

**The Effect of Diet and Exercise Interventions for
the Treatment of Male Obesity Induced
Sub Fertility**

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Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Nicole McPherson

Wednesday, 10th September 2014

Abstract

Male overweight/obesity affects 70% of the adult Australian population with this rate 10% higher (80%) in men attending a South Australian fertility clinic, suggesting a link between male overweight/obesity and sub fertility. Male obesity alters the molecular structure of sperm, increasing sperm DNA damage and reactive oxygen species (ROS) and altering mitochondrial function. This perturbed sperm function leads to altered embryo quality (reduced blastocyst development, blastocyst cell numbers and blastocyst mitochondrial function) which subsequently reduces implantation and live birth rates. Rodent models of male obesity have further implicated male obesity in the development of adult chronic diseases, increasing the susceptibility of obesity, diabetes and sub fertility in offspring across two generations.

Limited published research has assessed the reversibility of male obesity induced sub fertility. Due to limitations inherited in human studies, the aim of this thesis was to establish if obesity induced sub fertility could be reversed utilising a rodent model of male obesity with short term diet and/or exercise interventions for proof of concept. Male mice were fed a high fat diet (HFD) containing 21% fat or a control diet (CD) containing 6% fat for a period of 8-10 weeks to increase adiposity, following HFD exposure mice were allocated to one of four treatment groups 1) diet intervention (HC, change to CD), 2) exercise intervention (HE, continuation of a HFD with 3 x 30 min swimming sessions a week), 3) combined diet/exercise intervention (HCE, change to a CD with swimming exercise) or 4) continuation of a HFD (HH) for a further 8-10 weeks. Mice allocated to the CD continued on the CD (CC) for intervention period.

Diet intervention with (HCE) or without (HC) exercise reduced bodyweight, adiposity, and serum cholesterol while exercise intervention alone (HE) maintained their original level of adiposity. All interventions had improvements to serum glucose and leptin regulation while exercise subsequently improved serum free fatty acids and C - reactive protein. All interventions restored sperm function (motility, morphology, mitochondrial function, ROS and DNA damage levels). Males were subsequently mated with super ovulated normal weight females for assessment of embryo quality. All interventions restored blastocyst cell numbers and day 18 fetal weights while, exercise with (HCE) or without (HE) as CD further restored embryo development. As early embryo and fetal health are predictors of subsequent offspring health, males were also mated with naturally cycling normal weight females to produce offspring. Diet intervention alone (HC) showed the biggest restorations to male offspring sperm function (motility, sperm binding, capacitation and mitochondrial function). In contrast, exercise intervention alone (HE) showed the biggest restoration to female offspring metabolic health (glucose and insulin sensitivity and adipose accumulation) while the remaining interventions (HC and HCE) had minimal impact. The improvements to female offspring metabolic health from exercise interventions in their fathers may be related to their partial restoration of sperm X-linked microRNA abundance (i.e. mir-503 and mir-465b-5p), with these microRNAs specifically targeting pathways important for early embryo development including cell cycle control and apoptosis.

Together these studies provided some of the first evidence for the reversibility of obesity related fertility issues in males, highlighting that it may be more about restoring systematic metabolic health rather than a reduction in adiposity, with the deciphering an epigenetic mechanism in sperm for transmission of effects to the embryo and offspring phenotypes. These studies will undoubtedly stimulate further research into other related molecular mechanisms and the

independent associations between obesity related metabolic changes and their relationships with male fertility.

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Publications arising from this Thesis

N. O. Palmer, H. W. Bakos, T. Fullston and M. Lane 'Impact of Obesity on Male Fertility, Sperm Function and Molecular Composition', **Spermatogenesis** (2012) 4 2 1 – 11'

N. O. Palmer, H.W. Bakos, J.A. Owens, B.P. Setchell and M.Lane (2012) 'Diet and exercise in an obese mouse fed a high fat diet improves metabolic health and reverses perturbed sperm function', **American Journal of Physiology Endocrinology and Metabolism**, Apr 1; 302 (7): E768-80

N. O. McPherson, H. W. Bakos, J .A. Owens, B. P. Setchell and M. Lane (2013) 'Improving metabolic health in obese male mice via diet and exercise restores embryo development and fetal growth', **PLOS One**; Aug 19;8(8):e71459

N. O. McPherson, T. Fullston, H. W. Bakos, B. P. Setchell and M. Lane (2014) 'An obese father's metabolic state, adiposity and reproductive capacity which are improved by diet and/or exercise indicates a son's reproductive health', **Fertility and Sterility**; Mar; 101 (3): 865-73

Abstracts arising from this Thesis

N. O. Palmer, H. W. Bakos, B. P. Setchell and M. Lane (2011) 'Diet and exercise improves sperm function in obese mice', SRB, Cairns

Oozoa Student Award Finalist, SRB Student Travel Grant, Robinson Institute Travel Grant

N. O. Palmer, H. W. Bakos, J. A. Owens, B. P. Setchell and M. Lane (2012) 'Exercise and to a lesser extent a low fat diet in obese fathers restores metabolic health of subsequent offspring', Adelaide Post Graduate Research day, Wine Centre Adelaide

Prize Poster Obstetrics and Gynaecology

N. O. Palmer, Hassan W Bakos, Julie A Owens, Brian P Setchell and Michelle Lane (2012) 'Exercise and to a lesser extent a low fat diet in obese fathers restores metabolic health of subsequent offspring', Biology of Reproduction, SSR 2012, Penn State University

Larry Ewing Memorial Trainee Travel Fund

N. O. McPherson, H. W. Bakos, J. A. Owens, B. P. Setchell and M. Lane (2013) 'Exercise and diet interventions in obese fathers restores early embryo development and fetal weights, improving insulin sensitivity and reducing adipocyte cell size in subsequent female offspring', SRB, Sydney

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N. O. McPherson, H. W. Bakos, B. P. Setchell and M. Lane (2013) 'Metabolic status of fathers predicts sperm function in male offspring', International Congress of Andrology, Melbourne

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Robinson Institute research day, Adelaide

Prize Poster - Student

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1 Literature Review

Parts of this literature review have been published;

N. O. Palmer, H. W. Bakos, T. Fullston and M. Lane 'Impact of Obesity on Male Fertility, Sperm Function and Molecular Composition', *Spermatogenesis* (2012) 4 (2) 1 – 11.

1.1 INTRODUCTION

Obesity is a global health problem that is reaching epidemic proportions with 1.6 billion adults classified as overweight and an extra 500 million adults classified as obese (Nguyen & El-Serag, 2010). It accounts for 7.5% of the total burden of disease costing approximately \$21 billion dollars each year in Australia which is continuing to increase (Begg et al., 2007). Since the 1970's the rates of overweight and obese reproductive-age men has nearly tripled (Dixon & Waters, 2003) such that 70% of Australian adult men (>18 years) are now overweight or obese (ABS, 2012, 2013). This increase in obesity is coincident with an increase in male infertility as evidenced by the increase in couples seeking assisted reproductive technologies (ART) especially intra-cytoplasmic sperm injection (ICSI) for male factor infertility (Sunderam et al., 2009; Wang, 2008). There is an increasing awareness that male obesity reduces sperm quality, in particular altering the physical and molecular structure of spermatogenic cells in the testes and mature sperm (Du Plessis et al., 2010; Macdonald et al., 2010; Teerds et al., 2011). Furthermore, there is increasing evidence that paternal health cues can be passed to the next generation with male age associated with an increase in autistic spectrum disorders in offspring (Hultman et al., 2011) and paternal exposure to industrial chemicals is associated with increases in the incidences of offspring with congenital abnormalities (Chang, 2009; Cooper et al., 2010). Alarmingly, there is new evidence in animal models that paternal obesity increases the susceptibility of offspring to obesity and diabetes, suggesting another pathway for the amplification of these chronic diseases (Fullston et al., 2013; Ng et al., 2010). However, it is currently unknown if interventions to reduce paternal adiposity during peri-conception in fathers can reverse their sub fertility or to the health of the next generation.

1.2 CLASSIFICATION OF OVERWEIGHT AND OBESITY

The World Health Organisation (WHO) defines overweight and obesity as an abnormal or excessive fat accumulation that is likely to impair health (WHO, 2013). Body mass index (BMI) is the most common index for classifying a person as overweight or obese; defined as a person's weight (kg) divided by the square of their height (m) (kg/m^2) (WHO, 2013). It provides a useful population measure of overweight and obesity as it is the same for both sexes and all ages of adults, however can only be considered a guide, as the correlation between the level of adiposity and BMI can be different between individuals and doesn't always account for large muscle mass (WHO, 2013). The WHO has four main categories for classification of BMI; 1) Underweight is defined as a BMI $<18.5 \text{ kg/m}^2$, which is further split into three additional categories (severe thinness BMI $<16.0 \text{ kg/m}^2$, moderate thinness BMI $>16.0 \text{ kg/m}^2$ and $<16.9 \text{ kg/m}^2$ and mild thinness BMI $>17.0 \text{ kg/m}^2$ and $<18.5 \text{ kg/m}^2$); 2) Normal weight is defined as a BMI ranging between 18.5 and 24.9 kg/m^2 ; 3) Overweight (pre-obese) is defined as a BMI ranging between 25.0 and 29.9 kg/m^2 , and 4) obese is classified as a BMI $>30.0 \text{ kg/m}^2$. The obese class can be further classified into three categories to include obese class I (BMI ranging between 30.0 kg/m^2 and 34.9 kg/m^2), obese class II (BMI ranging between 35.0 kg/m^2 and 39.9 kg/m^2) and obese class III (BMI $\geq 40 \text{ kg/m}^2$).

1.3 MALE REPRODUCTIVE SYSTEMS

1.3.1 Testicular function and spermatogenesis

The adult testes can be divided into two main physiological compartments, 1) seminiferous tubules; which contain the developing germ cells (spermatogonium to late spermatids) and their supporting Sertoli cells which form the blood testes barrier and 2) interstitial tissue which contains testosterone producing Leydig cells, leucocytes, endothelial cells, connective tissue, blood

vessels and lymphatic vessels (Fig 1.1). The blood testes barrier is a protective barrier formed by tight, adhesion and gap junctions between Sertoli cells that isolates developing germ cells from toxic agents in circulating blood (Pelletier & Byers, 1992). The barrier can still allow small to medium molecular weight molecules through and controls the adluminal environment by influencing the composition of the luminal fluid, thereby regulating sperm production (Pelletier, 2011). This highly complex tissue is the site of spermatogenesis which commences at puberty.

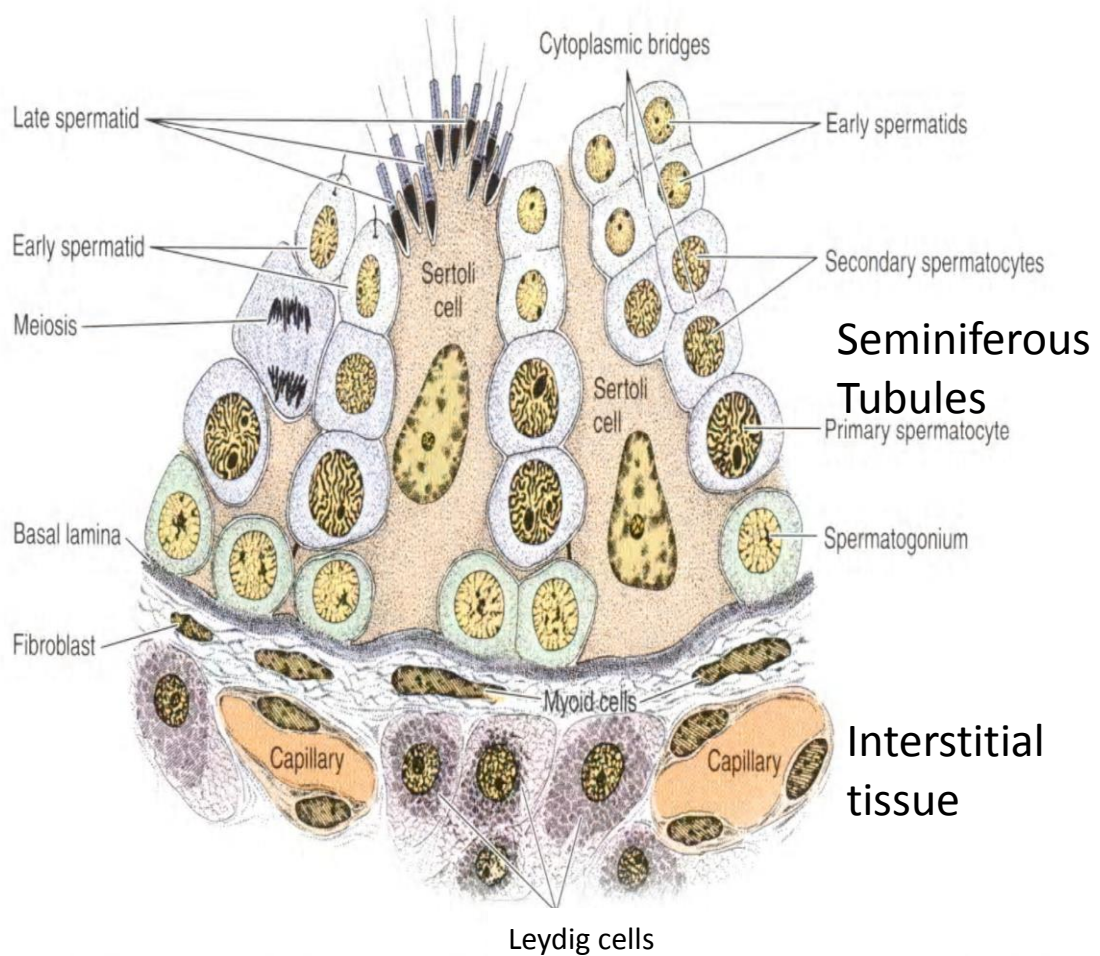


Figure 1.1 Cells of the testes

The testes are made up of two main compartments the seminiferous tubules and interstitial tissue which contain specific cell types, as indicated. Image adapted from (Johnson & Everitt, 2000).

Spermatogenesis is a highly complex and selective process that is characterised by three main phases; 1) mitotic proliferation, 2) meiotic division and 3) spermiogenesis (Fig 1.2) (Hess & De Franca, 2008). The first phase begins at puberty when somatic A1 spermatogonia divide from spermatogonial stem cells in the seminiferous tubules. These cells undergo a set number of mitotic divisions to duplicate chromosome number ($4n$) (2 in the human and 6 in the mouse) to form type B spermatogonia and then primary spermatocytes. These mitotic divisions are necessary to increase the overall number of cells that ultimately form viable sperm. The second phase begins with the first meiotic divisions of primary spermatocytes into secondary spermatocytes. The homologous chromosomes duplicated during mitosis in primary spermatocytes are paired on the metaphase plate. During this time translocations and exchange of genetic material can occur to create some level of genetic diversity. The first meiotic division is complete when the separation of homologous chromosomes leads to two secondary spermatocytes ($2n$). Prophase during the first meiotic division is prolonged; therefore spermatocytes are highly sensitive to damage during this phase. For example, the exposure to increased heat of primary spermatocytes (i.e. pachytene spermatocytes) causes incremental increases in the generation of reactive oxygen species (ROS) and the formation of DNA damage (Pino et al., 2013). The second meiotic division takes place when the secondary spermatocytes give rise to four haploid round spermatids ($1n$); two from each secondary spermatocyte halving the chromosome number.

The third phase of spermatogenesis involves dramatic repackaging of the nuclear compartment and structural changes to cells for effective delivery to the oocyte, a process known as spermiogenesis. One of the major structural changes during this process is the remodelling of the cytoplasm in each round spermatid resulting in elongation of the nucleus, shedding of the cytoplasm which removes the majority of the sperm's cytoplasmic scavenging enzymes and aids

the formation of the acrosome cap. Additionally changes include the formation of; the mid piece that contains the mitochondria (the energy powerhouse) and the flagellum that allows the sperm to move and the centriole that connects the mid-piece and tail to the head of the sperm is formed.

One of the marked and most important changes during this process is the repackaging of the DNA and condensation of the chromatin, known as protamination. Protamination involves the replacement of the histones first with transitional proteins and then with protamines and is required to enable tight packing of DNA within the sperm head, which aids in the protection against DNA damage in the absence of normal cellular defences that are greatly diminished by the shedding of the cytoplasm during spermatogenesis. However, the histone to protamine transition is incomplete with roughly 1% of histones remaining in mature murine sperm (Balhorn et al., 1977). Curiously, up to 15% of histones are retained in human mature sperm (Gatewood et al., 1987). There is evidence that the retention of these histones during protamination is not a random process with key pluripotency regulating genes remaining histone bound (ie *Nanog*, *Oct4* and *Sprouty*) (Farthing et al., 2008). Therefore these loci are capable of normal somatic cell histone modifications within mature sperm (i.e. methylation and acetylation) (Farthing et al., 2008). Chromatin condensation is necessary for fertilisation with incomplete histone removal associated with sub fertility (Aoki et al., 2006a; Aoki et al., 2006b; Aoki et al., 2005). The repackaging of histones to transition proteins to protamines occurs via single and double strand breaks to DNA which is mediated through histone acetylation/deacetylation (Grimes & Henderson, 1984; Marcon & Boissonneault, 2004; Meistrich et al., 1992). Due to this process, any disruptions to histone removal via environmental perturbations can result in abnormal protamination and increases in DNA damage during this phase. An example is paternal smoking which is associated with disruptions to protamine ratios in mature sperm (Hammadeh et al., 2010) and can impair subsequent embryo and pregnancy health (Venner et al., 2004). The

process from first mitotic division until late elongated spermatid release into the seminiferous lumen takes approximately 64 days in human and about 35 days in the mouse.

Once the sperm leave the lumen of the seminiferous tubules and enter the caput of the epididymis they are non-functional, in that they lack motility and the ability to fertilise an oocyte (Cornwall & Horsten, 2007). Further sperm maturation occurs during transit in the epididymis from caput to cauda where motility and fertilisation ability is acquired (Fig 1.2). This occurs through changes to sperm mitochondrial function and the addition and alteration of plasma surface proteins (i.e. GSTM5, HSPA4L and HSPA2) (Cornwall & Horsten, 2007; Dacheux et al., 2012). Sperm are again highly susceptible to environmental disruption during this transit as they have lost most of their cytoplasmic scavenging enzymes during the spermiogenesis phase (Aitken & Fisher, 1994; De Lamirande & Gagnon, 1995). Their plasma membrane contains large quantities of polyunsaturated fatty acids which are particularly susceptible to oxidative stress (Alvarez & Storey, 1995) and they have left the protective blood testes barrier. The entire process of spermatogenesis and sperm maturation is under tight regulation by hormones which is further discussed below.

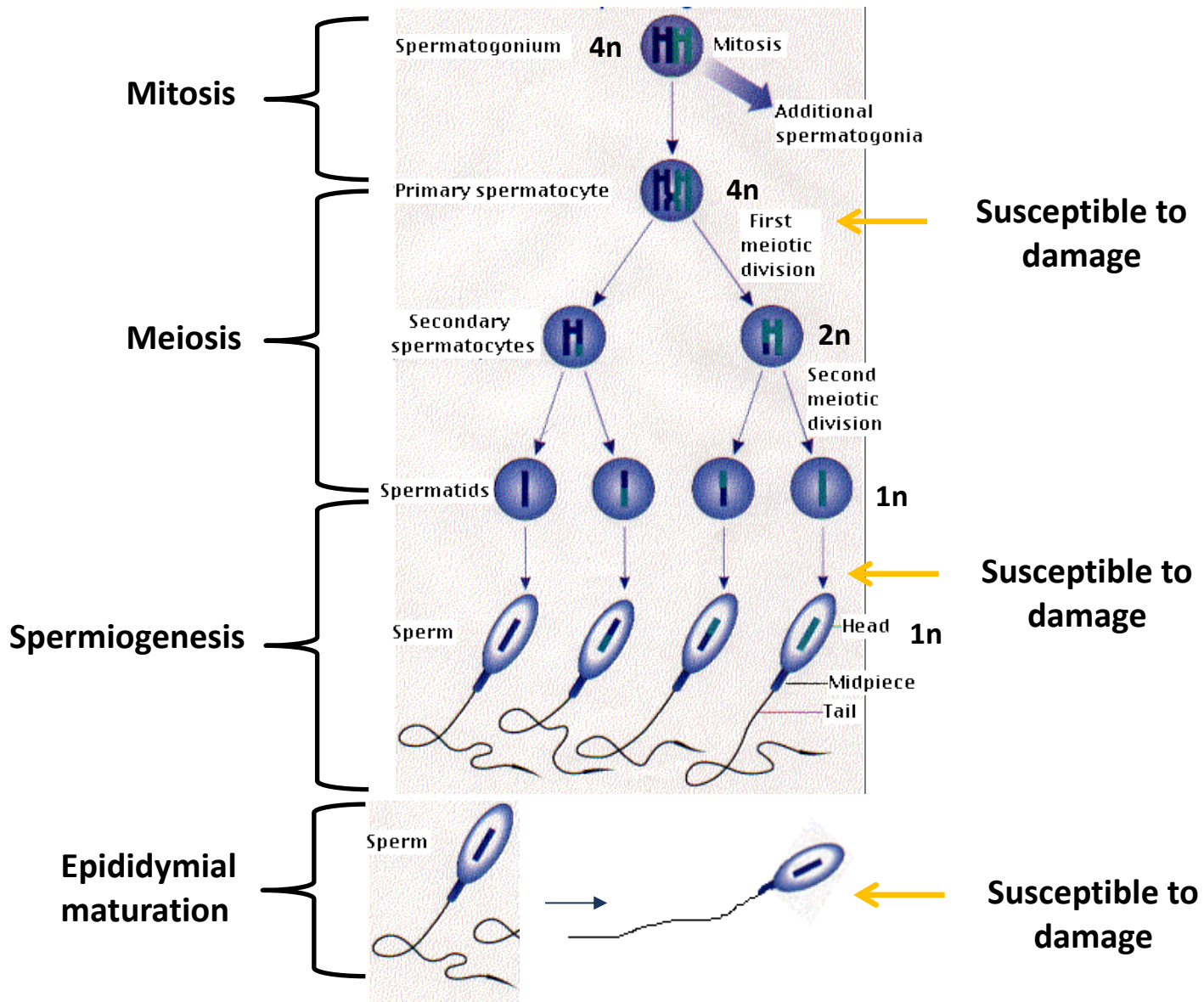


Figure 1.2 Stages of spermatogenesis

Spermatogenesis can be divided into 3 phases; phase 1) mitosis; phase 2) meiosis and phase 3) spermiogenesis. Additional sperm maturation also occurs in the epididymis. Sperm are most susceptible to damage during meiosis, spermiogenesis and epididymal maturation.

1.3.2 Hormonal regulation of spermatogenesis

Spermatogenesis is under the strict control of hormones, regulated by the hypothalamus, pituitary, as well as the Leydig and Sertoli cells of the testes (Fig 1.3) (Ruwanpura et al., 2010; Sofikitis et al., 2008). At puberty, the hypothalamus releases gonadotrophin releasing hormone (GnRH), which acts on the pituitary via pulsatile secretions into the portal system (Johnson & Everitt, 2000). Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are released by the anterior pituitary to act directly on the testes to stimulate somatic cell function and to support spermatogenesis. LH targets Leydig cells via the LH receptor, which activates the production of cyclic AMP (cAMP), promoting the transfer of cholesterol across the plasma membrane that results in the synthesis of testosterone (Midzak et al., 2009). Testosterone is either transported to the blood or acts on Sertoli cells via the androgen receptor (AR) where it is converted to either oestrogen or dihydrotestosterone (DHT) and transported to the tubular fluid (Johnson & Everitt, 2000). Testosterone is critical for the completion of meiosis, entry into and completion of spermiogenesis via stimulation of adhesion molecules and regulation of cell survival (Ruwanpura et al., 2010). Therefore testosterone is a commonly used surrogate marker for assessing male fertility (Corona et al., 2011, Handelsman & Swerdloff, 1985).

Similarly FSH targets Sertoli cells and activates multiple signalling pathways including cAMP and protein kinase, which activate downstream targets of spermatogenesis (Ruwanpura et al., 2010). FSH also activates the production of Sertoli cell specific cytokines and peptides (Inhibin B, Activin, Androgen binding protein (ABP)), which are important in controlling spermatogenesis through regulation of spermatogenic cell division (Johnson & Everitt, 2000). Thus FSH and testosterone act synergistically on Sertoli cells to promote spermatogenesis. Both FSH and LH secretion are under negative feedback from testosterone, oestrogen and inhibin B at both the level of the hypothalamus and anterior pituitary affecting the release of GnRH and consequently

a decrease in frequency of LH and FSH pulsatile release (Johnson & Everitt, 2000). In contrast, inhibin B can also directly inhibit FSH secretion from the anterior pituitary (Ruwanpura et al., 2010). As FSH concentrations are related to the loss of germinal elements in the testes, FSH concentrations are commonly used clinically as a marker of spermatogenesis (Medras et al., 2010).

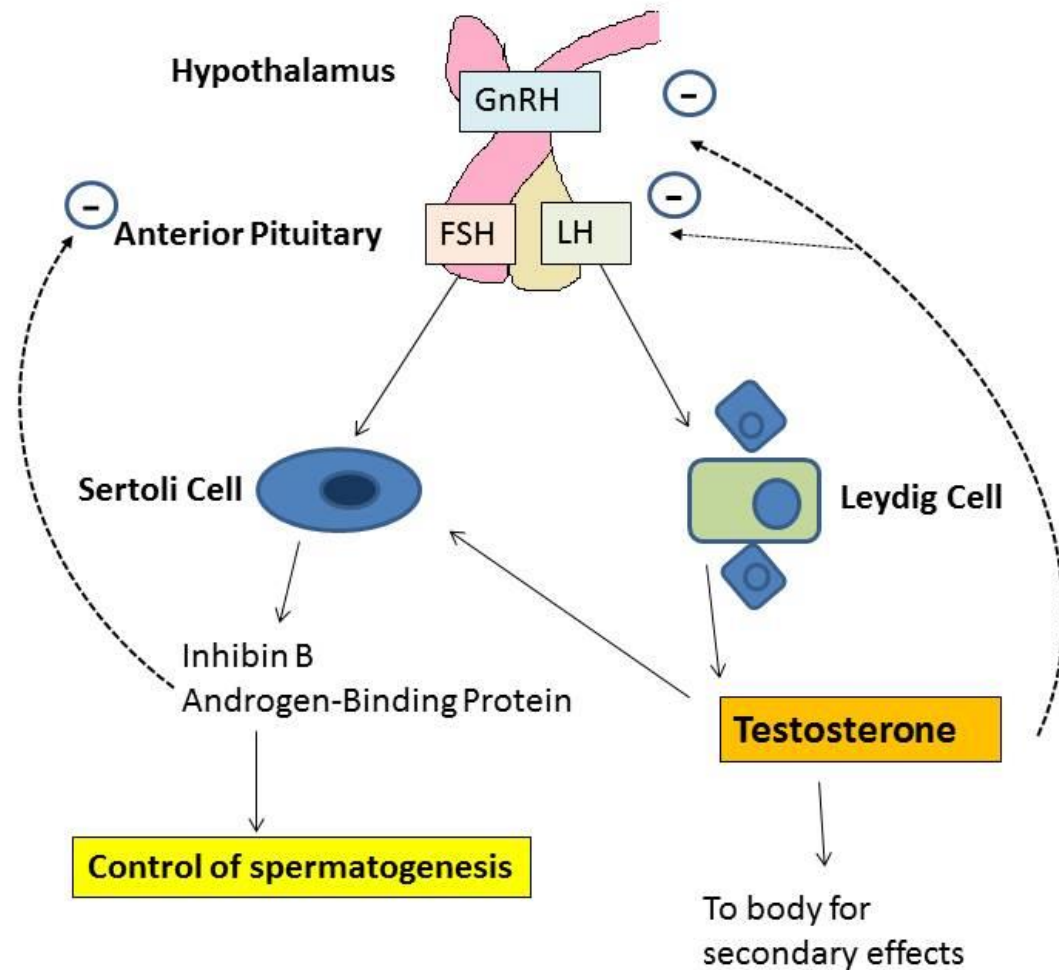


Figure 1.3 Hormonal regulation of spermatogenesis

GnRH is secreted by the hypothalamus and controls the secretion of LH/FSH from the anterior pituitary. LH targets Leydig cells to produce testosterone and FSH together with testosterone acts on Sertoli cells to aid in regulation of spermatogenesis

1.4 SPERM CONTRIBUTION TO EARLY EMBRYO DEVELOPMENT

1.4.1 Post ejaculation sperm activation

Although sperm obtain the ability to fertilise an oocyte in the epididymis, further post ejaculation sperm activation occurs within the female reproductive tract in preparation for sperm to fertilise the oocyte. Two processes occur during this time; 1) capacitation and 2) hyperactivation (Suarez, 2008b). Capacitation involves changes to the sperm's plasma membrane, which includes activation of soluble adenylyl cyclase and protein kinase (Signorelli et al., 2012), shedding of cholesterol and phosphorylation of proteins (i.e. AKAP4, AKAP3, VCP and CABYR) (Visconti et al., 2011). This process prepares sperm to elicit an acrosome reaction and therefore renders the sperm responsive to the signals encountered by the cumulus cells and oocyte (De Jonge, 2005). Hyperactivation is a change in the flagella beating that involves an increase in beat amplitude and increased whiplashing beats that propels the sperm forward. Hyperactivation allows sperm to swim through viscous substances such as mucus and oviduct fluid, assist in the penetration of cumulus cells surrounding the oocyte and the oocyte's zona pellucida (Suarez, 2008a). Unless sperm have undergone both capacitation and hyperactivation they cannot be released from the oviduct endothelium (Demott & Suarez, 1992). The changes to female reproductive hormones around ovulation have been hypothesised to be a trigger for sperm capacitation and hyperactivation, although it has been shown that sperm can exhibit similar process in *in vitro* conditions (Fraser, 2010; Suarez, 2008a).

1.4.2 Fertilisation

Fertilisation can be characterised by three main process; 1) induction of the acrosome reaction by sperm , 2) binding of sperm to oocyte zona pellucida and 3) fusion of sperm and oocyte (Wassarman, 1999). Acrosome intact sperm bind to zona pellucida glycoproteins on the oocyte via proteins located on the plasma membrane of sperm head. Currently, as many as two dozen

different sperm proteins/glycoproteins have been implicated in the binding of sperm to oocytes (e.g. zona receptor kinase, galactose-binding protein and sperm agglutination antigen -1) (Wassarman, 1999). Once sperm have successfully bound to the zona pellucida of the oocyte the acrosome reaction is induced by zona pellucida protein 3 (ZP3) activating G proteins in sperm, opening Ca^{2+} ion channels and changing the internal pH (Wassarman, 1999). Morphologically the acrosome reaction is seen as multiple fusions between the outer acrosome membrane and plasma membrane. This exposes the inner acrosomal membrane and acrosome contents (Cardullo & Florman, 1993). Penetration of the zona pellucida is likely achieved by a combination of sperm motility and enzymatic hydrolysis (Bedford, 1998). Acrosome reacted sperm then bind to and fuse with the oocyte's plasma membrane occurring at the microvillous surface (Wassarman et al., 2001). Fusion of the sperm to the oolema activates the oocyte to resume meiosis II that is mediated by intracellular Ca^{2+} oscillations, which are instrumental in initiating the embryo developmental program (Saunders et al., 2002).

1.4.3 Early embryo development

The sperm have an important role in fertilisation that extends beyond the delivery of the haploid genome. At fertilisation, in humans the proximal centrioles from the sperm become the centre for the sperm aster, which bring together the male and female pronuclei, however this tends to be species specific with aster formation in mouse derived from maternal proteins (Sutovsky & Schatten, 2000). Sperm also harbour a vast array of mRNA and small non-coding RNAs which appear to influence first stage cleavage events, embryo development and even offspring phenotypes (Jodar et al., 2013; Sandler et al., 2013). Additionally, a recent report also suggests that there is likely paternal transmission of a small proportion of mitochondrial DNA to the oocyte and resultant embryo, which had long been thought to be engulfed by autophagosomes and undergo lysosomal degradation at fertilisation (Kidgotko et al., 2013). This paternal mitochondrial DNA was present in a number of offspring organs (i.e. liver, kidneys, stomach, heart, intestines

and seminal glands), suggesting it may be functional at least in the rodent model (Kidgotko et al., 2013). Many sperm components including inner acrosomal membrane, axonema and accessory fibers in the sperm tail are digested by oocyte multivesicular bodies, lysosomes and ubiquitin-mediated proteasomes and do not contribute to embryo development (Yanagimachi, 2005). The remodelling of protamine wound chromatin back to histone variants within the male pronucleus is mediated by oocyte specific peptides and molecules (Monardes et al., 2005). After histone assembly, the chromatin in the male pronucleus becomes hydroxyl-methylated and decondenses (Zhang et al., 2012), allowing for additional proteins to be incorporated from the ooplasm necessary for DNA replication and demethylation during early cleavage (Mclay & Clarke, 2003; Zhang et al., 2012). If the sperm chromatin is damaged (i.e. DNA strand breaks) through disrupted spermatogenesis or environmental exposures, it is thought that the oocyte is equipped with machinery to actively repair the damage, however this results in a longer time to first and second cleavage events and subsequent blastocyst development via delays in paternal genome replication (Gawecka et al., 2013; Tesarik et al., 2004). Once both the maternal and paternal pronuclei fuse at syngamy active transcription and translation of the paternal genome is not apparent until the zygote (transcription) to two cell embryo (translation) in mouse (Matsumoto et al., 1994) and the four (transcription) to eight cell embryo (translation) in humans (Tesarik et al., 1986a, 1988). Previous studies have reported repeat ART failure from paternal effects such as increased sperm DNA damage and altered protamine ratios, implying that successful completion of embryogenesis requires active transcription and translation of both the maternal and paternal genomes (Mcgrath & Solter, 1984).

1.5 DIAGNOSIS OF MALE SUB FERTILITY

One in 20 Australia men are considered sub fertile with defective sperm function the most common cause (Iammarrone et al., 2003). Sub fertility is generally defined as decreased fertility or a decreased chance of getting pregnant, but not a complete inability to get pregnant and is often mistaken and used interchangeably with infertility. While advancements in ART such as ICSI have allowed for the bypass of defective sperm function (at least in terms of count, motility and morphology), male sub fertility is increasing worldwide (Hwang et al., 2010). The WHO laboratory manual for examining and processing human semen 2010 (WHO, 2010) provides the most universal criteria for the diagnosis of male sub fertility (Table 1.1). It identifies two major quantifiable attributes of semen; one refers to the total number of sperm and its nature (i.e. vitality, motility and morphology) and the second to the total fluid volume and composition contributed by the various accessory glands (2010).

Table 1.1 Lower reference limits (5th centiles and their 95% confidence intervals) for semen characteristics and diagnosis of male sub fertility

Parameter	Lower reference limits and confidence intervals
Semen volume (ml)	≥1.5 (1.4-1.7)
Total sperm number (10 ⁶ per ejaculate)	≥39 (33-46)
Sperm concentration (10 ⁶ per ml)	≥15 (12-16)
Total motility (progressive + non progressive %)	≥40 (38-42)
Progressive motility (%)	≥32 (31-34)
Vitality (live spermatozoa, %)	≥58 (55-63)
Sperm morphology (normal forms, %)	≥4 (3.0-4.0)

Extracted from the 2010 WHO laboratory manual for the examination and processing of human semen (5th Edition) (WHO, 2010).

More specialised tests looking at sperm chromatin integrity (terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), single cell gel electrophoresis assay (comet assay) and sperm

chromatin structure assay (SCSA)), sperm binding (hyaluronan binding test (HBT) and hamster zona assay), reactive oxygen species (formazan production, dichlorofluorescein diacetate (DCFDA)), round cell staining (CD45 leukocyte staining) and assessment of the acrosome reaction (Lectin PNA) which are deployed to determine more subtle functional and genetic/epigenetic characteristics. These techniques are yet to be routinely recommended by the WHO for the assessment of male fertility, and as such their use is clinic dependent (WHO, 2010). Blood concentrations of sex hormones (predominately testosterone, FSH, Inhibin B and oestrogen) are also commonly used for assessing gonadal function as they have been related to male sub fertility (Section 1.3.2 and (Hwang et al., 2011).

1.6 IMPACT OF MALE OBESITY ON SPERM FUNCTION AND HEALTH

It has been widely established that environmental factors such as smoking, alcohol, illicit drug use, some prescription drugs, supplements (in particular steroids) and industrial chemicals can negatively affect sperm quality (Sharpe, 2000, 2010). However, only more recently, has it been appreciated that BMI in men is also a predictor of male reproductive function (Hammoud et al., 2008a; Macdonald et al., 2010; Sermondade et al., 2013b).

1.6.1 WHO sperm parameters

There have been several studies that have investigated the impact of male obesity on traditional WHO sperm parameters used in a semen analysis, namely sperm concentration, sperm motility and sperm morphology (summarised in Table 1.2). There are many contradicting reports as to whether there is an impact of male BMI on sperm parameters, for example 18 out of the 36 papers reported a decrease in sperm counts. A decrease in sperm motility, especially progressive motility was reported in 13 out of 31 reports and a decrease in the percentage of sperm with normal morphology was only reported in 9 out of 25 papers (Table 1.2). The

discrepancies observed in the literature likely result from several limitations that are inherent in human studies. Firstly, these studies can be confounded by lifestyle factors such as smoking, alcohol consumption and recreational drug use as well as co-pathologies such as metabolic syndrome, all which can impair sperm function. Secondly, the majority of studies originate from fertility clinics, where patient cohorts are frequently biased toward sub fertile men, which may also confound findings. Thirdly, some studies rely on self-reporting of parameters such as lifestyle factors and BMI, which can lead to inaccurate reporting. A recent systematic review established that there was a J-shaped curve association between male BMI and abnormal sperm count, with overweight and obese men associated with increased rates of oligozoospermia (low sperm count) and azoospermia (no sperm) through evaluation of 21 studies (Sermondade et al., 2013a). This study contradicted the previous systematic review in 2010 which stated no effect existed between these factors (Macdonald et al., 2010). The earlier systematic review drew its conclusions based on five studies as data from other studies could not be consolidated and therefore may have underestimated the effect of male overweight and obesity on sperm count.

Due to the difficulties in interpreting data from human studies, rodent models of male obesity have now been established to assess the impact of male obesity on sperm function; however it is necessary to be aware of the differences between species, which have been highlighted above. Rodent studies have demonstrated that males fed a high fat diet to induce obesity had reduced sperm motility, decreased sperm counts with increases in epididymal sperm transit time and a decrease in percentage of sperm with normal morphology (Bakos et al., 2011b; Chen et al., 2013; Fernandes et al., 2012; Fernandez et al., 2011; Ghanayem et al., 2010; Palmer et al., 2011; Vendramini et al., 2013). However, it should be noted that a number of these studies had significant reductions in testosterone, altered glucose homeostasis, different diet compositions

and diet exposures in the high fat diet fed groups which could be exacerbated to the changes observed.

Table 1.2 Summary of studies investigating male obesity and its effect on WHO sperm parameters.

	Concentration	Motility	Morphology
(Strain et al., 1982)	Decreased	No change	n/a
(Jensen et al., 2004)	Decreased	No change	Decreased
(Magnusdottir et al., 2005)	Decreased	Decreased	n/a
(Fejes et al., 2005)	Decreased	No change	No change
(Kolozsar et al., 2005)	Decreased	n/a	n/a
(Kort et al., 2006)	Decreased *	Decreased *	Decreased *
(Qin et al., 2007)	No change	No change	No change
(Hammoud et al., 2008b)	Decreased	Decreased	Decreased
(Pauli et al., 2008)	No change	No change	No change
(Aggerholm et al., 2008)	No change	No change	No change
(Nicopoulou et al., 2009)	Decreased	n/a	n/a
(Hofny et al., 2009)	Decreased	Decreased	Decreased
(Stewart et al., 2009)	Decreased	n/a	n/a
(Chavarro et al., 2010a)	No change	No change	No change
(Shayeb et al., 2011)	No change	No change	Decreased
(Kolozsar et al., 2005)	Decreased	n/a	n/a
(Sekhavat & Moein, 2010)	Decreased	Decreased	n/a
(Paasch et al., 2010)	Decreased	No change	Decreased
(Tunc et al., 2011)	Decreased	No change	No change
(Rybar et al., 2011)	No change	No change	No change
(Bakos et al., 2011a)	Decreased	Decreased	No change
(Kriegel et al., 2009)	No change	No change	Decreased
(Fariello et al., 2012)	No change	Decreased	No change
(Dupont et al., 2013)	No change	Decreased	No change
(Hajshafiha et al., 2013)	Decreased	n/a	n/a
(Sermondade et al., 2013a)	No change	No change	No change
(Merhi et al., 2013)	No change	No change	n/a
(Thomas et al., 2013)	No change	Decreased	Decreased
(Pyttel et al., 2012)	n/a	Decreased	n/a
(Colaci et al., 2012)	No change	No change	No change
(Hammiche et al., 2012)	Decreased	Decreased	n/a
(Fariello et al., 2012)	Decreased	Decreased	No change
(Eskandar et al., 2012)	No change	No change	No change
(Shayeb et al., 2011)	No change	No change	Decreased
(Rybar et al., 2011)	No change	No change	No change
(La Vignera et al., 2012b)	No change	Decreased	No change

*Significant for Normal Motile Sperm (NMS) = volume*concentration*%motility*%morphology. N/A = not assessed

1.6.2 Male obesity on sperm DNA integrity and oxidative stress

While routine WHO sperm parameters (sperm concentration and motility) are important measures of male fertility it is becoming increasingly apparent that the molecular structure and content of the sperm are equally important for the ability of a sperm to generate a healthy term pregnancy. Human sperm DNA integrity is important for successful fertilisation and normal embryonic development, as evidenced by sperm with poor DNA integrity being negatively correlated with successful pregnancies (Bakos et al., 2008; Brahem et al., 2011; Gallagher et al., 1993; Kumar et al., 2012; Thomson et al., 2011). Furthermore, sperm oxidative stress correlates with decreased sperm motility, increased sperm DNA damage, decreased rates of acrosome reaction and lower embryo implantation rates following IVF (Aitken & Baker, 2006; Aziz et al., 2004; Zorn et al., 2003). Numerous human studies have determined that a relationship between obesity and reduced sperm DNA integrity exists (8 out of 9 studies show an increase), despite the use of a variety of different methodologies to measure sperm DNA integrity namely TUNEL, COMET and SCSA (Chavarro et al., 2010b; Dupont et al., 2013; Fariello et al., 2012; Kort et al., 2006; La Vignera et al., 2012b; Paasch et al., 2010; Rybar et al., 2011; Thomas et al., 2013; Tunc et al., 2011). Increased sperm DNA damage has further been confirmed in rodent models of obesity (Bakos et al., 2011b; Chen et al., 2013; Vendramini et al., 2013). Only three studies to date, one human (Tunc et al., 2011) and two rodent (Bakos et al., 2011b; Chen et al., 2013) have directly linked levels of sperm oxidative stress with male obesity, concluding that a positive association exists between increasing adiposity and increased sperm oxidative stress. Therefore, it appears that male obesity is associated with significant perturbations to the molecular composition of sperm, which has implications not only for sperm function but also may have lasting impacts on the resultant embryo. Both the relationship of ROS with DNA damage and the

biology behind the lack of antioxidant protection in sperm may be important in understanding the effect of obesity on male sub fertility.

1.6.3 Male obesity on additional markers of sperm function

Sperm parameters including sperm binding and sperm mitochondrial health have also been used to assess functional properties of sperm. Currently only two human studies (Sermondade et al., 2013a; Wegner et al., 2010) and one rodent model (Bakos et al., 2011b) have measured sperm binding in relation to male obesity. To date the human studies contradict each other with one (Wegner et al., 2010) showing decreased sperm binding to hyaluronan coated slides as BMI increased and the other (Sermondade et al., 2013a) reporting no change to sperm binding to human zona pellucida as a result of increasing BMI. The reasons for these discrepancies could be related to either differences in the populations of men selected in each study ((Wegner et al., 2010) included all types and cause of infertility) or the methods for determining sperm binding (HBT assay as assessed in (Wegner et al., 2010) only provides an indirect assessment sperm binding). In contrast, the single study to date using a rodent model of male obesity found that mice fed a high fat diet had reduced sperm binding to mouse oocytes which was directly related to reduced sperm capacitation and reduced fertilisation (Bakos et al., 2011b). These studies further demonstrate the lack of coherence, and the need for more controlled studies to determine if male obesity is associated with reduced sperm binding.

Two recent studies in humans have now assessed the effects of male obesity on sperm mitochondrial output (Fariello et al., 2012; La Vignera et al., 2012b). Both studies, using two different methods (JC-1 and mitochondrial C oxidise), found that sperm exhibited lower mitochondrial activity and lower mitochondrial membrane potential with increasing male BMI. It is hypothesised that a cascade of events occur as a result of environmental insult beginning with impaired mitochondrial function ultimately resulting in the generation of increased ROS and

initiation of DNA damage (Aitken & Curry, 2011; Aitken et al., 2012; Aitken & Koppers, 2011). Therefore, the increased rates of DNA damage and alterations to sperm motility described in obese males maybe related to dysfunctional sperm mitochondria.

1.6.4 Proposed mechanisms by which obesity alters sperm function

The mechanisms by which obesity affects male sperm function are yet to be fully determined. A number of hypotheses have been suggested including dysregulation of key hormones vital for normalisation of the HPG axis, metabolic syndrome associated with obesity, adipose tissue as a hormone regulator, increases in scrotal heat, seminal fluid composition, sperm proteomics/epigenetic profiles and obesity related genetic disorders.

1.6.4.1 Altered hormone concentrations

Examining the effect of obesity on the hormone regulation of spermatogenesis is underpinned by the hypothesis that the HPG axis is deregulated by obesity (Fig 1.4). Several studies document that increased male BMI is associated with reduced plasma concentrations of sex hormone binding globulin (sHBG), therefore testosterone, and a concomitant increased plasma concentration of oestrogen (Aggerholm et al., 2008; Chavarro et al., 2010b; Fejes et al., 2006; Hinz et al., 2010; Hofny et al., 2010; Jensen et al., 2004; La Vignera et al., 2012b; Paasch et al., 2010; Pauli et al., 2008; Ramlau-Hansen et al., 2010; Tunc et al., 2011). Decreased testosterone and increased oestrogen have long been associated with sub fertility and reduced sperm counts through disruption of the negative feedback loop of the HPG axis and are therefore common clinical markers of fertility (Handelsman & Swerdloff, 1985). The Sertoli cell is the only somatic cell in direct contact with the developing germ cell providing both physical and nutritional support, therefore are of particular interest in male sub fertility. Adhesion of the developing germ cells to the Sertoli cells is dependent on testosterone, with decreased testosterone leading to retention and phagocytosis of mature spermatids thereby reducing sperm counts (Kerr et al., 1993a; Kerr et al., 1993b). Other hormones involved in the regulation of Sertoli cell function and

spermatogenesis, such as FSH/LH ratios, inhibin B and SHBG concentrations have all been observed to be decreased in males with increased BMI (Aggerholm et al., 2008; Chavarro et al., 2010b; Du Plessis et al., 2010; Macdonald et al., 2010; Paasch et al., 2010; Ramlau-Hansen et al., 2010; Teerds et al., 2011). Mouse knockout models which result in loss of FSH or impairments in FSH receptor are associated with decreased testis weight, and sperm output due to a reduction in Sertoli cell numbers (Dierich et al., 1998; Kumar et al., 1997). Therefore, it remains plausible that the decreased sperm counts observed in male obesity are at least in part a result of a perturbation to the HPG axis through testosterone and oestrogen which reduce Sertoli cell function.

1.6.4.2 Metabolic syndrome

Hyperinsulinemia and hyperglycemia are common comorbidities in obese males and are also confounding factors in many rodent studies of male obesity (Ghanayem et al., 2010; Ng et al., 2010; Palmer et al., 2012). Hyperinsulinemia and hyperglycemia have been shown to have an inhibitory effect on sperm quantity and quality (in isolation of obesity) and therefore could both be contributing to the reduced fertility seen in obese men and explain some discrepancies in the current literature on sperm count, motility and morphology (Kasturi et al., 2008; La Vignera et al., 2012a). Common perturbations to sperm function in obesity such as decreased count, increased ROS and sperm DNA damage are also prevalent in diabetic men (Amaral et al., 2008; La Vignera et al., 2012a). High circulating concentrations of insulin is suggested as one possible mechanism for changes to sperm parameters, with increased insulin reducing the production of SHBG in the liver thereby indirectly increasing the amount of active unbound oestrogens and testosterone (not bound by SHBG) in the blood stream (Fig 1.4) (Laing et al., 1998). The decreased concentrations of SHBG to sustain homeostatic concentrations of testosterone could contribute to the decreased concentrations of testosterone and decreased sperm counts seen in diabetic men. Further, increasing concentrations of circulating glucose have also been shown to reduce the amount of

LH released by the anterior pituitary in sheep (Clarke et al., 1990; Medina et al., 1998) and therefore could contribute to the impaired HPG axis and altered sperm function seen in both diabetic and overweight or obese men. Additionally, there is emerging evidence that low testosterone concentrations might also induce aspects of metabolic syndrome and therefore obesity may not be the direct cause of reduced sperm counts seen in these men but a symptom of the same low testosterone (Akishita et al., 2010; Haring et al., 2009; Kupelian et al., 2006).

1.6.4.3 Increased adipose tissue and hormone regulation

Elevated oestrogens in obese men may in part result from an increased mass of white adipose tissue. White adipose tissue is responsible for aromatase activity and adipose derived hormones and adipokines, which are shown to be elevated in obese men (Wake et al., 2007). The aromatase cytochrome P450 enzyme, is produced by many tissues including adipose tissue and testicular Leydig cells, and in men actively converts testosterone to oestrogens (Cohen, 1999; Meinhardt & Mullis, 2002). With obesity it is suggested that elevated oestrogen concentrations may result from an increased conversion of androgens to oestrogens by white adipose tissue therefore contributing to the increased plasma oestrogens sometimes seen (Fig 1.4) (Cohen, 2008; Kley et al., 1980; Ruige, 2011). Another key hormone produced by white adipose tissue is leptin, which plays a pivotal role in the regulation of energy intake and expenditure (Gonzalez Jimenez et al., 2010; Machleidt & Lehnert, 2011). Leptin mainly targets receptors in the hypothalamus by counteracting the effects of neuropeptide Y. However, leptin receptors have recently been discovered in ovaries and testes, functioning to regulate the HPG axis (Ahima et al., 1996; Chehab et al., 1997; Finn et al., 1998; Mounzih et al., 1997; Pinilla et al., 1999). Specifically, increased concentrations of leptin significantly decreased the production of testosterone from Leydig cells (Caprio et al., 1999). Taken together this suggests that elevated leptin concentrations commonly found in obese males (Caro et al., 1996) could alter the HPG axis, thus contributing to the decreased testosterone and sperm counts observed.

