The effect of a high sucrose diet on ovarian morphology: an age-matched generational study

By

Jaudon Ron Foiret

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Supervisor: Prof SH Kotzé

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Declaration

By submitting this thesis, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Abstract

Metabolic syndrome (MetS) is rapidly becoming an epidemic in society, affecting between 10% and 40% of Western populations. High-fat and refined sugar diets have been implicated in the increased prevalence of insulin resistance, obesity and dyslipidaemia, the hallmarks of MetS. Risk factors of MetS have been correlated with decreased reproductive potential and suboptimal pregnancy outcomes, while predisposing offspring to a MetS state in adulthood.

Therefore, this study aimed to assess the effects of a high-sucrose diet on the reproductive potential and mating outcomes of albino Wistar rats, and their offspring using a foetal programming model.

Female nulliparous albino Wistar rats (n=28) were randomly divided into a high-sucrose feed group (HSF) (n=19) and a control-feed group (CF) (n=9). All animals in this study were housed in standard rat cages in a temperature and humidity-controlled environment on a reverse 12-hour dark/light cycle with free access to water and respective feeds. Diets consisted of 68% carbohydrate consisting of either sucrose (HSF) or corn starch (CF). Maternal feeding commenced four weeks prior to mating with unexposed males. Maternal metabolic profile and mating outcomes were recorded. Maternal animals were euthanised and the ovaries harvested immediately after their offspring were weaned. The offspring were randomly divided into three groups; HSF/HSF (pups from HSF dam maintained on high-sucrose feed) (n=6), CF/CF (pups from CF dam maintained on control-feed) (n=6) and HSF/CF (pups from HSF dam and maintained on control-feed) (n=4). Pups were maintained on their respective feed for 10 weeks to achieve an age match comparison with dams. All animal's ovaries were harvested, formalin-fixed and paraffin-embedded, routinely stained and histologically evaluated for follicle type and numbers, follicle development, and morphological changes.

Results indicated no overt hyperglycaemia or obesity in any group, however a significant ($p<0.01$) decrease in mean body mass (MBM) was observed in the HSF and HSF/HSF groups when compared to their respective controls. Mating was deleteriously affected, with HSF dams birthing fewer and significantly lighter offspring. End point metabolic profiles of pups, indicated no significant differences in fasting blood glucose level, however the HSF/HSF MBM was found to be significantly decreased. An intermediate metabolic profile was observed in the HSF/CF group. Histological examination indicated a significant decrease in numbers of functional follicles in any sucrose feed group, with varying degrees of indicative morphological changes.

Metabolic profiles of all animals, although not overtly pathological, displayed dysregulation in energy balance. This is hypothesised to be a result of adaptations in hepatic fructose metabolism and the protective effects of oestrogen. Effects on reproductive potential and ovarian morphology in this study appear to be as result of gonadotropic hormone dysregulation mediated by metabolic status. Foetal programming by means of high-sucrose diet was confirmed in this study with HSF/CF being deleteriously affected despite control feed postnatal diet.

This study demonstrated the deleterious effects of a high-sucrose diet on maternal reproductive health and its compounding effects on their offspring. Deductions from this research emphasise the importance of maternal diet beyond overt MetS risk factors and can be applied in family planning.

Keywords: Maternal nutrition, sucrose diet, ovarian morphology and foetal programming

Opsomming

Metaboliese sindroom (MetS) is vinnig besig om ’n epidemie in die samelewing te word, en affekteer tussen 10% en 40% van Westerse populasies. Diëte met ’n hoë vet en verfynde suikerinhoud word geïmpliseer by die kenmerke van MetS, naamlik ’n toename in insulien weerstandigheid, vetsug en dislipidemie. Daar is verder ook ’n korrelasie tussen die risikofaktore van MetS, ’n afname in reproduktiewe potensiaal en ’n sub-optimale uitkoms met swangerskap gevind, terwyl dit ook die nakomelinge predisponeer tot MetS as volwassenes.

Die doelwit van die studie was om die effek van ’n hoë sukrose dieet op die voortplantingspotensiaal en die uitkomste van paring in albino Wistar rotte, te bepaal, sowel as die effek op die nakomelinge met behulp van ’n fetale programmeringsmodel.

