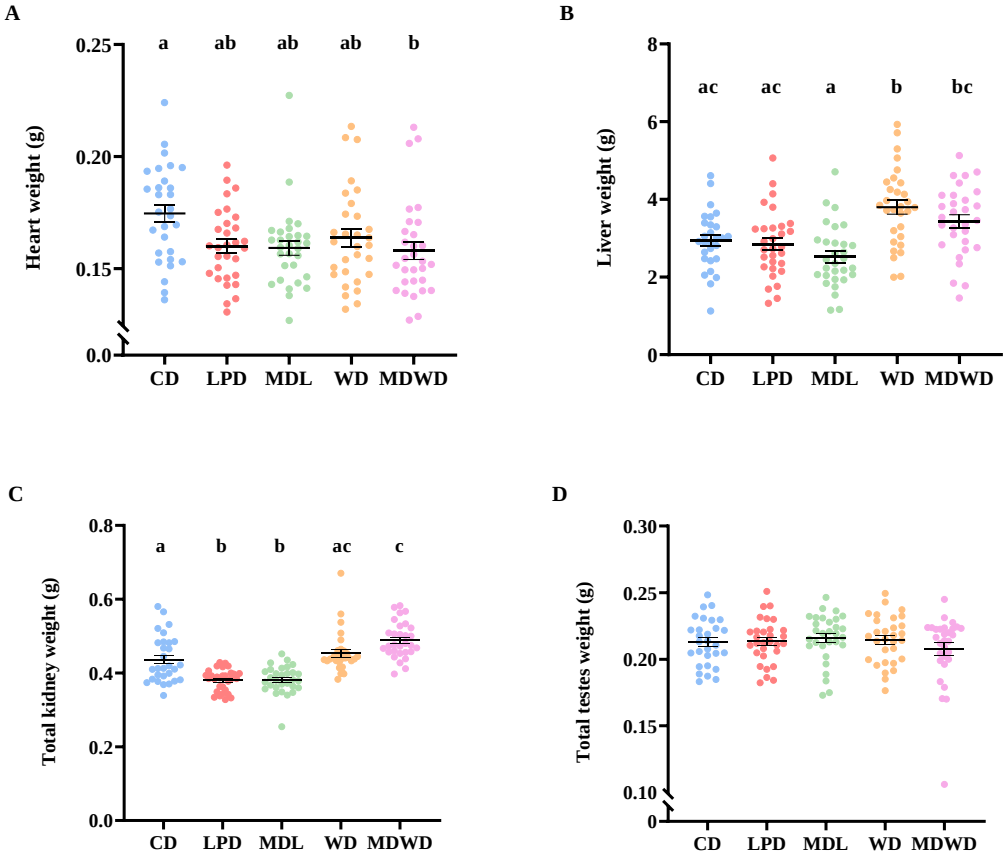

Figure 3.13: Dietary intake alters raw organ weights. Graphs represent the differences in raw heart (A), liver (B), kidneys (C) and testes (D) end-of-study weights. Means are denoted by black bars ± SEM. D'Agostino and Pearson's test was used to identify normal distribution. One-way ANOVA was used with Tukey's post-hoc test for normally distributed sets (B) and Kruskal-Wallis with Dunn's post-hoc test was used for non-parametric data (A, C, D). Bars without letters in common are statistically significant (n=30/group).

When looking at the organ to body weight ratios, no significant differences were observed in the heart to total body weight ratio across all study groups (*Figure*

3.14A). On the other hand, WD and MDWD males were observed to have proportionally larger livers than the other study groups. As shown in *Figure 3.14B*, WD had a greater liver to body weight ratio than CD ($p \leq 0.0001$), LPD ($p \leq 0.0001$), and MDL ($p \leq 0.0001$). MDWD also had a significantly increased liver to body weight ratio when compared to CD ($p \leq 0.004$), LPD ($p \leq 0.05$), and MDL ($p \leq 0.0001$). In addition, MDWD had a greater kidney to body weight ratio than CD, LPD, and MDL ($p \leq 0.0001$; *Figure 3.14C*). WD-fed males also exhibited proportionately heavier kidneys compared to LPD ($p \leq 0.001$) and MDL ($p \leq 0.05$). When comparing the testes weight to body weight ratios, only MDL-fed males showed significantly heavier testes proportionate to their body weight compared to CD ($p \leq 0.05$; *Figure 3.14D*). No other significant differences were observed when comparing testes to body weight ratios across the different groups.

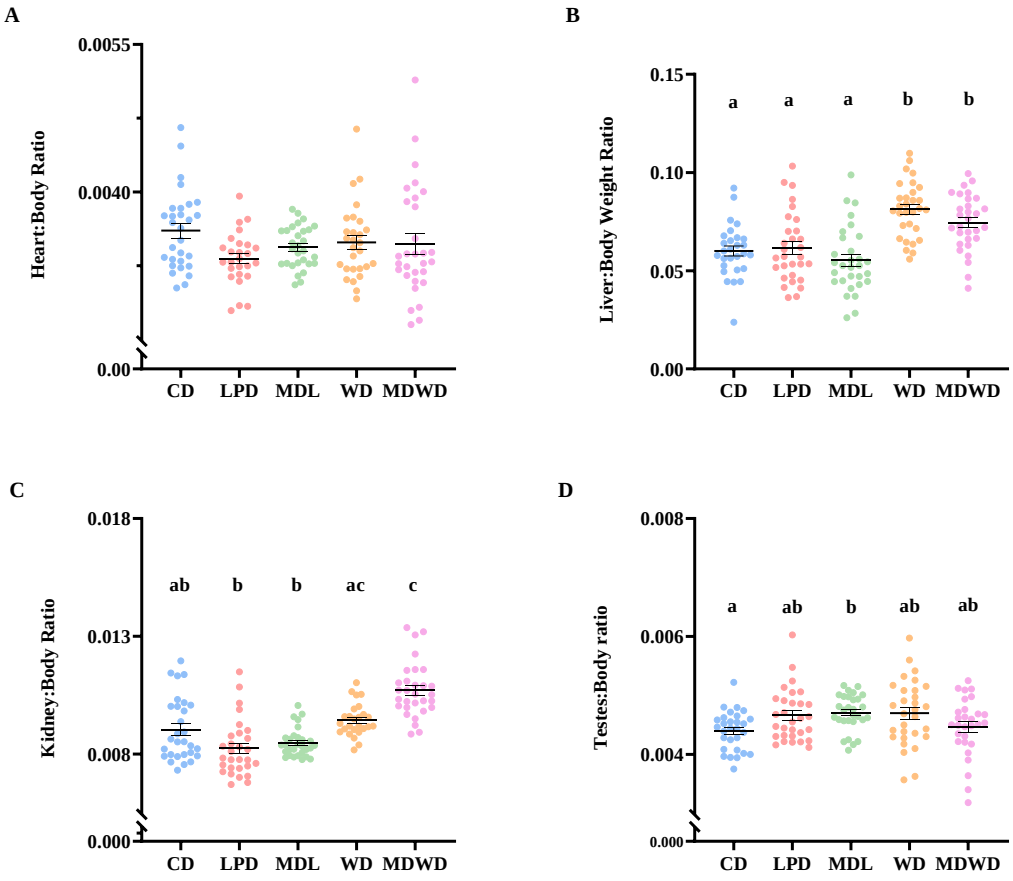

Figure 3.14: Dietary regimens influence organ to body weight ratios. Graphs represent the differences in end-of-study organ to body weight ratios. Means are denoted by black bars ± SEM. D'Agostino and Pearson's test was used to identify normally distributed data sets. One-way ANOVA was used with Tukey's post-hoc test for normally distributed sets (B) and Kruskal-Wallis with Dunn's post-hoc test was used for non-parametric data (A, C, D). Outliers were identified using GraphPad Prism's ROUT method. Different letters denote statistical significance (n=30/group).

3.4.5 Dietary intake's effect on male crude carcass weight

The total weight of organs and tissues collected from each male was subtracted from each of their total weights at time of sacrifice, resulting in a crude carcass weight (comprised predominantly of skin, bone, brain, and muscle). While

measurement of individual components of the carcass weight was not performed (i.e brains, bones), when analysing the pooled crude carcass weight data across all batches, it was observed that male mice from the MDL, WD, and MDWD groups exhibited lighter carcasses compared to those from the control group CD as shown in *Figure 3.15*.

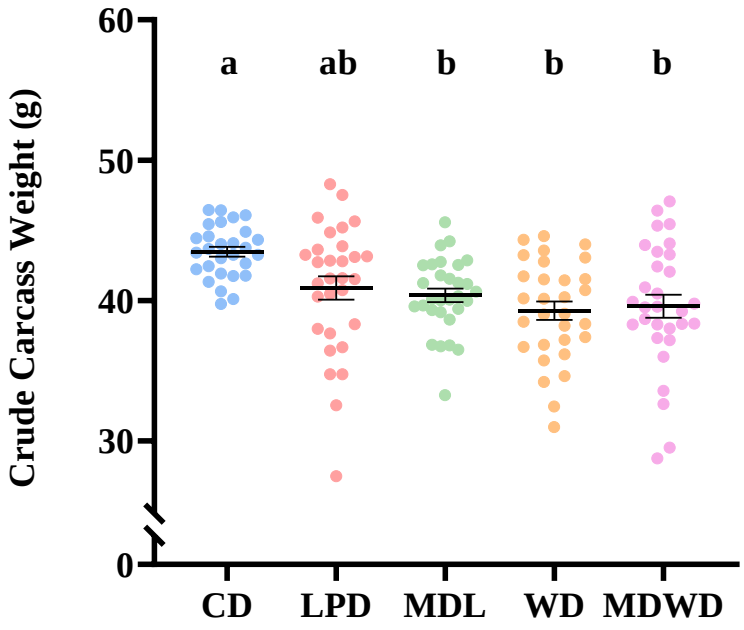

Figure 3.15: Male mouse crude carcass weight impacted by diet. Figure shows the impact of the different diets on carcass weight, which was calculated by subtracting the weights of harvested organs from the total body weight upon sacrifice. Individual points represent individual animals (n=30/group). Means are denoted by the black bars ± SEM. Bars without letters in common are statistically significant. Analysis was performed using Kruskal-Wallis test followed by Dunn's post-hoc test.

3.4.3 The impact of sub-optimal diet on fat deposition

In addition to the collection of different organs, gonadal fat was collected from the male mice upon sacrifice and was weighed before storage. Inguinal, intra-

scapular, and peri-renal fat pads were additionally collected from batch 4 animals as fat pad collection was not performed for the previous batches. Raw fat pad weights were also compared across the five different study groups, revealing significant differences in total gonadal fat pad weight across multiple groups (*Figure 3.16A*). MDL showed a significantly greater gonadal fat pad weight than CD ($p \leq 0.05$), whereas both WD and MDWD displayed significantly greater gonadal fat pad weights than the control group, respectively ($p \leq 0.0001$). WD and MDWD males also exhibited significantly greater gonadal fat weight than LPD males, respectively ($p \leq 0.005$). However, no significant differences were observed in the raw weights of inguinal (*Figure 3.16B*), intra-scapular (*Figure 3.16C*), or peri-renal (*Figure 3.16D*) fat pads across the study groups.

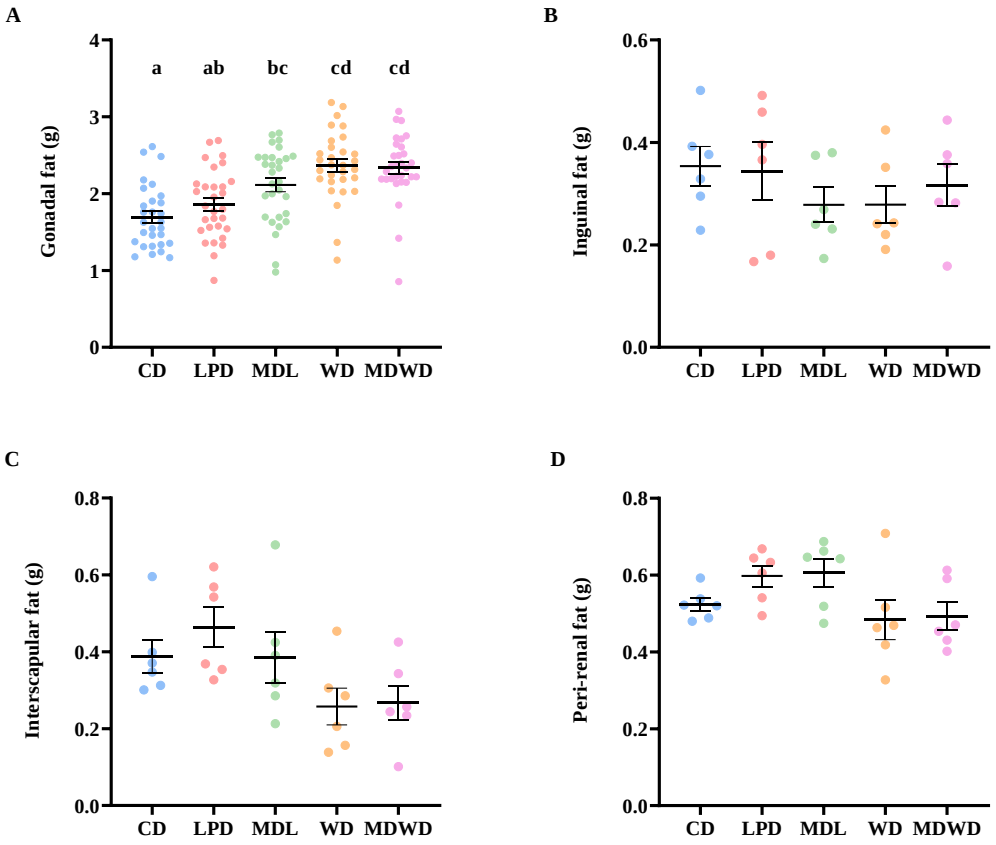

Figure 3.16: Sub-optimal diet influences raw fat pad weights. Graph shows raw gonadal (A), inguinal (B), interscapular (C), and peri-renal (D) fat pad weights across the different diet groups. Individual points represent individual male mice. D'Agostino and Pearson normality test was performed followed by one-way ANOVA analysis for normally distributed data with Tukey's post-hoc test (B, C). Kruskal-Wallis analysis was performed with Dunn's post-hoc test for non-parametric data sets (A, D). Means are denoted by the black bars ± SEM. Different letters denote statistical significance (n=29-30/group; n=6/group in batch 4). *The n number per group in this analysis is 6, which may be a limiting sample size.

Figure 3.17 shows the fat pad to body weight ratios across the different groups. No significant differences were observed in gonadal fat to body weight ratios between CD and LPD. However, MDL, WD, and MDWD had significantly

increased fat to body weight ratios relative to CD (*p≤0.001, p≤0.0001 and p≤0.0001*, respectively) and both WD and MDWD groups exhibited a significantly larger gonadal fat to body weight ratio than LPD (*p≤0.01*) (*Figure 3.17A*). No significant differences were observed in inguinal fat and intra-scapular fat pads to body weight ratios from batch 4 animals as shown in *Figure 3.16B* and *C*, respectively. On the other hand, MDL males displayed an increased peri-renal fat to body weight ratio compared to WD males in batch 4 (*p≤0.05*) (*Figure 3.17D*).

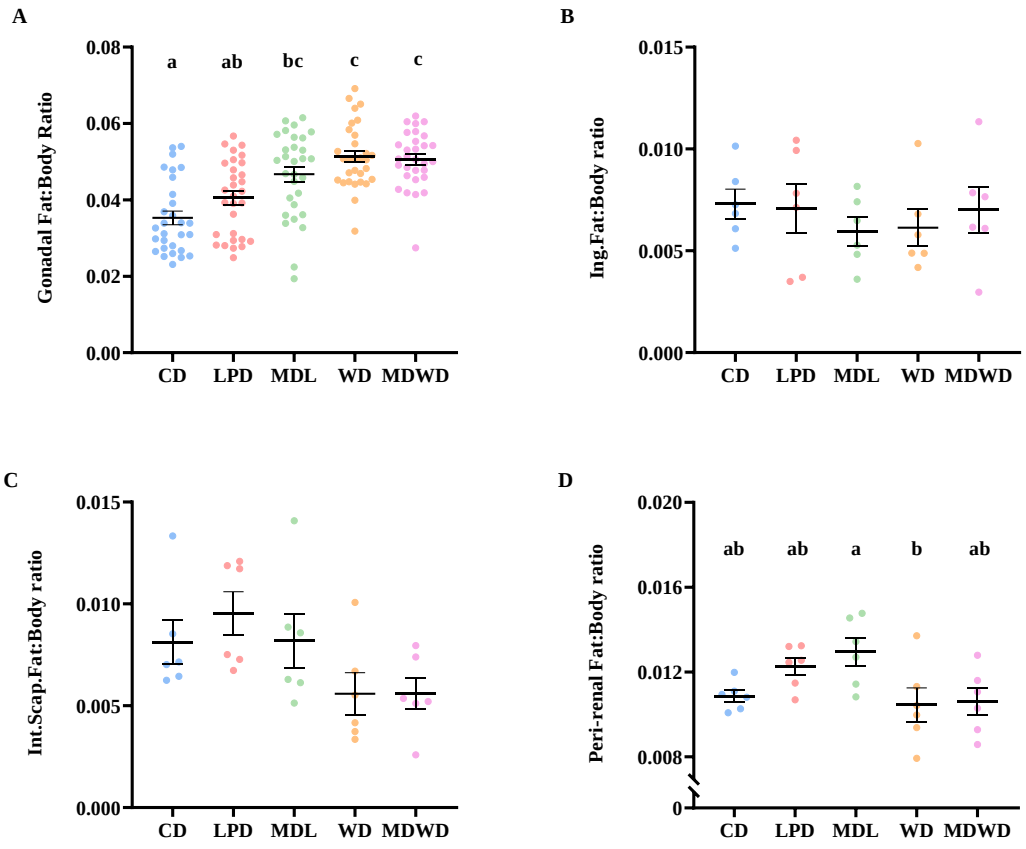

Figure 3.17: Sub-optimal diet affects fat pad to body weight ratios. Graph shows gonadal (A), inguinal (Ing. Fat; B), interscapular (Int. Scap. fat; C), and peri-renal (D) fat pads to total body weight ratios. D'Agostino and Pearson normality test was used followed by one-way ANOVA for normally distributed data with Tukey's post-hoc test (B, D). Kruskal-Wallis analysis was used with Dunn's post-hoc test for non-parametric data sets (A, C). Means are denoted by the black bars ± SEM. Different letters denote statistical significance (n=29-30/group; n=6/group in batch 4). *The n number per group in this analysis is 6, which may be a limiting sample size.

The total fat pad weight in batch 4 animals was significantly greater in MDWD males than CD males ($p \leq 0.05$; *Figure 3.18A*) while the total fat weight to body weight ratio was greater in MDWD than both CD and LPD ($p \leq 0.05$; *Figure 3.18B*).

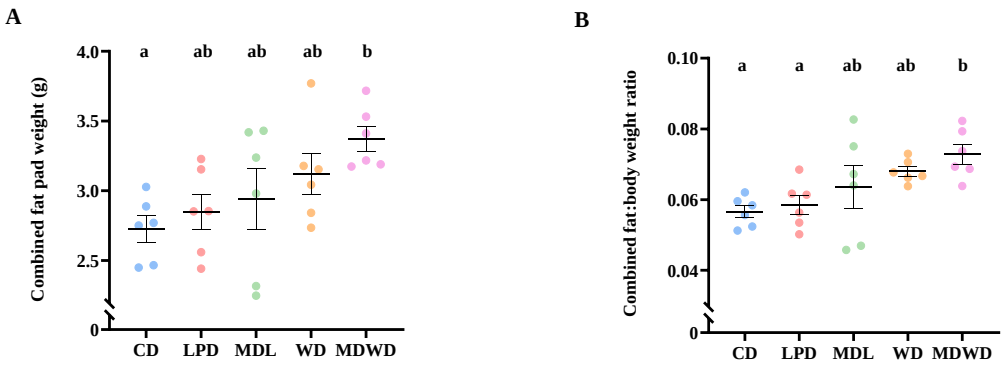

Figure 3.18: Sub-optimal diet influences fat deposition. Graph shows raw total fat pad weight (A) and total fat pad weight to total body weight ratio in batch 4 males. Individual points represent individual male mice. D'Agostino and Pearson normality test was performed followed by one-way ANOVA analysis with Tukey's post-hoc test. Means are denoted by the black bars ± SEM. Different letters denote statistical significance (n=6/group).

3.4.4 Sub-optimal diet does not influence seminal vesicle to body weight ratio or seminal fluid yield.

No significant difference was observed in seminal vesicle to total body weight ratio across all study groups (*Figure 3.19A*). Furthermore, the different dietary regimens do not have a statistically significant impact on the seminal fluid yield collected from seminal vesicles upon animal sacrifice (*Figure 3.19B*).

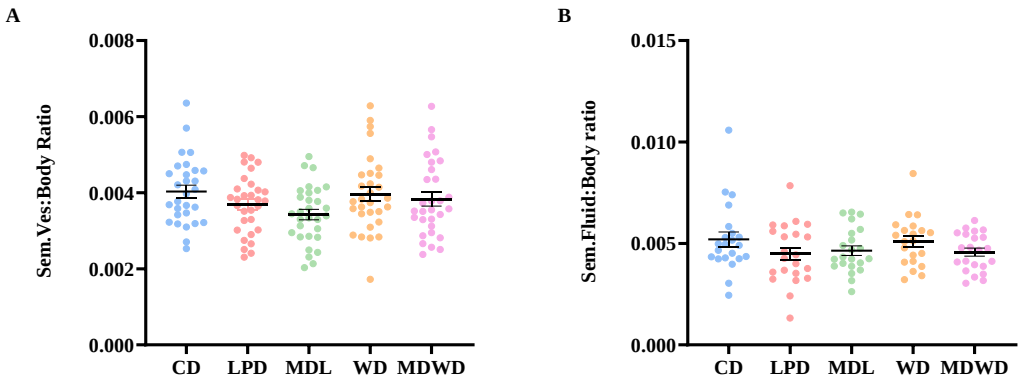

Figure 3.19: Effect of diet on seminal vesicle to body weight ratio and seminal fluid yield. Graphs represent the ratios of both seminal vesicle (n=30/group) and seminal fluid (n=22/group; total n=110) to total body weight at time of sacrifice. Individual points represent individual animals. Means are denoted by the black bars ± SEM. One-way ANOVA with Tukey's post hoc test was carried out for normally distributed data sets after passing the D'Agostino and Pearson normality test (A). Kruskal-Wallis with Dunn's post hoc test was used for data that failed the normality test (B).

3.4.5 Serum and liver metabolites are influenced by dietary intake.

3.4.5.1 Liver tissue assays

Using the values obtained from the appropriate respective standards, standard curves were plotted for cholesterol, free fatty acid (FFA), and triglyceride (TG). The concentration of each was determined from its respective plotted standard curve. Both WD and MDWD groups displayed a significantly increased level of liver tissue cholesterol compared to the control group CD ($p \leq 0.05$ and $p \leq 0.005$, respectively), whereas MDWD males displayed an increased liver tissue cholesterol than LPD ($p \leq 0.01$) and MDL ($p \leq 0.05$) as represented in *Figure 3.20A*. Additionally, FFA ELISA on liver tissue revealed that WD-fed males displayed significantly increased levels of FFAs compared to CD ($p \leq 0.05$), LPD

($p \leq 0.005$), and MDL ($p \leq 0.01$) as shown in *Figure 3.20B*. However, no significant differences were observed in total liver tissue TG levels between the study groups (*Figure 3.20C*).

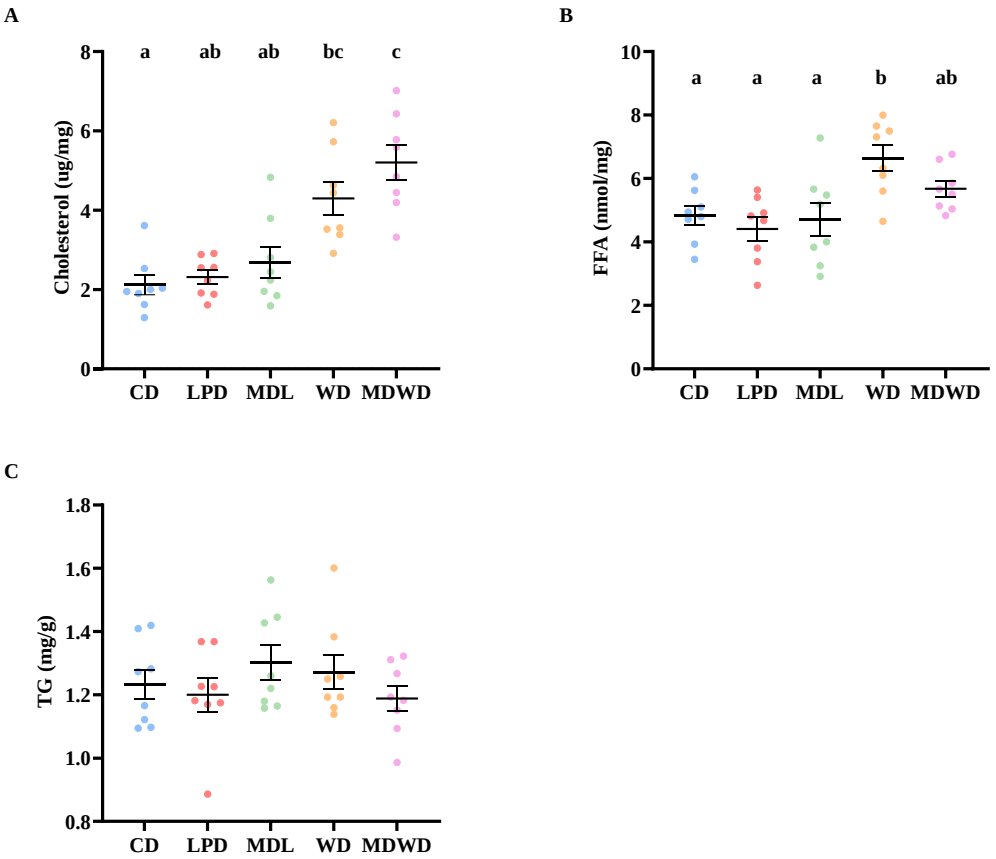

Figure 3.20: Sub-optimal diet impacts key lipid metabolism products in liver tissue. Graphs show liver tissue ELISAs of cholesterol (A), FFA (B), and TG (C) in male mice. Data is represented as mean ± SEM. D'Agostino and Pearson normality test was performed followed by one-way ANOVA with Tukey's post-hoc test (B) for normally distributed data and Kruskal-Wallis followed by Dunn's post-hoc test for non-parametric data (A, C); n=8/group.

3.4.5.2 Glucose and insulin

Standard curves for both serum glucose and insulin were plotted using the appropriate values of the respective standards, from which the concentrations of glucose and insulin were determined. The measurement of total glucose and insulin levels was carried out by means of ELISAs on the collected serum samples from the stud males. While the assays were performed on one batch, both assays showed no significant differences between glucose and insulin levels across all five groups (*Figure 3.21A, B*).

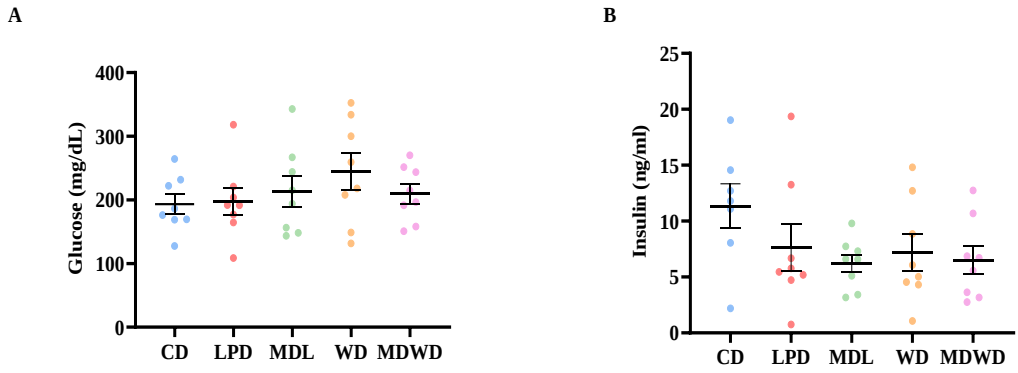

Figure 3.21: Serum glucose and insulin levels. Graphs show serum ELISAs of glucose (A) and insulin (B) in male mice. Data is represented as mean ± SEM. D'Agostino and Pearson normality test was performed followed by one-way ANOVA with Tukey's post-hoc test (A) for normally distributed data and Kruskal-Wallis followed by Dunn's post-hoc test for non-parametric data (B); n=8/group.

The glucose to insulin ratio is a useful metric utilised as a measure of insulin sensitivity. In this study, no significant differences were observed in the glucose to insulin ratio between the study groups (*Figure 3.22*).

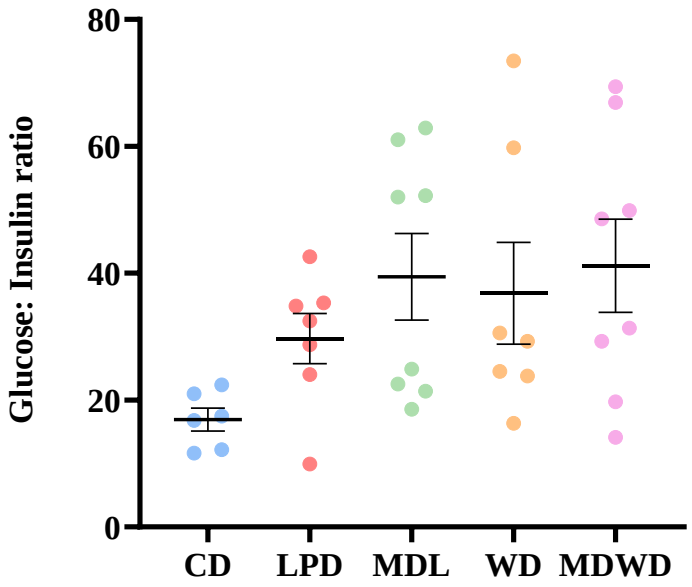

Figure 3.22: Serum glucose to insulin ratio. Graph shows the ratio of serum glucose to insulin in male mice. Data is represented as mean ± SEM. D'Agostino and Pearson normality test was performed followed by Kruskal-Wallis and Dunn's post-hoc test; n=8/group. Outliers were identified using GraphPad's ROUT method (3 outliers removed).

Table 3.1 summarises and highlights key findings from the assessment and analysis of stud male mouse weight and organ data. These findings include the body weight progression, weight at the end of the study period, organ and gonadal fat weight, and liver metabolite levels.

	Key findings
1	*Body weight progression is altered between weeks 7 and 15 of the study between the different batches of animals.*
2	*MDL mice were significantly lighter than CD at the end of the study period*
3	*WD and MDWD have significantly increased liver:body weight ratio compared to CD.*
4	*WD and MDWD have significantly increased gonadal adiposity compared to CD.*
5	*WD and MDWD have decreased crude carcass weight compared to CD, with no change to total body weight.*
6	*WD and MDWD have increased hepatic cholesterol levels compared to CD.*
7	*WD has increased hepatic FFA levels compared to CD, with no change in TG levels.*

Table 3. 1: Summary of key findings from the analysis of male weight and organ data.

3.4.6 Gut bacterial populations are influenced by diet.

Bioinformatics was carried out on the DNA sequencing data followed by statistical analysis using PRISM. Analysis of phylogenetic diversity was carried out using Faith's Phylogenetic Diversity (PD) metric, which is defined as the sum of the branch lengths of a phylogenetic tree connecting all species spanning the set (Faith, 1992). No statistical significance was observed between the groups with regard to phylogenetic diversity (*Figure 3.23A*). In addition, species evenness was assessed using Pielou's evenness index (Pielou, 1966). No significant differences were observed between the groups, however (*Figure 3.23B*).

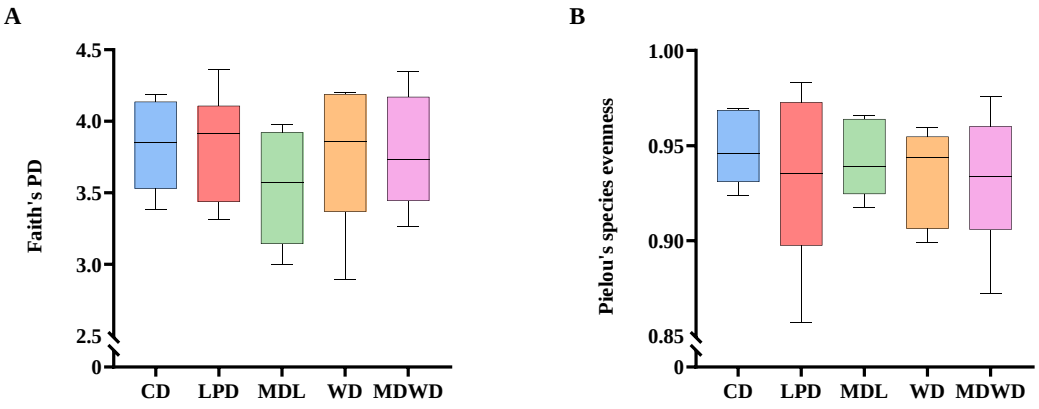

Figure 3.23: Impact of suboptimal diet on phylogenetic diversity and species evenness. Graphs represent Faith's phylogenetic diversity (A) and Pielou's species evenness (B) (n=6/group). Data is represented as mean ± SEM. D'Agostino and Pearson normality test was performed followed Kruskal-Wallis analysis with Dunn's post-hoc test. A p value ≤ 0.05 was deemed significant.

Analysis of phylum percentage distribution across groups yielded no significant differences between the groups in the analysis of the percentage distribution of

Bacteroidetes (*Figure 3.24A*), Firmicutes (*Figure 3.24B*), and Defferibacteres (*Figure 3.24C*). However, significant differences were observed in MDWD-fed males, who exhibited a greater percentage of gut Actinobacterium compared to the MDL group (*Figure 3.24D*; $p \leq 0.05$). Moreover, MDWD displayed a greater percentage of Proteobacteria of its total population count compared to the other study groups ($p \leq 0.0005$ vs CD, LPD, MDL; $p \leq 0.05$ vs WD) as presented in *Figure 3.24E*. Analysis of the percentage distribution of TM7, also known as Saccharibacteria, revealed a significantly decreased percentage in MDWD compared to LPD ($p \leq 0.05$) with no other significant differences across the study groups (*Figure 3.24F*). A significantly increased percentage of Tenericutes was observed in LPD-fed males compared to WD, of which no sample recorded a distribution of Tenericutes ($p \leq 0.005$; *Figure 3.24G*). Furthermore, a significantly greater percentage of Verrucomicrobia was observed in WD compared to both CD and LPD, both of which recorded no distribution of this phylum ($p \leq 0.05$; *Figure 3.24H*).

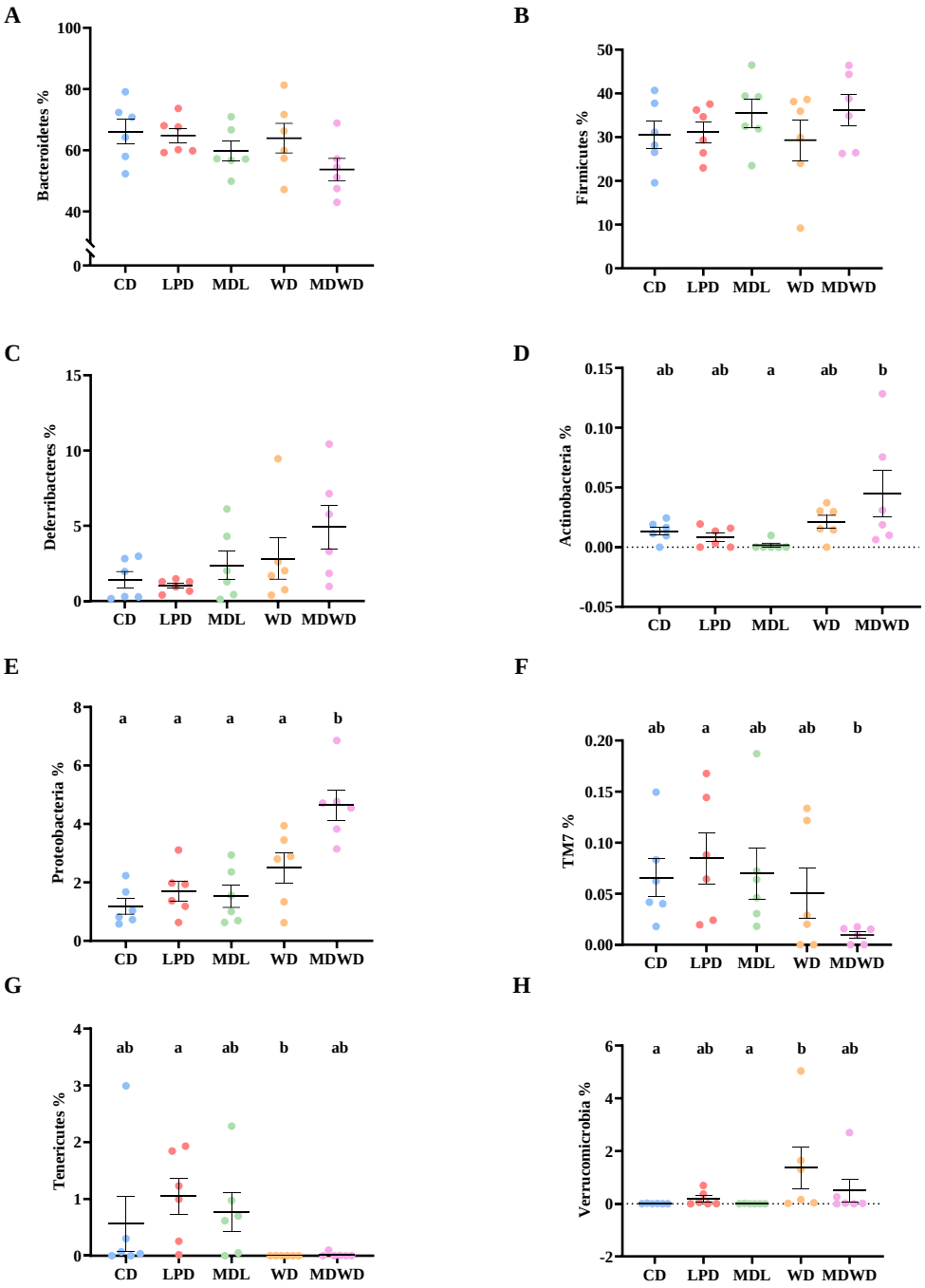

Figure 3.24: Graphs represent phylum distributions in the sequenced stool DNA (n=6/group). Data is represented as mean ± SEM. One-way ANOVA (+ Tukey's post-hoc test) was used for normally distributed data (A, B, E) and Kruskal-Wallis (+ Dunn's post-hoc test) was used for non-parametric data (C, D, F, G, H).