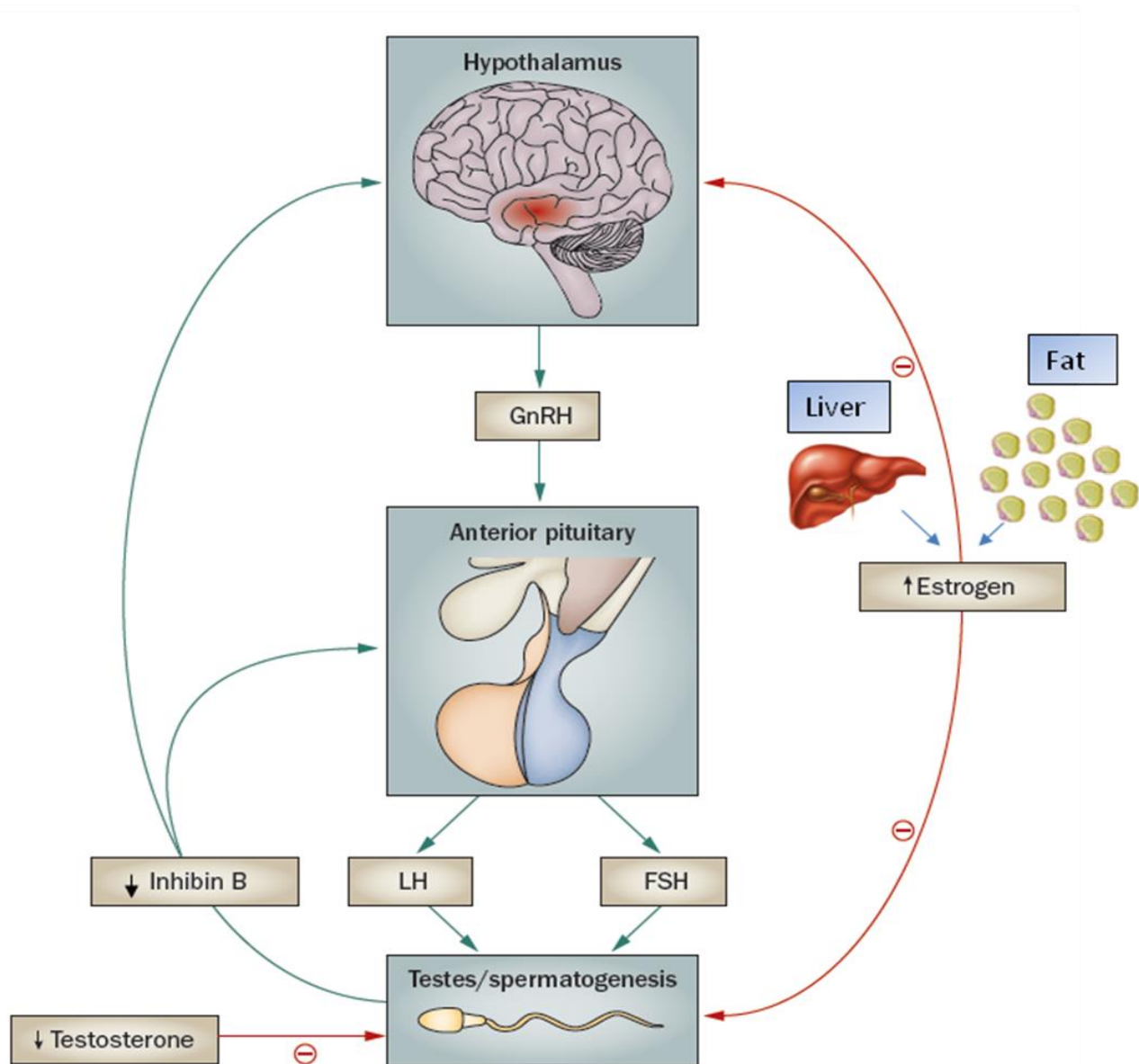


Figure 1.4 Potential changes to hormonal regulation resulting from male obesity

Due to the increase in oestrogens produced by the liver and adipocytes negative feedback of oestrogen to both the hypothalamus and anterior pituitary may cause a decrease release of LH and FSH and therefore reduce the production of testosterone and inhibin B altering spermatogenesis. Picture modified from (Du Plessis et al., 2010).

1.6.4.4 Increased adipose tissue on testicular temperature

One suggested side effect of obesity that may contribute to altered sperm parameters is raised gonadal heat resulting from increased scrotal adiposity. The process of spermatogenesis is highly sensitive to heat, with optimal temperature ranging between 34-35°C in humans (Robinson et al., 1968). Increased testicular heat is associated with reduced sperm motility, increased sperm DNA damage and increased sperm oxidative stress (Paul et al., 2008a; Paul et al., 2008b; Shiraishi et al., 2010). Changes to testicular temperature can occur via a number of pathologies (i.e. varicoceles), increased scrotal adiposity or environmental disturbances (i.e. prolonged bike riding) and are associated with reduced sperm function and increased sub fertility (Ivell, 2007; Mariotti et al., 2011; Setchell, 1998; Shafik & Olfat, 1981; Southorn, 2002; Wise et al., 2011; Yaeram et al., 2006). It is noteworthy that increased sperm DNA damage and oxidative stress were restored following the surgical removal of scrotal fat in obese men (Shafik & Olfat, 1981).

1.6.4.5 Changes to seminal plasma composition

Growing evidence suggests that seminal plasma may help modulate sperm function and their ability to interact with the female reproductive tract (Rodriguez-Martinez et al., 2011). Therefore any changes to seminal plasma composition as a result of obesity may interfere with these processes. Fructose concentrations are significantly higher in seminal plasma of overweight and obese men (Martini et al., 2010) while adiponectin, progranulin and alpha-glucosidase concentrations are significantly lower (Martini et al., 2010; Thomas et al., 2013). Fructose is a carbohydrate transported into sperm and is one of its main energy sources (Bucci et al., 2011). Whether the high fructose concentrations in seminal plasma of obese males can explain the changes to sperm mitochondrial function remains to be determined. Alpha-glucosidase is an enzyme that is involved in sperm motility acquisition (Cooper, 1990) and its decreased concentrations in the seminal plasma of patients with high BMI may contribute to their reported motility defects. The functional roles of the adipokines (adiponectin and progranulin) in seminal

plasma and on sperm function remains unknown, however it is hypothesised that increased levels of adipocytes from increased epididymal fat associated with obesity may increase secretion of these molecules into epididymal fluid and therefore alter sperm function (Thomas et al., 2013).

1.6.4.6 Changes to sperm proteomics

Due to the numbers of physical sperm changes described as a result of obesity, it is highly likely that sperm proteomic profiles may be altered in obese males. One study has examined the proteomic profile of sperm from diabetic, obese non-diabetic and men of normal BMI without diabetes (Kriegel et al., 2009). A number of key proteins associated with perturbed sperm function were altered in sperm from obese men which have been previously linked with asthenozoospermia (Semenogelin 1), suppression of sperm motility at the time of ejaculation (Semenogelin 1 and 2), capacitation (AKAP4), defective sperm function and loss of cell integrity (ODFP2/2). Additionally it has been found that the lipid phosphatidylcholine and lysophosphatidylcholine ratios are compromised in sperm of obese class II men (BMI ranging between 35.0 kg/m² and 39.9 kg/m²) (Pyttel et al., 2012). More studies investigating the changes in sperm proteomics due to obesity are needed to establish the molecular pathways altered in the testes.

1.6.4.7 Genetic disorders

A number of genetic diseases that display perturbed male fertility are also associated with increases in adiposity. Genetic disorders such as Klinefelter, Prader-Willi or Larence-Moon-Bardet-Biedel syndromes all display both obesity and sub fertility phenotypes to varying degrees. For example, one of the genes inactive in Prader-Willi syndrome (*Mage12*) is highly expressed in the hypothalamus causing impaired HPG regulation, as well as obesity in *Mage12* null mice (Tennese & Wevrick, 2011). Additionally the length of the aromatase *CYP19A1* short tandem repeat polymorphism is associated with reduced sperm counts, increased oestrogens and

decreased oestrogen/testosterone ratios in obese individuals from the general population (Hammoud et al., 2010a; Hammoud et al., 2010b).

1.6.4.8 *Erectile dysfunction*

Erectile dysfunction is common in male sub fertility and and increased prevalence of erectile problems is associated with poor lifestyle choices (Christensen et al., 2011). Overweight and obese patients made up approximately 76% of the men who report erectile dysfunction and/or a decrease in libido (Pauli et al., 2008) suggestive of a negative interaction between obesity, reproductive hormones and sexual function.

1.7 PATERNAL PROGRAMMING

The Barker hypothesis for the fetal origins of adult disease was first proposed in 1992 (Hales & Barker, 1992), and provided some of the first evidence that the weight of a baby at birth and infancy could predict the risk of coronary heart disease later in life. These observations implied that the transfer of nutrients from the mother to baby could have long term implications for the health of their child. There is now a large amount of evidence that maternal health factors during pregnancy including smoking, alcohol, illicit drugs and over nutrition (i.e. obesity) alter fetal growth parameters, with increases in non-communicable diseases observed in the next generation (Behl et al., 2013; Foltran et al., 2011; Sithisarn et al., 2012; Symonds et al., 2013). Maternal obesity in humans negatively effects oocyte quality and increases the risk of macrosomia in the fetus as well as increased obesity and diabetes in offspring (Grindler & Moley, 2013; Li et al., 2011; Mission et al., 2013).

Paternal health at the time of conception has now additionally been shown to affect the health of subsequent children, extending the concept of development programming of adult disease to

include a paternal contribution in the early life origins of disease. A number of paternal health factors including smoking, advanced age and exposure to industrial chemicals have been associated with increased rates of cancer, mental disorders (including autism and congenital abnormalities) in subsequent offspring (El-Helaly et al., 2011; Lee et al., 2009; Van Balkom et al., 2012). Additionally, rodent models have demonstrated that direct insults to sperm molecular function either through inducing sperm DNA damage or impaired fetal testes development via altered intrauterine environments from mothers, is associated with increases in gamete and somatic cell DNA damage (Adiga et al., 2010), impaired reproductive function (Fullston et al., 2012) and changes to body composition in first generation offspring (Dunn & Bale, 2011). These studies provide evidence that environmental perturbations that alter sperm function can impact the health of the next generation presumably by changes to the molecular constitution of sperm. More recently there has been an increasing awareness that male obesity at conception, known to alter sperm function, can also additionally affect early embryo, pregnancy and alter offspring health.

1.7.1 Paternal obesity programs embryos, pregnancy and offspring health

1.7.1.1 Male obesity, fertilisation and pregnancy

There is mounting evidence that male obesity is implicated in affecting pregnancy outcomes in addition to the reported maternal contributors. An overweight or obese male partner, with a female of normal body mass index (BMI), have an increased odds ratio for time to conceive compared with couples with normal weight male partners (Nguyen et al., 2007; Ramlau-Hansen et al., 2007). A small number of clinical studies suggest similar outcomes, with obesity in males associated with decreased pregnancy rates and an increase in pregnancy loss in couples undergoing ART (Bakos et al., 2011a; Colaci et al., 2012; Hinz et al., 2010; Keltz et al., 2010; Merhi et al., 2013; Ramasamy et al., 2013). In part, this effect appears to be due to, reduced blastocyst development, sperm binding and fertilisation rates during *in vitro* fertilisation (IVF),

when the male partner is overweight or obese (Bakos et al., 2011a; Colaci et al., 2012; Hwang et al., 2011; Merhi et al., 2013). Although there are reports of reduced pregnancy rates with obese males during ICSI cycles (Colaci et al., 2012; Ramasamy et al., 2013), more studies would be welcomed on this topic as limitations regarding sample size, cycle numbers, known factor infertility and the use of either IVF or ICSI are potential cofounders. This is suggested by Keltz et al (Keltz et al., 2010), as these researchers did not identify the same reductions to fertilisation and embryo development when sperm were injected directly into the oocyte suggesting that the process of ICSI was bypassing the impairment of the sperm to bind and fertilise. This is perhaps not surprising as rodent models of obesity have shown impaired capacitation and sperm binding when males are fed high fat diet (Bakos et al., 2011b) suggesting that post ejaculation maturation was altered and this may be bypassed by ICSI. Studies including ART patients have established that male obesity at the time of conception impairs embryo development to the blastocyst stage, therefore reducing implantation and live birth rates. These findings are paralleled by rodent models of male obesity (Binder et al., 2012a; Binder et al., 2012b; Ghanayem et al., 2010; Mitchell et al., 2011) which additionally investigated invasive molecular markers of embryo viability, which are not possible to conduct in humans due to ethical considerations. When male mice are fed a high fat diet, embryos have extended 1st, 2nd and 3rd cleavage times, reduced cavitation and compaction (Binder et al., 2012a; Binder et al., 2012b; Mitchell et al., 2011) and also reduced cell number of the inner cell mass and trophectoderm cell numbers in the late stage blastocysts (Binder et al., 2012a; Binder et al., 2012b; Mitchell et al., 2011). This caused functional reductions in outgrowth when embryos were plated onto a fibronectin cell layer (Binder et al., 2012a). The changes to embryo development, cell number and function have been hypothesised to be caused by alterations to mitochondrial function. There is evidence that embryos produced by high fat fed male rodents displaying reduced mitochondrial membrane potential suggestive of uncoupling of the mitochondrial electron transport chain

(Binder et al., 2012a) and increases in cellular glycolysis with an increased rate of glucose and increased production of lactate (Binder et al., 2012b). Both of these adverse mitochondrial phenotypes in the developing embryo have previously been shown to alter implantation and fetal growth (Lane & Gardner, 2005; Wakefield et al., 2011). The additionally reported changes to embryo cell number, metabolism and function from rodent models of male obesity are likely causing the reduced implantation and pregnancy rates seen in both human and animal models. Taken together, data from animal models of obesity and human clinical studies suggest that male obesity negatively affects embryo quality, implantation, and pregnancy establishment and live birth outcomes.

1.7.1.2 Male obesity and altered metabolic health in offspring

Epidemiological studies have concluded that in addition to the impairments to embryos and early pregnancy, obese fathers are more likely to father an obese child (Danielzik et al., 2002; Li et al., 2009). However, it must be noted that the extent of the individual contributions of genetic, epigenetic and environment cannot be separated due to the common raising environment shared by both father and child. Due to this confounding factor in human studies, two rodent studies have now been published that establish the direct effect of paternal high fat diet (HFD) on the metabolic health of the next generation in the absence (Fullston et al., 2013) and presence of altered glucose homeostasis (Ng et al., 2010) in founders. In the absence of altered glucose homeostasis, founder males fed a HFD who were mated with normal weight females fed standard chow produced F₁ offspring that were heavier, with F₁ females displaying increases in adiposity and serum triglycerides who themselves produced F₂ males with increased adiposity (Fullston et al., 2013). Similarly, F₁ females and their F₂ female and male offspring had compromised metabolic health (reduced glucose tolerance/clearance and insulin sensitivity) as they aged compared to those born by a control founder, demonstrating that the effect of paternal obesity could extend to impact the metabolic health of two generations (Fullston et al., 2013).

These changes to metabolic health in F₁ female offspring were likely due to altered pancreatic function with increased insulin secretion, reduced pancreatic islet cell size and alterations to methylation and gene profiles in their pancreas relating to insulin and glucose metabolism, ATP binding, cytostructure and intracellular transport noted (Ng et al., 2010). Additionally F₁ males born to founders fed a HFD displayed altered glucose and insulin sensitivity, however this was not evident until later in life, with only F₂ females from this lineage showing impaired insulin tolerance (Fullston et al., 2013). Taken together, these data suggest that paternal obesity at the time of conception has a marked effect on offspring metabolic health, which seems to be exacerbated in female offspring and can extend into two generations.

1.7.1.3 Male obesity and altered reproductive health in offspring

In addition to the reported changes to metabolic health in offspring from an obese father, one study in a rodent model of male obesity has also reported a negative effect to the reproductive health of offspring extending two generations (Fullston et al., 2012). In the absence of altered glucose homeostasis in HFD fed founders, F₁ males displayed reduced sperm function including reduced motility and increases in sperm DNA damage and ROS levels. These F₁ males went on to produce F₂ male offspring with similar impaired reproductive health with reduced sperm motility and altered mitochondrial function and increased ROS in oocytes in their F₂ daughters. F₁ daughters of founders fed a HFD displayed altered oocyte health with reduced meiotic progression and altered mitochondrial membrane potential. Their F₂ male offspring, however showed severe sub fertility phenotypes, with reduced testes weights, serum testosterone, reduced sperm motility and increased ROS, whilst reduced metabolic output of oocytes was reported in F₂ females. However, it should be noted that F₁ females born to HFD fed founders at mating had increases in adiposity, were glucose intolerant and insulin insensitive, which could have contributed to the F₂ results from this lineage (Li et al., 2011). This study provided the first evidence that altered fertility as seen in male obesity can be transmitted through two generations

of offspring. Whether paternal obesity in humans is shown to also affect the reproductive health of their children and grandchildren is still to be established. However the increasing reliance on ART methods to achieve pregnancy in western societies suggests a likely environmental factor (Sunderam et al., 2009; Wang, 2008), which obesity is a prime candidate.

1.7.2 Proposed transmission of altered offspring health

Paternal health cues are likely transmitted to the next generation via the sperm (Youngson & Whitelaw, 2011). Due to the lack of cytoplasmic scavenging enzymes, sperm are highly susceptible to environmental stress and therefore are susceptible to changes to their epigenetic or genetic content. Several studies examining trans-generational transmission (Fullston et al., 2012; Ng et al., 2010) have proposed epigenetic modifications to the sperm through changes to mRNA and non-coding RNAs, methylation or acetylation content. Currently there is little known of changes to sperm molecular composition induced by obesity.

1.7.2.1 mRNA and non-coding RNAs

It is now evident that mature sperm contain a regulated suite of both mRNA and other non-coding RNA that are suggested to be important for normal fertilisation and subsequent embryonic development, with active transcription and translation occurring in the sperm's mitochondria (Dadoune, 2009; Lalancette et al., 2008; Lalancette et al., 2009; Ostermeier et al., 2004). Although it is not yet clear what the precise role these RNAs play, it has been proven that these RNA can cause phenotypic change in resultant offspring after injection into oocytes, albeit at amounts that far exceed physiological levels (Sone et al., 2005). While to date there is little known about mRNA abundance in sperm from obese males, one rodent model of obesity and diabetes has shown significant down regulation of genes important for male fertility including *Crem*, *Dhh*, and *Sh2b1* within testes compared to lean controls (Ghanayem et al., 2010). Additionally genes encoding metabolic sensing proteins (*Sirts*) were also down-regulated in testes of rodents fed a HFD (Palmer et al., 2011).

Mature sperm also contain high levels of small non-coding RNAs including silencing RNAs (siRNAs), microRNAs and piwi-interacting RNA (piRNAs) (Krawetz et al., 2011). Small non coding microRNAs are 20-22 nucleotides (nt) in length and contain an abundance of stop codons and generally lack open reading frames (Costa, 2005). They regulate at the level of both transcription and translation via control of chromatin organisation, mRNA stability and protein synthesis. It is apparent that small non coding RNAs have a role in the oocyte and can influence the first stage cleavage, embryo development and the phenotype of subsequent offspring (Jodar et al., 2013; Sendler et al., 2013). Alteration of microRNA abundance in the male pronucleus of recently fertilised zygotes can alter offspring phenotypes, depending on the ratios of microRNAs injected (Rassoulzadegan et al., 2006). Obesity is known to alter the microRNA profile of islet cells and adipose tissue (Williams & Mitchell, 2012), however only one study to date has assessed microRNA profiles of sperm and testes in a rodent model of male obesity (Fullston et al., 2013). A high fat diet altered the expression of 23 microRNAs in mouse testes, with four of these altered microRNAs (mmu-mir196a, 205-5p, 133b-3p and 340-5p) convergent on pathways specific for metabolic disease, ROS production, DNA replication, NF- κ B signalling, p53 signalling, lipid metabolism, spermatogenesis and embryo development. Interestingly the same four microRNAs which were also dysregulated in mature sperm from the same animals (Fullston et al., 2013). The direct impact of these sperm mRNA and microRNAs changes to both fertilisation and embryo quality remains to be determined.

1.7.2.2 Methylation

Methylation of DNA and histones is dynamic during spermatogenesis and is vital for the normal processes of spermiogenesis and fundamental for a successful pregnancy. Changes to sperm methylation are required and essential for X chromosome inactivation during meiosis and for the establishment of paternally imprinted genes in sperm (Goto & Monk, 1998; Ooi & Henikoff, 2007).

Generally, hypermethylated DNA at promoter regions inhibits gene expression by excluding transcription factor binding. In contrast, hypomethylation generally allows increased access of transcription factors to the DNA and increases gene expression. It is estimated that 96% of the genomic CpGs in sperm DNA are methylated, although there are site specific variations of methylation in mature sperm (Molaro et al., 2011). Analysis throughout human spermatogenesis has determined that DNA methyltransferase proteins (DNMT 1, 3A, 3B) are present in developing germ cells, with gene knockout strategies resulting in changes to sperm methylation and in some cases sperm function (Jenkins & Carrell, 2012). Stage specific changes to nuclear localisation of DNMT proteins during spermatogenesis coincide with the establishment of methylation imprints. Subsequent maintenance of these imprints occurs throughout the remainder of spermatogenesis suggesting methylation imprints are key molecular events during spermatogenesis (Marques et al., 2011). There is some evidence that the methylation status of sperm DNA is associated with sub fertility. Hypomethylation of imprinted genes and repeat elements in sperm have been linked with reduced pregnancy success and correlate with increased sperm DNA damage in males undergoing fertility treatment (El Hajj et al., 2011; Minor et al., 2011; Nanassy & Carrell, 2011; Tunc & Tremellen, 2009). Additionally, altered levels of methylation in the promoter regions of genes such as *MTHFR* are associated with decreased sperm function. Further, imprinted regions such as *H19* and *ALU* repeat elements are more likely to be hypomethylated in sub fertile men (El Hajj et al., 2011; Minor et al., 2011). Environmental exposures of males have also been linked with changes to methylation status of sperm. Toxins such as exposure to 5-aza-2'-deoxycytidine, tamoxifen and chemotherapy agents disturb the *de novo* methylation activity in sperm as shown in animal models (Barton et al., 2005; Oakes et al., 2007; Pathak et al., 2009). This aberrant methylation was observed at imprinted regions such as *IGF2* and *H19*. Subsequently, this led to a disruption of the DNA methylation reprogramming of the male pronucleus, which in turn increased post-implantation pregnancy loss (Barton et al., 2005; Oakes et al., 2007; Pathak et al.,

2009). Moreover, excessive alcohol consumption in men has been associated with site-specific hypomethylation in sperm (Ouko et al., 2009), a finding confirmed in animal models (Bielawski et al., 2002). Excessive paternal alcohol consumption impacts negatively on offspring prenatal growth and also alters the methylation status of offspring DNA (Knezovich & Ramsay, 2012; Stouder et al., 2011). To date there is little information as to the impact of male obesity on the methylation status of sperm. Only one study has assessed the global methylation patterns of testes and mature sperm in HFD fed rodents, finding diet induced obesity caused hypomethylation of elongating spermatids and DNA extracted from testes (Fullston et al., 2013). Additionally, it was found in humans that the paternally imprinted *IGF2* methylation status of cord blood could be directly related to the adiposity level of their fathers providing the first evidence that male adiposity can alter the *IGF2* methylation pattern in pregnancy (Soubry et al., 2013). Whether the metabolic and reproductive changes observed in offspring as well as reduced fertilisation and increased pregnancy loss induced from paternal obesity results from alterations to *de novo* methylation patterns of developmental genes in the male germ line is yet to be determined.

1.7.2.3 Acetylation

Histone hyperacetylation is essential to relax chromatin and allows for the repair of the DNA double and single strand breaks that result from the removal of histones to protamines during spermiogenesis (Gaucher et al., 2010). Thus, alterations to histone acetylation at developmentally important loci, which preferentially retain histones, due to environmental cues could result in epigenetic modifications to sperm that might form the basis of paternal programming of offspring (Hammoud et al., 2011). The N-terminus of histones is a key region attracting post-translational modifications such as acetylation. Acetylated histones in mature sperm are thought to represent epigenetic marks capable of transmission to the oocyte during fertilisation and regulate gene expression in early embryogenesis, with entire nucleosomes

transmitted to the zygote unadulterated from their sperm configuration (Brykczynska et al., 2010; Erkek et al., 2013). Interestingly, key pluripotency genes are reportedly associated with retained histones that are proposed to be in readiness for immediate activation of expression of these genes post fertilisation (Farthing et al., 2008). Studies exploring the roles of histone deacetylases (HDAC) found that germ cells treated with HDAC inhibitors, resulted in premature hyperacetylation of late round spermatids (Awe & Renkawitz-Pohl, 2010; Fenic et al., 2004; Hazzouri et al., 2000). The functional consequence of this early hyperacetylation is still to be fully understood, however studies indicate that an increased rate of DNA damage occurs as a result (Fenic et al., 2004; Marcon & Boissonneault, 2004). Interestingly, male mice fed a HFD displayed hyperacetylation in late round spermatids which also correlated with increased DNA damage in the germ cells (Palmer et al., 2011). Alterations to sperm histone acetylation correlates with poor protamination, which in turn positively correlates with increased DNA damage in mature sperm and therefore potentially contributes to poor sperm parameters observed in obese males (Aoki & Carrell, 2003; Aoki et al., 2006a; Aoki et al., 2005). This suggests that alterations to histone acetylation represent a potential epigenetic basis for the programming observed in resultant embryos and offspring born from obese males (Jenkins & Carrell, 2012).

1.8 REVERSIBILITY

Whilst it is becoming clear that male obesity has negative impacts on fertility, sperm function and potentially long-term disease burden of offspring there is emerging evidence that that these effects might be reversible. Weight loss in obese men via bariatric surgery, can improve hormone concentrations (testosterone, inhibin B, SHBG and oestrogens), erectile dysfunction and does not to interfere with sperm function (Bastounis et al., 1998; Reis et al., 2010; Reis et al., 2012; Savastano et al., 2013). There have been two case studies showing that aromatase inhibitors

(i.e. anastrozole) in obese men can restore testosterone, LH, FSH and oestrogen levels as well as sperm function (count, motility and morphology) (Roth et al., 2008; Stephens & Polotsky, 2013). Animal models of male obesity have shown improvements to both metabolic health (glucose, insulin and cholesterol concentrations) and fertility measures (increased sperm count, increased sperm motility, decreased sperm ROS and DNA damage) with intake of selenium enriched probiotics, olive oil and metformin (diabetes medication) (Chen et al., 2013; Ibrahim et al., 2011; Saez Lancellotti et al., 2013). While these pharmacological approaches have had some positive impacts for reversing altered hormone concentrations and sperm function in male obesity, the most effective and least invasive method to reduce obesity is through simple lifestyle interventions such as changes to diet and/or increases in physical activity. Whether, diet and/or exercise intervention in obese males can improve sperm function, impaired embryo quality and offspring pathology is largely unknown (Fig 1.5).

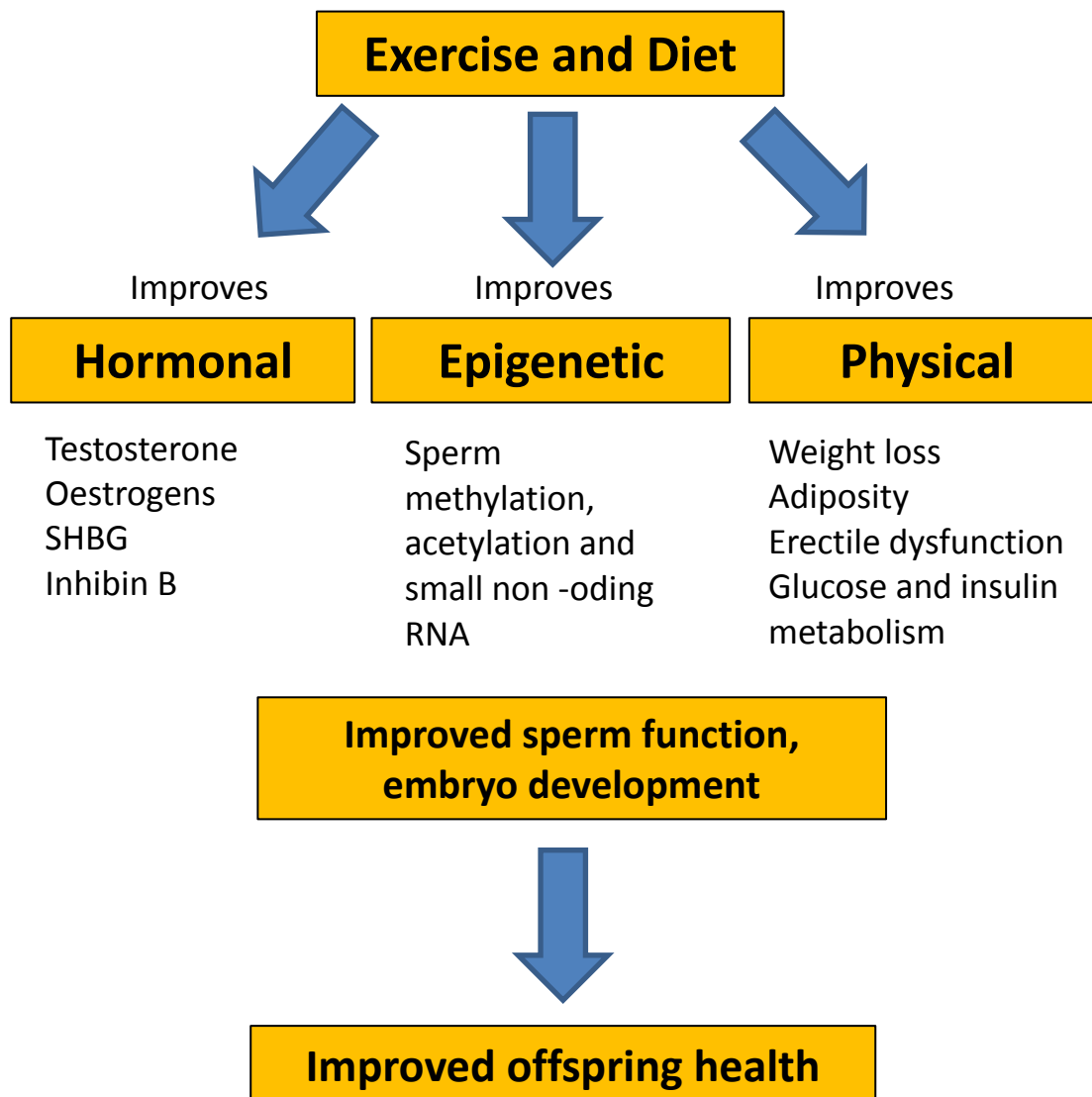


Figure 1.5: Hypothesis for the improvement to fertility in obese males via weight loss through diet and exercise interventions

Diet and exercise interventions may normalise hormone regulation, adiposity, and epigenetic markers of sperm function, improving sperm parameters and early embryo development likely influencing improved offspring health.

1.8.1 Diet and exercise

Unlike most health pathologies obesity can be reversed with changes to lifestyle having profound beneficial effects to the health of individuals. It is broadly accepted that diet and lifestyle changes such as exercise and decreased caloric intake can decrease fat storage and improve cardiovascular and metabolic health (Van Dorsten & Lindley, 2008). A number of studies have reported normalisation of glucose and insulin homeostasis (Beltaifa et al., 2011; Jankiewicz-Wika et al., 2011; Umpierre et al., 2011) as well the HPG axis (Hammoud et al., 2009; Kasturi et al., 2008; Strain et al., 1988) post weight loss in overweight and obese men. Therefore, weight loss through diet and exercise in obese males may improve sperm function, embryo development and offspring health; however limited studies have been performed to test this hypothesis.

1.8.1.1 Changes to HPG axis regulation

There is a general consensus that diet and exercise interventions can reduce body weight in obese men and restore the HPG axis. Men who lost weight through low caloric diet and/or scheduled exercise programs experienced increased, testosterone, inhibin B and SHBG (Kasturi et al., 2008; Kaukua et al., 2003; Strain et al., 1988; Tymchuk et al., 1998) common hormone biomarkers altered as a result of a high BMI. Whether these improvements are likely from reduced aromatase activity and/or changes to leptin concentrations from decreased white adipose tissue or increases to aerobic fitness from exercise are still to be determined. However, together these studies indicate that weight loss via diet and/or exercise interventions can restore androgens concentrations and therefore, likely improve sperm function.

1.8.1.2 Glucose and insulin regulation

As mentioned above, male obesity is commonly associated with hyperinsulinemia and hyperglycemia, which can additionally affect sex steroids and sperm function (Kasturi et al., 2008; La Vignera et al., 2012a). There have been a number of studies in both humans and animal models reporting improvements to glucose and insulin regulation from weight loss in obese

males. Exercise interventions including the run walk system reduced blood glucose, insulin and leptin concentrations in obese patients (Beltaifa et al., 2011). Resistance training in combination with aerobic exercise in obese males with type II diabetes resulted in reduced glycated haemoglobin concentrations (hbA(1c)) (Umpierre et al., 2011). Additionally, numerous animal models have shown improved glucose and insulin regulation in obese males via diet and exercise interventions (Jung et al., 2013; Shaker & Sadik, 2013; Wagener et al., 2012). The reduction/normalisation of glucose and insulin concentrations observed in obese men may contribute to the improved serum sex hormones or have direct effects (i.e. reduction in diabetic symptoms) in these men and therefore, is likely to be beneficial for sperm function.

1.8.1.3 *Erectile dysfunction*

The reported erectile dysfunction and decreased sexual function, as a result of male obesity in humans can be shown to be reversed by weight loss through diet and exercise interventions (Hannan et al., 2009; Meldrum et al., 2012). Similar reported benefits can be confirmed in a rodent models of male obesity with exercise interventions (La Favor et al., 2013). These studies suggest that sexual dysfunction in obese men is likely as a result of increased scrotal adiposity, antioxidant stress and associated metabolic changes and not solely related to reproductive hormone concentrations. However, this cannot be entirely confirmed as diet and exercise interventions reducing weight loss tend to also improve circulating serum testosterone and SHBG concentrations in these same men.

1.8.1.4 *Sperm parameters*

Currently only one study has assessed the effects of diet and exercise interventions on sperm function in obese men. This study examined 43 obese men (20-59 years) during a 14 week residential weight loss program based on healthy diet and daily exercise (Hakonsen et al., 2011). The study demonstrated significant improvements to total sperm count, sperm motility and normal sperm morphology in those men who lost the greatest amounts of weight. Interestingly,

no changes were seen in levels of sperm DNA damage prior to or after the intervention; however it should be noted that this was only a pilot study and the sample numbers were low and no changes to sperm DNA damage were seen at the beginning of the study between BMI ranges. Never the less, this study provides the first evidence that scheduled diet and exercise programs in obese men in order reduced adiposity can improve sperm function.

1.8.1.5 Epigenetic regulators methylation, acetylation and RNAs

Male obesity has been widely documented to change the RNA, methylation and acetylation profiles of a variety of tissues (e.g. adipose tissue, liver, pancreas etc.) (Barres & Zierath, 2011; Funato et al., 2011; Sun et al., 2013; Tian et al., 2013). Less is known about the effect of weight loss through diet and exercise on these targeted tissues. Two studies have assessed the microRNA content in serum and methylation profiles of white blood cells in men undergoing weight loss programs (Milagro et al., 2011; Ortega et al., 2013). The microRNA content in the serum of obese males was altered, most notably the dysregulation of microRNAs that target components of cell cycle regulation and apoptosis pathways (Ortega et al., 2013), similar to those microRNA pathways altered in sperm from obese male rodents (Fullston et al., 2013). Interestingly, the abundance of these particular microRNAs in serum was restored through weight loss, providing evidence that interventions to health can change the microRNA content. Whether weight loss interventions could also restore the microRNA content of testes and sperm and therefore alter embryo and offspring health remains unknown. Additionally, restoration of hypermethylation of genes in white blood cells, including *H19*, *APOA2*, *NTF3* and *TGFB1* important for metabolic regulation were found after caloric restriction in previously obese men (Milagro et al., 2011). Interestingly, the level of increased hypermethylation was dependent on responsiveness to the weight loss treatment. Again whether weight loss interventions could restore the hypomethylation patterns of sperm produced by high fat fed rodents is unknown.

1.9 CONCLUSION

The current literature lacks studies investigating the extent of the reversibility of male sub fertility caused by obesity and its downstream programming effects to pregnancy and offspring. Improved hypothalamic regulation, glucose and insulin sensitivity by weight loss through diet and exercise has been reported in humans. Less information is known about the effects of weight loss via diet and exercise interventions to sperm parameters, and currently no information is known about the reversibility of weight loss on altered embryo development and offspring outcomes. Studies in controlled animal models of male obesity (without additional lifestyle factors that confound human studies) are needed to establish if weight loss through diet and/or exercise interventions can improve sperm perturbations, restore embryo quality, subsequent offspring health and the possible mechanisms involved. Additional information is needed in regards to intervention combinations and amount of weight loss required to obtain desired improvements to reproductive function to direct future human population based investigations.

1.10 RESEARCH HYPOTHESIS AND AIMS

As discussed, the reversibility of paternal obesity on sperm function, embryo development and offspring health by diet and/or exercise weight loss interventions is mostly unknown. Due to the reported improvements to hormone concentrations, metabolic health and changes to epigenetic regulators in other tissues via diet and exercise interventions, this thesis will test the following overarching hypotheses;

- *Weight loss through diet and exercise in obese males will decrease adiposity and therefore improve sperm function increasing subsequent fertilisation and embryo development rates.*

- *Weight loss through diet and exercise in obese males will restore the epigenetic status of sperm and therefore improve the metabolic health and reproductive health of offspring.*

Aim 1: To establish an animal model of obesity with the addition of diet and/or exercise interventions and determine if weight loss via these methods can restore sperm function.

Aim 2: To use the same animal model of obesity plus diet and/or exercise interventions to determine if improvements to early embryo and fetal development could also be established.

These aims will determine if perturbed sperm function and early embryo development resulting from male obesity can be restored via diet and/or exercise interventions.

Aim 3: To assess the capacity of diet and/or exercise interventions in founders to improve F₁ male sperm function.

Rodent models of male obesity have shown that sperm function of F₁ males is impaired (motility, ROS and DNA damage), this aim will determine if interventions in obese fathers can restore sperm function in their male offspring.

Aim 4: To test the ability of diet and/or exercise interventions in founder males to improve F₁ female metabolic health.

Due to the reported worsened phenotype of first generation female offspring metabolic health in with founder HFD feeding. This aim will determine if interventions to obese fathers can restore metabolic health in their female offspring as they age.

Aim 5: To determine whether the microRNA profile of sperm after diet and/exercise interventions in obese males is restored; focusing on X-linked specific microRNAs to establish potential epigenetic links between paternal health and the metabolic health of their female offspring.

Aims 3, 4 and 5 will potentially will provide information regarding a potential novel intervention window for improving the metabolic health and reproductive health in subsequent generations through interventions to their father's health peri-conception.

These studies will add to the current knowledge of paternal obesity and fertility, especially addressing the knowledge gap pertaining to the capacity of adverse outcomes to sperm function, embryo development and offspring health that result from male obesity and whether restoration via diet and exercise interventions in the father is possible. These studies in rodents will provide evidence for planning and initiation of targeted human based studies.

1.11 REFERENCES

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2 Diet and exercise in an obese mouse fed a high fat diet improves metabolic health and reverses perturbed sperm function

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3 Improving metabolic health in obese male mice via diet and exercise restores embryo development and fetal growth

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3.2 LINKING TO CHAPTER 3

The data in chapter 2 has demonstrated that all diet and/or exercise interventions in obese males fed a high fat diet restored measures of sperm function, thus it was hypothesised that these diet and/or exercise interventions would also restore early embryo and fetal health which are similarly perturbed by paternal obesity. Early embryo and fetal health was therefore determined by mating obese males subjected to the same diet and exercise interventions as chapter 2 with control diet fed female mice to assess embryo development. These embryos were subsequently transferred to normal weight control diet fed mothers for development *in utero* and assessment of early pregnancy. Findings from further investigating this hypothesis are discussed in this chapter.

3.3 ABSTRACT

Paternal obesity is now clearly associated with or causal of impaired embryo and fetal development and reduced pregnancy rates in humans and rodents. This appears to be a result of reduced blastocyst potential. Whether these adverse embryo and fetal outcomes can be ameliorated by interventions to reduce paternal obesity has not been established. Here, male mice fed a high fat diet (HFD) to induce obesity were used, to determine if early embryo and fetal development is improved by interventions of diet (CD) and/or exercise to reduce adiposity and improve metabolism. Exercise and to a lesser extent CD in obese males improved embryo development rates, with increased cell to cell contacts in the compacting embryo measured by E-cadherin in exercise interventions and subsequently, increased blastocyst trophoctoderm (TE), inner cell mass (ICM) and epiblast cell numbers. Implantation rates and fetal development from resulting blastocysts were also improved by exercise in obese males. Additionally, all interventions to obese males increased fetal weight, with CD alone and exercise alone, also increasing fetal crown-rump length. Measures of embryo and fetal development correlated with paternal measures of glycaemia, insulin action and serum lipids regardless of paternal adiposity or intervention, suggesting a link between paternal metabolic health and subsequent embryo and fetal development. This is the first study to show that improvements to metabolic health of obese males through diet and exercise can improve embryo and fetal development, suggesting such interventions are likely to improve offspring health.

3.4 INTRODUCTION

Worldwide obesity is epidemic, with 200 million men and 300 million women over the age of 20 currently classified as obese (WHO, 2012). While, maternal obesity is well established to adversely affect the oocyte and negatively impact the establishment of pregnancy (Marquard et al., 2011; Pinborg et al., 2011; Veleva et al., 2008), there is now mounting evidence that paternal obesity is also implicated in gamete health and pregnancy outcomes (Bakos et al., 2011a; Hinz et al., 2010; Keltz et al., 2010). An overweight or obese male with a female partner of normal body mass index (BMI), has an increased odds ratio for a longer time to conceive, compared with couples where both are of normal weight (Nguyen et al., 2007; Ramlau-Hansen et al., 2007). Studies of couples undergoing assisted reproductive technology (ART) have established that male obesity is associated with reduced pregnancy rates and increased pregnancy loss (Bakos et al., 2011a; Hinz et al., 2010; Keltz et al., 2010). This phenomenon seems to be as a result of reduced sperm binding and fertilisation rates, as well as impaired blastocyst development (Bakos et al., 2011a; Binder et al., 2012a; Hwang et al., 2011).

Similarly, in experimental rodent models of paternal obesity where males are fed a high fat diet (HFD) to induce obesity with or without impaired glucose control, perturbed sperm function, with reduced sperm motility, increased oxidative stress and DNA damage are seen (Bakos et al., 2011b; Fernandez et al., 2011; Palmer et al., 2012). When these obese males were then mated to normal weight females, they exhibited impaired embryo development, and reduced implantation and live birth rates (Binder et al., 2012a; Ghanayem et al., 2010; Mitchell et al., 2011).

Recently it has been established that exercise and caloric restriction in obese males can reduce adiposity and improve sexual function. One study in humans examined 43 obese men who were placed

on a 14 week diet and exercise weight loss program and demonstrated improved total sperm count and morphology in those men who lost the greatest weight (Hakonsen et al., 2011). Gastric bypass surgery and weight loss in obese men have shown similar results with improvements to sex hormone profiles and sexual function (Reis et al., 2012). Additionally, diet and exercise interventions in a mouse model of male obesity induced by a HFD has recently shown that sperm function was highly correlated with their metabolic health, with normalisation of serum glucose, cholesterol, triglycerides, free fatty acids (FFA) and insulin, associated with restoration of sperm motility, morphology, oxidative stress, DNA damage and sperm binding (Palmer et al., 2012)

Thus weight loss strategies have shown promise in restoring sperm quality of obese males in both rodents and humans, but no studies to date have determined whether weight loss and improved metabolic state in obese males can reverse their associated impaired embryo quality and pregnancy outcomes. Therefore, I induced obesity in male mice with additional diet and/ or exercise interventions, previously reported to reduce adiposity, improve metabolic state and sperm function (Palmer et al., 2012) and determined if early embryo, fetal development and pregnancy rate can be restored. We hypothesise that weight loss and or an improvement to metabolic health via diet and exercise interventions in obese males will improve subsequent embryo and fetal health similar to levels of normal weight males.

3.5 METHODS

3.5.1 Ethics Statement

This study was carried out in strict accordance with the Australian code of practice for the care and use of animals for scientific purposes. The use and care of all animals used in the study was approved by the Animal Ethics Committee of The University of Adelaide.

3.5.2 Animals and Diet

Five week old male C57BL6 mice (n=40) were randomly assigned to one of two diets for an initial period of 9 weeks: 1) control diet (CD) (SF04-057; Specialty Feeds, Perth, Australia, (Table S3.1); or 2) a high fat diet (HFD) high in fat and nutrient matched (SF00-219; Specialty Feeds, Perth, Australia, Table S3.1). Diets used in the study have been previously shown to increase adiposity in male mice, without affecting glucose tolerance after 9 weeks (Bakos et al., 2010; Brake et al., 2006; Mitchell et al., 2011; Palmer et al., 2011b). After the initial feeding period, males allocated to the HFD were further allocated to one of the following interventions for a further period of 9 weeks: 1) continuation of a HFD (HH) (n=8); 2) change to a CD (HC) (n=8); 3) continuation of a HFD with exercise (HE) (n=8); 4) change to a CD with exercise (HCE) (n=8). Mice allocated to the CD during the initial feeding period were also fed a CD during the intervention period as a baseline control (CC) (n=7). These interventions have previously been shown to reduce adiposity in those obese males that undergo diet interventions and to improve metabolic parameters in those obese males that undergo exercise and or diet interventions (Palmer et al., 2012). Animals were individually housed for the entire study and fed *ad libitum*.

3.5.3 Exercise Intervention (Swimming)

The swimming exercise regimen was followed as previously described (Palmer et al., 2012). Briefly male mice were placed into tank containing warm water at a constant temperature of $32^{\circ}\text{C} \pm 1^{\circ}\text{C}$ to swim freely for the set time period. For the first 2 weeks mice swam for 3x15min periods over 7 days with one days rest in between swimming sessions (Andreazzi et al., 2009). This allowed time for the mice to become accustomed to exercise regimen and the swimming tank. For the remainder of the intervention (6 weeks) mice swam for 3x30 min training sessions each week with at least one days rest in between swimming sessions to simulate light exercise. This imposes light exercise in comparison with a moderate training program in which mice freely swam for 60 min, 5 days a week, for 18weeks (Napoli et al., 2004).

3.5.4 Body Composition

Individual body weights of the males were recorded weekly during the pre and post intervention periods. At pre intervention week 9 (14 weeks of age) and at the end of intervention week 9 (23 weeks of age) whole body composition of lean mass, bone density, adiposity were measured by a dual-emission X-ray absorptiometry machine (DEXA) (Piximus, Ge Lunar, Wisconsin, USA) as previously described (Palmer et al., 2012). Additionally, selected organs (liver, kidneys and pancreas) and reproductive tissues (gonadal fat, testes, seminal vesicles) were collected and weighed at the end of the intervention period (intervention week 9, 23 weeks of age) after males were humanely killed by cervical dislocation post cardiac puncture.

3.5.5 Serum Metabolite Analysis

Blood was sampled from the tail vein after six hours fasting, at the end of pre intervention week 9 (14 weeks of age) and post intervention week 9 (23 weeks of age), and blood glucose was measured by a glucometer (Hemocue, Angelholm, Sweden). At the end of the intervention period (intervention week 9, 23 weeks of age), a fasted blood sample was obtained via cardiac puncture under anaesthetic with 2% Isoflurane (1-chloro-2,2,2-trifluoroethylidifluoromethylether) (Veterinary Companies of Australia, Kings Park, Australia) (44 hr post swimming training for exercise intervention mice) for measurement of serum cholesterol, FFAs, triglycerides and leptin. Serum cholesterol, FFAs and triglycerides were measured by enzymatic analysis on a Hitachi 912 automated sample system, as previously described (Palmer et al., 2012) and serum leptin concentrations were determined using a mouse leptin ELISA kit (Cat# 90030) as per the manufacturer's instructions (Crystal Chem Inc, Downer Grove, USA).

3.5.6 Intraperitoneal Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

A GTT and ITT was performed at the beginning of intervention week 6 and 7 respectively (20 and 21 weeks of age respectively) after 6 h of fasting by intra-peritoneal (IP) injection of 2 g/kg of 25% D-glucose solution for GTT or during a fed state by IP injection of 0.75 IU of human insulin (Actapid®, Novo Nordisk, Bagsvaerd, Denmark) for a ITT. GTT and ITT were performed on rest days for exercise intervention mice (HE and HCE). Glucose concentrations were measured using a glucometer (Hemocue, Angelholm, Sweden) at time points 0 (pre-bolus basal), 15, 30, 60 and 120 min on whole blood collected from the tail vein through a tail cut. Data were expressed as mean blood glucose concentration per group as area under curve (AUC) for glucose and area above the curve (AAC) for insulin.

3.5.7 Embryo Collection

3-4 week old C57BL6 female mice who were fed standard chow (Table S3.1) were given an intraperitoneal (IP) injection of 5 IU Pregnant Mare's Serum Gonadotropin (PMSG) (Folligon, Invervet, Bendigo, Australia), followed 48h later with an IP injection of 5 IU human Chorionic Gonadotropin (hCG) (Pregnyl, Organon, Sydney, Australia) to induce ovulation as per (Gardner, 2004; Nagy, 2003). Following the hCG injection female mice were individually placed with a C57BL6 male between intervention weeks 7-9 (21-23 weeks of age). Each male (n=8 HH, HC, HE, HCE and n=7 CC) had the opportunity to mate with 6 super ovulated females at independent times. Successful mating was assessed the following morning by the presence of a vaginal plug. There was no significant difference between mating rates between groups. Successful mating occurred in 6 HH and HCE males and 7 CC, HC and HE males. Each individual male produced between 30 to 80 zygotes from 2-4 females (Table S3.2). Males that underwent swimming exercise (HE and HCE) were mated on alternate days to exercise and each male from all treatment groups had a least 1 days rest in between mating. At 22-24 hr post hCG female mice were humanly killed by cervical dislocation and cumulus enclosed zygotes were collected and placed in MOPS at 37°C and denuded of cumulus cells by 1 min incubation with 0.5 mg/ml hyaluronidase. Zygotes were washed twice in MOPS and once in G1 medium before culture in G1 media (Lane & Gardner, 1997).

3.5.8 Embryo Culture

Embryos were cultured in groups of 10 in 20µl drops of G1 under paraffin oil (Merek, New Jersey, USA) for 48 h at 37°C in 5% O₂, 6% CO₂ and 89% N₂. Embryos were then washed and cultured in medium G2 for a further 48 h to the blastocyst stage. On-time embryo development was assessed at 43 hr post hCG (day 2, cleavage), 67 hr post hCG (day 3, compaction), 98 hr post hCG (day 4, early blastocyst) and 115 hr post hCG (day 5, late blastocyst). All embryo culture dishes were prepared 4 hr prior to

embryo culture to allow for gassing and temperature equilibration with 24 replicate experiments performed.