Vroulike, nullipareuse albino Wistar rotte (N=28) is lukraak ingedeel in ’n hoë sukrose voedingsgroep (HSF) (n=19) en ’n kontrole voedingsgroep (CF) (n=9). Alle diere in die studie is in standaard rothokke gehuisves, in ’n temperatuur en humiditeit gekontroleerde omgewing met ’n 12-uur donker/lig siklus, en met vrye toegang tot water en die onderskeie voere. Diëte het 68% koolhidrate bevat, wat bestaan het uit sukrose (HSF) of mieliestysel (CF). Voeding van vroulike diere het vier weke voor paring met manlike diere wat nie blootgestel is nie, begin. Vroulike diere se metaboliese profiele en die resultate van paring is aangeteken. Onmiddellik nadat die kleintjies gespeen is, is die vroulike diere getermineer en die ovaria geoes. Die kleintjies is lukraak in drie groepe verdeel; HSF/HSF (kleintjies vanaf HSF moeders is op hoë sukrose voedings behou) (n=6), CF/CF (kleintjies vanaf CF moeders het voortgegaan met kontrole voedings) (n=6), en HSF/CF (kleintjies vanaf HSF moeders het voortgegaan met kontrole voedings) (n=4). Kleintjies is vir 10 weke op die onderskeie voere gehou totdat ‘n ouderdom soortgelyk aan dié van die moederlike diere bereik is. Alle diere se ovaria is geoes, in formalien gefikseer en in paraffien ingebed, het roetine kleuring ondergaan, en is histologies geëvalueer vir tipe en aantal follikels, follikel ontwikkeling en morfologiese veranderinge.

Resultate het geen uitgesproke hiperglisemie of obesiteit in enige groep getoon nie, maar ’n betekenisvolle ($p<0.01$) afname in gemiddelde liggaamsmassa (MBM) is in die HSF en HSF/HSF groepe waargeneem, in vergelyking met die onderskeie kontroles. Paring is nadelig beïnvloed, met HSF moeders wat geboorte gegee het aan kleiner getalle kleintjies, met ’n betekenisvolle laer geboortegewig. Eindpunt metaboliese profiele van die kleintjies het geen betekenisvolle verskille in vastende bloedglukosevlakke getoon nie, maar die HSF/HSF MBM was betekenisvol laer. ’n Intermediêre metaboliese profiel is waargeneem

in die HSF/CF groep. Histologiese ondersoek het ’n betekenisvolle afname in die getal funksionele follikels in die groepe wat met sukrose gevoer is, getoon, met aanduidings van variërende grade van beduidende morfologiese veranderinge.

Metaboliese profiele van alle diere het ’n wangereguleerde energiebalans getoon, alhoewel nie uitermatig patologies nie. Die hipotese is dat dit die resultaat van aanpassings in fruktose metabolisme in die lewer, asook die beskermende effekte van estrogeen, is. Dit blyk uit die studie dat die effekte op die voortplantingspotensiaal en ovariale morfologie die gevolge van wanregulasie van die gonadotropiese hormone is, wat deur die metaboliese status bewerkstellig is. Fetale programmering deur middel van ’n hoë sukrose dieet is in die studie bevestig, met HSF/CF wat nadelig geaffekteer is ten spyte van ’n gekontroleerde postnatale dieet.

Die studie toon die nadelige effekte van ’n hoë sukrose dieet op die moederlike voortplantingsgesondheid, asook die saamgestelde effekte op die nakomelinge. Bo en behalwe uitgesproke MetS risiko faktore, beklemtoon gevolgtrekkings vanuit die navorsing die belang van die dieet wat deur die moeder gevolg word, en kan in gesinsbeplanning toegepas word.

Sleutelwoorde: Moederlike voeding, sukrose dieet, ovariale morfologie en fetale programmering.