3.5 DISCUSSION

In this study, high fat diet feeding with or without supplementation did not cause an increase in total body weight in male mice, but increased gonadal adiposity while decreasing crude carcass weight, suggesting a potential loss of muscle mass at the expense of excess fat accumulation. Furthermore, both WD and MDWD displayed significantly increased liver to total body weight ratios compared to CD. Furthermore, both high fat diet groups WD and MDWD had significantly increased levels of liver cholesterol compared to CD. WD also had increased levels of hepatic free fatty acids compared to the control group. MDL, on the other hand, had significantly decreased total body weight at the end of the study compared to the control group, in addition to increased gonadal adiposity. Additionally, both low protein diet groups LPD and MDL had significantly decreased total kidney weight compared to CD.

The results show that while total weight progression over the course of the study period was not significantly altered, the progression trajectory tended to differ across the different groups. During the first seven weeks of the study, the weight progression of males in all five groups was observed to be not statistically different. However, the growth trajectory of each group varied from study week 7 onwards until time of sacrifice. While the changes in weight throughout the study period did not show any statistical significance, it is worth noting the unique patterns in which each diet group behaved. For instance, no significant differences in total body weight were observed between the groups between week 1 and week 7 of the study, however, MDL displayed significantly decreased body weight compared to CD on week 15 of the study. On week 23 of the study, both MDL and WD males exhibited significantly reduced body

weights when compared to CD males, suggesting that certain changes in anthropometric indices such as body weight are more amplified over longer-term feeding compared to short-term feeding. Weight progression in different batches was also significantly different in certain groups such as MDL, WD, and MDWD between week 7 and week 15 of the study. These inter-batch differences however normalise by the latter weeks of the study until the experiment's endpoint. It is difficult to attribute these changes throughout the study to one single factor, however, whether it is the dietary regimen itself or animal behaviour or metabolism. It stands as a limitation for this particular aspect of the study that animal food intake was not recorded, as it would have provided an extra index of insight into male mouse eating patterns and could explain the differences in weight progression between the batches between week 7 and week 15 of the study. The mechanisms behind the variations in body weight are not currently clear, but it is suggested that dietary fat may play a larger role in being a determining factor of variability observed in a C57BL/6 model due to the increased expression of 5-hydroxytryptamine (5-HT; serotonin) receptor genes in addition to the dopamine opioid signalling pathways in C56BL/6 mouse hypothalamus (Hu et al., 2018; Wu et al., 2022). In a recent study, Karlsson et al. (2021) showed that a disruption in the dopamine-opioid interactions is associated with obesity in men and can act as a mediator for excess energy intake (Karlsson et al., 2021). Whether dietary fat plays a more significant role in mediating excess energy intake is not conclusive, as low protein diets have been linked to inducing overeating and excess energy intake as a compensatory measure to increase protein intake in rodents (Blais et al., 2018) and potentially humans (Apolzan et al., 2007; Du et al., 2000; White et al., 2000). It is suggested

that the fat-free body mass is less influenced by dietary regimens than fat mass (Yang et al., 2014), which may imply that adiposity acts as a driver for the variability in growth trajectory when subject to different diets. Miller and German (1999) report that isocaloric low protein diet feeding (4 % casein) in a rat model significantly altered the animals' growth trajectories while not necessarily affecting the final adult size. This altered growth trajectory was characterised by the low protein diet group having lower growth decay, a lower instantaneous initial rate of growth, a lower maximum growth rate, and a significantly longer growth duration (365.1 days) compared to the control group (91.4 days) (Miller and German, 1999). The unique growth patterns observed despite the trajectories reaching a total body weight of no significant difference across certain groups is of interest. Between weeks 1 and 7 of the study, no differences in total body weight were observed. However, at weeks 15 and 23, MDL mice were significantly lighter than CD mice, with no difference observed between LPD and the control, suggesting a role for methyl donor supplementation on the decrease in total body weight after 15 weeks of feeding. While methyl donor supplementation has been shown to reduce fatty liver in rodents fed a high fat diet through modifying the fatty acid synthase promoter DNA methylation patterns (Cordero et al., 2013), there is little evidence on the impact of methyl donors on body weight progression when supplemented to a diet deficient in protein. It has been reported that methyl donor supplementation does not impact fat deposition in adipose tissue, but only hepatic fat accumulation in a HFD-fed rat model (Cordero et al., 2013). Methyl donor supplementation in this study resulted in similarly increased gonadal adiposity compared to the control group as WD, suggesting that the increase in adiposity

is driven by WD and not supplementation. The suggested mechanism behind the effect of methyl donors on the reduction of fatty liver development, as hypothesised by Cordero et al. (2013) involves epigenetic modification such as DNA hyper- or hypomethylation, thereby inducing changes in gene expression patterns. The authors observed an altered methylation pattern at the fatty acid synthase (FASN) gene promoter in response to both an obesogenic diet and to methyl donor supplementation (Cordero et al., 2013). Methyl donor supplementation also influences histone modifications and chromatin remodelling. Dahlhoff et al. (2014) demonstrated that, in a mouse model, methyl donor supplementation prevented the progression of liver steatosis by means of altering key 1-carbon metabolites such as S-adenosyl-methionine and S-adenosyl-homocysteine, and a significant increase in AMPK-α and increased activity of β-hydroxyacyl-CoA dehydrogenase (β-HAD), which suggests an increase in the flux through pathways of fatty acid oxidation (Dahlhoff et al., 2014). The authors also report a decreased level of liver fatty acids compared to the high fat diet group, which is also observed in the current study, as MDWD does not have a significantly increased hepatic fatty acid level compared to CD, whereas WD does. A number of studies investigate methyl donor supplementation and report its role in the promotion of metabolic health in overweight adolescents (Poursalehi et al., 2022) in addition to the prevention of transgenerational disorders and diseases such as obesity (Waterland et al., 2008) and fatty liver disease (Cordero et al., 2014).

While the total body weight at time of sacrifice of WD and MDWD males tended to be lower than that of CD males, no statistical significance was observed. When examining the crude carcass weights, however, WD and both the MDL

and MDWD groups displayed a relatively reduced weight compared to the control group. This may suggest a reduction in lean (muscle, skin and/or bone) mass with a relative increase in adiposity. However, this study did not collect an accurate measurement of carcass weight and muscle mass. A number of studies in mice have observed that high fat diet feeding resulted in muscle wasting, characterised by loss of muscle mass and weakness (Abrigo et al., 2016; Sishi et al., 2011; Sousa et al., 2021). The loss of body (lean mass) weight in methyl donor groups could potentially be related to the AMP-activated protein kinase (AMPK) pathway. In mice, AMPK activation has been shown to play a crucial role in regulating weight loss and protection against obesity (Pollard et al., 2019). Interestingly, the activation of AMPK through an AMPK-derived peptide resulted in reductions in body weight, glucose, and insulin levels, but increased liver weight and adiposity due to hepatic fat retention in an obesogenic mouse model (Chapnik et al., 2014). Looking at the role of methyl donors in a high fat diet mouse model, Dahlhoff et al. (2014) examined the role of supplementation on fatty acid oxidation and suggested that dietary methyl donors contribute to the activation of the AMPK pathway (Dahlhoff et al., 2014). The findings in this study could potentially be linked to high fat diet-induced muscle wasting combined with methyl donor-induced activation of AMPK, resulting in relative reductions in carcass weight. However, analysis of the AMPK pathway as well as hepatic and skeletal muscle gene expression is necessary to confirm this hypothesis.

The results also show that an HFD, with or without supplementation (i.e WD and MDWD) plays a primary role in increased liver weight. Mice from both the WD and MDWD groups exhibited a significantly greater liver to body weight

ratio compared to the other diet groups in this study. This finding is consistent with others in the literature, which report increased liver weight and liver steatosis in response to high fat diet feeding in rodent models (Rusdiana et al., 2022; VanSaun et al., 2009; Varani et al., 2022). The liver is a principal organ playing a crucial role in the metabolism of carbohydrates, proteins, and fat and its regulation. Excess fat leading to excess weight and ultimately obesity is a risk factor associated with a fatty liver. Steatosis, or fat build-up in the liver, while considered by some to be a benign accumulation of fat (Ekstedt et al., 2006; Musso et al., 2011), is now recognised as a manifestation of metabolic syndrome in the liver (Yki-Järvinen, 2010). Such increase in liver weight is often reported in cases of high fat diet feeding and is closely linked to non-alcoholic fatty liver disease (NAFLD) development, a disease that could potentially progress into nonalcoholic steatohepatitis (NASH), a more progressed type of NAFLD characterised by liver damage in addition to fat accumulation. It is important to note that there is yet to be an animal model used encompassing the entirety of the human NASH spectrum (Zhong et al., 2020). While our WD and MDWD models exhibit proportionally heavier livers compared to the control group CD, it is important to accurately label the phenotype as observed, as NAFLD diagnosis involves the assessment of steatosis in addition to other key histopathological findings observed in NAFLD such as hepatocyte degeneration, apoptosis, and inflammation. This data confirms findings in the literature reporting greater liver weights in response to high fat diet feeding, with or without the induction of obesity.

Interestingly, supplementation of WD with methyl donors and other vitamins and minerals does not ameliorate or work to counteract the impact of WD on the

liver or adiposity. Although methyl donor supplementation has been shown to help alleviate symptoms of fatty liver disease including slowing of the progression of liver fat accumulation in both rat (Codero et al., 2012) and cow models (Zang et al., 2019), additional evidence is needed in order to fully characterise the role of such supplementation in cases of fatty livers in human models, as evidence from randomised controlled trials in humans is inconclusive. For instance, Talari et al. (2022) found no significant differences in liver steatosis, and key liver enzymes such as alanine transaminase (ALT) between study participants with NAFLD given a vitamin B_{12} intervention and those given a placebo. The authors report some significant differences within the intervention group, such as liver steatosis, but not when compared to the placebo group (Talari et al., 2022). However, vitamin B_{12} supplementation did significantly decrease levels of serum homocysteine compared to the control group, suggesting potential beneficial effects on decreasing hyperhomocysteinemia in NAFLD cases (Talari et al., 2022).

Weight gain and liver fat deposition induced by a feeding of high fat diets has also been shown to be associated with an increased level of hepatic oxidative stress in addition to insulin resistance (Milagro et al., 2006). Preventative and alleviative measures to counteract the development of disease associated with increased body and liver weight with increased liver adiposity have been a subject of study as well. For instance, in addition to the role of supplementation – which is yet to be fully elucidated –, exercise has been shown to significantly reduce liver lipid accumulation, steatosis, and other high fat diet-associated symptoms such as obesity and hepatocyte hypertrophy (Huang et al., 2022). There remains, however, a gap in our understanding of the mechanisms behind

liver fat accumulation with or without obesity and the role of certain macronutrients such as proteins, carbohydrates, and fats in the regulation of liver fat content (Parry and Hodson, 2017). Some studies suggest a greater role of dietary fat in the development of diseases such as NAFLD than that of dietary fructose in rodent models (Jensen et al., 2018). However, it is difficult to confirm the role of any macronutrient without examining them in isolation, with a known baseline from which studies would be able to compare and attribute function to each macronutrient present, whether it is fat, carbohydrate, or protein.

The results also show that WD significantly increased kidney to body weight ratios compared to both LPD and MDL while MDWD increased the kidney to body weight ratios compared to both LPD and MDL with the addition of CD. While MDWD tends to have a greater kidney to body weight ratio compared to WD, the result is not statistically significant. Evidence regarding changes to kidney weight in response to sub-optimal diet is not strong in the literature and further investigation into renal physiology in response to diet is still needed to bridge the gap in our understanding. However, it has been shown that kidney weight increases with increased BMI (Tsuboi et al., 2017), and that the weight of kidneys from autopsies of obese individuals tend to be significantly greater (Mandal et al., 2012). Animal model studies have also found that high fat diet-induced obesity not only alters body and kidney weight, but also promotes renal damage characterised by tubular abnormalities, glomerular atrophy, necrosis and inflammation (Altunkaynak et al., 2008; Deji et al., 2009; Laurentius et al., 2019). In other studies that utilise a moderately high fat diet (MHFD) where fat (majority of which is lard, as opposed to the anhydrous milk fat used in the current study) constitutes 45 % of the total caloric supply (Castro-Rodriguez et

al., 2020) inducing significant obesity, body weight increase was reported without an increase in kidney weight. However, the kidney weight to body weight ratio was reported to have been reduced in response to the MHFD treatment (Sánchez-Navarro et al., 2021). These findings contradict results reported by other studies investigating the impact of an obesity-inducing high fat diet on renal integrity and function. Moreover, the results observed by Sánchez-Navarro et al. (2021) are contradictory to the ones in this study, where chronic feeding of a high fat diet (i.e WD and MDWD) lead to an increase in the kidney to body weight ratio compared to LPD, MDL, and CD, LPD, and MDL, respectively. However, it is worth noting that the high fat diets used in this study did not induce significant changes in total body weight at time of sacrifice compared to CD.

Furthermore, the results show that both WD and MDWD exhibit a significantly increased ratio of gonadal white adipose tissue weight to total body weight in addition to a significant relative decrease in crude carcass weight compared to CD. This agrees with findings in the literature, as high fat diet feeding is associated with increased fat deposition, as the homeostatic balance between energy intake and expenditure is tipped in favour of intake. An increase in gonadal adiposity is reported in response to variations of high fat diets, such as the modified standard American diet (mSAD) used by Chehade et al. (2022). The mSAD consists of 12.3 % protein (12.2 % kcal), 49.5 % carbohydrate (49.2 % kcal), and 17.3 % fat (38.6% kcal), and high fructose corn syrup being the main source of carbohydrates (Chehade et al., 2022). A number of saturated and unsaturated fat sources were used for this diet including palm oil, corn oil, soybean oil, lard, cottonseed oil, beef tallow, and anhydrous milk fat (Chehade

et al., 2022). Significant increases in white adipose depots in the gonadal region were also reported in response to a cafeteria HFD in a female obese rat model (Amengual-Caldera et al., 2013). On the other hand, minimal differences were observed in the different adipose tissue pads collected from batch 4 of male mice. No significant differences were observed in intrascapular and inguinal fat pads across the different groups in this specific batch. However, MDL mice exhibited a relatively increased peri-renal fat to body weight ratio compared to WD, converse to what is expected and what is found in the literature. However, it is important to note that the sample size for this cohort in this experiment was n=6 per group, totalling 30 males across all five study groups. Fat pads were not collected from animals of previous batches and collection only began after batch 4 mice were maintained at the animal unit. Therefore, the limited sample size may not necessarily be representative of a population or the total n number of this study, and the results need to be interpreted with prudence and caution. A larger sample size with the same experimental design and parameters is recommended for a more accurate analysis of deposition of adipose tissue.

The results show a significant increase in gonadal adiposity, a relatively lower crude carcass weight, and no change in total body weight at time of sacrifice in the high fat diet groups, WD and MDWD. No significant increase in body weight was observed, indicating that WD and MDWD do not induce obesity in the C57BL/6 mice used in this study. These findings, when observed in isolation provide a limited understanding of the impact of sub-optimal diets on the physiology of adult male mice. However, when observed altogether, these findings suggest that in response to the high fat, high sugar diets (WD and MDWD), fat deposition occurs at the expense of lean mass. Further investigation

in the form of either percutaneous liver biopsy (Starekova and Reeder, 2020) or a non-invasive MRI (Lv et al., 2018) is recommended to confirm whether fat deposition is underlying the increase in liver to body weight ratio. The findings indicating a loss of lean mass in favour of fat deposition is also suggestive of what is known as 'normal weight obesity', or NWO. NWO is a more difficult candidate type of obesity to diagnose, as BMI in NWO is normal. However, normal weight obesity is also characterised by a high fat percentage and is closely associated with cardiometabolic risk factors (Franco et al., 2016; Mohammadian Khonsari et al., 2022). The emergence of this candidate syndrome is relatively recent, and the amount of evidence available both in animal models and humans is not currently sufficient. However, recent studies have investigated generating normal weight obesity models in rodent models exposed to a high fat diet since early life (Maejima et al., 2020). Furthermore, some human studies have previously investigated the relationship between NWO and CVD risk factors (De Lorenzo et al., 2006). However, the evidence is still lacking and more studies into normal weight obesity and its underlying mechanisms as well as its evolution into high-BMI obesity are of importance.

In addition to the increase in adiposity and relative decrease in crude carcass mass, both WD and MDWD exhibit significantly increased levels of liver tissue cholesterol compared to CD. Additionally, a significantly increased level of FFAs in the liver was observed in WD males compared to CD, LPD, and MDL. While MDWD had a relatively greater level of liver FFAs compared to the other study groups with the exclusion of WD, the differences weren't statistically significant. Additionally, the level of triglycerides in hepatic tissue was not observed to be significantly different between the study groups. Fatty acid

metabolism occurs in the liver, where under normal circumstances, the liver exports triglycerides as very low-density lipoproteins (VLDL) particles that serve to deliver fatty acids to muscle and adipose tissue (Alves-Bezerra and Cohen, 2017). The excess accumulation of free fatty acids in the liver is believed to be a risk factor for fatty liver disease. Excess amounts of FFAs in the liver can cause the formation of several toxic metabolites such as ceramides, diacylglycerols, and lysophosphatidylcholine which can lead to hepatocyte injury (Neuschwander-Tetri, 2010). Furthermore, said metabolites can act as disruptors to the mitochondrial oxidative respiratory chain, leading to an increase in reactive oxygen species (ROS) production and ROS-mediated oxidative stress in the liver (Paris et al., 2001). This is often accompanied by an increase in liver cholesterol levels, which contribute to oxidative and endoplasmic reticulum stress which in turn promote the release of pro-inflammatory cytokines that elevate the risk of hepatocyte apoptosis (Püschel and Henkel, 2018). An increased level of TGs in the liver is strongly believed to be associated with NAFLD and markers of other diseases such as diabetes and hyperlipidaemia and insulin resistance (Kawano and Cohen, 2013). No significant differences being observed in both serum glucose and insulin in this study indicates a preliminary absence of insulin resistance or an indication of diabetes. However, it is worth noting that both glucose and insulin assays were carried out on serum taken at time of sacrifice and not at a consistent fasting level.

While the elevated TG levels are considered to be a “hallmark” of NAFLD by most (Tomizawa et al., 2014), some studies have suggested that such a level of TGs in the liver may actually be the result of a protective mechanism against

FFA-induced lipotoxicity (McClain, Barve and Deaciuc, 2007). Listenberger et al. (2003) found that cultured cell viability is not decreased in response to an increase in TG after exposure to unsaturated FFAs, whereas cellular apoptosis markedly increased in response to incubation with saturated fatty acids (SFAs) with no TG accumulation. Another study suggests that TGs may be a mere "innocent bystander" in the development of NAFLD, while FFAs and their metabolites are the drivers of the disease's progression (Liu et al., 2016). Emerging evidence indeed suggests the role of hepatic TGs as the "good fat" and the role of hepatic FFAs as the "bad" fat (Alkhouri et al., 2009). Normal levels of TGs coupled with increased levels of FFAs in the liver were interestingly observed in cases of alcoholic liver disease (ALD) but not in NAFLD (Mavrelis et al., 2007). Elevated levels of cholesterol in both WD and MDWD in addition to elevated levels of liver FFAs in WD and the relative increase in liver size may be indicative of abnormal lipid metabolism as a precursor to fatty liver disease. However, the normal levels of liver TG suggest that the excess fat is not being stored in the liver, but in adipose tissues as was observed in the two groups. However, it is possible that WD males are in the early stages of NAFLD and therefore do not exhibit the full spectrum of associated symptoms. It is of interest to examine the effects of WD chronically and to assess the level of hepatic steatosis in the WD males. Unfortunately, this study does not examine the degree of liver steatosis and therefore no accurate diagnosis could be made.

The supplementation of methyl donors to WD seems to ameliorate the increased levels of FFAs in the liver. This finding is in line with others in the literature, as the supplementation of methyl donors has been found to reduce fatty liver by

modifying the FA synthase DNA methylation profile (Cordero et al., 2012) and partially reverse excess lipid accumulation in the liver (Cordero et al., 2011). Further investigation into liver function enzyme activity, hepatic fatty acid profiles, and serum levels of FFAs and TGs is crucial for a more accurate characterisation of the effect of sub-optimal diet and methyl donor supplementation on adult male metabolic health in this study.

The gut microbiome is significantly involved in its host's metabolic and immune systems, their development, and risk of disease. Therefore, the recent emergence of studies focused on characterising the role of the microbiome to its pathogenesis and how it interacts with external and environmental factors is unsurprising. Recent research into the influence of dietary intake on metabolic and physiological health and overall wellbeing has identified the gut microbiome as another factor affected by the changes in what individuals consume as part of their diets. The composition of the gut microbiome is unique to and differs between individuals, and its dysfunction has been linked to a lack of appropriate nutritional intake among other factors (Murphy et al., 2015).

Analysis of sequenced lower gut stool DNA has identified a total of 8 phyla across all study groups, the most prevalent of which are Bacteroidetes, which is known to comprise around 50 % of the gut microbiome (Ettinger, 2017). While no significant differences were found between the groups in the percentage distribution of Bacteroidetes, other phyla such as Proteobacteria were observed to be significantly increased in MDWD compared to the other study groups. Similarly, Andújar-Tenorio et al. (2022) found an increase in Proteobacteria in their high fat diet mouse model. Interestingly, while WD in this study does have a relatively higher percentage of Proteobacteria compared to CD, the difference

is not statistically significant. However, the addition of methyl donors to this high fat high sugar diet resulted in a significant increase in the Proteobacteria population. It is unknown whether this increase is specifically caused by the supplementation of methyl donors, but methyl donors have been shown to alter the composition of gut microbiome populations. In most cases, methyl donor supplementation is associated with positive and restorative outcomes such as altering offspring gut microbiota by increasing both diversity and uniformity with an increase in the phylum Firmicutes to result in improved weaning weights in a porcine model (He et al., 2021) for example. However, the evidence in the literature is conflicting with regard to the role of methyl donor supplementation, as some studies report that methyl donor supplementation results in the alteration of gut microbiota that exacerbated a septic mortality (Yu et al., 2021).

An increase of Proteobacteria, as observed in MDWD, is associated with different intestinal and extraintestinal diseases in humans such as inflammatory bowel disease, asthma, and chronic obstructive pulmonary disease (Rizzatti et al., 2017). In murine studies, interestingly, an increase in Proteobacteria numbers has been shown to be indicative of gut-liver axis induced liver steatosis, a known marker of NAFLD (Vasques-Monteiro et al., 2021). Another interesting finding that Vasques-Monteiro et al. (2021) reported is gut dysbiosis and fatty liver even without the presence of overweight in their high fructose C57BL/6 model. It is crucial to pose the question of how well the murine microbiome translates to the human microbiome. The mouse is a very reliable study model in many different aspects, as highlighted by the wealth of studies in the literature. With regard to the microbiome, however, as with other aspects of physiology and anatomy, it is important to acknowledge the degree of

dissimilarity between the model and the system that is modelled, as even well-controlled microbiota studies show a degree of variation due to different factors such as the experimental setup, genetic backgrounds, and environmental conditions (Nguyen et al., 2015). Therefore, when reporting observations and findings, it is of importance to proceed to making conclusions with caution, as they may not always be translatable to humans.

3.6 CONCLUSIONS, STRENGTHS, AND LIMITATIONS

One of the limitations of this study was the time constraints that could not allow for further investigation into the impact of suboptimal diet on adult male metabolic health and growth profile. This study does not measure animal food consumption either on a weekly or a daily basis and does not measure the levels of certain hormones associated with energy intake such as leptin and ghrelin. Additionally, some of the analyses in this study feature a limited sample size such as the case of the collected fat pads from batch 4, where the sample size was 6 per group. This could potentially be a limiting factor and therefore interpretation should be made with caution. Furthermore, another limitation to this study is that blood was only collected from the animals at time of sacrifice, introducing variability to serum assays, as the results are not reflective of fasting levels of metabolites. Due to time constraints, this study was not able to explore lipid profiles and hepatic gene expression or liver histology to further examine potential liver steatosis or development of fatty liver. This study, however, manages to highlight the impact of suboptimal diets such as low protein and high fat (with or without supplementation) on mouse anthropometrics, adiposity, and hepatic metabolite levels in addition to growth trajectory over the course of 25 weeks. This study also utilises 16S DNA sequencing to determine the impact of dietary intake on gut microbiota. Another strength of this study is the use of multiple batches of mice, yielding a sample size of 30 animals per group over four batches with the same dietary regimens implemented.

CHAPTER FOUR: DIETARY INTAKE AND ITS IMPACT ON TESTICULAR AND EPIDIDYMAL HISTOLOGY AND GLOBAL TESTICULAR GENE EXPRESSION

4.1 INTRODUCTION

With recent trends of declining sperm count (Carlsen et al., 1992; Levine et al., 2017) paralleling those of increased prevalence of obesity in men, studies have aimed to characterise the effect of overnutrition on different reproductive parameters. However, inconclusiveness and controversy exist in the literature, likely due to inherent confounding factors in human studies. However, some studies report a negative correlation between increased BMI and semen parameters in men, including decreased sperm count (Sermondade et al., 2013), concentration (Belloc et al., 2014; Jensen et al., 2004), and motility (Hammoud et al., 2008b). Impairment of hormonal profiles have also been attributed to increased weight, notably a decrease in levels of testosterone, inhibin B and an increase in levels of oestradiol in males (Chavarro et al., 2010; Jensen et al., 2004). Overnutrition-induced endocrine dysregulation is also thought to exacerbate impairments in spermatogenesis and sperm quality (Hammoud et al., 2008a) in addition to increased sperm DNA damage (Dupont et al., 2013; Kort et al., 2006).

Overnutrition by means of consumption of high fat and high sugar diets has also been shown to impair mitochondrial respiration through reduction in adenosine triphosphate (ATP) synthesis and increased oxidative damage by means of

elevated levels of reactive oxygen species (ROS) (Ferramosca et al., 2016; Chianese and Pierantoni, 2021). Furthermore, excess accumulation of adipose tissue results in the increased activity of the enzyme aromatase which converts testosterone to oestradiol (Zhao et al., 2014). This has also been associated with Leydig cell mitochondrial dysfunction (Rovira-Llopis et al., 2017; Lunetti et al., 2021), ultimately leading to increased ROS production, thereby adversely affecting germ cells and mature spermatozoa (Saez Lancellotti et al., 2010; Bobjer et al., 2012). Such overnutrition has also been shown to alter testicular gene expression to potentially affect crucial biological processes such as the blood-testis barrier (BTB), immunity, inflammation, and DNA methylation in gametes (Zhang et al., 2023). Furthermore, altered testicular gene expression was also linked to an HFD-induced dysbiosis of the gut microbiome in a mouse model, which subsequently impairs spermatogenesis (Ding et al., 2020). The authors suggest that such effects are mediated by an observed increase in the level of endotoxin which correlates with epididymal inflammation, thereby impairing sperm motility (Ding et al., 2020). While a number of studies in animal models have reported altered gene expression profiles in the testes in response to dietary intake such as high fat diets (Feng et al., 2021; Jarvis et al., 2020), the pathways and mechanisms by which HFD is associated with having a deleterious role on male physiology and reproductive fitness are still relatively unknown (Jarvis et al., 2020).

Compared to the impact of overnutrition, the role that undernutrition plays in regulating spermatogenesis, as well as sperm and semen quality, is relatively under-investigated, despite the state of undernutrition or malnutrition being widely prevalent in today's world (WHO, 2021). However, over the past two

decades, more studies focused on investigating the role of undernutrition and sub-optimal dietary intake on male reproductive fitness have emerged. Some evidence remains contradictory, however, especially with regard to protein in diets (Ferramosca and Zara, 2022). In a 2004 paper investigating the effects of undernutrition on sexual function in a rat model, Santos et al. (2004) reported that the impact of undernutrition on reproduction is less related to low protein in the diet and instead more to caloric restriction. On the other hand, diets low in protein have been identified as potential risk factors for male-factor infertility, as they have been linked to significantly reduced testis, epididymis, and seminal vesicle weights in addition to decreased levels of serum testosterone in rat models (Ajuogo et al., 2020). Furthermore, paternal low protein diet can influence offspring development in an epigenetically mediated manner, as was shown by Carone et al., (2010). In a low protein diet mouse model, Carone et al., (2010) discovered that paternal low protein diet feeding resulted in an increase in the relative levels of saturated free fatty acids and saturated (and monounsaturated) triglycerides in the offspring. This was also reflected in gene expression profiles corresponding to perturbed cholesterol and lipid metabolism transcription factors such as sterol regulatory element binding proteins (SREBP) and peroxisome proliferator-activated receptor alpha (PPARα), two key regulators in cholesterol synthesis and fatty acid oxidation, respectively (Carone et al., 2010).

With evidence in the literature associating diets such as low protein diets with altered DNA methylation status, the role of methyl donor supplementation is brought into question. Previous studies have examined the role of methyl donor supplementation (and/or deficiency) such as folic acid in avian (Wu et al., 2019)

and murine (Lambrot et al., 2013; Swayne et al., 2012) models. Folate supplementation in a paternal avian model revealed significant alterations in offspring liver metabolites involved in lipid and glucose metabolism, with altered glycolysis, pentose phosphate, and sucrose metabolism across different generations (Wu et al., 2019). Lambrot and colleagues discovered that a low intake in dietary folate resulted in decreased pregnancy rates in mice compared to controls, with a two-fold increase in post-implantation embryonic loss (Lambrot et al., 2013). Swayne et al. (2012) reported that mice weaned to a folate-deficient diet exhibited a greater level of DNA fragmentation compared to control diet-fed mice. Similar observations have been made in humans, as randomised controlled trials have reported reductions in sperm DNA damage after folic acid supplementation (Boonyarangkul et al., 2015; Raigani et al., 2014). The impact of methyl donors has – to a lesser extent – been studied on the testis in response to dietary modification. We have recently shown that supplementing a low protein diet with methyl donors in a C57BL/6 mouse model results in a decreased relative expression of the pro-apoptotic genes Bcl-2 associated X-protein (*Bax*), Caspase 1 (*Casp1*), and the Fas cell surface death receptor (*Fas*) (Morgan et al., 2020). In addition, we observed that methyl donor supplementation resulted in reduced telomere lengths but with increased testicular telomerase activity. Telomere length progressively increases from spermatogonial stem cells (SSCs) to spermatozoa throughout spermatogenesis (Ozturk, 2015). As cells with the longest telomeres possess the lowest levels of telomerase activity (Tanemura et al., 2005), the findings reported by Morgan et al. (2020) suggest a potential overcompensation of telomerase in the methyl donor group to promote the elongation of premeiotic spermatocyte telomeres

(Ozturk, 2015). The number of studies examining the influence of methyl donor supplementation on the testicular environment, however, is very limited, and further investigation is needed to appropriately characterise the role of methyl donors. Alterations to sperm DNA integrity, motility and vitality, in addition to changes in testicular gene expression are indicative of a change in the testicular environment itself. This includes changes affecting the process of spermatogenesis in addition to cell subpopulations in the testis. To better define the influence of dietary intake on the testicular environment, it is important to understand the fundamentals of the testicular environment and the process of spermatogenesis, and the cell populations present within the seminiferous tubules.

As different aspects of our physiology are impacted by external and lifestyle factors, the observed role of suboptimal diet on sperm quality may point to underlying alterations in testicular physiology and gene expression, leading to impaired spermatogenesis. Significantly reduced spermatogenic cell proliferation, with significantly decreased seminiferous epithelium height was observed in testes of a high fat diet rat model (Campos-Silva et al., 2015). Similarly, observing alterations in morphological seminiferous tubule parameters, others also reported that HFD-fed sedentary rats exhibited significantly reduced seminiferous tubule diameter and increased frequency of atypical tubules (Ibáñez et al., 2017). In line with these observations, a more recent study reported stark morphological changes in HFD-fed rats, highlighting atrophic tubules with diminished spermatogenic cells (Yang et al., 2018). However, evidence of the correlation between dietary intake and such observations in the literature is still inconclusive, as some studies have reported

contrasting findings. For instance, despite observing significantly reduced sperm parameters, Vigueras-Villaseñor et al. (2011) reported no significant differences in seminiferous tubule area or cytoarchitecture in 35 % HFD-fed rats. In a study investigating a long term (5 months) HFD feeding regimen, mouse testes showed a dramatic deterioration in histoarchitecture characterised by seminiferous tubule disruption, vacuolisation, and tubule atrophy (Ghosh and Mukherjee, 2018). Furthermore, it has been reported that in Sprague-Dawley rats, HFD feeding for 9 weeks resulted in disrupted adhesion between spermatogenic and Sertoli cells, with impairment to cell junctions between Sertoli cells (Liu at al., 2014). Furthermore, Liu et al. (2014) observed Sertoli cell atrophy. Similarly, in a C57BL/6 mouse model fed a high fat diet for a period of 10 weeks, disrupted cell adhesion between germ cells and Sertoli cells was observed in addition to seminiferous epithelial atrophy (Fan et al., 2015). Additionally, the study reports a compromised BTB in the same model, which coincided with impaired sperm parameters (Fan et al., 2015). Other studies observed significant reductions in Sertoli cell numbers in Wistar rat testes after only a 6-week HFD feeding regimen, in addition to significantly reduced tubule and lumen diameter but no change to the number of spermatogonia (Matuszewska et al., 2020). Since the reporting of the different deleterious effects suboptimal diet consumption has on reproductive fitness, several studies have investigated means by which such effects can be reversed or ameliorated. For instance, antioxidant supplementation has been shown to impart a protective role, as, compared to HFD, antioxidant supplementation has been shown to result in significantly increased sperm count, motility, viability in addition to normal morphology in rodents (Mortazavi et al., 2014). Furthermore, the study

reports improved testicular histology, with significantly increased spermatogonia and Sertoli cells (Mortazavi et al., 2014).

Although relatively less extensively explored than overnutrition, the impact of undernutrition on testicular histology, specifically LPD, has also been investigated. One study evaluating rats fed a LPD (9 % protein) found normal testicular histology with no alterations to seminiferous tubule diameters compared to controls (Glass et al., 1979). This result was replicated years later using 10 % protein diets (Vawdaw and Mandlwana, 1990). However, a further restricted 5 % protein LPD was shown to cause a significant decrease in seminiferous tubule diameter in addition to significantly reduced numbers of Sertoli cells and spermatogonia (Vawdaw and Mandlwana, 1990). In contrast, recently Morgan et al. (2020) reported significantly increased seminiferous tubule cross-section area, perimeter, and epithelial area in 9% LPD-fed mice compared to the control diet group (18 % protein) or methyl-donor supplemented LPD-fed mice. Despite these morphological alterations, no differences in expression of specific cell markers for spermatocytes, spermatids or Sertoli cells were observed. LPD-fed males did however display significantly decreased seminiferous tubule apoptosis (Morgan et al., 2020).

While there is evidence in the literature indicating an influence of dietary intake on testicular function, relatively little is known about the impact on the epididymis. The epididymis plays an essential role in the sperm cell cycle, as spermatozoa complete their maturation in the epididymis, where they also acquire their motility and fertilisation capacity (Breton et al., 2019). Recently, Li et al. (2022) showed that a high fat diet can cause epididymal oxidative stress in a mouse model, potentially leading to impairments in sperm maturation and

fertility (Li et al., 2022). A more recent study also examined the effect of high fat diet feeding on male Wistar rats for a period of 5 weeks, citing increased oxidative stress in all epididymal segments in addition to increased apoptosis in both the corpus and caudal regions of the epididymis (Falvo et al., 2023). The evidence in the literature is, however, inconclusive due to the lack of sufficient evidence examining testis and/or epididymis histology and function in response to dietary modification and supplementation.