3.5.9 E-cadherin in Embryos

The localisation of E-cadherin in embryos was determined by a modified immunofluorescence protocol as previously described (Harrouk et al., 2000). Briefly, 8-cell embryos were collected at 67 hr post hCG, fixed in 4% paraformaldehyde overnight and stored in 0.1M glycine. Embryos were permeabilised in 0.25% TritonX-100 (PBS-TX) for 45 min and placed into blocking solution (1:10 donkey serum) and primary antibody (1:200, Rabbit anti E-cadherin, Abcam, Cambridge, UK; ab53033) at 4°C overnight. The following day embryos were washed through PBS-TX and placed into secondary antibody (1:200, Donkey anti Rabbit Alex 488, Life Technologies, Invitrogen, Mulgrave, Australia; A-21206) at 37°C for 2 hr. Following another wash in PBS-TX embryos were placed in 0.25mg/ml of propidium iodide (PI) for 5 min to stain the nucleus. For a negative control the primary antibody was omitted from the reaction. Embryos were loaded in glycerol and allowed to settle to the bottom before been imaged blinded by the same individual using confocal microscopy. E-cadherin localisation were determined by characterising if 8-cell embryos had either 1 of 3 staining patterns observed during confocal z-sectioning ; 1) E-cadherin at cell to cell contacts (Fig 3.1A), 2) E-cadherin at both cell to cell contacts and cytoplasm (Fig 3.1B) and 3) E-cadherin only in the cytoplasm (Fig 3.1C). The numbers of embryos for each staining pattern for each treatment group were expressed as a percentage.

3.5.10 Blastocyst DNA Damage

The number of apoptotic cells in each blastocyst were determined at day 5 of embryo development (115 hr post hCG) using TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling) (In Situ Cell Death detection kit, Roche Molecular Biochemicals, Indianapolis, IN) as described previously

(Kamjoo et al., 2002). The numbers of apoptotic cells were expressed as a percentage of total cell number for each blastocyst. A positive control was used where blastocysts were incubated in the presence of DNase I (Invitrogen, Mulgrave, Australia) and a negative control where blastocysts were incubated only in the fluorescent buffer.

3.5.11 Assessment of ICM, Trophectoderm and Epiblast Cell Number (Nanog and Oct4 Staining)

To determine the number of epiblast cells within the ICM, day 5 blastocysts were further grown in the above culturing system for an additional 24 hr (Day 6, 139 hr post hCG) to allow time for epiblast cells to be detected in the ICM by Nanog and Oct4 antibodies and stained using our previously published method (Campbell et al., 2012). Briefly blastocysts were fixed in 4% paraformaldehyde overnight at 4°C and neutralised in 0.1M glycine. Blastocysts were permeabilised in PBS-TX for 15 min at room temperature (RT) and blocked overnight at 4°C in 10% normal donkey serum (Sigma, D9663). Blastocysts were then incubated in primary antibodies rabbit anti Nanog (Cozmo Bio, Tokyo, Japan; REC-RCAB0002P) (1:200) and goat anti Oct4 (Santa Cruz, California, USA; SC-8628) (1:100) for 1½ hr at 37°C. Following washing in PBS-TX blastocysts were incubated in secondary antibodies donkey anti rabbit Alexa 488 (Life Technologies, Invitrogen, Mulgrave, Australia; A-21206) (1:100) and donkey anti goat Alexa 594 (Life Technologies, Invitrogen, Mulgrave, Australia; A-11058) (1:100) for 2 hr at RT. To further determine cell nuclei blastocyst were incubated in DAPI for 2-3 min at RT before being loaded in glycerol and counted using fluorescent microscopy. Total cell number (TCN) was assessed by nuclei stained DAPI; inner cell mass (ICM) cell number nuclei also stained positive for Oct4, and epiblast cell number nuclei additionally stained positive for Nanog. Trophectoderm cell number (TE) was determined by subtracting ICM from TCN. For a negative control the primary antibodies were omitted from the reaction.

3.5.12 Embryo Transfer

Morphologically similar expanded or hatched blastocysts (115 h post HCG) were transferred into day 4 pseudo pregnant 10 week old Swiss female mice (-1 day asynchronous). Embryos from each treatment were randomly allocated to each uterine horn with each mother having six blastocysts from 2 different treatment groups (transferred to each uterine horn). Twelve to eighteen embryos were transferred into 2-3 mothers per father from 5 HE and HCE and 6 CC, HH and HC males. Mothers were maintained on standard chow (Table S3.1) until day 18 of pregnancy. On day 18 of pregnancy, mothers were humanely killed by cervical dislocation and implantations, numbers of fetuses and resorptions were determined per uterine horn for calculation of implantation rates, percentage of fetuses and percentage of fetuses per total number of implantations. Fetuses and placentas were dissected and removed of connective tissue, umbilical cords and maternal arterial space (apostrophe) for placentas. Crown rump length and weights of resultant fetuses and placentas were measured.

3.5.13 Statistical Analysis

All data were expressed as mean \pm SEM and checked for normality using a Kolmogorov-Smirnov test and equal variance using a Levene's test. All statistical analysis was performed in SPSS (SPSS Version 18, SPSS Inc., Chicago, USA). A p value <0.05 was considered to be significant.

Weight gain data, body composition and DEXA body composition in our founder males were analysed by a one way ANOVA with an LSD post hoc test.

Embryo cell development, embryo DNA damage and embryo cell number were expressed per father and analysed by a one way ANOVA with a LSD post hoc test between groups. Nanog staining was analysed by Mann-Whitney U test between groups as the data are not normally distributed.

Fetal weight and size, placental weight and size and fetal to placental weight ratios there was no interaction between litter size and fetal weights and therefore fetuses and placentas were expressed per

father and analysed by a univariate generalized linear model with a LSD post hoc test between groups. Cohort of founders was fitted as a covariate.

Implantation rates and E-cadherin staining patterns were analysed by a Chi Square between groups.

Correlations between founder's serum metabolites and embryo and fetal health were determined by a Pearson's Rho test and expressed as the correlation coefficient (CCO) along with its corresponding p value and number of observations. To determine if paternal adiposity was contributing to the correlations of serum metabolites to embryo and fetal health additional partial correlations controlling for paternal adiposity were also included.

3.6 RESULTS

3.6.1 Effect of Diet and Exercise on Embryo Development

There was no change in cleavage rates to the 2-cell stage between any of the treatment groups ($p>0.05$, Table 3.1). Males fed a HH produced a decreased number of embryos at the 8-cell/compacting stage on day 3 compared with CC ($p<0.05$, Table 3.1), with combined diet and exercise intervention (HCE) advancing development to the 8 cell/compacting stage compared with HH and HC ($p<0.05$, Table 3.1). Diet alone (HC) or exercise alone (HE) did not alter development to the 8-cell/compacting stage, compared with HH ($p>0.05$, Table 3.1). This advancement in embryo development from CC males was also evident on day 4 with reduced numbers of early blastocysts contributing to total blastocyst numbers compared with HH males ($p<0.05$, Table 3.1). All interventions (HC, HE and HCE) displayed advanced blastocyst development on day 4 with increasing numbers of hatching blastocysts compared with HH ($p<0.05$, Table 3.1) and a decrease in expanded blastocysts compared with CC ($p<0.05$, Table 3.1). By day 5 of embryo development exercise interventions (HE and HCE) maintained their increased numbers of hatching blastocysts compared with HH ($p<0.05$, Table 3.1). There was no difference between total blastocysts, expanded blastocysts or hatching blastocysts between the remaining treatment groups (HC, CC and HH) indicating a slower rate of development in embryos from HH males, rather than a failure to develop.

3.6.2 Effect of Diet and Exercise on Blastocyst Cell Numbers and DNA Damage

Blastocysts produced by males from the HH group had lower total cell numbers on day 5, compared with those of CC males ($p<0.05$, Table 3.2). All interventions (HC, HE and HCE) increased total blastocyst cell number on day 5, compared with HH ($p<0.05$, Table 3.2), restoring this to be comparable with the CC group ($p>0.05$, Table 3.2). This reduction in cell number from the HH was not as a result

from increased apoptosis as there was no difference in the number or percentage of TUNEL positive cells in the blastocyst across groups ($p>0.05$, Table 3.2).

3.6.3 Effect of Diet and Exercise on Epiblast, ICM and Trophectoderm Cell Number

Embryos were further cultured for an additional 24 hr (day 6) to allow time for epiblast cells to be detected in the ICM by Nanog and Oct4 antibodies. All interventions (HC, HE and HCE) and CC had increased cell numbers (TCN and TE) compared with HH, similar to that observed on day 5 ($p<0.05$, Table 3.2). ICM and epiblast cell numbers were increased in blastocysts from the CC males compared with HH ($p<0.05$, Table 3.2) with combined diet and exercise interventions (HCE) also having an increase ICM cell numbers compared with HH ($p<0.05$, Table 3.2). Diet alone (HC) and exercise alone (HE) resulted in ICM and epiblast cell number not different to CC ($p>0.05$, Table 3.2). There was no change in the proportion of cells or percentage of cells allocated to the trophectoderm, ICM or epiblast between any of the treatment groups ($p>0.05$, Table 3.2).

3.6.4 Effect of Diet and Exercise on Embryo Cell to Cell Contact

To further evaluate the effect of intervention treatments on rates of compaction after embryonic genome activation (after 2-cell stage) and therefore activation of the paternal contribution to the embryo, the localisation of E-cadherin (a marker of cell to cell contact) and one of the first paternally translated proteins within the developing 8-cell embryo (Fleming et al., 2001), was assessed. CC and HE males had more 8-cell embryos with E-cadherin located at cell to cell contacts, compared with the other treatment groups (HH, HC and HCE, $p<0.05$, Fig 3.2A). HCE males had more 8-cell embryos with E-cadherin located to both cell to cell contacts and cytoplasm compared with CC and HE males ($p<0.05$, Fig 3.2B), but similar numbers compared with HH or HC males ($p>0.05$, Fig 3.2B). HC males had more 8-cell embryos with E-cadherin located to the cytoplasm compared with HCE males ($p<0.05$, Fig 3.2C).

There was no change to the proportion of 8-cell embryos with E-cadherin located to the cytoplasm between the remaining treatment groups (HH, HC, HE, $p>0.05$, Fig 3.2C).

3.6.5 Effect of Diet and Exercise on Implantation and Fetal Viability

Blastocyst viability was assessed by transferring embryos to recipient mothers. Blastocysts produced from HH had reduced implantation rates compared with blastocysts from CC ($p<0.05$, Table 3.3). The combined diet and exercise intervention (HCE) was the only intervention to improve implantation rates compared with HH ($p=0.08$, Table 3.3) with diet alone (HC) and exercise alone (HE) showing no improvements. There was no difference in fetal development, as assessed by the number of viable fetuses per the total number of blastocyst transferred, between any of the treatment groups ($p>0.05$, Table 3.3). Exercise intervention alone (HE) increased the percentage of implantations that resulted in viable fetuses, compared with CC and diet intervention alone (HC) ($p<0.05$, Table 3.3), with the remaining groups (HH and HCE) showing no change. HH had decreased fetal weights, lengths and placental weight compared with CC ($p<0.05$, Table 3.3). Any form of diet and/or exercise intervention (HC, HE, HCE) increased fetal weights compared with HH (HE and HCE $p<0.05$ and HC $p=0.08$, Table 3.3); with diet alone (HC) and exercise alone (HE) additionally increasing crown-rump length compared with HH ($p<0.05$, Table 3.3). There was no change in placental weights between treatment groups (HC, HE and HCE) and HH ($p>0.05$, Table 3.3), however exercise alone did decrease placental weights compared with CC ($p=0.08$, Table 3.3). There was no change in fetal to placenta weights ratio between any of the groups ($p>0.05$, Table 3.3).

3.6.6 Associations between Paternal Metabolic and Hormonal state and Embryo and Fetal Growth and Development

Diet interventions (HC and HCE) reduced founder male body weight (HC $-2.37 \pm 0.79\text{g}$ and HCE $-3.19 \pm 0.75\text{g}$), levels of adiposity ($p < 0.01$), serum cholesterol ($p < 0.05$), serum leptin ($p < 0.05$), increased lean mass ($p < 0.05$) and improved glucose tolerance ($p < 0.05$) but maintained insulin resistance as measured by AAC compared with HH males (Table S3.3 and Table S3.4). Those receiving exercise intervention alone (HE) maintained their pre intervention body weight ($-0.28 \pm 0.75\text{g}$) and level of adiposity (Table S3.3) with serum cholesterol and insulin resistance remaining comparable to HH males ($p > 0.05$, Table S3.4) however, had a significant reduction in serum leptin concentrations ($p < 0.05$, Table S3.4). Interventions including exercise (HE and HCE) reduced fasting serum glucose (HE $p < 0.05$ and HCE $p = 0.08$) with an increased clearance of glucose after a GTT compared with HH males ($p < 0.05$, Table S3.4).

Associations with metabolic and hormonal measures in founder males with embryo and fetal outcomes were further examined to identify any potentially influential factors due to the differences in embryo and fetal health observed in the intervention combinations. Paternal serum cholesterol correlated negatively with blastocyst cell number on day 5 (CCO -0.17 , $p = 0.01$, $n = 191$) and day 6 embryos (CCO -0.52 , $p < 0.01$, $n = 216$), as well as TE cell number (CCO -0.54 , $p < 0.01$, $n = 216$), but positively with the percentage of DNA damaged cells in blastocysts (CCO 0.42 , $p = 0.01$, $n = 191$). Paternal serum FFA correlated negatively with fetal to placental weight ratio (CCO -0.30 , $p = 0.01$, $n = 64$) and percentage of ICM cells (CCO -0.50 , $p = 0.01$, $n = 210$), but positively with the ratio of epiblast cells to ICM cells (CCO 0.51 , $p < 0.01$, $n = 209$) and the percentage of TE cells (CCO 0.51 , $p < 0.01$, $n = 216$). Paternal serum triglyceride correlated negatively with both total cell number (CCO -0.44 , $p < 0.01$, $n = 216$) and TE cell numbers (CCO -0.41 , $p < 0.01$, $n = 216$). Paternal insulin sensitivity (as measured by AAC during ITT) correlated negatively with the percentage of TE cells (CCO -0.44 , $p < 0.01$, $n = 216$) and percentage of

DNA damaged cells in blastocysts (CCO -0.20, $p < 0.01$, $n = 191$), but positively with the percentage of ICM cells (CCO 0.43, $p < 0.01$, $n = 210$). Paternal fasting serum glucose correlated negatively with fetal to placental weight ratio (CCO -0.36, $p < 0.01$, $n = 64$) and positively with placental weight (CCO 0.21, $p = 0.05$, $n = 64$). Additionally paternal adiposity correlated negatively with both TCN (CCO -0.51, $p < 0.01$, $n = 216$) and TE cell number (CCO -0.49, $p < 0.01$, $n = 216$) and positively with the percentage of DNA damaged cells in blastocysts (CCO 0.31, $p = 0.03$, $n = 191$).

When controlled for paternal adiposity the majority of the above correlations still held, indicating that paternal lipid status, glycaemia and insulin sensitivity are correlated to embryo and fetal health independently of paternal adiposity (Table S3.5).

3.7 DISCUSSION

There is emerging evidence that paternal obesity and related metabolic changes are associated with reduced embryo development and pregnancy establishment in both humans and rodent models (Bakos et al., 2011a; Binder et al., 2012b; Ghanayem et al., 2010; Hinz et al., 2010; Keltz et al., 2010; Mitchell et al., 2011). Here I show for the first time, using a well characterised model of diet induced obesity in the mouse, that diet and exercise interventions in the obese father, which either reduces adiposity or improves their metabolic state at the time of conception, improves subsequent embryo, pregnancy and fetal health. Previous studies of interventions to reverse obesity and metabolic syndrome in males had focused on influences on restoration of hormone profiles, sperm function and sexual dysfunction (Bastounis et al., 1998; Hakonsen et al., 2011; Hammoud et al., 2009; Kasturi et al., 2008; Kaukua et al., 2003; Reis et al., 2012; Strain et al., 1988), but none had examined effects on embryo development and pregnancy establishment. Using diet induced obesity in male mice; I showed that the negative impact of paternal obesity on blastocyst development, blastocyst cell numbers, as well as subsequent implantation rate and fetal growth can be reversed through improvements to paternal metabolic health via diet and/or exercise. Further, the metabolic status of the father, including lipid status, glycaemia and insulin sensitivity are strongly associated with markers of blastocyst and fetal development or growth, independently of paternal adiposity or exposure to dietary or exercise interventions.

It has previously been shown and confirmed by this study that males fed a HFD produce embryos that have delayed development, with longer times to 1st, 2nd and 3rd cleavage and cavitation (Binder et al., 2012a; Binder et al., 2012b) with a reduction in the numbers of on time developing embryos at the compacting, early blastocyst and late blastocyst stage (Mitchell et al., 2011). Here I show for the first time that improvements to adiposity and metabolism via diet and/or exercise improve embryo development rates and blastocyst cell numbers in previous obese male mice. Specifically, the improved

embryo development seen by those mice that underwent interventions involving exercise (HE and HCE) was evident after activation of the embryonic genome (in the mouse at the 2-cell stage); with more embryos reaching the 8 cell/compacting stage on day 3 and similarly for blastocysts on day 4 and day 5. In humans it has been shown that embryos that have successful implantation develop to the 8 cell stage faster than those embryos that couldn't implant (Dal Canto et al., 2012) suggestive of improved implantation capability of embryos produced from exercise males.

Furthermore, we also confirmed the reductions in blastocyst cell numbers, including reduced numbers of ICM and TE cells, which have been observed from males fed a HFD (Binder et al., 2012a; Mitchell et al., 2011). All diet and exercise interventions in obese male mice, increased blastocyst total cell numbers on both day 5 and day 6 of in vitro embryo development with improvements to both TE and to a lesser extent ICM cells. The late blastocyst is made up of two main cell types: 1; trophoderm cells, which are necessary for embryo implantation and invasion and the establishment of the placenta; and 2; ICM cells, which can be split into epiblast cells that form the fetus and the primitive endoderm, that forms the extra embryonic tissue (Dard et al., 2008). Changes to the numbers of cells or the distribution of the cells within the blastocyst can adversely affect the developing fetus, for an example, a reduction in numbers of ICM cells in a maternal diabetic rat model associated with fetal growth restriction (Lea et al., 1996). Additionally in rodents blastocyst cell numbers as well as the proportion of cells within the late blastocysts have also been shown to positively correlate with implantation and pregnancy rates (Lane & Gardner, 1997). This suggests that exercise and diet intervention in obese males could potentially improve implantation and live birth rates, by restoring cell allocation and mitosis in the blastocyst.

In a clinical ART setting, obese males are associated with an increased rate of miscarriage, which may be as a result of sac only pregnancies (Bakos et al., 2011a) implying that cells within the embryo that

form the fetus (i.e. epiblast cells) are impaired or missing. Here we directly show for the first time that diet induced obesity reduces the number of epiblast cells within the developing embryo and could potentially provide an explanation for the increased miscarriage rate seen in association with obese men (Bakos et al., 2011a; Hinz et al., 2010; Keltz et al., 2010). Although diet and/or exercise interventions did not significantly improve epiblast cells number compared with HFD (HH) or CD (CC) they were still slightly increased compared to HFD mice, again suggesting likely improvements to implantation and pregnancy establishment.

Previous studies have also shown that blastocyst apoptosis is another marker of embryo quality, with increasing apoptosis associated with reduced embryo survival, implantation rates and increases in early miscarriage (Aitken et al., 2010; Bakos et al., 2008; Dada et al., 2010; Zribi et al., 2011). Interestingly, in the current study, no differences were observed in relation to the levels of blastocyst apoptosis between the diet and exercise interventions or between HFD (HH) and CD (CC). This is contradictory to those findings found in Mitchell et al (Mitchell et al., 2011), where male mice fed a HFD for a period of 8 weeks showed increased rates of apoptosis in the late blastocysts. The differences in outcomes with the current study could be strain (C57BL6 compared with C57BL6xCBAF1) and age specific (23 weeks of age compared with 13 weeks) or related to the increased length of exposure to both the CD and HFD diets in the current study versus the previous study (18 weeks compared with 8 weeks).

The current study showed for the first time that diet induced obesity resulted in changed localisation patterns of E-cadherin in compacting embryos, with more 8 cells having E-cadherin located in the cytoplasm from males fed a HFD, indicating a potential delay in establishment of the junctional communication between the blastomeres. However this potential delay was improved by exercise interventions with increased in E-cadherin observed in cell to cell contacts from these groups. The

process of compaction in the developing embryo, involves polarisation of the cells, cell flattening and junctional communication (Chen et al., 2010). E-cadherin is required in the developing embryo for the above processes, to allow compaction and cell division whilst also coordinating the cellular allocation and spatial segregation of the ICM and trophectoderm, with E-cadherin mutants unable to form a trophectoderm (Fleming et al., 2001; Kan et al., 2007; Larue et al., 1994). During embryo polarisation E-cadherin moves from the cytoplasm and becomes restricted to cell to cell contacts where it is stabilised before embryo compaction (Pey et al., 1998). It therefore appears that E-cadherin in embryos localised in the cytoplasm compared with cell to cell contacts, is indicative of a delay in this polarisation and compaction. Changes to the localisation and levels of E-cadherin in compacting embryos where males were treated with cyclophosphamide (Harrouk et al., 2000), resulted in half of all compacting embryos with little or no cell contacts, with the immune reactivity of E-cadherin reduced and a significant delay in embryo development (Harrouk et al., 2000). The improvements seen in those males that underwent exercise interventions suggests an advancement in development of compaction which may be contributing to the improvements in development and cell numbers seen in embryos derived from these males.

Previous studies in humans undergoing assisted reproductive technologies suggest that male obesity reduces implantation and therefore live birth rates (Bakos et al., 2011a; Hinz et al., 2010; Keltz et al., 2010). Consistent with results observed in a previous rodent study of male obesity and embryo quality (Binder et al., 2012a; Mitchell et al., 2011), the current study confirmed that males fed a high fat diet for the entire study had reduced implantation rates assessed at day 18 of gestation after embryo transfer, compared with those fed a CD. Interestingly, only the combined diet and exercise intervention (HCE) group improved implantation rates compared with males fed a HFD for the entire study. However it should be mentioned that morphological similar blastocysts from each group were used in embryo transfers which can predict implantation rates (Dal Canto et al., 2012) and suggests that evaluation of a

combined approach to weight loss in a clinical setting to evaluate the impact on implantation and therefore live birth rates is warranted.

Fetal development and size are not only determinants of a healthy pregnancy but can also pre-determine the likelihood of developing adult chronic disease (Barker, 2004; Johnson & Schoeni, 2011). Paternal exposure to high levels of x-rays as well as fathers' occupation is associated with small for gestational age babies independently of other confounding factors (Shea et al., 1997; Shea & Little, 1997), showing that paternal health at the time of conception can influence fetal health outcomes. Consistent with previous studies in rodents (Binder et al., 2012a; Ng et al., 2010) our HFD fed mice (HH) produced smaller fetuses compared with those fed a CD (CC), which has been linked with impaired metabolic and reproductive health in subsequent offspring (Fullston et al., 2012; Ng et al., 2010). Interestingly, all interventions (HC, HE, HCE) increased fetal weights compared to males fed a HFD (HH) with diet alone (HC) and exercise alone (HE) additionally increasing fetal lengths. This suggests that offspring born from obese fathers that underwent diet and/or exercise interventions may have a reduced susceptibility to develop adult chronic diseases.

It is becoming increasingly evident that at least some changes to embryo and fetal health must be resultant from the sperm health at the time of conception. Previous studies have shown that poor sperm quality including increased levels of DNA damage and/or reactive oxygen species are associated with reduced fertilisation, impaired embryonic development and increased pregnancy loss (Aitken et al., 2010; Bakos et al., 2008; Dada et al., 2010; Gharagozloo & Aitken, 2011; Kumar et al., 2012; Tunc et al., 2010; Zribi et al., 2011). Increased levels of DNA damage and ROS in sperm are commonly found in obese males (Du Plessis et al., 2010; Hammoud et al., 2008; Macdonald et al., 2010). Using this same model it has previously been shown that diet and exercise interventions reduced both DNA damage and

ROS levels in sperm of obese male mice (Palmer et al., 2012). The improved embryo and fetal health seen in the current study may therefore be as a result of improvements to sperm DNA damage and/or ROS levels in sperm. In humans, diet and exercise interventions as well as gastric bypass surgeries have been shown to improve other sperm measures such as count, motility as well as sexual function in previously overweight and obese men (Hakonsen et al., 2011; Reis et al., 2012). Further, it has been shown that the proteomic, microRNA and methylome content of sperm including the RNA content of testes from obese rodents are also altered (Daxinger & Whitelaw, 2012; Fullston et al., 2013; Ghanayem et al., 2010; Kriegel et al., 2009; Palmer et al., 2011a; Youngson & Whitelaw, 2011) with sperm molecular signatures previously been shown to impact embryo quality (Filkowski et al., 2010; Yan et al., 2008). This may be contributing to the impaired sperm and embryo development seen in obese men. It is therefore possible that improvements to embryo and fetal health seen in the current study are resultant from additional improvements to sperm epigenetic molecular structure including changes to histone methylation and microRNA content. The specific types of molecular signatures currently altered in sperm as a result of obesity and then restored by diet and exercise interventions are currently unknown. However, a recent study in humans showed that microRNAs in serum previously altered by obesity could be restored through weight loss (Ortega et al., 2013), suggesting that a similar restoration could likely occur in sperm and explain the improvements in our study.

As seen in our previous study (Palmer et al., 2012) mice that underwent diet interventions (HC and HCE) had reduced levels of adiposity, improved serum cholesterol, FFAs, glucose tolerance and additionally serum leptin concentrations. Those receiving exercise intervention only (HE) were still obese maintaining their pre intervention level of adiposity, serum cholesterol, and impaired response to insulin, however had significant reductions in serum leptin concentrations which are consistent to findings found previously in obese males (Esteghamati et al., 2010; Villareal et al., 2011). Interventions including exercise (HE and HCE) reduced fasting serum glucose with an increased clearance of glucose

after a GTT. This is likely as a result from increased glucose utilisation by skeletal muscle (reviewed by Ivy, 1997). Using association analysis between the father's body composition and metabolic parameters, the data in this study suggests that the adverse embryo and fetal health due to diet induced obesity may not only be resultant from increased adiposity but additionally whole body metabolic changes. Irrespective of father's treatment or adiposity, carbohydrate and lipid profiles showed the strongest correlations to embryo and fetal health, with reduced cholesterol, and FFAs associated with improved blastocyst cell numbers and reduced cellular apoptosis. Additionally, glucose and insulin metabolism were also found to be associated with improved embryo cell numbers and fetal and placental weight ratios.

Due to lack of cytoplasmic scavenging enzymes and high concentrations of polyunsaturated fatty acids in their plasma membrane, sperm are highly susceptible to oxidative stress and damage (Aitken & Baker, 2002; Aitken & Curry, 2011; Alvarez & Storey, 1995). Exposure to increasing concentrations of both cholesterol and fatty acids in human and animal sperm in vitro has previously been shown to cause increased mitochondrial ROS (Koppers et al., 2010) reduced sperm motility, capacitation and fertilisation (Saez et al., 2011; Saez Lancellotti et al., 2010). In particular, cholesterol is found in the sperm membrane and its concentrations within the membrane affects fluidity and helps determine motility, capacitation and acrosome reaction (Haidl & Opper, 1997; Jones, 1998), all important processes required for successful fertilisation (Johnson & Everitt, 2000). Hypercholesterolemia in rabbits showed that the transport ion channels were open in the epididymis between the circulating lipids and the sperm micro environment (Koppers et al., 2010) and that the sperm from these rabbits displayed reduced motility and capacitation likely caused from increasing sperm ROS. It is therefore plausible that high circulating plasma cholesterol, triglycerides and FFAs as seen in male obesity cause changes to sperm membrane dynamics and directly alter the fluidity of the plasma membrane allowing the sperm to be more susceptible to ROS and DNA damage. Increased levels of ROS have been shown to change the

global methylation profile of sperm (Tunc & Tremellen, 2009), with hypomethylation of imprinted genes and repeat elements in sperm linked with reduced pregnancy success and increased sperm DNA damage (El Hajj et al., 2011; Minor et al., 2011; Nanassy & Carrell, 2011; Tunc & Tremellen, 2009). It is therefore, possible that elevated paternal plasma glucose and lipids may in this way modifies the epigenetic profile of sperm, which is then passed onto the newly fertilised embryo, causing a delay in embryo development via alterations to paternal chromatin remodelling and indirectly reducing embryo cell numbers and implantation rates.

This is the first study to show that the impaired embryo and fetal development commonly seen in obese males can be reversed by improving their metabolic profile via exercise and diet. This study also provides the first direct evidence that the metabolic profile of obese fathers maybe a better indicator for determining the health of the resultant embryo and fetus than adiposity alone. Therefore, this study shows that exercise and diet interventions could be a combined approach to target sub fertility in overweight and obese men by improving embryo development and therefore subsequent pregnancy health. Future studies in the human are needed to determine if similar improvements can occur.

3.8 TABLES

Table 3.1: The effect of diet and exercise of obese males on embryo development

Diet/Intervention	CC	HH	HC	HE	HCE
Day 2					
Cleavage (%)	88.6 ± 5.3	88.9 ± 4.7	89.2 ± 2.5	90.3 ± 3.7	94.3 ± 6.2
Day 3					
8 cell/compacting (%)	71.4 ± 5.2 ^a	54.2 ± 5.5 ^b	58.2 ± 5.4 ^b	64.3 ± 4.9 ^{ab}	72.0 ± 5.6 ^a
Compacting (%)	14.7 ± 3.7 ^{ab}	15.7 ± 3.9 ^{ab}	9.6 ± 3.7 ^a	8.4 ± 3.5 ^a	22.2 ± 4.0 ^b
Day 4					
Total blastocyst (%)	75.5 ± 5.0	74.1 ± 5.3	74.7 ± 5.1	82.2 ± 4.7	86.2 ± 5.4
Early blastocyst (%)	10.3 ± 3.6 ^a	20.7 ± 4.1 ^b	13.8 ± 3.8 ^{ab}	16.1 ± 3.0 ^{ab}	12.2 ± 3.5 ^{ab}
Expanded blastocyst (%)	27.3 ± 3.1 ^a	21.3 ± 3.2 ^{ab}	14.0 ± 3.1 ^b	13.9 ± 2.9 ^b	14.4 ± 3.3 ^b
Hatching blastocyst (%)	45.3 ± 5.0 ^{ab}	34.3 ± 5.3 ^b	47.9 ± 5.1 ^{ac}	53.0 ± 4.7 ^{ac}	59.2 ± 5.4 ^c
Day 5					
Total blastocyst (%)	86.4 ± 4.5	83.1 ± 4.7	83.2 ± 4.5	84.1 ± 4.2	87.5 ± 4.8
Expanded blastocyst (%)	19.6 ± 3.6	21.1 ± 3.8	16.5 ± 3.7	10.9 ± 3.4	12.3 ± 3.9
Hatching blastocyst (%)	65.2 ± 5.0 ^{ab}	55.4 ± 5.3 ^a	61.9 ± 4.9 ^{ab}	72.4 ± 4.7 ^b	74.5 ± 5.6 ^b

Embryo development is expressed as percentage of embryos ± SEM at each stage of development per father. Data represents 6 HH and HCE males and 7 CC, HC and HCE males which is representative of 300 embryos per treatment group (for exact breakdown of embryos per father see supplementary table 2). On day 4 total blastocysts is made up of early, expanded and hatching blastocysts, while on day 5 of on time embryo development total blastocyst is made up of a combination of expanded and hatching blastocysts. Different letters denote significance at $p < 0.05$.

Table 3.2: The effect of diet and exercise of obese males on blastocyst development

Diet/Intervention	CC	HH	HC	HE	HCE
Day 5					
Total cell number	76.7 ± 3.8 ^a	64.3 ± 3.6 ^b	75.3 ± 4.2 ^a	74.9 ± 3.5 ^a	86.6 ± 5.7 ^a
TUNEL positive cells	3.73 ± 1.10	4.54 ± 1.00	3.50 ± 0.87	3.76 ± 0.87	3.59 ± 0.50
DNA damage (%)	4.86 ± 1.38	7.03 ± 1.60	4.65 ± 1.15	5.01 ± 1.12	4.15 ± 0.51
Day 6					
Total cell number (TCN)	98.3 ± 6.3 ^a	84.0 ± 7.6 ^b	100.0 ± 3.7 ^a	96.3 ± 3.5 ^a	99.7 ± 6.2 ^a
Trophectoderm cell number	83.4 ± 5.5 ^a	72.1 ± 7.3 ^b	86.6 ± 1.3 ^a	83.2 ± 3.7 ^a	85.6 ± 5.3 ^a
ICM cell number	15.0 ± 1.29 ^a	12.0 ± 1.04 ^b	12.9 ± 1.05 ^{ab}	13.6 ± 0.53 ^{ab}	14.2 ± 1.04 ^a
Epiblast cell number	7.2 ± 0.71 ^a	5.7 ± 0.80 ^b	6.2 ± 0.73 ^{ab}	6.2 ± 0.45 ^{ab}	6.6 ± 0.84 ^{ab}
Trophectoderm (% of TCN)	84.7 ± 0.96	85.4 ± 1.48	86.6 ± 0.78	85.6 ± 0.95	85.5 ± 0.64
ICM (% of TCN)	15.3 ± 0.96	14.8 ± 1.41	13.4 ± 1.39	14.4 ± 0.95	14.5 ± 0.64
Epiblast (% of ICM)	46.2 ± 4.50	46.7 ± 5.99	47.1 ± 3.63	46.6 ± 3.90	45.7 ± 3.37

Data are expressed as mean ± SEM per father. Data represents 6 HH and HCE males and 7 CC, HC and HCE males which is representative of 5-8 embryos per father per group. Different letters denote significance at $p < 0.05$.

Table 3.3: The effect of diet and exercise of obese males on subsequent implantation and fetal development

Diet/Intervention	CC	HH	HC	HE	HCE
Implantation (%)	98.5 ^a	83.3 ^b	85.9 ^{bc}	84.7 ^{bc}	93.1 ^{ac[^]}
Fetal (%)	15.3	23.6	14.4	26.4	16.0
Fetal/Implantation (%)	14.5 ^a	24.2 ^{ab}	14.6 ^a	27.7 ^b	15.9 ^{ab}
Fetal weight (mg)	773 ± 31 ^a	701 ± 28 ^b	776 ± 42 ^{a[^]}	816 ± 38 ^a	818 ± 31 ^a
Crown-Rump length (mm)	18.4 ± 0.4 ^a	17.4 ± 0.3 ^b	18.6 ± 0.5 ^a	18.5 ± 0.3 ^a	18.0 ± 0.4 ^{ab}
Placental weight (mg)	113 ± 3 ^a	102 ± 6 ^b	109 ± 8 ^{ab}	104 ± 5 ^{b*}	102 ± 10 ^{ab}
Fetal : placenta ratio	6.8 ± 0.5	7.0 ± 0.5	7.3 ± 0.3	7.7 ± 0.3	7.4 ± 0.2

Data are expressed as mean ± SEM per father. Implantation and fetal development data represents 5 HE and HCE and 6 CC, HH and HC males which is representative of 12-18 embryos transferred per father for each group. Fetal and placenta data represents 5 HE and HCE and 6 CC, HH and HC males which is representative of at least 10 fetuses per treatment group (for exact breakdown of number of fetuses per father see supplementary table 2). Different letters denote significance at $p < 0.05$. [^]different to HH at $p = 0.08$ and *different to CC at $p = 0.08$. Implantation (%) refers to the percentage of blastocyst that implanted into the uterus resultant from total number of embryos transferred determine by fetal numbers and resorptions. Fetal (%) refers to the percentage of embryos that formed fetus resultant for the total number of embryos transferred. Fetal/implantation (%) refers to the percentage of fetuses that formed from the total number of successful implantations.

3.9 FIGURES

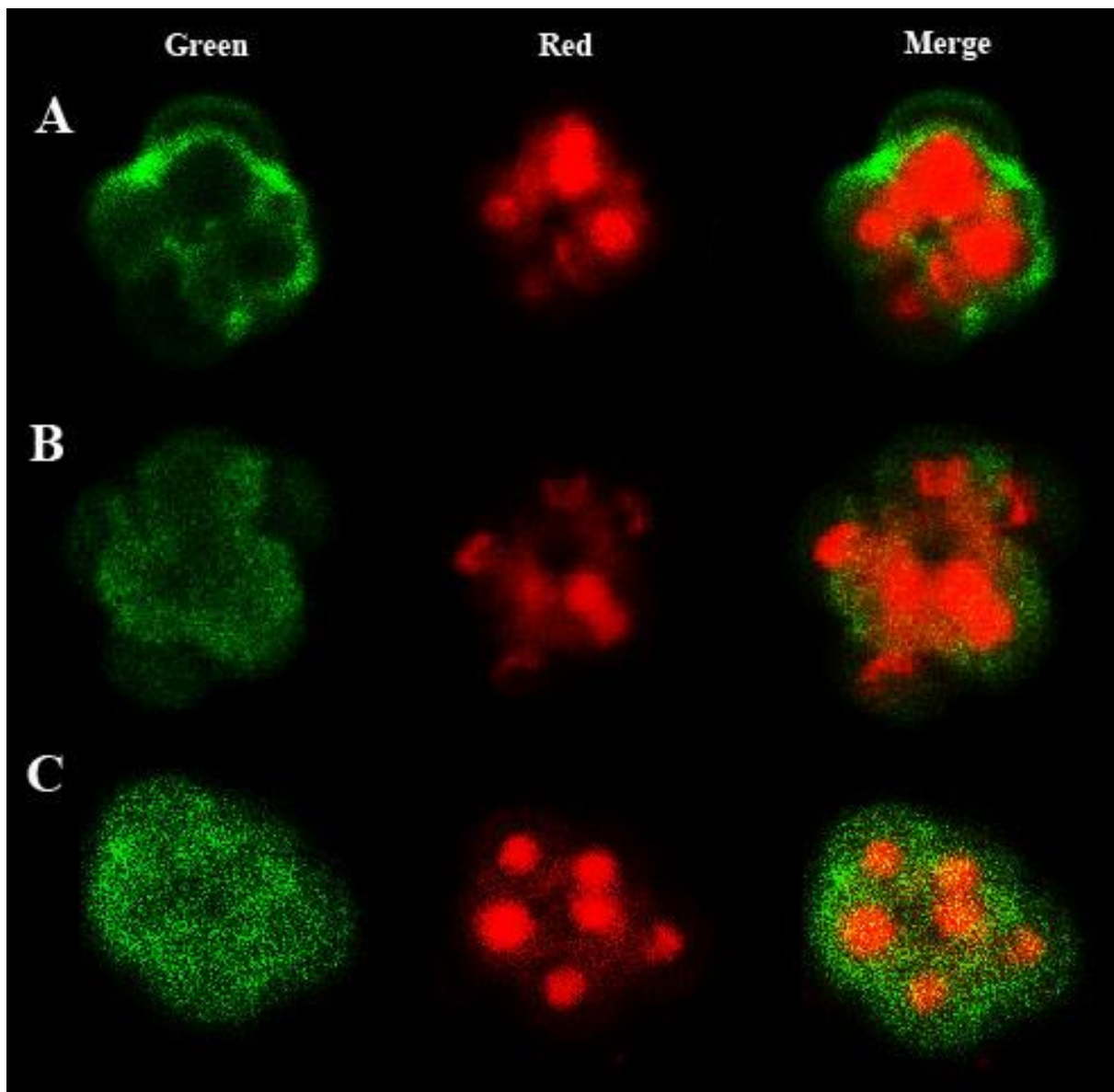


Figure 3.1: E-cadherin staining patterns

A E-cadherin localisation to cell to cell contacts. **B** E-cadherin localisation to both cell to cell contacts and cytoplasm. **C** E-cadherin localisation to the cytoplasm. Green images represent E-cadherin staining, red images represent nuclear staining and merge images represent a compression of both. Pictures are representative of a single z-section through embryos.

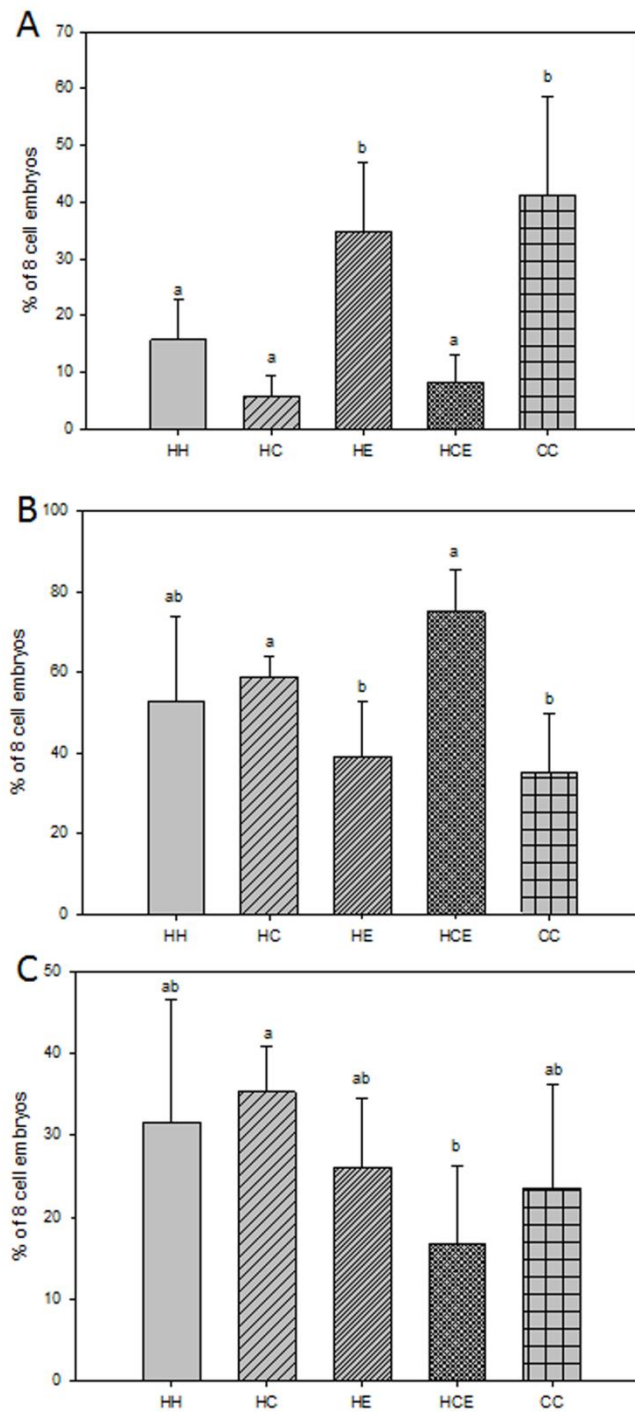


Figure 3.2: The effect of diet and exercise of obese males on e-cadherin staining patterns in compacting embryos

A The percentage of 8 cell embryos with E-cadherin localised to cell to cell contacts. **B** The percentage of 8 cell embryos with E-cadherin localised to both cell to cell contacts and cytoplasm. **C** The percentage of 8 cell embryos with E-cadherin localised to the cytoplasm. Data represents 3 HH, HCE and 4 CC, HC and HE males which is representative of 3-7 embryos per father per group.

3.10 SUPPLEMENTARY TABLES

Table S3.1: Composition of animal diets

Ingredients	CD (SF04-057) Control Diet	HFD (SF00-219) Harlan Teklad TD88137 Equival	Standard Chow (Irradiated Rat and Mouse Diet)
Sucrose (g/100g)	34.1	34.1	-
Casein (Acid) (g/100g)	19.5	19.5	-
Canola Oil (g/100g)	6.0	-	-
Clarified Butter (g/100g)	-	21.0	-
Cellulose (g/100g)	5.0	5.0	-
Wheat starch (g/100g)	30.5	15.5	-
Minerals (g/100g)	4.9	4.9	-
Digestible energy (MJ/kg)	16.1	19.4	14.0
Digestible energy from lipids (%)	21.0	40.0	4.8
Digestible energy form protein (%)	14.0	17.0	20.0
Digestible energy from carbohydrates (%)	65.0	43.0	74.4

CD = Control diet and HFD = High fat diet. Standard Chow is the standard mouse food supplied by the University of Adelaide's Animal House

Table S3.2: Numbers of embryos and pups derived from each father

Father ID	Group	Number of successful matting's	Number of embryos derived from each super ovulated mother	Number of pups derived from each father
1	HH	4	35 11 18 37	2
2	HH	2	26 15	2
3	HH	2	25 5	4
4	HH	3	18 36 13	2
5	HH	2	56 8	2
6	HH	2	39 16	1
7	HC	3	25 21 25	2
8	HC	2	30 8	1
9	HC	2	6 34	3
10	HC	3	6 22 27	3
11	HC	2	22 19	1
12	HC	2	38 6	0
13	HC	2	23 6	1
14	HE	2	31 36	2
15	HE	3	42 24 19	4
16	HE	3	21 5 28	3
17	HE	2	23 32	2
18	HE	2	35 26	0

19	HE	2	17 18	0
20	HE	2	37 4	4
21	HCE	2	27 7	0
22	HCE	4	23 5 21 27	4
23	HCE	2	18 14	2
24	HCE	3	37 12 18	1
25	HCE	2	37 5	3
26	HCE	2	6 37	1
27	CC	3	15 16 27	2
28	CC	3	19 4 9	2
29	CC	2	15 14	2
30	CC	2	25 7	1
31	CC	3	25 10 30	2
32	CC	3	5 29 14	1
33	CC	2	12 17	0

Table S3.3: The effect of diet and exercise on founder male body composition after intervention

Diet/Intervention	CC	HH	HC	HE	HCE
Pre Intervention					
Weight (g)	26.6 ± 0.5 ^a	32.7 ± 0.9 ^b	32.3 ± 1.8 ^b	31.8 ± 1.2 ^b	32.4 ± 1.5 ^b
Total Adiposity #	14.6 ± 0.9 ^a	25.4 ± 1.7 ^b	24.8 ± 2.8 ^b	24.5 ± 1.6 ^b	25.3 ± 1.5 ^b
Post Intervention					
Weight (g)	29.9 ± 1.3 ^a	35.1 ± 1.3 ^b	30.2 ± 1.2 ^a	31.6 ± 1.2 ^a	29.3 ± 1.2 ^a
% of body weight #					
Total Adiposity	16.7 ± 1.7 ^a	28.0 ± 1.7 ^b	18.1 ± 1.6 ^a	22.3 ± 1.6 ^c	16.8 ± 1.6 ^a
Lean Mass	77.2 ± 2.0 ^a	69.0 ± 2.0 ^b	77.7 ± 1.8 ^a	73.9 ± 1.8 ^{a*}	79.9 ± 1.8 ^a
Total Bone	1.33 ± 0.05 ^a	1.16 ± 0.05 ^{bc}	1.26 ± 0.04 ^{ab}	1.13 ± 0.04 ^c	1.32 ± 0.04 ^a
Adiposity					
Gonadal	2.50 ± 0.36 ^a	4.66 ± 0.36 ^b	2.90 ± 0.34 ^a	3.82 ± 0.33 ^{b^}	2.44 ± 0.33 ^a
Organs					
Liver	4.19 ± 0.30	4.09 ± 0.30	4.27 ± 0.29	3.64 ± 0.28	3.77 ± 0.28
Pancreas	0.48 ± 0.04	0.45 ± 0.04	0.45 ± 0.03	0.50 ± 0.03	0.47 ± 0.03
Kidneys	1.36 ± 0.06 ^{ab}	1.27 ± 0.06 ^a	1.40 ± 0.06 ^{ab}	1.26 ± 0.06 ^a	1.45 ± 0.06 ^b
Reproductive Organs					
Testes	0.55 ± 0.04	0.49 ± 0.04	0.55 ± 0.03	0.51 ± 0.03	0.57 ± 0.03
Seminal Vesicles	1.30 ± 0.10	1.13 ± 0.11	1.18 ± 0.10	1.20 ± 0.10	1.32 ± 0.10

Data are expressed as mean ± SEM per male. # measured by DEXA. Adiposity and organs data are representative of 7 CC and HCE males and 8 HH, HC and HE males. Different letters denote significance at $p < 0.05$. [^]Different to HC at $p = 0.07$. ^{*}Different to HH at $p = 0.08$.

Table S3.4: The effect of diet and exercise on founder male serum metabolites after intervention

Diet/Intervention	CC	HH	HC	HE	HCE
Glucose (mmol/L ⁻¹)	10.2 ± 0.6 ^{ab}	10.7 ± 0.6 ^a	9.7 ± 0.5 ^{ab}	8.8 ± 0.05 ^b	9.4 ± 0.5 ^{b[^]}
Cholesterol (mmol/L ⁻¹)	3.12 ± 0.4 ^a	4.42 ± 0.4 ^b	3.10 ± 0.4 ^a	4.01 ± 0.4 ^{b[*]}	2.65 ± 0.4 ^a
FFA (mmol/L ⁻¹)	0.92 ± 0.09	0.88 ± 0.09	0.84 ± 0.08	0.87 ± 0.08	0.81 ± 0.09
Triglycerides (mmol/L ⁻¹)	0.65 ± 0.05	0.67 ± 0.05	0.58 ± 0.04	0.68 ± 0.05	0.57 ± 0.04
Leptin (ng/mL ⁻¹)	3.4 ± 1.5 ^a	16.3 ± 1.4 ^b	5.3 ± 1.0 ^a	5.2 ± 1.5 ^a	3.9 ± 1.6 ^b
Glucose (AUC)	1827 ± 137 ^{ab}	2102 ± 138 ^b	1680 ± 129 ^a	1640 ± 128 ^a	1770 ± 129 ^{a[^]}
Insulin (AAC)	140 ± 13.8 ^a	100 ± 13.7 ^{bc}	116 ± 12.9 ^{ab}	80 ± 12.8 ^c	112 ± 12.7 ^{abc}

Data are expressed as mean ± SEM per male. Serum glucose, cholesterol, FFA, triglyceride, glucose (AUC) and insulin (AAC) concentrations are representative of 7 CC and HCE males and 8 HH, HC and HE males. For serum leptin concentrations 4 males per treatment group were measured. Different letters denote significance at p<0.05. [^]different to HH at p=0.08. ^{*}different to CC and HC at p=0.07.

Table S3.5: Correlations between founder metabolite concentrations and blastocyst and fetal health independent of founder adiposity

Founder Metabolite	Glucose (mmol/L⁻¹)	Cholesterol (mmol/L⁻¹)	Triglycerides (mmol/L⁻¹)*	FFA (mmol/L⁻¹)	Insulin (AAC)*
DNA damage cell in blastocyst		0.19, p<0.01			-0.19, p<0.01
Blastocyst trophoctoderm cell number		-0.35, p=0.02			-0.42, p<0.01
Blastocyst inner cell mass cell number			-0.24, p<0.01		0.47, p<0.01
Fetal to placental weight ratio	-0.42, p<0.01			-0.31, p<0.01	
Fetal placental weight	0.23, p=0.04				
Fetal length	-0.28, p=0.05				

Data are expressed at Pearson Correlation coefficient with corresponding p value. n=216 observations for embryo correlations and n=64 observations for fetal and placental correlations.

*Correlations refer to percentage of cells within blastocyst not total cell numbers.