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Wendy Foiret and the late Michael Foiret

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List of Abbreviations

ATP	Adenosine triphosphate
AgRP	Agouti-related peptide
BMI	Body mass index
CF	Control feed group (dams)
CF/CF	Control feed / Control feed (pups)
CRP	C-reactive protein
CVD	Cardiovascular disease
DAB	3,3'-diaminobenzidine tetrahydrochloride hydrate
ER	Epitope retrieval
ER α	Oestrogen receptor alpha
FBGL	Fasting blood glucose level
FFA	Free fatty acids
FSH	Follicle stimulating hormone
GABA	Gamma-amino butyric acid
GDF-9	Growth differentiation factor 9
GLUT	Glucose transporter
GWAS	Genome wide association study
GnRH	Gonadotropin-releasing hormone
H&E	Haematoxylin and eosin
HDL	High density lipoprotein
HPA	Hypothalamic pituitary adrenal axis
HPG	Hypothalamic pituitary gonadal axis
HSF/CF	High sucrose feed / Control feed (pup)
HSF	High sucrose feed group (dams)
HSF/HSF	High sucrose feed / High sucrose feed (pups)
IDL	Intermediate density lipoprotein
IgG	Immunoglobulin G
IL-6	Interleukin 6
IR	Insulin resistance
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LH	Luteinising hormone
MetS	Metabolic syndrome
NBF	Neutral buffered formalin

NO	Nitrogen oxide
NOS	Nitrogen oxide synthase
NPY	Neuropeptide Y
OGTT	Oral glucose tolerance test
OS	Oxidative stress
PCOS	Polycystic ovarian syndrome
Poly-HRP	Horseradish peroxidase and dextran polymer
POMC	proopiomelanocortin
PMN	Polymorphonuclear
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCF	Stem cell factor
SANS	South African National Standards
SHBG	Sex hormone binding globulin
SNP	Single nucleotide polymorphism
STZ	Streptozotocin
T2DM	Type 2 diabetes mellitus
TBARS	Thiobarbituric acid-reactive substances
TA	Tunica albuginea
TG	Triglyceride
TNFα	Tumour necrosis factor alpha
VLDL	Very low-density lipoprotein
WBC	White blood cells
WHO	World Health Organization
oxLDL	Oxidised low-density lipoproteins

Chapter 1: Introduction

1.1 Background

Metabolic syndrome (MetS) and its risk factors are rapidly rising to epidemic proportions in western society. An American study in 2002 determined that 23.7% of the population were diagnosed with MetS with male and females affected equally (Ford, Giles and Dietz, 2002). However, in some population groups the prevalence in females was found to be more than twice that of males (Al Awlaqi, Alkhayat and Hammadeh, 2016). Risk factors of MetS include insulin resistance (IR), obesity and dyslipidaemia and result in an altered metabolic profile (Huang, 2009).

Associated risk factors of MetS are known to exert both individual and compound effects (Sookoian and Pirola, 2011) on the reproductive system leading to infertility, sub-optimal pregnancy outcomes and poor foetal health (Diamanti-Kandarakis and Bergiele, 2001; Michalakis *et al.*, 2013; Talmor and Dunphy, 2015). Hyperinsulinemia is found to cause modifications of the reproductive system indirectly at the level of the hypothalamus as well as direct inhibition of gonadotropic hormones and steroidogenesis at the level of the ovary (Evanthia *et al.*, 1999; Budak *et al.*, 2006). Obesity and dyslipidaemia are associated with poor rates of conception and poor foetal health (Michalakis *et al.*, 2013). In combination these factors lead to a systemic inflammatory state and result in multiple tissue level complications.

Changes in follicles numbers and morphology of the ovary are in direct relation to the reproductive potential of an individual. It is well established that alterations to gonadotropic hormones result in dysregulation of reproductive cycling and ultimately follicle numbers and ovarian morphology (Dixon *et al.*, 2014; Fontana and Della Torre, 2016). Additionally, systemic inflammatory and glucohomeostatic changes are known to lead to increased levels of oxidative stress and can result in the inhibition of intraovarian follicle recruitment and morphological changes within the ovary (McGee and Hsueh, 2000).

Links between metabolism and reproduction are well established yet not fully understood (Fontana and Della Torre, 2016). Theories of glucotoxicity in hypothalamic neurons causing dysregulation of gonadotropin releasing hormone (Roa *et al.*, 2006; Roa, Navarro and Tena-Sempere, 2011), as well as potential hepatic alterations leading to poor hepatic steroidogenesis modulation have been assessed in rats with varying success (Fontana and Della Torre, 2016). Highlighted in these studies are the variable nature of response to different feeding models as well as sexual dimorphism in MetS induction (Kim *et al.*, 2013).