4.2 AIMS AND OBJECTIVES

While studies have examined the impact of paternal dietary intake on fertility and subsequent offspring development, the influence dietary modification – with or without supplementation – on the testicular environment itself warrants further study. This study hypothesises that a sub-optimal dietary intake regimen could impair the testicular environment as a whole, which subsequently impacts reproductive fitness, spermatogenesis, and sperm integrity. Furthermore, this study hypothesises that methyl donor supplementation can – to an extent – counteract some of the adverse effects caused by sub-optimal diet consumption. In order to adequately characterise the impact of suboptimal diet on testicular and epidydimal histology and understand the mechanisms underlying this impact, this study aims to:

- Define the impact of suboptimal diet on testicular and epididymal cytoarchitecture.
- Examine the influence of dietary intake on global testicular gene expression patterns.
- Determine the effect of suboptimal diet on seminiferous tubule cell populations.

4.3 METHODOLOGY

4.3.1 Animal treatments

As detailed in *section 2.1*, male mice were randomly assigned to five different groups each fed a different experimental diet (n=8 per group within each batch). The composition of all the diets fed to the stud males are listed in *Appendix A1*. Testes were weighed and processed either for histological assessment following fixation or snap freezing whereas epididymides were either fixed in formalin as described in *section 2.3*.

4.3.2 Histology

Three sets of testis sections were generated for each sample (see *section 2.4* for detailed description of tissue cutting process), with each being used to assess a separate feature. One set was stained with haematoxylin and eosin (H&E) for the assessment of gross testicular morphology (see *section 2.3.1* for a detailed description of staining procedure), and two sets for immunohistochemistry (IHC) staining (see *section 2.3.2* for full staining protocol details). From each embedded epididymis, sections were taken to be used for morphological assessment by H&E staining.

4.3.2.1 Haematoxylin & Eosin staining

For morphological assessment, the slides were stained with haematoxylin and eosin (H&E). The sections were de-waxed in xylene (Fisher Scientific) then rehydrated in decreasing concentrations of ethanol (100 %, 95 %, 70 %; Honeywell) as detailed in *section 2.4.1*. The slides were then stained with Haematoxylin and Eosin (H&E) as detailed in *Section 2.4.1*. Cover slips were

mounted on the slides with DPX (Sigma-Aldrich®). The slides were left to dry overnight in a fume hood. The staining protocol is summarised in *Table 2.1*.

4.3.2.2 Immunohistochemistry staining

A set of 5 µm testis sections were dewaxed using xylene as described in *Table 2.1* and rehydrated into PBS. Sections were then equilibrated at room temperature in 10 mM citrate buffer (diluted from a 0.1 M stock solution made from tri-sodium citrate (dihydrate) (Sigma-Aldrich®) with distilled water; pH = 6.0). The slides were equilibrated in an antigen retrieval chamber for 5 to 10 minutes at room temperature as described in detail in *section 2.4.2*. To prevent non-specific binding of antibodies or other reagents to the tissue, a blocking solution was prepared and applied to the slides as detailed in *section 2.4.2*. As previously discussed, one limitation to this experiment is the absence of a non-specific IgG antibody control to further add to the accuracy and integrity of the resultant data.

As detailed in *section 2.4.*2, secondary antibodies were applied before the slides were incubated at room temperature for 1 hour, protected from light. After incubation, the slides were washed twice in PBST for 5 minutes each. The secondary antibody Alexa Fluor 488 nm (GREEN) Streptavidin conjugate (Invitrogen™, S-32354) was applied to the slides with SOX-9 at a concentration of 1:200 for 30 minutes at room temperature before undergoing two more 5-minute PBST washes. Finally, the slides were mounted with Antifade Mounting Media with DAPI (Vectashield®) before being covered with glass coverslips and sealed with transparent nail polish. The slides were then left to dry and were refrigerated overnight before use as described in *section 2.4.2*.

For the detection of PLZF in the testis, the staining had to be run separately from SOX-9 in order to avoid antibody cross-reactivity, as both primary antibodies were raised in the same species (rabbit). A different set of testis sections was used with a rabbit polyclonal Anti-PLZF antibody - N-terminal (Abcam®, ab189849) as a primary antibody (1:3000) in 5 % BSA and 10 % NGS. The staining protocol was carried out for SOX-9 and PLZF as it was for DDX4. The primary antibodies used in addition to their target cells are summarised in *Table 4.1*.

Antibody	**Target**	**Cell type**	**Channel**
Anti-DDX4 antibody (mouse anti-DDX4/MVH (Vasa)	DDX4	Germ cells	TxRed (Red)
Rabbit anti-SOX-9 Antibody	SOX-9	Sertoli cells	FITC (Green)
Rabbit polyclonal Anti-PLZF antibody	PLZF	Spermatogonia	TxRed (Red)

Table 4. 1: Primary antibodies and their targets used in this study.

4.3.2.3 Imaging

Images for H&E sections were taken using a Leica DMRB microscope, which enabled the scanning of an entire slide to create high resolution images at 20x magnification. The open-source image processing package Fiji was used for image analysis. For each testis sample, an average of 50 seminiferous tubule cross-sections were analysed. The tubules were analysed for total tubule area (µm²), total perimeter (µm), lumen area (µm²), epithelial area (µm²), and lumen

percentage (%). The area of the epithelium was calculated by the subtraction of the lumen area from the total area. Furthermore, the lumen percentage was worked out from the ratio of lumen area over the total area ((lumen area/total area) *100). The measured parameters in seminiferous tubules are highlighted in *Figure 2.2*. After imaging and measurement using Fiji, the slides were then unblinded and the data analysed.

For the analysis of testicular SOX-9, DDX4 and PLZF expression, sections were viewed and imaged using a Nikon Eclipse 90i fluorescent microscope with the mercury-fiber illuminator Nikon Intensilight C-HGFI and the Hamamatsu ORCA-ER Digital camera Model C4742-80. Images were taken at 10 x magnification, capturing an average of about 5-6 tubules per field. Close-ups were taken at 20 x magnification, capturing about 2-3 tubules per field. Image viewing and exposure was captured using Volocity software. Images were captured on three channels, both separately and merged with their respective exposure settings: DAPI (Blue; 200 ms), FITC (Green; 220 ms), and TxRed (Red; 150 ms). To retain consistency, all images of all samples were taken using the above exposure settings.

Grid imaging was carried out, where the stage was moved in a fixed pattern to ensure images of the entirety of each tissue were taken. Cut-off points were marked using landmark tubules and artifacts in a microscopic field. Images from each grid were generated and saved for analysis. Total numbers of SOX-9 positive cells, DDX4-positive cells and PLZF-positive cells were counted and analysed for cell distribution across tubules, tubule size, as well as positive cells in relation to each other. SOX-9 and PLZF cells were manually counted in each sample whereas DDX4-positive cells were counted using the automated cell

counter plugin in Fiji. Tubule area was averaged per field for immunohistochemical analysis. Representative images are showcased in *Figures 2.3* and *2.4* in *section 2.4.3*.

4.3.3 RNA extraction

Total RNA was extracted from frozen testicular tissues using the RNeasy® Plus Mini Kit (QIAGEN®) in accordance with the manufacturer's instructions and as described in *section 2.5*. Testes samples were homogenised with RLT buffer then centrifuged at full speed. After centrifugation, the column was discarded, and 1 volume of 70 % ethanol was added to the flow-through and mixed by pipetting. Up to 700 µl of each sample was transferred to RNeasy spin columns in supplied 2 ml collection tubes. The tubes were centrifuged for 15 seconds at ≥ 8000 *g* at RT. The flow-through was discarded and 700 µl of Buffer RW1 was added into each spin column to be centrifuged for 15 seconds at ≥ 8000 *g* at RT before 500 µl of Buffer RPE was added to the spin columns followed by centrifugation for 15 seconds at ≥ 8000 *g*. This step was repeated and the tubes were centrifuged for 2 minutes at ≥ 8000 *g* at RT. After discarding the flow-through, the columns were placed in new 2 ml collection tubes and were centrifuged at full speed for 1 minute in order to further dry the spin column membranes. A volume of 30 µl of RNase-free water (QIAGEN®) was added directly to the spin column membrane of each tube, followed by centrifugation for 1 minute at ≥ 8000 *g* at RT to elute the RNA. The flow-through was collected and 1 µl of RNA was used to measure total RNA concentration and purity ratios using the NanoDrop™ Spectrophotometer (Lab Tech).

4.3.4 Microarray

Samples of testes RNA were diluted to 100 ng/µl using the same RNAse-free water used in the elution step in *section 2.4* and made into 20 µl aliquots for microarray preparation. The samples were taken to the Nottingham Arabidopsis Stock Centre (NASC; School of Biosciences, Sutton Bonington Campus), where the RNA was quality control checked. RNA integrity was assessed using microfluidic analysis via an Agilent Bioanalyser 2100 (Agilent), using the RNA Integrity Number (RIN) for evaluation of RNA integrity (Agilent). The average RIN for the RNA samples sent for Microarray analysis was 9.91. GeneChip™ WT PLUS Reagent Kit Manual Target Preparation for GeneChip™ Whole Transcript (WT) Expression Arrays (ThermoFisher Scientific) was used to generate a complete transcriptome coverage. First-strand cDNA was synthesised from the extracted RNA using Reverse Transcriptase (RT), followed by a second-strand cDNA synthesis using DNA Polymerase and RNAse H. Complementary RNA (cRNA) was synthesised by *in vitro* transcription (IVT) of the second-strand cDNA using T7 RNA polymerase. cRNA was then purified by incubating the samples so they bind to a volume of Purification Beads (Thermo Fisher) before being placed on a magnetic stand in order to capture the Beads. The supernatant is then discarded carefully. The Purification Beads are then washed twice with an 80 % ethanol wash before being air-dried on a magnetic plate. The cRNA is then eluted using 27 µl of nuclease-free water that is preheated to 65º C. The tubes are then mixed before being placed on the magnetic plate to capture the Purification Beads. The supernatant is transferred into a provided nuclease-free tube for each sample and placed on ice in preparation for the 2nd cycle single-strand cDNA synthesis by reverse

transcription of cRNA. The cRNA template was then hydrolysed using RNase H leaving only a single-strand cDNA (ss-cDNA). In preparation for fragmentation and labelling, the cDNA strand was purified using the same method used to purify the cRNA but with the elution in a volume of 30 µl of nuclease-free water. The purified ss-cDNA is fragmented by uracil-DNA glycosylase (UDG; provided) and apurinic/apyrimidinic endonuclease 1 (APE 1; provided) at the unnatural Deoxyuridine Triphosphate (dUTP) residues and breaks the DNA strand. The fragmented cDNA is labelled by terminal deoxynucleotidyl transferase (TdT) using the provided DNA Labelling Reagent. After labelling with biotin, the strand is hybridised to DNA probes on Clariom™ S Assay Mouse GeneChip™ (ThermoFisher Scientific).

4.3.5 Statistics

For the statistical analysis of the data, GraphPad PRISM 9 was used. Outliers in data sets were identified using PRISM's Robust Regression and Outlier Removal (ROUT) method and data was statistically analysed with the outliers excluded from the data sets. The data sets were tested for normality using D'Agostino & Pearson normality test. Normally distributed data were analysed using one-way ANOVAs with Tukey's post hoc test and non-normally distributed data were analysed using non-parametric Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons. Area Under the Curve (AUC) analysis was carried out for animal weight progression data. Total gene lists of microarray data sets were generated through Partek Genomics Suite (Partek®). False detection rate (FDR) and fold change thresholding were set to 0.05 and 1.1, respectively. Gene lists were sorted by p value and fold change to determine the top genes of each diet group comparison (CD vs. LPD, CD vs. WD, etc.).

Preliminary pathway and enrichment analysis was carried out on microarray data sets using Gene Set Enrichment Analysis (GSEA) functional enrichment analysis tools such as WebGestalt (WEB-based Gene SeT AnaLysis Toolkit) in addition to functional annotation using DAVID (The Database for Annotation, Visualization and Integrated Discovery). Two-way ANOVA was used to analyse array differential gene expression across the different diet groups. A p value ≤ 0.05 was deemed statistically significant for this study.

4.4 RESULTS

4.4.1 Sub-optimal diet minimally influences gross testicular morphology.

Representative H&E image from the control group with a respective close-up on tubule morphology is represented in *Figure 4.1*. Analysis of H&E-stained testicular tissue sections revealed no significant differences between all five groups as presented in *Figure 4.2* in total tubule area (*A*), total perimeter (*B*), epithelial area (*C*), lumen area (*D*) as well as lumen ratio (*E*). High variability was observed in the MDWD group across all parameters.

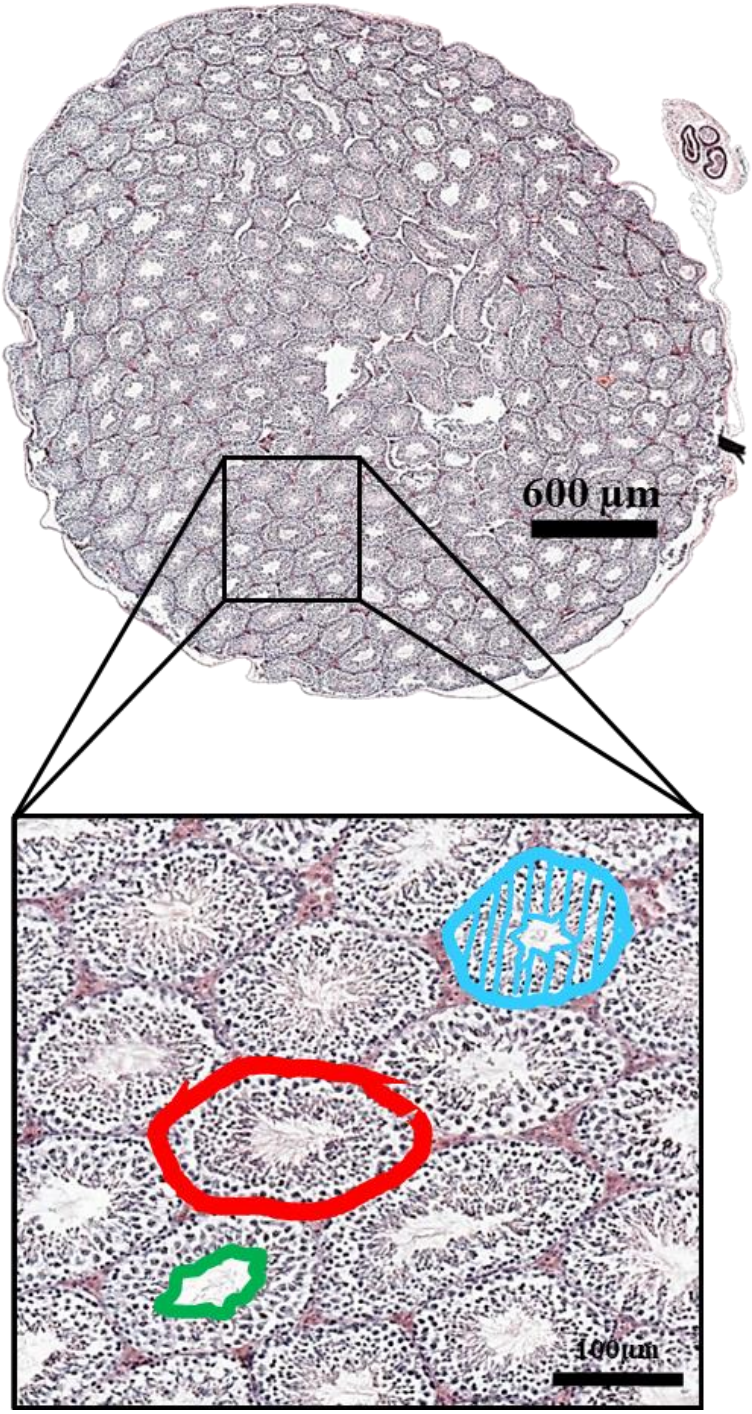

Figure 4.1: Testicular morphology representative images. Figure demonstrates representative image of H&E staining on testis from CD. Top image is representative of the whole testis; 20 x magnification images inset demonstrate tubule morphology in greater detail. Red outlined area represents the area of a seminiferous tubule. The green outlined area is the seminiferous tubule lumen. The area in blue represents the epithelial area of the tubule.

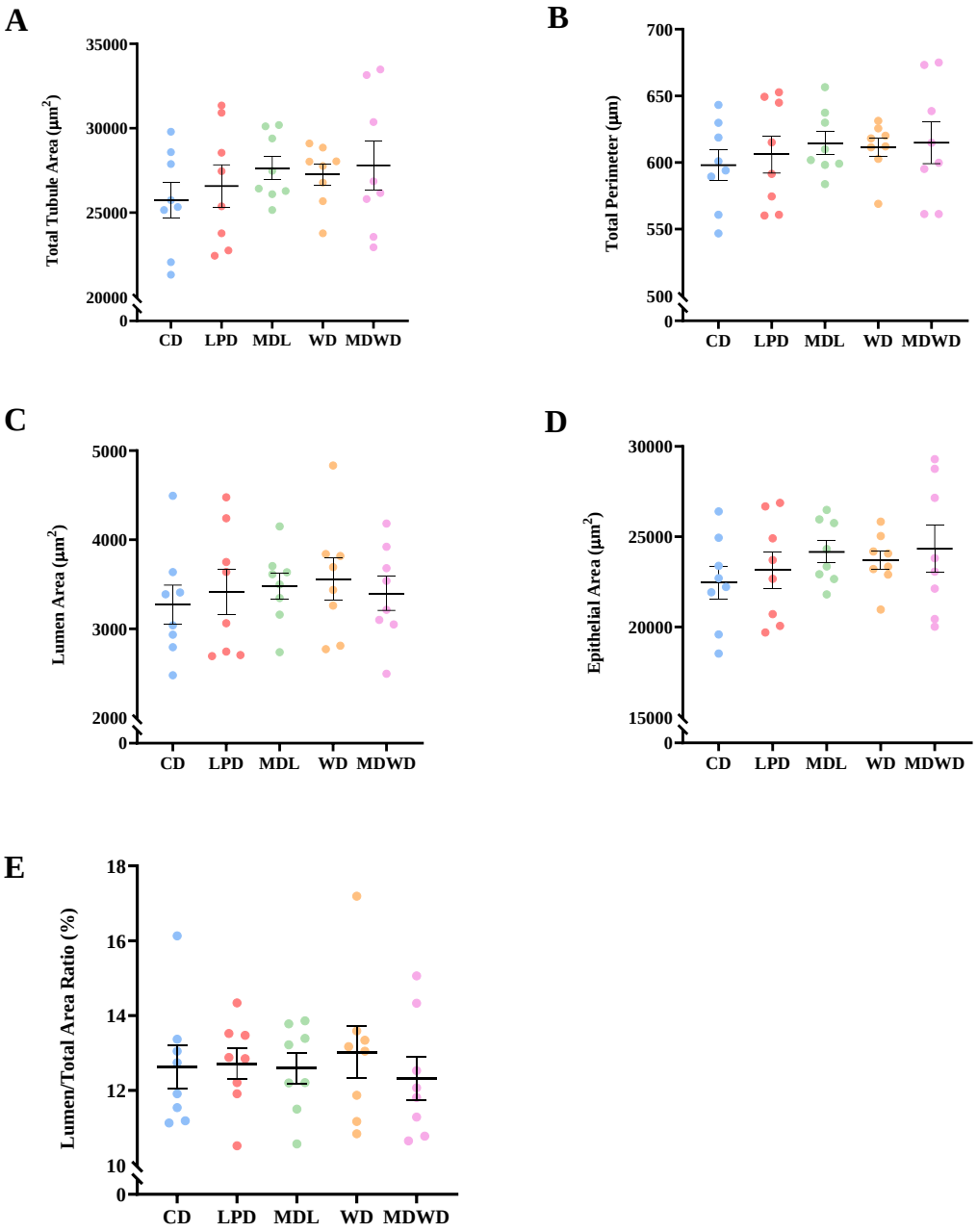

Figure 4.2: Sub-optimal diet impact on seminiferous tubule morphometry and histology. Figure shows mean seminiferous tubule area (A), mean tubule perimeter (B), mean tubule lumen area (C), mean area of tubule epithelium (D) and tubule lumen ratio (E). Error bars represent mean ± SEM. A one-way ANOVA with Tukey's post-hoc test was used for statistical analysis after confirmation of normality (n=8/group, with each data pointer presenting the average value for one male).

Differences in seminiferous tubule areas were also represented in a frequency distribution curve, highlighting the individual pattern of each of the study groups (*Figure 4.3*). Though not statistically significant, all study groups displayed increased mean total seminiferous tubule area compared to the control group., where the pattern of total tubule area is shifted from the median tubule area of the CD group.

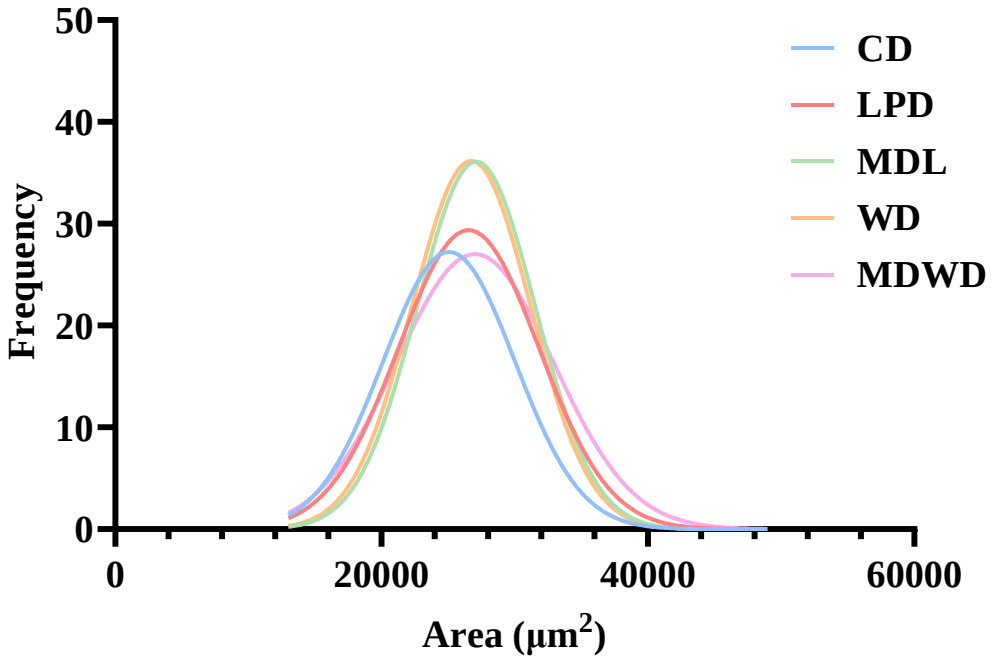

Figure 4.3: Frequency distribution histogram of total seminiferous tubule area of the different study groups.

4.4.2 A high fat, high sugar diet significantly influences abnormal seminiferous tubule cytoarchitecture.

Seminiferous tubule abnormalities such as spermatogenic cell sloughing, germ cell depletion and Sertoli-cell-only tubules were also examined across sections

of the five diet groups. In testes of males fed the high fat, high sugar diet groups WD and MDWD, abnormal/atypical seminiferous tubules were observed significantly more than all other diet groups (*Figure 4.5*). *Figure 4.4* shows representative images of tubule abnormalities observed.

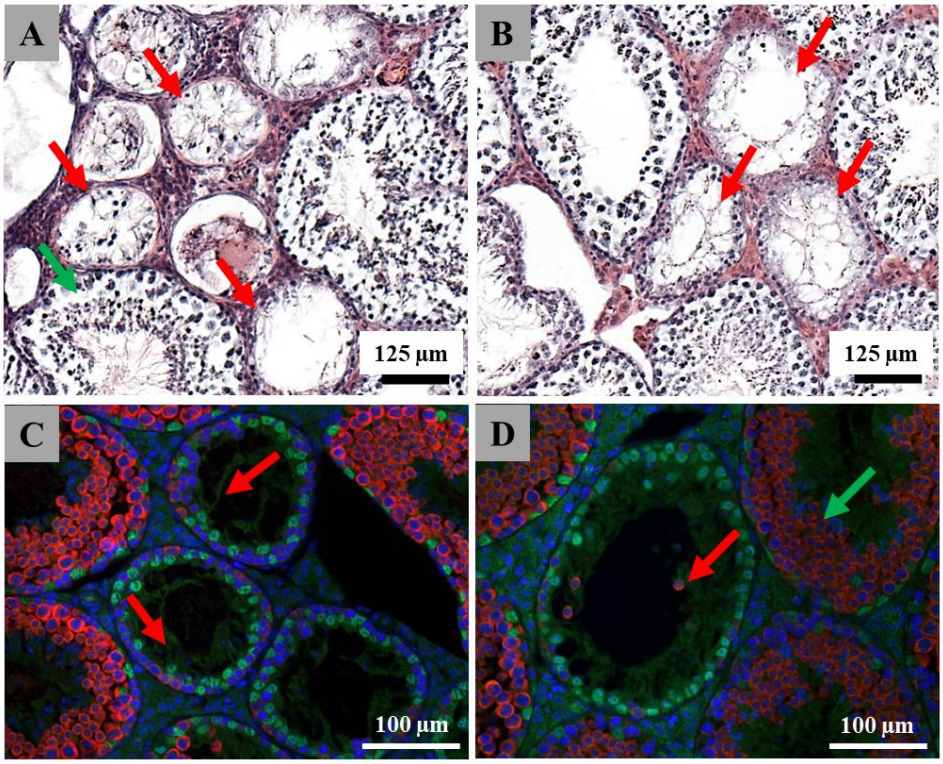

Figure 4. 4: Tubule abnormalities representative images of Sertoli-only tubules and germ cell loss from WD and MDWD groups. Top images are H&E-stained (A, B) with tubules featuring germ cell loss and epithelial sloughing in addition to the widening of the intratubular space. Bottom images (C, D) are immunostained testis tissues for SOX-9 (Green, denoting Sertoli cells), DDX4 (Red, denoting germ cells), and DAPI (Blue, denoting nucleated cells) featuring germ cell loss and Sertoli cell only tubules. Red arrows indicate abnormal tubules with cellular loss (A, B, C, D) and epithelial disruption (A, B; separation from basement membrane, epithelial sloughing), and Sertoli cell only features (C, D). Green arrows depict tubules with normal morphology.

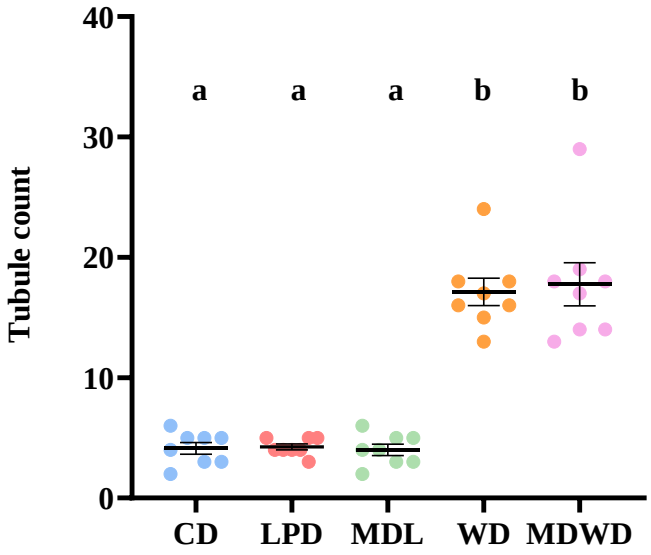

Figure 4.5: Frequency of seminiferous tubule abnormalities per whole testis tissue cross section between the different groups ± SEM (n=8/group; each dot represents the mean value for one male). The abnormalities analysed include cell separation from basement membrane, Sertoli cell only tubules, epithelial degeneration, and epithelial sloughing. D'Agostino and Pearson normality test was carried out to test the distribution of the data sets. Kruskal-Wallis followed by Dunn's post-hoc test was carried out for the analysis of the data sets.

4.4.3 Dietary intake does not influence number of Sertoli cells but impacts spermatogonial stem cell (SSC) populations.

The total number of Sertoli cells, germ cells, and total nucleated cells within the seminiferous tubules of each testis section was obtained through ImageJ2 analysis and was analysed as a function of the number of assessed tubules in each sample. Representative images of SOX9 and DDX4 staining are shown in *Figure 4.6*, and representative images of PLZF staining are shown in *Figure 4.7*.

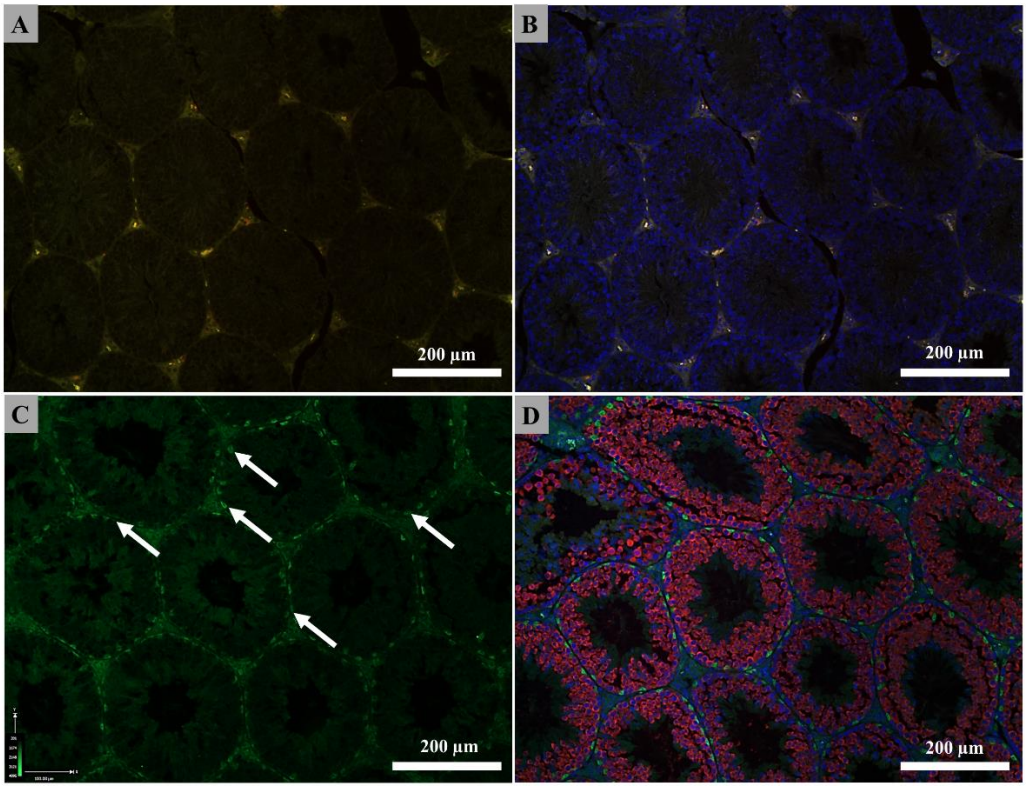

Figure 4. 6: Representative images of immunostaining negative controls versus positive samples. Images show a negative control sample with no primary or secondary antibodies with the FITC and TxRED channels (A) and the channels merged with DAPI (B), FITC channel with positively stained Sertoli cells (C; as indicated by the arrows), and a merge if all 3 channels in a positive sample (D).

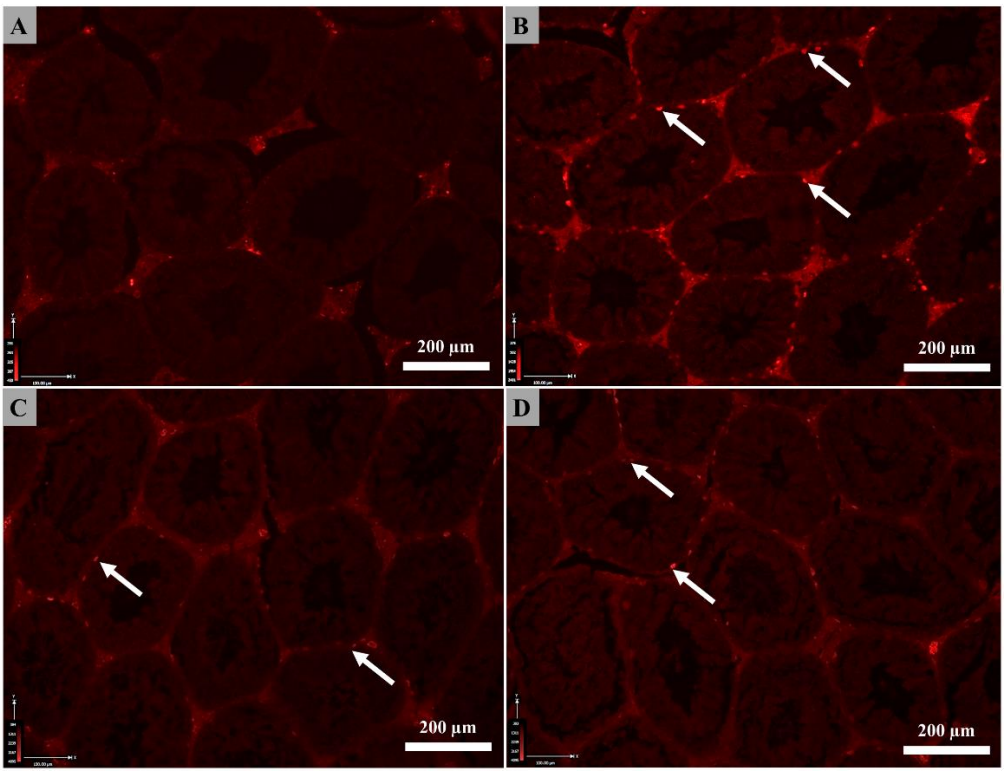

Figure 4. 7: Anti-PLZF staining in testis tissue. Images depict control sample (A) and positive samples from CD (B), LPD (C), and WD (D). Arrows point to PLZF+ cells in the testis tissue.

No significant differences were observed between the diet groups with regard to total number of SOX-9-positive cells counted manually (*Figure 4.8A*), DDX4-positive cells (*Figure 4.8B*) and total nucleated cells counterstained with DAPI (*Figure 4.8C*).

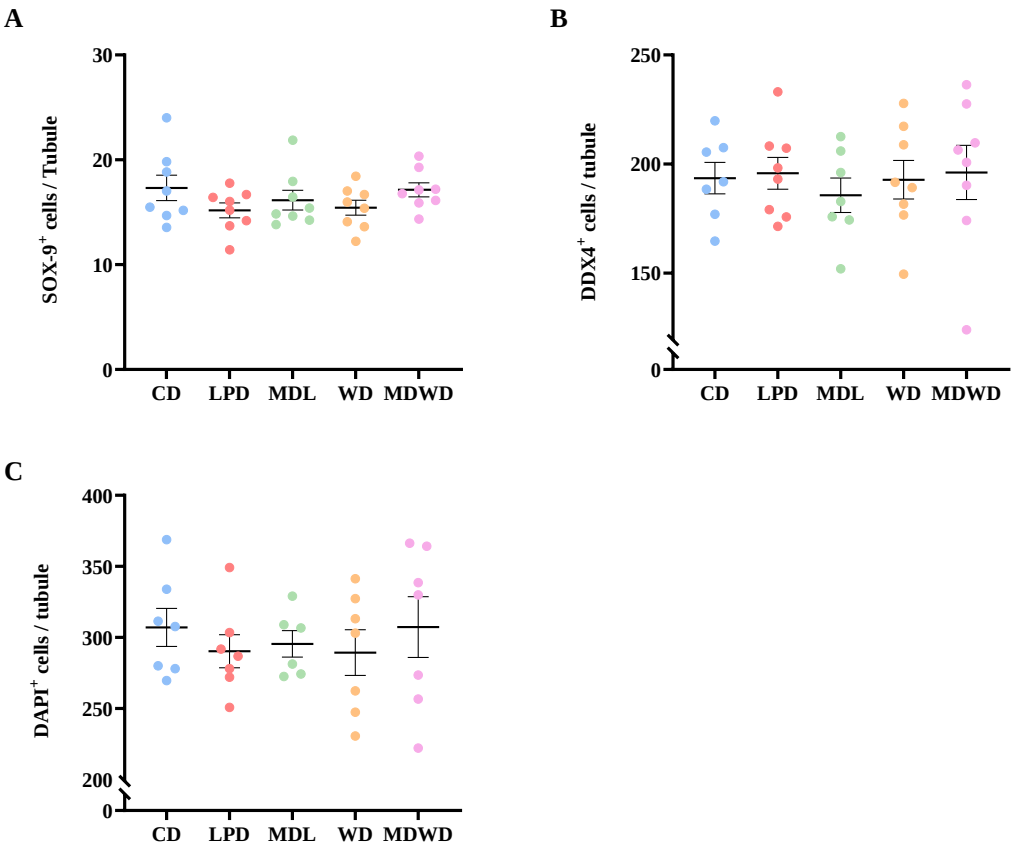

Figure 4. 8: Number of DDX4/SOX9-positive cells per seminiferous tubules per sample. Number of SOX-9-positive cells (A), DDX4-positive cells (B), and DAPI-positive cells (C) per seminiferous tubule per sample. D'Agostino and Pearson test was carried out to test for normality and one-way ANOVA with Tukey's post-hoc test was used in the analysis of the normally distributed data sets (B, C) and Kruskal-Wallis followed by Dunn's post-hoc test for nonparametric data (A) with error bars representing mean ± SEM (n=7-8/group).