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3.12 CHAPTER 3 PUBLISHED PAPER

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Improving Metabolic Health in Obese Male Mice via Diet and Exercise Restores Embryo Development and Fetal Growth

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Abstract

Paternal obesity is now clearly associated with or causal of impaired embryo and fetal development and reduced pregnancy rates in humans and rodents. This appears to be a result of reduced blastocyst potential. Whether these adverse embryo and fetal outcomes can be ameliorated by interventions to reduce paternal obesity has not been established. Here, male mice fed a high fat diet (HFD) to induce obesity were used, to determine if early embryo and fetal development is improved by interventions of diet (CD) and/or exercise to reduce adiposity and improve metabolism. Exercise and to a lesser extent CD in obese males improved embryo development rates, with increased cell to cell contacts in the compacting embryo measured by E-cadherin in exercise interventions and subsequently, increased blastocyst trophoblast (TE), inner cell mass (ICM) and epiblast cell numbers. Implantation rates and fetal development from resulting blastocysts were also improved by exercise in obese males. Additionally, all interventions to obese males increased fetal weight, with CD alone and exercise alone, also increasing fetal crown-rump length. Measures of embryo and fetal development correlated with paternal measures of glycaemia, insulin action and serum lipids regardless of paternal adiposity or intervention, suggesting a link between paternal metabolic health and subsequent embryo and fetal development. This is the first study to show that improvements to metabolic health of obese males through diet and exercise can improve embryo and fetal development, suggesting such interventions are likely to improve offspring health.

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Introduction

Worldwide obesity is epidemic, with 200 million men and 300 million women over the age of 20 currently classified as obese [1]. While, maternal obesity is well established to adversely affect the oocyte and negatively impact the establishment of pregnancy [2–4], there is now mounting evidence that paternal obesity is also implicated in gamete health and pregnancy outcomes [5–7]. An overweight or obese male with a female partner of normal body mass index (BMI), has an increased odds ratio for a longer time to conceive, compared with couples where both are of normal weight [8,9]. Studies of couples undergoing assisted reproductive technology (ART) have established that male obesity is associated with reduced pregnancy rates and increased pregnancy loss [5–7]. This phenomenon seems to be as a result of reduced sperm binding and fertilisation rates, as well as impaired blastocyst development [5,10,11].

Similarly, in experimental rodent models of paternal obesity where males are fed a high fat diet (HFD) to induce obesity with or without impaired glucose control, perturbed sperm function, with reduced sperm motility, increased oxidative stress and DNA

damage are seen [12–14]. When these obese males were then mated to normal weight females, they exhibited impaired embryo development, and reduced implantation and live birth rates [11,15,16].

Recently it has been established that exercise and caloric restriction in obese males can reduce adiposity and improve sexual function. One study in humans examined 45 obese men who were placed on a 14 week diet and exercise weight loss program and demonstrated improved total sperm count and morphology in those men who lost the greatest weight [17]. Gastric bypass surgery and weight loss in obese men have shown similar results with improvements to sex hormone profiles and sexual function [18]. Additionally, diet and exercise interventions in a mouse model of male obesity induced by a HFD has recently shown that sperm function was highly correlated with their metabolic health, with normalisation of serum glucose, cholesterol, triglycerides, free fatty acids (FFA) and insulin, associated with restoration of sperm motility, morphology, oxidative stress, DNA damage and sperm binding [13].

Thus weight loss strategies have shown promise in restoring sperm quality of obese males in both rodents and humans, but no

studies to date have determined whether weight loss and improved metabolic state in obese males can reverse their associated impaired embryo health and pregnancy outcomes. We have therefore induced obesity in male mice with additional diet and/or exercise interventions, previously reported to reduce adiposity, improve metabolic state and sperm function [13] and determined if early embryo, fetal development and pregnancy rate can be restored. We hypothesise, that weight loss and/or an improvement to metabolic health via diet and exercise interventions in obese males will improve subsequent embryo and fetal health similar to levels of normal weight males.

Methods

Ethics Statement

This study was carried out in strict accordance with the Australian code of practice for the care and use of animals for scientific purposes. The use and care of all animals used in the study was approved by the Animal Ethics Committee of The University of Adelaide.

Animals and Diet

Five week old male C57BL6 mice ($n=40$) were randomly assigned to one of two diets for an initial period of 9 weeks: 1) control diet (CD) (SF04-057; Specialty Feeds, Perth, Australia, Table S1); or 2) a high fat diet (HFD) high in fat and nutrient matched (SF00-219; Specialty Feeds, Perth, Australia, Table S1). Diets used in the study have been previously shown to increase adiposity in male mice, without affecting glucose tolerance after 9 weeks [15,19–21]. After the initial feeding period, males allocated to the HFD were further allocated to one of the following interventions for a further period of 9 weeks: 1) continuation of a HFD (HH) ($n=8$); 2) change to a CD (HC) ($n=8$); 3) continuation of a HFD with exercise (HE) ($n=8$); 4) change to a CD with exercise (HCE) ($n=8$). Mice allocated to the CD during the initial feeding period were also fed a CD during the intervention period as a baseline control (CC) ($n=7$). These interventions have previously been shown to reduce adiposity in those obese males that undergo diet interventions and to improve metabolic parameters in those obese males that undergo exercise and/or diet interventions [13]. Animals were individually housed for the entire study and fed ad lib.

Exercise Intervention (Swimming)

The swimming exercise regime was followed as previously described [13]. Briefly male mice were placed into tank containing warm water at a constant temperature of $32^{\circ}\text{C}\pm 1^{\circ}\text{C}$ to swim freely for the set time period. For the first 2 weeks mice swam for 3×15 min periods over 7 days with one days rest in between swimming sessions [22]. This allowed time for the mice to become accustomed to exercise regime and the swimming tank. For the remainder of the intervention (6 weeks) mice swam for 3×30 min training sessions each week with at least one days rest in between swimming sessions to simulate light exercise. This imposes light exercise in comparison with a moderate training program in which mice freely swam for 60 min, 5 days a week, for 18 weeks [23].

Body Composition

Individual body weights of the males were recorded weekly during the pre and post intervention periods. At pre intervention week 9 (14 weeks of age) and at the end of intervention week 9 (23 weeks of age) whole body composition of lean mass, bone density, adiposity were measured by a dual-emission X-ray absorptiometry machine (DEXA) (Picimus, Ge Lunar, Wisconsin,

USA) as previously described [13]. Additionally, selected organs (liver, kidneys and pancreas) and reproductive tissues (gonadal fat, testes, seminal vesicles) were collected and weighed at the end of the intervention period (intervention week 9, 23 weeks of age) after males were humanely killed by cervical dislocation post cardiac puncture.

Serum Metabolite Analysis

Blood was sampled from the tail vein after six hours fasting, at the end of pre intervention week 9 (14 weeks of age) and post intervention week 9 (23 weeks of age), and blood glucose was measured by a glucometer (Hemocue, Angelholm, Sweden). At the end of the intervention period (intervention week 9, 23 weeks of age), a fasted blood sample was obtained via cardiac puncture under anaesthetic with 2% Isoflurane (1-chloro-2,2,2-trifluoroethylidifluoromethyl ether) (Veterinary Companies of Australia, Kings Park, Australia) (44 hr post swimming training for exercise intervention mice) for measurement of serum cholesterol, FFAs, triglycerides and leptin. Serum cholesterol, FFAs and triglycerides were measured by enzymatic analysis on a Hitachi 912 automated sample system, as previously described [13] and serum leptin levels were determined using a mouse leptin ELISA kit (Cat# 90030) as per the manufacturer's instructions (Crystal Chem Inc, Downer Grove, USA).

Intraperitoneal Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

A GTT and ITT was performed at the beginning of intervention week 6 and 7 respectively (20 and 21 weeks of age respectively) after 6 h of fasting by intra-peritoneal (IP) injection of 2 g/kg of 25% D-glucose solution for GTT or during a fed state by IP injection of 0.75 IU of human insulin (Actapid[®], Novo Nordisk, Bagsvaerd, Denmark) for a ITT. GTT and ITT were performed on rest days for exercise intervention mice (HE and HCE). Glucose concentrations were measured using a glucometer (Hemocue, Angelholm, Sweden) at time points 0 (pre-bolus basal), 15, 30, 60 and 120 min on whole blood collected from the tail vein through a tail cut. Data were expressed as mean blood glucose concentration per group as area under curve (AUC) for glucose and area above the curve (AAC) for insulin.

Embryo Collection

3–4 week old C57BL6 female mice who were fed standard chow (Table S1) were given an intraperitoneal (IP) injection of 5 IU Pregnant Mare's Serum Gonadotropin (PMSG) (Folligon, Intervet, Bendigo, Australia), followed 48 h later with an IP injection of 5 IU human Chorionic Gonadotropin (hCG) (Pregnyl, Organon, Sydney, Australia) to induce ovulation as per [24,25]. Following the hCG injection female mice were individually placed with a C57BL6 male between intervention weeks 7–9 (21–23 weeks of age). Each male ($n=8$ HH, HC, HE, HCE and $n=7$ CC) had the opportunity to mate with 6 super ovulated females at independent times. Successful mating was assessed the following morning by the presence of a vaginal plug. There was no significant difference between mating rates between groups. Successful mating occurred in 6 HH and HCE males and 7 CC, HC and HE males. Each individual male produced between 30 to 80 zygotes from 2–4 females (Table S2). Males that under went swimming exercise (HE and HCE) were mated on alternate days to exercise and each male from all treatment groups had a least 1 days rest in between mating. At 22–24 h post hCG female mice were humanely killed by cervical dislocation and, cumulus enclosed zygotes were collected and placed in MOPS at 37°C and denuded

of cumulus cells by 1 min incubation with 0.5 mg/ml hyaluronidase. Zygotes were washed twice in MOPs and once in G1 medium before culture in G1 media [26].

Embryo Culture

Embryos were cultured in groups of 10 in 20 μ l drops of G1 under paraffin oil (Merek, New Jersey, USA) for 48 h at 37°C in 5% O₂, 6% CO₂ and 89% N₂. Embryos were then washed and cultured in medium G2 for a further 48 h to the blastocyst stage. On-time embryo development was assessed at 43 h post hCG (day 2, cleavage), 67 h post hCG (day 3, compaction), 98 h post hCG (day 4, early blastocyst) and 115 h post hCG (day 5, late blastocyst). All embryo culture dishes were prepared 4 h prior to embryo culture to allow for gassing and temperature equilibration with 24 replicate experiments performed.

E-cadherin in Embryos

The localisation of E-cadherin in embryos was determined by a modified immunofluorescence protocol as previously described [27]. Briefly, 8-cell embryos were collected at 67 h post hCG, fixed in 4% paraformaldehyde overnight and stored in 0.1 M glycine. Embryos were permeabilised in 0.25% TritonX-100 (PBS-TX) for 45 min and placed into blocking solution (1:10 donkey serum) and primary antibody (1:200, Rabbit anti E-cadherin, Abcam, Cambridge, UK; ab33033) at 4°C overnight. The following day embryos were washed through PBS-TX and placed into secondary antibody (1:200, Donkey anti Rabbit Alex 488, Life Technologies, Invitrogen, Mulgrave, Australia; A-21206) at 37°C for 2 h. Following another wash in PBS-TX embryos were placed in 0.25 mg/ml of propidium iodide (PI) for 5 min to stain the nucleus. For a negative control the primary antibody was omitted from the reaction. Embryos were loaded in glycerol and allowed to settle to the bottom before being imaged blinded by the same individual using confocal microscopy. E-cadherin localisation were determined by characterising if 8-cell embryos had either 1 of 3 staining patterns observed during confocal z-sectioning; 1) E-cadherin at cell to cell contacts (Fig. 1A), 2) E-cadherin at both cell to cell contacts and cytoplasm (Fig. 1B) and 3) E-cadherin only in the cytoplasm (Fig. 1C). The numbers of embryos for each staining pattern for each treatment group were expressed as a percentage.

Blastocyst DNA Damage

The number of apoptotic cells in each blastocyst were determined at day 5 of embryo development (115 h post hCG) using TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling) (In Situ Cell Death detection kit, Roche Molecular Biochemicals, Indianapolis, IN) as described previously [28]. The numbers of apoptotic cells were expressed as a percentage of total cell number for each blastocyst. A positive control was used where blastocyst were incubated in the presence of DNase I (Invitrogen, Mulgrave, Australia) and a negative control where blastocysts were incubated only in the fluorescent buffer.

Assessment of ICM, Trophectoderm and Epiblast Cell Number (Nanog and Oct4 Staining)

To determine the number of epiblast cells within the ICM, day 5 blastocysts were further grown in the above culturing system for an additional 24 hours (Day 6, 139 h post hCG) to allow time for epiblast cells to be detected in the ICM by Nanog and Oct4 antibodies and stained using our previously published method [29]. Briefly blastocysts were fixed in 4% paraformaldehyde overnight at 4°C and neutralised in 0.1 M glycine. Blastocysts

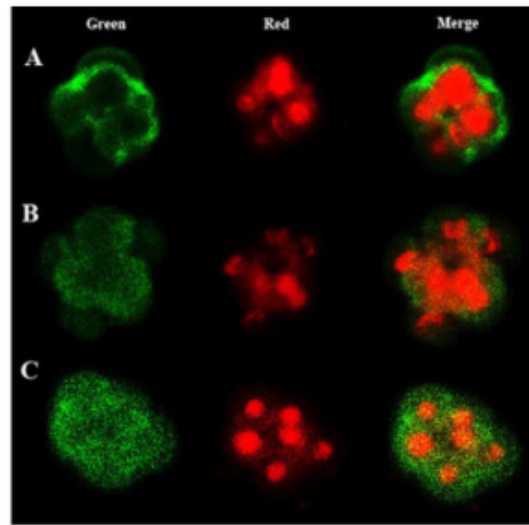


Figure 1. E-cadherin Staining Patterns. A E-cadherin localisation to cell to cell contacts. B E-cadherin localisation to both cell to cell contacts and cytoplasm. C E-cadherin localisation to the cytoplasm. Green images represent E-cadherin staining, red images represent nuclear staining and merge images represent a compression of both. Pictures are representative of a single z-section through embryos. doi:10.1371/journal.pone.0071459.g001

were permeabilised in PBS-TX for 15 min at room temperature (RT) and blocked overnight at 4°C in 10% normal donkey serum (Sigma, D9663). Blastocysts were then incubated in primary antibodies rabbit anti Nanog (Cozmo Bio, Tokyo, Japan; REC-RCAB0002P) (1:200) and goat anti Oct4 (Santa Cruz, California, USA; SC-8628) (1:100) for 1 1/2 h at 37°C. Following washing in PBS-TX blastocysts were incubated in secondary antibodies donkey anti rabbit Alexa 488 (Life Technologies, Invitrogen, Mulgrave, Australia; A-21206) (1:100) and donkey anti goat Alexa 594 (Life Technologies, Invitrogen, Mulgrave, Australia; A-11058) (1:100) for 2 h at RT. To further determine cell nuclei blastocyst were incubated in DAPI for 2–3 min at RT before being loaded in glycerol and counted using fluorescent microscopy. Total cell number (TCN) was assessed by nuclei stained DAPI; inner cell mass (ICM) cell number nuclei also stained positive for Oct4, and epiblast cell number nuclei additionally stained positive for Nanog. Trophectoderm cell number (TE) was determined by subtracting ICM from TCN. For a negative control the primary antibodies were omitted from the reaction.

Embryo Transfer

Morphological similar expanded or hatched blastocysts (115 h post HCG) were transferred into day 4 pseudo pregnant 10 week old Swiss female mice (–1 day asynchronous). Embryos from each treatment were randomly allocated to each uterine horn with each mother having six blastocysts from 2 different treatment groups (transferred to each uterine horn). Twelve to eighteen embryos were transferred into 2–3 mothers per father from 5 HE and HCE and 6 CC, HH and HC males. Mothers were maintained on standard chow (Table S1) until day 18 of pregnancy. On day 18 of pregnancy, mothers were humanely killed by cervical dislocation and implantations, numbers of fetuses and resorptions were determined per uterine horn for calculation of implantation rates,

percentage of fetuses and percentage of fetuses per total number of implantations. Fetuses and placentas were dissected and removed of connective tissue, umbilical cords and maternal arterial space (apostrophe) for placentas. Crown rump length and weights of resultant fetuses and placentas were measured.

Statistical Analysis

All data were expressed as mean \pm SEM and checked for normality using a Kolmogorov-Smirnov test and equal variance using a Levene's test. All statistical analysis was performed in SPSS (SPSS Version 18, SPSS Inc., Chicago, USA). A p value <0.05 was considered to be significant.

Weight gain data, body composition and DEXA body composition in our founder males were analysed by a one way ANOVA with an LSD post hoc test.

Embryo cell development, embryo DNA damage and embryo cell number were expressed per father and analysed by a one way ANOVA with a LSD post hoc test between groups. Nanog staining was analysed by Mann-Whitney U test between groups as the data are not normally distributed.

Fetal weight and size, placental weight and size and fetal to placental weight ratios there was no interaction between litter size and fetal weights and therefore fetuses and placentas were expressed per father and analysed by a univariate generalized linear model with a LSD post hoc test between groups. Cohort of founders was fitted as a covariate.

Implantation rates and E-cadherin staining patterns were analysed by a Chi Square between groups.

Correlations between founder's serum metabolites and embryo and fetal health were determined by a Pearson's Rho test and expressed as the correlation coefficient (CCO) along with its corresponding p value and number of observations. To determine if paternal adiposity was contributing to the correlations of serum metabolites to embryo and fetal health additional partial correlations controlling for paternal adiposity were also included.

Results

Effect of Diet and Exercise on Embryo Development

There was no change in cleavage rates to the 2-cell stage between any of the treatment groups ($p>0.05$, Table 1). Males fed a HH produced a decreased number of embryos at the 8-cell/compacting stage on day 3 compared with CC ($p<0.05$, Table 1), with combined diet and exercise intervention (HCE) advancing development to the 8 cell/compacting stage compared with HH and HC ($p<0.05$, Table 1). Diet alone (HC) or exercise alone (HE) did not alter development to the 8-cell/compacting stage, compared with HH ($p>0.05$, Table 1). This advancement in embryo development from CC males was also evident on day 4 with reduced numbers of early blastocysts contributing to total blastocyst numbers compared with HH males ($p<0.05$, Table 1). All interventions (HC, HE and HCE) displayed advanced blastocyst development on day 4 with increasing numbers of hatching blastocysts compared with HH ($p<0.05$, Table 1) and a decrease in expanded blastocysts compared with CC ($p<0.05$, Table 1). By day 5 of embryo development exercise interventions (HE and HCE) maintained their increased numbers of hatching blastocysts compared with HH ($p<0.05$, Table 1). There was no difference between total blastocysts, expanded blastocysts or hatching blastocysts between the remaining treatment groups (HC, CC and HH) indicating a slower rate of development in embryos from HH males, rather than a failure to develop.

Table 1. The Effect of Diet and Exercise of Obese Males on Embryo Development.

Diet/ Intervention	CC	HH	HC	HE	HCE
Day 2					
Cleavage	88.6 \pm 5.3	88.9 \pm 4.7	89.2 \pm 2.5	90.3 \pm 3.7	94.3 \pm 6.2
Day 3					
8 cell/compacting	71.4 \pm 5.2 ^a	54.2 \pm 5.5 ^b	58.2 \pm 5.4 ^b	64.3 \pm 4.9 ^{ab}	72.0 \pm 5.6 ^a
Compacting	14.7 \pm 3.7 ^{ab}	15.7 \pm 3.9 ^{ab}	9.6 \pm 3.7 ^a	8.4 \pm 3.5 ^a	22.2 \pm 4.0 ^b
Day 4					
Total blastocyst	75.5 \pm 5.0	74.1 \pm 5.3	74.7 \pm 5.1	82.2 \pm 4.7	86.2 \pm 5.4
Early blastocyst	10.3 \pm 3.6 ^a	20.7 \pm 4.1 ^b	13.8 \pm 3.8 ^{ab}	16.1 \pm 3.0 ^{ab}	12.2 \pm 3.5 ^{ab}
Expanded blastocyst	27.3 \pm 3.1 ^a	21.3 \pm 3.2 ^{ab}	14.0 \pm 3.1 ^a	13.9 \pm 2.9 ^a	14.4 \pm 3.3 ^a
Hatching blastocyst	45.3 \pm 5.0 ^{ab}	34.3 \pm 5.3 ^b	47.9 \pm 5.1 ^{ac}	53.0 \pm 4.7 ^{ac}	59.2 \pm 5.4 ^c
Day 5					
Total blastocyst	86.4 \pm 4.5	83.1 \pm 4.7	83.2 \pm 4.5	84.1 \pm 4.2	87.5 \pm 4.8
Expanded blastocyst	19.6 \pm 3.6	21.1 \pm 3.8	16.5 \pm 3.7	10.9 \pm 3.4	12.3 \pm 3.9
Hatching blastocyst	65.2 \pm 5.0 ^{ab}	55.4 \pm 5.3 ^a	61.9 \pm 4.9 ^{ab}	72.4 \pm 4.7 ^b	74.5 \pm 5.6 ^b

Embryo development is expressed as percentage of embryos \pm SEM at each stage of development per father. Data represents 6 HH and HCE males and 7 CC, HC and HCE males which is representative of at least 300 embryos per treatment group (for exact breakdown of embryos per father see Table S2). On day 4 total blastocysts is made up of early, expanded and hatching blastocysts, while on day 5 of on time embryo development total blastocyst is made up of a combination of expanded and hatching blastocysts. Different letters denote significance at $p<0.05$.

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Effect of Diet and Exercise on Blastocyst Cell Numbers and DNA Damage

Blastocysts produced by males from the HH group had lower total cell numbers on day 5, compared with those of CC males ($p<0.05$, Table 2). All interventions (HC, HE and HCE) increased total blastocyst cell number on day 5, compared with HH ($p<0.05$, Table 2), restoring this to be comparable with the CC group ($p>0.05$, Table 2). This reduction in cell number from the HH was not as a result from increased apoptosis as there was no difference in the number or percentage of TUNEL positive cells in the blastocyst across groups ($p>0.05$, Table 2).

Effect of Diet and Exercise on Epiblast, ICM and Trophoctoderm Cell Number

Embryos were further cultured for an additionally 24 hrs (day 6) to allow time for epiblast cells to be detected in the ICM by Nanog and Oct4 antibodies. All interventions (HC, HE and HCE) and CC had increased cell numbers (TCN and TE) compared with HH, similar to that observed on day 5 ($p<0.05$, Table 2). ICM and epiblast cell numbers were increased in blastocysts from the CC males compared with HH ($p<0.05$, Table 2) with combined diet and exercise interventions (HCE) also having an increase ICM cell numbers compared with HH ($p<0.05$, Table 2). Diet alone (HC) and exercise alone (HE) resulted in ICM and epiblast cell number not different to CC ($p>0.05$, Table 2). There was no

Table 2. The Effect of Diet and Exercise of Obese Males on Blastocyst Development.

Diet/Intervention	CC	HH	HC	HE	HCE
Day 5					
Total cell number	76.7±3.8 ^a	64.3±3.6 ^b	75.3±4.2 ^a	74.9±3.5 ^a	86.6±5.7 ^a
TUNEL positive cells	3.73±1.10	4.54±1.00	3.50±0.87	3.76±0.87	3.59±0.50
DNA damage (%)	4.86±1.38	7.03±1.60	4.65±1.15	5.01±1.12	4.15±0.51
Day 6					
Total cell number (TCN)	98.3±6.3 ^a	84.0±7.6 ^b	100.0±3.7 ^a	96.3±3.5 ^a	99.7±6.2 ^a
Trophectoderm cell number	83.4±5.5 ^a	72.1±7.3 ^b	86.6±1.3 ^a	83.2±3.7 ^a	85.6±5.3 ^a
ICM cell number	15.0±1.29 ^a	12.0±1.04 ^b	12.9±1.05 ^{ab}	13.6±0.53 ^{ab}	14.2±1.04 ^a
Epiblast cell number	7.2±0.71 ^a	5.7±0.80 ^b	6.2±0.73 ^{ab}	6.2±0.45 ^{ab}	6.6±0.84 ^{ab}
Trophectoderm (% of TCN)	84.7±0.96	85.4±1.48	86.6±0.78	85.6±0.95	85.5±0.64
ICM (% of TCN)	15.3±0.96	14.8±1.41	13.4±1.39	14.4±0.95	14.5±0.64
Epiblast (% of ICM)	46.2±4.50	46.7±5.99	47.1±3.63	46.6±3.90	45.7±3.37

Data is expressed as mean ± SEM per father. Data represents 6 HH and HCE males and 7 CC, HC and HCE males which is representative of 5–8 embryos per father per group. Different letters denote significance at $p < 0.05$.
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change in the proportion of cells or percentage of cells allocated to the trophoblast, ICM or epiblast between any of the treatment groups ($p > 0.05$, Table 2).

Effect of Diet and Exercise on Embryo Cell to Cell Contact

To further evaluate the effect of intervention treatments on rates of compaction after embryonic genome activation (after 2-cell stage) and therefore activation of the paternal contribution to the embryo, the localisation of E-cadherin (a marker of cell to cell contact) and one of the first paternally translated proteins within the developing 8-cell embryo [30], was assessed. CC and HE males had more 8-cell embryos with E-cadherin located at cell to cell contacts, compared with the other treatment groups (HH, HC and HCE, $p < 0.05$, Fig. 2A). HCE males had more 8-cell embryos with E-cadherin located to both cell to cell contacts and cytoplasm compared with CC and HE males ($p < 0.05$, Fig. 2B), but similar numbers compared with HH or HC males ($p > 0.05$, Fig. 2B). HC males had more 8-cell embryos with E-cadherin located to the cytoplasm compared with HCE males ($p < 0.05$, Fig. 2C). There was no change to the proportion of 8-cell embryos with E-cadherin located to the cytoplasm between the remaining treatment groups (HH, HC, HE, $p > 0.05$, Fig. 2C).

Effect of Diet and Exercise on Implantation and Fetal Viability

Blastocyst viability was assessed by transferring embryos to recipient mothers. Blastocysts produced from HH had reduced implantation rates compared with blastocysts from CC ($p < 0.05$, Table 3). The combined diet and exercise intervention (HCE) was the only intervention to improve implantation rates compared with HH ($p = 0.08$, Table 3) with diet alone (HC) and exercise alone (HE) showing no improvements. There was no difference in fetal development, as assessed by the number of viable fetuses per the total number of blastocyst transferred, between any of the treatment groups ($p > 0.05$, Table 3). Exercise intervention alone (HE) increased the percentage of implantations that resulted in viable fetuses, compared with CC and diet intervention alone (HC) ($p < 0.05$, Table 3), with the remaining groups (HH and HCE) showing no change. HH had decreased fetal weights, lengths and placental weight compared with CC ($p < 0.05$, Table 3). Any form

of diet and/or exercise intervention (HC, HE, HCE) increased fetal weights compared with HH (HE and HCE $p < 0.05$ and HC $p = 0.08$, Table 3); with diet alone (HC) and exercise alone (HE) additionally increasing crown-rump length compared with HH ($p < 0.05$, Table 3). There was no change in placental weights between treatment groups (HC, HE and HCE) and HH ($p > 0.05$, Table 3), however exercise alone did decrease placental weights compared with CC ($p = 0.08$, Table 3). There was no change in fetal to placenta weights ratio between any of the groups ($p > 0.05$, Table 3).

Associations between Paternal Metabolic and Hormonal state and Embryo and Fetal Growth and Development

Diet interventions (HC and HCE) reduced founder male body weight (HC -2.37 ± 0.79 g and HCE -3.19 ± 0.75 g), levels of adiposity ($p < 0.01$), serum cholesterol ($p < 0.05$), serum leptin ($p < 0.05$), increased lean mass ($p < 0.05$) and improved glucose tolerance ($p < 0.05$) but maintained insulin resistance as measured by AAC compared with HH males (Table S3 and Table S4). Those receiving exercise intervention alone (HE) maintained their pre intervention body weight (-0.28 ± 0.75 g) and level of adiposity (Table S3) with serum cholesterol and insulin resistance remaining comparable to HH males ($p > 0.05$, Table S4) however, had a significant reduction in serum leptin levels ($p < 0.05$, Table S4). Interventions including exercise (HE and HCE) reduced fasting serum glucose (HE $p < 0.05$ and HCE $p = 0.08$) with an increased clearance of glucose after a GTT compared with HH males ($p < 0.05$, Table S4).

Associations with metabolic and hormonal measures in founder males with embryo and fetal outcomes were further examined to identify any potentially influential factors due to the differences in embryo and fetal health observed in the intervention combinations. Paternal serum cholesterol correlated negatively with blastocyst cell number on day 5 (CCO -0.17 , $p = 0.01$, $n = 191$) and day 6 embryos (CCO -0.52 , $p < 0.01$, $n = 216$), as well as TE cell number (CCO -0.54 , $p < 0.01$, $n = 216$), but positively with the percentage of DNA damaged cells in blastocysts (CCO 0.42 , $p = 0.01$, $n = 191$). Paternal serum FFA correlated negatively with fetal to placental weight ratio (CCO -0.30 , $p = 0.01$, $n = 64$) and percentage of ICM cells (CCO -0.50 , $p = 0.01$, $n = 210$), but

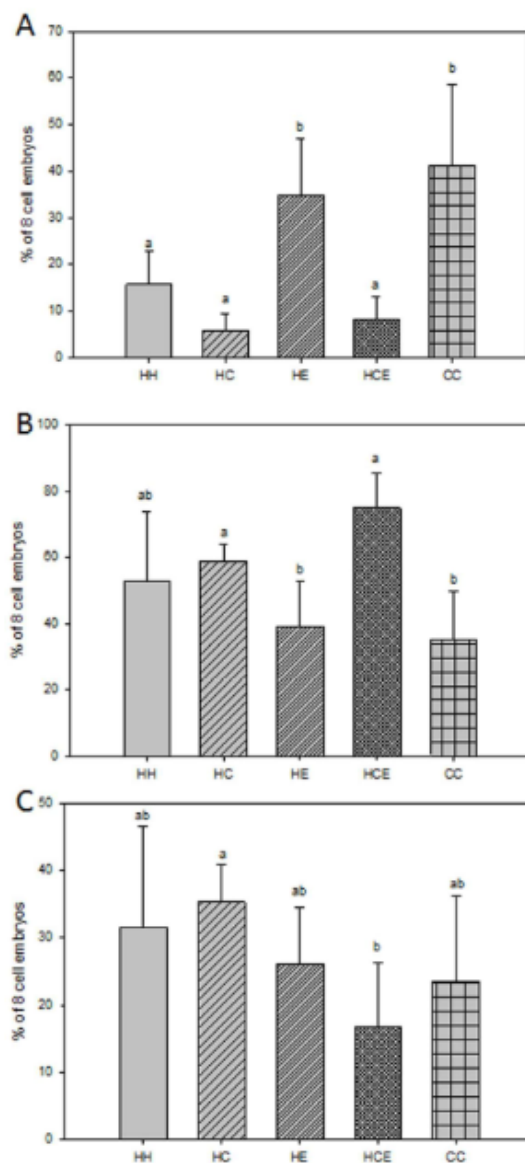


Figure 2. The Effect of Diet and Exercise of Obese Males on E-cadherin Staining Patterns in Compacting Embryos. **A** The percentage of 8 cell embryos with E-cadherin localised to cell to cell contacts. **B** The percentage of 8 cell embryos with E-cadherin localised to both cell to cell contacts and cytoplasm. **C** The percentage of 8 cell embryos with E-cadherin localised to the cytoplasm. Data represents 3 HH, HCE and 4 CC, HC and HE males which is representative of 3–7 embryos per father per group. doi:10.1371/journal.pone.0071459.g002

positively with the ratio of epiblast cells to ICM cells (CCO 0.51, $p < 0.01$, $n = 209$) and the percentage of TE cells (CCO 0.51, $p < 0.01$, $n = 216$). Paternal serum triglyceride correlated negatively with both total cell number (CCO -0.44 , $p < 0.01$, $n = 216$) and TE cell numbers (CCO -0.41 , $p < 0.01$, $n = 216$). Paternal insulin

Table 3. The Effect of Diet and Exercise of Obese Males on Subsequent Implantation and Fetal Development.

Diet/ Intervention	CC	HH	HC	HE	HCE
Implantation (%)	98.5 ^a	83.3 ^b	85.9 ^{bc}	84.7 ^{bc}	93.1 ^{ac}
Fetal (%)	15.3	23.6	14.4	26.4	16.0
Fetal/implantation (%)	14.5 ^a	24.2 ^{ab}	14.6 ^a	27.7 ^b	15.9 ^{ab}
Fetal weight (mg)	773±31 ^a	701±26 ^b	776±42 ^a	816±38 ^a	818±31 ^a
Crown-Rump length (mm)	18.4±0.4 ^a	17.4±0.3 ^b	18.6±0.5 ^a	18.5±0.3 ^a	18.0±0.4 ^{ab}
Placental weight (mg)	113±3 ^a	102±6 ^b	109±8 ^{ab}	104±5 ^{ab}	102±10 ^{ab}
Fetal:placenta ratio	6.8±0.5	7.0±0.5	7.3±0.3	7.7±0.3	7.4±0.2

Data is expressed as mean ± SEM per father. Implantation and fetal development data represents 5 HE and HCE and 6 CC, HH and HC males which is representative of 12–18 embryos transferred per father for each group. Fetal and placenta data represents 5 HE and HCE and 6 CC, HH and HC males which is representative of at least 10 fetuses per treatment group (for exact breakdown of number of fetuses per father see Table S2). Different letters denote significance at $p < 0.05$, different to HH at $p = 0.08$ and different to CC at $p = 0.08$.

Implantation (%) refers to the percentage of blastocyst that implanted into the uterus resultant from total number of embryos transferred determine by fetal numbers and resorptions. Fetal (%) refers to the percentage of embryos that formed fetus resultant for the total number of embryos transferred. Fetal/implantation (%) refers to the percentage of fetuses that formed from the total number of successful implantations. doi:10.1371/journal.pone.0071459.t003

sensitivity (as measured by AAC during ITT) correlated negatively with the percentage of TE cells (CCO -0.44 , $p < 0.01$, $n = 216$) and percentage of DNA damaged cells in blastocysts (CCO -0.20 , $p < 0.01$, $n = 191$), but positively with the percentage of ICM cells (CCO 0.43, $p < 0.01$, $n = 210$). Paternal fasting serum glucose correlated negatively with fetal to placental weight ratio (CCO -0.36 , $p < 0.01$, $n = 64$) and positively with placental weight (CCO 0.21, $p = 0.05$, $n = 64$). Additionally paternal adiposity correlated negatively with both ICN (CCO -0.51 , $p < 0.01$, $n = 216$) and TE cell number (CCO -0.49 , $p < 0.01$, $n = 216$) and positively with the percentage of DNA damaged cells in blastocysts (CCO 0.31, $p = 0.03$, $n = 191$).

When controlled for paternal adiposity the majority of the above correlations still held, indicating that paternal lipid status, glycaemia and insulin sensitivity are correlated to embryo and fetal health independently of paternal adiposity (Table S5).

Discussion

There is emerging evidence that paternal obesity and related metabolic changes are associated with reduced embryo development and pregnancy establishment in both humans and rodent models [5–7,15,16,31]. Here we show for the first time, using a well characterised model of diet induced obesity in the mouse, that diet and exercise interventions in the obese father, which either reduces adiposity or improves their metabolic state at the time of conception, improves subsequent embryo, pregnancy and fetal health. Previous studies of interventions to reverse obesity and metabolic syndrome in males had focused on influences on restoration of hormone profiles, sperm function and sexual dysfunction [17,18,32–36], but none had examined effects on

embryo development and pregnancy establishment. Using diet induced obesity in male mice; we showed that the negative impact of paternal obesity on blastocyst development, blastocyst cell numbers, as well as subsequent implantation rate and fetal growth can be reversed through improvements to paternal metabolic health via diet and/or exercise. Further, the metabolic status of the father, including lipid status, glycaemia and insulin sensitivity are strongly associated with markers of blastocyst and fetal development or growth, independently of paternal adiposity or exposure to dietary or exercise interventions.

It has previously been shown and confirmed by this study that males fed a HFD produce embryos that have delayed development, with longer times to 1st, 2nd and 3rd cleavage and cavitation [11,31] with a reduction in the numbers of on time developing embryos at the compacting, early blastocyst and late blastocyst stage [15]. Here we show for the first time that improvements to adiposity and metabolism via diet and/or exercise improve embryo development rates and blastocyst cell numbers in previous obese male mice. Specifically, the improved embryo development seen by those mice that underwent interventions involving exercise (HE and HCE) was evident after activation of the embryonic genome (in the mouse at the 2-cell stage); with more embryos reaching the 8 cell/compacting stage on day 3 and similarly for blastocysts on day 4 and day 5. In humans it has been shown that embryos that have successful implantation develop to the 8 cell stage faster than those embryos that couldn't implant [37] suggestive of improved implantation capability of embryos produced from exercise males.

Furthermore, we also confirmed the reductions in blastocyst cell numbers, including reduced numbers of ICM and TE cells, which have been observed from males fed a HFD [11,15]. All diet and exercise interventions in obese male mice, increased blastocyst total cell numbers on both day 5 and day 6 of *in vitro* embryo development with improvements to both TE and to a lesser extent ICM cells. The late blastocyst is made up of two main cell types: 1; trophoblast cells, which are necessary for embryo implantation and invasion and the establishment of the placenta; and 2; ICM cells, which can be split into epiblast cells that form the fetus and the primitive endoderm, that forms the extra embryonic tissue [38]. Changes to the numbers of cells or the distribution of the cells within the blastocyst can adversely affect the developing fetus, for an example, a reduction in numbers of ICM cells in a maternal diabetic rat model associated with fetal growth restriction [39]. Additionally in rodents blastocyst cell numbers as well as the proportion of cells within the late blastocysts have also been shown to positively correlate with implantation and pregnancy rates [26]. This suggests that exercise and diet intervention in obese males could potentially improve implantation and live birth rates, by restoring cell allocation and mitosis in the blastocyst.

In a clinical ART setting, obese males are associated with an increased rate of miscarriage, which may be as a result of sac only pregnancies [5] implying that cells within the embryo that form the fetus (i.e. epiblast cells) are impaired or missing. Here we directly show for the first time that diet induced obesity reduces the number of epiblast cells within the developing embryo and could potentially provide an explanation for the increased miscarriage rate seen in association with obese men [5–7]. Although diet and/or exercise interventions did not significantly improve epiblast cells number compared with HFD (HH) or CD (CC) they were still slightly increased compared to HFD mice, again suggesting likely improvements to implantation and pregnancy establishment.

Previous studies have also shown that blastocyst apoptosis is another marker of embryo health, with increasing apoptosis associated with reduced embryo survival, implantation rates and

increases in early miscarriage [40–43]. Interestingly, in the current study, no differences were observed in relation to the levels of blastocyst apoptosis between the diet and exercise interventions or between HFD (HH) and CD (CC). This is contradictory to those findings found in Mitchell et al [15], where male mice fed a HFD for a period of 8 weeks showed increased rates of apoptosis in the late blastocysts. The differences in outcomes with the current study could be strain (C57BL6 compared with C57BL6xCBAF1) and age specific (25 weeks of age compared with 13 weeks) or related to the increased length of exposure to both the CD and HFD diets in the current study versus the previous study (18 weeks compared with 8 weeks).

The current study showed for the first time that diet induced obesity resulted in changed localisation patterns of E-cadherin in compacting embryos, with more 8 cells having E-cadherin located in the cytoplasm from males fed a HFD, indicating a potential delay in establishment of the junctional communication between the blastomeres. However this potential delay was improved by exercise interventions with increased in E-cadherin observed in cell to cell contacts from these groups. The process of compaction in the developing embryo, involves polarisation of the cells, cell flattening and junctional communication [44]. E-cadherin is required in the developing embryo for the above processes, to allow compaction and cell division whilst also coordinating the cellular allocation and spatial segregation of the ICM and trophoblast, with E-cadherin mutants unable to form a trophoblast [30,45,46]. During embryo polarisation E-cadherin moves from the cytoplasm and becomes restricted to cell to cell contacts where it is stabilised before embryo compaction [47]. It therefore appears that E-cadherin in embryos localised in the cytoplasm compared with cell to cell contacts, is indicative of a delay in this polarisation and compaction. Changes to the localisation and levels of E-cadherin in compacting embryos where males were treated with cyclophosphamide [27], resulted in half of all compacting embryos with little or no cell contacts, with the immune reactivity of E-cadherin reduced and a significant delay in embryo development [27]. The improvements seen in those males that underwent exercise interventions suggests an advancement in development of compaction which may be contributing to the improvements in development and cell numbers seen in embryos derived from these males.

Previous studies in humans undergoing assisted reproductive technologies suggest that male obesity reduces implantation and therefore live birth rates [5–7]. Consistent with results observed in a previous rodent study of male obesity and embryo health [11,15], the current study confirmed that males fed a high fat diet for the entire study had reduced implantation rates assessed at day 18 of gestation after embryo transfer, compared with those fed a CD. Interestingly, only the combined diet and exercise intervention (HCE) group improved implantation rates compared with males fed a HFD for the entire study. However it should be mentioned that morphological similar blastocysts from each group were used in embryo transfers which can predict implantation rates [37] and suggests that evaluation of a combined approach to weight loss in a clinical setting to evaluate the impact on implantation and therefore live birth rates is warranted.

Fetal development and size are not only determinants of a healthy pregnancy but can also pre determine the likelihood of developing adult chronic disease [48,49]. Paternal exposure to high levels of x-rays as well as fathers' occupation is associated with small for gestational age babies independently of other confounding factors [50,51], showing that paternal health at the time of conception can influence fetal health outcomes. Consistent with previous studies in rodents [11,52] our HFD fed mice (HH)

produced smaller fetuses compared with those fed a CD (CC), which has been linked with impaired metabolic and reproductive health in subsequent offspring [52,53]. Interestingly, all interventions (HC, HE, HCE) increased fetal weights compared to males fed a HFD (HH) with diet alone (HC) and exercise alone (HE) additionally increasing fetal lengths. This suggests that offspring born from obese fathers that underwent diet and/or exercise interventions may have a reduced susceptibility to develop adult chronic diseases.

It is becoming increasingly evident that at least some changes to embryo and fetal health must be resultant from the sperm health at the time of conception. Previous studies have shown that poor sperm quality including increased levels of DNA damage and/or reactive oxygen species are associated with reduced fertilisation, impaired embryonic development and increased pregnancy loss [40–43,54–56]. Increased levels of DNA damage and ROS in sperm are commonly found in obese males [57–59]. Using this same model it has previously been shown that diet and exercise interventions reduced both DNA damage and ROS levels in sperm of obese male mice [13]. The improved embryo and fetal health seen in the current study may therefore be as a result of improvements to sperm DNA damage and/or ROS levels in sperm. In humans, diet and exercise interventions as well as gastric bypass surgeries have been shown to improve other sperm measures such as count, motility as well as sexual function in previously overweight and obese men [17,18]. Further, it has been shown that the proteomic, microRNA (miRNA) and methylome content of sperm including the RNA content of testes from obese rodents are also altered [16,60–64] molecular sperm signatures that have previously been shown to impact embryo health and quality [65,66]. This may be contributing to the impaired sperm and embryo development seen in obese men. It is therefore possible that improvements to embryo and fetal health seen in the current study are resultant from additional improvements to sperm epigenetic molecular structure including changes to histone methylation and miRNA content. The specific types of molecular signatures currently altered in sperm as a result of obesity and then restored by diet and exercise interventions are currently unknown. However, a recent study in humans showed that miRNAs in serum previously altered by obesity could be restored through weight loss [67], suggesting that a similar restoration could likely occur in sperm and explain the improvements in our study.

As seen in our previous study [13] mice that underwent diet interventions (HC and HCE) had reduced levels of adiposity, improved serum cholesterol, FFAs, glucose tolerance and additionally serum leptin levels. Those receiving exercise intervention only (HE) were still obese maintaining their pre intervention level of adiposity, serum cholesterol, and impaired response to insulin, however had significant reductions in serum leptin levels which are consistent to findings found previously in obese males [68,69]. Interventions including exercise (HE and HCE) reduced fasting serum glucose with an increased clearance of glucose after a GTT. This is likely as a result from increased glucose utilisation by skeletal muscle (reviewed by [70]). Using association analysis between the father's body composition and metabolic parameters, the data in this study suggests that the adverse embryo and fetal health due to diet induced obesity may not only be resultant from increased adiposity but additionally whole body metabolic changes. Irrespective of father's treatment or adiposity, carbohydrate and lipid profiles showed the strongest correlations to embryo and fetal health, with reduced cholesterol, and FFAs associated with improved blastocyst cell numbers and reduced cellular apoptosis. Additionally, glucose and insulin metabolism

were also found to be associated with improved embryo cell numbers and fetal and placental weight ratios.

Due to lack of cytoplasmic scavenging enzymes and high levels of polyunsaturated fatty acids in their plasma membrane, sperm are highly susceptible to oxidative stress and damage [71–73]. Exposure to increasing levels of both cholesterol and fatty acids in human and animal sperm *in vitro* has previously been shown to cause increased mitochondrial ROS [74] reduced sperm motility, capacitation and fertilisation [75,76]. In particular, cholesterol is found in the sperm membrane and its levels within the membrane affects fluidity and helps determine motility, capacitation and acrosome reaction [77,78], all important process required for successful fertilisation [79]. Hypercholesterolemia in rabbits showed that the transport ion channels were open in the epididymis between the circulating lipids and the sperm micro environment [74] and that the sperm from these rabbits displayed reduced motility and capacitation likely caused from increasing sperm ROS. It is therefore plausible that high circulating plasma cholesterol, triglycerides and FFAs as seen in male obesity cause changes to sperm membrane dynamics and directly alter the fluidity of the plasma membrane allowing the sperm to be more susceptible ROS and DNA damage. Increased levels of ROS have been shown to change the global methylation profile of sperm [80], with hypomethylation of imprinted genes and repeat elements in sperm linked with reduced pregnancy success and increased sperm DNA damage [80–83]. It is therefore, possible that elevated paternal plasma glucose and lipids may in this way modifies the epigenetic profile of sperm, which is then passed onto the newly fertilised embryo, causing a delay in embryo development via alterations to paternal chromatin remodelling and indirectly reducing embryo cell numbers and implantation rates.

This is the first study to show that the impaired embryo and fetal development commonly seen in obese males can be reversed by improving their metabolic profile via exercise and diet. This study also provides the first direct evidence that the metabolic profile of obese fathers maybe a better indicator for determining the health of the resultant embryo and fetus than adiposity alone. Therefore, this study shows that exercise and diet interventions could be a combined approach to target sub fertility in overweight and obese men by improving embryo development and therefore subsequent pregnancy health. Future studies in the human are needed to determine if similar improvements can occur.

Supporting Information

Table S1 Composition of Animal Diets.
(DOC)

Table S2 Numbers of Embryos and Pups Derived from each Father.
(DOC)

Table S3 The Effect of Diet and Exercise on Founder Male Body Composition after Intervention.
(DOC)

Table S4 The Effect of Diet and Exercise on Founder Male Serum Metabolites after Intervention.
(DOC)

Table S5 Correlations of Founder Metabolism on Blastocyst and Fetal Health Independent of Founder Adiposity.
(DOC)

Author Contributions

Conceived and designed the experiments: NOM JAO ML. Performed the experiments: NOM ML. Analyzed the data: NOM. Contributed reagents/

materials/analysis tools: JAO ML. Wrote the paper: NOM. Edited the manuscript: HWB JAO BPS ML. Interpreted the data: NOM JAO ML.

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4 An obese father's metabolic state, adiposity and reproductive capacity indicate a son's reproductive health

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4.2 LINKING TO CHAPTER 4

Worldwide diagnosis of male sub fertility is increasing especially in Western societies. Rodent models of male obesity have shown that sperm function of F₁ males was impaired (motility, ROS and DNA damage) and persisted into F₂ generation males, which may contribute to rising male sub fertility in Western societies. Therefore, lifestyle interventions applied to obese men may provide an unappreciated novel intervention window to maximise the reproductive function of the next generation of males. Additionally embryo growth and early fetal health are determinants of subsequent adult health and can be used as predictors for determining likelihood of development of adult chronic diseases (i.e. birth weight and diabetes). Since all exercise interventions in obese founders restored embryo development, embryo total cell numbers and fetal weights, while diet interventions restored embryo total cell numbers and fetal weights it was further hypothesised that diet and/or exercise in obese founders would restore sperm function in subsequent male offspring. Founder obese males were again allocated to either continuation of a HFD or one of the diet and/or exercise interventions and naturally mated with natural cycling normal weight control diet fed mothers to produce offspring. Male offspring were monitored to establish if there were improvements to their reproductive function after interventions to founder males.

4.3 CAPSULE

Diet and exercise interventions in obese founder males improve the reproductive health of male offspring with a father's metabolic and reproductive status predicting his son's reproductive health.

4.4 ABSTRACT

Objective: To determine if dietary and exercise regimes in obese males can provide a novel intervention window for improving the reproductive health of the next generation.

Design: Experimental animal study

Setting: University research facilities

Animal(s): C57BL6 male and female mice

Intervention(s): Mice were fed a control diet (CD, 6% fat) or a high fat diet (HFD, 21% fat) for 9 weeks. After the initial feeding HFD males were allocated to diet and/or exercise interventions for a further 9 weeks. Post intervention males were mated with females fed standard chow (4% fat) before and during pregnancy.

Main Outcome Measures: F1 sperm motility, count, morphology, capacitation, mitochondrial function, and sperm binding and weight of reproductive organs.

Results: Our primary finding was that diet intervention alone in founders improved offspring sperm motility and mitochondrial markers of sperm health (decreased ROS and mitochondrial membrane potential) ultimately improving sperm binding. Sperm binding and capacitation was also improved in F1 males born to combined diet and exercise intervention in founders. Founder sperm parameters and metabolic measures as a response to the diet and/or exercise, (i.e. lipid/glucose homeostasis, sperm count and morphology) correlated with offspring's sperm function independent of founder treatment. This implicates paternal metabolic and reproductive status in predicting male offspring's reproductive function.

Conclusion: This is the first study to show that improvements to both metabolic (lipids, glucose and insulin sensitivity) and reproductive function (sperm motility and morphology) in obese fathers via diet and exercise interventions can improve subsequent reproductive health in offspring.

4.5 INTRODUCTION

Peri-conception paternal health has been shown to influence the health of subsequent children. For example paternal smoking, age and occupational chemical exposure are associated with an increased risk of impaired child health (El-Helaly et al., 2011; Lee et al., 2009; Van Balkom et al., 2012). Recent epidemiological studies demonstrate that paternal nutritional status and obesity are correlated with altered child health outcomes (Danielzik et al., 2002; Li et al., 2009). However, human studies are confounded by the common environmental exposures shared by both father and child.

Recent rodent models of paternal obesity have demonstrated that male offspring reproductive function was impaired, as evident by increased sperm intracellular reactive oxygen species (ROS), reduced sperm motility and reduced sperm binding (Fullston et al., 2012). Interestingly the same impairments persisted into second generation males (Fullston et al., 2012). The relevance of these findings are highlighted in western societies, as currently 70% of reproductive aged men are overweight or obese (ABS, 2013) suggesting likely changes to offspring reproductive health. Therefore, lifestyle changes in obese men may provide an unappreciated novel intervention window to maximise the reproductive function of the next generation.

Diet and exercise interventions in obese males have recently been shown to improve sperm parameters. Both gastric bypass surgery and weight loss through scheduled diet and exercise programs have resulted in improvements to sex hormone profiles, sexual function, total sperm count and

morphology in men who lost the greatest amount of weight (Hakonsen et al., 2011; Reis et al., 2012). The extent to which either the metabolic profile or adiposity enacted these outcomes remains to be investigated. In a mouse model of male obesity diet and exercise interventions normalised levels of sperm ROS, DNA damage and sperm binding (Palmer et al., 2012), parameters which subsequently improved embryo development and fetal size (McPherson et al., 2013).