Foetal programming as a result of dietary intervention has been well studied in small animals, as well as epidemiology using multiple mixed fat and sugar feeding models proving that poor maternal diet is sufficient to predispose offspring to adulthood illness (Aiken and Ozanne, 2014; Aiken, Tarry-Adkins and Ozanne, 2016; Khanal and Nielsen, 2017). Mechanisms by which this occur are only partially elucidated and believed to be multifactorial, as varying diets

led to different results. Furthermore, literature is limited regarding the isolated transgenerational effects of sucrose, and the reproductive potential of the resultant offspring, particularly potential ovarian and follicular morphological changes.

This study makes use of a novel sucrose diet foetal programming model in Wistar rats, to evaluate the effects of a high-sucrose diet on the metabolic profile and reproductive potential of dams and their offspring at the same age.

1.2 Research questions

- Does a high sucrose diet have an effect on the metabolic profile, mating outcomes and ovarian morphology in Wistar rats?
- Does a high sucrose maternal diet have transgenerational effects on offspring at the same age?

1.3 Aim

Primary:

Identify, describe and quantify changes in the metabolic profile, mating outcomes and ovarian morphology of Wistar rats maintained on a post-weaning high sucrose diet.

Secondary:

Identify, describe and quantify changes in the metabolic profile and ovarian morphology of age matched Wistar rats born of a high sucrose foetal programming model on a post-weaning high sucrose diet.

1.4 Objectives

- Compare age-matched metabolic data of dams and pups for control and experimental groups:
    - Body mass
    - Blood glucose levels
- Assess mating outcomes of dams and comparing on the basis of:
    - Size of litter
    - Sex ratio
    - Body mass of pups
- Macroscopically examine the ovaries of all animals to compare:
    - Ovarian mass

- Microscopically examine all ovaries to describe and compare:
    - Follicle numbers
    - Follicular development by mean of stem cell factor staining
    - Morphological changes in ovaries

Chapter 2: Literature Review

2.1 Metabolic syndrome

2.1.1 Background

Metabolic syndrome (MetS) is rapidly rising to epidemic proportions, and thus resulted in increased attention from the scientific community (O'Neill and O'Driscoll, 2015). Gerald Reaven was the first individual to present the concept of MetS, then referred to as 'syndrome X' which had the development of coronary heart disease and Type 2 diabetes mellitus (T2DM) as central features (Reaven, 1988; Kassi *et al.*, 2011). Definitions of MetS are highly variable causing inconsistent and unreliable reports of prevalence of the syndrome (Huang, 2009).
Metabolic Syndrome (MetS) is detailed by the presentation of multiple risk factors, which in combination, will have a detrimental effect on health (Huang, 2009). Risk factors include insulin resistance (IR), central obesity, hypertension, dyslipidaemia and microalbuminuria (Huang, 2009; O'Neill and O'Driscoll, 2015). Health agencies and institutes have various criteria for the specific combinations of risk factors, as well as specific definitions of the risk factors themselves, used in defining MetS. However, in most definitions IR and central obesity are vital requirements for a diagnosis of MetS. The criteria for diagnosing MetS as stipulated by the World Health Organization (WHO) requires IR as an absolute requirement with two additional risk factors (WHO, 1999).

Sookoian and Pirola, (2011) suggest that the effects of MetS are not only a cumulative result of its risk factors, but rather that, in combination, the clinical outworking of these risk factors are amplified. Additional, studies have proposed that individuals with MetS have a fivefold risk of developing Type 2 Diabetes mellitus (T2DM) and twice the risk of developing cardiovascular diseases (CVD)(O'Neill and O'Driscoll, 2015).

2.1.2 Genetic components

Genome wide association studies (GWAS) have been conducted in search of single nucleotide polymorphisms (SNPs) that could account for MetS as a whole (Zabaneh and Balding, 2010). While a single SNP was not found, it has been postulated that five SNPs in the apolipoprotein A-V, lipoprotein lipase and cholesteryl ester transfer protein genes were identified to correlate with the development of MetS in populations of European origin (Kraja *et al.*, 2011). In addition, studies have highlighted various other groupings of SNPs correlating with MetS in differing populations, indicating a possible population specificity for genetic predispositions of MetS (Zabaneh and Balding, 2010). In most of these cases SNPs are found to be in close proximity to genes responsible for or play a role in lipid metabolism and IR (O'Neill and O'Driscoll, 2015).