No significant differences were observed in the total number of SOX-9-positive cells per microscopic field between the five groups (*Figure 4.9A*). Additionally, no significant differences were observed in DDX4-positive cells per field (*Figure 4.9B*) as well as DAPI-positive cells per field (*Figure 4.9C*). However,

both LPD and WD groups displayed a significantly decreased number of PLZF-positive cells per microscopic field (*Figure 4.9D*) compared to the control group.

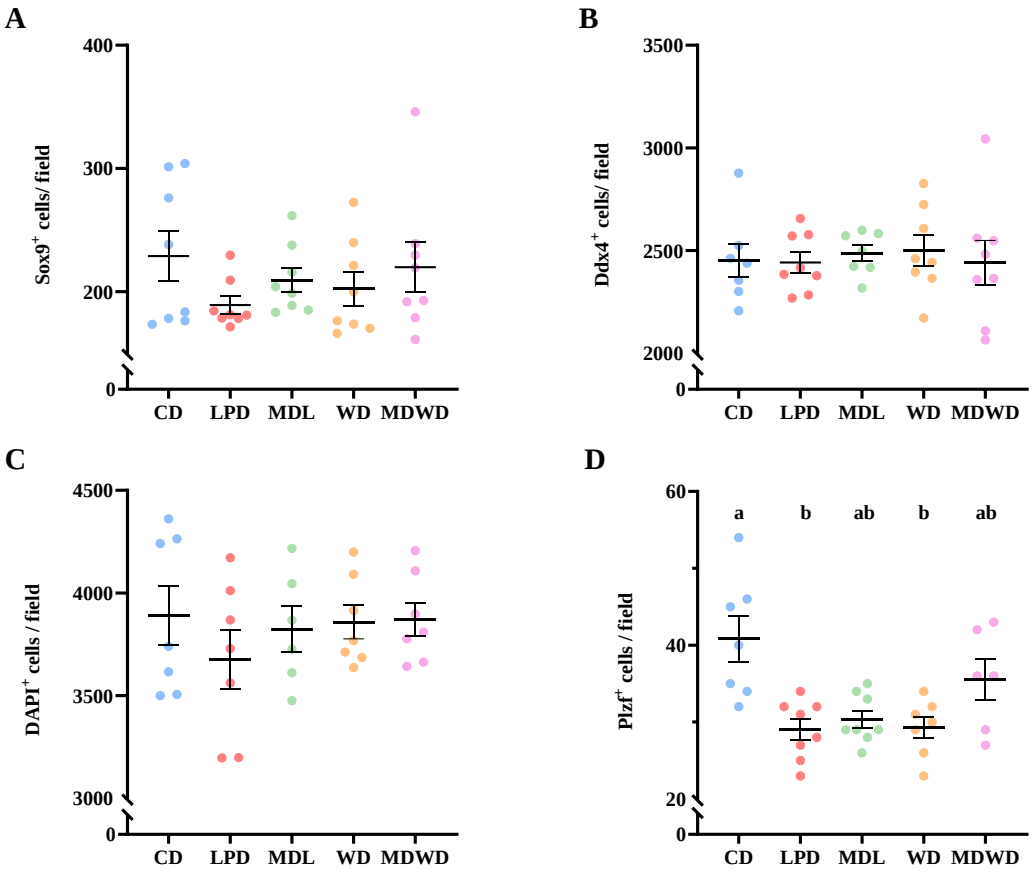

Figure 4. 9: Number of cells per microscopic field. Number of SOX-9-positive cells/field (A), DDX4-positive cells/field (B), DAPI-positive cells/field (C), and PLZF-positive cells/field (D). D'Agostino and Pearson normality test was carried out followed by one-way ANOVA for normally distributed data sets and Tukey's post-hoc test (B, C). Kruskal-Wallis test followed by Dunn's post-hoc test was carried out on data sets that did not pass the normality test (A, D). Samples are represented as individual points ± SEM (n=7-8/group).

Table 4.2 below summarises key findings in this study, highlighting the results observed of dietary intake and its impact on testicular histology, both through H&E staining and immunohistochemistry.

	Key Findings
1	*Dietary intake has minimal impact on testicular tubule area*
2	*LPD and WD have a significantly decreased population of PLZF^{+} cells*
3	*Methyl donor supplementation normalises spermatogonial stem cell population, with no difference between MDL, MDWD, and CD*
4	*HFD (WD and MDWD) groups influence abnormalities in testicular morphology, including epithelial sloughing and tubule degeneration*
5	*LPD and MDL have no significant impact on testicular abnormalities*

Table 4. 2: Key findings with regard to dietary intake and its influence on testicular histology.

4.4.4 Global testicular gene expression patterns are influenced by dietary intake.

Analysis of microarrays prepared from total testes RNA was performed using Partek® Genomics Suite® analysis software. Comparative analysis between the five diet groups was performed using standard One-way ANOVAs. Top gene

lists were generated according to the following chosen parameters: False Detection Rate (0.05 FDR), $p \leq 0.05$, and a fold change > 1.1.

Preliminary pathway analysis was performed using web-based functional annotation bioinformatics microarray analysis tools such as The Database for Annotation, Visualisation and Integrated Discovery (DAVID) (Huang et al., 2007) and WEB-based GEneSeTAnaLysis Toolkit (WebGestalt) (Liao et al., 2019). Analysis of total gene lists between the study groups compared to CD are represented in *Figure 4.11*. A total of 910 genes were differentially expressed between LPD and CD (*Figure 4.10A*), 2959 genes between MDL and CD (*Figure 4.10B*), 3503 genes between WD and CD (*Figure 4.10C*), and 3425 genes between MDWD and CD (*Figure 4.10D*).

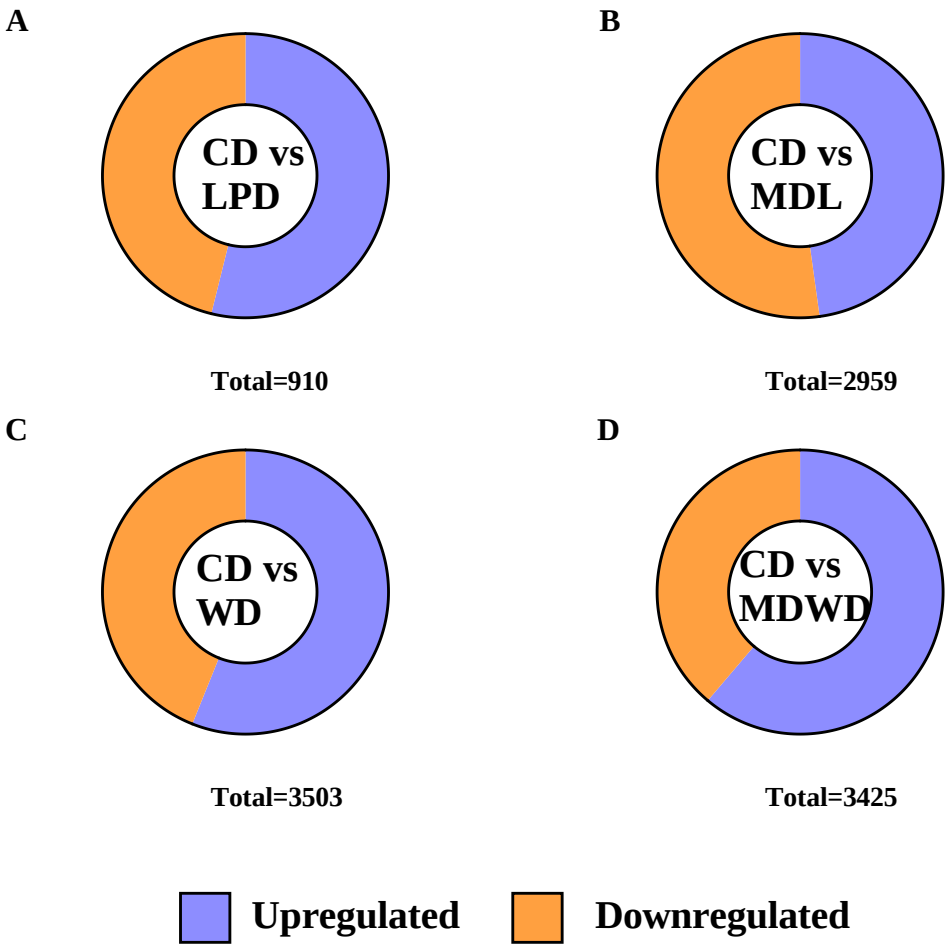

Figure 4. 10: Differential testicular expression of genes across study groups. Pie charts showing the differential expression of genes across study groups compared to the control group CD, with different colours denoting upregulated and downregulated genes.

Analysis of significantly differentially expressed genes between study groups using WebGestalt identified certain pathways and phenotypes implicated by these changes in gene expression. Compared to CD, testes of LPD-fed males displayed an upregulation of genes associated with non-coding RNA (ncRNA) metabolic processes such as cysteinyl-tRNA synthetase (*Cars*), tryptophanyl-

tRNA synthetase (*Wars*), and two members of the Argonuate RNA-induced silencing complex (RISC) family, *Ago1* and *Ago4* (*Table 4.3*).

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Cars1	Cysteinyl-tRNA synthetase	≤0.05	1.15
Wars1	Tryptophanyl-tRNA synthetase	≤0.05	1.1
Ago1	Argonaute RISC catalytic subunit 1	≤0.05	1.2
Ago4	Argonaute RISC catalytic subunit 4	≤0.01	1.16

Table 4. 3: LPD vs CD (ncRNA metabolic process). Gene Set Enrichment Analysis (GSEA) of testicular gene expression (LPD vs CD) using Geneontology database Biological Proces noRedundant through WebGestalt. Positive fold change indicates relative overexpression compared to CD.

Conversely, a downregulation of genes enriched for ncRNA metabolic processes was observed in MDL compared to CD, with significantly reduced expression notable genes such as tRNA methyltransferases and mitochondrial tRNA translation regulators. These include tRNA methyltransferase 6 (*Trmt6*), poly(A)-specific ribonuclease (deadenylation nuclease) (*Parn*), mitochondrial methionyl-tRNA formyltransferase (*Mtfmt*), methyltransferase-like 15 (*Mettl15*), and argonaute RISC catalytic subunit 1 (*Ago1*) (*Table 4.4*).

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Trmt6	tRNA methyltransferase 6	≤0.05	-1.26
Parn	Poly(A)-specific ribonuclease	≤0.005	-1.5
Mtfmt	Methionyl-tRNA formyltransferase	≤0.05	1.15
Mettl15	Methyltransferase-like 15	≤0.05	-1.4
Ago1	Argonaute RISC catalytic subunit 1	≤0.05	-1.2

Table 4. 4: MDL vs CD (ncRNA metabolic processes). Gene Set Enrichment Analysis (GSEA) of testicular gene expression (MDL vs CD) using Geneontology database Biological Proces noRedundant through WebGestalt. Negative fold change indicates relative underexpression compared to CD.

MDL also upregulated a number of members of the cadherin and claudin gene family that are involved in cell-cell adhesion and plasma-membrane adhesion. These include cadherin 7 (*Cdh7*), cadherin 8 (*Cdh8*), cadherin 10 (*Cdh10*), cadherin 16 (*Cdh16*), cadherin 13 (*Cdh13*), claudin 2 (*Cldn2*) and claudin 14 (*Cldn14*) (*Table 4.5*).

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Cdh7	Cadherin 7	≤0.05	1.4
Cdh8	Cadherin 8	≤0.05	1.35
Cdh10	Cadherin 10	≤0.05	1.25
Cdh13	Cadherin 13	≤0.05	1.146
Cdh16	Cadherin 16	≤0.05	1.35
Cldn2	Claudin 2	≤0.01	1.34
Cldn14	Claudin 14	≤0.05	1.23

Table 4. 5: MDL vs CD (cell adhesion). Gene Set Enrichment Analysis (GSEA) of testicular gene expression (MDL vs CD) using Geneontology database Biological Proces noRedundant through WebGestalt. Positive fold change indicates relative overexpression compared to CD.

When comparing MDL and LPD, a number of genes involved in spermatogenesis and embryonic development are downregulated in MDL, such as protection of telomeres 1B (*pot1b*; *p≤0.01*; fold change = -1.6), androgen receptor (*Ar*; *p≤0.005*; fold change = -1.5), HECT, UBA and WWE domain containing 1 (*Huwe1*; *p≤0.01*; fold change = -1.9), spermine synthetase (*Sms*; *p≤0.005*; fold change = -1.67), and PWP1 homolog (S. cerevisiae) (*Pwp1*; *p≤0.01*; fold change = -1.2). Furthermore, MDL significantly upregulated the mRNA expression levels of the glial cell derived neurotrophic factor (*Gdnf*)

gene, which is involved in spermatogonial stem cell renewal and proliferation, compared to both LPD ($p \leq 0.001$; fold change = 1.67) and CD ($p \leq 0.05$; fold change = 1.4) (*Table 4.6*).

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Potb1	Protection of telomeres 1B	≤0.01	-1.6
Ar	Androgen receptor	≤0.005	-1.5
Huwe1	HECT, UBA and WWE domain containing 1	≤0.01	-1.9
Sms	Spermine synthetase	≤0.005	-1.67
Pwp1	PWP1 homolog (S. cerevisiae)	≤0.01	-1.2
Gdnf	Glial cell derived neurotrophic factor	≤0.001	1.67

Table 4. 6: Gene Set Enrichment Analysis (GSEA) of testicular gene expression (MDL vs LPD) through WebGestalt. Positive fold change indicates relative overexpression compared to LPD. Negative fold change indicates relative under-expression compared to LPD.

WD-fed mice displayed an upregulation of genes such as claudin 8 (*Cldn8*), claudin 11 (*Cldn11*), claudin 14 (*Cldn14*) and claudin 23 (*Cldn23*) in addition to cadherin 1 (*Cdh1*), vascular cell adhesion molecule 1 (*Vcam1*) and filamin

alpha (*Flna*), which are associated with cell adhesion and tight junction maintenance and BTB assembly (*Table 4.7*).

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Cldn8	Claudin 8	≤0.05	1.32
Cldn11	Claudin 11	≤0.05	1.2
Cldn14	Claudin 14	≤0.05	1.23
Cldn23	Claudin 23	≤0.005	1.3
Cdh1	Cadherin 1	≤0.05	1.3
Vcam1	Vascular cell adhesion molecule 1	≤0.05	1.3
Flna	Filamin alpha	≤0.05	-1.35

Table 4. 7: WD vs CD (cell adhesion). Gene Set Enrichment Analysis (GSEA) of testicular gene expression (WD vs CD) using KEGG pathway database through WebGestalt. Positive fold change indicates relative overexpression compared with CD. Negative fold change indicates relative under-expression compared with CD.

In addition, overexpression of endothelial lipase (*Lipg*) and monoglyceride lipase (*Mgll*), and the downregulation of hormone sensitive lipase (*Lipe*) associated with glycerolipid metabolic pathways and abnormal triglyceride metabolism was observed compared with CD, with the overexpression of calcium regulation genes such as calcitonin-related polypeptide alpha (*Calca)* (*Table 4.8*).

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Lipg	Lipase G (endothelial)	≤0.05	1.26
Mgll	Monoglyceride lipase	<0.0001	1.32
Lipe	Hormone sensitive lipase	≤0.05	-1.12
Calca	Calcitonin/calcitonin-related polypeptide, alpha	≤0.05	1.2641

Table 4. 8: WD vs CD (triglyceride, glycerolipid, and calcium metabolism). Gene Set Enrichment Analysis (GSEA) of testicular gene expression (WD vs CD) using Mammalian phenotype Ontology through WebGestalt. Positive fold change indicates relative overexpression compared to CD while negative fold change indicates relative under-expression compared with CD.

Additionally, genes such as paraoxonase 3 (*Pon3*), mitochondrial superoxide dismutase 2 (*Sod2*), and peroxiredoxin 3 (*Prdx3*) associated with abnormal

mitochondrial physiology and regulation of oxidative stress were upregulated with WD (*Table 4.9*).

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Sod2	Superoxide dismutase 2 (mitochondrial)	≤0.005	1.18
Pon3	Paraoxonase 3	≤0.05	1.24
Prdx3	Peroxiredoxin 3	≤0.05	1.23

Table 4. 9: WD vs CD (oxidative stress response). Gene Set Enrichment Analysis (GSEA) of testicular gene expression (WD vs CD) using Mammalian phenotype Ontology through WebGestalt. Positive fold change indicates relative overexpression compared to CD.

WD testis also exhibited a downregulation of key genes involved in the 1-carbon metabolism pathway, namely phosphatidylethanolamine N-methyltransferase (*Pemt*) and DNA methyltransferase 1 (*Dnmt1*) in addition to an upregulation of betaine-homocysteine S-methyltransferase (*Bhmt*) (*Table 4.10*) and a downregulation of histone modification regulators remodelling and spacing factor 1 (*Rsf1*; *p≤0.05*; fold change = -1.4) and histone aminotransferase 1 (*Hat1*; *p≤0.05*; fold change = -1.32). Furthermore, protamine 1 (*Prm1*; *p≤0.005*; fold change -1.1) and SRY-Box transcription factor 30 (*Sox30*; *p≤0.001*; fold change = -1.2) were downregulated in WD testis compared to CD.

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Dnmt1	DNA methyltransferase 1	≤0.0005	-1.3
Pemt	Phosphatidylethanolamine N-methyltransferase	≤0.05	-1.2
Bhmt	Betaine-homocysteine S-methyltransferase	≤0.05	1.2
Rsf1	Remodelling and spacing factor 1	≤0.05	-1.4
Hat1	Histone aminotransferase 1	≤0.05	-1.32
Prm1	Protamine 1	≤0.005	-1.1
Sox30	SRY-Box transcription factor 30	≤0.001	-1.2

Table 4. 10: WD vs CD (1-carbon metabolism). Differential expression of key 1-carbon metabolism genes between WD and CD. Positive fold change indicates relative overexpression compared to CD. Negative fold change indicates relative under-expression compared to CD.

Compared to CD, testes of MDWD males displayed no significant changes in the expression levels of claudin and cadherin genes, but a downregulation of filamin alpha (*Flna*; *p≤0.001*; fold change = -1.5), a gene associated with actin

filament organisation and actin cytoskeleton reorganisation. MDWD additionally downregulated *Pemt* and *Dnmt1* and upregulated *Bhmt* (*Table 4.11*). The expression of key genes in the process of spermatogenesis was also altered in MDWD compared to CD, as array analysis revealed the upregulation of protamine 1 (*Prm1; p≤0.05*; fold change = 1.05) and the downregulation of SRY-Box transcription factor 30 (*Sox30*; *p≤0.05*; fold change = -1.1). Furthermore, superoxide dismutase 2 (mitochondrial) (*Sod2; p≤0.05*; fold change = 1.2*)* and Paraoxonase 3 (*Pon3; p≤0.05*; fold change = 1.35) were upregulated in MDWD similar to WD compared to CD. However, no significant differential expression of *Prdx3* was observed in MDWD. Similar to WD, MDWD also upregulated monoacylglycerol lipase (*Mgll; p≤0.005*; fold change = 1.16) and downregulated Lipase E (*Lipe; p≤0.01*; fold change = -1.14), both important genes in glycerolipid and triglyceride metabolism.

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Dnmt1	DNA methyltransferase 1	≤0.001	-1.2
Pemt	phosphatidylethanolamine N-methyltransferase	≤0.001	-1.3
Bhmt	Betaine-homocysteine S-methyltransferase	≤0.05	1.2
Sod2	Superoxide dismutase 2 (mitochondrial)	≤0.05	1.2
Pon3	Paraoxonase 3	≤0.05	1.35
Mgll	Monoacylglycerol lipase	≤0.005	1.16
Lipe	Lipase E, Hormone Sensitive Type	≤0.01	-1.14

Table 4. 11: MDWD vs CD. Differential expression of key 1-Carbon metabolism, oxidative stress, and lipid metabolism genes between MDWD and CD. Positive fold change indicates relative overexpression compared to CD; negative fold change indicates relative under-expression compared with CD.

Analysis of MDWD compared to WD data sets revealed the overexpression of sperm adhesion molecule 1 (*Spam1*), equatorin (*Eqtn*) and synaptonemal complex protein 2 (*Sycp2*). In addition, an overexpression of SRY-Box

Transcription Factor 30 (*Sox30*) was observed in MDWD compared to WD (*Table 4.12*).

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Spam1	Sperm adhesion molecule 1	≤0.01	1.26
Eqtn	Equatorin	≤0.05	1.2
Sycp2	Synaptonemal complex protein 2	≤0.01	1.4
Sox30	Oviductal glycoprotein 1	≤0.05	1.02

Table 4. 12: MDWD vs WD (spermatogenesis, sperm function). Differential expression of key sperm function genes between MDWD and WD. Positive fold change indicates relative overexpression compared to WD.

Furthermore, a number of chromatin modification and remodelling genes were observed to be upregulated in MDWD compared to WD, such as remodelling and spacing factor 1 (*Rsf1*), histone aminotransferase 1 (*Hat1*) and snail family zinc finger 2 (*Snai2*) (*Table 4.13*).

Gene symbol	***Gene Name***	***P value***	***Fold Change***
Rsf1	remodelling and spacing factor 1	≤0.01	1.44
Hat1	histone aminotransferase 1	≤0.05	1.2
Snai2	snail family zinc finger 2	≤0.001	1.43

Table 4. 13: MDWD vs WD (histone modification and chromatin remodelling). Differential expression of key histone and chromatin remodelling genes between MDWD and WD. Positive fold change indicates relative overexpression compared to WD.

4.4.5 Sub-optimal diet influences epididymal tubule histology.

Histological analysis of H&E-stained epididymal tissue – representative images presented in *Figure 4.11* – revealed significant differences between both high fat, high sugar groups with regard to visible widening of intracellular space and visible reduction in sperm density in the lumen, as WD had significantly more atypical epididymal tubules compared to MDWD. Tubules with noticeably wider intratubular space, empty tubules, tubules with visibly lower density of cells in the lumen area, and tubules with sloughed membrane cells were flagged as atypical in this study.

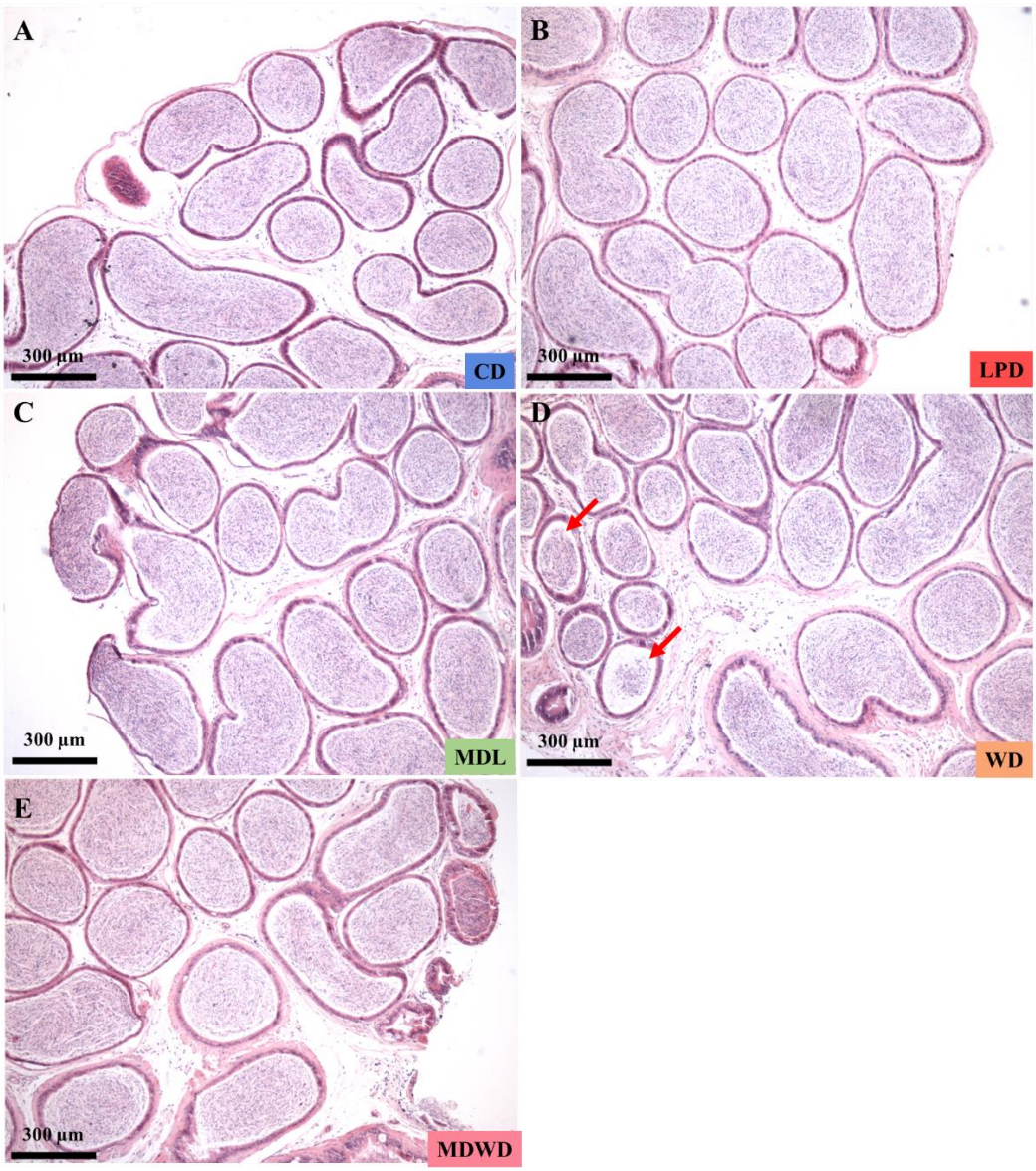

Figure 4. 11: Representative epididymal histology images stained with H&E. Representative images show CD (A), LPD (B), MDL (C), WD (D) and MDWD (E) hematoxylin and eosin stained caudal epididymal cross sections taken at 10x magnification. Red arrows in WD sample indicate lower cell density and membrane sloughing.

Examples of epididymal tubules displaying atypical features are highlighted in *Figure 4.12*. The WD-derived tubules presented in *Figure 4.12* express a reduction in lumen density and wider intratubular space unlike that of normal tubules found within the same tissues or tissues of the different diet groups shown in *Figure 4.11*. Some histological artifacts are also present as a result of tissue processing, cutting, and staining. However, these artifacts are distinguishable from tubules with atypical features such as those identified by the red arrows in *Figure 4.12*.

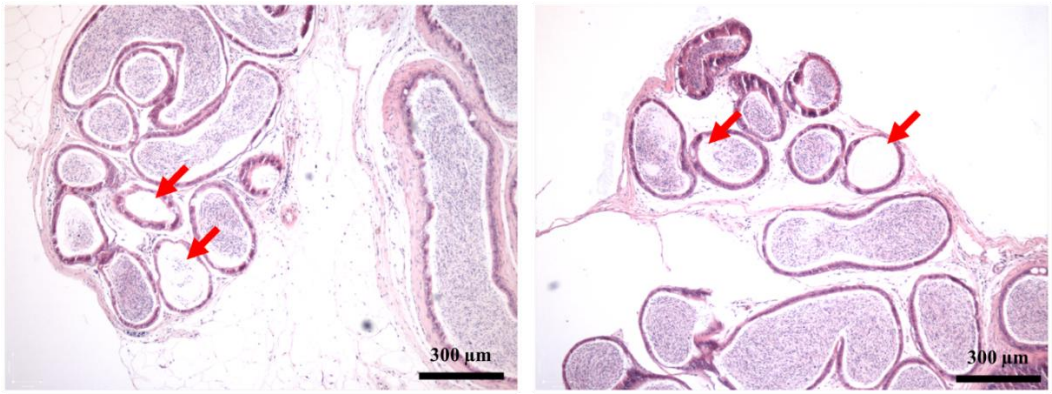

Figure 4. 12: Representative images of atypical epididymal histology. Images depict WD tubules displaying reduction in lumen density and widening of intratubular space as indicated by the red arrows.

MDWD displayed significantly fewer atypical tubules compared to WD (*Figure 4.12*). However, no other significant differences were observed between the different study groups. Representative images of atypical epididymal histology are presented in *Figure 4.13*.

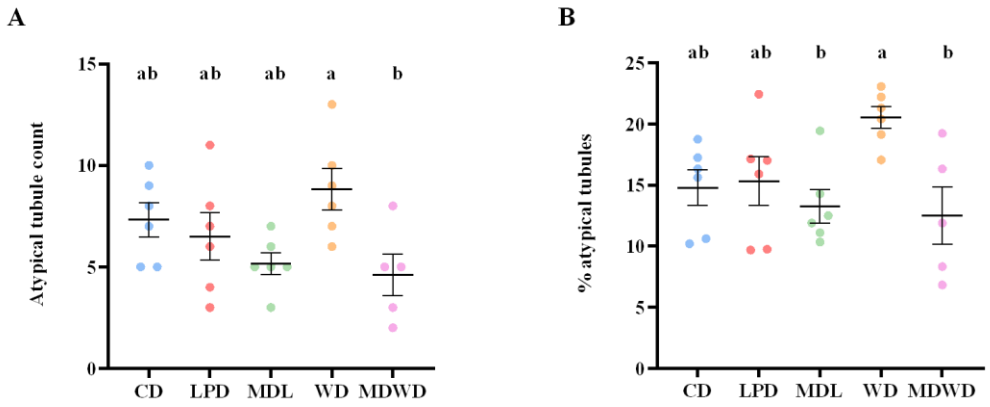

Figure 4. 13: Atypical epididymal tubules per sample (n=6/group). Figures show total number of atypical tubules per sample (A) and the proportion of atypical tubules per total number of tubules per sample (B). Proportion of atypical tubules (B) is presented as a percentage of the total number of tubules in each epididymal tissue. D'Agostino and Pearson normality test followed by one-way ANOVA analysis with Tukey's post-hoc test was carried out. Data points represent individual samples with mean ± SEM.

4.5 DISCUSSION

Suboptimal diet consumption has been linked to a number of diseases such as coronary artery disease (CAD) and type 2 diabetes. Additionally, reproductive health has also been shown to be adversely affected by suboptimal diet (Pascoal et al., 2022). It is therefore of importance to investigate the impact of nutrition on reproductive fitness in greater depth. The strong link between maternal diet, reproductive health, and altered offspring development is well-characterised in the literature (Ashworth et al., 2009; Cetin et al., 2010; Dunford et al., 2014; Kereliuk et al., 2017). However, the role that nutrition plays in influencing male reproductive fitness including testicular morphology and function warrants further elucidation. To provide a clearer understanding of the impact of diet on male reproductive fitness, this study aimed to characterise the effect of suboptimal dietary intake with or without supplementation with key nutrients and vitamins on testicular morphology, cell type abundance and global gene expression patterns.

This study shows that feeding adult male mice either an over (WD) or undernutrition (LPD) regimen, with or without methyl donors, did not significantly affect testicular tubule total area, epithelial area, or lumen area. Vawdaw and Mandlwana (1990) discovered that feeding rats a protein restricted diet (0 % and 5 %) results in reduced seminiferous tubule diameter. On the other hand, tubules from low protein diet-fed male mice displayed significantly increased total tubule and epithelial area (Morgan et al., 2020). Crean et al., (2023) observed no significant differences in seminiferous tubule diameter across a panel of 10 experimental diets including varying protein content percentages. The data in this study contradicts findings in some studies but

mirrors that of others, suggesting a need for further characterisation of the impact of diet on seminiferous tubule morphology. However, seminiferous tubule area, diameter and epithelial height differences could potentially be directly correlated with the spermatogenic activity of the testis, as was reported by França and Russell (1998) in a male boar model. Whether this translates to mouse models is not currently known, however. While no significant changes to gross seminiferous tubule morphological parameters were observed, testes of WD and MDWD-fed males displayed a significantly increased number of abnormalities affecting tubule cytoarchitecture compared to the other study groups. These abnormalities included loss of germ cells and loose arrangement of spermatogenic cells, epithelial sloughing germ cell sloughing and separation from the basement membrane, as well as enlargement in the intratubular space. Erdemir et al. (2012) showed that exposing rats to a high fat diet for a period of 10 weeks causes a decrease in the Johnsen score of high fat diet group testis, with the changes potentially being oxidative stress mediated. The Johnsen criteria, a ten-point score system used for the quantification of spermatogenesis according to the cells along the seminiferous tubules. A score of 10 indicates maximum spermatogenesis activity, while a score of 1 indicates an absence of germ cells (Teixeira et al., 2019). Unfortunately, the current study did not assess the Johnsen score for spermatogenesis between the diet groups due to time constraints. Moreover, Liu and colleagues reported that high fat diet (20 % fat) exposure causes seminiferous tubules of Sprague-Dawley rats to display abnormal structures such as the disruption of the epithelium (Liu et al., 2014). Furthermore, Campos-Silva et al. (2015) reported decreased cellular proliferation in seminiferous tubules from rats fed a high fat diet compared to

rats fed a standard chow diet. However, these findings are not in line with the findings of Vigueras-Villasenor et al. (2011), whose study reported no abnormal seminiferous tubule structures from 21 to 90 days in rats fed a high fat diet (35 % fat). A more recent study found significant changes in testicular epithelium height and tubule diameter in rats fed a high fat diet for 30 days then fed a normal diet from 60 days onwards in addition to treadmill exercise (Ibáñez et al., 2017). Our findings are in line with those of Erdemir et al. (2012) and Liu et al. (2014) with regard to atypical tubule cytoarchitecture in response to high fat diet feeding. A more recent investigation by Yang et al. (2018) reported pathological changes to seminiferous tubules in rats fed a high fat diet. These changes include seminiferous tubule atrophy and distortion as well as loss of spermatogenic cells. These changes are also in line with our findings in both WD and MDWD testes. In a study investigating the impact of chronic high fat diet feeding on a C57BL/6 mouse model, Funes et al. (2019) also reported altered testicular morphology in response to HFD, characterised by impaired seminiferous epithelium and testicular vacuolisation, two abnormal tubule features observed in the current study. Funes et al. (2019) attributed testicular interstitial space abnormalities such as enlargement and presence of foamy cells to excess inflammation and lipid deposition, which is supported by their observation of increased levels of testicular cholesterol. Additionally, the authors reported systemic oxidative stress and inflammation, which is another potential cause for abnormal testicular histology (Funes et al., 2019).

In this current study, both WD and MDWD displayed increased testicular expression of Monoglyceride Lipase (*Mgll*) and a simultaneous under-expression of Hormone Sensitive Lipase (*Lipe*) as well as increased hepatic

cholesterol content, suggesting abnormal cholesterol metabolism. Hormone Sensitive Lipase (HSL), encoded by *Lipe*, plays a crucial role in fatty acid metabolism, as it hydrolyses acylglycerols (tri-, di-, and mono-diacylglycerols), cholesterol esters and other lipids in a number of tissues (Kraemer and Shen, 2002; Lampidonios et al., 2011). The knockout of *Lipe* in mice resulted in azoospermia and significant disruption to testicular energy metabolism (Wang et al., 2017). Disrupted lipid metabolism gene expression patterns resulting in impaired testicular energy metabolism could potentially result in adverse downstream alterations that manifest histologically. It is therefore possible for the interstitial testicular abnormalities observed in both WD and MDWD to be, in part, promoted by abnormal cholesterol metabolism in the testis leading to interstitial lipid accumulation. It is not possible to confirm the deposition of lipids in the interstitial space in the testis tissues in this study, however, as any accumulated lipid dissolves through the process of tissue fixing and processing. MGLL is a metabolic enzyme that serves to transform triglycerides into FFAs and is involved in tumour signalling (Zhang et al., 2021). The overexpression of *Mgll* has been observed in cancer cells, and the suppression of the gene results in a decrease in the proliferation, migration, and invasion of melanoma (Tan et al., 2023) and hepatic carcinoma (Rajasekaran et al., 2016) cells.

Preliminary microarray pathway analysis through WebGestalt revealed that, compared to CD, LPD upregulated genes enriched for non-coding RNA metabolic processes, such as cysteinyl-tRNA synthetase (*Cars1*) and tryptophanyl-tRNA synthetase (*Wars1*) in addition to the upregulation of *Ago1* and *Ago4*, two members of the Argonuate family of RNA-induced silencing complexes (RISC). It is established that sperm RNA can essentially be shaped

according to the environment and lifestyle which the individual is exposed to, and consequently modify offspring phenotypes (Zhang et al., 2019). Non-coding RNA (ncRNA) have emerged as crucial components in mechanisms regulating metabolic, immune, and developmental processes (Weikard et al., 2018). A number of studies report the impact of non-coding RNA on an individual's epigenetic status in response to certain diets (An et al., 2017; Klastrup et al., 2019; Weikard et al., 2018). For instance, Chen et al. (2016) showed that sperm transfer RNA-derived small RNAs (tsRNAs) display RNA modifications and alterations in gene expression profiles in a male mouse model exposed to a high fat diet. Furthermore, Chen et al. (2016) investigated the transgenerational impact of this diet by injecting sperm tsRNA fractions into normal zygotes. The group observed metabolic disorders in F1 offspring as well as altered gene expression of metabolic pathways in early embryos. These changes were reported to be independent of DNA methylation, thus reinforcing the role of tsRNAs as a potential epigenetic mediator of metabolic disorder inheritance induced by diet (Chen et al., 2016). In the current study, LPD testis displayed an increased mRNA expression of different tRNA synthetase genes such as *Cars1* and *Wars1* in addition to the upregulation of *Ago1* and *Ago4*, two members of the Argonuate family of RNA-induced silencing complexes (RISC). *Ago1* is known to bind to micro-RNAs and inhibit complementary mRNA translation (Gómez Acuña et al., 2020). While *Ago1* overexpression closely related to progression of diseases such as colon (Li et al., 2010), breast (Sung et al., 2011), and lung (Kim et al., 2010) cancer, it has been discovered that stable overexpression of *Ago1* exerts tumour suppressor properties in a neuroblastoma cell line (Parisi et al., 2011). Parisi et al. (2011) found that the overexpression of

*A*go1 in a stable manner induced a slower cell cycle coupled with a stronger apoptotic response to ultraviolet (UV) irradiation (Parisi et al., 2011). Furthermore, LPD upregulated the expression of *Ago4*, another member of the RISC family which also binds to small RNAs and also plays a regulatory role in the mouse germ cell line (Modzelewski et al., 2012). *Ago4* knockout mouse models initiate meiosis earlier than those expressing normal *Ago4* with a significant reduction in associated miRNAs (Modzelewski et al., 2012). While both *Ago1* and *Ago4* have significantly reduced expression in MDL compared to LPD, only *Ago1* is significantly downregulated in MDL compared to CD, with a fold change of -1.08. This suggests that methyl donor supplementation to LPD normalises the mRNA expression levels of *Ago1* and *Ago4* in the testis.