Altogether, the evidence highlights the potential of weight loss strategies to restore sperm function of obese males in both rodent models and humans. To date no studies have determined whether weight loss and improved metabolic status in obese males can reverse the adverse reproductive effects in their male offspring. I therefore used a mouse model of male obesity to assess the hypothesis that a reduction in adiposity and/or an improvement to metabolic health via diet and exercise in obese fathers will improve reproductive health in their male offspring.

4.6 MATERIALS AND METHODS

4.6.1 Founder Animals and Diet

Five week old male C57BL6 mice (n=40) were randomly assigned to one of two diets for an initial period of 9 weeks: 1) control diet (CD; n=8) (SF04-057; Specialty Feeds, Perth, Australia); or 2) a high fat diet (HFD; n=32) high in fat and nutrient matched (SF00-219; Specialty Feeds, Perth, Australia) (Supplementary Table 4.1). The HFD used in the study has been previously shown to increase adiposity and impair sperm function compared with the nutritionally matched CD (Bakos et al., 2011; Brake et al., 2006; Mitchell et al., 2011; Palmer et al., 2011). After the initial feeding period, males allocated to the HFD were randomly allocated to one of the following interventions for a further 9 weeks: 1) continuation of a HFD (HH) (n=8); 2) change to a CD (HC) (n=8); 3) continuation of a HFD with exercise (HE) (n=8); 4) change to a CD with exercise (HCE) (n=8). Mice allocated to the CD during the initial feeding period continued to be fed a CD during the intervention period as a baseline control (CC). The previously described swimming intervention regime simulates light exercise (Mcpherson et al., 2013; Palmer et al., 2012) and demonstrated not to cause additional stress whereby the mice are given gradual acclimatisation to the full exercise program over two weeks (Palmer et al., 2012). Male body weights were recorded weekly both pre and post intervention. Metabolic status of founder males was obtained via fasted glucose tolerance test (GTT, expressed as area under curve (AUC)), fed insulin tolerance test (ITT, expressed as area above curve (AAC)) at 7 and 8 weeks respectively as per (Palmer et al., 2012) and fasting post mortem plasma measures of cholesterol, free fatty acids (FFA), glucose and triglycerides as previously described in (Gatford et al., 2009). Animals were individually housed in a 12:12 h dark light cycle for the entire study, fed *ad libitum* and given free access to water. The use and care of all animals used in the study was approved by the Animal Ethics Committee of The University of Adelaide.

4.6.2 Generation/Sampling of F1 Males

At 7 weeks post intervention (21 weeks of age) founder males were paired with 2 normal weight 8-10 week C57BL6 females for a maximum period of 8 nights. Female mice were housed with founder males during the dark cycle only and separated and maintained standard chow during the light cycle. Successful mating was assessed the following morning by the presence of a vaginal plug. HH founders had a reduced number of successful matings compared with HCE and CC founders ($p < 0.05$, data not shown). After successful mating female mice were group housed until day 15 of pregnancy and then individually housed until offspring were weaned. Females were maintained on standard chow during pregnancy and post birth. Females were allowed to pup and at weaning, the first male sexed was sampled from each litter for measurements of reproductive. For the CC group 8 F1 males were sampled from 8 litters representing 6 founders. For the HH group 10 F1 males were sampled from 10 litters representing 7 founders. For the HC treatment 10 F1 males were sampled from 10 litters representing 7 founders. For the HE treatment 10 F1 males were sampled from 10 litters representing 7 founders and for the HCE treatment 8 F1 males were sampled from 8 litters representing 6 founders. F1 males were group housed independently of founder treatment and maintained on standard chow.

4.6.3 Sperm Collection, Count, Motility and Morphology Analysis

Sperm were collected immediately post mortem from the cauda epididymis and ductus deferens and expressed into 1 ml of G-IVF medium (Vitrolife, Gothenberg, Sweden) and incubated for at least 10 min in 6% CO₂ and 5% O₂ at 37°C (Bakos et al., 2011). Sperm count, motility and morphology were assessed blinded in accordance with WHO guidelines (WHO, 2010), with at least 200 sperm from each sample measured. Sperm motility was assessed by classifying sperm as either progressive motile, non-progressive motile or immotile. Motility was expressed as a percentage for both progressive motile and total motility (combination of both progressive motile and non-progressive motile sperm). Sperm morphology was assessed on samples fixed with methanol:acetone (3:1) and stained with haematoxylin

and eosin. Sperm morphology of individual sperm were scored as normal, tail defect or head defect as per (Palmer et al., 2012) and expressed as a percentage of each form.

4.6.4 F1 Male Sperm Binding

The numbers of sperm bound to the zona pellucida of an MII oocyte were assessed as described in (Bakos, 2011). At least 10 oocytes were analysed per sperm sample.

4.6.5 F1 Male Sperm Capacitation and Acrosome Reaction

Capacitation and acrosome reaction were measured using *Arachis Hypogaea* (peanut) agglutinin (Lectin PNA; Molecular Probes, Eugene, USA) as previously described (Baker et al., 2004; Bakos et al., 2011). A minimum of 200 sperm were counted per sample. The proportion of non-capacitated, capacitated and acrosome reacted sperm were expressed as a percentage.

F1 Male Sperm Mitochondrial ROS assay & Vitality Measure

The intracellular generation of mitochondrial ROS was determined using MitoSox Red (MSR; Molecular Probes, Eugene, USA) and SytoxGreen (Molecular Probes) as previously described (Koppers et al., 2010). Both negative (sperm incubated only in SytoxGreen) and positive (sperm incubated in 1500 μM of H_2O_2) controls were conducted. MSR and SytoxGreen fluorescence was measured on a FACSCanto flow cytometer (BD Bioscience, North Ryde, Australia). Non-specific sperm events were gated out and 20,000 cells were examined per sample. MSR results were expressed as percent of live sperm positive for MSR. Vitality was measured as the percentage of sperm that did not display SytoxGreen fluorescence.

4.6.6 F1 Male Sperm Mitochondrial Membrane Potential (MMP) (JC-1)

Sperm mitochondrial membrane potential was determined by using the ratiometric dye JC-1 (Molecular Probes, Eugene, USA) as previously described (2010). A negative control was also included whereby sperm were incubated in 10 μ M of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to dissipate membrane potential before incubation in JC-1. JC-1 and PI fluorescence were measured on a FACSCanto flow cytometer. Non-specific sperm events were gated out and 20,000 cells were examined per sample. Results were expressed as the percent of live sperm positive for a high JC-1 reading.

4.6.7 Adiposity and Reproductive Organ Measurement

Founder males at pre intervention week 9 (14 weeks of age) and post intervention week 8 (22 weeks of age) and 10 weeks of age for F1 male offspring, total adiposity was measured by a dual-emission X-ray absorptiometry machine (DEXA) (Piximus, Ge Lunar, Wisconsin, USA) as previously described (Nagy & Clair, 2000). At intervention week 9 (23 weeks of age) for founder males and 10 weeks of age for F1 males gonadal fat, testes and seminal vesicles were dissected and weighed post mortem. All dissections and weighing were performed blinded to treatment group and performed by the same investigator.

4.6.8 Testosterone Analysis

At intervention week 9 for founder males (23 weeks of age) and 10 weeks of age for F1 males, serum testosterone was measured by a stable-isotope dilution liquid chromatography coupled with tandem mass spectrometry as previously described (Bakos et al., 2011; Mcnamara et al., 2010).

4.6.9 Statistical Analysis

All data were expressed as mean \pm SEM and checked for normality using a Kolmogorov-Smirnov test and equal variance using a Levene's test. All statistical analysis was performed in SPSS (SPSS Version 18, SPSS Inc., Chicago, USA) with an observed power of $\geq 80\%$. A p value < 0.05 was considered to be significant.

Founder Measures

Founder reproductive and metabolic changes were determined by a linear mixed model. Cohort of animals and replicate were fitted as covariates

F1 Male Measures

To compare F1 male offspring sperm parameters, reproductive organs and testosterone concentrations across the 5 treatments, linear mixed effect models were fitted. In the model father ID was included as a random effect to adjust for dependence in results between offspring from the same father and litter size as a dependent variable to compared litter size variations between and within the 5 treatments.

Correlations between F1 male offspring reproductive health and founder metabolic and reproductive health were determined by multiple regression analysis and corrected for multiple observations.

4.7 RESULTS

4.7.1 Effect of Diet and Exercise on Founder Metabolism and Sperm Parameters

Founders fed a HFD during the pre-intervention period had a 20.4% increase in body weight compared with founders fed a control diet (31.6 ± 0.69 vs. 26.3 ± 0.47 , $p < 0.05$). Post intervention CC, HH and HE founders continued to gain weight (13.6%, 15.8% and 6.4% respectively, Table 4.1), while interventions involving diet (HC and HCE) reduced founder body weight (-4.4% and -11.0% respectively, Table 4.1). Founder male post-intervention metabolic and reproductive phenotypes recapitulated previous findings (Mcpherson et al., 2013; Palmer et al., 2012). Diet interventions (HC and HCE) reduced adiposity (total and gonadal), serum cholesterol and glucose tolerance compared with HH founders ($p < 0.05$, Table 4.1). Founders who received exercise intervention alone (HE) maintained their increased serum cholesterol and insulin resistance (Table 4.1) while sustaining their pre intervention level of adiposity such that is was still increased compared with CC founders ($p < 0.05$, Table 4.1), however reduced compared with HH founders ($p < 0.05$, Table 4.1). Interventions including exercise (HE and HCE) improved glucose clearance compared with HH founders ($p < 0.05$, Table 4.1) with HE founders also reducing fasting glucose ($p < 0.05$, Table 4.1). All interventions reduced the number of sperm with tail defects compared with HH founders ($p < 0.05$, Table 4.1) while exercise interventions in founders (HE and HCE) additionally restored sperm motility ($p < 0.05$, Table 4.1). Diet intervention alone also increased serum testosterone concentrations compared with HH founders ($p < 0.05$, Table 4.1) restoring it to concentrations of CC founders.

4.7.2 Effect of Founder Diet and Exercise on F1 Conventional Sperm Parameters

HH founders produced F1 males with reduced progressively motile sperm compared with males born to CC founders ($p < 0.05$, Table 4.2). Diet intervention alone in founders (HC) increased the percentage of progressively motile sperm in F1 males compared with F1 males born by HH founders ($p < 0.05$, Table 4.2), restoring to level that of F1 males born by CC founders while exercise interventions in founders

with (HCE) or without dietary intervention (HE) did not improve percentage of progressively motile sperm in F1 males (Table 4.2). There was no change in the total proportion of motile sperm or proportion of immotile sperm from F1 males born from any founder treatment group (Table 4.2). Founder treatment group had no effect on the proportion of normal sperm morphology in F1 males (Table 4.2), although the proportion of head and tail defects in sperm were altered among groups ($p < 0.05$, Table 4.2). HH founders produced F1 males with reduced sperm counts compared with F1 males born by CC founders ($p < 0.05$, Table 4.2). There were no significant improvements to F1 sperm count from diet and/or exercise interventions in founders compared with F1 male born by HH founders (Table 4.2).

4.7.3 Effect of Founder Diet and Exercise on F1 Sperm Capacitation and Oocyte Binding

HH founders did not produce F1 males with any difference in sperm capacitation compared with CC founders (CC), concordant with previous reports using shorter feeding periods (Fullston et al., 2012). However, all diet and/or exercise interventions in founders (HC, HE and HCE) increased the percentage of capacitated F1 sperm compared with F1 males born to HH or CC founders ($p < 0.05$, Table 4.2), conversely reducing non capacitated and acrosome reacted sperm (Table 4.2).

HH founders produced F1 males with reduced sperm binding compared with F1 males born to CC founders ($p < 0.05$, Table 4.2). Diet intervention with (HCE) or without (HC) exercise in founders increased sperm binding compared with F1 males born from HH founders ($p < 0.05$, Table 4.2) with F1 males born to combined diet and exercise (HCE) intervention in founders exceeding numbers of F1 males born by CC founders ($p < 0.05$, Table 4.2). In contrast, exercise alone intervention (HE) in founders did not improve sperm binding in F1 males compared to born to HH founders ($p > 0.05$, Table 4.2).

4.7.4 Effect of Founder Diet and Exercise on F1 Sperm Mitochondrial Parameters

There was no effect of HH founders on F1 levels of sperm ROS compared with F1 males born to CC founders, which was previously reported in shorter feeding periods (Fullston et al., 2012). Diet intervention alone in founders (HC) reduced MSR positive sperm in F1 males compared with F1 males born from CC, HE and HCE founders ($p < 0.05$, Table 4.2). Once more a founder intervention limited solely to diet (HC) reduced the proportion of F1 sperm positive for high JC-1 compared with F1 males born to HH, and HCE founders ($p < 0.05$, Table 4.2), while exercise intervention alone (HE) had no effect (Table 4.2).

4.7.5 Effect of Founder Diet and Exercise on F1 Male Reproductive Body Composition and Serum Testosterone

There was no effect of founder treatment on F1 male body composition as assessed by total body weight, total adiposity, gonadal adiposity, seminal vesicle weights, testes weights or serum testosterone concentration ($p > 0.05$, Table 4.3). However, it must be noted that the similarities in testosterone results in offspring to that of their fathers was further confirmed by founder serum testosterone positively correlating with offspring serum testosterone (0.693, $p < 0.001$, Table 4.4).

4.7.6 Correlations of Founder Adiposity and Metabolic status with F1 Reproductive Function

We have previously shown that founder metabolites independent to treatment group correlated with founder male sperm function, embryo development and early fetal health markers (Mcpherson et al., 2013; Palmer et al., 2012) with perturbed sperm function seen in F1 males produced by founders with increased adiposity and serum cholesterol (Fullston et al., 2012). Given the variations of founder metabolites within treatments, the varied improvements to F1 male sperm parameters across treatments and the systematic changes to whole body physiology from obesity impacting on spermatogenesis (i.e.

hyperglycaemia) we further examined if measures of founder metabolic health correlated with F1 male reproductive measures. We hypothesised that the differing levels of adiposity and metabolic health in founders both between and within treatment groups could help explain their son's reproductive measures. Correlations highlighted that F1 male reproductive health (sperm motility, sperm binding, sperm count, total body weight, testes weights, seminal vesicle weights, gonadal adiposity and testosterone levels) is sensitive to founder gonadal adiposity, serum FFA/triglyceride/cholesterol concentrations, glucose tolerance and insulin sensitivity ($p < 0.05$, Table 4.4).

4.7.7 Correlations of Founder Reproductive Function with F1 Reproductive Function

Commonly observed sub-fertility phenotypes seen in obese men, such as reduced sperm motility, count and morphology have been associated with chromatin and epigenetic modifications in sperm (Hammoud et al., 2011; Iranpour et al., 2000). We hypothesised that sperm parameters in founders may indicate epigenetic modifications in sperm which might form the basis for their offspring inheriting similar sub fertility phenotypes. Correlations revealed that an F1 male's reproductive measures are also sensitive to a founder male's reproductive health (Table 4.4). For example founder sperm levels of normal morphology correlated positively with F1 testes weights ($p = 0.04$) and negatively with F1 sperm positive for MSR ($p < 0.01$, Table 4.4).

4.8 DISCUSSION

To date the end points for assessing the reversibility of paternal obesity and associated co-morbidities have been limited to hormone profiles (Bastounis et al., 1998; Hammoud et al., 2009; Strain et al., 1988), sperm function (Hakonsen et al., 2011; Reis et al., 2012) and extended as far as early embryo development and quality in rodent models of paternal obesity (McPherson et al., 2013). This is the first study to further determine if the altered reproductive phenotypes in male offspring that results from an obese father can be improved by lifestyle interventions in the father. I demonstrate that diet and/or exercise interventions in obese fathers can improve the reproductive health of male offspring in a mouse model, demonstrating that adiposity, metabolic and reproductive status of fathers influences their son's reproductive health. If translatable to the human, these data suggests a potential novel pre-conception intervention window for obese fathers to improve their male offspring's reproductive health via lifestyle interventions.

Sperm motility and concentration are standard parameters for assessing male fertility (WHO, 2010). While additional measures of sperm capacitation and binding (Franken & Oehninger, 2006; Liu et al., 2004) can be indications of sperm function, due to the essential nature of these processes for penetration into the oocyte during fertilisation (Johnson & Everitt, 2000). Further, sperm ROS concentrations are a further marker of sperm health, with increased levels in human sperm correlating with reduced fertilisation, impaired embryonic development and pregnancy loss (Dada et al., 2010; Gharagozloo & Aitken, 2011; Tunc et al., 2010; Zribi et al., 2011).

In a mouse model, it has been previously reported that in addition to effects on founder male sperm, male offspring born from HFD fathers also had reduced sperm motility, reduced sperm binding and

increased levels of ROS (Fullston et al., 2012). This study recapitulated the reduced sperm motility and sperm binding of male offspring born from HFD (HH) fathers, although we did not report similar increases in levels of ROS. This could be due to a number of reasons including duration of founder exposure to diet (10 weeks vs. 18 weeks) or different ROS detection method (DCFDA vs. MitoSox Red).

Diet intervention alone in obese founders restored adiposity, glucose homeostasis and cholesterol concentrations similar to human studies (Klop et al., 2013). These founders produced male offspring with improvements to sperm mitochondrial health (ROS and membrane potential) which were sometimes below levels of controls (CC), increased sperm motility, binding and capacitation compared with F1 males born to HH founders. This suggests that simple caloric restriction in obese males restores adiposity as well as glucose/lipid metabolism and can improve subsequent male offspring sperm function, which would potentially increase embryo development and pregnancy rates for this F1 generation. Combined diet and exercise interventions (HCE) in founders also increased the functional measure of sperm binding in their male offspring likely resulting from increased sperm capacitation and improvements to sperm motility which again suggests likely improvements to fertilisation.

Interestingly, the continuation of a HFD with an exercise intervention in founders (HE) showed the least improvements to offspring sperm function, with only slight improvements to sperm capacitation compared with F1 males from HH founders. Founder males in this group, maintained their pre intervention level of adiposity compared with founders undergoing diet interventions (HC or HCE) and controls (CC), indicating that the exercise regime saw the similar amount of calories expended as was ingested. HE founders also maintained their increased serum cholesterols likely resulting from the lipid dense diet consumed. This is a similar phenotype to that observed in exercise intervention alone in obese humans which have shown that exercise alone does not reduce adiposity levels (Dwyer-Lindgren

et al., 2013). This implies that improvements to these aspects of sperm function in male offspring maybe related to the adipose state and cholesterol concentrations of their father.

Increased scrotal heat due to increased adiposity in humans is associated with reduced sperm motility, morphology, increased sperm DNA damage and increased sperm oxidative stress (Paul et al., 2008a; Paul et al., 2008b; Shiraishi et al., 2010), parameters which have been independently linked with epigenetic changes to sperm (Hammoud et al., 2011; Iranpour et al., 2000; Tunc & Tremellen, 2009). Therefore as our males fed a HFD with (HE) maintain gonadal adiposity, increased scrotal heat could potentially underpin the transmission of altered offspring health seen. However, it should be noted that offspring from males fed a HFD with exercise (HE) interventions did have some improvements in sperm function (motility and morphology), suggesting that heat may not be the sole mechanism for the adverse changes to offspring sperm function seen in the current study.

The precise mechanisms that are responsible for the transmission of altered offspring health due to paternal obesity remain unidentified. Epigenetic, molecular and functional changes within the sperm either through changes to epigenetic marks to sperm DNA and sperm chromatin structure are clearly implicated. Direct insults to founder male sperm DNA such as irradiation induced sperm DNA damage, or gamete and somatic cell DNA damage induced by impaired intra-uterine environments from mothers (Adiga et al., 2010) leads to impaired reproductive function (Fullston et al., 2012) and changes to body composition in first generation offspring (Dunn & Bale, 2011). This links the molecular composition of father's sperm at the time of conception to the health of the next generation. There is emerging evidence that increased adiposity in males impacts the epigenetic status of their sperm. Correlations with markers of founder metabolic health and offspring sperm function determined that founder gonadal adiposity negatively correlated with offspring's sperm count and positively with the proportion of non-

progressive motile sperm independent of treatment group. Global measures of methylation in testes and elongating spermatids showed that DNA from cells were hypomethylated in obese male mice (Fullston et al., 2013). Obesity additionally alters the methylation status of DNA in other tissues (Barres & Zierath, 2011). Whether the changes to reproductive function in our male offspring results from a global alteration to de novo methylation or site specific methylation at paternally imprinting gene loci in the obese father's sperm, remains to be determined. Sperm also harbour a vast array of small non-coding RNAs which are thought to be important for early embryo and fetal development (Liu et al., 2012) and have been previously shown to be altered in sperm of obese rodents (Fullston et al., 2013). Interestingly, changes to circulating serum microRNAs caused from obesity were restored through weight loss (Ortega et al., 2013) providing evidence that interventions to health can change the microRNA content of specific tissues. Whether the diet interventions (HC and HCE) which induced weight loss could also restore the microRNA content of testes and sperm and identify a potential part of the mechanism that might improve reproductive function in offspring remains to be determined.

I have previously demonstrated that metabolic markers in fathers including plasma lipids, glucose homeostasis and insulin sensitivity can impact on both their sperm function and F1 embryo development independently of adiposity (Mcpherson et al., 2013; Palmer et al., 2012). In this study, founder plasma measures of glucose, insulin and fatty acid metabolism which showed the biggest restoration in diet interventions displayed the strongest correlations with male offspring sperm function and body composition including total body weight, testes and seminal vesicle weights. Increased circulating concentrations of serum lipids in men, have been associated with increased ROS in sperm (Koppers et al., 2010) and changes to the global methylation of sperm DNA (Tunc & Tremellen, 2009). Additionally fasting plasma insulin concentrations in fathers can predict umbilical cord insulin concentrations and therefore offspring fetal size (Shields et al., 2006). Together these data suggest that pre-conception paternal metabolic status may alter the epigenetic signature of sperm, programming

offspring phenotypes thereby providing part explanations for our correlations between markers of paternal metabolic state with their son's reproductive phenotypes.

Recently it has been demonstrated that the proteomic, mRNA and microRNA content of sperm/testes are altered in obese rodents (Daxinger & Whitelaw, 2012; Fullston et al., 2013; Ghanayem et al., 2010; Kriegel et al., 2009; Palmer et al., 2011; Youngson & Whitelaw, 2011). In agreement with the previous study (Palmer et al., 2012), I report here that diet and exercise interventions in founders improves their sperm function, suggesting lifestyle interventions may at least in part restore the micro-molecular environment of testes and/or epididymis thereby restoring the molecular makeup of sperm. The concept that diet and exercise interventions restore the micro-environments of reproductive organs and therefore restores the molecular determinants responsible for programming offspring health in sperm is supported by the associations of founder sperm parameters of morphology, count and motility with F1 reproductive parameters of sperm morphology, capacitation, testes weights and serum testosterone concentrations.

The model described of feeding a HFD to induce obesity mimics some aspects of human obesity including increased adiposity serum cholesterol and altered glucose and insulin homeostasis (Klop et al., 2013), with diet and exercise interventions used previously shown to restore sperm function and DNA integrity (Palmer et al., 2012), similar to those reported improvements to sperm parameters found in diet and exercise interventions in human studies (Hakonsen et al., 2011). Due to the similar metabolic and sperm function phenotypes of both rodent models of obesity and obese humans with diet and exercise interventions suggests that the changes and or improvements to the testicular microenvironment is likely similar and therefore the molecular changes proposed to founder sperm to induce F1 male offspring phenotypes could act through similar pathways and be translatable to humans. However the differences in sperm molecular makeup between both mouse and humans still need to be

noted. For example it has been proposed that human sperm are much more sensitive to environmental perturbations than mouse sperm due to their higher concentrations of histone retention (~15% human (Gatewood et al., 1987) compared with ~1% in the mouse (Balhorn et al., 1977)) which are capable of normal histone modifications (Farthing et al., 2008) and are retained at loci that contain genes important for early embryogenesis (Farthing et al., 2008). The similarities between both human and rodent models of male obesity suggest that these results would likely be translatable to humans; however confirmation studies in a human cohort would still be warranted.

This report shows that impaired offspring reproductive health resulting from paternal obesity can be improved through weight loss and restoration of metabolic health in fathers via diet interventions. Additionally paternal markers of adiposity, metabolism and reproductive function may also indicate their son's reproductive function, thus potentially highlighting a novel intervention window for improving reproductive health outcomes in the next generation. The direct sperm molecular mechanism responsible for this improvement transmitted via the father and if this can be replicated in humans to improve reproductive outcomes in the next generation, warrants further investigation.

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4.11 TABLES

Table 4.1: Effect of diet and exercise on founder adiposity, serum metabolites and reproductive measures

	Diet/Intervention				
	CC	HH	HC	HE	HCE
Pre intervention					
Body weight (g)	26.3 ± 0.47 ^a	31.5 ± 0.82 ^b	31.8 ± 1.5 ^b	30.8 ± 1.3 ^b	32.6 ± 1.9 ^b
Total adiposity (% of body weight)	14.6 ± 0.92 ^a	24.7 ± 1.7 ^b	24.7 ± 2.0 ^b	24.5 ± 1.7 ^b	26.0 ± 1.6 ^b
Post intervention					
Final body weight (g)	29.9 ± 0.58 ^a	36.3 ± 1.14 ^b	30.4 ± 1.6 ^a	32.8 ± 1.2 ^a	29.2 ± 1.1 ^a
Total adiposity (% of body weight)	16.4 ± 0.88 ^a	28.4 ± 2.00 ^b	18.1 ± 2.15 ^a	24.0 ± 1.19 ^c	16.8 ± 1.18 ^a
Gonadal Adiposity (% total body weight)	2.46 ± 0.2 ^a	4.82 ± 0.28 ^b	2.82 ± 0.27 ^a	4.06 ± 0.27 ^c	2.77 ± 0.28 ^a
Glucose (mmol/L)	10.0 ± 0.51 ^{ab}	10.6 ± 0.42 ^a	9.8 ± 0.47 ^{ab}	9.4 ± 0.48 ^b	9.7 ± 0.44 ^{ab}
Cholesterol (mmol/L)	3.24 ± 0.36 ^{ac}	4.11 ± 0.30 ^b	3.17 ± 0.34 ^{ac}	3.82 ± 0.34 ^{ab}	2.39 ± 0.36 ^c
FFA (mmol/L)	0.69 ± 0.05 ^a	0.64 ± 0.04 ^{ab}	0.61 ± 0.04 ^{ab}	0.65 ± 0.04 ^{ab}	0.55 ± 0.05 ^b
Triglycerides (mmol/L)	0.77 ± 0.06	0.83 ± 0.05	0.78 ± 0.06	0.83 ± 0.06	0.80 ± 0.06
Glucose (AUC)	1797 ± 122 ^a	2111 ± 100 ^b	1814 ± 123 ^a	1695 ± 113 ^a	1787 ± 103 ^a
Insulin (AAC)	167 ± 13.4 ^a	121 ± 10.1 ^b	135 ± 12.2 ^{ab}	75 ± 12.4 ^c	111 ± 12.2 ^b
Reproductive Measures					
Testosterone (ng/L)	0.36 ± 0.14 ^a	0.10 ± 0.03 ^b	0.38 ± 0.15 ^a	0.28 ± 0.11 ^{ab}	0.17 ± 0.04 ^{ab}
Testes weights (g)	0.16 ± 0.05	0.17 ± 0.04	0.16 ± 0.04	0.16 ± 0.03	0.16 ± 0.05
Seminal vesicle weights (g)	0.37 ± 0.03	0.39 ± 0.03	0.36 ± 0.02	0.34 ± 0.03	0.36 ± 0.03
Sperm count (10 ⁶)	21.7 ± 3.1	20.0 ± 3.3	16.9 ± 3.1	22.2 ± 2.9	22.1 ± 3.3
Progressive sperm motility (%)	31.9 ± 5.7	26.2 ± 6.1	28.9 ± 5.7	38.6 ± 5.3	39.8 ± 6.1
Total sperm motility (%)	71.3 ± 3.9 ^a	58.3 ± 4.2 ^b	66.6 ± 3.9 ^{ab}	74.5 ± 3.6 ^a	72.9 ± 4.2 ^a
Sperm normal forms (%)	56.9 ± 1.8 ^a	55.9 ± 3.1 ^a	59.3 ± 1.9 ^{ab}	64.1 ± 2.2 ^b	58.7 ± 3.7 ^{ab}
Sperm with tail defects (%)	30.8 ± 2.1 ^a	36.4 ± 3.0 ^b	29.9 ± 1.3 ^a	29.7 ± 2.7 ^a	30.7 ± 2.5 ^a
Sperm with head defects (%)	12.2 ± 2.4 ^a	8.6 ± 1.9 ^{ab}	10.6 ± 1.1 ^{ab}	6.1 ± 0.7 ^b	10.51 ± 0.7 ^{ab}

Data is representative of 8 founder males per treatment group with sperm morphology representative of 4 founder males per treatment group and expressed per male. Testes weights were a combined weight of both left and right testis. Data was analysed by a GLM with cohort and replicated fitted as covariates. Different letters denote significance at $p < 0.05$.

Table 4.2: Effect of founder diet and exercise on f1 sperm function

F1 Sperm Measures	Founder Diet/Intervention				
	CC	HH	HC	HE	HCE
Conventional Sperm Parameters					
Progressive sperm motility (%)	32.2 ± 6.4 ^a	18.1 ± 5.1 ^b	31.2 ± 6.0 ^a	22.1 ± 6.0 ^{ab}	23.0 ± 5.6 ^{ab}
Total sperm motility (%)	53.1 ± 5.2	45.9 ± 4.3	49.2 ± 4.9	45.6 ± 4.6	46.9 ± 4.4
Immotile sperm (%)	48.1 ± 4.7	54.5 ± 3.7	48.6 ± 4.7	56.7 ± 4.3	54.6 ± 4.1
Sperm normal morphology (%)	56.7 ± 4.3	50.6 ± 3.6	50.7 ± 4.1	51.8 ± 4.0	49.1 ± 3.7
Sperm head defect (%)	10.8 ± 1.5 ^{ab}	11.6 ± 1.3 ^{ab}	14.8 ± 1.5 ^a	9.4 ± 1.4 ^b	10.4 ± 1.3 ^b
Sperm tail defect (%)	32.3 ± 3.4 ^a	37.7 ± 2.8 ^{ab}	34.4 ± 3.3 ^{ab}	38.6 ± 3.2 ^{ab}	40.4 ± 2.9 ^b
Sperm count (10 ⁶)	12.3 ± 1.1 ^a	10.0 ± 0.9 ^b	11.1 ± 1.2 ^{ab}	8.2 ± 1.0 ^b	10.2 ± 1.1 ^{ab}
Sperm Capacitation and Oocyte binding					
Sperm non capacitated (%)	2.36 ± 0.38 ^{ab}	3.04 ± 0.30 ^a	2.05 ± 0.34 ^b	1.54 ± 0.31 ^b	1.27 ± 0.33 ^b
Sperm capacitated (%)	94.3 ± 0.6 ^a	94.6 ± 0.5 ^a	96.5 ± 0.6 ^b	96.2 ± 0.5 ^b	96.6 ± 0.6 ^b
Sperm acrosome reacted (%)	3.33 ± 0.39 ^a	2.35 ± 0.25 ^{ac}	1.42 ± 0.28 ^{bc}	2.24 ± 0.26 ^c	2.12 ± 0.64 ^c
Sperm bound to MII oocyte	48.0 ± 1.2 ^a	37.6 ± 1.3 ^b	42.9 ± 1.5 ^c	40.7 ± 1.2 ^{bc}	52.9 ± 1.5 ^d
Sperm Mitochondrial Function					
Sperm positive for MSR (%)	61.3 ± 4.3 ^a	57.1 ± 3.6 ^{ab}	49.6 ± 4.0 ^b	60.5 ± 4.0 ^a	60.8 ± 3.6 ^a
Vitality (%)	58.8 ± 4.9	60.7 ± 4.1	65.5 ± 4.6	63.0 ± 4.3	56.9 ± 4.1
Sperm positive for high JC-1 (%)	52.4 ± 4.1 ^{ab}	55.6 ± 3.4 ^a	48.1 ± 3.8 ^b	51.3 ± 3.8 ^{ab}	60.3 ± 3.3 ^a

8 F1 CC and HCE males and 10 HH, HC and HE males were analysed and expressed per animal representative of 6 CC and HCE founders and 7 HH, HC and HE founders. MSR H2O2 positive control was 73.4% and MSR negative control was 4.5%. CCCP negative control for high JC-1 was 17.1% and PI only negative control for high JC-1 was 2.7%. Data was analysed by a linear mixed effects model with father ID added as a random effect and litter size as a fixed variable. Different letters denote significance at p<0.05.

Table 4.3: Effect of founder diet and exercise on f1 male reproductive organs and testosterone

F1 weight	Founder Diet/Intervention				
	CC	HH	HC	HE	HCE
Total body weight (g)	22.0 ± 0.3	22.1 ± 0.3	21.8 ± 0.3	21.3 ± 0.3	22.7 ± 0.3
Total adiposity (g) [#]	1.65 ± 0.06	1.57 ± 0.07	1.51 ± 0.07	1.51 ± 0.06	1.53 ± 0.06
Gonadal adiposity (g) [#]	0.146 ± 0.016	0.152 ± 0.014	0.158 ± 0.016	0.168 ± 0.015	0.145 ± 0.016
Testes (g) [#]	0.145 ± 0.003	0.155 ± 0.003	0.154 ± 0.003	0.149 ± 0.003	0.153 ± 0.003
Seminal Vesicles (g) [#]	0.149 ± 0.010	0.155 ± 0.009	0.42 ± 0.011	0.150 ± 0.010	0.165 ± 0.010
Testosterone (ng/L)	0.167 ± 0.061	0.109 ± 0.054	0.166 ± 0.066	0.112 ± 0.073	0.114 ± 0.057

8 F1 CC and HCE males and 10 F1 HH, HC and HE males were analysed and expressed per male representative of 6 CC and HCE founders and 7 HH, HC and HE founders. Testes weights were a combined weight of both left and right testis. [#]No significant differences in total or gonadal adiposity, testes or seminal vesicle weights when expressed as percentage of total body weight. Data was analysed by a linear mixed effects model with father ID added as a random effect and litter size as a fixed variable.

Table 4.4: Founder metabolic and reproductive health correlate with f1 reproductive measures

F1 Measure	Founder Measure	Correlation Coefficient	P Value
<i>Sperm Function</i>			
Progressive sperm motility (%)	FFA (mmol/L)	-0.557	0.024
Non progressive sperm motility (%)	Gonadal adiposity (%)	0.489	0.045
Sperm head defect (%)	Sperm count (10 ⁶)	-0.539	0.029
Sperm positive for MSR (%)	Progressive sperm motility (%)	-0.570	0.027
	Total motile sperm (%)	-0.829	<0.001
	Sperm normal morphology (%)	-0.664	0.009
	Sperm head defect (%)	0.474	0.060
Sperm bound to MII oocyte	FFA (mmol/L)	-0.529	0.038
	Glucose (mmol/L)	-0.482	0.056
	Glucose (AUC)	-0.551	0.032
	Seminal vesicle weights (g)	0.479	0.058
Sperm non capacitated (%)	Progressive sperm motility (%)	-0.565	0.028
	Total motile sperm (%)	-0.820	<0.001
	Sperm normal morphology (%)	-0.475	0.060
Sperm capacitated (%)	Progressive sperm motility (%)	0.572	0.026
	Total motile sperm (%)	0.621	0.016
	Sperm count (10 ⁶)	-0.574	0.026
Sperm acrosome reacted (%)	Sperm count (10 ⁶)	0.693	0.006
Sperm count (10 ⁶)	FFA (mmol/L)	-0.503	0.040
	Triglycerides (mmol/L)	-0.440	0.060
	Gonadal adiposity (%)	-0.468	0.050
	Progressive sperm motility (%)	0.808	<0.001
	Testes weights (g)	0.644	0.009
<i>Reproductive Organs and Testosterone</i>			
Total body weight (g)	Glucose (mmol/L)	-0.512	0.025
	Glucose (AUC)	-0.487	0.033
	Total motile sperm (%)	-0.433	0.050
Testes (%)	Glucose (mmol/L)	0.466	0.040
	Glucose (AUC)	0.521	0.023
	Progressive sperm motility (%)	0.607	0.008
	Sperm normal morphology (%)	0.623	0.040
Seminal vesicles (g)	Glucose (mmol/L)	-0.430	0.046
	Glucose (AUC)	-0.443	0.049
	Seminal vesicles (g)	0.478	0.036
Gonadal adiposity (%)	FFA (mmol/L)	0.487	0.033
	Cholesterol (mmol/L)	0.486	0.040
Testosterone (ng/L)	Sperm normal morphology (%)	-0.606	0.024
	Sperm tail defect (%)	0.517	0.052
	Testosterone (ng/L)	0.693	<0.001

Correlations were determined by multiple regression analysis and corrected for multiple observations.

4.12 SUPPLEMENTARY TABLE

Table S4.1: Composition of animal diets

Ingredients	CD (SF04-057)	HFD (SF00-219)
	Control Diet	Harlan Teklad TD88137 Equival
Sucrose (g/100g)	34.1	34.1
Casein (Acid) (g/100g)	19.5	19.5
Canola Oil (g/100g)	6.0	-
Clarified Butter (g/100g)	-	21.0
Cellulose (g/100g)	5.0	5.0
Wheat starch (g/100g)	30.5	15.5
Minerals (g/100g)	4.9	4.9
Digestible energy (MJ/kg)	16.1	19.4
Digestible energy from lipids (%)	21.0	40.0
Digestible energy form protein (%)	14.0	17.0
Digestible energy from carbohydrates (%)	65.0	43.0

CD = Control diet (6% fat) and HFD = high fat diet (21% fat).

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5 Preconception diet and exercise interventions in obese fathers rescues sperm microRNA profile, insulin resistance and obesity in female offspring

This chapter is written for submission,

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5.2 STATEMENT OF AUTHORSHIP

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5.3 LINKING TO CHAPTER 5

The data in Chapter 4 focussed on understanding if diet and/or exercise interventions in the father could improve the known negative impact on their son's reproductive function. A second reported consequence of male obesity in rodents is perturbed female offspring metabolic health via increased adiposity and pancreatic islet cell dysfunction, which persists in 2nd generation offspring through the maternal lineage. The incidence of adolescent obesity and type II diabetes has tripled in the past 30 years in Western communities; therefore novel interventions targeting preconception father's health provide an additional window of opportunity for improving the metabolic health of subsequent generations with a particular emphasis at least in rodent models on female offspring health. The transmission of altered metabolic health in female offspring must be delivered by the sperm at fertilisation. Changes to sperm microRNAs due to male obesity have previously been shown to converge on pathways regulating metabolic disease, ROS production, DNA replication, NF-κB signalling, p53 signalling, lipid metabolism, spermatogenesis and embryo development. Therefore determining if preconception diet/exercise in obese fathers may restore altered microRNA profile specifically targeting X-linked microRNAs will begin to unravel the underlying molecular mechanisms for altered offspring transmission. Therefore this study assessed the effects of diet and intervention in the obese father on the metabolic phenotype of their daughters and further established whether preconception diet and/or exercise interventions in obese fathers could restore X-linked sperm microRNAs.

5.4 ABSTRACT

Obesity and type II diabetes are increasingly prevalent across all demographics. Paternal obesity in humans and rodents has been shown to increase obesity and alter insulin sensitivity in female offspring. It remains to be determined whether detrimental offspring phenotypes can be reversed through targeted lifestyle interventions in the father. We demonstrate using a mouse model that diet and/or exercise interventions in obese founder males restored insulin sensitivity and adiposity in female offspring. Founder diet and/or exercise also restored X-linked microRNA abundance in sperm. These microRNAs target genes that regulate cell cycle and apoptosis, pathways central to oocyte activation and early embryogenesis following fertilisation. Additionally, founder glycaemia which were improved by diet/exercise interventions also predicted sperm microRNA profiles as well as offspring phenotypes independent of adiposity. Thus improvements to paternal metabolic health during specific sperm maturation windows can partially ameliorate aberrant epigenetic signals in sperm and improve the health of subsequent female offspring. We demonstrated that peri-conception targeting of the father's health improves the health of female offspring, reducing metabolic syndrome and obesity, providing a novel intervention window for addressing obesity and type II diabetes in the next generation.

5.5 ONE SENTENCE SUMMARY

Diet and exercise interventions in obese fathers can partially restore insulin sensitivity and adiposity in female offspring concomitant with microRNAs profile of sperm.

5.6 INTRODUCTION

In the past 30 years the incidence of obesity has more than doubled in children (4-11 years) and tripled in adolescence (12-19 years) (Ogden, et al., 2012). Concomitantly, the prevalence of type II diabetes among children and adolescents has increased 33% in the past decade and predicted to increase another 33% by 2050 (Imperatore, et al., 2012). Entering adulthood these children are more likely to be obese, have type II diabetes and have increased risks of cancer, stroke, heart disease and osteoarthritis (Ogden, et al., 2012). 80% of obese children with type II diabetes have one affected parent (Ogden, et al., 2012), suggesting shared genetics, a shared environment or developmental programming by maternal or paternal health at the earliest stages of life. It is widely accepted that maternal BMI and glucose status pre-conception, during gestation or lactation can program children for obesity and diabetes (O'Reilly, et al., 2013). Recent studies now suggest that paternal health at conception can also impact children's health. Obese fathers are more likely to father an obese child (Li, et al., 2009) with altered IGF-2 methylation patterns in cord blood (Soubry, et al., 2013). Diet induced male obesity in the mouse, induces obesity, insulin resistance and glucose intolerance in female offspring through two generations, while in the rat, when accompanied by diabetes, diet induced obesity impairs glucose tolerance due to pancreatic islet dysfunction in female offspring (Fullston, et al., 2013, Ng, et al., 2010). These are some of the first evidence that paternal metabolic health at conception programs adverse metabolic health outcomes in female offspring. However, the capacity for these metabolic phenotypes to be rescued through short term interventions in the father is unknown.

In Western societies, greater than 70% of reproductively aged men are overweight or obese (i.e. 74% in USA) (Flegal, et al., 2012). Weight loss via diet and exercise interventions in obese men is known to improve glucose control and insulin action (Beltaifa, et al., 2011), reproductive function (i.e. sexual function, hormone profiles and basic sperm parameters) (Hakonsen, et al., 2011) and induce epigenetic

modifications (i.e. methylation and microRNA profiles of white blood cells in circulating blood (Ortega, et al., 2013, Milagro, et al., 2011). Diet and/or exercise interventions in rodent models of male obesity reduces adiposity, improves metabolic profiles and sperm function (Palmer, et al., 2012). Concomitantly, this normalizes early embryo and fetal development (McPherson, et al., 2013), demonstrating that improved peri-conception founder metabolic health rescues the early embryo and fetus from otherwise adverse outcomes. Here I show that improvements to metabolic health and/or adiposity status and X-linked microRNAs in sperm of obese fathers by short term diet and exercise interventions rescue female offspring from programmed insulin resistance, glucose intolerance and obesity, therefore identifying a novel intervention target and window during spermatogenesis, for improving obesity and metabolic syndrome in the next generation.

5.7 ADIPOSITY AND METABOLIC HEALTH OF OFFSPRING IN RESPONSE TO FOUNDER DIET AND/OR EXERCISE INTERVENTIONS

5.7.1 High Fat Diet (HH):

5.7.1.1 Founders

Feeding of a high fat diet (HFD, Table S5.1) for 18 weeks increased weight (48%) (Fig S5.1), induced obesity (39%), hypercholesterolemia (30%), hyperinsulinemia (ITT, 67%), hyperleptinemia (75%), increased plasma corticosterone (15%) and induced a pro-inflammatory state (21%, $p < 0.05$, Table S5.2), similar to previously reported phenotypes for this model (Palmer, et al., 2012, McPherson, et al., 2013).

5.7.1.2 Offspring

Female offspring of HH founders gained more weight during the neonatal and post natal period and were heavier than female offspring of CC founders by day 12, which persisted until weaning ($p < 0.05$,

Fig S5.2). Consistent with the previous mouse study (Fullston, et al., 2013) HH female offspring accumulated more site specific adiposity depots over age (gonadal, renal, and dorsal) to be increased by 19% at 28 weeks ($p < 0.05$, Fig 5.1A, 5.1B, 5.1C and 5.1D). Although in HH female offspring, increased site specific adipose depot masses were not evident until 28 weeks, hypertrophic adipocytes ($p < 0.05$, Fig 5.1E) and a decrease in plasma adiponectin occurred as early as 10 weeks ($p < 0.05$, Fig 5.1F). Intriguingly, this altered adipocyte phenotype prior to the onset of obesity, is consistent with a recent observation in mice, that a paternal HFD changes the transcriptome of adipose tissue in young female offspring, which are associated with pathways typical of obesity, including mitochondrial and cellular response to stress, telomerase signalling, cell death and survival, cell cycle, cellular growth and proliferation and cancer (Ng, et al., 2014). HH female offspring were insulin resistant at both 9 and 17 weeks ($p < 0.05$, Fig 5.2A, 5.2B, 5.2D) and secreted more insulin during a glucose challenge, while maintaining normal glycaemia at 16 weeks ($p < 0.05$, Fig 5.2F, 5.2H, 5.2I, 5.2J, 5.2K, 5.2L) consistent with previous findings (Fullston, et al., 2013, Ng, et al., 2010). HH female offspring also had increased plasma triglycerides at both 10 and 18 weeks ($p < 0.05$, Fig 5.1G). By 27-28 weeks, the insulin resistance and hypertriglyceridemia of HH female offspring was similar to that of CC female offspring (Fig 5.2C, 5.2D, 5.1G and Table S5.4). As C57BL6 mice are prone to metabolic disease (Rossmeisl, et al., 2003) it appears that the genetic propensity for metabolic disease in this strain was made evident earlier in female offspring born to HH founders, suggesting an increased rapid deterioration of these metabolic measures over age from founder HFD feeding.

5.7.2 Diet alone (HC):

5.7.2.1 Founders

Diet intervention alone in founders normalized weight (Fig S5.1), adiposity, plasma insulin, cholesterol and leptin, but did not normalize plasma corticosterone (26%) or C - reactive protein (15%) which both remained elevated (Table S5.2).

5.7.2.2 Offspring

HC increased weight gain during the neonatal and post natal periods in female offspring, towards that of CC offspring (Fig S5.2D), however HC exhibited a reduced body weight compared with female offspring of HH and CC founders until 4 weeks ($p < 0.05$, Fig S5.3C). This smaller pre weaning size was associated with increased adipose depots at 8 weeks (gonadal and dorsal) compared with both HH and CC female offspring ($p < 0.05$, Fig 5.1B and Table S5.3), which was normalized by 18 weeks (Fig 5.1B) and reduced by 28 weeks compared with HH female offspring ($p < 0.05$, Fig 5.1B) even though total adiposity remained increased compared with HH female offspring ($p < 0.05$, Table S5.5). Adipocyte cell size was restored to that of CC offspring at all ages (Fig 5.2C), with concomitant restoration of plasma adiponectin at 18 weeks (Fig 5.2D). Female offspring had partial restoration of insulin secretion at 16 weeks of age maintaining their glucose concentrations during a glucose challenge (Fig 5.2I, 5.2J, 5.2K and 5.2L), however were hyperglycemic in a fasted state compared to CC female offspring ($p < 0.05$, Table S5.4), which was reversed by 28 weeks ($p < 0.05$, Table S5.5). Females remained insulin resistant at 9 weeks ($p < 0.05$, Fig 5.2E and 5.2H) which was partially restored at 17 weeks being not different to either CC or HH female offspring (Fig 5.2F and 5.2H). Partial restoration of plasma triglycerides was also evident at both 10 and 18 weeks (Fig 5.1G), with significant reductions to plasma cholesterol compared with CC female offspring at 18 and 28 weeks ($p < 0.05$, Table 5.S4 and 5.S5) with increases to FFAs also evident at 18 weeks ($p < 0.05$, Table S5.4). Interestingly, HC female offspring had increased corticosterone at 18 weeks ($p < 0.05$, Table S5.4), which was not present at either 10 or 28 weeks (Table S5.3 and S5.5). These data imply that while there were some improvements to female offspring site specific adiposity, adipocyte size and health through diet interventions in their fathers, residual impacts of founder HFD was still evident in the programming of female offspring metabolic syndromes.

5.7.3 Exercise alone (HE):

5.7.3.1 Founders

Exercise intervention in founders normalize body weight (Fig S5.1) however maintained increased adiposity (24%), hyperinsulinemia (28%) and hypercholesterolemia (23%) (Table S5.2). Although these founders did reduce plasma leptin and C - reactive protein concentrations to those of CC with partially reductions to plasma corticosterone (Table S5.2). Additionally both fasting plasma glucose and glucose tolerance were improved compared with both HH and CC founders (Table S5.2).

5.7.3.2 Offspring

Female offspring born to HE founders had restoration of day 5 and 7 neonatal weights (Fig S5.2B) however were heavier than CC female offspring by 21 days old ($p < 0.05$, Fig S5.2B) and had an increase in weight gained pre weaning ($p < 0.05$, Fig S5.2D). This increased weight gain was also evident between 8 and 16 weeks ($p < 0.05$, Fig S5.3C) although post weaning weights were not altered (Fig S5.3A). HE female offspring had increased site specific adipose masses at 10 weeks (gonadal and renal) compared with CC female offspring ($p < 0.05$, Fig 5.1B and 5.1D), however these were restored by 28 weeks (gonadal, omental and dorsal) and significantly reduced compared to HH female offspring ($p < 0.05$, Fig 5.1B, Table S5.5). These changes to adipose mass were mirrored by adipocyte cell size, with hypertrophic adipocytes at 10 weeks (Fig 5.1E) but full restoration to that of CC offspring by 18 weeks (Fig 5.1E) with improvements to adipocyte specific adiponectin concentrations evident as early as 10 weeks (Fig 5.1F). Additionally fasting leptin concentrations were also reduced in HE female offspring at 28 weeks compared with HH female offspring ($p < 0.05$, Table S5.5) likely as a result of their smaller adipocytes (Fig 5.1E). Females had lower fasting glucose concentrations at 10 weeks which coincided with improved glucose tolerance at 8 weeks of age (Fig 5.2E, 5.2H and Table S5.3) and restoration of insulin secretion during a glucose challenge at 16 weeks (Fig 5.2I, 5.2J, 5.2K, 5.2L). This improved glucose regulation and fasting glucose concentrations were maintained at 26 to 28 weeks but only compared with CC offspring ($p < 0.05$, Fig 5.2G, 5.2F and Table S5.5). Insulin sensitivity was

restored as early as 9 weeks of age (Fig 5.2A, 5.2B and 5.2D) with further reductions in fasting insulin compared with HH offspring at 28 weeks ($p < 0.05$, Table S5.5). Female offspring born to HE founders also has improvements to fasting lipids, with partial restoration of triglycerides at 10 weeks (Fig 5.1G) with full restoration by 18 weeks which coincided with additional improvements to FFAs (Table S5.4). By 27-28 weeks their improved insulin sensitivity was lost (Figure 5.1C and 5.1D), which explains in part their increases to plasma FFA and triglycerides observed (Fig 5.1G and Table S5.5). Similar to HC female offspring, HE female offspring also had increases in corticosterone at 18 weeks ($p < 0.05$, Table S5.4) which was not present at either 10 or 28 weeks (Table S5.3 and S5.5).