2.1.3 Major components of metabolic syndrome

2.1.3.1 Insulin resistance

Insulin resistance is characterised by impaired insulin-mediated glucose uptake in cells (Petersen *et al.*, 2007). The term insulin resistance is often used interchangeably with hyperinsulinemia or can be defined as an impaired glucose tolerance (Roberts, Hevener and Barnard, 2013). In Gerald Reavens's initial hypothesis (Reaven, 1988), great importance was placed on the central role of IR in the development of MetS and has since been supported by various studies (Petersen *et al.*, 2007; Moran *et al.*, 2008; Romeo, Lee and Shoelson, 2012) .

Insulin is a vital hormone in the regulation of blood glucose homeostasis, with additional anabolic functions with regards to tissue growth and development. Blood glucose levels are conserved through various mechanisms, which include glucose production by the liver, through glycogenolysis, and glucose uptake by peripheral tissues such as skeletal muscle, liver and adipose tissue (Petersen *et al.*, 2007; Roberts, Hevener and Barnard, 2013). Uptake of glucose in the cell is mediated by various transmembrane proteins known as glucose transporters (GLUT). Current literature suggests that there are more than 14 different GLUTs, stratified into 3 different subgroups according to gene sequence similarities. Class I glucose transporters (GLUT1 - GLUT4) are predominantly expressed in glucoregulatory tissue such as adipose and skeletal muscle (Roberts, Hevener and Barnard, 2013). All GLUT isoforms have specific functions in hexose (sugars containing 6 carbon atoms) metabolism (Petersen *et al.*, 2007).

Insulin mediated glucose uptake is achieved by insulin binding to surface receptors which triggers a signalling cascade (Bryant, Govers and James, 2002; Watson and Pessin, 2007), resulting in the redistribution of GLUT4 transporters to the plasmalemma (Watson and Pessin, 2007). This allows for increased uptake of glucose into the cell to be stored as glycogen or metabolised to produce adenosine triphosphate (ATP) (Roberts, Hevener and Barnard, 2013). As seen in Figure 2.1.

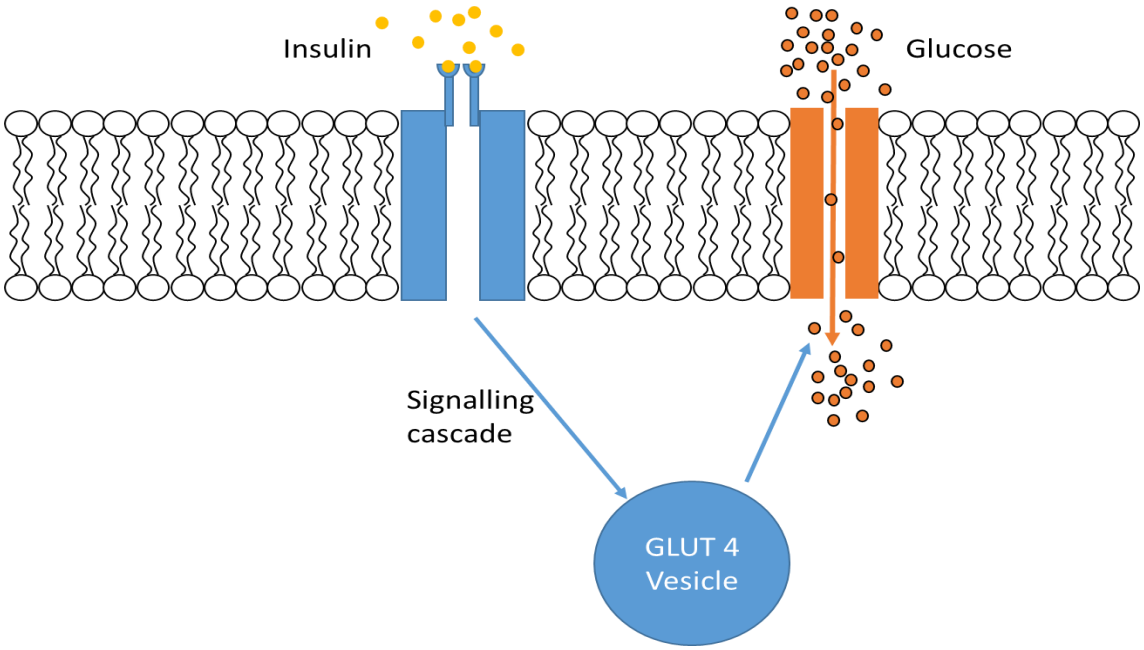

Figure 2.1: The insulin stimulated translocation of glucose transporters to the cell membrane. Adapted from Watson and Pessin, (2007).