MDL, on the other hand, downregulated genes enriched for ncRNA metabolic processes compared to CD, by reducing the mRNA expression of tRNA transferases such as *Trmt6* and *Mtfmt* in addition to *Mettl15*, a methyltransferase-like protein coding gene. While testicular expression of these genes is not well documented, studies in cancer cell lines revealed that the inhibition of TRMT6 leads to the suppression of cell invasion, proliferation, and migration in glioma (Wang et al., 2021). *Mtfmt* was shown to be upregulated in mouse livers in response to high fat diet feeding (Sun et al., 2023). While homozygous knockout mice experienced embryonic death, heterozygous mice exhibited increased glucose tolerance and a reduction in high fat diet-induced inflammation (Sun et al., 2023). However, in a human cell line, MTFMT silencing resulted in compromised mitochondrial integrity (Seo et al., 2020). It is unclear whether the downregulation of *Mtfmt* in the testis adversely impacts downstream processes. The inhibition of METTL15 in human cell lines also

results in the disruption of mitochondrial function by means of impairing *de novo* protein synthesis (Van Haute et al., 2019). While MDL and LPD both respectively downregulate and upregulate genes enriched for ncRNA metabolic processes, the two diet groups seem to alter the expression of independent genes without much overlap if any at all.

In fact, changes attributed to epigenetics that are observed in adult offspring phenotypes such as cardiovascular and metabolic (Watkins and Sinclair, 2014) or glucose intolerance (Watkins et al., 2018) in mouse models could be mediated by ncRNA. This remains to be investigated, as the mechanisms underlying sperm RNA structures in addition to the interaction of RNA with DNA and other epigenetic factors remain largely unknown (Zhang et al., 2019). Another potential factor that influences that could also be the gut microbiome, as recent studies have shown that gut bacteria produce certain short chain fatty acids which can have an impact on an individual's epigenetic status (Krautkramer et al. 2016). The change to an individual's epigenetic status could therefore be attributed to a number of different factors working together to elicit certain changes.

The level of mRNA expression of the androgen receptor (*Ar*) was significantly decreased in MDL compared to both CD and LPD. Mayer et al. (2018) report on the regulation of androgen receptor mRNA by the levels of testicular and peritubular levels of androgens in humans. Additionally, the importance of androgen receptor signalling also extends to encapsulate Sertoli cell function (Wang et al., 2022). While the current study did not examine the levels of androgens in mouse serum or testis, the data does not show any significant difference in mRNA levels of any of the key testosterone synthesis and secretion

genes such as Steroidogenic Acute Regulatory Protein (*Star*), Hydroxy-delta-5-steroid Dehydrogenase, 3 Beta- and Steroid Delta-Isomerase 1 (*Hsd3b1*), or Luteinizing Hormone/Choriogonadotropin Receptor (*Lhcgr*). Interestingly, MDL upregulated a number of genes belonging to the cadherin and claudin families, such as *Cdh7*, *Cdh8*, *Cdh13*, *Cldn2*, and *Cldn14*. Whether the upregulation of the cadherin and claudin genes is a result of a downregulation of the androgen receptor in the testis and disrupted Sertoli cell tight junction integrity is not currently known, however. It is worth noting, however, that the expression of N-cadherin (*Cdh2*), a major role player in BTB tight junction maintenance (Piprek et al., 2020) is not altered in MDL.

The relative mRNA expression level of *Parn* is also reduced in MDL testis compared to both CD and LPD. *Parn* is a ribonuclease that plays a role in controlling mRNA stability and gene expression (Balatsos et al, 2012). Using knockout cell line models, Benyelles et al. (2019) reported that the knockout of *Parn* modifies telomere length in human cell lines and induces embryonic lethality in mouse cell lines (Benyelles et al., 2019). Heterozygous PARN mutations have been linked to shorter telomeres in human pulmonary disease (Juge et al., 2017; Stuart et al., 2015). Compared to LPD, MDL downregulated the expression of *Pot1b*, a telomeric binding protein that plays a crucial role in the maintenance and protection of telomere ends (He et al., 2006). In a recent study in mouse embryonic fibroblasts, Gu et al. (2021) showed that *Pot1b* is a promoter of telomere lengthening. Additionally, compared to LPD, testicular expression of *Pwp1* is reduced in MDL. In heterozygous mouse cell line knockout models, the depletion of *Pwp1* caused significant and rapid telomere shortening (Yu et al., 2019). Previous research from our lab recently showed that

methyl donor supplementation to a low protein diet results in shorter telomere lengths in mouse testes (Morgan et al., 2020). This finding coincides with the results in this study showing the downregulation of *Parn*, *Pot1b* and *Pwp1* in the MDL group, suggesting that telomere shortening in methyl donor supplemented low protein diets is influenced by the suppression of these genes. Testicular gene expression profiles did not seem to overlap between MDL and LPD when compared to CD. Even when both diet groups display altered differential gene expression enriched for the same biological processes, the genes within the altered pathways and processes are unique to each diet group. Furthermore, while LPD seems to alter testicular gene expression patterns, the alterations are relatively modest when compared to the other diet groups that exhibit three times the total number of differentially expressed genes compared to LPD relative to the control group. This could suggest that protein restriction is a lesser dietary insult than an overnutrition with high fat, high sugar diets, and that the addition of methyl donors alters gene expression at a similar rate in the testis, which is seen in the difference between MDL and LPD.

Furthermore, while the data presented in this study does not report a decrease in spermatogenic cell numbers in seminiferous tubules, it does reveal a significant decrease in PLZF-positive cells in both the LPD and WD groups, indicating a possible loss in spermatogonial stem cell population when compared to the control group. Over time, seminiferous tubules without a spermatogonial stem cell population would lose maturing spermatogenic cells in a process called maturation depletion, eventually resulting in Sertoli-cell only tubules (Vidal and Whitney, 2014). Parker et al. (2021) investigated the role of glial cell line-derived neurotrophic factor (GDNF), a product of Sertoli cells that is crucial for

establishing and maintaining the spermatogonial stem cell pool and found that mouse testes deprived of GDNF signalling were histologically similar to human testes with Sertoli cell-only (SCO) syndrome. Microarray analysis of global testicular RNA revealed an upregulation of *Gdnf* in MDL compared to CD. A downregulation of *Gdnf* was observed in LPD, though not statistically significant. In mouse testis, the lack of GDNF leads to the impairment of spermatogonial stem cell renewal and thus a reduction in the stem cell pool, while the overexpression of GDNF results in spermatogonial accumulation (Meng et al., 2000). This is mirrored by the overexpression of *Gdnf* in MDL testis in addition to the distribution of $PLZF^+$ spermatogonia in the seminiferous tubules. While LPD had significantly decreased number of PLZF-positive cells compared to CD, this was not observed in MDL. This suggests that methyl donor supplementation to a low protein diet induces *Gdnf* overexpression, leading to increased SSC renewal. Interestingly, no significant change in the differential expression of *Gdnf* was observed between either of the high fat diet groups and CD. Indeed, while WD testis expressed significantly fewer $PLZF^+$ cells, no change was observed in the expression of *Gdnf*. Methyl donor supplementation seems to play a role in the recovery and renewal of the spermatogonial population, as no significant differences were observed in the number of $PLZF^+$ cells in the MDWD group compared to CD.

While the results of this study do not show a significant abundance of Sertoli-only tubules between the groups, testes from WD and MDWD display an increased frequency of abnormal and atypical tubules, of which there are Sertoli cell-only in addition to tubules with epithelial sloughing and vacuolisation. While the supplementation of WD with methyl donors normalises the population

of spermatogonial stem cells, it did not alter the frequency of abnormal tubules. This observation suggests that the development of seminiferous tubule abnormalities in both WD and MDWD groups is not fully dependent on PLZF-positive cells by means of impaired spermatogenesis, but additional mechanisms, such as abnormal mitochondrial respiration and cell cycles as well as cell adhesion and tight junction maintenance, processes displaying positive enrichment compared to CD testes. Interestingly, while the results revealed a significant increase in abnormal seminiferous tubule histology, no change in the number of Sertoli cells across the different diet groups was observed. The function, not the number, of Sertoli cells has been reported to be influenced by undernutrition (underfeeding) in a male sheep model, with disruption of tight-junction morphology and regression of Sertoli cell maturity leading to an impairment of Sertoli cell function and spermatogenesis (Guan et al., 2014). This suggests that the function of Sertoli cells in these groups may be disrupted independently of the number of cells. In fact, high fat diets have been shown to impair spermatogenesis through altering glucose and lipid metabolism in rat Sertoli cells (Luo et al., 2020). Additional mechanisms may include disruptions to the Sertoli cell cytoskeleton and impairment of the Sertoli-Sertoli tight junctions (Johnson, 2014). In WD testis, the results revealed an overexpression of several key genes mediating tight junction morphology and cytoskeleton integrity. This included several members of the claudin family such as *Cldn8*, *Cldn23*, *Cldn14*, and *Cldn11*. Claudins play a pivotal role in the maintenance of the BTB and are thus essential for spermatogenesis. While its mechanisms are not well understood, the overexpression of claudins is known to disrupt cell-cell adhesion and promote the progression of certain cancers (Lal-Nag and Morin,

2009; Li, 2021). In fact, the overexpression of many claudins has been reported in a number of tumours (Ding et al., 2013; Gowrikumar et al., 2019; Leech et al., 2013). In the testis, overexpression of *Cldn11* is linked to impaired spermatogenesis in men (Chiba et al., 2012; Fink et al., 2009; Nah et al., 2011; Stammler et al., 2016). WD testis also displayed an overexpression of *Cdh1*, a gene coding for CDH1 (previously known as E-cadherin), a transmembrane glycoprotein that plays a role in cell adhesion and is also involved in cancer formation through both increased and reduced expression (Fujita et al., 2009; Sonne et al., 2006; Xie et al., 2022). In the mouse testes, however, Tokuda et al. (2007) identified CDH1 as a specific marker for undifferentiated type A spermatogonia. In humans, CDH1 expression was not detected in adult testis but on the surface of epithelial cells in the epididymis (Andersson et al., 1994). The overexpression of *Vcam1* was also recorded in WD testis compared to CD. *Vcam1* (also known as CD106) is a known Leydig-cell specific gene that plays a role in testicular immunoregulation and cellular binding to lymphocytes (Sainio-Pöllänen et al., 1997; Veräjänkorva et al., 2003). Overexpression of *Vcam1* was observed in testis of rats undergoing autoimmune orchitis, or testicular inflammation (Guazzone et al., 2012). In MDWD, no significant changes in differential expression of cell adhesion regulators such as claudins or E-cadherin were observed. Filamin A (*Flna*) was under-expressed in both WD and MDWD compared to CD, further implicating compromised BTB integrity. Evidence in the literature on the role of *Flna* in the testis is rather lacking, however, its role in the assembly of the BTB during postnatal development has been reported in rat testis (Su et al., 2012). Additionally, FLNA expression was described in mouse testis in both germ cells and peritubular cells (Jarvis et al.,

2020) and human testicular peritubular cells (Flenkenthaler et al., 2014). Impaired functionality of FLNA is associated with the development and metastasis of a number of cancers (Welter et al., 2020). Taken together, while the findings in this study suggest that methyl donor supplementation inhibits the overexpression of cell adhesion and tight junction genes involved in BTB impairment induced by WD, MDWD testis still display significant abnormalities to testicular histology, suggesting that testicular damage may not be reversible by methyl donor supplementation within the duration of the study.

The data revealed an overexpression of oxidative stress-related genes *Pon3*, *Sod2,* and *Prdx3* in WD testis. Paraoxonase 3, or *Pon3,* while relatively under-investigated compared to other paraoxonase enzymes, is found to be overexpressed in a number of different tumours in humans (Whitaker et al., 2013). Furthermore, it serves to reduce mitochondrial superoxide formation (Schweikert et al., 2012). The expression of *Sod2* acts as a ROS scavenger in response to increased oxidative stress (Aitken and Roman, 2008; Kampfer et al., 2012). In a study examining *Sod2* function in response to diet-induced obesity, Liu et al., (2013) showed that the mice with an overexpression of *Sod2* were protected from high fat diet induced glucose intolerance (Liu et al., 2013). In a similar fashion *Prdx3* has been shown to improve glucose tolerance in a mouse model through the reduction of mitochondrial hydrogen peroxidase (Chen et al., 2008). Both *Pon3* and *Sod2* were similarly overexpressed in MDWD testis with a relatively greater fold change compared to CD, potentially indicating increased antioxidative activity in MDWD. The expression of *Prdx3* was not significantly altered in MDWD, however. The overexpression of these genes in WD suggests

an increase in oxidative stress in the testis, and that damage to the testicular cytoarchitecture could be mediated by an increase in ROS.

When comparing MDWD to WD, the differential expression of several key genes is observed and may thus further characterise the role of methyl donors when supplemented to a high fat high sugar diet. MDWD altered the expression of key 1-Carbon metabolism genes such as *Rsf1*, *Hat1*, and *Snai2*. *Rsf1* was downregulated in WD compared to CD, but its expression was not significantly altered in MDWD when compared to CD. However, the expression of *Rsf1* in MDWD was significantly increased in MDWD compared to WD. While the overexpression of *Rsf1* has been reported in a number of cancers and is often associated with poor prognosis (Cai et al., 2021), when normally expressed, it is required for a number of different regulatory functions including the facilitation of DNA strand break repair, suppression of oncogene transcription, and gene silencing histone modifications (Zhang et al., 2017). While the expression of *Rsf1* in the testis or how it is impacted by dietary intake has not been documented, its deficiency has been linked to impaired apoptotic function in response to excessive DNA strand breaks in the mouse brain (Min et al., 2018). The authors also observed changes in histone modifications which likely lead to altered chromatin structure (Min et al., 2018; Min et al., 2021). In the current study, methyl donor supplementation normalises *Rsf1* gene expression in the testis, suggesting a protective role against disrupted DNA fragmentation. Histone acetyltransferase 1 (*Hat1*), a key gene involved in histone modification, is also upregulated in MDWD compared to WD. Compared to CD, the expression of *Hat1* in WD was suppressed, whereas this was not observed in MDWD when compared to the control group. *Hat1* expression was found to be

significantly decreased in testis of sterile male cattle-yaks compared to non-sterile yaks, suggesting that a decrease in testicular histone acetylation could be one of the underlying causes of sterility in the cattle-yak hybrid species (Sun et al., 2022). *Hat1* has also been identified as a key player in spermatogenesis in *Eriocheir sinensis* (Chinese mitten crab) species, as its expression helped maintain histone acetylation and exerts a protective effect on nuclear chromatin (Liu et al., 2023; preprint). The expression of *Hat1* is normalised in MDWD compared to its suppression in WD without methyl donor supplementation. *Snai2* expression was not significantly altered in WD compared to CD. However, in MDWD, *Snai2* was significantly overexpressed when compared to both CD and WD. Snail transcription factors regulate cellular transitions during a number of developmental processes such as embryogenesis, spermatogenesis, and tumorigenesis (Zhou et al., 2019). The overexpression of *Snai2* has been reported in a number of cancers and is linked to poor prognosis (Coll-Bonfill et al., 2016). *Snai2* is also established as a primary regulator of adult stem cell function and differentiation in the mammary epithelium (Guo et al., 2012; Nassour et al., 2012; Ye et al., 2015). More recently, Gross et al., (2019) discovered that *Snai2* plays a pivotal role in the facilitation of DNA damage repair in mouse mammary epithelial cells (Gross et al., 2019). While the expression of *Snai2* in the testis is not well documented, its upregulation in MDWD may suggest that methyl donor supplementation induces an over-compensatory activity in the repair of DNA fragmentation. This is supported by the suppression of *Rsf1* in WD testis and the upregulation of oxidative stress-related genes. The long-term implications of the overexpression of *Snai2* in MDWD are uncertain, however, as the overexpression of *Snai2* has been

associated with tumorigenesis in humans and animal models (Lamouille et al., 2013).

In MDWD, the mRNA expression of sperm- and spermatogenesis-related genes was significantly increased compared to WD. The expression of these genes in either WD or MDWD, however, was not significantly altered when compared to the control group. Sperm Adhesion Molecule 1 (*Spam1*) was significantly overexpressed in MDWD testis when compared to WD. *Spam1* codes for an important sperm surface protein that is synthesised in both the testis and the epididymis independently with differing transcriptional regulation (Zhang and Martin-DeLeon., 2001). It plays a major role in sperm cumulus penetration during the process of fertilisation and has been identified as a marker for sperm maturation (Chen et al., 2006). The expression of *Spam1* is altered by dietary intake, as Yang et al. (2020) discovered that in mice fed a high cholesterol diet (3 %), the testicular mRNA expression of *Spam1* was reduced by 56 % compared to mice fed a standard chow diet (Yang et al., 2020). While a reduced expression of *Spam1* may be indicative of disrupted sperm maturation, WD testis in this study did not express a decreased level of *Spam1* mRNA expression. It is worth highlighting that Yang et al., (2020) used a 3 % cholesterol diet in their study, whereas half that amount of cholesterol (1.5 %) is used in the WD model in the current study, which may explain the unaltered expression level of *Spam1*. MDWD, however, had a significantly increased expression of *Spam1* mRNA compared to WD, suggesting that the influence of methyl donors on spermatogenesis extends to sperm maturation and is perhaps an adaptive response to the altered state of spermatogenesis in WD. Significantly increased levels of Equatorin (*Eqtn*) mRNA expression were observed in MDWD

compared to WD, despite neither of the groups having a significantly altered expression of *Eqtn* compared to CD. Equatorin is a sperm-specific acrosomal membrane protein that is involved in sperm acrosome reaction and fusion to the oolemma during fertilisation (Hao et al., 2014; Yamatoya et al., 2009). The role of *Eqtn* was further characterised by Ito et al. (2018) who, in a comparative study in a mouse *Eqtn*-knockout model, discovered that while *Eqtn* $^{-/-}$ sperm possessed normal morphology and membrane-penetrative capacity, they had significantly reduced fertility. Furthermore, fetuses of *Eqtn* $^{-/-}$ fathers had significantly decreased body weight, indicating a potential role of *Eqtn* in embryonic development (Ito et al., 2018). Furthermore, Synaptonemal Complex Protein 2 (*Sycp2*) was significantly upregulated in MDWD compared to WD. However, when both diet groups were compared to CD, no significant altered mRNA expression level of *Sycp2* was observed. *Sycp2* is a crucial structural element of the axial/lateral components of synaptonemal complexes (SCs) during meiosis and is pivotal for normal function of meiotic chromosome synapsis during both oocyte and spermatocyte development (Fraune et al., 2012). Knockout of the *Sycp2* gene in mouse models results in male sterility and female subfertility (Yang et al., 2006). Additionally, its crucial role in early meiotic recombination was further highlighted in a recent study in which no spermatids or spermatozoa were detected in *Sycp* $^{-/-}$ zebrafish testis (Takemoto et al., 2020). The expression of *Sox30* was significantly reduced in both WD and MDWD compared to CD. However, when compared to WD, MDWD had significantly greater level of mRNA expression of *Sox30*. *Sox30* is an essential transcription factor in spermiogenesis, and its absence in mouse models results in arrested spermiogenesis followed by spermatid apoptosis (Zhang et al., 2018).

Furthermore, another key role that *Sox30* plays is the regulation of spermatid-specific long non-coding and protein-coding RNA genes (Zhang et al., 2018). The expression of *Sox30* is not only observed in germ cells but in Sertoli cells too. The hypomethylation of *Sox30* is associated with its increased expression in the testis, whereas a hypermethylated state in organs such as the ovaries results in the lack of *Sox30* expression (Han et al., 2014). The deletion of *Sox30* in mice results in impaired testicular development and spermatogenesis and infertility (Han et al., 2019). Han et al. (2014) were able to restore *Sox30* expression in different cell lines following demethylation, confirming that epigenetic regulation in the form of methylation contributes to *Sox30* silencing (Han et al., 2014). Here in this study, results show that *Sox30* expression is suppressed in response to high fat diet feeding, and that methyl donor supplementation contributes to the relative normalisation of *Sox30* mRNA expression in the testis, potentially through epigenetically mediated activation. A summary of altered gene expression levels in different models in the literature is highlighted in *Table 4.12*.

Gene	Model	Result	Study
Lipe	Knockout (mouse)	Azoospermia & disrupted energy metabolism in testis	Wang et al. (2017)
Ago1	Stable overexpression (human cell line)	Tumour suppressor properties in a neuroblastoma cell line	Parisi et al., 2011
Ago4	Knockout (mouse)	Initiates meiosis earlier than non-knockout models	Modzelewski et al., 2012
Mtfmt	Knockout (mouse)	Embryonic death	Sun et al., 2023
Mettl	Inhibition (human cell line)	Disruption of mitochondrial function	Van Haute et al., 2019
Parn	Knockout	Modifies telomere length (human), embryonic lethality (mouse)	Benyelles et al., 2019
Pwp1	Knockout (mouse)	Rapid telomere shortening	Yu et al., 2019
Cldn11	Overexpression (human testes)	Impaired spermatogenesis	Chiba et al., 2012
Vcam1	Overexpression (rat testis)	Testicular inflammation	Guazzone et al., 2012
Sod2	Overexpression (mouse)	Protection from high fat diet induced glucose intolerance	Liu et al., 2013
Sox30	Knockout (mouse)	Arrested spermiogenesis and spermatid apoptosis	Zhang et al., 2018

Table 4. 14: Gene expression and knockout studies in the literature.

Feeding male mice a high fat diet has also been shown to reduce plasma testosterone levels in rodents, which might be another possible explanation behind the alterations observed in testicular tubule morphology (Cano et al., 2008). It is worth highlighting that findings in the literature are not consistent, as the impact of high fat diets on seminiferous tubule morphology is dependent on multiple factors such as fat content in diets, duration of feeding, whether a state of obesity was induced or not as well as testicular aging. It has been shown that testicular aging in rats causes germ cell loss and what seems to be a total or partial regression of seminiferous tubules (Syed and Hecht, 2001). The mechanisms by which high fat diet helps induce such changes in seminiferous tubule cytoarchitecture, and whether or not it is linked to more rapid testicular aging remain unclear.

High fat diet has been shown to have a number of implications on adult male reproductive wellbeing. For instance, high fat diets are associated with low levels of testosterone in both men (Minguez-Alarcón et al., 2017; Nagata1 et al., 2000) as well as rodents fed for 12 weeks (Cui and Guan, 2016). Moreover, elevated levels of sperm DNA fragmentation have been reported in response to high fat diets in men (La Vignera et al., 2012). Studies in mouse models also show significant increase in levels of sperm DNA fragmentation (Bakos et al., 2011) and abnormal embryo development (Mitchell et al., 2011). In the current study, WD upregulated genes involved in perturbed calcium regulation as well as abnormal mitochondrial physiology. Serum calcium levels have been shown to be linked to sex steroid hormone levels (Alexandre, 2005). On the other hand, testosterone has been shown to cause an increase in intracellular calcium levels in rodent cardiac cells (Vicencio et al., 2006). Disrupted calcium levels and

mitochondrial physiology thus might be associated to reduced serum testosterone levels caused by the high fat diet. Furthermore, high fat diets have been associated with increased apoptosis in Leydig cell populations, leading to reduced levels of testosterone (Amrolia et al., 1988; Lu et al., 2003; Rato et al., 2014) and spermatogenic cells (Chen et al., 2011; Ghosh and Mukherjee, 2018).

The findings in this study confirm further observations in the literature in both mouse and rat models with regard to the impact of high fat diets on testicular cytoarchitecture. WD and MDWD resulted in an increased number of seminiferous tubule abnormalities compared to the other diet groups, notably epithelial disruption, germ cell loss, and sloughing of the basement membrane. Despite the increase in abnormal tubule number, the number of spermatogenic and Sertoli cells remains unchanged, indicating a possible impact on cell function rather than number. This hypothesis is supported by the gene expression patterns in WD and MDWD testes, which show an upregulation of genes involved in abnormal cell cycle, abnormal mitochondrial physiology, and calcium regulation which is a necessary component in the regulation of Sertoli cell and epithelial junctions in the seminiferous tubules. When looking at the epididymal histology, the supplementation of methyl donors to WD may potentially cause a decrease in the number of atypical tubules in the caudal epididymis; however, the results obtained in this study are not statistically significant. It is worth noting that he number of studies investigating the impact of dietary modification on epididymal histology remains limited. In a recent study in high fat diet-fed mice, Li et al. (2022) discovered an increased level of oxidative stress in the epididymis, which may give rise to impairments in sperm maturation and fertility (Li et al., 2022). More recently, Falvo and colleagues

confirmed those findings in a Wistar rat model fed a high fat diet over a 5-week period, highlighting an increase in oxidative stress in all regions of the epididymis as well as elevated levels of apoptosis in the corpus and caudal segments of the epididymis (Falvo et al., 2023). Increasing evidence suggests that oxidative stress and damage can impair DNA methylation in the testis (Sharma et al., 2019). It is also established that lifestyle factors such as dietary intake can influence spermatogenesis through promoting oxidative stress in the testis, as is demonstrated by Luo et al. (2019) in their study of diet-induced obesity in a rat model. In the present study, methyl donor supplementation significantly reduced the number of atypical epididymal tubules. Though not statistically significant, methyl donor supplementation also relatively reduced epididymal abnormalities when supplemented to a low protein diet. It is therefore conceivable that methyl donor supplementation could reduce the effect of epididymal oxidative damage by means of DNA re-methylation. An additional mechanism of oxidative stress mitigation through methyl donor supplementation is by means of their antioxidative properties, as it is evidenced that methyl donors such as vitamin B_{12} (van de Lagemaat et al., 2019), folate (Sijilmassi, 2019), and betaine (Alirezaei et al., 2015) could play antioxidative roles. However, further study is required to test this hypothesis.

4.6 CONCLUSIONS, STRENGTHS, AND LIMITATIONS

Taken together, this data suggests that high fat diet consumption may induce aberrations in seminiferous tubule histology through compromised BTB integrity and an increase in oxidative damage. The decrease in spermatogonial stem cell population leads to disruptions in the normal spermatogenic cycle and potentially sperm DNA integrity when coupled with altered expression of oxidative stress related genes. Epididymal damage may be caused by an increase in oxidative damage beyond the repair capacity of the epididymal antioxidative system (Flaherty, 2019). It is also worth noting that histological artifacts from tissue processing may be present and impact certain aspects of the analysis, particularly that of cell density. Therefore, further experiments aimed at examining epididymal damage may be necessary to establish a clearer picture.

Methyl donor supplementation may play a role in mitigating oxidative stress-related damage and facilitating DNA repair potentially through different epigenetic mechanisms involving histone modification and chromatin remodelling. Additionally, methyl donor supplementation may play a role in restoring spermatogonial stem cell populations and restoring normal spermatogenesis gene expression function and BTB integrity. However, supplementation does not seem to influence testicular tissue cytoarchitecture in this study. In fact, methyl donors result in uniquely different changes to adult male testis depending on the diet to which they are supplemented to. Global testicular gene expression analysis revealed four distinct gene expression profiles with relatively minimal overlap. Future investigations aiming to characterise the role of methyl donor supplementation on testicular function are recommended to examine high fat diet feeding and supplementation

independently and in combination over a more extended duration. Furthermore, germ cell marker testing in the epididymis would help determine the impact on spermatogenesis and sperm maturation. A deeper insight into testicular cell populations and their individual gene expression patterns offers an effective characterisation of the mechanisms underlying impact of diet – with or without supplementation – on testicular function. One limitation of this study is the time constraints, chiefly brought on by the COVID-19 pandemic lockdown, limiting the capability of carrying out further experimentation such as sperm RNA sequencing, RT-qPCR analysis and validation on testicular and epididymal tissue, and immunostaining of different proteins expressed in the testis or the epididymis. Moreover, the Johnsen scoring method was also not used for the analysis of the testis and their abnormalities due to the same constraints brought upon by the COVID-19 pandemic and the limited timeframe available for analysis, writing, and submission. However, this data still succeeds in shedding further light on the influence of nutrition on the testicular environment. It also contributes to the growing body of evidence showing that dietary intake, with or without supplementation, plays a significant role in altering testicular gene expression patterns and histology, potentially with longer term impact on spermatogenesis.

CHAPTER FIVE: THE IMPACT OF SUB-OPTIMAL PATERNAL DIET ON MATERNAL GESTATIONAL WEIGHT AND FETAL GROWTH

5.1 INTRODUCTION

The role that maternal diet plays in shaping her reproductive health and subsequent embryonic and offspring development is a well-established in the literature. In fact, a number of studies in animal models (Mennitti et al., 2022; Watkins et al., 2011) as well as epidemiological studies and meta-analyses in humans (Chia et al., 2019; Wang et al., 2022) have reported that maternal consumption of poor diets such as high fat or low protein diets before and during pregnancy not only perturbs pregnancy outcomes, such as live birth rates, but also adversely impacts offspring development and increases their predisposition to diseases such as obesity, cardiovascular disease, and type 2 diabetes. Physiological changes during maternal gestation require significant adaptive responses in maternal immune, metabolic, reproductive, and cardiovascular functions (Soma-Pillay et al., 2016). Perturbations and disruptions to a mother's health during gestation have been shown to elicit both short- and long-term consequences including gestational diabetes and preeclampsia, which are thought to be associated to fetal overgrowth and intrauterine growth restriction respectively (Brett et al., 2014; Kingdom et al., 2018). Proper early placental establishment is crucial with regard to these complications in addition to the modulation of fetal development (Sferruzzi-Perri & Camm 2016). The majority of available research on placental development and integrity throughout pregnancy has focused on factors such as maternal diet, age, and the critical

maternal-fetal interactions and how they impact subsequent offspring development through placenta-mediated mechanisms. However, the placenta is a biparental organ, with its genetic makeup being contributed to by both mother and father (Wang et al., 2013). It is therefore conceivable that paternal factors such as diet and metabolic health during periconception/conception could also influence placental development and subsequently impact both maternal and offspring health through placentally-mediated mechanisms. For instance, in mice, paternal low protein diet has been shown to induce a significant increase in the placental junctional zone and a reduction in the labyrinth zone in a C57BL/6 mouse model (Watkins et al., 2017). And while the significance of placental glycogen flux is not yet fully understood, fluctuations in humans have been linked to pregnancy complications such as gestational diabetes and preeclampsia (Akison et al., 2017). Furthermore, today, we know from studies in animal models (Binder et al., 2015; Carone et al., 2010; Lambrot et al., 2013; Watkins et al., 2018) as well as human cohorts (Burke et al., 2005; Kasman et al., 2020) that paternal diet and physiology plays a significant role in altering embryonic development and adult offspring health.

Since then, studies have aimed to also identify and characterise the mechanisms by which offspring health is altered by paternal diet during the periconception period, identifying certain markers such as sncRNA populations (Chen et al., 2016), sperm DNA methylation profiles (Soubry et al., 2013), and histone modifications (Wang et al., 2019) as the possible mechanisms behind the epigenetic regulation of fertility and paternal programming of offspring development.

Interestingly, another potential mechanism of paternal programming of offspring health is mediated through the seminal plasma and not the sperm itself. Studies have highlighted the role of seminal plasma in modulating the maternal uterine environment during the preimplantation period independent of sperm-mediated mechanisms. In a paternal low protein diet model, seminal plasma from males fed a LPD were found to program metabolic dysfunction in the offspring despite the sperm being derived from control diet-fed males (Watkins et al., 2018). Bromfield et al. (2014) also highlighted the importance of seminal plasma by demonstrating that removing seminal plasma around conception significantly impacts embryo development and subsequent offspring health. Furthermore, the authors transferred embryos from females mated with control males to ones mated with males with removed seminal plasma, resulting in offspring with increased adult adiposity, thereby highlighting the role of seminal plasma in essentially priming the uterine environment (Bromfield et al., 2014). In a recent study on a paternal high fat diet mouse model, Schjenken et al., (2021) found that high fat diet significantly increased the volume of seminal vesicles in addition to decreased levels of cytokines and transforming growth factor beta (TGF-β) isoforms, all of which were implicated in modulating the immune response of the maternal uterine environment (Morgan and Watkins, 2021; Schjenken et al., 2021).

5.2 AIMS AND OBJECTIVES

The impact of maternal dietary intake on fetal growth and offspring development has become well established in recent decades. Our understanding of the role that the father plays in influencing the growth of his offspring as well as his impact on the maternal reproductive environment during gestation still require further elucidation. This study hypothesises that sub-optimal paternal diet could influence offspring health by indirectly influencing maternal gestational health, or vice versa. Furthermore, this study hypothesises that the supplementation of methyl donors could potentially ameliorate certain adverse effects caused by paternal consumption of sub-optimal diets. The aims of this study are to:

- Assess fetal morphometric indices in response to paternal diet.
- Examine the impact of paternal diet with or without supplementation on fetal and placental growth.
- Determine the role of methyl donor supplementation in the context of high fat high sugar or low protein nutritional state on late gestation.

5.3 METHODOLOGY

5.3.1 Animals and mating

Virgin 8-week-old male C57BL/6 mice (n=40/batch) and 5 to 9-week-old female C57BL/6 mice were maintained described in *section 2.1*. The composition of all the diets fed to the stud males are detailed in *Appendix A1*.

As detailed in *section 2.2*, vaginal plugs were checked the following morning, and the presence of a vaginal plug taken as an indication of successful mating. Females positive for plugs were maintained on regular chow *ad libitum* until late gestation (day 17, term being day 19) when they would be culled via cervical dislocation.

5.3.2 Tissue Harvesting

As described in *section 2.2*, female mice were weighed following cull prior to the removal and weighing of the uterus (including all individual concepti). Maternal liver, heart, and kidneys were collected, weighed and snap frozen. One kidney (left) was fixed in 10 % formalin for later histological use while the other (right) was snap frozen and was stored at -80º C.

After the collection of maternal tissue, the uterus was cut open and the conceptuses were removed into labelled dishes based on the position of each fetus on either uterine horn. Conceptuses were weighed before dissection, with this weight being comprised of the weight of the fetus, yolk sac, and placenta. Each component of the conceptus (fetus, yolk sac, placenta) was weighed individually. Fetal organs (heart, liver, kidneys) were weighed and snap frozen.

5.3.3 Statistics

GraphPad PRISM 9 was used for the statistical analysis of the data. Outliers in data sets were identified using PRISM's Robust Regression and Outlier Removal (ROUT) method and data was statistically analysed with the outliers excluded from the data sets as necessary. Normality testing was carried out using D'Agostino & Pearson test. Normally distributed data were analysed using one-way ANOVAs with Tukey's post hoc test and non-normally distributed data were analysed using non-parametric Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons. A p value ≤ 0.05 was deemed statistically significant for this study.

5.4 RESULTS

5.4.1 Paternal diet has minimal impact on maternal gestational weight.

After being culled via cervical dislocation, female mice at day 17.5 of pregnancy were weighed before being dissected. No significant differences were observed in maternal starting weight (maternal starting weight recording was not performed prior to the last batch of females) (*Figure 5.1*).

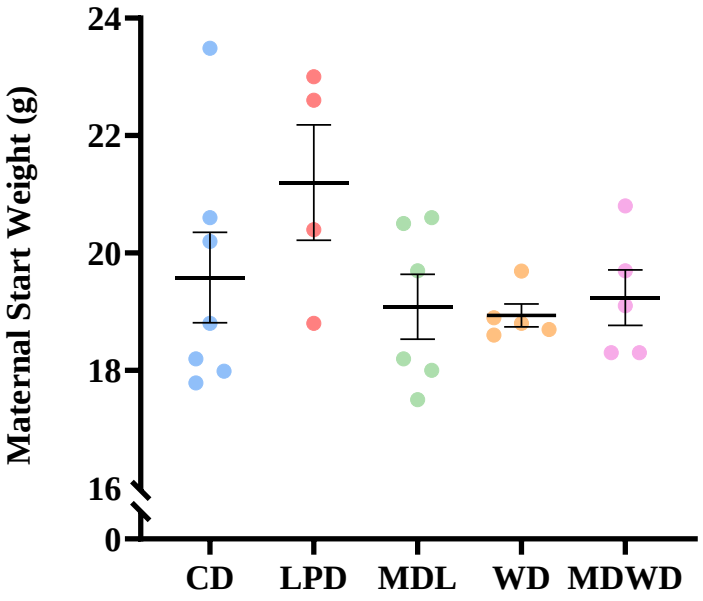

Figure 5.1: Maternal starting weight (n=4-7/group). D'Agostino & Pearson test was carried out followed by One-way ANOVA and Tukey's post-hoc tests. Individual dams are presented as data points on each graph ± SEM. Maternal starting weight data was only collected for successfully mated and culled females from the last batch.