5.7.4 Combined diet/exercise (HCE):

5.7.4.1 Founders

Combined diet/exercise interventions in founders restored weight (Fig S5.1), adiposity, plasma cholesterol, leptin and C - reactive protein, improved glucose concentrations and glucose tolerance compared with both HH and control founders, however maintained increased corticosterone (20%) (Table S5.2).

5.7.4.2 Offspring

Female offspring born to HCE founders had restored day 5 neonatal weights (Fig S5.1C), although total weight gained pre weaning was significantly increased compared with CC and HH female offspring ($p < 0.05$, Fig S5.1D). This increased growth rate continued post weaning (Fig S5.2B) although total body weight was not different at each weekly time point (Fig S5.2A). Total adiposity was decreased at 10 weeks compared to HH offspring ($p < 0.05$, Table S5.3), however with aging individual adipose depots increased in mass such that by 28 weeks were not different to HH female offspring (Table S5.5). Changes to adipocyte health were similar to the changes found in adiposity, with restoration of size and adiponectin concentrations at 10 weeks (Fig 5.1B and 5.1C) with restoration of adiponectin concentrations only maintained until 18 weeks (Fig 5.1C), although increases to adipocyte cell size were

seen at this same time (Fig 5.1B). Female offspring had limited improvements to insulin secretion at 16 weeks of age secreting more insulin to maintain glucose concentrations during a glucose challenge (Fig 5.2I, 5.2J, 5.2K and 5.2L), however by 28 weeks females had lower fasting glucose concentrations compared with CC female offspring ($p < 0.05$, Table S5.5). Insulin sensitivity was restored in female offspring at 9 weeks (Figure, 5.2A and 5.2D), however, this was not maintained at 17 weeks (Fig 5.2B and 5.2D). Female offspring had increases to plasma cholesterol at 10 weeks (Table S5.3), as well as liver weights and plasma triglycerides at 18 weeks (Table S5.4), suggesting that their reduced insulin sensitivity may have been caused by increased lipid accumulation in adipocytes and adipose accumulation in the liver thereby altering metabolic function. However, by 26-28 weeks fasted FFAs, insulin and triglycerides were restored which coincided with normal weight gain between 16 weeks and 28 weeks (Figure S5.2D and Table S5.5). Similar to both HC and HE female offspring, HCE female offspring also had increased corticosterone at 18 weeks ($p < 0.05$, Table S5.4) which was not present at either 10 or 28 weeks (Table S5.3 and S5.5).

5.8 PATERNAL EPIGENETIC SIGNALS: SPERM MICRORNA PROFILES

Any improvements to offspring health transmitted through the father must be delivered to the developing embryo through contents of the ejaculate at mating (i.e. sperm and seminal plasma). Sperm deliver a vast array of RNAs, including microRNAs to the oocyte at fertilisation that can influence first cleavage stage, embryo development and the phenotype of subsequent offspring (Jodar, et al., 2013). Due to the transmission pattern of the most acute metabolic phenotypes seen in our original study (Fullston, et al., 2013) (grandfather to daughter to grandson) and that a HFD altered microRNA abundance in sperm (Fullston, et al., 2013) I assessed the levels of X-linked sperm microRNA in males fed a CD or HFD for 10 weeks by array card (Table S5.6). Eight X-linked sperm microRNAs (Table S5.7) were shown to have differential abundance ($p \leq 0.05$; fold change ≥ 1.5). The abundance of these eight X-linked diet

sensitive microRNAs were then analysed in a cohort of diet and exercise intervention mice to determine if prolonged high fat exposure altered the abundance of these microRNAs and whether diet and/or exercise could restore levels. 4/8 microRNAs (mir-503, mir-534-3p, mir-456b-5p, mir-652) had altered abundance with prolonged HFD exposure (HH) compared with controls (CC) ($p < 0.04$, Fig 5.4A, 5.4B, 5.4C and 5.4D), with only 2/4 microRNAs (mir-543-3p and mir-456-5p) showing the same direction of change. The remaining 4/8 microRNAs (mir-871, mir-465a-3p, mir-743b-3p and mir-883a-5p) altered abundance levels were not altered in prolonged HFD exposure (HH) (Fig S5.4) but were altered in diet and/or exercise intervention groups (HC, HE and HCE). This suggests a unique sperm microRNA profile that is sensitive to diet, diet duration and change in diet and exercise.

MiR-503 abundance was restored in sperm from founders undergoing exercise intervention (HE and HCE, Fig 5.4A). MiR-503 has confirmed cyclin family mRNA targets involved in cell cycle regulation, critical for G1 to S phase progression (Table S5.7). Cyclins are required after fertilisation for oocyte meiotic resumption and first stage cleavage events in the developing embryo (Wolgemuth, 2011). HFD fed male rodents produced embryos with delayed cleavage and cavitation (Binder, et al., 2012), which exercise interventions restored (McPherson, et al., 2013). This normalization of embryo development rate from exercise interventions in obese founders may be partly related to enhanced oocyte activation and cell cycle control potentially initiated through changes to sperm miR-503. Mir-542-3p abundance was restored only in those founders undergoing combined diet and exercise interventions (Fig 5.4B). MiR-542-3p targets BIRC5, an inhibitor of apoptosis, and when up regulated in zebra fish embryos, apoptosis in the inner cell mass is increased (Ma, et al., 2009). Interestingly, combined diet and exercise was the only intervention to restore the number of inner cell mass cells in the developing embryo (McPherson, et al., 2013), which may be related to restoration of sperm miR-542-3p at fertilisation at least for female embryos. Although mir-465b-5p has currently no confirmed gene targets, it is highly abundant in neonatal ovaries, implicating a role in ovarian development in the developing

fetus (Ahn, et al., 2010). Interestingly poor oocyte quality was found in female offspring born to HFD fed founders (Fullston, et al., 2012), concomitant with a reduced abundance of sperm miR-465b-5p. MiR-465b-5p was restored in founders undergoing diet intervention (HC and HCE) and up-regulated further in exercise intervention alone (HE, Fig 5.4C), suggesting that changes to sperm miR-465b-5p may restore altered embryogenesis caused by male obesity and HFD feeding in female embryos (Dintilhac, et al., 2004). Sperm mir-652 abundance was refractory to founder diet and/or exercise intervention (Fig 5.4D). Given that diet/exercise intervention did not fully rescue female offspring phenotype and microRNAs form one part of a complex epigenetic landscape we therefore did not expect to see complete restoration of the sperm microRNA profile.

Nevertheless improvement to X-linked sperm microRNA abundance was primarily seen in founders that underwent exercise intervention (HE and HCE) and could partly underpin the improvements to early embryo development and health reported (McPherson, et al., 2013), which in its self is a predictor for subsequent offspring metabolic health (Stegers-Theunissen, et al., 2013). Thus providing a potential epigenetic signal in sperm that programs improvements to adiposity and insulin sensitivity recorded for female offspring in this study, although the precise molecular mechanisms underpinning the mode of action remains uncharacterized.

5.9 PATERNAL GLYCAEMIA AND CORTICOSTERONE IS ASSOCIATED WITH SPERM MICRORNA LEVELS AND FEMALE OFFSPRING PHENOTYPES

To dissect why the remaining 4/8 microRNAs had differential abundance limited to diet and/or exercise intervention (and not prolonged HFD founders) (Figure S5.4) I performed correlations between founder metabolites and hormones and sperm microRNA abundance. Founder fasting glucose concentrations

and glucose tolerance, improved by diet and/or exercise intervention, but not in CC or HH founders (Table S5.2) correlated with two of the X-linked sperm microRNAs not found to be altered by HH feeding (i.e. mir-871 and mir-743b-3p, Table 5.1). Interestingly, founder fasting glucose concentrations and glucose tolerance also correlated with measures of female offspring post-weaning weight and glucose tolerance (Table 5.1), implicating paternal glucose metabolic state independent of adiposity, also in the alteration of sperm microRNA abundance and potential programming of offspring health.

Additionally besides improved glucose homeostasis, founders undergoing exercise intervention alone were the only group to have corticosterone concentrations similar to controls (CC) (Table S5.2) and who produced female offspring with the greatest restoration of metabolic health. A recent study reported that founder male rodent exposure to a single high intensity stressor was associated with reduced body weight in female offspring only (Hoyer, et al., 2013). Moreover female offspring pre-weaning size, post weaning adiposity depots and insulin tolerance were strongly correlated with founder corticosterone concentrations (Table 5.1). Although there were no direct correlations to founder sperm microRNA levels, this still suggests that paternal stress may also influence female offspring phenotypes likely through a different sperm mediator.

5.10 CONCLUDING REMARKS

There is now clear evidence that father's peri-conception health can program the health of the subsequent generation (El-Helaly, et al., 2011, van Balkom, et al., 2012, Lee, et al., 2009). This study provides some of the first evidence that lifestyle interventions to restore metabolic health in father's can reverse sperm microRNA levels which target confirmed genes necessary for successful embryogenesis and can partially normalize female offspring predisposition to obesity and metabolic disease. This

implies that exposures to environmental perturbations throughout spermatogenesis are able to program/reprogram signals in sperm to alter offspring phenotype. With this study showing that these programmed signals to offspring are reversible and that there are potential for timely intervention opportunities during spermatogenesis for reprogramming. Additionally the partial restorations of F₁ female offspring phenotypes are likely to further restore F₂ offspring metabolic health due to the pathway of multigenerational, transmission of metabolic consequences of grandfather paternal obesity that we recently reported (Fullston, et al., 2013). Whether similar benefits to male offspring metabolic health are achieved through diet and exercise interventions in obese fathers is still to be determined. Furthermore, I have identified that paternal glycaemia and stress as key mediators for offspring programming and could be the focus of targeted interventions that maybe alternatives to diet/exercise as it's potentially the change in key metabolites that is the driver and not the way in which this occurs. Future directions will be to identify critical periods of paternal environmental exposures during spermatogenesis and the key metabolic signals and mediating factor(s) within the testes to develop novel and more targeted interventions to prevent/overcome adverse paternal developmental programming.

These findings extend the concept of developmental programming of adult disease to include a paternal role in the early life origins of disease. I have identified a novel intervention window for the reversibility of adverse offspring health through interventions in the father, providing a potential circuit breaker for the generational amplification of obesity/metabolic syndrome in future generations.

5.11 ACKNOWLEDGMENTS:

We would like to thank Thomas Sullivan biostatistical manager of the Statistics Division Data Management & Analysis Centre, at the University of Adelaide, for his contribution to statistical design and aid in statistical analysis for the project. All authors contributed to the experimental design and editing of the manuscript. NOM performed the experiments and wrote the manuscript.

5.12 MATERIALS AND METHODS

5.12.1 Founder Animals and Diet

Five week old male C57BL6 mice (n=40) were randomly assigned to one of two diets for an initial feeding period of 9 weeks: 1) control diet (CD) (SF04-057; Specialty Feeds, Perth, Australia); or 2) a high fat diet (HFD) containing 21% fat and nutrient matched (SF00-219; Specialty Feeds, Perth, Australia) (Table S5.1). Diets used in the study have been previously shown to increase adiposity (Brake, et al., 2006, Mitchell, et al., 2011, Bakos, et al., 2011, Palmer, et al., 2011). After the initial feeding period, males fed the HFD were then allocated to one of the following interventions for a further period of 9 weeks: 1) continuation of a HFD (HH) (n=8); 2) change to a CD (HC) (n=8); 3) continuation of a HFD with exercise (HE) (n=8); 4) change to a CD with exercise (HCE) (n=8). Mice allocated to the CD during the initial feeding period were also fed a CD during the intervention period to be used as a baseline control (CC) (n=7). This length of exposure to these interventions has been previously shown to reduce adiposity in those males that undergo diet interventions and to improve metabolic parameters in those males that undergo exercise or exercise combined with diet interventions (Palmer, et al., 2012). Animals were individually housed in a 12:12 dark light cycle for the entire study and fed *ad libitum*. The use and care of all animals used in the study was approved by the Animal Ethics Committee of The University of Adelaide.

5.12.2 Exercise Intervention (Swimming)

The swimming exercise regimen was conducted as described previously (Palmer, et al., 2012).

5.12.3 Founder Body Composition

Founder body weights were recorded weekly during both initial and post intervention periods. At pre intervention week 9 and intervention week 8 whole body composition of adiposity were measured by a dual-emission X-ray absorptiometry machine (DEXA) (Piximus, Ge Lunar, Wisconsin, USA) as previously described in (Nagy, et al., 2000). At 23 weeks of age adiposity (gonadal adiposity, omental adiposity, retro peritoneal adiposity, peritoneal adiposity and dorsal adiposity) was calculated, in addition testes, seminal vesicles, liver, kidneys and pancreas were collected, weighed in a blinded manner by the same individual.

5.12.4 Founder Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

Intervention week 7 a GTT was performed after 6 h of fasting by intra-peritoneal (IP) injection of 2 g/kg of 25% D-glucose solution. ITT was performed at intervention week 8 during a fed state by IP injection of 0.75 IU of human insulin (Actapid®, Novo Nordisk, Bagsvaerd, Denmark). Tail blood glucose concentrations were measured using a glucometer (Hemocue, Angelholm, Sweden) at time points 0 (pre-bolus basal), 15, 30, 60 and 120 min. Both GTT and ITT were performed in female offspring where their oestrus cycle stage was unknown, however as females were housed in groups irrespective of treatment we believe oestrus stage would be equally distributed between groups. Data were expressed as mean blood glucose concentration per group as area under curve (AUC) for glucose and area above the curve (AAC) for insulin.

5.12.5 Natural Mating to produce F1 females

At intervention week 7 (21 weeks of age), founder males had the opportunity to mate with 2-3 normal weight 10 week old C57BL6 females during a maximum period of 8 days. Female mice were exposed to founder males during the dark cycle and separated from the males and maintained on standard chow during the light cycle (Table S5.1). Successfully mating was assessed the following morning by the presence of a vaginal plug (Table S5.8). There were no differences in time to mate or gestational length between treatment groups (Table S5.9), however HH founders had a reduced number of successful matings compared with HCE and CC founders ($p < 0.05$, Table S9), with HCE founders having a high number of successful mating compared HC and HE founders ($p < 0.05$, Table S5.9). After successful mating female mice were group housed until day 15 of pregnancy where they were individually housed until offspring were weaned. Mothers were maintained on standard chow during pregnancy and post birth (Table S5.1). Mothers were allowed to give birth and at weaning (day 21 of life) female offspring were separated from their mothers for use in measurements of adult metabolic health. HC founders increased litter size compared with all interventions ($p < 0.05$, Table S5.9), while HCE founders reduced litter sizes compared with HH and HE founders ($p < 0.05$, Table S5.9). Interestingly, HCE founders had a change in the sex ratio of pups producing less female offspring (Table S5.9). Post weaning, F1 females were group housed independently of founder treatment and maintained on standard chow. For each independent measure of metabolic health one female was sampled per litter as per (Lazic, et al., 2013) to reduce litter effects.

5.12.6 F1 Female Body Composition

Pre weaning body weights were recorded on days 5, 7, 10, 12, 14 and 21 post birth and identification of individual pups was determined by a foot pad tattoo. Post weaning females received an identifiable ear tag and body weights were recorded up until 28 weeks of age. At 8 weeks, 16 weeks and 26 weeks of age 10 females from 10 litters representative of 7 founders per treatment group underwent a DEXA as

described above. At 10 weeks, 18 weeks and 28 weeks of age an additional 7-8 females from 7-8 litters representative of at least 5 founders per treatment group underwent a full post mortem where adiposity (gonadal adiposity, omental adiposity, retro peritoneal adiposity, peritoneal adiposity and dorsal adiposity) was calculated, in addition liver, kidneys and pancreas were collected, weighed and performed in a blinded manner by the same individual.

5.12.7 F1 Female GTT and ITT

At 8-9 weeks, 16-17 weeks and 26-27 weeks of age 10 females from 10 litter's representative of 7 founders per treatment group underwent repeated measures glucose and insulin challenges respectively as described above. A 5 day break was given to each mouse between each test at each time point.

5.12.8 F1 Female Insulin Response during a GTT

At 16 weeks of age, 7 females from 7 litter's representative 4 founder males underwent insulin testing during a GTT. At time points 0 (pre-bolus basal), 15, 30, 60 min an additional 50ul of blood was obtained via the tail vein using a paster pipette. Whole blood was spun at 4000 rpm for 5 min and between 5-10 μ l of plasma was removed and frozen at -20°C until further testing. Insulin concentrations were determined by an Ultra-Sensitive Mouse Insulin ELISA Kit (#90080, Crystal Chem Inc, Downer Grove, Illinois, USA) as per manufacturer's instructions. Data were expressed as mean whole blood insulin concentration per group as area under curve (AUC) and insulin secretion relative to the glucose stimulus at 0, 15, 30, 60 min.

5.12.9 Metabolites and Hormone Analysis

At 9 weeks post intervention for founder males and weeks 10, 18 and 28 of age for F1 females (n=7-8 offspring per group) overnight fasting blood plasma was collected post mortem by a cardiac puncture under anaesthetic with 2% Isoflurane(1-chloro-2,2,2-trifluoroethyldifluoromethylether, Veterinary Companies of Australia, Kings Park, Australia) for founders or 5% Avertin (2-2-2 Tribromethanol, Sigma Aldrich, St Louis, Missouri, USA) for F1 females. Plasma cholesterol, free fatty acids (FFA), glucose and triglycerides were measured on a Cobas Integra 400 plus automated sampler system (Roche, Basel, Switzerland). Plasma insulin, leptin, adiponectin, corticosterone and C-reactive protein (CRP) concentrations were measured by either an Ultra Sensitive Mouse Insulin ELISA Kit (#90080, Crystal Chem Inc, Downer Grove, Illinois, USA), Mouse Leptin ELISA Kit (#90030, Crystal Chem Inc, Downer Grove, Illinois, USA), a Quantikine ELISA mouse adiponectin/Acrp30 Kit (#MRP300, R&D Systems, Minneapolis, USA), a corticosterone ELISA Kit (#KA0468, Abnova, Taipei City, Taiwan), or by a CRP mouse ELISA kit (#KA2467, Abnova, Taipei City, Taiwan) as per the manufactures instructions.

5.12.10 Histology of F1 Female Gonadal Adiposity

F1 female gonadal adiposity was fixed overnight in 4% paraformaldehyde and stored in 70% ethanol until further use. Gonadal adiposity was embedded in wax using standard methods and 7 μ m sections were cut and heat fixed onto super frost slides. Each slide contained four 7 μ m sections 50 μ m apart. Slides were dewaxed, rehydrated in ethanol dilutions and stained with haematoxylin and eosin as per standard methods. Slides were mounted in DPX mounting media and imaged using a NanoZoomer slide scanner (Hamamatsu Photonics, Sunayama-cho, Naka-ku, Japan). The areas of 100 adipocytes from at least 5 different sections per animal were measured using NanoZoomer NDP viewer software. Adipocyte size was determined in 6 female offspring representative of 6 litters from at least 5 founders at 10, 18 and 28 weeks.

5.12.11 MicroRNA analysis of Founder Sperm

Sperm were obtained from the vas deferens immediately following animal euthanasia. The sperm collection process included a light microscopy purity assessment, whereby samples were confirmed to be >99% sperm by cell count. Total RNA was isolated from $4-8 \times 10^6$ sperm with TRI reagent, using glycogen as an RNA carrier, and concentration and quality of RNA were assessed by spectrophotometry (Nanodrop, Thermo Scientific, Waltham, USA). Due to the transmission of the worsened female metabolic health phenotypes reported by paternal obesity (10 weeks on diet) through subsequent generations described in the original study (Fullston, et al., 2013) (grandfather to daughter to grandson). X-linked MicroRNA targets were chosen from previous microRNA expression profiling of founder CD and HFD sperm (15 weeks of age, 10 weeks on diet, matched to original study) by a 384-well microfluidic TagMan Rodent MicroRNA Array cards v. 3.0 amplified on a 7900 HT Real Time PCR system (data not shown). Differentially expressed X-linked microRNAs were chosen based on ranking using LIMMA in R, after raw Ct data were quantile normalized using the normQpcrQuantile function of the R qPCRNorm package (Table S5.6). Micro RNAs were chosen if they displayed a p value ≤ 0.05 with a fold change ≥ 1.5 or ≤ -1.5 relative to CC. Validation of these microRNAs in founder sperm post intervention (18 weeks on diet) was performed by qRT-PCR using multiplexed TaqMan primers (Applied Biosystems, Foster City, USA) and the RT product pre-amplified with Megaplex PreAmp Primers (Applied Biosystems), on a 7900 HT Real Time PCR system, including NTC and noRT controls. MicroRNA expression fold change in samples were determined by $\Delta\Delta C_T$, with data normalized to the geometric mean of mir-10a, which was determined to be invariable and ubiquitous endogenous control by cel-miR normalization of the original array data (data not shown). All data are expressed as relative fold change to controls (CC).

5.12.12 Statistical Analysis

All data were expressed as mean \pm SEM and checked for normality using a Kolmogorov-Smirnov test and equal variance using a Levene's test. Statistical analysis was performed in SPSS (SPSS Version 18, SPSS Inc., Chicago, USA) with AUC and AAC calculated in GraphPad Prism (GraphPad Software, San Diego, USA). A p value <0.05 was considered to be significant.

Founder metabolites, body composition, weight gain/loss and sperm microRNAs were measured using a linear mixed effect model. In the model cohorts of animals were fitted as a covariate and age was fitted as a fixed effect when appropriate.

Founder matting rates, time to mate, gestational length, litter size and sex ratios were expressed per father and analysed by a Fisher's Exact Test.

F1 female pre and post weaning body weights were analysed using repeated measures ANOVA. In the model father ID and mother ID were included as a random effect to adjust for dependence in results between offspring from the same father and mother and litter size as a fixed variable to compared litter size variations between and within treatments.

F1 female post weaning adipocyte cell size and post mortems were matched for litter size and analysed using a linear mixed effect model. In the model father ID was included as a random effect to adjust for dependence in results between offspring from the same father, litter size as a fixed variable to compared litter size variations between and within treatments and age as a fixed variable to compared changes over the three age points.

F1 female ITT, GTT and DEXA composition were matched for litter size and analysed using repeated measures ANOVA. In the model father ID was included as a random effect to adjust for dependence in results between offspring from the same father and litter size as a fixed variable to compared litter size variations between and within treatments.

Correlations of founder fasting plasma glucose concentrations, glucose AUC, to founder sperm microRNA ($\Delta\Delta C_T$) were determined by a Pearsons Correlation. Correlations of female offspring measures to founder fasting glucose concentrations, glucose AUC and plasma corticosterone were determined by multiple regression analysis controlling for multiple offspring per father.

5.13 TABLES

Table 5.1: Paternal glycaemia and plasma corticosterone correlate with founder sperm microRNAs and female offspring phenotypes independent of treatment

	Founder Measure	Correlation Coefficient and P Value
Founder Sperm microRNA ($\Delta\Delta C_T$)		
mir-532-3p	Glucose (AUC)	-0.410, p=0.02
mir-542-3p	Plasma Glucose (mmol/L ⁻¹)	0.445, p<0.01
mir-871	Glucose (AUC)	-0.461, p<0.01
	Plasma Glucose (mmol/L ⁻¹)	-0.351, p=0.03
mir-465a3p	Glucose (AUC)	-0.361, p=0.02
mir-743b-3p	Glucose (AUC)	-0.342, p=0.03
mir-883a-5p	Glucose (AUC)	-0.342, p=0.03
Female offspring measures		
Pre-weaning body weight		
Day 5 total body weight	Plasma Corticosterone (ng/mL ⁻¹)	-0.391, p<0.01
Day 7 total body weight	Plasma Corticosterone (ng/mL ⁻¹)	-0.353, p<0.01
Day 10 total body weight	Plasma Corticosterone (ng/mL ⁻¹)	-0.359, p<0.01
Day 12 total body weight	Plasma Corticosterone (ng/mL ⁻¹)	-0.277, p<0.01
Day 14 total body weight	Plasma Corticosterone (ng/mL ⁻¹)	-0.268, p<0.01
Day 21 total body weight	Plasma Glucose (mmol/L ⁻¹)	0.155, p=0.03
	Glucose (AUC)	0.255, p<0.01
	Plasma Corticosterone (ng/mL ⁻¹)	-0.287, p<0.01
8-10 weeks		
Glucose (AUC)	Plasma Glucose (mmol/L ⁻¹)	0.299, p<0.01
Insulin Tolerance (AAC)	Glucose (AUC)	-0.209, p=0.05
Renal adiposity (%)	Plasma Corticosterone (ng/mL ⁻¹)	0.322, p=0.03
Sum of Adiposity deposits (%)	Plasma Corticosterone (ng/mL ⁻¹)	0.290, p=0.05
16-18 weeks		
Glucose (AUC)	Plasma Glucose (mmol/L ⁻¹)	-0.306, p=0.02
	Glucose (AUC)	-0.262, p=0.04
Total body weight (g)	Plasma Glucose (mmol/L ⁻¹)	0.256, p=0.05
Fasting plasma insulin (ng/mL ⁻¹)	Plasma Corticosterone (ng/mL ⁻¹)	0.745, p<0.01
26-28 weeks		
Total body weight (g)	Plasma Glucose (mmol/L ⁻¹)	0.284, p=0.03
Fasting plasma Insulin (ng/mL ⁻¹)	Plasma Corticosterone (ng/mL ⁻¹)	0.373, p=0.02

All interventions (HC, HE and HCE) improved glucose tolerance while exercise interventions (HE and HCE) improved fasting glucose concentrations compared with both HH and CC founders. Corticosterone was increased by HFD feeding (HH) and only partially restored in exercise intervention alone (HE) with levels not different to both CC and HH founders. Founder glucose regulation and fasting plasma glucose concentrations associates with founder sperm microRNA ($\Delta\Delta C_T$) as assessed by a Persons Correlation. Associations of female offspring measures to founder glycaemia and corticosterone and were assessed by multiple regression analysis controlling for multiple offspring per father. Sum of adiposity depots include (gonadal, omental, renal and dorsal fat masses). No other founder metabolic measure correlated with founder sperm microRNAs or female offspring phenotypes.

5.14 FIGURES

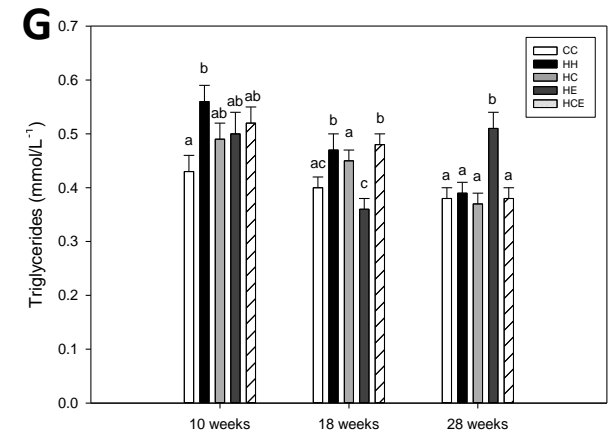
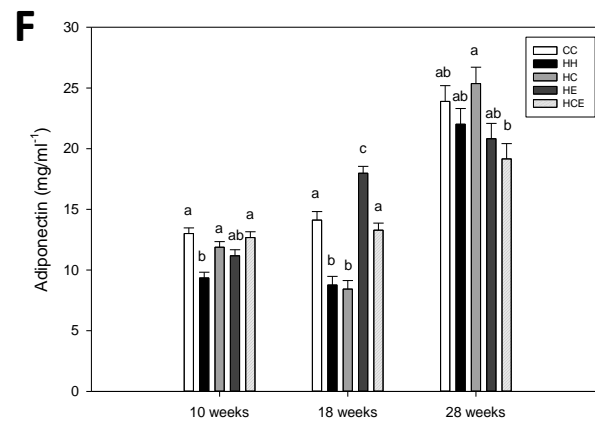
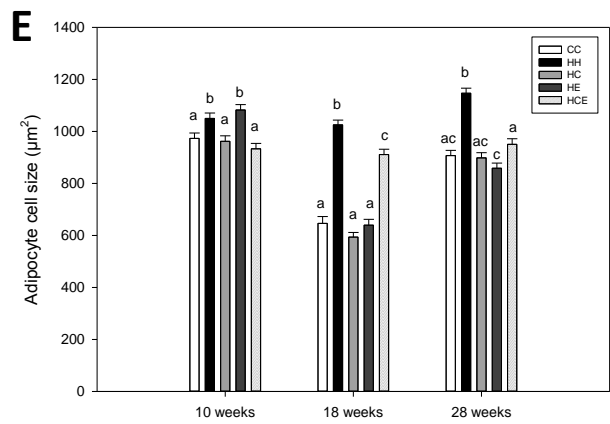
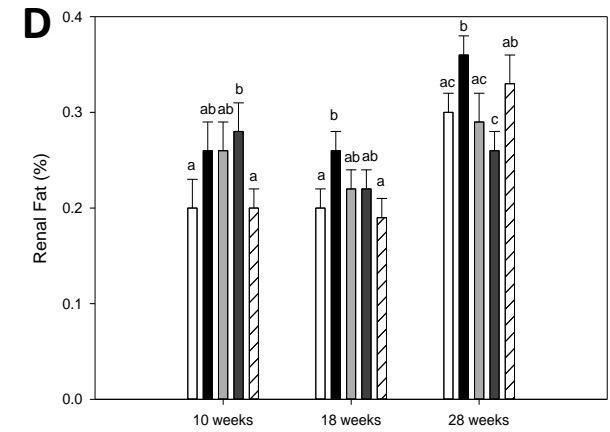
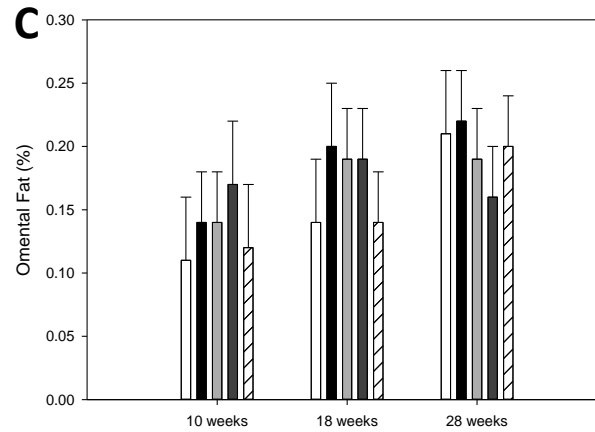
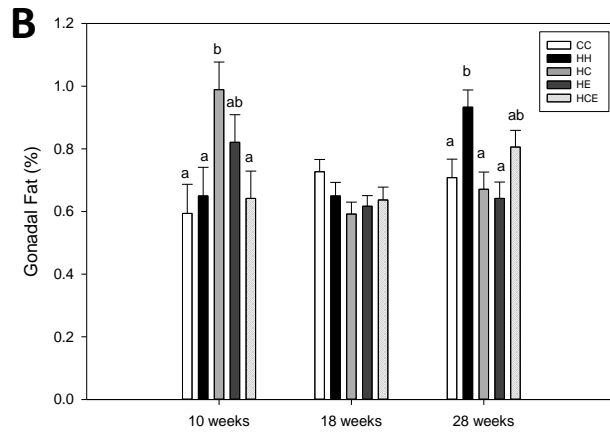
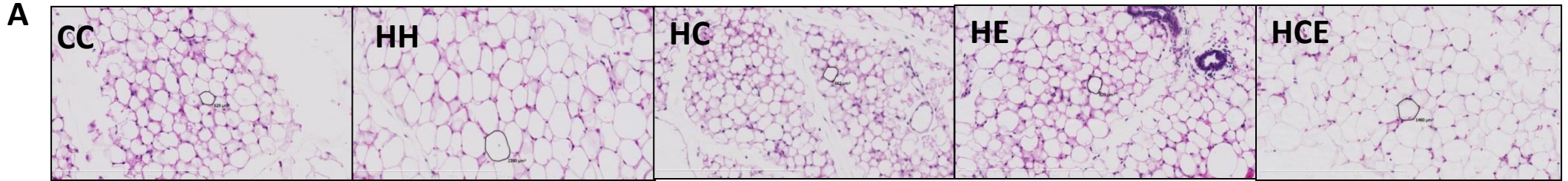


Figure 5.1: The effect of diet and exercise in diet induced paternal obesity on adiposity and plasma triglyceride in female offspring

A Representative pictures of adipocyte cells from gonadal adiposity stained with haematoxylin and eosin at 17 weeks of age per treatment group. **B** Gonadal adiposity as a percentage of body weight with age. **C** Omental adiposity as a percentage of body weight with age. **D** Renal adiposity as a percentage of body weight with age. **E** Adipocyte cell size of the gonadal adipose depot with age. **F** Plasma adiponectin with age. **G** Plasma triglyceride with age. Different letters denote significance at $p < 0.05$. Data was matched for litter size and analysed using linear mixed effects model with father ID added as a random factor and litter size and age as a fixed effect. Adipocyte cell size was determined in 6 female offspring representative of 6 litters from at least 5 founders at 10, 18 and 28 weeks. Adiposity as a percentage of body weight and plasma adiponectin and triglycerides was determined in at least 7 female offspring from at least 7 litters from at least 5 founder males at 10, 18 and 28 weeks.

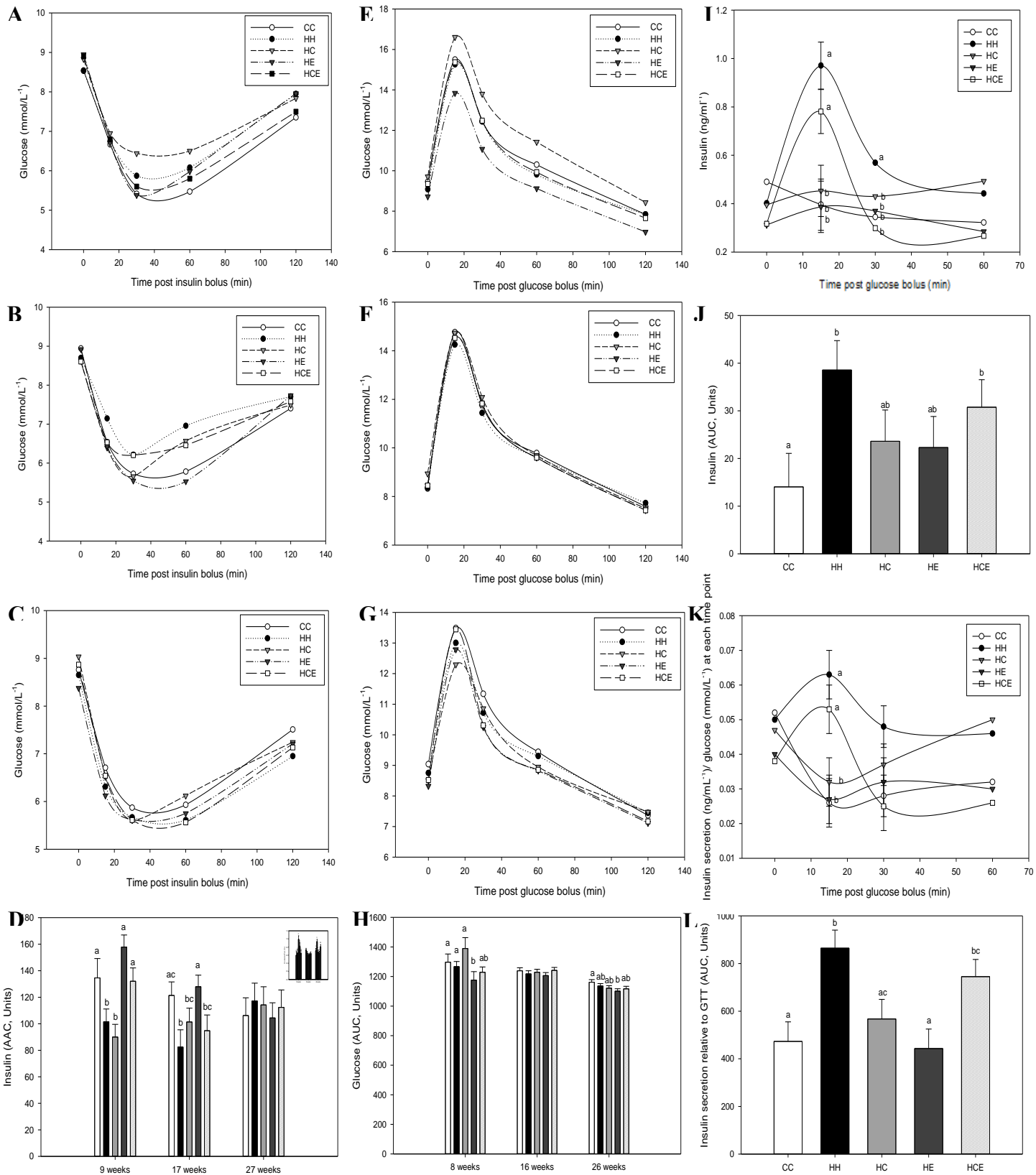


Figure 5.2: The effects of diet and exercise in diet induced paternal obesity on insulin sensitivity, glucose tolerance and insulin secretion in female offspring

A Insulin sensitivity as assessed by insulin tolerance test (ITT, 0.75IU) at 9 weeks. **B** Insulin sensitivity as assessed by ITT at 17 weeks. **C** Insulin sensitivity as assessed by ITT at 27 weeks. **D** Insulin sensitivity (glucose area during ITT, AAC, Units) with age. **E** Glucose tolerance as assessed by glucose tolerance test (GTT, 2g/kg) at 8 weeks. **F** Glucose tolerance assessed by GTT at 16 weeks. **G** Glucose tolerance assessed by GTT at 26 week. **H** Glucose area under the curve (AUC, Units) during GTT over age. **I** Insulin secretion during GTT at 16 weeks. **J** Insulin secretion AUC during GTT at 16 weeks. **K** Insulin secretion relative to GTT (insulin ng/mL^{-1} / glucose mmol/L^{-1}) at 0, 15, 30 and 60 min during the GTT) at 16 weeks. **L** Insulin secretion AUC relative to GTT. Different letters denote significance at $p < 0.05$. Data was matched for litter size and analyzed using repeated measures ANOVA or linear mixed effects modelling with father ID added as a random factor and litter size as a fixed effect. ITT and GTT data represents 10 females from 10 litter's representative of 7 males per treatment group. Insulin secretion during a GTT represents 7 females from 7 litter's representative of 4 males per treatment group.

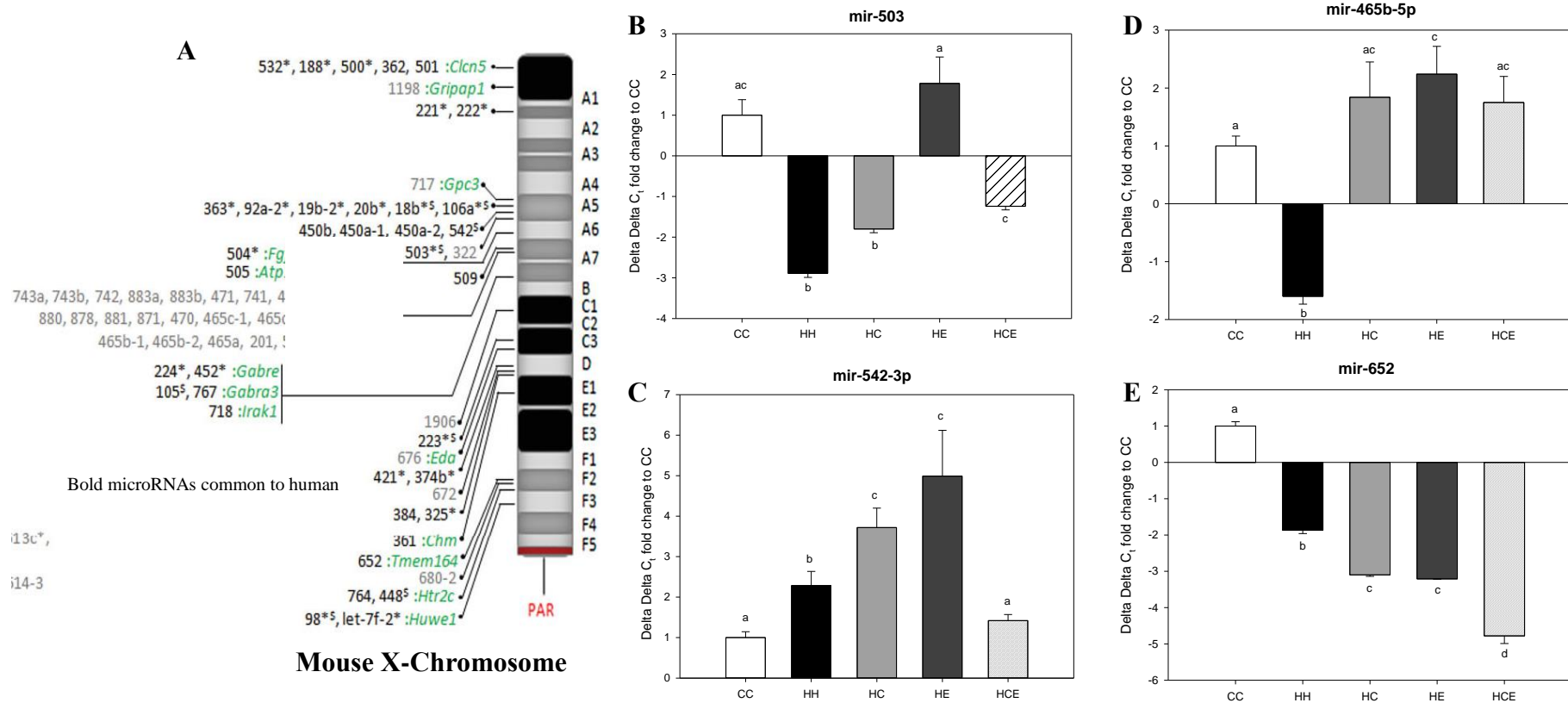


Figure 5.3: The effect of diet and exercise interventions in diet induced paternal obesity on x-linked sperm microRNAs

A Chromosomal localization of X-linked mouse microRNAs, adapted from (Pinheiro, et al., 2011). Fold change abundance of **B** mir-503, **C** mir-542-3p, **D** mir-465b-5p, **E** mir-652, in founder sperm compared with CC founders. Data are representative of n=6 HH and CC founders and n=8 HC, HE and HCE founders.

5.15 SUPPLEMENTARY TABLES

Table S5.1: Composition of animal diets

Ingredients	CD (SF04-057) Control Diet	HFD (SF00-219) Harlan Teklad TD88137 Equivalent	Standard Chow (Irradiated Rat and Mouse Diet)
Sucrose (g/100g)	34.1	34.1	-
Casein (Acid) (g/100g)	19.5	19.5	-
Canola Oil (g/100g)	6.0	-	-
Clarified Butter (g/100g)	-	21.0	-
Cellulose (g/100g)	5.0	5.0	-
Wheat starch (g/100g)	30.5	15.5	-
Minerals (g/100g)	4.9	4.9	-
Digestible energy (MJ/kg)	16.1	19.4	14.0
Digestible energy from lipids (%)	21.0	40.0	4.8
Digestible energy from protein (%)	14.0	17.0	20.0
Digestible energy from carbohydrates (%)	65.0	43.0	74.4

CD = Control diet and HFD = High fat diet. Standard Chow is the standard mouse food supplied by the University of Adelaide's Animal House for maintaining breeding colonies.

Table S5.2: Effect of diet and exercise on founder body composition, metabolism and hormones

	CC	HH	HC	HE	HCE
Pre Intervention					
Total body weight	26.2 ± 1.4 ^a	31.5 ± 1.3 ^b	31.8 ± 1.2 ^b	30.8 ± 1.3 ^b	32.7 ± 1.3 ^b
DEXA body composition					
Total Adiposity (g)	3.92 ± 0.83 ^a	7.84 ± 0.77 ^b	8.00 ± 0.73 ^b	7.68 ± 0.77 ^b	8.59 ± 0.83 ^b
Total Adiposity (%)	14.8 ± 1.7 ^a	24.8 ± 1.5 ^b	24.5 ± 1.4 ^b	24.5 ± 1.5 ^b	25.8 ± 1.7 ^b
Lean Mass (g)	20.0 ± 0.65 ^a	21.7 ± 0.61 ^b	22.0 ± 0.58 ^b	21.6 ± 0.61 ^b	22.4 ± 0.65 ^b
Lean Mass (%)	76.4 ± 1.3 ^a	69.1 ± 1.3 ^b	69.7 ± 1.2 ^b	70.2 ± 1.3 ^b	69.0 ± 1.4 ^b
Bone (g)	0.38 ± 0.01 ^a	0.36 ± 0.02 ^a	0.38 ± 0.01 ^a	0.37 ± 0.02 ^a	0.37 ± 0.02 ^a
Bone (%)	1.39 ± 0.04 ^a	1.16 ± 0.04 ^b	1.20 ± 0.03 ^b	1.21 ± 0.04 ^b	1.26 ± 0.03 ^b
Glucose (mmol/L ⁻¹)	12.2 ± 0.58 ^a	11.6 ± 0.55 ^a	11.3 ± 0.51 ^a	11.1 ± 0.55 ^a	12.3 ± 0.59 ^a
Post Intervention					
Total body weight	30.0 ± 1.15 ^a	36.2 ± 1.15 ^b	29.3 ± 1.15 ^a	31.9 ± 1.10 ^a	29.3 ± 1.10 ^a
DEXA body composition					
Total Adiposity (g)	5.04 ± 0.70 ^a	10.2 ± 0.70 ^b	5.05 ± 0.69 ^a	7.26 ± 0.64 ^c	4.94 ± 0.65 ^a
Total Adiposity (%)	17.5 ± 1.66 ^a	28.5 ± 1.66 ^b	17.1 ± 1.65 ^a	22.9 ± 1.56 ^c	17.1 ± 1.55 ^a
Lean Mass (g)	23.2 ± 0.59 ^a	24.9 ± 0.58 ^b	23.1 ± 0.56 ^a	23.4 ± 0.54 ^{ab}	23.3 ± 0.53 ^a
Lean Mass (%)	81.1 ± 1.67 ^a	70.3 ± 1.67 ^b	81.5 ± 1.66 ^a	75.8 ± 1.56 ^{ab}	81.5 ± 1.55 ^a
Bone (g)	0.40 ± 0.02 ^{ab}	0.42 ± 0.02 ^b	0.37 ± 0.02 ^{ab}	0.36 ± 0.01 ^a	0.38 ± 0.02 ^{ab}
Bone (%)	1.40 ± 0.05 ^a	1.18 ± 0.05 ^b	1.32 ± 0.05 ^a	1.17 ± 0.04 ^b	1.34 ± 0.04 ^a
Post mortem body composition					
Gonadal Adiposity (%)	2.46 ± 0.2 ^a	4.82 ± 0.28 ^b	2.82 ± 0.27 ^a	4.06 ± 0.27 ^c	2.77 ± 0.28 ^a
Omental Adiposity (%)	0.59 ± 0.07 ^{ac}	0.97 ± 0.08 ^b	0.49 ± 0.07 ^a	0.73 ± 0.08 ^c	0.56 ± 0.07 ^{ac}
Kidney Adiposity (%)	0.90 ± 0.11 ^a	1.53 ± 0.10 ^b	1.01 ± 0.11 ^a	1.39 ± 0.10 ^b	0.89 ± 0.10 ^a
Dorsal Adiposity (%)	0.88 ± 0.08 ^a	1.09 ± 0.08 ^b	0.85 ± 0.07 ^a	1.12 ± 0.07 ^b	0.95 ± 0.08 ^{ab}
Sum of Adiposity deposits (%)	4.83 ± 0.47 ^a	8.40 ± 0.48 ^b	5.17 ± 0.46 ^a	7.29 ± 0.46 ^c	5.16 ± 0.47 ^a
Liver (g)	1.18 ± 0.10 ^a	1.55 ± 0.10 ^b	1.29 ± 0.10 ^{ab}	1.29 ± 0.11 ^{ab}	1.12 ± 0.11 ^a
Liver (%)	4.15 ± 0.24 ^a	4.54 ± 0.24 ^a	4.36 ± 0.23 ^a	4.04 ± 0.23 ^a	3.99 ± 0.22 ^a
Pancreas (g)	0.13 ± 0.01 ^a	0.15 ± 0.01 ^a	0.13 ± 0.01 ^a	0.15 ± 0.01 ^a	0.13 ± 0.01 ^a
Pancreas (%)	0.46 ± 0.03 ^a	0.43 ± 0.03 ^a	0.44 ± 0.02 ^a	0.47 ± 0.02 ^a	0.45 ± 0.03 ^a
Kidneys (g)	0.39 ± 0.01 ^a	0.42 ± 0.02 ^a	0.40 ± 0.01 ^a	0.39 ± 0.01 ^a	0.39 ± 0.02 ^a
Kidneys (%)	1.36 ± 0.03 ^a	1.21 ± 0.05 ^b	1.39 ± 0.05 ^a	1.23 ± 0.04 ^b	1.41 ± 0.04 ^a
Soleus (mg)	14.0 ± 4.0 ^a	11.0 ± 4.0 ^a	17.0 ± 4.2 ^a	11.0 ± 4.1 ^a	9.0 ± 3.9 ^a
Vats lat (mg)	133 ± 5.0 ^{ab}	130.0 ± 6.0 ^{ab}	129.0 ± 6.0 ^a	144 ± 5.0 ^b	128.0 ± 6.0 ^a
Glucose tolerance, Insulin tolerance, Fasting plasma metabolites and hormones					
Glucose Tolerance GTT (AUC)	1943 ± 101 ^{ab}	2089 ± 102 ^a	1743 ± 97 ^{bc}	1607 ± 92 ^c	1667 ± 98 ^c
Insulin Tolerance ITT (AAC)	148.2 ± 14.7 ^a	100.4 ± 14.6 ^b	136.3 ± 14.5 ^{ab}	107.2 ± 14.0 ^b	126.5 ± 15.0 ^{ab}
Glucose (mmol/L ⁻¹)	10.4 ± 0.41 ^{ab}	10.9 ± 0.41 ^a	10.3 ± 0.39 ^{ab}	8.99 ± 0.39 ^b	9.73 ± 0.39 ^{bc}
Insulin (ng/mL ⁻¹)	0.24 ± 0.17 ^{ab}	0.51 ± 0.19 ^a	0.14 ± 0.18 ^b	0.43 ± 0.16 ^{ab}	0.36 ± 0.20 ^{ab}

Cholesterol (mmol/L ⁻¹)	3.12 ± 0.36 ^a	4.45 ± 0.4 ^b	3.00 ± 0.39 ^a	4.03 ± 0.34 ^b	2.25 ± 0.43 ^a
FFA (mmol/L ⁻¹)	0.87 ± 0.05 ^{ab}	0.92 ± 0.05 ^a	0.82 ± 0.05 ^{ab}	0.86 ± 0.04 ^{ab}	0.75 ± 0.05 ^b
Triglycerides (mmol/L ⁻¹)	0.65 ± 0.05 ^a	0.67 ± 0.06 ^a	0.60 ± 0.06 ^a	0.68 ± 0.05 ^a	0.56 ± 0.06 ^a
Leptin (ng/mL ⁻¹)	2.94 ± 1.20 ^a	11.8 ± 1.30 ^b	3.68 ± 1.28 ^a	5.07 ± 1.12 ^a	2.58 ± 1.41 ^a
Corticosterone (ng/mL ⁻¹)	398 ± 37 ^a	470 ± 29 ^{bc}	538 ± 30 ^b	435 ± 28 ^{ac}	493 ± 29 ^{bc}
CRP (mg/mL ⁻¹)	8.01 ± 0.61 ^a	10.13 ± 0.57 ^b	9.37 ± 0.53 ^{ab}	8.44 ± 0.53 ^a	8.01 ± 0.57 ^a

Data are representative of 8 HH, HC, HE and HCE founder males and 7 CC founder males. Data was analysed by a linear mixed effects model with cohort of animals fitted as a covariate and age of animal as a fixed factor when appropriate.