Insulin resistance can be fundamentally understood as a decrease in cellular insulin sensitivity, resulting in reduced glucose uptake (Bandyopadhyay *et al.*, 2005). In early stages of IR development, pancreatic β-cells are stimulated to secrete excess insulin to adjust for the reduced insulin sensitivity. However, β-cells are incapable of fully compensating for this reduced insulin sensitivity, which results in a hyperglycaemic condition (Petersen *et al.*, 2007). Thresholds for β-cell compensation vary for individuals, resulting insulin resistant individuals, having varying levels of glucose tolerance (Petersen *et al.*, 2007).

The pathogenesis of IR is well described, with multiple mechanisms being identified as possible causative factors. Abnormal lipid supply (Hirosumi *et al.*, 2002; Schmitz-peiffer, 2002), and metabolic substrate alterations (Arkan *et al.*, 2005; Turban and Hajduch, 2011) lead to chronic tissue inflammation and promote the development of IR. Reports indicate that the build-up of these bioactive lipids in peripheral tissue promote pro-inflammatory signalling pathways, which alters integral phosphorylation events in the insulin signalling cascade (Arkan *et al.*, 2005; Bandyopadhyay *et al.*, 2005). These post-receptor defects are regarded as the chief impairment in the development of IR (Roberts, Hevener and Barnard, 2013).

2.1.3.2 Dyslipidaemia

Dyslipidaemia is a broad term that describes a dysfunctional maintenance of lipids in an individual (Ruotolo and Howard, 2002). Individuals with MetS are often found to have increased levels of plasma triglycerides and small dense low-density lipoproteins (LDLs), with decreased levels of high-density lipoproteins (HDLs) (Sparks and Sparks, 1994). Due to the

integrated nature of lipoprotein metabolism, it is hypothesised that a common metabolic defect explains all the lipid changes in the metabolic syndrome (Ruotolo and Howard, 2002).

Hepatic overproduction of very low-density lipoproteins (VLDLs) plays a central role in the dyslipidaemic state within IR (Ruotolo and Howard, 2002). Metabolic irregularities affecting hepatic VLDL regulation include increased hepatic glucose production, glucose intolerance and excessive free fatty acid (FFA) release from the liver, muscle and adipose tissue respectively (Phillips *et al.*, 2002; Gibbons, 2004).

Hormone sensitive lipase (HSL) is an insulin responsive enzyme that acts to regulate the release of FFA in adipocytes, by modulating the hydrolysis of triglycerides (TGs) to their FFA and glyceride components (Meijssen *et al.*, 2001). In a healthy individual, insulin results in the suppression of the HSL enzyme, resulting in decreased FFA and glyceride production. However, in the insulin resistant state, the HSL enzyme is over stimulated and results in increased FFA and glyceride plasma levels (Sparks and Sparks, 1994).

Excessive levels of FFA in hepatic circulation result in the increased production of VLDLs in an effort to facilitate the transport of the FFAs. A number of the newly produced VLDLs are immediately removed from circulation by the hepatic lipase enzyme. Lipoprotein lipase (LPL) within the peripheral tissues bind the circulating VLDLs and cause the release of TGs into these tissues, causing the VLDL to transition into an intermediate lipoprotein (IDL) (Gibbons, 2004).

Hepatic lipase subsequently acts on these IDLs and converts them to LDLs. Low-density lipoproteins are prone to oxidation and glycation whereby they become detrimental to tissue. Alternatively, LDLs can be acted upon by cholesterol esterase transfer enzyme, whereby LDLs become TG-rich LDLs (Ruotolo and Howard, 2002; Kotsovassilis and Bei, 2003). These TG-rich LDLs in turn release FFA and monoglycerides into the liver by the action of hepatic lipase forming a small dense LDLs. Small dense LDLs have decreased affinity for LPL, and increased endothelial permeability leading to the development of atherosclerotic plaque in blood vessels (Kotsovassilis and Bei, 2003).