Additionally, no significant differences were observed in maternal weight at time of culling (*Figure 5.2A*). Upon dissection, maternal organs were collected

and weighed. No significant differences were observed in maternal heart (*Figure 5.2B*), liver (*Figure 5.2C*), and total kidney weights (*Figure 5.2D*).

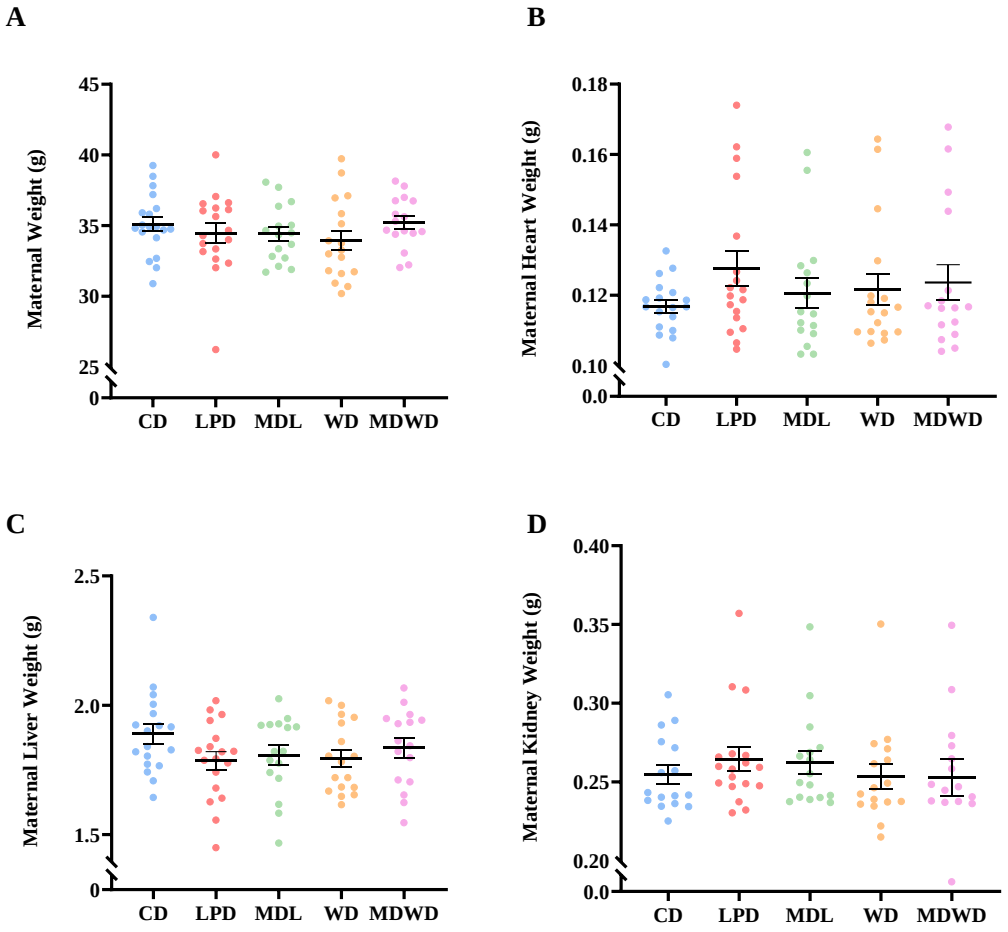

Figure 5.2: Maternal weights at time of sacrifice showing (A) maternal heart weights (B), liver weights (C), and total kidney weights (D) (n=16-19 dams/group). D'Agostino & Pearson test was carried out followed by One-way ANOVA and Tukey's post-hoc tests for normally distributed data (A) and Kruskal-Wallis with Dunn's post-hoc test for the other data sets (B, C, D). Individual dams are presented as data points on each graph ± SEM.

Furthermore, no significant differences were observed in organ to body weight ratios when looking at maternal heart, liver, and total kidney weights as represented in *Figure 5.3A*, *B*, and *C* respectively.

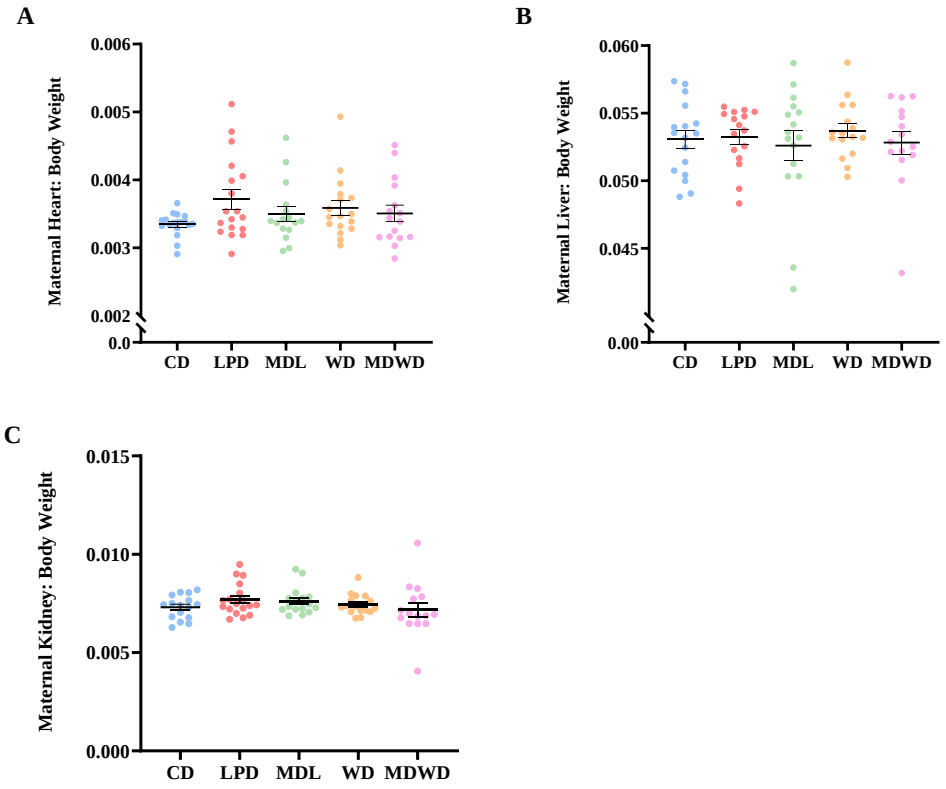

Figure 5.3: Maternal organ to body weight ratios. Graphs represent maternal heart to total body weight ratio (A), liver to body weight ratios (B), and total kidney to body weight ratios (C) (n= 15-18 dams/group ± SEM). D'Agostino & Pearson normality test was carried out to test data distribution. Kruskal-Wallis analysis followed by Dunn's post-hoc test were carried out on all three data sets after the identification of outliers using Prism's ROUT method.

5.4.2 Paternal nutrition does not significantly influence litter size or uterine weight.

Female mice were dissected after being weighed and the whole uterus was removed from each individual animal, with each uterus being weighed before dissection. The total weight of each uterus was subtracted from the weight of each respective female mouse at time of sacrifice to calculate the weight of each dam independent of its litter. No significant differences were recorded in whole uterus weight (*Figure 5.4A*), whole uterus to total body weight ratio (*Figure 5.4B*), or the weight of the dams independent of uterine and litter weight (*Figure 5.4C*). Furthermore, litter size was also compared between the dams mated to stud males of each of the study groups to see if paternal dietary intake plays a role in influencing litter size in dams maintained on a standard rodent chow diet. Differences in litter size between the groups, however, were not statistically significant (*Figure 5.4D*).

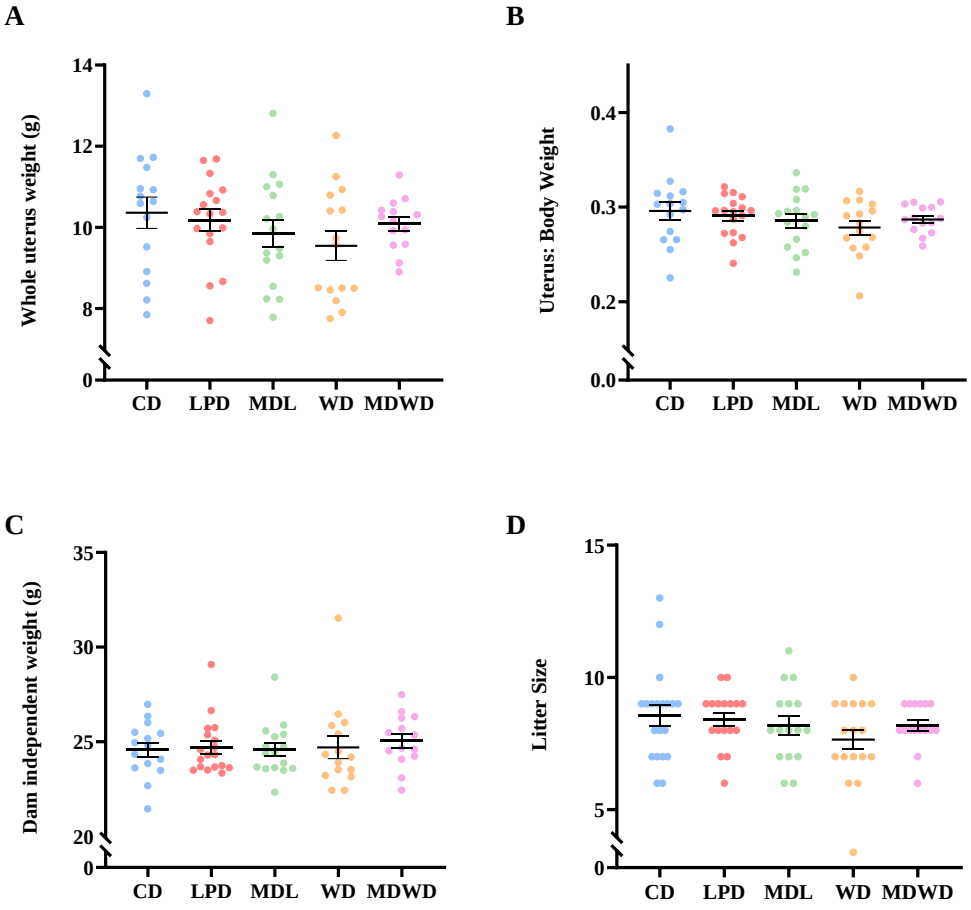

Figure 5.4: Maternal uterine weight, weight ratio, and litter size. Graphs represent total uterine weight (A), uterus to body weight ratio (B), dam weight independent of whole uterus (C), and total litter size (D) ± SEM (A, B, C, n=15-18/group; D, n=16-20/group). Data sets were tested for normal distribution using D'Agostino & Pearson test. Kruskal-Wallis test followed by Dunn's post-hoc test was carried out to analyse the data.

5.4.3 The impact of paternal dietary intake on fetal and placental growth.

Upon uterine dissection, each conceptus from each litter was carefully removed and placed in labelled dishes. Conceptuses were weighed before being delicately dissected for the removal and weighing of the fetuses, placentas, and yolk sacs. No significant differences in conceptus weights were observed (*Figure 5.5A*).

However, upon the removal and weighing of the fetuses, it was revealed that LPD and MDL fetuses were significantly lighter than those of the CD and WD groups (*Figure 5*.5B; $p \leq 0.05$). Whole placental weight was also compared between the different experimental groups, revealing the MDWD group to have significantly heavier placentas compared to MDL (*Figure 5.5C*; $p \leq 0.05$). When looking at the weights of the yolk sacs between the different study groups, the MDL group displayed significantly reduced yolk sac weights compared to CD, LPD, and MDWD (*Figure 5.5D*).

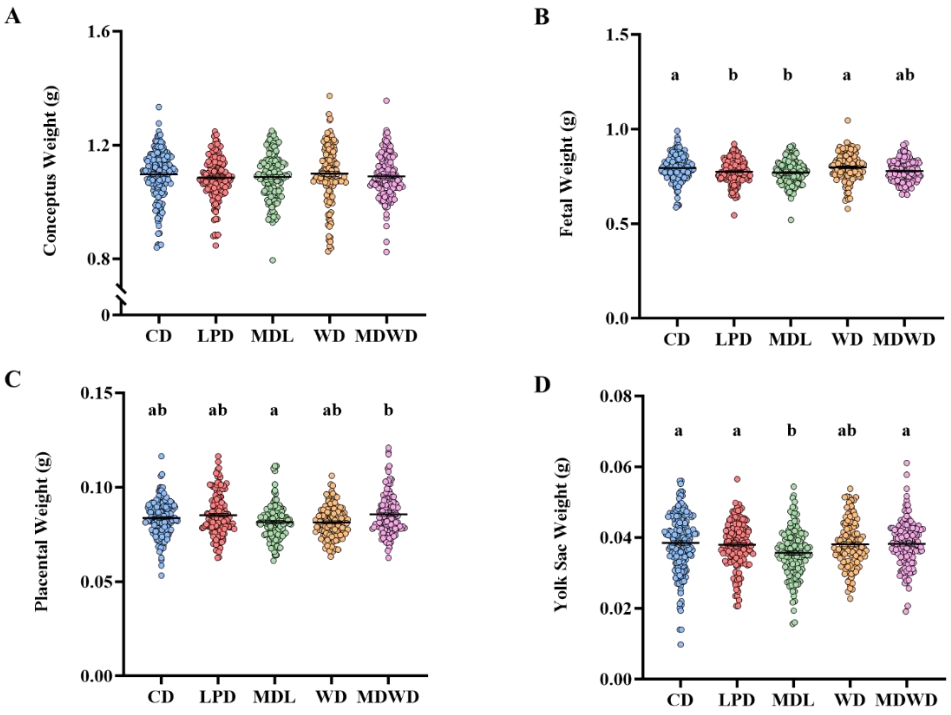

Figure 5.5: Conceptus fetal, placental, yolk sac weights. Graphs represent conceptus weight (A), fetal weight (B), placental weights (C), and yolk sac weights (D) between the five groups ± SEM. Data sets were assessed for normality using D'Agostino & Pearson test. Kruskal-Wallis followed by Dunn's post-hoc test was carried out to analyse the data sets.

When looking at fetal weights as a ratio of placental weights, as an indicator of placental efficiency, significant differences are observed between the study groups. WD-group samples displayed a significantly increased ratio of fetal body weight to placental weight compared to the other study groups (*Figure 5.6*).

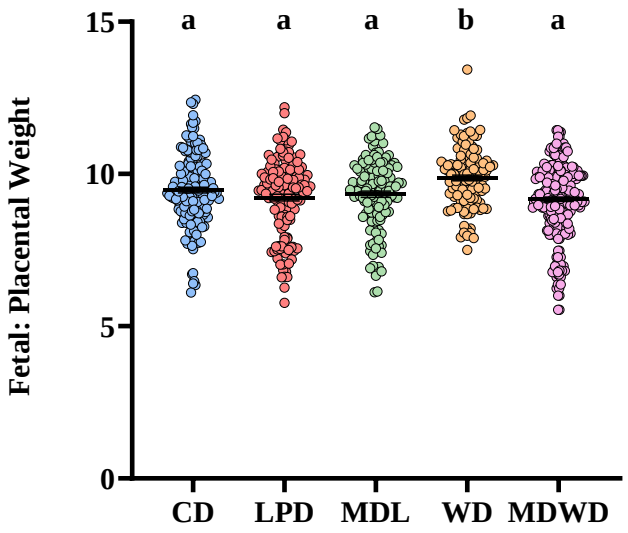

Figure 5.6: Fetal to placental weight ratios. Graphs show fetal body weight as a ratio to placental weight ± SEM. Kruskal-Wallis analysis followed by Dunn's post-hoc test were carried out on this data set.

Fetuses were then dissected, and their hearts and livers were weighed. No significant differences were observed in fetal heart or liver weights between the different groups (*Figure 5.7A*, *B*).

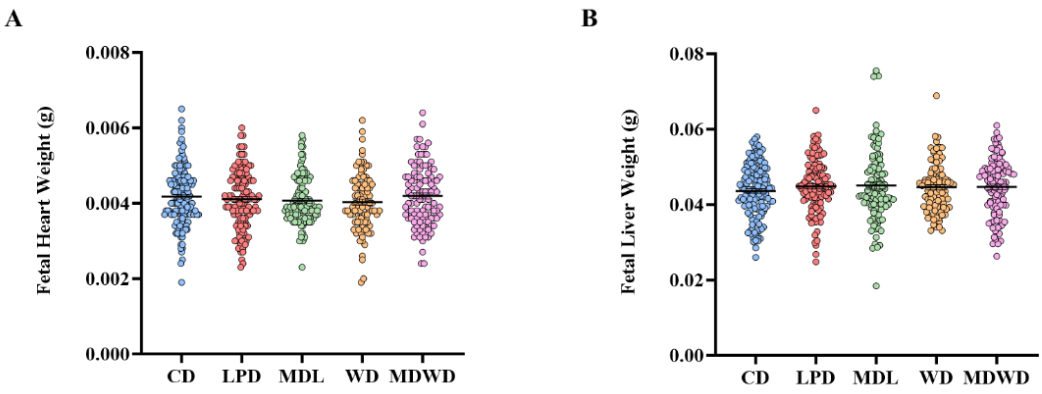

Figure 5.7: Fetal raw organ weight. Graphs represent fetal heart weight (A) and fetal liver weights (B) across the different groups ± SEM. D'Agostino & Pearson normality test was used followed by Kruskal-Wallis analysis (+ Dunn's post-hoc test).

Fetal organ weights were also analysed as a ratio of total body weight, revealing no significant differences in fetal heart to body weight ratio (*Figure 5.8A*) but showing a significantly increased liver to body weight ratio in the LPD and MDWD groups compared to the control group CD (*Figure 5.8B*).

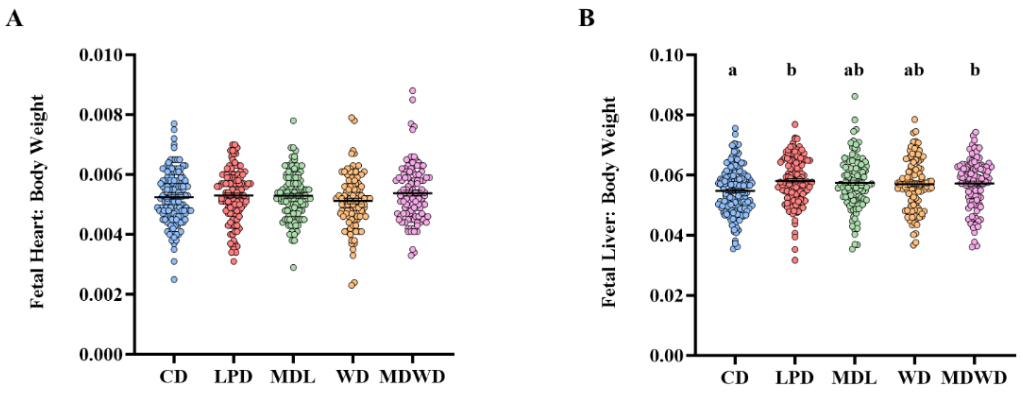

Figure 5.8: Fetal organ weight as a ratio of body weight. Graphs show fetal heart (A) and fetal liver (B) weights as a ratio of total body weight ± SEM. D'Agostino & Pearson normality test and Kruskal-Wallis analysis, followed by Dunn's post-hoc test, were carried out.

5.5 DISCUSSION

In the current study, no difference was observed in maternal late-gestation weight in response to being mated with males from any of the experimental diet groups. Additionally, paternal diet did not appear to have an effect on maternal heart, liver, or kidney weights during late pregnancy, or their respective ratios to maternal total body weight. It is worth highlighting that research into the impact of paternal diet on maternal gestational health is relatively in its infancy. Moreover, beyond our understanding of the impact of paternal sperm and seminal plasma on the uterine environment and placental growth, there is a clear need for further study in this particular area. Litter size was also not significantly altered between the groups in response to the different paternal diets. This observation confirms other findings in murine studies in the literature such as paternal high fat diet (Masuyama et al., 2016), paternal LPD (Watkins et al., 2018) and paternal dietary restriction (McPherson et al., 2016) or supplementing LPD with methyl donors (Morgan et al., 2020). Additionally, no significant changes to uterine weight and its respective ratio to total dam body weight was observed either. In recent years, evidence of the impact of paternal suboptimal diet on the maternal uterine environment has become more abundant, with recent animal model studies reporting that paternal LPD (Watkins et al., 2018) and high fat diet (Schjenken et al., 2021) can influence maternal uterine immune responses through seminal plasma mediated mechanisms at conception. While paternal diet has more recently been known to impact offspring health through altering maternal uterine cytokine production and reproductive tract immune response through seminal plasma (Schjenken et al., 2021), there is no reported incidence of an impact on the uterine weight itself.

While dietary intake in this animal model does not have an effect on either paternal or maternal fertility or reproductive fitness, it does impact fetal and placental development, as also observed by Watkins et al. (2018) in a paternal LPD model, Wu et al., (2021) in a high fat diet-induced obesity model, and Ryan et al. (2017) with a methyl donor-supplemented model. Previous studies in mouse models showed that paternal LPD feeding results in the increased placental mRNA expression levels of key glucose transporters (*Slc2a1*, *Slc2a4*) in addition to calcium (*Atp2b1*) and amino acid (*Slc38a2*, *Slc38a4*) and programmed fetal overgrowth during maternal late gestation (Day 17), thereby increasing offspring predisposition to adult-onset cardiometabolic disorders (Watkins et al., 2017). In this current study, LPD-derived fetuses were significantly lighter than those of the CD group. This contradicts previous observations made by Morgan et al., (2021) and Watkins et al. (2017), who found significant increases to fetal weight in response to paternal LPD. The discrepancy in fetal weight may be influenced by different factors such as the litter sex ratio, as it has been observed that diet can program birth weight in a sex specific manner. Female offspring of LPD-fed male mice were shown to have significantly reduced birth weights compared to those from fathers fed a control diet (da Cruz et al., 2018). The authors report that this phenotype is associated with an altered differential expression of key miRNAs (miR-92a, miR-200c, and miR-451a) in female offspring mammary glands involved in the regulation of the AMPK energy signalling pathway (da Cruz et al., 2018). Indeed, miRNAs play a pivotal role as small RNA molecules in the epigenetic programming of offspring health. As late gestational fetal weight can be a strong indicator of birth weight, which is associated with adult-onset diseases such as

ischemic heart disease (Barker and Osmond, 1986), it is conceivable that suboptimal paternal diet can program fetal weight and subsequent offspring birth weight through a sex-specific manner. While offspring sexing was not carried out in the current study due to time constraints and offspring sexing not being one of the primary objectives of the study as a whole and stands as a possible limitation to this analysis, it is possible that fetal weight in response to paternal LPD is influenced by a greater ratio of female to male offspring. This hypothesis is supported by previous observations made in a mouse model, where paternal LPD feeding was linked to a reduced litter male to female ratio (Watkins and Sinclair, 2014). Additionally, caloric restriction in female mice results in a male-favoured litter sex ratio (Rosenfield, 2011). This has been mirrored on the paternal side in a study by McPherson et al., (2016) who also reported that dietary restriction results in altered male to female sex ratio, with diet restricted fathers siring more male offspring. While the number of studies confirming or denying such findings is relatively insufficient to adequately produce strong evidence, this phenomenon could be the result of altered X/Y chromosome bearing sperm which thus disrupts individual sperm function (Edwards and Cameron., 2014). This, however, remains a hypothesis and requires further elucidation by means of substantial evidence. Additionally, the altered litter sex ratio could also be explained by LPD influencing the DNA content between X or Y spermatozoa, thereby differentially influencing sperm motility and swimming pattern (Johnson et al., 1989; Johnson et al., 1994). However, the findings made in these studies are not conclusive. Another possible mechanism behind the altered litter sex ratio could also be LPD semen influencing the maternal uterine environment in a manner more favourable to female embryos

(Rahman and Pang, 2020; Rosenfield, 2011), unlike that of strictly dietary restriction regimens, possibly through nutritional availability and the differences in nutritional demands between male and female embryos (Tamimi et al., 2003).

When looking at supplementing LPD with methyl donors, it was revealed that supplementation had no significant effect on fetal body weight, as MDL fetuses were similarly significantly lighter than CD fetuses. This could either be explained by methyl donors having no effect on fetal body weight, or that methyl donor supplementation influences litter sex ratio to subsequently influence fetal bodyweight epigenetically by means of altered differential sperm gene expression profiles. An additional potential explanation could be in the form of methyl donors having an opposing effect, thereby balancing out the observed outcomes. However, a recent study by Gross et al. (2020) found that methionine supplementation in rams does not significantly influence overall sex ratio in the F1 generation compared to the control diet. This may potentially indicate that methyl donors simply do not influence litter sex ratio and that the relatively reduced fetal weight is instead driven by protein malnutrition. As there is little published evidence on the impact of paternal low protein diet on litter sex ratio, and even less evidence on the effect of methyl donor supplementation, it is not possible to come to a definitive conclusion and so this study can only hypothesise about the changes in fetal sex ratio. Fetuses derived from the WD group did not display any change in weight when compared to CD, but they were significantly heavier than both low protein diet (LPD and MDL) groups, whereas no significant difference was observed between both methyl donor-supplemented groups MDL and MDWD. While significant reductions in fetal weight and birth weights have been shown in response to high fat diet induced

obesity in a number of studies in rodent models (Binder et al., 2012; Binder et al., 2015; McPherson et al., 2013), the impact of high fat feeding without obesity is relatively less documented. Interestingly, the likelihood of both small for gestational age (SGA) and macrosomia has been reported in a paternal obesity model in humans (Campbell and McPherson, 2019). In other population-based human studies, significant correlations between paternal obesity and offspring macrosomia have been reported (Yang et al., 2015). On the other hand, McCowan et al. (2011) reported that obese men have a higher likelihood of having SGA than men who weren't obese. Sun et al. (2022) recently reported that paternal pre-pregnancy obesity and overweight are negatively correlated with offspring birth weight. On the other hand, Xu et al. (2021) observed that paternal BMI was positively correlated with increased birth weight in a sex-specific manner, with the correlation being only observed in male neonates. The authors also note that paternal body weight was not correlated with the risk of macrosomia (Xu et al., 2021). Lin et al. (2022) recently published a prospective cohort study that included over 7,000 women with singleton pregnancies to investigate fetal growth and pregnancy complications in response to paternal BMI. The authors found that as paternal BMI increased, fetal and placental weight also showed a positive trend. However, Lin et al. (2022) reported that these differences were not observed in the obese group and concluded that paternal high BMI has relatively little effect on average fetal birth weight as opposed to other indices such as SGA and preeclampsia. While a study with over 7,000 participants offers robust evidence of these observations, evidence in other human studies remain relatively contradictory. Data from animal models, on the other hand, seems to be in agreement on the impact on obesity on fetal growth.

Further study into the nature of this relationship is needed, with appropriately characterising the role of high fat diet feeding prior to the induction of obesity on fetal growth. The observed impact of paternal overnutrition on altered fetal weight in a sex-specific manner could be mediated by sperm or seminal plasma mediated pathways. For instance, a recent study in a C57BL/6 mouse model fed a high fat diet (45 % fat) showed that paternal high fat diet consumption alters sperm microRNA content in addition to influencing histone protein expression and modified placental mRNA expression of nutrient transporters (Claycombe-Larson et al., 2020). In fact, it is possible that paternal overnutrition programs offspring birth weight through epigenetically mediated mechanisms involving sperm noncoding RNA and histone modifications (Noor et al., 2019). The impact on placental nutrient transport could also significantly influence fetal weight, as impaired placental transport of key nutrients required for healthy fetal growth could result in fetal growth restriction (Gaccioli and Lager, 2016). Low birth weight is associated with a number of adult-onset diseases including hypertension, type 2 diabetes, and ischemic heart disease (Barker and Osmond, 1986; Neel, 1962), and high birth weight is programmed for increased risk of adult-onset diseases such as cancer (Thornburg et al., 2010). It is therefore of great importance to better understand the mechanisms underlying paternal programming of fetal weight and overall growth, as birth weight is a significant determinant of offspring health and development.

Placental weight was not significantly altered between CD and any of the study groups. This deviates from the findings made by Watkins et al. (2017) who observed reduced placental weights in response to paternal LPD. Additionally, observations made by Binder and colleagues in 2012 and 2015 show that

paternal obesity, induced by high fat diet feeding, is associated with reduced placental weight in a rodent model (Binder et al., 2012; Binder et al., 2015). Babies born with a significantly increased or decreased placental to birth weight ratios have an increased risk of developing adult-onset chronic diseases (Thornburg and Marshall, 2015). This programming of placental development – and subsequent offspring health – could be mediated by a number of possible mechanisms including sperm epigenetic status. The altered state of embryonic development could also possibly influence placental physiology by means of nutritional transport and compensatory and adaptive responses to embryonic nutritional demands.

The only significant difference observed in placental weight in this study was between both methyl donor groups, where MDWD fetuses had significantly increased placental weights compared to MDL, raising the question of whether methyl donors impart relatively unique influences depending on the diet to which they are supplemented to. Another question raised by this observation is whether the difference is driven by either of the sub-optimal diets LPD and WD. The differences between those and the control, however, are not statistically significant. In a study examining paternal folate status on epigenetic programming in a mouse model, Lambrot et al. (2013) discovered that sperm differential methylation profiles in folate deficiency is implicated in chronic diseases such as cancer and diabetes. However, folate deficiency had no significant impact on placental size and weight, and only two of the differentially methylated genes identified in the sperm showed altered differential expression in the placenta with no change in methylation patterns (Lambrot et al., 2013). This led the authors to hypothesise the involvement of other mechanisms such

as chromatin modification and histone methylation (Lambrot et al., 2013). The findings made by Lambrot and colleagues (2013) with regard to placental weight are not in line with those made by Kim et al., (2011) who observed significantly reduced placental weight in offspring of folate-deficient male rats. Whether the discrepancy is due to the study design, animal model, or experimental procedures is not known. In a mouse model with the same LPD and methyl donor supplementation regimen as the one used in this study, Morgan et al. (2021) recently provided evidence that methyl donor supplementation may reduce or help prevent LPD-induced fetal overgrowth, as the authors observed significantly reduced fetal weight in the methyl donor group compared to the LPD group accompanied by significantly reduced placental weight in both groups compared to the control group. LPD-induced fetal overgrowth could be due to the increased placental expression of the imprinted genes *Cdkn1c* and *Grb10* as observed by Watkins et al. (2017). Imprinted genes are expressed in a monoallelic manner (either paternally or maternally) while the other copy is suppressed, and a number of these imprinted genes has been identified and associated with fetal growth and placental development (Peters, 2014). The under-expression of *Cdkn1c* is linked to increased placental size (Tunster et al., 2011), and the impaired function of *Grb10* is associated with fetal and placental overgrowth (Charalambous et al., 2010). The findings reported by Morgan et al. (2021) are not mirrored here, as LPD does not induce fetal overgrowth in this study, but the opposite of that. This perhaps suggests that while methyl donor supplementation may be implicated in the prevention of LPD-induced fetal overgrowth – perhaps through influencing the methylation profiles of placentally-expressed imprinted genes (Ly et al., 2017) – the same preventative

effect is not observed in a case where fetal overgrowth is not a factor, and that different underlying mechanisms such as histone modification and chromatin remodelling are involved in regulating each process. As methyl donors can either increase (Waterland, 2006) or decrease DNA methylation (Gross et al., 2020), the supplementation of methyl donors to LPD to counteract fetal overgrowth could potentially alter the methylation patterns of imprinted genes such as *Grb10* and *Cdkn1c*, thereby normalising their expression levels compared to LPD. When fetal overgrowth is not observed, however, it is possible that the expression levels of these imprinted genes is not significantly altered and therefore methyl donor supplementation does not influence their relative expression levels. As this study unfortunately does not examine sperm DNA/RNA methylation or the methylation and expression levels of imprinted genes or histone modifications in the placenta, further investigation into that is required. Paternal low intake of methyl donors such as choline, betaine, methionine, and folate, have been associated with longer gestation and altered birth weight in humans (Martín-Calvo et al., 2019; Pauwels et al., 2017). In the study by Pauwels et al. (2017), however, paternal betaine/methionine intake was negatively correlated with fetal birth weight, whereas paternal choline intake had a positive correlation with birth weight. The authors, however, do not explain these differences in adequate detail, but only explain that a possible mechanism that underlies these findings involves DNA methylation during spermatogenesis which then influences the sperm genome and pregnancy outcomes (Pauwels et al., 2017). It is worth highlighting that choline intake had a positive correlation with birth weight while its metabolite, betaine, had a negative correlation in the study by Pauwels et al. (2017). An inverse

relationship between choline and betaine has previously been described (Golzarand et al., 2022), and one explanation as to the opposite effects observed in a case of metabolic syndrome, for instance, is impaired or disrupted mitochondrial choline oxidation to betaine (Ueland, 2010). Whether there is any disruption to the choline to betaine oxidation process in cases of paternal dietary modification models is unknown but also possibly unlikely unless impaired oxidation is observed outside metabolic disorders. Despite altered fetal weight observed in LPD and MDL compared to CD in addition to the reduced placental weight in MDL compared to MDWD, no significant differences were observed in fetal to placental weight ratio between these groups. However, the fetal to placental weight ratio was significantly increased in WD compared to all other study groups. This suggests a potential upregulation in the net flux of nutrients to each fetus per gram of placenta (Hayward et al., 2016).

Further examination of conceptus components (the combined weight of the fetus, the placenta, and yolk sac) revealed significant reductions in yolk sac weight in MDL compared to CD, LPD, and MDWD. This finding is not mirrored in the study by Morgan et al. (2021) who found no significant differences in yolk sac weights in a mouse model fed either a control diet, a LPD, or a LPD supplemented with methyl donors. In rodents, the yolk sac plays a crucial role in embryo nutrition and enrichment through the absorption and transfer of both macro and micronutrients, with damage to its normal function being associated with damage to the embryo/growing fetus (Ornoy and Miller, 2023). While it is known that yolk sac function can be impaired through toxic substances and anti-yolk sac antibodies for example (Ornoy et al., 2003), the impact of factors such

as dietary intake on yolk sac function and its implications on fetal development is unknown and requires further investigation.

Fetal dissection and organ weighing revealed no significant differences in fetal heart and liver weights between the study groups. However, both LPD and MDWD had significantly increased fetal liver to body weight ratio compared to CD. This could potentially represent a manifestation of altered offspring liver metabolism in response to paternal diet, as previous studies have shown that both paternal LPD (Watkins et al., 2018) and high fat diet (Aizawa et al., 2022) alter offspring hepatic gene expression as well as metabolism profiles and predisposition to metabolic disorders such as NAFLD, in addition to increased liver weight after high fat diet exposure (Aizawa et al., 2022). Supplementation of LPD with methyl donors seems to have contributed to the normalisation of the fetal liver to body weight ratio. However, methyl donors induced an opposite effect when supplemented to WD, as MDWD – but not WD – had a significantly increased fetal liver to body weight ratio compared to CD. This could potentially highlight the unique mode of action of methyl donors which induces a different influence depending on the existing nutritional state they are supplemented to but could also highlight the inconsistency of evidence between different models. Methyl donors seem to counteract some of the effects of a Western diet by normalising testicular mRNA levels of genes involved in tight junction maintenance, spermatogenesis, and oxidative stress response. Supplementing methyl donors to a low protein diet, on the other hand, may play a role in the acceleration of cell cycles in the testis in addition to telomere shortening. Furthermore, while paternal LPD upregulates genes enriched for noncoding RNA metabolic processes, MDL downregulates *other* genes enriched for the

same biological process, indicating a lack of overlap between the two. Further highlighting the different effects imparted by methyl donor supplementation is their impact on placental weight in this study. MDL placentas were significantly lighter than control placentas, whereas MDWD placentas were heavier, with no significant differences in fetal to placental weight ratio observed between the two groups. Since placental weight between LPD and WD is not significantly different, and since both MD diets contain the same methyl donors at identical quantities, it is therefore plausible that the differences in placental weights are driven by the methyl donors' interaction with either LPD or WD. Methyl donor supplementation is shown to improve liver triglyceride accumulation resultant from the consumption of obesogenic diets in HFD rat models (Cordero et al., 2011b; Cordero et al., 2013) in addition to reducing offspring fat mass and altering miRNA profiles (Chleilat et al., 2021). While the increased fetal liver to body weight ratio could potentially signify an exacerbated phenotypic manifestation of impaired hepatic metabolism, a detailed look into fetal hepatic gene expression would provide a better understanding of the mechanisms underlying this finding.