Different letters denote significance at $p < 0.05$.

Table S5.3: Effect of founder diet and exercise interventions on f1 female body composition, metabolites and hormones at 10 weeks of age

	CC	HH	HC	HE	HCE
Total body weight	18.4 ± 0.5 ^{ab}	18.4 ± 0.4 ^{ab}	17.5 ± 0.4 ^a	18.8 ± 0.4 ^b	18.5 ± 0.5 ^b
DEXA body composition					
Total Adiposity (g)	1.52 ± 0.08 ^a	1.55 ± 0.08 ^a	1.45 ± 0.08 ^a	1.51 ± 0.07 ^a	1.43 ± 0.08 ^a
Total Adiposity (%)	9.30 ± 0.48 ^{ab}	9.74 ± 0.45 ^a	9.31 ± 0.46 ^{ab}	9.38 ± 0.39 ^{ab}	8.59 ± 0.45 ^b
Lean Mass (g)	13.8 ± 0.27 ^{ab}	13.6 ± 0.26 ^b	13.3 ± 0.26 ^b	13.6 ± 0.23 ^b	14.2 ± 0.26 ^a
Lean Mass (%)	84.4 ± 0.80 ^a	84.2 ± 0.75 ^a	84.2 ± 0.77 ^a	83.6 ± 0.67 ^a	85.1 ± 0.75 ^a
Bone (g)	0.28 ± 0.01 ^{ab}	0.28 ± 0.01 ^{ab}	0.26 ± 0.01 ^a	0.27 ± 0.01 ^a	0.29 ± 0.01 ^b
Bone (%)	1.71 ± 0.03 ^{ab}	1.75 ± 0.03 ^a	1.64 ± 0.03 ^{bc}	1.65 ± 0.03 ^c	1.70 ± 0.03 ^{abc}
Post mortem body composition					
Dorsal Adiposity (%)	0.48 ± 0.03 ^{ab}	0.46 ± 0.03 ^a	0.56 ± 0.04 ^b	0.52 ± 0.04 ^{ab}	0.44 ± 0.03 ^b
Sum of Adiposity deposits (%)	1.38 ± 0.18 ^a	1.52 ± 0.17 ^{ab}	1.95 ± 0.17 ^c	1.81 ± 0.17 ^{bc}	1.38 ± 0.18 ^a
Liver (g)	0.71 ± 0.03 ^a	0.71 ± 0.03 ^a	0.72 ± 0.03 ^a	0.71 ± 0.03 ^a	0.72 ± 0.03 ^a
Liver (%)	3.87 ± 0.16 ^a	3.86 ± 0.15 ^a	4.10 ± 0.15 ^a	3.78 ± 0.14 ^a	3.88 ± 0.15 ^a
Pancreas (g)	0.11 ± 0.01 ^a	0.12 ± 0.01 ^a	0.12 ± 0.01 ^a	0.13 ± 0.01 ^a	0.13 ± 0.01 ^a
Pancreas (%)	0.61 ± 0.05 ^a	0.64 ± 0.05 ^a	0.66 ± 0.04 ^a	0.69 ± 0.05 ^a	0.68 ± 0.05 ^a
Kidneys (g)	0.19 ± 0.01 ^a	0.20 ± 0.01 ^a	0.18 ± 0.01 ^a	0.20 ± 0.01 ^a	0.19 ± 0.01 ^a
Kidneys (%)	1.03 ± 0.04 ^a	1.07 ± 0.04 ^a	1.01 ± 0.03 ^a	1.05 ± 0.04 ^a	1.00 ± 0.04 ^a
Soleus (mg)	6.94 ± 0.63 ^a	5.51 ± 0.43 ^b	6.33 ± 0.80 ^{ab}	5.38 ± 0.68 ^{ab}	6.42 ± 0.79 ^{ab}
Vats lat (mg)	88.6 ± 5.9 ^a	94.6 ± 6.1 ^a	93.7 ± 3.8 ^a	101.4 ± 5.2 ^a	101.5 ± 5.6 ^a
Metabolites and hormones					
Glucose (mmol/L ⁻¹)	9.39 ± 0.25 ^a	9.39 ± 0.23 ^a	9.23 ± 0.23 ^a	8.56 ± 0.24 ^b	9.39 ± 0.23 ^a
Cholesterol (mmol/L ⁻¹)	1.76 ± 0.09 ^a	1.82 ± 0.09 ^a	1.84 ± 0.09 ^a	1.65 ± 0.09 ^a	2.10 ± 0.09 ^b
FFA (mmol/L ⁻¹)	0.66 ± 0.04 ^a	0.73 ± 0.04 ^a	0.70 ± 0.05 ^a	0.63 ± 0.04 ^a	0.66 ± 0.05 ^a
Insulin (ng/mL ⁻¹)	0.46 ± 0.14 ^a	0.33 ± 0.15 ^a	0.41 ± 0.15 ^a	0.49 ± 0.13 ^a	0.36 ± 0.15 ^a
Leptin (ng/mL ⁻¹)	0.38 ± 0.12 ^a	0.31 ± 0.07 ^a	0.42 ± 0.06 ^a	0.41 ± 0.13 ^a	0.32 ± 0.11 ^a
Corticosterone (ng/mL ⁻¹)	871 ± 94 ^a	932 ± 93 ^a	985 ± 93 ^a	1059 ± 91 ^a	935 ± 91 ^a

DEXA data represents 10 females from 10 litter's representative of n=7 HH, HC, HE, HCE and CC males. Post mortem and metabolite data represents 7 females from 7 litters representative of n=6 HE and n=5 CC males and 8 females from 8 litters representative of n=7 HC, n=6 HH and HCE males. Data was matched for litter size and analysed using either repeated measures ANOVA or linear mixed effects model with father ID as a random effect and litter size and age as a fixed effect. Different letters denote significance at p<0.05. Total body weight was measured at post mortem. DEXA data was analysed to body weight recorded at DEXA.

Table S5.4: Effect of founder diet and exercise interventions on f1 female body composition, metabolites and hormones at 18 weeks of age

	CC	HH	HC	HE	HCE
Total body weight	19.8 ± 0.12 ^{ab}	20.0 ± 0.19 ^a	19.4 ± 0.18 ^{ab}	19.3 ± 0.18 ^b	19.8 ± 0.19 ^{ab}
DEXA body composition					
Total adiposity (g)	1.58 ± 0.04 ^a	1.66 ± 0.04 ^a	1.66 ± 0.04 ^a	1.64 ± 0.03 ^a	1.66 ± 0.04 ^a
Total adiposity (%)	7.69 ± 0.23 ^a	8.10 ± 0.21 ^{ab}	8.23 ± 0.27 ^b	8.39 ± 0.19 ^b	8.14 ± 0.21 ^{ab}
Lean Mass (g)	17.6 ± 0.31 ^a	17.5 ± 0.29 ^a	17.3 ± 0.29 ^{ab}	16.8 ± 0.26 ^b	17.6 ± 0.29 ^a
Lean Mass (%)	85.8 ± 0.86 ^a	85.3 ± 0.81 ^a	86.1 ± 0.72 ^a	85.1 ± 0.71 ^a	86.5 ± 0.81 ^a
Bone (g)	0.40 ± 0.01 ^a	0.39 ± 0.01 ^a	0.38 ± 0.01 ^a	0.38 ± 0.03 ^a	0.40 ± 0.02 ^a
Bone (%)	1.93 ± 0.07 ^a	1.89 ± 0.08 ^a	1.91 ± 0.07 ^a	1.96 ± 0.06 ^a	1.97 ± 0.07 ^a
Post mortem body composition					
Dorsal Adiposity (%)	0.40 ± 0.02 ^a	0.43 ± 0.03 ^a	0.37 ± 0.02 ^a	0.38 ± 0.03 ^a	0.42 ± 0.02 ^a
Sum of Adiposity deposits (%)	1.44 ± 0.13 ^a	1.53 ± 0.13 ^a	1.37 ± 0.13 ^a	1.40 ± 0.12 ^a	1.39 ± 0.12 ^a
Liver (g)	0.75 ± 0.03 ^a	0.74 ± 0.03 ^a	0.70 ± 0.02 ^a	0.78 ± 0.02 ^{ab}	0.83 ± 0.02 ^b
Liver (%)	3.78 ± 0.12 ^{ac}	3.70 ± 0.13 ^a	3.62 ± 0.12 ^a	4.00 ± 0.13 ^{bc}	4.19 ± 0.13 ^b
Pancreas (g)	0.12 ± 0.01 ^a	0.14 ± 0.01 ^a	0.14 ± 0.00 ^a	0.13 ± 0.01 ^a	0.13 ± 0.01 ^a
Pancreas (%)	0.62 ± 0.03 ^a	0.68 ± 0.05 ^a	0.70 ± 0.04 ^a	0.66 ± 0.03 ^a	0.65 ± 0.04 ^a
Kidneys (g)	0.19 ± 0.01 ^a	0.22 ± 0.01 ^b	0.20 ± 0.01 ^a	0.21 ± 0.01 ^{ab}	0.21 ± 0.00 ^{ab}
Kidneys (%)	0.96 ± 0.05 ^a	1.06 ± 0.05 ^b	1.00 ± 0.04 ^{ab}	1.06 ± 0.04 ^b	1.04 ± 0.03 ^{ab}
Soleus (mg)	7.72 ± 0.65 ^a	7.82 ± 0.68 ^a	7.26 ± 0.76	7.67 ± 0.64 ^a	7.84 ± 0.36 ^a
Vats lat (mg)	115.9 ± 3.6 ^{ab}	120.9 ± 5.4 ^a	101.9 ± 2.0 ^c	120.7 ± 4.0 ^a	108.2 ± 3.8 ^{bc}
Metabolites and hormones					
Glucose (mmol/L ⁻¹)	8.18 ± 0.22 ^a	8.41 ± 0.23 ^{ab}	8.90 ± 0.22 ^b	8.46 ± 0.22 ^{ab}	8.45 ± 0.21 ^{ab}
Cholesterol (mmol/L ⁻¹)	1.92 ± 0.10 ^a	1.72 ± 0.11 ^{ab}	1.66 ± 0.09 ^b	1.83 ± 0.08 ^{ab}	1.96 ± 0.09 ^a
FFA (mmol/L ⁻¹)	0.57 ± 0.05 ^{ac}	0.64 ± 0.05 ^a	0.82 ± 0.05 ^b	0.55 ± 0.05 ^c	0.69 ± 0.05 ^{ab}
Insulin (ng/mL ⁻¹)	0.24 ± 0.09 ^a	0.12 ± 0.10 ^a	0.20 ± 0.09 ^a	0.12 ± 0.08 ^a	0.10 ± 0.07 ^a
Leptin (ng/mL ⁻¹)	0.49 ± 0.22 ^a	0.61 ± 0.16 ^a	0.52 ± 0.18 ^a	0.53 ± 0.18 ^a	0.52 ± 0.20 ^a
Corticosterone (ng/mL ⁻¹)	537 ± 106 ^a	685 ± 110 ^{ab}	849 ± 101 ^b	856 ± 87 ^b	864 ± 89 ^b

DEXA data represents 10 females from 10 litter's representative of n=7 HH, HC, HE, HCE and CC males. Post mortem and metabolite data represents 7 females from 7 litters representative of n=6 HH, HC, HCE and CC males and 8 females from 8 litters representative of n=5 HE males. Data was matched for litter size and analysed using either repeated measures ANOVA or linear mixed effects model with father ID as a random effect and litter size and age as a fixed effect. Different letters denote significance at p<0.05. Total body weight was measured at post mortem. DEXA data was analysed to body weight recorded at DEXA.

Table S5.5: Effect of founder diet and exercise interventions on f1 female body composition, metabolites and hormones at 28 weeks of age

	CC	HH	HC	HE	HCE
Total body weight	22.2 ± 0.37 ^a	22.7 ± 0.33 ^a	22.4 ± 0.34 ^a	22.1 ± 0.29 ^a	22.3 ± 0.34 ^a
DEXA body composition					
Total Adiposity (g)	1.72 ± 0.04 ^a	1.74 ± 0.03 ^a	1.81 ± 0.03 ^a	1.76 ± 0.03 ^a	1.81 ± 0.04 ^a
Total Adiposity (%)	7.76 ± 0.15 ^{ab}	7.69 ± 0.12 ^a	8.10 ± 0.13 ^b	7.94 ± 0.12 ^{ab}	8.13 ± 0.13 ^b
Lean Mass (g)	19.3 ± 0.32 ^a	19.6 ± 0.28 ^a	19.5 ± 0.29 ^a	18.3 ± 0.25 ^b	19.2 ± 0.29 ^a
Lean Mass (%)	86.9 ± 0.51 ^a	86.2 ± 0.45 ^a	86.9 ± 0.46 ^a	83.1 ± 0.41 ^b	86.2 ± 0.47 ^a
Bone (g)	0.44 ± 0.01 ^a	0.44 ± 0.01 ^a	0.45 ± 0.01 ^a	0.45 ± 0.01 ^a	0.45 ± 0.01 ^a
Bone (%)	2.00 ± 0.03 ^{ab}	1.94 ± 0.03 ^a	2.00 ± 0.03 ^{ab}	2.05 ± 0.03 ^b	2.02 ± 0.03 ^{ab}
Post mortem body composition					
Dorsal Adiposity (%)	0.40 ± 0.02 ^a	0.47 ± 0.01 ^b	0.41 ± 0.01 ^a	0.42 ± 0.01 ^a	0.45 ± 0.01 ^{ab}
Sum of Adiposity deposits (%)	1.62 ± 0.10 ^a	1.98 ± 0.11 ^b	1.55 ± 0.09 ^a	1.48 ± 0.09 ^a	1.78 ± 0.10 ^{ab}
Liver (g)	0.77 ± 0.01 ^{ac}	0.86 ± 0.02 ^b	0.74 ± 0.01 ^a	0.82 ± 0.01 ^{bc}	0.85 ± 0.02 ^b
Liver (%)	3.82 ± 0.08 ^{ac}	4.05 ± 0.09 ^a	3.60 ± 0.08 ^c	3.94 ± 0.07 ^{ab}	4.15 ± 0.08 ^b
Pancreas (g)	0.16 ± 0.01 ^a	0.17 ± 0.01 ^{ab}	0.19 ± 0.01 ^b	0.16 ± 0.01 ^a	0.16 ± 0.01 ^a
Pancreas (%)	0.77 ± 0.04 ^a	0.82 ± 0.04 ^{ab}	0.91 ± 0.04 ^b	0.75 ± 0.03 ^a	0.76 ± 0.02 ^a
Kidneys (g)	0.22 ± 0.01 ^a	0.24 ± 0.01 ^b	0.23 ± 0.01 ^{ab}	0.22 ± 0.02 ^{ab}	0.23 ± 0.01 ^{ab}
Kidneys (%)	1.06 ± 0.04 ^a	1.14 ± 0.05 ^a	1.10 ± 0.04 ^a	1.07 ± 0.04 ^a	1.13 ± 0.04 ^a
Soleus (mg)	8.23 ± 0.54 ^a	8.38 ± 0.35 ^a	8.46 ± 0.75 ^a	9.10 ± 0.71 ^a	7.92 ± 0.40 ^a
Vats lat (mg)	122.7 ± 6.5 ^a	139.5 ± 5.5 ^a	133.2 ± 6.0 ^a	123.3 ± 5.4 ^a	125.7 ± 5.0 ^a
Metabolites and hormones					
Glucose (mmol/L ⁻¹)	9.13 ± 0.17 ^a	8.80 ± 0.18 ^{ab}	8.54 ± 0.18 ^{bc}	8.29 ± 0.18 ^c	8.64 ± 0.17 ^{bc}
Cholesterol (mmol/L ⁻¹)	2.25 ± 0.09 ^a	2.14 ± 0.10 ^a	1.90 ± 0.08 ^b	2.15 ± 0.09 ^b	2.15 ± 0.09 ^b
FFA (mmol/L ⁻¹)	0.78 ± 0.05 ^a	0.65 ± 0.04 ^a	0.67 ± 0.04 ^a	0.86 ± 0.06 ^b	0.50 ± 0.05 ^c
Insulin (ng/mL ⁻¹)	0.58 ± 0.18 ^{ab}	1.28 ± 0.19 ^a	0.97 ± 0.17 ^{ac}	0.35 ± 0.17 ^{bc}	0.14 ± 0.18 ^b
Leptin (ng/mL ⁻¹)	1.16 ± 0.38 ^a	0.73 ± 0.13 ^a	0.79 ± 0.16 ^a	0.35 ± 0.06 ^b	0.80 ± 0.12 ^a
Corticosterone (ng/mL ⁻¹)	715 ± 11 ^a	754 ± 12 ^a	738 ± 10 ^a	733 ± 11 ^a	750 ± 10 ^a

DEXA data represents 10 females from 10 litter's representative of n=7 HH, HC, HE, HCE and CC males. Post mortem and metabolite data represents 7 females from 7 litters representative of n=7 HH and HC and n=5 CC males and 8 females from 8 litters representative of n=7 HE and n=6 HCE males. Data was matched for litter size and analysed using either repeated measures ANOVA or linear mixed effects model with father ID as a random effect and litter size and age as a fixed effect. Different letters denote significance at p<0.05. Total body weight was measured at post mortem. DEXA data was analysed to body weight recorded during DEXA.

Table S5.6: X-linked microRNAs in Sperm from CD and HFD Founders 10 weeks pre-Intervention

TaqMan Array (10 weeks on diet)					
MicroRNA	CD (mean CT relative to mir-10a)	HFD (mean CT relative to mir-10a)	$\Delta\Delta C_T$ Fold change	Regulation (compared to CD)	P Value
mir-503	15.4 ± 0.5	11.5 ± 0.2	15.1	Up	0.003
mir-871	5.4 ± 0.1	6.0 ± 0.1	-1.5	Down	0.009
mir-542-3p	23.9 ± 2.2	14.4 ± 0.4	756.5	Up	0.02
mir-465b-5p	-0.95 ± 0.31	0.12 ± 0.08	-2.1	Down	0.02
mir-465a-3p	2.5 ± 0.3	3.4 ± 0.2	-1.9	Down	0.03
mir-743b-3p	4.1 ± 0.3	4.9 ± 0.1	-1.7	Down	0.04
mir-652	4.1 ± 0.1	3.4 ± 0.2	1.6	Up	0.05
mir-883a-5p	10.2 ± 0.2	11.0 ± 0.3	-1.7	Down	0.05
mir-223	-1.1 ± 0.6	0.4 ± 0.1	-4.4	Down	0.07
mir-532-3p	4.2 ± 0.2	4.8 ± 0.1	-1.5	Down	0.08
mir-741	4.0 ± 0.3	4.6 ± 0.2	-1.6	Down	0.09
mir-20b	1.8 ± 0.2	2.0 ± 0.1	-1.1	-	0.1
mir-332	7.5 ± 0.2	7.0 ± 0.3	1.6	-	0.11
mir-500	11.9 ± 0.7	10.6 ± 0.3	2.6	-	0.16
mir-98	11.1 ± 0.2	10.1 ± 0.5	2	-	0.2
mir-743a	2.2 ± 0.2	2.6 ± 0.2	-1.3	-	0.21
mir-672	2.7 ± 0.5	3.6 ± 0.4	-1.8	-	0.23
mir-19b	-4.3 ± 0.1	-4.1 ± 0.1	1.2	-	0.23
mir-1198	6.9 ± 0.4	7.7 ± 0.3	-1.7	-	0.27
let-7f	5.1 ± 0.4	5.6 ± 0.2	-1.5	-	0.27
mir-471	10.4 ± 0.4	9.4 ± 0.5	1.7	-	0.31
mir-471	10.1 ± 0.4	9.3 ± 0.5	1.7	-	0.31
mir-106a	1.2 ± 0.2	1.7 ± 0.3	-1.3	-	0.32
mir-764-3p	5.4 ± 0.6	4.5 ± 0.5	1.8	-	0.34
mir-92a	5.5 ± 0.6	4.7 ± 0.9	1.8	-	0.34
mir-717	5.3 ± 0.6	4.5 ± 0.5	1.8	-	0.35
mir-718	5.3 ± 0.6	4.5 ± 0.5	1.8	-	0.35
mir-767	5.4 ± 0.6	4.6 ± 0.5	1.8	-	0.35
mir-384-5p	8.8 ± 0.4	7.7 ± 0.2	1.4	-	0.36
mir-362-3p	8.1 ± 0.4	7.7 ± 0.2	1.4	-	0.36
mir-452	21.5 ± 3.5	17.5 ± 1.4	15.2	-	0.42
mir-221	6.1 ± 1.2	5.1 ± 0.2	2	-	0.45
mir-878-5p	4.8 ± 0.2	5.0 ± 0.1	-1.1	-	0.48
mir-105	11.6 ± 0.9	12.6 ± 0.7	-2	-	0.49
mir-878	4.8 ± 0.2	5.0 ± 0.1	-1.1	-	0.49
mir-504	26.7 ± 0.9	23.7 ± 2.7	7.9	-	0.53
mir-547	8.5 ± 0.3	8.2 ± 0.1	1.2	-	0.53
mir-351	22.3 ± 1.7	20.4 ± 2.5	4	-	0.54
mir-680	6.4 ± 0.4	6.1 ± 0.1	1.2	-	0.55
mir-361	7.3 ± 0.4	7.6 ± 0.1	-1.2	-	0.56
mir-463	5.8 ± 0.9	6.4 ± 0.6	-1.6	-	0.57
mir-470	4.7 ± 1.1	5.4 ± 0.5	-1.6	-	0.58
mir-880	11.5 ± 0.3	11.0 ± 0.6	1.4	-	0.59
mir-883b-3p	4.2 ± 0.3	4.3 ± 0.1	-1.1	-	0.63
mir-742	2.3 ± 0.9	2.8 ± 0.6	-1.4	-	0.68
mir-450b-3p	12.3 ± 0.3	12.9 ± 0.8	-1.5	-	0.68
mir-363	15.0 ± 0.8	15.3 ± 0.6	-1.2	-	0.78
mir-465c	4.1 ± 0.5	4.0 ± 0.6	1.1	-	0.93

Table continues

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mir-676	9.4 ± 0.4	9.5 ± 0.1	1	-	0.95
mir-224	5.4 ± 0.6	5.4 ± 0.3	1	-	0.99
mir-188-3p	ND	ND	-	-	-
mir-18b	ND	ND	-	-	-
mir-1906	ND	ND	-	-	-
mir-201	ND	ND	-	-	-
mir-448	ND	ND	-	-	-
mir-505	ND	ND	-	-	-

All microRNA is specific to mouse (mmu-mir). ND = not detected. Gray cells represent miRNAs that were confirmed using

Taqman qPCR assays post-intervention as determined by $p \leq 0.05$ with a fold change of ≥ 1.5 or ≤ -1.5 .

Table S5.7: Confirmed gene targets of x-linked microRNAs change in sperm of HH founders

mir-RNA	Confirmed Gene Targets	Gene function
mir-503 (Xu et al., 2013) (Zhou et al., 2013) (Cheng et al., 2012) (Zhou and Wang, 2011)	<i>CDKN1A</i>	This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1.
	<i>CCNF</i>	This gene encodes a member of the cyclin family. Cyclins are important regulators of cell cycle transitions through their ability to bind and activate cyclin-dependent protein kinases.
	<i>WEE1</i>	This gene encodes a nuclear protein, which is a tyrosine kinase belonging to the Ser/Thr family of protein kinases. This protein catalyzes the inhibitory tyrosine phosphorylation of CDC2/cyclin B kinase, and appears to coordinate the transition between DNA replication and mitosis by protecting the nucleus from cytoplasmically activated CDC2 kinase.
	<i>CCNE2</i>	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK2. This cyclin has been shown to specifically interact with CIP/KIP family of CDK inhibitors, and plays a role in cell cycle G1/S transition. The expression of this gene peaks at the G1-S phase.
	<i>CCNE1</i>	This cyclin forms a complex with and functions as a regulatory subunit of CDK2, whose activity is required for cell cycle G1/S transition. This protein accumulates at the G1-S phase boundary and is degraded as cells progress through S phase.
	<i>CDC25A</i>	CDC25A is required for progression from G1 to the S phase of the cell cycle. It activates the cyclin-dependent kinase CDC2 by removing two phosphate groups. CDC25A is specifically degraded in response to DNA damage, which prevents cells with chromosomal abnormalities from progressing through cell division.
	<i>AGO1</i>	This gene encodes a member of the Argonaute family of proteins which play a role in RNA interference. The encoded protein is highly basic, and contains a PAZ domain and a PIWI domain. It may interact with dicer1 and play a role in short-interfering-RNA-mediated gene silencing.
	<i>ATF6</i>	This gene encodes a transcription factor that activates target genes for the unfolded protein response (UPR) during endoplasmic reticulum (ER) stress. Although it is a transcription factor, this protein is unusual in that it is synthesized as a transmembrane protein that is embedded in the ER. It functions as an ER stress sensor/transducer, and following ER stress-induced proteolysis, it functions as a nuclear transcription factor via a cis-acting ER stress response element (ERSE) that is present in the promoters of genes encoding ER chaperones.

<i>ANLN</i>	It is a 124 kDa scaffolding protein involved in <u>cytokinesis</u> . It is found in high concentrations near the <u>cleavage furrow</u> and coincides with <u>RhoA</u> , the key regulator of contractile-ring formation
<i>CDC14A</i>	The protein encoded by this gene is a member of the dual specificity protein tyrosine phosphatase family. It is highly similar to <i>Saccharomyces cerevisiae</i> Cdc14, a protein tyrosine phosphatase involved in the exit of cell mitosis and initiation of DNA replication, suggesting a role in cell cycle control.
<i>CCND1</i>	This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition.
<i>Inha</i>	This gene encodes the alpha subunit of inhibins A and B protein complexes. These complexes negatively regulate follicle stimulating hormone secretion from the pituitary gland. Inhibins have also been implicated in regulating numerous cellular processes including cell proliferation, apoptosis, immune response and hormone secretion.
<i>Inhba</i>	The inhibin beta A subunit joins the alpha subunit to form a pituitary FSH secretion inhibitor. Inhibin has been shown to regulate gonadal stromal cell proliferation negatively and to have tumor-suppressor activity.
<i>Cyp19a1</i>	This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the endoplasmic reticulum and catalyzes the last steps of oestrogen biosynthesis, three successive hydroxylations of the A ring of androgens.
<i>Lhcgr</i>	This gene encodes the receptor for both luteinizing hormone and choriogonadotropin. This receptor belongs to the G-protein coupled receptor 1 family, and its activity is mediated by G proteins which activate adenylate cyclase.
<i>Esr2</i>	This gene encodes a member of the family of oestrogen receptors and superfamily of nuclear receptor transcription factors. The gene product contains an N-terminal DNA binding domain and C-terminal ligand binding domain and is localized to the nucleus, cytoplasm, and mitochondria. Upon binding to 17beta-estradiol or related ligands, the encoded protein forms homo- or hetero-dimers that interact with specific DNA sequences to activate transcription.
<i>Ar</i>	The androgen receptor gene is more than 90 kb long and codes for a protein that has 3 major functional domains: the N-terminal domain, DNA-binding domain, and androgen-binding domain. The protein functions as a steroid-hormone activated transcription factor. Upon binding the hormone ligand, the receptor dissociates from accessory proteins, translocates into the nucleus, dimerizes, and then stimulates transcription of androgen responsive genes.

<i>Cdkn1b</i>	The encoded protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1. The degradation of this protein, which is triggered by its CDK dependent phosphorylation and subsequent ubiquitination by SCF complexes, is required for the cellular transition from quiescence to the proliferative state.
<i>Casp3</i>	This gene encodes a protein which is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes which undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme.
<i>Ccnd2</i>	This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition.
<i>Bcl2</i>	This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes.
<i>Fshr</i>	The protein encoded by this gene belongs to family 1 of G-protein coupled receptors. It is the receptor for follicle stimulating hormone and functions in gonad development.
<i>Acvr2b</i>	Activins are dimeric growth and differentiation factors which belong to the transforming growth factor-beta (TGF-beta) superfamily of structurally related signaling proteins. Activins signal through a heteromeric complex of receptor serine kinases which include at least two type I (I and IB) and two type II (II and IIB) receptors. These receptors are all transmembrane proteins, composed of a ligand-binding extracellular domain with cysteine-rich region, a transmembrane domain, and a cytoplasmic domain with predicted serine/threonine specificity. Type I receptors are essential for signaling; and type II receptors are required for binding ligands and for expression of type I receptors.
<i>Inhbb</i>	The inhibin beta B subunit joins the alpha subunit to form a pituitary FSH secretion inhibitor. Inhibin has been shown to regulate gonadal stromal cell proliferation negatively and to have tumour-suppressor activity.
<i>CD40</i>	The protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor has been found to be essential in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation.
<i>FGF2</i>	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members bind heparin and possess broad mitogenic and angiogenic activities. This protein has been implicated in diverse biological processes, such as limb and nervous system development, wound healing, and tumor growth.

	<i>VEGFA</i>	This gene is a member of the PDGF/VEGF growth factor family and encodes a protein that is often found as a disulfide linked homodimer. This protein is a glycosylated mitogen that specifically acts on endothelial cells and has various effects, including mediating increased vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis.
mir-532-3p	<i>TRAPPC2P1</i>	This gene has been described as a transcribed retropseudogene (or retro-xaptonuon) based on its structure which lacks most of the introns of <i>SEDL</i> and the detection of transcripts from this locus and is important in cellular ion transport.
mir-456b-5p	<i>N/A</i>	No confirmed targets
mir-652	<i>SLC7A1</i>	High-affinity, low capacity permease involved in the transport of the cationic amino acids (arginine, lysine and ornithine) in non-hepatic tissues. May also function as an ecotropic retroviral leukemia receptor.
	<i>HOXA9</i>	Expression of these proteins is spatially and temporally regulated during embryonic development. This gene is part of the A cluster on chromosome 7 and encodes a DNA-binding transcription factor which may regulate gene expression, morphogenesis, and differentiation.

Confirmed gene targets were established from miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>) with confirmation paper references. Gene function summaries were obtained by the NCBI website gene search (<http://www.ncbi.nlm.nih.gov/gene>).

Table S5.8: Number of successful mating's per founder male

Founder treatment group	Animal ID	Total number of females successfully mated	Successful matting's (%)
CC	34	2/2	100
CC	35	1/2	50
CC	36	2/2	100
CC	37	2/2	100
CC	38	3/3	100
CC	39	1/3	50
CC	40	1/3	50
HH	1	3/3	100
HH	2	1/3	33
HH	3	1/3	33
HH	4	1/3	33
HH	5	1/3	33
HH	6	2/2	100
HH	7	1/2	50
HH	8	0/2	0
HC	9	3/3	100
HC	10	2/3	66
HC	11	3/3	100
HC	12	1/3	33
HC	13	0/2	0
HC	14	1/2	50
HC	15	2/2	100
HC	16	2/2	100
HE	17	1/3	33
HE	18	1/3	33
HE	19	1/3	33
HE	20	2/3	66
HE	21	2/2	100
HE	22	2/2	100
HE	23	0/2	0
HE	24	2/2	100
HCE	25	3/3	100
HCE	26	3/3	100
HCE	27	3/3	100
HCE	28	3/3	100
HCE	29	2/2	100
HCE	30	2/2	100
HCE	31	2/2	100
HCE	33	1/2	50

Table S5.9: Effect of diet and exercise on founder time to mate, mating rates, litter size and sex ratio

	CC	HH	HC	HE	HCE
Time to mate (days)	2.44 ± 0.35	3.16 ± 0.40	2.91 ± 0.39	2.90 ± 0.35	3.14 ± 0.39
Successful mating (%)	78.5 ^{ab}	47.8 ^c	68.6 ^{bc}	58.1 ^c	93.8 ^a
Gestational length (days)	20.0 ± 0.1	20.0 ± 0.1	19.8 ± 0.1	19.8 ± 0.1	19.9 ± 0.1
Litter size	5.80 ± 0.24 ^{ac}	6.35 ± 0.22 ^a	7.04 ± 0.24 ^b	6.32 ± 0.21 ^a	5.72 ± 0.22 ^c
Female offspring (%)	51 ^{ab}	47 ^{ab}	54 ^a	56 ^a	36 ^b

Data are representative of 8 HH, HC, HE and HCE founder males and 7 CC founder males. Data are expressed per father and was analysed by a Fishes Exact Test. Different letters denote significance at $p < 0.05$.

5.16 SUPPLEMENTARY FIGURES

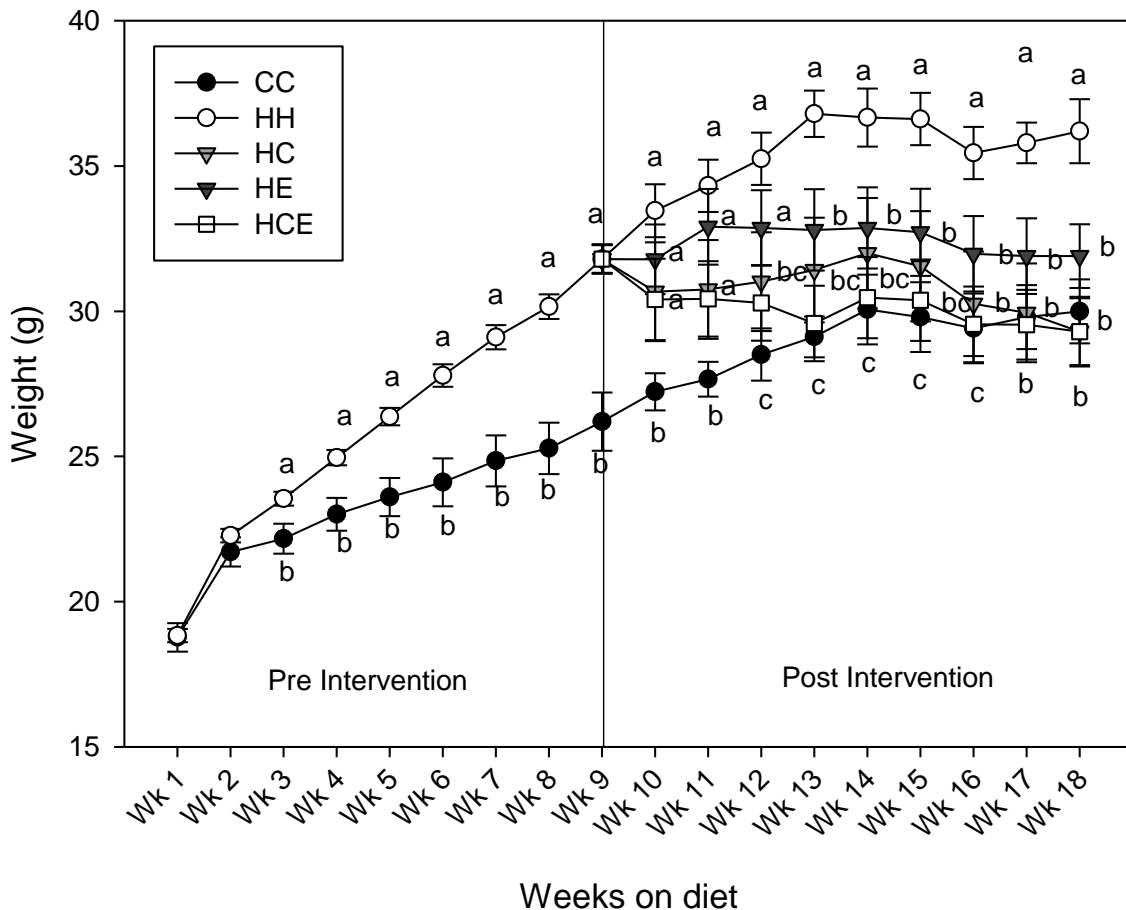


Figure S5.1: Paternal diet and exercise as obesity interventions on growth rate pre and post intervention

Pre intervention (1-9 weeks) and post intervention (10-18 weeks) growth curves for founder males. Data are representative of 8 HH, HC, HE and HCE founder males and 7 CC founder males. Data was analysed by Repeated Measure ANOVA and expressed at mean ± SEM. Different letters denote significance at p<0.05.

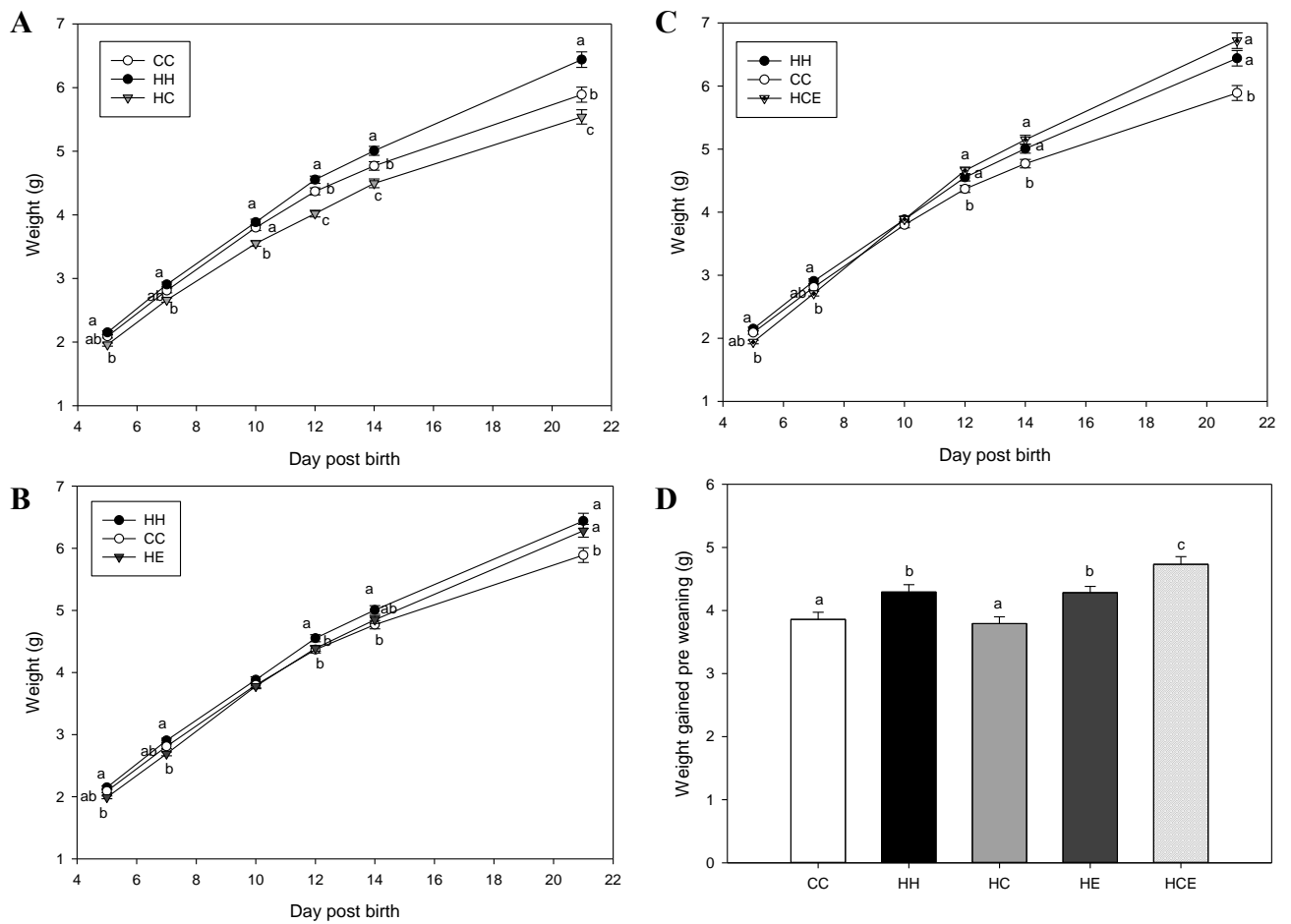


Figure S5.2: The effect of paternal diet and exercise as obesity interventions on female offspring pre-weaning growth

A Pre-weaning growth of female offspring born from diet alone intervention (HC), **B** Pre weaning growth of female offspring born from exercise alone intervention (HE), **C** Pre weaning growth of female offspring born from combined diet and exercise intervention (HCE), compared with offspring born from controls (CC) and high fat diet controls (HH). **D** Total amount of pre-weaning weight gained (day 5 until day 21 of life). Different letters denote significance at $p < 0.05$. Data was analysed using repeated measures ANOVA with father ID and mother ID added as a random factor and litter size as a fixed factor. Data are representative of at least 30 female offspring representing 10 HH litters from 7 fathers, 14 HC litters from 7 fathers, 11 HE litters from 7 fathers, 19 HCE litters from 8 fathers and 12 CC litters from 8 fathers.

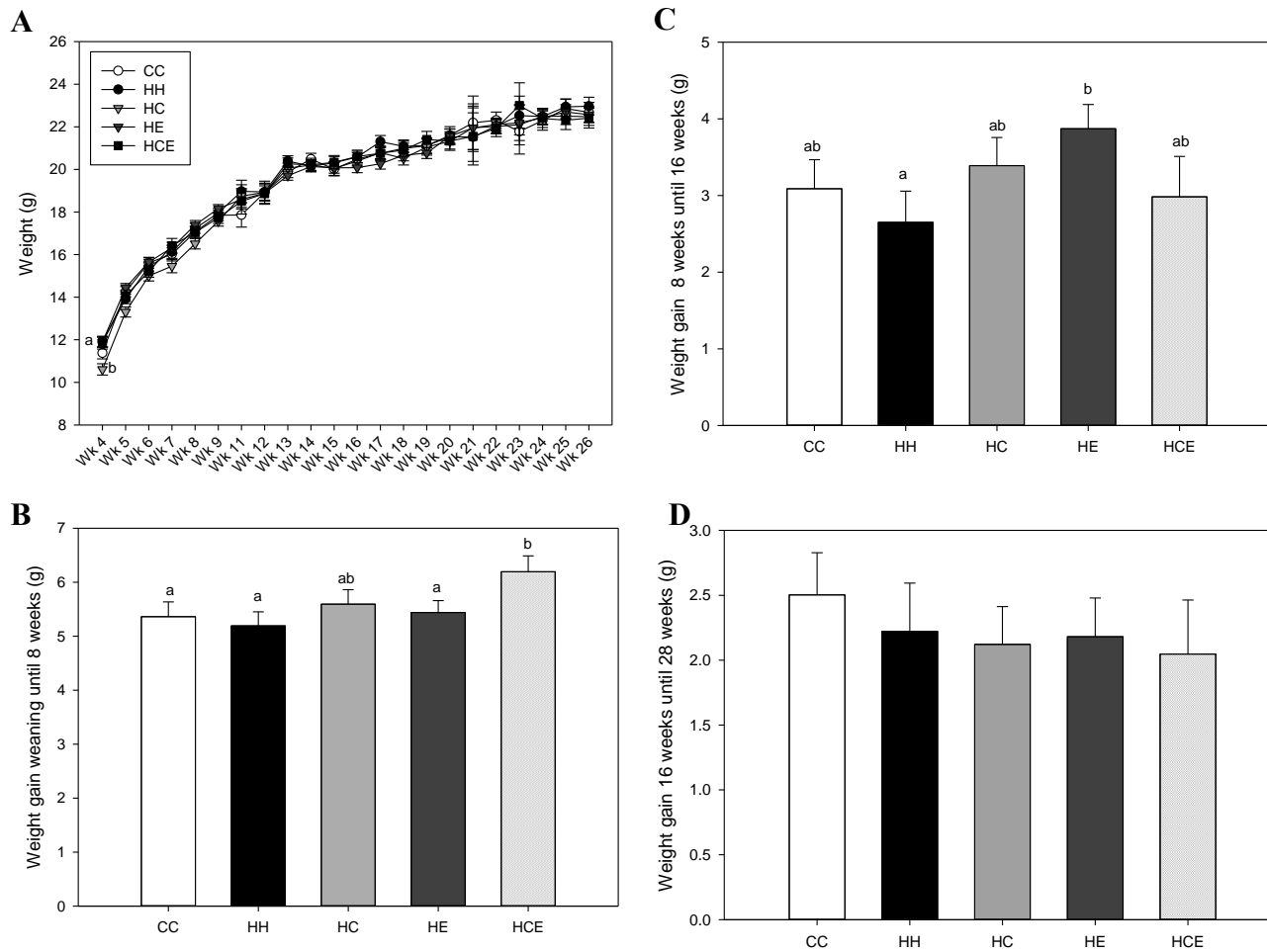


Figure S5.3: The effect of paternal diet and exercise interventions as obesity interventions on female offspring post weaning growth

A Post weaning growth of offspring from 4 weeks until 26 weeks. **B** Female offspring weight gained between weaning and 8 weeks of age. **C** Female offspring weight gained between 8 weeks and 16 weeks of age. **D** Female offspring weight gained between 16 weeks and 28 weeks of age. Data are analysed using repeated measures ANOVA with father ID and mother ID added as a random factor and litter size as a fixed factor. Data were representative of at least 28 female offspring representing 10 HH litters from 7 fathers, 14 HC litters from 7 fathers, 11 HE litters from 7 fathers, 19 HCE litters from 8 fathers and 12 CC litters from 8 fathers.

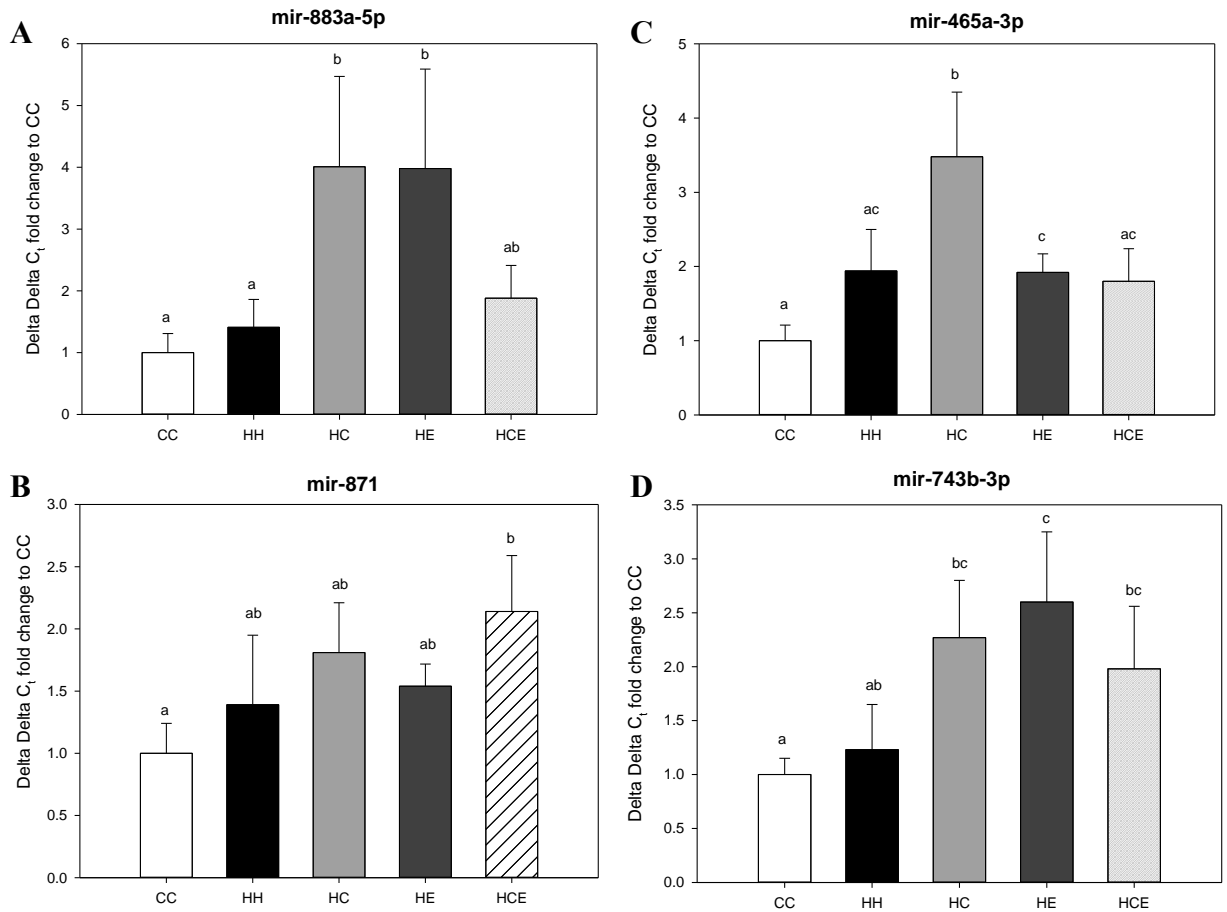


Figure S5.4: The effect of paternal diet and exercise as obesity interventions on x-linked sperm microRNAs

Fold change abundance of **A** mir-883a-5p, **B** mir-871, **C** mir-465a-3p, **D** mir-743b-3p, in founder sperm compared with CC founders. Data are expressed as mean \pm SEM. Different letters denote significance at $p < 0.05$. Data was analysed using linear mixed effects models with cohort as a covariate. Data are representative of $n=6$ HH and CC founders and $n=8$ HC, HE and HCE founders.

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6 Final Discussion

6.1 INTRODUCTION

There is general consensus that male obesity alters the molecular composition and function of sperm, particularly increasing ROS generation, which subsequently leads to associative increased sperm DNA damage and reduced sperm mitochondrial function (Chavarro et al., 2010; Dupont et al., 2013; Fariello et al., 2012; Kort et al., 2006; La Vignera et al., 2012b; Paasch et al., 2010; Rybar et al., 2011; Thomas et al., 2013; Tunc et al., 2011). Recently it has been shown in both human and animal models that paternal obesity at conception alters the developing embryo, reducing blastocyst development, cell numbers and mitochondrial function, which ultimately leads to reduced implantation and live birth rates (Bakos et al., 2011; Binder et al., 2012a; Binder et al., 2012b; Mitchell et al., 2011). Emerging evidence in rodent models of male obesity additionally describe ongoing impacts to the next generation with increased susceptibility to diabetes, obesity and sub fertility (Danielzik et al., 2002; Fullston et al., 2012; Fullston et al., 2013; Li et al., 2009; Ng et al., 2010). Together these data suggest male obesity not only negatively affects both the metabolic health and fertility of fathers, but also similarly affects the health of the next generation. Therefore investigation of interventions aimed at reducing paternal obesity, such as diet and exercise, and the downstream improvements to male fertility, embryo and offspring health were warranted.

Multi-generational studies that aim to investigate the legacy of paternal health in the human are complex and difficult to conduct. This is due to extended generation times (for assessing children's health as they age) and controlling for confounding factors is also difficult. Such confounders include maternal health during pregnancy (i.e. pre-eclampsia, gestational diabetes and adiposity status) which are known to alter fetal development and offspring health (Galjaard et al., 2013; O'reilly & Reynolds, 2013). Furthermore, paternal lifestyle factors including smoking, alcohol consumption and prescription/illicit drug use have been documented to perturb the molecular and functional status of sperm (Sharpe,

2010). Animal models are therefore useful tools which can be more readily controlled for confounding factors, mitigating many of the above limitations. Thus this study focussed on a rodent model of male diet induced paternal obesity whereby diet and exercise interventions were used to determine if the negative effect of obesity on sperm, embryo, pregnancy and offspring health could be restored.