2.1.3.2 Central obesity

Central obesity is considered one of the key cluster factors in the diagnosis of MetS (Björntorp, 2009; Al Awlaqi, Alkhayat and Hammadeh, 2016). It is predicted that by the year 2030 approximately half of the world's adult population will be classified as obese (Paley and Johnson, 2018). Although a cohort of obese metabolically stable individuals does exist, obesity is largely seen as a precursor / indicator of MetS. Conversely a non-obese MetS cohort exists in which muscle to fat proportionality are often considered as key factors in the development of metabolic dysfunction (Paley and Johnson, 2018).

Insulin resistance and central obesity are closely linked, with the development of ectopic fat in peripheral tissue being highly correlated with IR development in the MetS state (Snel *et al.*, 2012). It is hypothesised that systemic inflammation leads to the formation of enlarged and dysfunctional adipocytes, which in turn secrete additional pro-inflammatory prostaglandins and cytokines such as C-reactive protein (CRP), interleukin-6 (IL6), tumour necrosis factor alpha (TNF-α) and leptin. This increased inflammatory state promotes the development of T2DM and hyperlipidaemia which results in poor cardiovascular health (Arkan *et al.*, 2005; Björntorp, 2009; Paley and Johnson, 2018).

Excess adiposity and more specifically the systemic inflammation are reported to cause additional complications in humans. These complications include dysfunctional vascular neogenesis, leading to hypoxia of tissues, which has been attributed to increased levels of leptin. Hypoxic conditions contribute to the inflammatory state which in turn also increase levels of oxidative stress (Arkan *et al.*, 2005; Paley and Johnson, 2018).

Cortisol levels have also been found to be elevated in individuals with MetS, especially with the risk factors of central obesity and IR (Ruotolo and Howard, 2002). This indicates a derangement in the hypothalamic-pituitary-adrenal (HPA) axis. Excess cortisol drives processes of gluconeogenesis as a stress response, causing the cycles of inflammation and oxidative stress. Additionally, low grade inflammatory markers are hypothesised to be activators of the HPA axis, forming a positive feedback loop (Haffner *et al.*, 1988).

2.1.4 Metabolic syndrome and fertility

2.1.4.1 Metabolism and oestrogen

Fertility in females has been found to be strongly linked to the energy stores of an individual and the competency of the individual's metabolism (Al Awlaqi, Alkhayat and Hammadeh, 2016). Links between fertility and metabolism do not develop over time, but energy balance underpins the overall onset of puberty, albeit by mechanisms poorly understood at present (Al Awlaqi, Alkhayat and Hammadeh, 2016). Conditions of dysfunctional or irregular energy balance and metabolic stress have been found to affect fertility in females (Schneider, 2004; Torre *et al.*, 2014; Fontana and Della Torre, 2016). These conditions include MetS and its individual risk factors; obesity, IR and dyslipidaemia.

Oestrogen and its related receptors play a central role in the link between energy metabolism and reproduction (Schneider, 2004; Torre *et al.*, 2014; Fontana and Della Torre, 2016). The functions of oestrogen in female reproduction are well studied, however oestrogenic control of metabolism has only recently become a desired topic of research (D'Eon *et al.*, 2005; Riant *et al.*, 2009). Oestrogen receptor alpha (ER α) is of particular interest as ER α knockout mice

show not only reproductive deficits, but also increased food intake and weight gain (Lundholm *et al.*, 2008).

Oestrogen is known to attenuate the expression of neuropeptide Y (NPY) and agouti-related protein (AgRP) in the hypothalamus, which are both appetite stimulating (orexigenic) agents (Kalamatianos *et al.*, 2008). An increase in hypothalamic oestrogen leads to decreased in food intake, increased energy expenditure and promotion of subcutaneous fat storage over visceral fat storage (Tchernof *et al.*, 2004; Lundholm *et al.*, 2008; Stubbins *et al.*, 2012). Additionally, oestrogen conveys a potentiation of the anorexigenic neuropeptide secretion by the pro-opiomelanocortin (POMC) neurons. Peripheral leptin and ghrelin release have been shown to compound these effects (Olofsson, Pierce and Xu, 2009).