Another potential mechanism of paternal programming contained within the seminal plasma is the seminal microbiome, a population of microbiota residing in the seminal plasma of males that has been revealed to be modifiable by diet (Baud et al., 2019). The exact role of seminal microbiota in influencing semen, sperm, or subsequent offspring health is yet to be defined, Whether the seminal microbiome imparts any significant influence on this phenomenon observed in the maternal uterine environment and offspring development is yet to be examined. A number of the studies surrounding the seminal microbiome explore

its role in influencing sperm kinetic parameters such as motility (Dardmeh et al., 2017; Valcarce et al., 2017). However, the seminal microbiome additionally can be in essence exchanged between partners through unprotected sexual intercourse, with factors such as the frequency of sexual intercourse and the number of partners being linked to vaginal normal flora and incidences of bacterial vaginosis (Brotman et al., 2010; Plummer et al., 2018). This suggests that a partner's reproductive microbiota could potentially affect that of the other. With the mother's reproductive microbiota being closely related to that of the neonate (Mueller et al., 2015), it is therefore conceivable that at the time of conception, a male's metabolic status could modify his seminal microbiome, in turn affecting the female reproductive microbiota (Eid et al., 2019). While this offers a novel mechanism of paternal programming of offspring health, this has yet to be demonstrated in the literature.

5.6 CONCLUSIONS, STRENGTHS, AND LIMITATIONS

Overall, this study provides insight into the impact of paternal suboptimal dietary intake on fetal growth. Moreover, it challenges our existing knowledge on the impact of paternal low protein diet by offering a potentially different perspective on its effect on fetal weight during maternal late gestation. However, a few limitations to this study stand out. While this study covers fetal and maternal growth and organ weights, it does not go into detail with regard to fetal and placental gene expression, maternal metabolism, or fetal and maternal microbiome. It is also worth noting that despite the fact that all females in this study underwent the same treatment and are of the same strain with minimal variation, maternal factors still exist and can introduce variability to fetal data. The study also does not assess adult offspring development and predisposition to diseases and abnormalities and therefore does not determine the impact of paternal methyl donor supplementation to either low protein or high fat high sugar diets. The primary limiting factors to this study are time and resources available to cover the different aspects of maternal and fetal responses to paternal diet. With that in mind, this study raises valuable questions and hypotheses for future studies to address, thus furthering our understanding of the influence of paternal dietary intake on fetal and maternal wellbeing.

CHAPTER SIX: FUTURE PERSPECTIVES

Poor diet has been linked to a number of diseases such as coronary artery disease (CAD) and type 2 diabetes. Additionally, reproductive health is also adversely affected by poor diet. It is therefore necessary to investigate the impact of nutritional intake on reproductive fitness in greater depth. The strong link between maternal diet, reproductive health, and altered offspring development is well-characterised in the literature by a number of studies throughout the past five decades (Ashworth et al., 2009; Cetin et al., 2010; Dunford et al., 2014; Kereliuk et al., 2017; Martin et al., 2010; Rando and Simmons, 2015; Sen et al., 2012; Sinclair and Watkins, 2013). However, despite paternal programming moving more to the forefront of DOHaD research, the role that paternal nutrition plays in influencing male reproductive and metabolic health, as well as offspring development, warrants further investigation. To provide a clearer understanding of the impact of paternal diet on male reproductive health, this study aimed to characterise the effect of suboptimal diet on testicular morphology and global gene expression in addition to adult male metabolic fitness as well as fetal development.

While this study has shown that feeding adult male mice a modified diet has minimal impact on male fertility, testis of WD and MD-WD males displayed a significantly increased number of tubule abnormalities compared to the other diet groups. The atypical seminiferous tubules exhibited a loss of germ cells, vacuolisation, and epithelial sloughing. This finding coincides with others in the literature, as study by Liu and colleagues reported that high fat diet (20 % fat) exposure causes seminiferous tubules of Sprague-Dawley rats to display abnormal structures such as the disruption of the epithelium (Liu et al., 2014).

Furthermore, Campos-Silva et al. (2015) reported decreased cellular proliferation in seminiferous tubules from rats fed a high fat diet compared to rats fed a normal chow diet. This reduced cell proliferation observed by Campos-Silva et al. (2015) could potentially be due to a compromised spermatogonial stem cell population, which is mirrored in the WD group in this study, as WD-testis express a significantly reduced number of $PLZF^{+}$ cells. Yang et al. (2018) observed pathological changes to seminiferous tubules in rats fed a high fat diet, characterised by seminiferous tubule atrophy and distortion as well as loss of spermatogenic cells, which agrees with the findings in this current study. However, these findings are not in line with the observations made by Vigueras-Villasenor et al. (2011), who found no abnormal seminiferous tubule structures from 21 to 90 days in rats fed a high fat diet (35 % fat). A recent study by the same group found significant changes to testicular epithelium height and tubule diameter in rats fed a high fat diet for 30 days then fed a normal diet from 60 days onwards in addition to treadmill exercise (Ibáñez et al., 2017), highlighting the malleability of testicular cytoarchitecture in response to dietary modification. Evidence in the literature lacks more consistency, as the impact of high fat diets on seminiferous tubule morphology is dependent on multiple factors including the fat content of the diets to which the animals are exposed as well as the duration of dietary exposure. The altered expression of different members of the claudins family in the testis in addition to altered expression of key glycerolipid and triglyceride regulators in WD suggests the compromised integrity of the blood-testis barrier in addition to abnormal glycerolipid metabolism. WD additionally upregulated key genes involved in oxidative stress regulation, potentially as an adaptive response to accumulating ROS in the testis.

While methyl donor supplementation did not alter the frequency of seminiferous tubule abnormalities compared to WD, it did restore the $PLZF^{+}$ spermatogonial cell population. It also restored normal epididymal histology, as fewer epididymal abnormalities were observed in MDWD compared to WD. This could potentially be attributed to the significantly more abundant spermatogonial stem cell population which could indicate fewer impairments to the process of spermatogenesis. Global testicular gene expression data supports this hypothesis, as MDWD normalises the expression of a number of key genes involved in cell cycle regulation and DNA damage repair such as *Rsf1* and *Hat1*.The absence or deficiency of methyl donors carries significant implications with regard to male reproductive fitness and fertility. Abnormal patterns of DNA methylation have been observed in men presenting with infertility compared to fertile men (Niemitz and Feinberg, 2004). Additionally, altered genomic imprinting – that is DNA methylation-mediated – has been reported more frequently in men with oligozoospermia and oligoasthenoteratozoospermia (Kitamura et al., 2015). In human ART, developmental arrest was observed in embryos of patients with hypo-spermatogenesis and significant hypomethylation at the H19 gene differentially methylated region (DMR) (Kobayashi et al., 2009; Montjean et al., 2013). The understanding of the role of methyl donors is thus crucial for the potential improvement of male reproductive health and subsequent embryo development. One of the most prominent methyl donors is folate (or Vitamin B_9) as previously described in this study. As demonstrated by Lambrot et al. (2013), folate deficiency results in altered sperm DNA methylation at genes involved in development and metabolic processes. In humans, a reduced concentration of

folic acid was found in seminal plasma of infertile men compared to fertile men (Wang et al., 2022). In the UK, over 15 % of men aged between 19 and 64 (adult age) were shown to have evidence of levels below the WHO threshold, indicating folate deficiency (Buttriss, 2015). It is therefore of importance to determine how supplementation with methyl donors such as folate can influence an individual's reproductive wellbeing. Folate supplementation was shown to reduce sperm damage, as recently reviewed by Hoek et al. (2020). However, this also raises the question of "how much is too much?". In a 2009 study, Yajnik et al. (2009) found that a high folate concentration during pregnancy results in offspring with an increased fat mass compared to mothers with relatively lower folate concentrations (Yajnik et al., 2009). High folate concentrations have also been implicated in natural killer cell cytotoxicity in postmenopausal women (Troen et al., 2006), suggesting that folate, potentially with other methyl donors, can play a dual role depending on whether it is supplemented to help normalise a deficiency or result in high folate concentrations. With the increasing marketing and use of multivitamin supplements (which often include folate/folic acid among other vitamins), it is of importance to be aware of potential risks associated with 'over-supplementation' and to resort to supplement use almost exclusively in cases of deficiency/insufficiency, such as pregnancy, where demand for folate increases and folate deficiencies could result in neural tube defects (NTDs) (Greenberg et al., 2011). While the impact of high folate on male reproductive fitness and more specifically sperm DNA integrity remains unclear, it is of interest to investigate the potential effects of 'over-supplementation' on male reproduction and fertility.

Interestingly, supplementation of the LPD with methyl donors did downregulate genes enriched for ncRNA metabolism, but there was minimal overlap between the LPD and MDL groups with regard to differentially expressed genes. When comparing the mRNA expression levels in MDL and MDWD testes to their respective low protein and high fat diets, MDWD had more overlap in testicular gene expression with WD than MDL had with LPD. This suggests that while the supplementation of methyl donors may normalise some effects in a low protein or a high fat diet, it also exerts its influence on global testicular gene expression in a unique manner, thereby creating a new gene expression profile. The different effects observed in MDL and MDWD suggest an inherent difference brought upon by either a low protein or a high fat high sugar nutritional state, each of which results in a different physiological, metabolic, or reproductive outcome. The restorative effects of methyl donor supplementation to either a low protein diet or a high fat diet have been previously described (Chleilat et al., 2021; Morgan et al., 2020) as discussed in this study, but the literature is still lacking with regard to the extent to which these effects are characterised. It is clear that each diet interacts differently with methyl donor supplementation thereby resulting in different outcomes. However, further studies are required to characterise the nature of these interactions.

A Western diet not only influences hepatic cholesterol and fatty acid metabolism in addition to testicular histological abnormalities, but also reduces the spermatogonial stem cell population. Testicular gene expression data suggests that WD impairs spermatogenesis and methyl donors could prove to be an effective means of mitigating this effect. Low protein diet consumption, on the other hand, while playing a much less dramatic role in influencing the testicular

environment compared to WD, similarly results in a smaller spermatogonial stem cell population despite the absence of histological abnormalities. LPD consumption results in the elevated mRNA expression of key small RNA-associated genes including *Ago1*, the stable overexpression of which is associated with slower cell cycles (Parisi et al., 2011), and *Ago4*, the inhibition of which induces early meiosis in mice (Modzelewski et al., 2012). Taken together, the findings in this study suggest that LPD may play a role in slowing down testis cell cycle rate. This also suggests that observations made regarding the role of protein restriction and longevity (Kitada et al., 2019) may extend to encompass the testicular environment. However, it is worth noting that there is a significant difference between stable and controlled protein restriction and malnutrition due to insufficient dietary protein intake. Solon-Biet et al. (2014) demonstrated in a mouse model that a low protein high carbohydrate (LPHC) diet but not calorie-restricted or nutritionally diluted diets, are associated with longevity. In their study, Solon-Biet and colleagues discovered that the maximum lifespan was observed in the group fed a diet with 5 % protein/75 % carbohydrates (Solon-Biet et al., 2014). In the following year, the same group published a study comparing a LPHC model (with 5 % protein) to a caloric restriction model and found that the LPHC group displayed improved glucose and lipid metabolism similar to mice exposed to 40 % caloric restriction (Solon-Biet et al., 2015). However, the studies published by Solon-Biet and colleagues do not examine the testicular environment and therefore the results observed in the LPD group in this current study cannot yet be attributed to metabolic improvements or preservation of lifespan. On the other hand, the overconsumption of proteins is on the rise globally. A 2016 World Resources

Institute-affiliated working paper by Ranganathan et al. (2016) reported that the global average daily protein intake per person was around 68 g, which was more than a third greater than the average daily requirement for adults. This can have several implications on health and disease, as a long-term overconsumption (above the daily recommended amount) of protein in adults is associated with increased risk of disrupted calcium homeostasis, renal and hepatic function disorder, cancer, and progression of CAD (Delimaris, 2013).

Supplementing a low protein diet with methyl donors did not dramatically alter the low protein state as it did to a high fat high sugar state, at least with regard to testicular histology. However, it created a unique testicular gene expression profile characterised by suppressed androgen receptor mRNA expression and the reduced expression of three crucial telomere maintenance-related genes, which is a possible explanation of the shorter testicular telomeres we have previously observed in methyl donor supplemented LPD (Morgan et al., 2021). Telomeres, noncoding and repeating sequences of DNA that serve to preserve genomic stability, shorten with age in the majority of proliferating tissues (Eisenberg et al., 2012). In sperm, however, in contrast with most other cell types, telomere length increases with age (Allsopp et al., 1992). Berneau et al. (2020) recently found that sperm telomere length positively correlates with *in vitro* fertilisation (IVF) success, but not with embryo cleavage or post-fertilisation outcomes such as birth weight (Berneau et al., 200). Another recent study found that the average sperm telomere length was smaller in infertile men compared to fertile men (Darmishonnejad et al., 2020). These recent findings change the current view of their role from markers for aging to markers of both aging and potentially fertility. It is worth noting that the observed reduction in

telomere-related genes in MDL comes from global testicular mRNA expression levels and not sperm in isolation.

Furthermore, the data revealed a significantly elevated mRNA expression level of *Gdnf*, which is correlated with spermatogonial cell accumulation. This, coupled with the inhibited expression of telomere-regulator genes and previous findings revealing shorter telomeres in mouse testis in response to MDL (Morgan et al., 2021) indicate potentially overactive stem cell renewal and accelerated cell cycle. Taken together, the data suggests that LPD may play a role in cell cycle slowing whereas supplementing LPD with methyl donors counteracts that and accelerates the process, potentially through epigenetically mediated ncRNA mechanisms involving mitochondrial tRNA translation and metabolism (Morscher et al., 2018; Zhou et al., 2021). The unaltered number of $PLZF^{+}$ cells in MDL, however, raises the hypothesis that a feeding regimen extending over an even longer period of time would further exacerbate the process and modify the testicular phenotype accordingly.

Not only does suboptimal paternal diet consumption influence the father's reproductive fitness, but as described in the literature, it also contributes to embryonic and offspring development. Watkins et al. (2018) showed that female mice mated with males fed a low protein diet displayed blunted uterine responses compared to controls in terms of cell signalling, vascular remodelling, immunological and preimplantation responses. Fetal development also requires placental communication and resource exchange between the mother and the offspring. Placental gene expression has been shown to be affected by paternal diet (Binder et al., 2015). A brief flow-chart summary of the findings in this study and potential mechanisms that may be underlying the observed results is

displayed in *Figure 6.1*. This diagram serves to highlight the different findings in this study throughout the Chapter 3, 4, and 5 with potential underlying mechanisms that mediate downstream effects on fetal growth and offspring development. This also visualises this study's contribution to broadening our knowledge in this area of research.

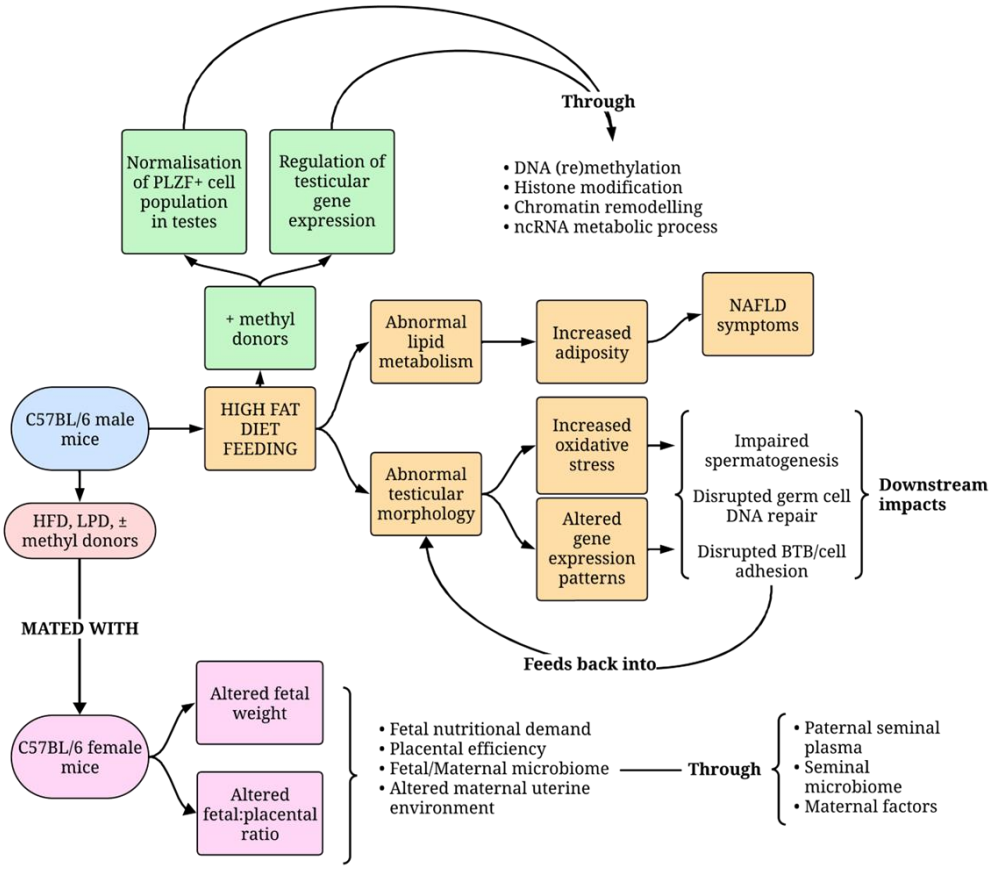

Figure 6. 1: Summarised representation of this study's findings with links to potential underlying mechanisms.

The findings in this study help shape future experiments to further our understanding of the impact of diet on testicular function and its underlying mechanisms. Such experiments encompass the assessment of damage to

testicular histology by means of immunohistochemical staining of more germ cell markers, assessment of lipid deposition in the testis, sperm epigenetic status through sequencing, and determining the level of liver steatosis either histologically or through ultrasound, among other experiments. This study provides new insight on the impact of dietary modification on adult male metabolic health and testicular function. It also offers new knowledge on the role methyl donors play when supplemented to either a low protein or a high fat high sugar diet. This study sheds light on the adverse effects of suboptimal dietary intake even without inducing obesity. Increased adiposity, or fat mass, without increased body weight is suggestive of a condition known as normal weight obesity, which is often underdiagnosed and under-investigated in today's world (Wijayatunga and Dhurandhar, 2021). Worldwide prevalence of normal weight obesity (NWO) was reported by De Lorenzo et al. (2016) to be around an estimated 10 %. However, more recent reviews report that the prevalence ranges between 4.5 % (Martinez et al., 2017) and 22 % (Ohlsson and Manjer, 2020). In normal weight adult populations, normal weight obesity prevalence ranges between 29 % (Correa-Rodríguez et al., 2020) to 46 % (Tayefi et al., 2019). In normal weight adults aged 60 and above in the US, the prevalence of normal weight obesity is estimated to be around 67 % (Batsis et al., 2016). In normal weight adolescents aged between 9 and 18, the prevalence of NWO is estimated to range between 42 % and 46 % (García-Hermoso et al., 2020; Olafsdottir et al., 2016). Altered body composition, oxidative stress, and inflammation in addition to sub-clinical cardiometabolic changes are observed in people with NWO compared to individuals with normal weight and normal fat mass (Wijayatunga and Dhurandhar, 2021). In a US population NWO study,

analysis from a National Health and Nutrition Examination Survey (NHANES) between 1988 and 1994 reported that people with NWO had a striking four-fold increase in the frequency of metabolic syndrome when compared with the low body fat group (Romero-Corral et al., 2009). The literature is lacking, however, with regard to standardising the diagnostic indices of this condition in both animal models and humans by means of adiposity cut-off values. This study shows that a high fat diet that does not induce obesity is sufficient to induce significant testicular damage and altered gene expression in addition to significantly increased adiposity and altered hepatic metabolic function. These findings suggest the beginnings of NAFLD, which is an increasingly prevalent disease among over 30 % of the adult population in the US (Fernando et al., 2019), 25 % in Europe (Bellentani et al., 2010), 25 % in Asia (Fan et al., 2015), and 13.5 % in sub-Saharan Africa (Spearman et al., 2021). While liver steatosis was not examined in this study, larger livers in response to the high fat high sugar diets are suggestive of liver inflammation, a typical symptom of NAFLD. This is supported by increased liver cholesterol (Malhotra et al., 2020) and increased adiposity (Pang et al., 2021), both of which are risk factors for the progression of NAFLD. These observations suggest that the consumption of a high fat high sugar diet may induce the progression of early-stage NAFLD without necessarily inducing obesity or weight gain. Assessment of liver steatosis either using ultrasound or histology is required to confirm this hypothesis, however. These findings could potentially inform studies in larger animal models focused on altered dietary intake on reproductive and metabolic fitness.

Furthermore, future investigations into the individual effects of different methyl donors such as folate, choline, betaine, and vitamin B_{12} on the testicular environment could be useful in determining what chiefly drives the effect of supplementation. This could be done through isolated feeding regimens using one supplement per group, followed by the assessment of cell populations in the testes using immunohistochemical techniques. Moreover, assessment of sperm epigenetic status is crucial, by means of RNA sequencing and RT-qPCR to obtain a deeper insight on the impact on altered epigenetic patterns and profiles. Controlled studies inducing obesity in one group and feeding a high fat diet without inducing obesity in another could also be an interesting way of determining the extent of damage NWO causes before reaching an obesogenic stage. The assessment of sperm oxidative stress levels and DNA damage could be a crucial way of addressing the impact of these diets on the testicular environment, and spermatogenesis specifically. These studies could also prove useful in the formulation of methyl donor cocktails in investigative studies to both expand our current knowledge and optimise interventions involving supplements. Moving forward, a better understanding of the impact dietary modification can have on the testicular environment and the role of methyl donors would help advise better strategies with regard to both animal model and human ART studies. Additionally, the observed effects of methyl donor supplementation on DNA repair and chromatin remodelling gene expression in the testis could inform future studies in larger male animal models, in which evidence is relatively lacking. The increasing use of multivitamin supplements (Mishra et al., 2021) is also put into perspective when looking at the results of this study and published studies in animal models and humans. As observed in

the results, supplementation may not always contribute to improved health, and as previously described, an increased level of methyl donors such as folate in pregnant women is linked to adverse outcomes in their offspring (Smith et al., 2008). It is therefore important to be aware of the risks associated with 'over-supplementation' when considering methyl donors preconceptionally or prior to ART treatment, to identify deficiency and address it with the appropriate level of supplementation and understand the intergenerational impact of supplemented dietary intake. For researchers, it is crucial to characterise the interaction of methyl donors with different nutritional states such as low protein or high fat and how that may impact human physiology, metabolism, and fertility. Findings in the current study provide new insight on the role of a low protein or a high fat high sugar nutritional state on metabolic and testicular function. Furthermore, this study contributes to the expanding bank of knowledge of the role of methyl donor supplementation on a suboptimal diet. When extrapolating data to larger animal models or to humans, however, it is always crucial to take a prudent approach and acknowledge the inherent differences between study models. With that in mind, this data adds to our understanding of the impact of dietary modification in a mouse model and helps shape future investigations in both mice and potentially larger models.

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APPENDICES

A1 Diet Compositions

A1.1 Control diet (CD) and Low Protein Diet (LPD) compositions.

	CD (g/kg)	***LPD (g/kg)***
Casein	180	90
Corn Oil	100	100
Starch Maize	425	485
Cellulose	50	50
Sucrose	213	243
Vitamins (AIN76)	5	5
MINERALS (AIN76)	20	20
Choline Chloride 50%	2	2
D,L-Methionine	5	5

Table A1: CD and LPD diet composition.

A1.2 Western Diet (WD) composition.

	WD (g/kg)
Sucrose	339.4
Milk Fat Anhydrous	200
Casein	195
Maltodextrin	100
Corn Starch	50
Cellulose	50
Corn oil	10
Calcium Carbonate	4
L-Cysteine	3
Choline Bitartarate	2
Cholesterol	1.5
Antioxidant	0.1
AIN 76A MX	35
AIN 76A – VX	10
Total	1000

Table A2: WD diet composition.

Specification	*% (w/w)*	*kcal/g*	*% kcal*
Crude fat	21.4	1.93	42
Crude protein	17.5	0.7	15
Crude Fibre	3.5	/	/
Ash	4.1	/	/
Carbohydrate	50.0	2.00	43
Total AFE		4.63	100

Nutrient Information: AFE = ATWATER FUEL ENERGY = Decimal fractions of Fat, Protein, Carbohydrate multiplied by 9, 4 & 4 respectively to give kcal AFE / g of diet. 1 MJ = 239.23 Kcal Nutrient figures on a fresh weight basis unless otherwise stated.

A1.3 Methyl donor cocktail supplemented to both LPD and WD.

Methyl donor	***Rate***
Choline chloride	5 g/kg
Betaine	15 g/kg
Methionine	7.5 g/kg
Folic acid	15 mg/kg
Vitamin B12	1.5 mg/kg

Table A3: Methyl donor supplement cocktail composition.

A2 Tissue Processing for H&E and IHC.

Station	***Reagent***	***Duration***
1	70 % alcohol	1 hr. 30 min
2	80 % alcohol	1 hr. 30 min.
3	96 % alcohol	1 hr. 30 min.
4	100 % alcohol	1 hr.
5	100 % alcohol	1 hr.
6	100 % alcohol	1 hr.
7	Xylene	1 hr.30 min.
8	Xylene	1 hr.30 min.
9	Paraffin	2 hr.
10	Paraffin	2 hr.

Table A4: Tissue processing protocol for testes & epididymis.

A3 Serum and liver assay plate map

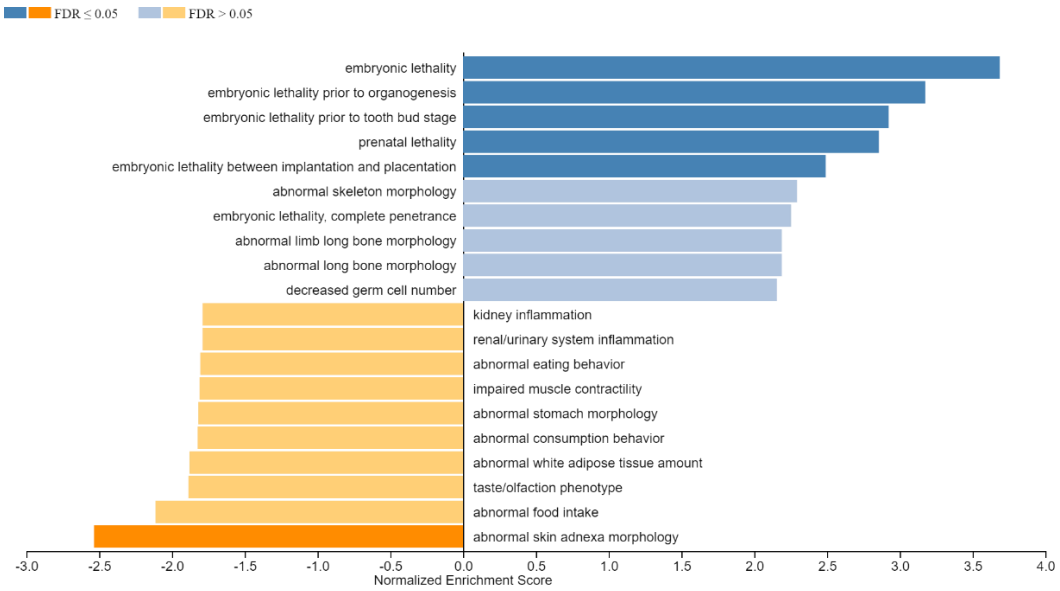
	1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***
A	Blank	Blank	WD4	WD4	LPD1	LPD1	MDWD 2	MDWD 2	LPD3	LPD3	CD8	CD8
B	STD 1	STD 1	MDL5	MDL5	MDWD 3	MDWD 3	WD5	WD5	MDL3	MDL3	WD2	WD2
C	STD 2	STD 2	WD8	WD8	MDL2	MDL2	LPD5	LPD5	CD2	CD2	WD3	WD3
D	STD 3	STD3	MDL8	MDL8	MDWD 4	MDWD 4	CD4	CD4	WD7	WD7	LPD2	LPD2
E	STD 4	STD 4	LPD6	LPD6	CD7	CD7	WD1	WD1	MDL1	MDL1	MDWD 7	MDWD 7
F	STD 5	STD 5	MDWD 1	MDWD 1	WD6	WD6	LPD4	LPD4	MDL7	MDL7	CD1	CD1
G	Back	Back	MDL6	MDL6	MDWD 8	MDWD 8	CD3	CD3	LPD8	LPD8	MDWD 5	MDWD 5
H			CD5	CD5	LPD7	LPD7	MDWD 6	MDWD 6	CD6	CD6	MDL4	MDL4

Table A5: ELISA plate map. Standards 1-5 were pipetted in increasing concentrations in duplicate. Samples were arranged randomly to minimise the impact of any pipetting errors. Each sample was run in duplicate in adjacent wells. Blank wells only contained a set volume of Assay Buffer. Some assays contained a background control provided by the manufacturer and the absorbance of the background wells was subtracted from the value of each sample to account for background absorbance in addition to the blank wells (STD = Standard).

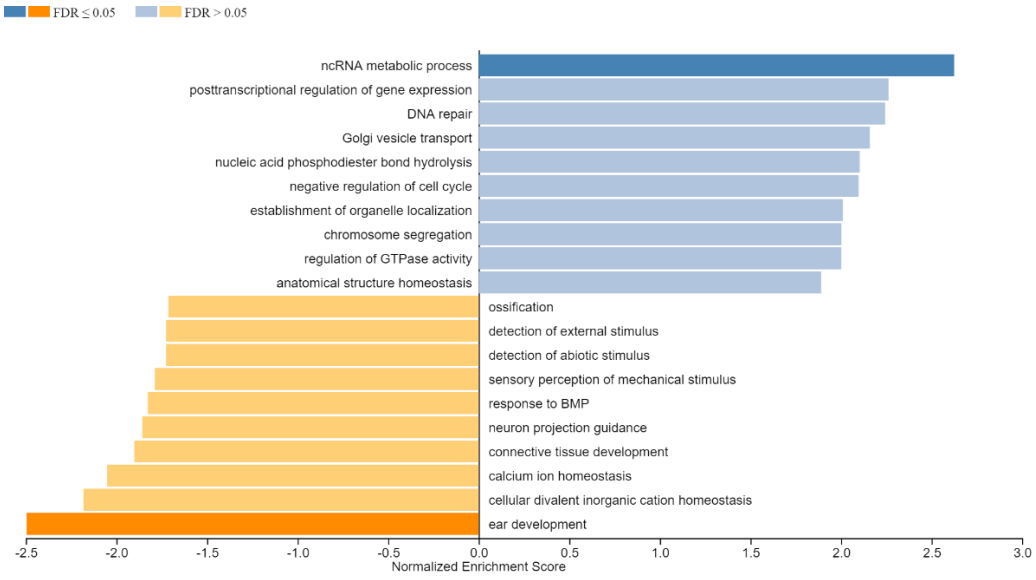
A4 WebGestalt biological process enrichment

A4.1 CD vs LPD

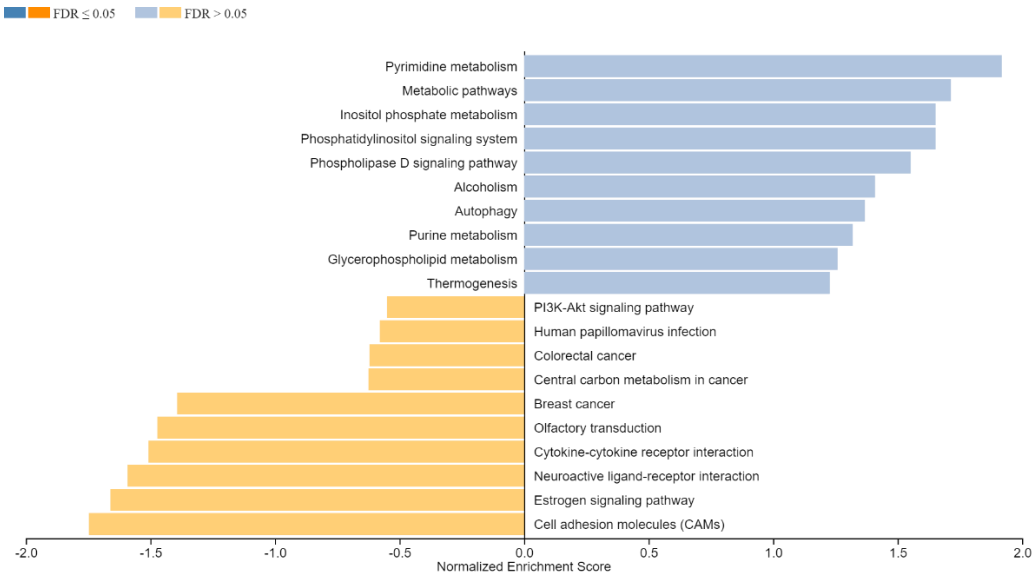
Phenotype



Geneontology

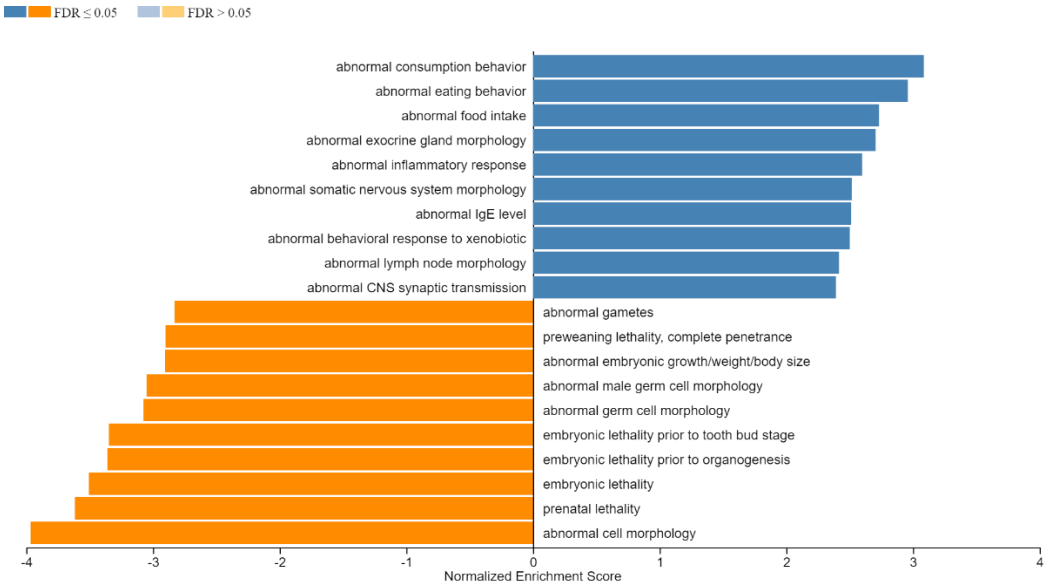


Pathway

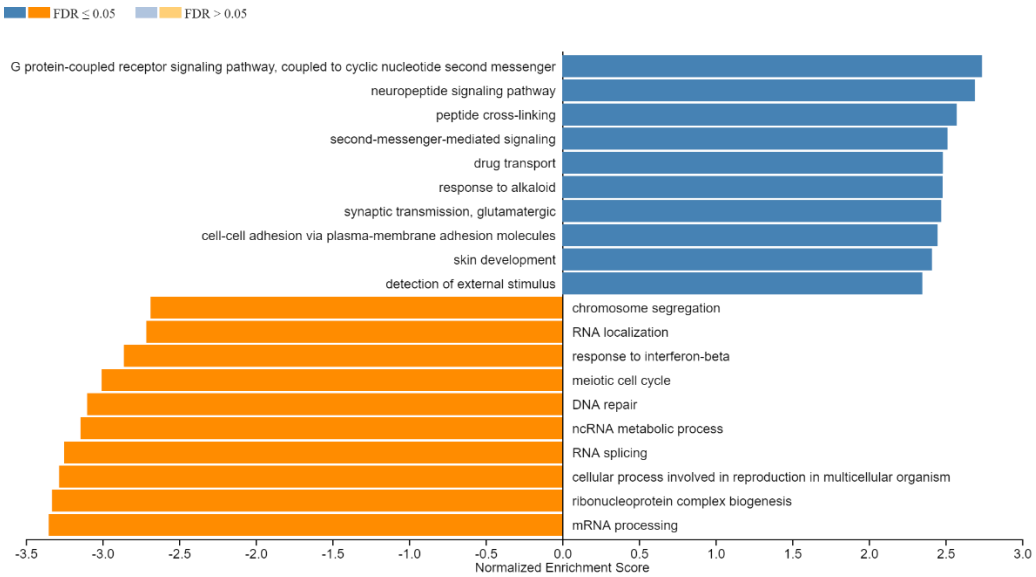

FDR ≤ 0.05
FDR > 0.05
Pyrimidine metabolism
Metabolic pathways
Inositol phosphate metabolism
Phosphatidylinositol signaling system
Phospholipase D signaling pathway
Alcoholism
Autophagy
Purine metabolism
Glycerophospholipid metabolism
Thermogenesis
PI3K-Akt signaling pathway
Human papillomavirus infection
Colorectal cancer
Central carbon metabolism in cancer
Breast cancer
Olfactory transduction
Cytokine-cytokine receptor interaction
Neuroactive ligand-receptor interaction
Estrogen signaling pathway
Cell adhesion molecules (CAMs)
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
Normalized Enrichment Score