6.2 DIET AND EXERCISE INTERVENTIONS IN OBESE FOUNDERS

6.2.1 Improvements to founder metabolic status

The obesity phenotype induced in this model resulted from feeding a semi pure high fat diet (21% fat) which is equivalent to the fat content of a western style fast food diet in humans. This period of high fat diet consumption in rodents caused many phenotypes that are common to aspects of human obesity including increased adiposity, alterations to serum metabolites (glucose and insulin), increased serum cholesterol, leptin and C-reactive protein concentrations (Klop et al., 2013). However, due to species differences there are some differences in body composition and the percentage of adiposity in rodents which would have to be modified for translation into humans. For instance in humans normal body fat percentages range from 18-25% in men with body fat percentages greater than 25% classified as overweight. The HH rodents of this study displayed body fat percentages between 22-25% as measured by DEXA, which was a 61% increase compared with the controls. Therefore the HH rodents used in these studies could be assessed as being equivalent to a human male with a fat percentage between 28-40%. But as DEXA is not the most accurate measure of adiposity, individual adipose depots (surrounding the adnominal organs) were also measured with a 200% increase recorded. Based on these individual adipose depots our HH rodents may be more related to human males with body fat percentages between 36-50%. The diet and exercise interventions used (either a change to a control diet with exercise (HCE) or without exercise (HC), or exercise alone whilst maintained on the high fat diet (HE; exercise constituted light controlled swimming) resulted in similar phenotypic outcomes as

studies of diet and exercise in interventions in obese humans. Both diet interventions (HC and HCE) in obese mice reduced adiposity, serum lipids and improved glucose tolerance. Interestingly, males on an exercise intervention alone (HE) maintained their pre intervention level of adiposity, implying that the amount of energy expended during exercise was similar to the calories ingested on the high fat diet. These mice also showed no improvements to serum cholesterol concentrations or insulin tolerance, similar to reported affects in human exercise alone intervention studies (Villareal et al., 2011). Interestingly both exercise interventions (HE and HCE) improved fasting glucose, FFA concentrations and glucose tolerance in males beyond that of controls (CC), likely a result of increased glucose uptake and oxidation by muscle and liver during exercise (Ivy, 1997; Rector et al., 2008). As this was an in vivo model of diet and exercise it was found that not all cohorts of intervention animals displayed exactly the same phenotypes. For example those founder animals used in chapter 2 who underwent exercise interventions (HE, HCE) had reductions to FFA concentrations, while those used in chapter 3 FFA concentrations were reduced but not significantly reduced. Reasons for these discrepancies could be multifactorial; 1) type of exercise intervention used – although forced swimming exercise was adopted in this study, some cohorts of male were better at this exercise regime than others and therefore some males may have exercised more than others; 2) semi pure rodent diet – the rodent high fat diet and control diets used were semi pure and therefore there could have been some minor changes in nutritional composition between batches and cohorts and 3) renovation of the animal house – during the study period the university's animal house was under renovation, with some cohorts experiencing 5 day weeks of disruptions associated with renovations while others experienced little or no disruption.

6.2.2 Restoration of sperm function and early embryo/fetal development

In Chapter 2, the impact of continued high fat diet feeding without intervention (HH) on sperm parameters was consistent with previous findings that saw negative changes to both sperm function (decreased motility, morphology, capacitation and oocyte binding) and molecular alterations, including

increased ROS and DNA damage. Chapter 3 demonstrated that these HH mice went on to produce embryos with delayed development, reduced cell numbers and alterations to cell-to-cell contacts in the compacting embryo. Interestingly all diet and exercise intervention combinations restored sperm function (motility, morphology, capacitation and sperm binding) and chromatin integrity (sperm ROS and DNA damage), which ultimately restored on-time embryo development, blastocyst cell number (a measure of mitosis) and partially restored cell-to-cell contacts in the compacting embryo (chapter 2 and chapter 3, Fig 6.1). Both sperm function and sperm chromatin integrity are required for successful fertilisation and early embryo development, whereby reductions to sperm motility, morphology and capacitation impair sperm binding and fertilisation (Aitken, 2013). The increased sperm binding observed after diet and/or exercise interventions was likely a result of restoration of sperm motility and capacitation. The improvements to embryo development from diet and/or exercise intervention are potentially due to improved sperm chromatin integrity, which would reduce the need for paternal DNA repair at fertilisation (Gawecka et al., 2013), thereby reducing any delay of the first and second cleavage events.

Blastocyst formation is regulated by both maternal and paternal proteins. The paternal genome is actively transcribed by the 2-cell stage in mouse embryos (4to 8-cell stage in human) and the first paternal proteins (i.e. E-cadherin) translated before embryo compaction (Matsumoto et al., 1994). Embryo compaction is required for spatial segregation of cells that will ultimately form the ICM (fetus and placenta) and trophectoderm (extra embryonic tissue) lineages and involves polarisation of cells, cells flattening and junctional communication which is regulated by proteins such as E-cadherin (Chen et al., 2010; Pey et al., 1998). The reduced on-time blastocyst development and reductions in both trophectoderm and ICM numbers seen in males fed a high fat diet without intervention (HH) may be related to increased numbers of 8-cell embryos with abnormal E-cadherin staining in the cytoplasm, which is indicative of a delay in polarisation and junctional communication (chapter 3). Additionally the

improvements to blastocyst formation and cell number in males who underwent exercise interventions (HE and HCE) may suggest restored embryo compaction with an increased number of 8-cell embryos with normal E-cadherin staining at cell-to-cell contacts, again indicative of increased cell polarisation and junctional communication. This suggests that E-cadherin may be a good marker for embryo quality, especially when assessing the impacts of a paternal environmental perturbation. As there were no differences in the proportion of cells within the blastocyst as a result of high fat diet feeding, the reduced numbers of cells in 96 h cultured blastocysts may also be related to their delayed development and a reduced cell division, which was restored by diet and/or exercise interventions.

Perturbations to the numbers of ICM and trophectoderm cells or the ratio of these cells within the blastocysts are related to reductions in implantation and fetal development (Lane & Gardner, 1997). Fetal growth and size are not only the cardinal determinants for a healthy pregnancy but are the key features of pre-determining the likelihood of developing non-communicable disease in later life, with small for gestational age at birth associated with increased rates of diabetes, cardiovascular disease and obesity in adulthood which likely initiated with their catch up growth in the post natal period (Barker, 2008; Crowther, 2012; Johnson & Schoeni, 2011). Therefore, in experiments in chapter 3, fetal growth and size was determined at embryonic day 18 after transfer of blastocysts produced by males to normal weight mothers. The results in chapter 3 confirmed previous findings in both obese rodent models and human *in vitro* studies, that report reduced blastocyst implantation rates and reduced fetal size produced from our obese high fat fed males (HH) (Binder et al., 2012a; Binder et al., 2012b; Mitchell et al., 2011). Interestingly, only the combined diet and exercise intervention group (HCE) restored implantation rates, and this intervention combination resulted in the biggest restoration of both ICM and trophectoderm cell numbers. The restoration from the combined diet/exercise intervention group (HCE) may be due to this group having the biggest effect on metabolic health, weight change/loss to that of the controls and not actually the type of intervention used. Future studies assessing weight loss vs types of

interventions are required to determine which the causative factor is; the intervention or weight loss/restoration of metabolic health. All intervention groups (diet and/or exercise) restored fetal weights to that observed in fetuses born from control males, however as the day 18 fetuses were not sexed it can't be determined if there were any sex specific effects which have been previously observed in rodent models of paternal obesity and fetal programming (Fullston et al., 2012; Ng et al., 2010). Although there were no differences between litter sizes or sex ratios following mating males fed a high fat (HH) compared to controls, a shift in sex ratios and litter sizes were observed in the combined diet and exercise intervention group (HCE, chapter 5). HCE males produced smaller litters with an increased proportion of male offspring suggesting that HCE males may have a change to the proportions of X/Y chromosome bearing sperm impairing their function (Edwards & Cameron, 2014). By post natal day 21 female offspring from HH fathers (chapter 5) were heavier having gained more weight in the pre weaning period compared to controls. This phenotype follows a similar pattern to that of SGA children who are born small and then display 'catch up growth'. Interestingly all interventions restored some pre weaning weights (post natal day 7, 10, 14), with diet intervention alone (HC) additionally fully restoring weight gained during the pre weaning period. This suggesting that offspring born from obese fathers that undergo diet and/or exercise interventions may have a reduced susceptibility to developing non-communicable diseases due to limiting this catch up growth recorded in offspring born from high fat fed male mice.

6.2.3 Partial rescue of offspring programming phenotype

The health and quality of the pre-implantation embryo can be used as an early predictor of offspring health (Calle et al., 2012). As diet and/or exercise interventions in obese founders restored both sperm function and chromatin integrity with concomitant improvements to both embryo development and early fetal weights, studies in chapters 4 and 5 went on to further establish if these improvements seen in pre-

implantation development would be translated into improved offspring metabolic and reproductive health.

The original rodent models of male obesity that assessed generational effects of metabolic syndrome in offspring, reported the first changes to glucose and insulin tolerance in F₁ female offspring at 14 weeks of age in the rat and 8 weeks in the mouse (Fullston et al., 2013; Ng et al., 2010). Although in the mouse model the F₁ male offspring did become glucose and insulin intolerant, this did not occur until later in age, and was less severe than the female phenotype (Fullston et al., 2013). Further the rat study reported no metabolic defects to male offspring born to fathers fed a high fat diet (Ng et al., 2010). Therefore, restoration of female metabolic health through interventions in obese founders was a focus of the studies in chapter 5. All combinations of interventions in obese founder males resulted in some restoration of their female offspring metabolic health, with exercise intervention alone (HE) in founders showing the greatest restoration of insulin sensitivity. Thereby, indicating that while there were some improvements to female F₁ metabolic health, there remained some residual impacts of the HFD or increased adiposity. Whether this partial restoration in female offspring resulted from the short intervention window in their father (8 weeks in this study) or if full restoration of offspring metabolic health could be achieved from a longer duration of intervention, remains to be determined. Additionally, the original rodent model determined that feeding a high fat diet to induce obesity in male mice perturbed the metabolic health across two generations (Fullston et al., 2013). As partial restoration of female offspring metabolic phenotypes was observed as a result of diet and/or exercise interventions in obese founders, further studies would be warranted to determine if this improvement would extend into the second generations.

Due to the altered F_1 sperm phenotypes that result of from being born to a father fed a HFD described in the original study (Fullston et al., 2012) and the increasing worldwide prevalence of male sub fertility, which implicates an environmental factor (Mendiola et al., 2013; Rolland et al., 2013; Swan et al., 2000), F_1 male reproductive health was the focus of chapter 4. Studies presented in chapter 4 replicated the negative effects of feeding a high fat diet to induce obesity in founder males on their F_1 son's sperm function, with reduced sperm motility and sperm binding found. Only diet interventions (HC and HCE) in founders appeared to improve the perturbed sperm function in male offspring born to an obese father, with exercise intervention alone (HE) only producing small improvements to F_1 sperm capacitation. However, there are significant differences in both body composition and metabolic health of founders who underwent exercise intervention alone (HE) compared with those founders that underwent diet interventions (HC and HCE), such as increased adiposity and serum cholesterol and triglycerides. Therefore male offspring's sperm function may be more related to the adipose state and cholesterol concentrations of their father as these were the parameters most improved by diet interventions. Interestingly, this phenomenon is not consistent with regards to the metabolic health of female offspring, with exercise intervention alone (HE) in founders showing the biggest restoration to female offspring metabolic health (chapter 5). However, further studies are required to determine if similar restoration to female offspring oocyte quality results, as this was demonstrated to be perturbed in the original study of founders fed a high fat diet (Fullston et al., 2012). One of the interesting findings to come from this study was the improvements to F_1 sperm capacitation and sperm mitochondrial function from diet intervention only founders (HC) beyond that of control offspring. Whether this improvement can be attributed to HC founders increased glucose regulation compared with control founders or another factor is still to be determined.

Taken together the studies in chapters 2, 3, 4 and 5 provide evidence that the effects of an overweight or obese male on sperm function, embryo development and pregnancy and offspring health are not

entirely permanent and can be at least in part reversed by restoration to normal adiposity levels and/or metabolic health via diet and/or exercise interventions (Fig 6.1). Studies in both animal models and humans of male obesity have reported improvements to both metabolic health and sperm function after surgical interventions, treatment with probiotics, olive oil supplementation and drugs to lower plasma glucose (Bastounis et al., 1998; Chen et al., 2013; Ibrahim et al., 2011; Reis et al., 2010; Reis et al., 2012; Saez Lancellotti et al., 2013; Savastano et al., 2013). To date only one small scale human study has assessed the impact of diet and exercise lifestyle interventions in obese males on sperm, using sperm parameters, demonstrating improvements to sperm count, motility and morphology in those men who lost the greatest amount of weight (Hakonsen et al., 2011). This result is slightly contradictory to the results found in this study as sperm parameters were also restored in those mice that underwent exercise intervention alone (HE) who had no reduction in weight and/or adiposity. Therefore confirmation of the results in chapters 2, 3, 4 and 5 via diet and/or exercise interventions in humans are a natural extension of these investigations to further determine if it's more about weight loss, a change in metabolic health or the type of intervention used to illicit an effect. Similar large human studies are required to determine if restoration of embryo, pregnancy and offspring outcomes are translatable to humans, thus providing evidence based targeted public health messages and policy for men prior to conception.

6.2.4 Partial normalisation of microRNA abundance in sperm – a potential mechanism

The magnified detrimental effect on female offspring metabolic health due to paternal obesity ((Fullston et al., 2013; Ng et al., 2010) and chapter 5), suggests a molecular mechanism in sperm that specifically targets female embryos. Sperm deliver more than just their genome at fertilisation, with mature sperm harbouring a vast pool of mRNA and small non coding RNAs that are delivered to the early embryo. Specifically sperm microRNAs have been shown to be necessary for the developing embryo and influence first stage cleavage events as well as the phenotype of subsequent offspring (Jodar et al.,

2013; Sandler et al., 2013). A recent study also showed microRNA levels in testes/sperm were altered in a similar mouse model of male diet induced obesity (Fullston et al., 2013). Furthermore, altered microRNA content in serum of obese men was restored by diet and exercise (Ortega et al., 2013). Therefore it is plausible that sperm microRNAs may be similarly restored by diet and exercise and therefore may provide a partial explanation for the improvements observed in embryo and offspring health as a result of diet and/or exercise interventions in obese males. Interestingly, loci encoding microRNAs are scattered across chromosomes in the human except for the Y chromosome, conversely many microRNA are encoded on the X chromosome. Therefore X-linked microRNAs were targeted in this study as a potential mechanism of father to daughter transmission.

Four X-linked microRNAs were shown in chapter 5 to have altered abundance in sperm of HH founders. In silico assessment of the experimentally confirmed gene targets of these microRNA implicated pathways that are important for early embryo development including cell cycle regulation and apoptosis. Such pathways are altered in embryos produced by HFD fed males (chapter 3), whereby rates of development and cell numbers were reduced, which therefore may be initiated through changes to microRNA cues delivered by sperm. It would be of interest to directly manipulate the levels of these X-linked microRNAs either through genetic mouse models of increased/reduced abundance in sperm or artificial injection into the male pronucleus and to study the effects during embryo development. Although there were no changes seen in the level of apoptosis in cells of blastocysts between treatments, HH founders had a significant increase in variance of apoptosis levels between HH derived embryos, with individual embryos showing up to 20% cell death. Given the demonstrated impact of X-linked microRNA it may be possible that male and female embryos would be differently affected, due to the possible dosage effects between females (2 X-chromosomes) vs. males (1 X-chromosome) and that X-chromosome inactivation does not occur until the blastocyst stage embryo. Thus sexing of embryos might show skewing toward apoptosis in female embryos. In any case it would be valuable to repeat

many of the measures described in chapter 3 on embryo, fetal and placental development with the addition of sexing tools to determine if there are indeed differential effects between male and female embryos.

Although all intervention groups showed some level of restoration of sperm microRNA abundance, exercise interventions (HE and HCE) showed the greatest improvements. The mechanism by which exercise restores these microRNAs not seen in diet interventions remains to be investigated. However it has been shown in humans that even short bouts of exercise with minimal weight loss can alter the microRNA profile of serum (Banzet et al., 2013; Sawada et al., 2013). It should be mentioned that exercise interventions in founders (HE and HCE) did result in the greatest improvements to embryo development, cell-to-cell contacts, embryo cell numbers and insulin sensitivity in female offspring at 9 weeks of age, suggesting that restoration of X-linked microRNA cues in sperm could be a causative mechanism. However, the improvements to offspring metabolism were not always consistent across age in exercise interventions (i.e. HCE) suggesting that the mechanism is multifactorial with restoration of sperm microRNAs likely part of a broader epigenetic and genetic landscape altered in sperm due to obesity. The direct mechanism by which sperm microRNAs exhibit their effect on the developing embryo and how they might initiate offspring phenotypes, the specific amount of weight loss to trigger a change in microRNA content in sperm or type of intervention requires further investigation.

Male Obesity

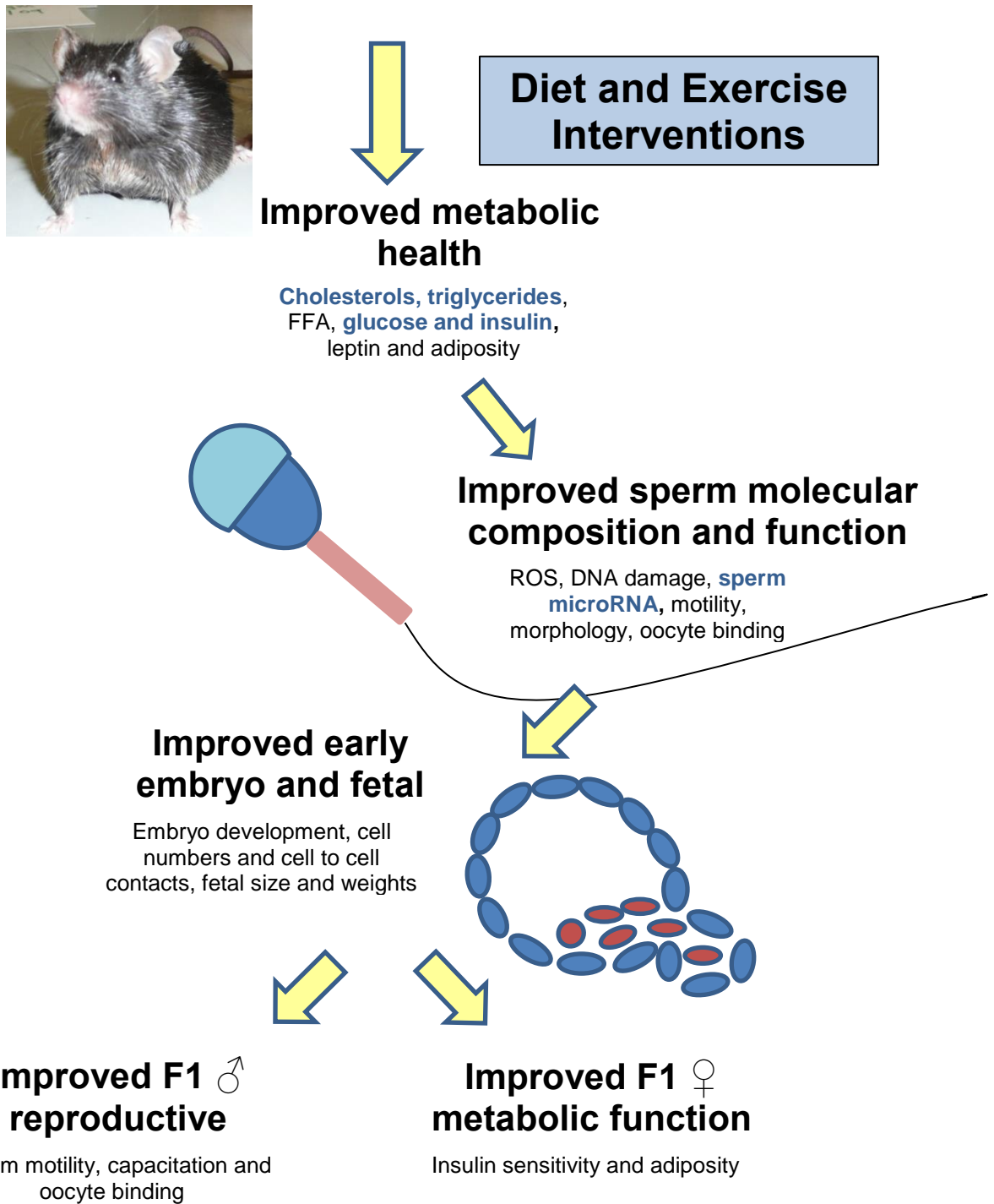


Fig 6.1 Summary of outcomes from diet and exercise interventions in obese males on their metabolic health, sperm function, subsequent early embryo development and offspring health. Emboldened words represent markers independent to treatment group that correlated with embryo, fetal and offspring outcomes.

6.3 DOES FOUNDER ADIPOSITY ALONE EXPLAIN THE OBSERVED EFFECTS?

The models of diet and/or exercise interventions used in this study resulted in groups of mice with varying degrees of adiposity and differences in metabolic health. For example two groups of mice with increased adiposity were established (HH and HE), with rodents undergoing exercise intervention alone (HE) showing an additional improvement to glucose regulation. This provided a model that could begin to dissect the individual contributions due to adiposity or metabolic health on founder male reproductive health. Although both the HH and the HE groups of animals had elevated levels of adiposity compared with control animals (CC), the males undergoing exercise intervention alone (HE) displayed some restoration of sperm function, embryo development and even female offspring metabolic health phenotypes. These observations support the hypothesis that increased adiposity may not be the sole driver of impaired reproductive function in obese males, with lipid and glucose regulation also influencing reproductive health. This may in part help explain some of the contradictions in the current literature about the effects of obesity on sperm function, as to date the majority of human studies have not assessed the metabolic health of obese males (i.e. glucose and insulin tolerance), typically relying solely on BMI.

Increased scrotal heat and therefore testicular temperature due to obesity has been proposed as a potential mechanism for the adverse sperm parameters described on obese males. Scrotal heat alone is associated with reduced sperm motility, increased sperm DNA damage and increased sperm oxidative stress (Paul et al., 2008a; Paul et al., 2008b; Shiraishi et al., 2010; Hammoud et al., 2011; Iranpour et al., 2000; Tunc & Tremellen, 2009). Interestingly, males fed a HFD with exercise (HE) intervention exhibited improved sperm function (motility and morphology), reduced sperm DNA damage and ROS, common sperm parameters normally elevated by heat exposure (Paul et al., 2008a; Paul et al., 2008b; Shiraishi et al., 2010) (chapter 2). This indicates that an increase in testicular temperature is an unlikely

mechanism for the changes to reproductive function described in this model. However, it is important to note that the differences in the location of rodent testes compared to human testes, which may result in some species specific differences. Mouse testes can retract and lower their testes within the abdominal cavity tightly regulating their temperature. In contrast, human testes are located in the scrotum (outside the abdominal wall) and function 2°C below body temperature (Reyes et al., 2012). Therefore, increased gonadal adiposity and a related increase in heat may have a bigger impact in humans, highlighted by improvements to sperm function and an increased incident of pregnancy found after scrotal lipectomies in obese men (Shafik & Olfat, 1981).

The results described in chapters 2, 3 and 5 reveal improved sperm function and offspring outcomes with exercise intervention alone (HE), suggesting that the positive benefits of exercise may be also override some of the negative effects of increased adiposity via improved metabolic health and inflammation. Single bouts of short term exercise before consumption of a high fat meal in obese adolescents reduce circulating lipids (Lee et al., 2013). Additionally the adipose specific hormone leptin, which can regulate the hypothalamus and testosterone, was also restored in obese founders undergoing exercise intervention alone (HE). This suggests that even though these mice still display increased adiposity compared with controls (CC), the secretory profiles and gene expression of their adipose tissue are likely changed due to the beneficial outcomes of exercise. This phenomenon has been reported in a human study of diet and exercise interventions in obese adolescents, with males who underwent exercise interventions without any alterations to levels of adiposity showing improvements to inflammatory pathways of their adipose tissues (Auerbach et al., 2013). Interestingly exercise intervention alone (HE) restored circulating C - reactive protein concentrations reducing the pro-inflammatory state that was associated with males fed a high fat diet without intervention (HH) (Chapter 5). Future studies should investigate whether inflammatory and metabolic pathways are

improved in adipose tissues of rodents undergoing exercise intervention alone (HE) to determine if functional pathways in their adipose tissue have indeed improved.

Despite improvements to reproductive function with exercise intervention alone (HE), correlations of founder adiposity independent of treatment group still displayed significant negative associations with blastocyst cell number, blastocyst apoptosis rates and F1 sperm motility/count (chapter 2 and 3). However, stronger associations were found between founder lipid, glucose and insulin status with sperm function, embryo development and offspring phenotypes, indicating that adiposity was not the sole contributing factor. Future studies assessing the impacts of increased adiposity with or without altered metabolic health in humans will help determine the role increased adiposity has on sperm function and whether this is a secondary phenotype to altered blood metabolites and a diet high in fat.

6.3.1 Are circulating lipids and metabolites a better biomarker for predicting altered sperm function and subsequent embryo and offspring health?

As previously mentioned, the strongest correlations (independent of adiposity and treatment group) to sperm function, embryo development and offspring health were with founder serum metabolites; in particular cholesterol, glucose and insulin. This suggests that circulating lipids and metabolites may be a better indicator of altered sperm function and early embryo development, then adiposity alone. Founder male glucose concentrations and response to a glucose challenge were shown to be key predictors of fetal size and offspring weights for both male and female offspring (chapter 3, 4 and 5). This has been previously described in rodent models of paternal hyperglycaemia, whereby fathers with high glucose concentrations have reduced offspring growth and weights into adulthood (Grasemann et al., 2012). Additionally, high circulating concentrations of glucose in men with type I diabetes, independent of obesity, reduced live birth rates (Sjoberg et al., 2013), increased sperm oxidative stress and increased

oxidative DNA adducts (Agbaje et al., 2008) via changes to oxidative stress clearly implicated as the causative pathways in sperm and testes (Mallidis et al., 2009). Similar correlations were found here with founder males response to a glucose bolus positively correlated to increased sperm DNA damage (chapter 2), which has been previously shown to negatively affect embryo development and pregnancy health (Bakos et al., 2008; Brahem et al., 2011; Gallagher et al., 1993; Kumar et al., 2012; Thomson et al., 2011). Therefore, suggesting that assessment of glucose metabolic state (fasting concentrations and glucose tolerance test) should be performed in males prior to conception to establish pregnancy risks for complications.

Elevated serum cholesterol concentrations are commonly seen in men with increased adiposity (Klop et al., 2013). This study correlated serum cholesterol concentration with impairment to measures of sperm function (chapter 2), embryo quality (chapter 3) and offspring outcomes (chapter 4). Increased serum cholesterol without marked increases to body weight in a rabbit model has previously been reported to cause sperm dysfunction with reduced sperm motility, count, morphology, capacitation and semen volume reported (Saez Lancellotti et al., 2010). These changes to sperm are proposed to occur in the epididymis where high concentrations of circulating cholesterols cause degradation in the proximal epididymis, leading to sperm morphological abnormalities, decreased motility and premature acrosome reaction in a rodent model (Saez et al., 2011). Additionally exposures to both increasing concentrations of cholesterols as well as FFA in human sperm in vitro caused increased levels of sperm oxidative stress (Koppers et al., 2010), which in itself can alter embryo and pregnancy outcomes (Aitken & Baker, 2006; Aziz et al., 2004; Zorn et al., 2003). Furthermore, seminal plasma with elevated saturated fatty acid concentrations has been negatively correlated to sperm motility and count (Attaman et al., 2012). Studies assessing the impact that seminal plasma metabolite concentration in normal weight, overweight and obese males has on sperm function and whether these could be improved through diet and exercise interventions or cholesterol lowering drugs are warranted.

6.3.2 How blood metabolites and lipid profiles associated with obesity might alter the epigenetic status of sperm

A two-step hypothesis for the development of DNA damage from environmental perturbations in human sperm has been proposed by Aitken and colleagues (Aitken & Curry, 2011; Aitken & De Luliis, 2010). The first step describes defective chromatin remodelling during spermiogenesis with sperm released from the lumen of the seminiferous tubules in an imperfect state, with altered protamination and other structural defects, creating a sperm cell that is vulnerable to attack. They further hypothesise a second step that as a result of poor protamination the sperm are vulnerable to DNA strand breaks that are likely mediated through oxidative stress, which increases sperm apoptosis and DNA fragmentation. The data presented here further implicates circulating metabolites, with or without adiposity, as one of the causative agents for creating the environment that leads to imperfect spermiogenesis. Additionally, it is not difficult to further speculate that similar environments might also result in perturbations to the epigenetic status of sperm (Fig 6.2). The data in this thesis ultimately leads to the theory that circulating metabolites have a multifactorial action and can alter 1) protamination and microRNA abundance during spermiogenesis, 2) change the epididymal microenvironment during sperm maturation and 3) directly alter the epigenetic state of sperm (Fig 6.2).

6.3.2.1 Protamination and microRNA abundance

Sperm protamination is usually incomplete resulting in approximately 1% of histones being retained in murine sperm (Balhorn et al., 1977) and up to 15% in human sperm (Gatewood et al., 1987). There is recent evidence that the retention of these histones is not a random process with key pluripotent genes necessary for early embryo development remaining histone bound (Farthing et al., 2008; Hammoud et al., 2011). Although there is a vast difference in the range of histone retention between mouse (1%) and humans (15%) the regions that are histone bound are conserved between mammals (Brykczynska et

al., 2010). The process of histone to protamine transition is reliant on histone acetylation, regulated by both histone deacetylases and histone acetylases (Gaucher et al., 2010). Histone acetylation presents as an epigenetic mark that is capable of being transmitted to the oocyte during fertilisation. Histone deacetylases have been demonstrated to be regulated by metabolic state in a mouse model of obesity, whereby increased cholesterol and triglycerides altered histone acetylation during spermiogenesis, changing the gene expression and protein levels of SIRT 6 a histone deacetylase (Palmer et al., 2011). This resulted in increased DNA damage in transitional spermatids, increased DNA damage and functional changes in mature sperm. Thus changes to the regulation of histone deacetylases via alterations to serum lipids could perturb or increase retention of histones and change histone acetylation that could be transmitted to the embryo, altering offspring phenotypes. Whether these changes to histone deacetylase can be restored via interventions that reduce circulating lipids (i.e. diet/exercise) is still to be established. However preliminary studies aimed at restoring elevated cholesterol concentrations via olive oil supplementation in rabbits, demonstrated improvements to sperm motility, capacitation and membrane integrity (Saez Lancellotti et al., 2013).

MicroRNAs are important for the regulation of spermatogenesis and are present during the final stages of sperm maturation and during fertilisation (Ostermeier et al., 2004). It was recently shown that microRNA can regulate metabolic state, specifically playing important roles in regulation of lipid and glucose homeostasis (Fernandez-Hernando et al., 2013), with increases to serum cholesterol and glucose concentrations associated with changes to circulating serum microRNAs (Karolina et al., 2012). Six X-linked sperm microRNAs significantly correlated with founder glucose tolerance (chapter 5), suggesting that alterations to sperm and testicular microRNAs may also be regulated by changes to metabolism. As sperm microRNAs can alter the phenotype of the embryo and subsequent offspring (Jodar et al., 2013), changes to the metabolic health of males results in changes to the abundance of sperm microRNAs, this may in part form the basis for impaired offspring phenotypes. Further studies

exploring how glucose homeostasis impacts on sperm microRNA abundance and the direct impact this might have within the paternal pronucleus for programming offspring phenotypes, remains to be assessed *in vivo* and *in vitro*. However, recent advancements in microRNA labelling technologies combined with time lapse microscopy and microinjection will allow for tracking of paternally inherited microRNAs throughout embryogenesis.

6.3.2.2 Epididymal microenvironment

It has established that epididymal endothelium transports proteins to the surface of sperm necessary for sperm maturation, occurring through epididymosomes (Sullivan et al., 2007). A recent study demonstrated that these epididymosomes also transport microRNAs to sperm during epididymal transit (Belleannee et al., 2013). Epididymal endothelial ion transporters operate between the circulating blood and epididymial lumen (Koppers et al., 2010; Saez et al., 2011), suggesting that increases to serum metabolites or changes to the secretions of the endothelium can change sperm membrane fluidity and therefore increase or reduce susceptibility to damage. Hypercholesterolemia has been shown to alter the structure of epididymal endothelial cells and potentially sperm function (Saez et al., 2011). This suggests that changes to circulating metabolites may alter epididymal endothelium function changing the epididymosomal content (i.e. microRNA) delivered to sperm and therefore subsequently altering the contents delivered to the oocyte at fertilisation. Further studies to assess the content of these epididymosomes, their modulation by circulating metabolites and the ultimate impact on sperm microRNA content are warranted.

6.3.2.3 Mature sperm

Both *in vivo* and *in vitro* studies have shown that increased exposures to cholesterol, FFAs, glucose and insulin alter sperm metabolism, reducing motility and increasing oxidative damage (Amaral et al., 2008; Koppers et al., 2010; La Vignera et al., 2012a). Due to the lack of cytoplasmic scavenging enzymes and high concentrations of polyunsaturated fatty acids sperm are highly susceptible to this oxidative damage. Increased levels of ROS are associated with changes to global methylation profiles

of sperm (Tunc & Tremellen, 2009). Further hypomethylation of imprinting genes and repeat elements in sperm is linked with reduced pregnancy success (El Hajj et al., 2011; Nanassy & Carrell, 2011). Additionally sperm harbour an oxidated form of 5-methylcytosine, namely 5-hydroxymethylcytosine (Jenkins et al., 2013) coined the '6th DNA base' (Cadet & Wagner, 2013), and has been demonstrated to be important during the early stages of pronuclear formation in the embryo (Salvaing et al., 2012). 5-methylcytosine has been shown to be oxidised to 5-hydroxymethylcytosine in the presence of ROS (Coulter et al., 2013). It is therefore plausible that changes to circulating metabolites may alter the oxidative state of the methyl side groups in sperm via elevated ROS, which is passed onto the newly fertilised embryo, potentially altering fetal health. Further studies assessing the ratio of 5-methylcytosine and 5-hydroxymethylcytosine in sperm on pregnancy outcomes, their changes due to altered metabolic profiles and increased ROS as well as how this alters the methylation patterns in the paternal pronucleus and early cleavage events warrants investigation.

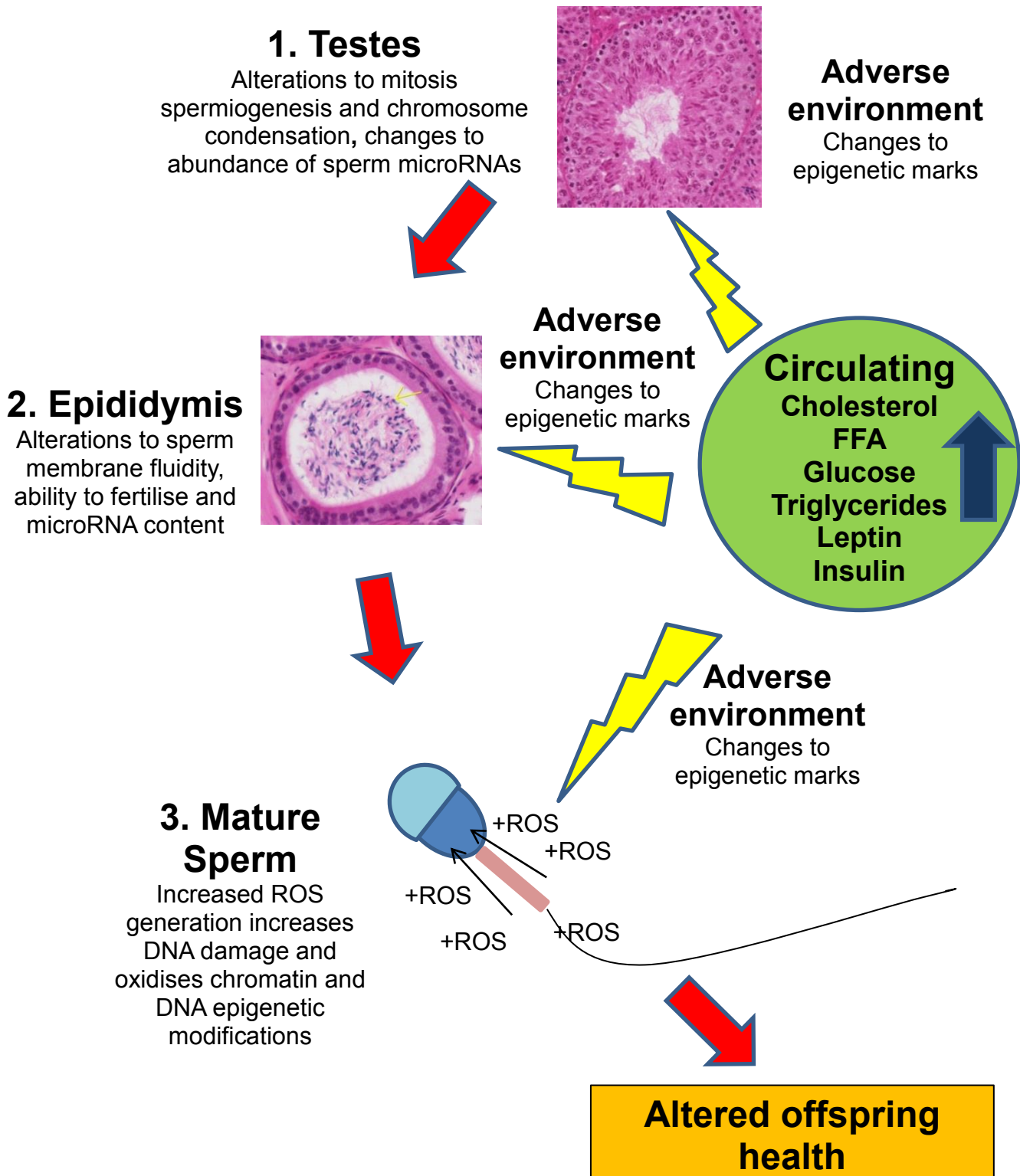


Figure 6.2 Hypothesis of how changes to circulating lipids and metabolites may change the epigenetic status of sperm that might ultimately form the basis for offspring programming

6.4 LIMITING FACTORS OF RODENT MODELS AND ALTERNATIVE PARADIGMS OF DIET AND EXERCISE INTERVENTIONS

6.4.1 Limitations

There are always limitations to the interpretation of data from animal models and extrapolation to human health. While the model used in this study did show some similarities to those seen in obese males, all findings will need to be confirmed in longitudinal human cohorts and clinical trials. Limitations of these studies include; 1) the use of a semi pure high fat diet – although the fat content of this diet was equivalent to a western fast food diet (21%), we know that other nutrients in excess (refined sugar, and carbohydrates) are also contributing to the world wide obesity epidemic in humans. To control for the level of fat in the high fat diet, the nutrient matched control diet had an increased level of carbohydrates in comparison. Whether this increase in carbohydrates in the control diet is also changing sperm function and quality needs to be determined. In addition many overweight/obese humans are nutrient poor even though they have increased caloric intake, while the semi pure rodent diet was nutrient rich. Further studies are required to determine types of nutrition and nutritional composition on male fertility. 2) The use of super ovulated pre pubertal females to generate embryos – is the most ethical way to generate embryos in the rodent as it reduces the number of animals needed to generate embryos. However, super ovulation results in an increased number of oocytes of reduced quality which would not have been normally ovulated. In the human the only scenario where superovulation is used, is during artificial reproductive technologies where an increasing number of overweight/obese males are present (Bakos et al., 2011) although it should be noted that the offspring studies used naturally ovulating females. 3) The use of swimming as exercise – rodents are not normally exposed to swimming in a natural environment and the use of an open water maze is commonly used by psychologists to determine anxiety in the mouse. The swimming regime used in this study was under different conditions to that used in psychological testing (i.e. water was warm and mice were acclimatised to the exercise over a two week period) and markers of stress i.e. corticosterone were assessed in all animals and found not

to be altered by swimming exercise. The swimming regime was chosen for number of reasons; i) created forced exercise – a number of trials using running wheels in HFD mice were unsuccessful where males did not run or use the wheel and ii) control the level of exercise – one benefit to swimming was controlling the level and amount of exercise each mouse received, which can be a limitation of a running wheel. In an ideal experiment an exercise only control on a CD would determine any negative effects of the swimming regime on sperm function. 4) Sperm collection ends in death in rodents – ideally assessing repeated sperm measures on the same animal after the initial high fat diet exposure and again multiple times during the intervention period, would provide the information required to determine if it's the intervention method or weight loss/restoration of metabolic health that is attributing to the improvements. While these studies could be conducted in humans where repeated semen sample could be collected and assessed, in rodents assessment of sperm function without maternal tract factors results in death of the animal. 5) The use of pre pubertal males – as I didn't want advanced paternal age to confound results after the 18-20 week pre intervention and intervention period, male mice were placed on the initial diets at 5-6 weeks of age. This start of diet would equate to 12-13 year old tween in the human and therefore questions the validity of extrapolation to human health. However many of the changes to sperm function described in chapter 2 mirror those seen in human studies.

6.4.2 Alternative paradigms

The model used in these studies is only one paradigm of high fat diet feeding with a fixed diet/exercise interventions. The next obvious question is; could other models of different nutritional feeding and durations, timing and or intensities of interventions result in a similar if not better outcome to male fertility. For example the fat content of the semi pure rodent diet used in these studies was saturated fat. Would we see similar negative effects to sperm quality utilising a different fat type (i.e. trans fat or polyunsaturated fats) or if we changed the main calorie component to refined sugars or would we be able to restore perturbed sperm function in animals fed a high fat diet for a prolonged period (i.e. six months to a year). Additionally, the duration, timing and or intensity of the exercise regime could answer

very different specific questions i.e. a shorter intervention period 6-10 days would target epididymal sperm maturation while a 5 week intervention period would target one round of spermatogenesis or a testes effect in the mouse. While intense training programs over a short time frame vs a light training program over a long time period would provide information on rapid weight loss vs. a sustained weight loss on male fertility.

6.5 CONCLUDING REMARKS AND FUTURE DIRECTIONS

The studies outlined in this thesis synthesise into two main findings; 1) targeted diet and exercise lifestyle interventions in obese males that improves metabolic health can restore sperm function, early embryo development and reduce the risk of diabetes, obesity and sub fertility in the next generation and 2) that serum glucose, insulin and lipid status may be better indicators than adiposity in fathers for predicting sperm function, embryo development as well as susceptibility of developing non-communicable disease in offspring.

The direct mechanism by which obesity and its associated co-morbidities are exerting their effects on the sperm, subsequent embryo, fetal health and how diet and exercise modulate these effects is clearly multifactorial. Epigenetic changes in sperm originating in the testes; epididymis and persisting into mature sperm are clearly implicated. Future studies assessing the direct changes to testes and epididymial microenvironments, related to obesity and altered metabolic health and how this is restored through interventions will be vital for improving the health of future generations.

Together these studies have shed light on the under-investigated reversibility of obesity related fertility pathologies in males. This will undoubtedly stimulate research into the molecular mechanisms and the associations between obesity related metabolic changes and their independent relationships with male

fertility. Additionally this thesis has provided the preliminary evidence base for human studies of diet and exercise interventions in obese males. Human studies will determine if the reproductive health improvements described in this rodent model can be translated, thus providing the basis for public health messages and policy targeted at males prior to conception.

6.6 REFERENCES

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7 Appendix

7.1 MODIFIED G-IVF COMPOSITION

Table 7.1: Media composition of modified G-IVF

Component	G-IVF (mM)	Amount
NaCl	100	5.83g
KCl	5.5	0.41g
NaHPO ₄ 2H ₂ O	0.36	0.05g
MgSO ₄ 7H ₂ O	1.8	0.44g
NaHCO ₃	2.0	2.101g
CaCl ₂ 2H ₂ O	1.0	0.147g
Glucose	1.0	0.18g
Fructose	1.0	0.18g
Na-Lactate	5.4	0.61g
Na-Pyruvate	0.1	0.035g
Glycyl-glutamine	0.5	0.0147g
Alanine	0.1	0.0089g
Asparagine	0.1	0.015g
Aspartate	0.1	0.0133g
Glutamate	0.1	10 ml
Glycine	0.1	0.0075g
Proline	0.1	0.0115g
Serine	0.1	0.0105g
Taurine	0.1	0.0125g

Methods

Weigh out all components into a 2ml beaker, add 890 ml of milli Q H₂O and dissolve ingredients until water is clear. Filter through a 0.25µm nylon filter and store at 4°C. Prior to use human serum albumin is added to a final concentration of 10%.

7.2 GLUCOSE AND FRUCTOSE UPTAKE EQUATIONS

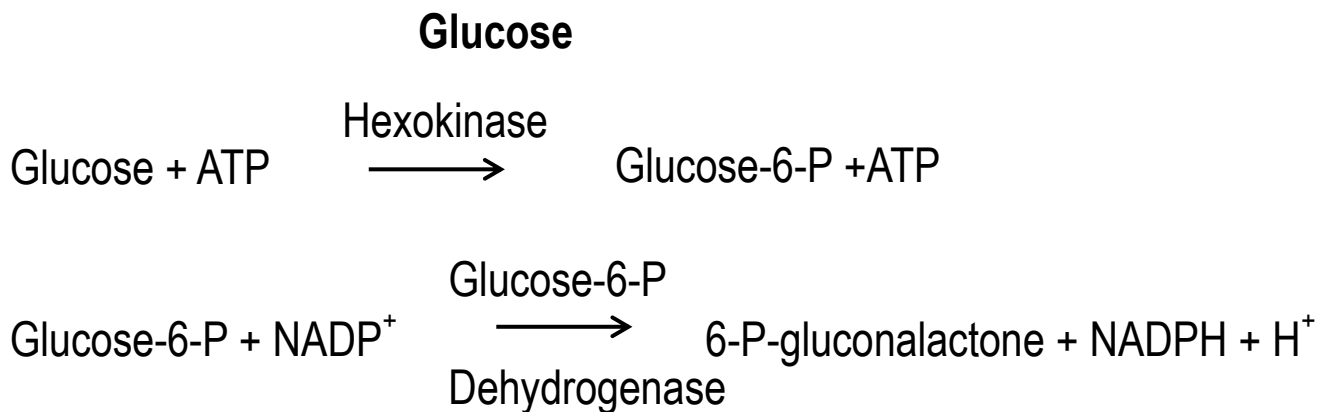


Figure 7.1 The two step equation illustrating the conversion of the non-fluorescent NADP^+ to the fluorescent NADPH

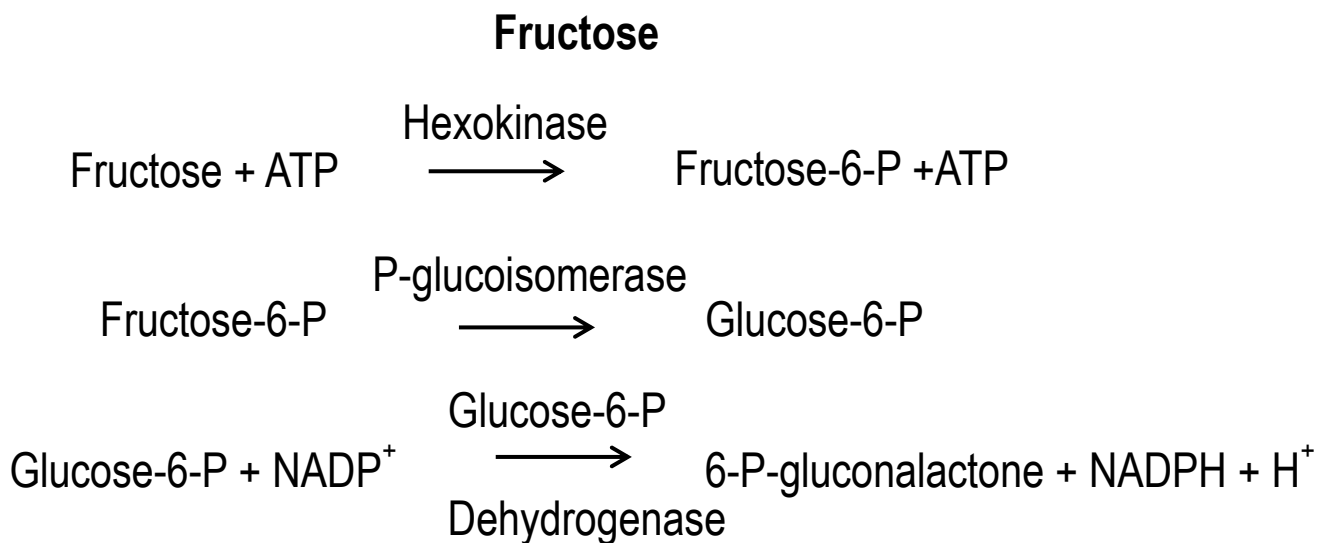


Figure 7.2 The three step equation illustrating the conversion of fructose-6-P to glucose-6-P, and then non-fluorescent NADP^+ to the fluorescent NADPH

7.3 PHOSPHATE BUFFERED SALINE

Dissolve 1 table of PBS (Sigma-Aldrich, P4417) into 200 ml of milliQ H₂O. Filter through a 0.25µm nylon filter and store at 4°C.

7.4 SALINE FOR INJECTION

0.9% sodium chloride. Add 0.9g of sodium chloride into 100ml of MilliQ water. Filter through a 0.25µm nylon filter and store at 4°C.

7.5 AVERTIN ANAESTHETIC

STOCK

Dissolve 0.5g of 2-2-2 tribromethanol (avertin) into 0.5ml of 2-methyl-2-butanol (Tert-amyl-alcohol). Store at 4°C.

WORKING SOLUTION

Add 240ul of Avertin stock solution into 10ml of saline for injection. Dissolve at 37°C for 4 hr.

7.6 TAQMAN PROBES

Table 7.2: TaqMan probes used for confirmation of microRNA PCRs in sperm

MicroRNA	Assay Name	Probe ID	Target Sequence
Mmu-mir-503	mmu-miR-503	002456	UAGCAGCGGGAACAGUACUGCAG
Mmu-mir-542-3p	hsa-miR-542-3p	001284	UGUGACAGAUUGAUAACUGAAA
Mmu-mir-652	hsa-miR-652	002352	AAUGGCGCCACUAGGGUUGUG
Mmu-mir-871	hsa-miR-871	002354	UAUUCAGAUUAGUGCCAGUCAUG
Mmu-mir-465b-5p	mmu-miR-465b-5p	002485	UAUUUAGAAUGGUGCUGAUCUG
Mmu-mir-465a-3p	mmu-miR-465a-3p	002040	GAUCAGGGCCUUUCUAAGUAGA
Mmu-mir-743b-3p	mmu-miR-743b-3p	002471	GAAAGACAUCAUGCUGAAUAGA
Mmu-mir-883a-5p	mmu-miR-883a-5p	002611	UGCUGAGAGAAGUAGCAGUUAC

All TaqMan probes were obtained from Applied Biosystems.