A4.2 CD vs MDL

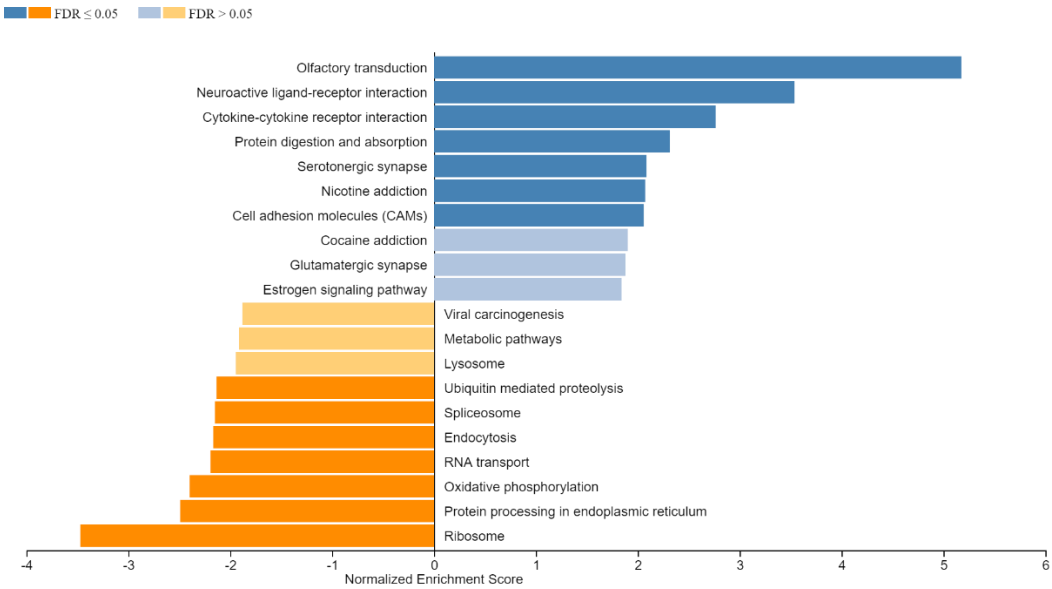
Phenotype



Geneontology

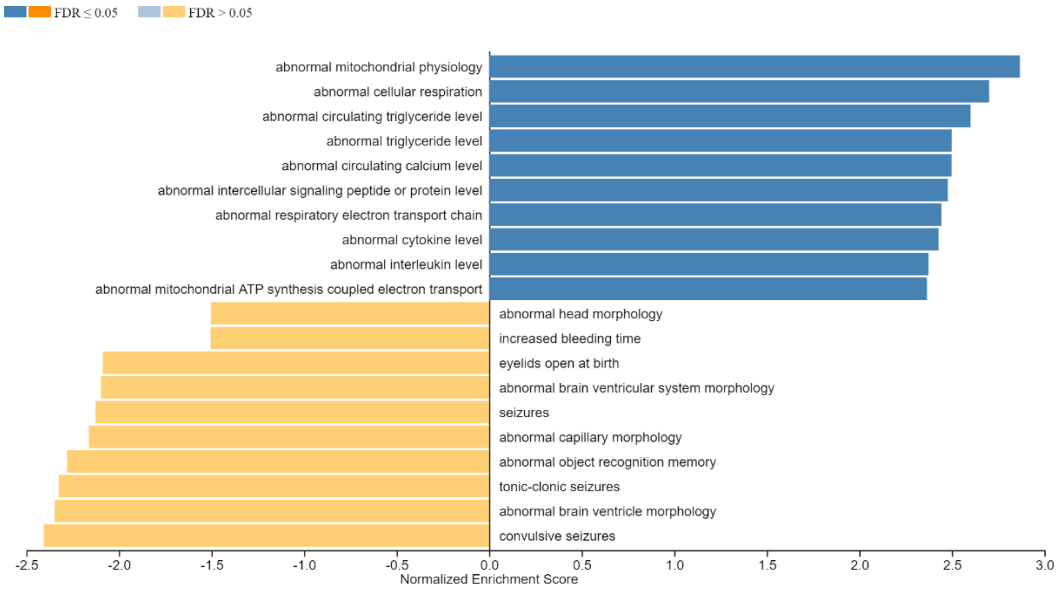


Pathway

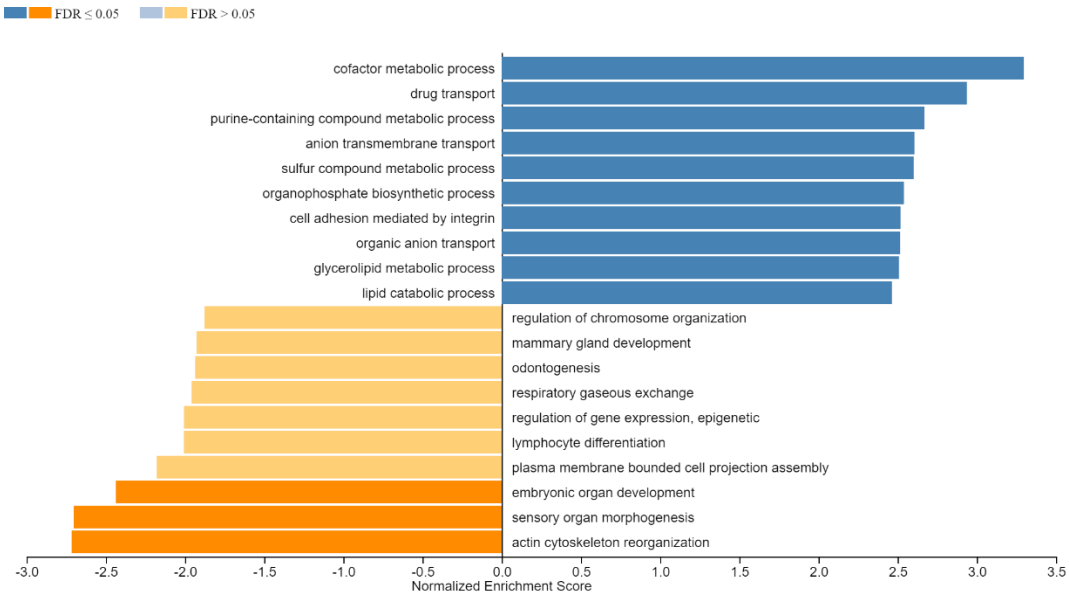

FDR ≤ 0.05
FDR > 0.05
Olfactory transduction
Neuroactive ligand-receptor interaction
Cytokine-cytokine receptor interaction
Protein digestion and absorption
Serotonergic synapse
Nicotine addiction
Cell adhesion molecules (CAMs)
Cocaine addiction
Glutamatergic synapse
Estrogen signaling pathway
Viral carcinogenesis
Metabolic pathways
Lysosome
Ubiquitin mediated proteolysis
Spliceosome
Endocytosis
RNA transport
Oxidative phosphorylation
Protein processing in endoplasmic reticulum
Ribosome
-4
-3
-2
-1
0
1
2
3
4
5
6
Normalized Enrichment Score

A4.3 CD vs WD

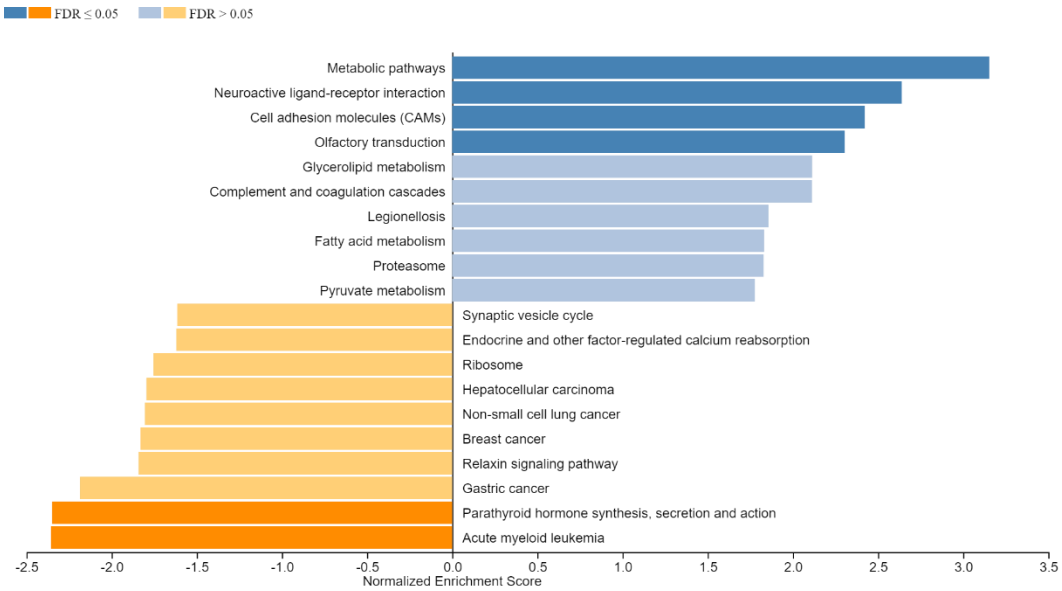
Phenotype



Geneontology

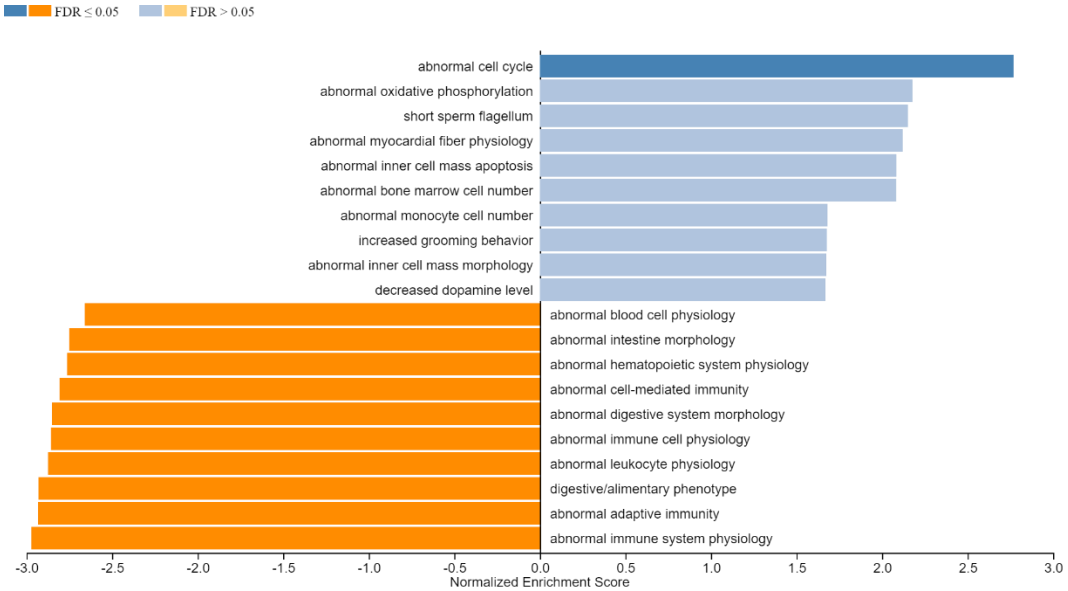


Pathway

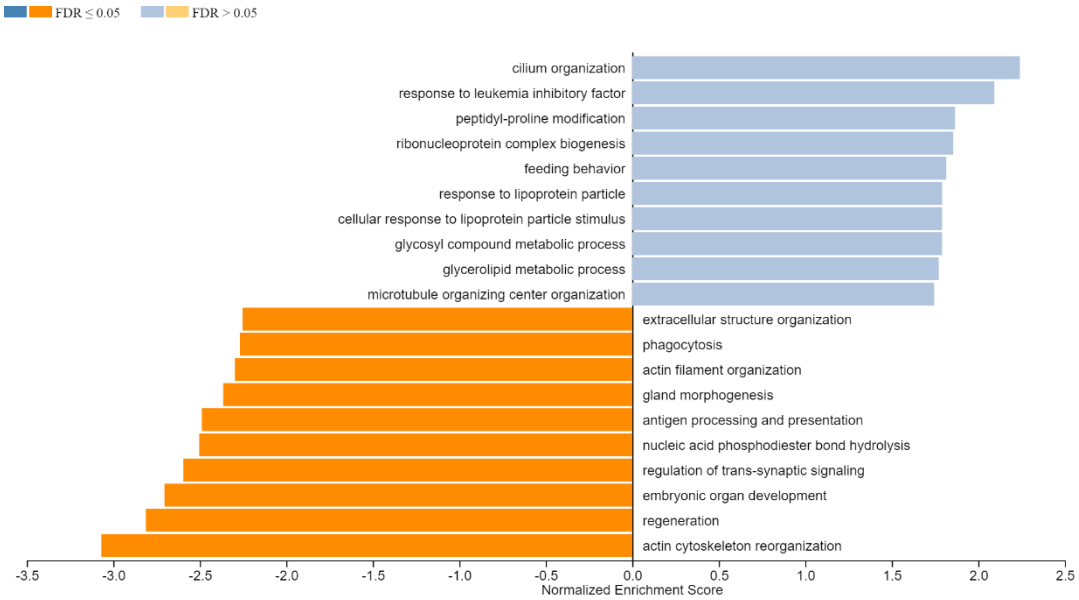

FDR ≤ 0.05
FDR > 0.05
Metabolic pathways
Neuroactive ligand-receptor interaction
Cell adhesion molecules (CAMs)
Olfactory transduction
Glycerolipid metabolism
Complement and coagulation cascades
Legionellosis
Fatty acid metabolism
Proteasome
Pyruvate metabolism
Synaptic vesicle cycle
Endocrine and other factor-regulated calcium reabsorption
Ribosome
Hepatocellular carcinoma
Non-small cell lung cancer
Breast cancer
Relaxin signaling pathway
Gastric cancer
Parathyroid hormone synthesis, secretion and action
Acute myeloid leukemia
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Normalized Enrichment Score

A4.4 CD vs MDWD

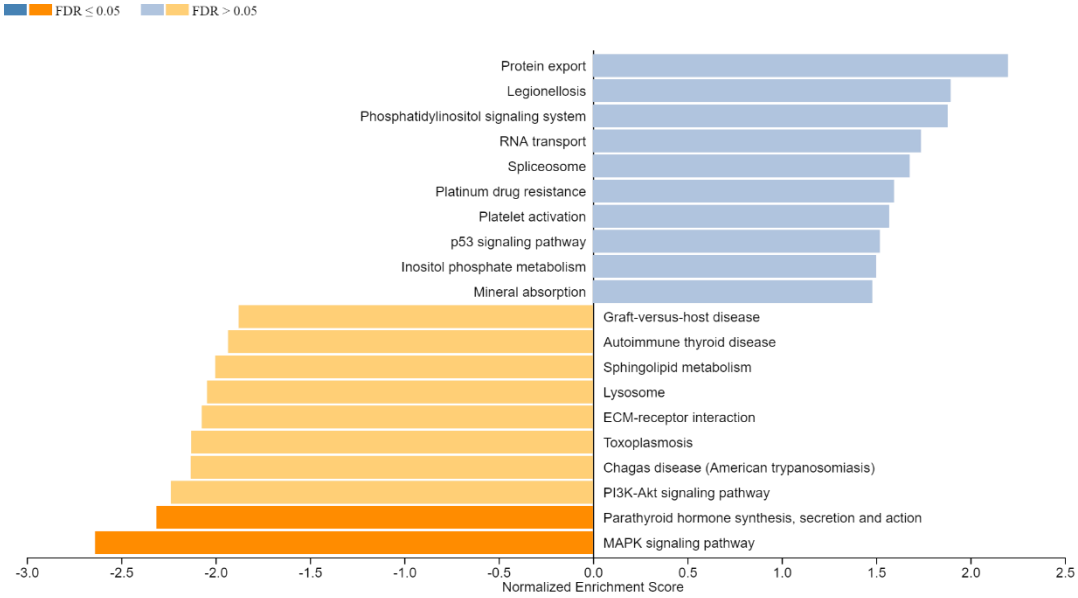
Phenotype



Geneontology

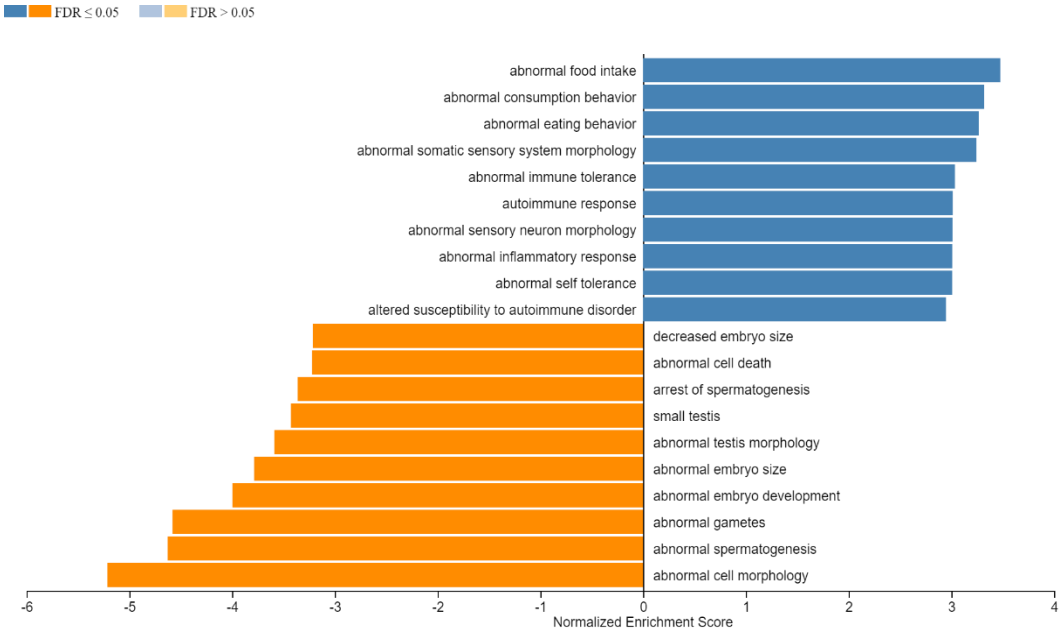


Pathway

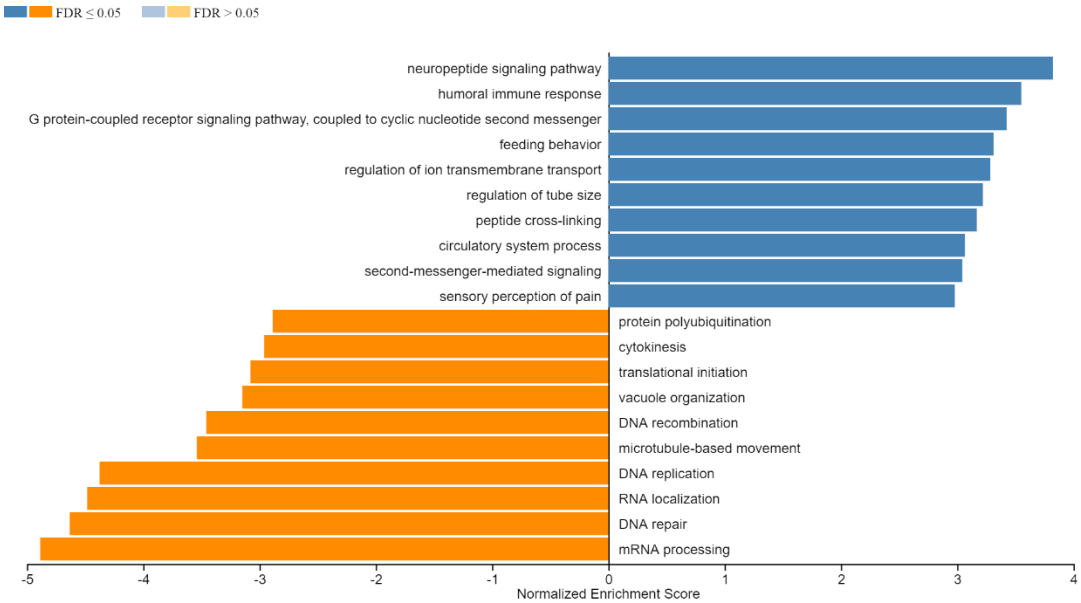

FDR ≤ 0.05
FDR > 0.05
Protein export
Legionellosis
Phosphatidylinositol signaling system
RNA transport
Spliceosome
Platinum drug resistance
Platelet activation
p53 signaling pathway
Inositol phosphate metabolism
Mineral absorption
Graft-versus-host disease
Autoimmune thyroid disease
Sphingolipid metabolism
Lysosome
ECM-receptor interaction
Toxoplasmosis
Chagas disease (American trypanosomiasis)
PI3K-Akt signaling pathway
Parathyroid hormone synthesis, secretion and action
MAPK signaling pathway
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
Normalized Enrichment Score

A4.5 LPD vs MDL

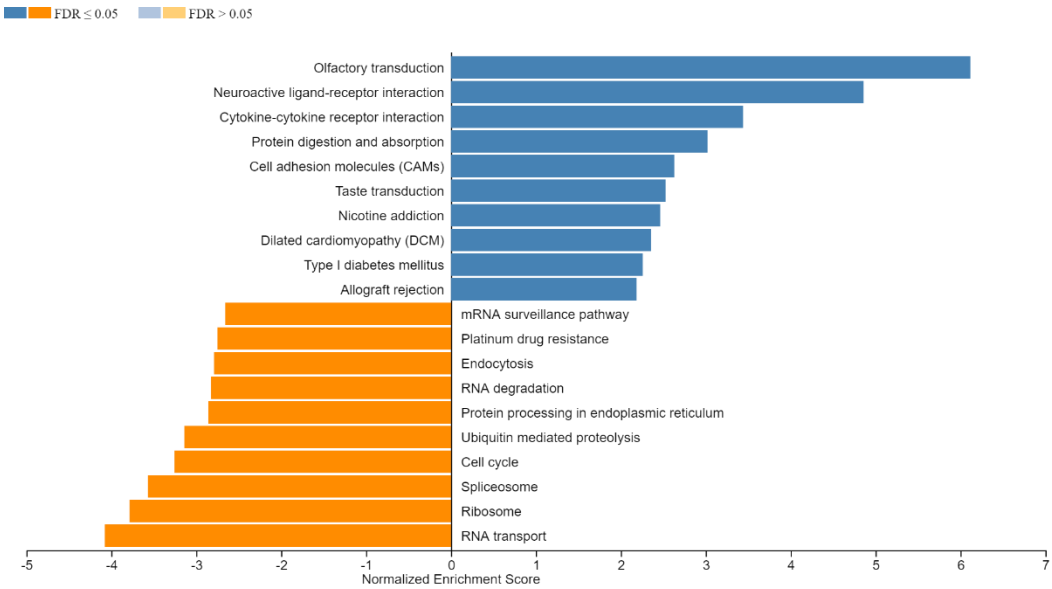
Phenotype



Geneontology

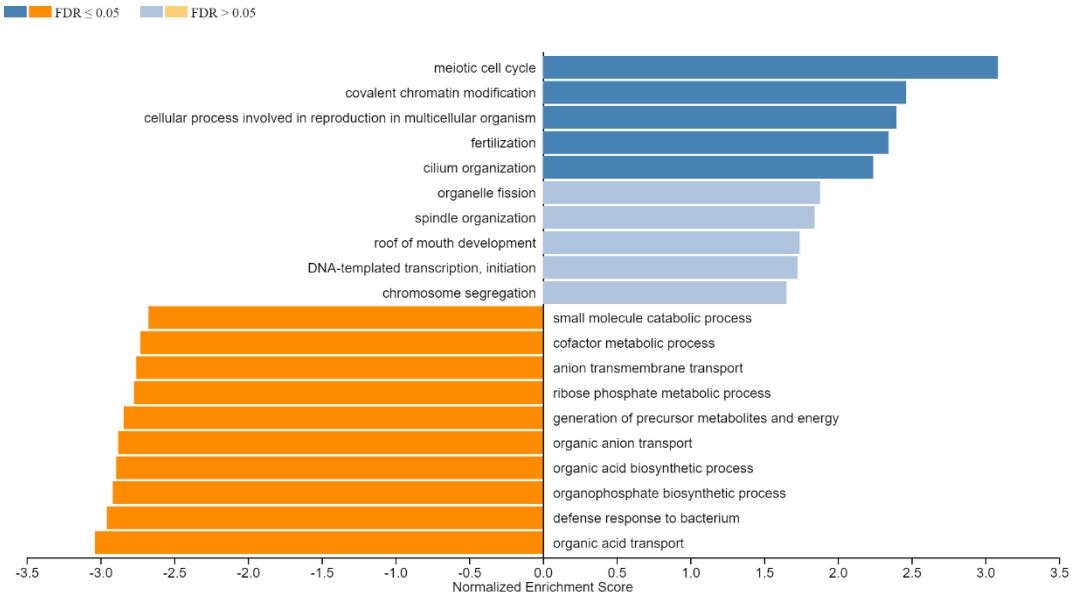


Pathway

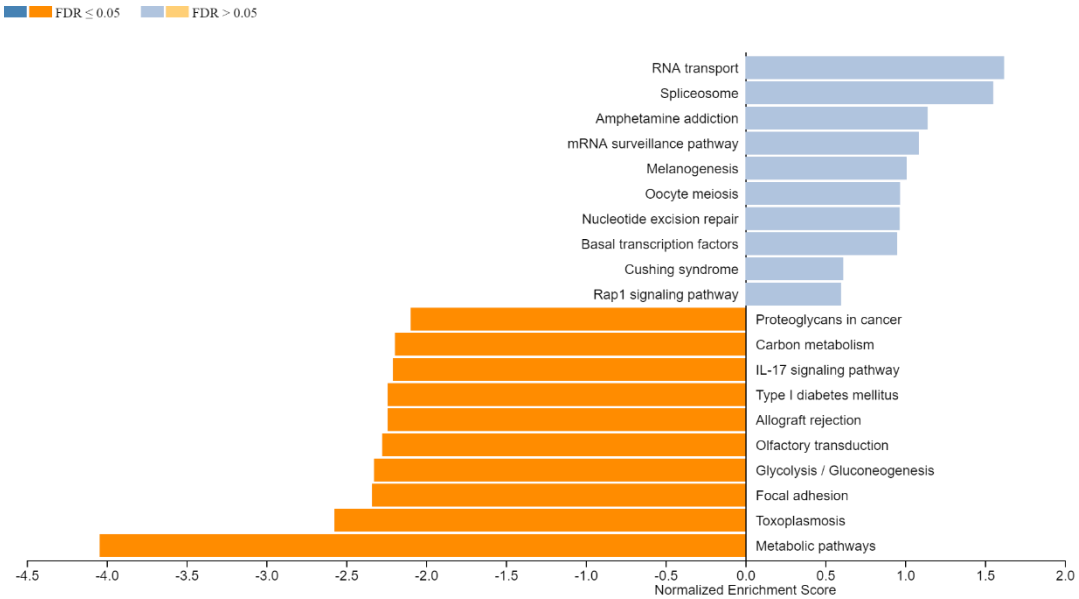

FDR ≤ 0.05
FDR > 0.05
Olfactory transduction
Neuroactive ligand-receptor interaction
Cytokine-cytokine receptor interaction
Protein digestion and absorption
Cell adhesion molecules (CAMs)
Taste transduction
Nicotine addiction
Dilated cardiomyopathy (DCM)
Type I diabetes mellitus
Allograft rejection
mRNA surveillance pathway
Platinum drug resistance
Endocytosis
RNA degradation
Protein processing in endoplasmic reticulum
Ubiquitin mediated proteolysis
Cell cycle
Spliceosome
Ribosome
RNA transport
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
Normalized Enrichment Score

A4.6 WD vs MDWD

Geneontology



Pathway



A5 Differential gene expression (array data from total testicular mRNA)

A5.1 CD vs LPD (Gene Ontology) ncRNA metabolic process.

Gene Symbol	*Gene Name*	*Fold Change*
Snapc1	Small nuclear RNA activating complex, polypeptide 1	1.2262
Polr1a	Polymerase (RNA) I polypeptide A	1.2124
Ago1	Argonuate RISC catalytic subunit 1	1.2094
Pwp2	PWP2 periodic tryptophan protein homolog	1.1794
Ago4	Argonuate RISC catalytic subunit 4	1.1615
Pwp0	PWP1 homolog, endonuclein	1.1614
Heatr1	HEAT repeat containing 1	1.1533
Cars	Cysteinyl-tRNA synthetase	1.1478
Elac2	elaC ribonuclease Z 2	1.1407
Toe1	Target of EGR1, member 1 (nuclear)	1.124
Wdr12	WD repeat domain 12	1.1136
Mtor	Mechanistic target of rapamycin kinase	1.1132
Dis3l	DIS3 like exosome 3’-5’ exoribonuclease	1.113
Ints2	Integrator complex subunit 2	1.1097
Bms1	BMS1, ribosome biogenesis factor	1.1097
Elp3	Elongator acetyltransferase complex subunit 3	1.1091
Wars	Tryptophanyl-tRNA synthetase	1.0928
Utp18	UTP18 small subunit processome component	1.085
Elp5	Elongator acetyltransferase complex subunit 5	1.0797
Tdrd1	Tudor domain containing 1	1.0738

A5.2 CD vs LPD (phenotype) embryo lethality

Gene Symbol	*Gene Name*	*Fold Change*
Slc40a1	Solute carrier family 40 (iron-regulated transporter), 1	1.3632
Rab23	RAB23, member RAS oncogene family	1.3262
Greb1l	Growth regulation by oestrogen in breast cancer-like	1.2555
Pprc1	Peroxisome proliferative activated receptor, gamma	1.2491
Erbb4	Erb-b2 receptor tyrosine kinase 4	1.2453
Aurkb	Aurora kinase B	1.211
Slc20a1	Solute carrier family 20, member 1	1.2081
Epb41l5	Erythrocyte membrane protein band 4.1 like 5	1.2035
Pcyt1a	Phosphate cytidyltransferase 1, choline, alpha isoform	1.1973
Bptf	Bromodomain PHD finger transcription factor	1.1851
Tro	Trophinin	1.1839
Dcps	De-capping enzyme, scavenger	1.1732
Ankrd17	Ankyrin repeat domain 17	1.1705
Pik3r4	Phosphoinositide-3-kinase regulatory subunit 4	1.167
Ncdn	Neurochondrin	1.1669
Snx13	Sorting nexin 13	1.1657
Hk2	Hexokinase 2	1.1603
Foxn3	Forkhead box N3	1.1582
Ipo11	Importin 11	1.1495
Kdm2b	Lysine (K)-specific demethylase 2B	1.1494

A5.3 CD vs MDL (phenotype) cell morphology, prenatal lethality

Gene Symbol	*Gene Name*	*Fold Change*
Huwe1	HECT, UBA, and WWE domain containing 1	-1.7627
Nrip1	Nuclear receptor interacting protein 1	-1.5377
Abcd3	ATP-binding cassette, sub-family D (ALD), member 3	-1.5362
Fetub	Fetuin beta	-1.4847
Amt	Aminomethyltransferase	-1.4814
Btbd18	BTB (POZ) domain containing 18	-1.4803
Lamb1	Laminin B1	-1.4641
Csk	c-src tyrosine kinase	-1.4641
Sms	Spermine synthase	-1.4536
Hspa9	Heat shock protein 9	-1.3887
Arhgap21	Rho GTPase activating protein 21	-1.3758
Sptssa	Serine palmitoyltransferase, small subunit A	-1.3749
Ar	Androgen receptor	-1.3719
Mcm4	Minichromosome maintenance complex component 4	-1.3658
Mcmdc2	Minichromosome maintenance domain containing 2	-1.3563
Pms2	PMS1 homolog2, mismatch repair system component	-1.3557
Arpc3	Actin related protein 2/3 complex, subunit 3	-1.3553
Fgf10	Fibroblast growth factor 10	-1.3549
Asah1	N-acylsphingosine amidohydrolase 1	-1.3378
Rhox13	Reproductive homeobox 13	-1.3324

A5.4 CD vs WD (phenotype) abnormal circulating calcium and triglyceride levels.

Gene Symbol	*Gene Name*	*Fold Change*
Tfap2b	Transcription factor AP-2 beta	1.529
Hsd3b7	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7	1.4535
Gpihbp1	GPI-anchored HDL-binding protein 1	1.3928
Mgll	Monoclyceride lipase	1.327
Enpp1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	1.3068
Mpzl3	Myelin protein zero-like 3	1.3033
Ppp1r3b	Protein phosphatase 1, regulatory subunit 3B	1.2804
C1qtnf1	C1q and tumour necrosis factor related protein 1	1.2798
Cd200	CD200 antigen	1.2732
Hspd1	Heat shock protein 1 (chaperonin)	1.2726
Lipg	Lipase, endothelial	1.2667
Calca	Calcitonin/calcitonin-related polypeptide, alpha	1.2641
Ffar1	Free fatty acid receptor 1	1.2628
Cox17	Cytochrome c oxidase assembly protein 17, copper chaperone	1.2563
Ndufs1	NADH:ubiquinone oxidoreductase core subunit S1	1.2512
Pon3	Paraoxonase 3	1.2448
Cdk5rap1	CDK5 regulatory subunit associated protein 1	1.2425
Prdx3	Peroxiredoxin 3	1.2397
Notch3	Notch 3	1.2361
Jak1	Janus kinase 1	1.2342

A5.5 CD vs MDWD (phenotype) abnormal cell cycle, immune response.

Gene Symbol	*Gene Name*	*Fold Change*
Atr	Ataxia telangiectasia and Rad3 related	1.3658
Rbm3	RNA binding motif (RNP1, RRM) protein 3	1.3394
Tmod3	Tropomodulin 3	1.3313
Brca2	Breast cancer 2, early onset	1.316
Aspm	Abnormal spindle microtubule assembly	1.282
Tnfrsf18	Tumour necrosis factor receptor superfamily, member 18	1.2806
Ranbp2	RAN binding protein 2	1.2707
Cacybp	Calcyclin binding protein	1.2628
Nbn	Nibrin	1.2526
Mdm2	Transformed mouse 3T3 cell double minute 2	1.235
Arrb2	Arrestin, beta 2	-1.7865
Cd74	CD74 antigen (invariant polypeptide of MHCII antigen-associated)	-1.5415
Elovl3	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	-1.5241
Pcgf2	Polycomb group ring finger 2	-1.5205
Irf7	Interferon regulatory factor 7	-1.4728
Cd68	CD68 antigen	-1.452
Mfge8	Milk fat globule-EGF factor 8 protein	--1.4394
H2-Ab1	Histocompatibility 2, class II antigen A, beta 1	-1.4355
Tnfrsfa1a	Tumour necrosis factor receptor superfamily, member 1a	-1.422
Irgm1	Immunity-related GTPase family M member 1	-1.4082

A5.6 CD vs MDWD (pathway) MAPK.

Gene Symbol	*Gene Name*	*Fold Change*
Arrb2	Arrestin, beta 2	-1.7865
Flna	Filamin, alpha	-1.5191
Tnfrsf1a	Tumour necrosis factor receptor superfamily, member 1a	-1.4422
Hras	Harvey rat sarcoma virus oncogene	-1.3644
Tab1	TGF-beta activated kinase 1/MAP3K7 binding protein 1	-1.3082
Fgf10	Fibroblast growth factor 10	-1.3036
Tgfb3	Transforming growth factor, beta 3	-1.3018
Dusp4	Dual specificity phosphatase 4	-1.282
Kit	KIT proto-oncogene receptor tyrosine kinase	-1.2745
Hgf	Hepatocyte growth factor	-1.2483
Igf2	Insulin-like growth factor 2	-1.2483
Bdnf	Brain derived neurotrophic factor	-1.2423
Map4k2	Mitogen-activated protein 4 kinase 2	-1.2351
Tgfb1	Transforming growth factor, beta 1	-1.2343
Map3k11	Mitogen-activated 3 kinase 11	-1.2321
Sos2	SOS Ras/Rho guanine nucleotide exchange factor 2	-1.2131
Rps6ka1	Ribosomal protein S6 kinase polypeptide 1	-1.2114
Braf	Braf transforming gene	-1.2065
Nr4a1	Nuclear receptor subfamily 4, group A, member 1	-1.1965
Cacng4	Calcium channel, voltage-dependent, gamma subunit 4	-1.1941

A5.7 LPD vs MDL (phenotype) Abnormal testis morphology.

Gene Symbol	*Gene Name*	*Fold Change*
Fancc	Fanconi anemia, complementation group C	-1.6819
Sms	Spermine synthase	-1.6734
Pms2	PMS1 homolog2, mismatch repair system component	-1.6485
Btbd18	BTB (POZ) domain containing 18	-1.6484
Pot1b	Protection of telomeres 1B	-1.6051
Exo1	Exonuclease1	-1.5254
Vps54	VPS54 GARP complex subunit	-1.5189
Rad21l	RAD21-like (S. pombe)	-1.5183
Npepps	Aminopeptidase puromycin sensitive	-1.5082
Mlh1	mutL homolog 1	-1.5071
Scml2	Scmpolycomb group protein like 2	-1.505
Ar	Androgen receptor	-1.5026
Hexb	Hexosaminidase B	-1.4961
4930447C04Rik	RIKEN cDNA 4930447C04 gene	-1.4819
AI481877	Expressed sequence AI481877	-1.4714
Snrpe	Small nuclear ribonucleoprotein E	-1.4442
Rnf8	Ring finger protein 8	-1.4398
Cks2	CDC28 protein kinase regulatory unit 2	-1.4109
Greb1l	Growth regulation by estrogen in breast cancer – like	-1.4077
Acox1	Acyl-coenzyme A oxidase 1, palmitoyl	-1.4075