Oestrogen is shown to have effects on pancreatic, hepatic and adipose tissue. In adipocytes, oestrogen has both a anti-lipogenic and pro-lipolytic effect (Torre *et al.*, 2014). In pancreatic β-cells, oestrogen promotes the biosynthesis of insulin and prevents lipid accumulation, therefore sparing them from the detrimental effects of lipotoxicity. Similarly, in the liver ER α is pivotal in the metabolism of fatty acids and cholesterol. Oestrogen receptor alpha in the liver has also been shown to be metabolically sensitive and facilitates the correct metabolic output to suit the current reproductive needs (Lundholm *et al.*, 2008).

2.1.4.2 Compromised metabolism and reproduction

It has been understood for many years that there is a link between energy balance and reproduction. The most widely studied scenario is undernutrition and its effects on reproduction. Underweight individuals, as represented by a body mass index (BMI) less than 19 kg/m^2, are shown to require four times the amount of time to conceive a child (Hassan and Killick, 2004). In developed countries, where food is abundant, eating disorders, and various other psychosomatic illnesses lead to identical outcomes (Devlin *et al.*, 1989; Sakurazawa *et al.*, 2013). Disruption of the hypothalamic control of FSH and LH, with altered steroidogenesis in the ovaries leads to the development of compromised reproductive cycles (Leyendecker and Wildt, 1984; Devlin *et al.*, 1989; Clegg, 2006; Fontana and Della Torre, 2016). In summary, in an energy poor environment, processes for life will be favoured above those of growth and reproduction (Schneider, 2004).

Similarly, in the case of obesity, reproductive deficits can be observed with the probability of conception decreasing for every unit if BMI increase over 29 kg/m^2 (Hassan and Killick, 2004; Van Der Steeg *et al.*, 2008). Other associated outcomes include infertility, suboptimal pregnancy outcomes and poor foetal health (Diamanti-Kandarakis and Bergiele, 2001; Michalakis *et al.*, 2013; Talmor and Dunphy, 2015). Although the mechanisms are not fully understood, obesity strongly correlates with the development of polycystic ovarian syndrome

(PCOS), a hormonal disorder, resulting in the production of ovarian cysts and impaired fertility (du Toit and Siebert, 2009; Michalakis *et al.*, 2013).

As previously stated, dyslipidaemia is often identified in obese individuals. Unbalanced levels of cholesterol and free fatty acids have been shown to affect oocyte development and various other factors at the level of the ovary and uterus (Bellver *et al.*, 2007, 2010). Della Torre *et al.* (2016) reports a hepatic ER α dependent modulation of cholesterol metabolism, indicating a bidirectional influence between metabolism and reproduction.

Insulin levels play multiple direct and indirect roles in hormonal control of reproduction. The liver, pancreas and adipose tissue secrete sex hormone binding globulin (SHBG), insulin, leptin and adiponectin respectively (Asuncion *et al.*, 2000; Budak *et al.*, 2006). These hormones work in concert to mediate follicular development and steroidogenesis by direct and indirect modulation (will be elaborated on in section 2.3 and Figure 2.3). In the case of IR however, this balance of hormones is skewed and leads to anomalies in the hypothalamic control of sex hormones as well as end product steroidogenesis (Budak *et al.*, 2006; Comninos, Jayasena and Dhillo, 2014). Furthermore, insulin is strongly correlated with the development of PCOS (Asuncion *et al.*, 2000). This is hypothesised to be due to the ability of insulin to act as an analogue for FSH, leading to hyper-stimulation of the ovaries (Asuncion *et al.*, 2000; Fontana and Della Torre, 2016).

2.1.5 Oxidative stress and the reproductive system

Reactive oxygen and nitrogen species (ROS and RNS) are momentary, highly reactive compounds formed as result of all oxygen dependent metabolic activity (Agarwal *et al.*, 2005; O'Neill and O'Driscoll, 2015). Oxidative stress (OS) occurs when there are excessive concentrations of the volatile compounds present, and may be due to an increase in ROS, an insufficiency in the antioxidant system, or a combination of the two (Roberts and Sindhu, 2009). Oxidative stress is considered key in the pathophysiology for most of the risk factors for MetS (Agarwal *et al.*, 2005). This has been shown in MetS patients presenting with decreased antioxidant protection and significant oxidative damage to tissues (Roberts and Sindhu, 2009). Additionally, OS has been positively correlated with visceral adiposity and total body fat percentage (Snel *et al.*, 2012). The Coronary Artery Risk Development in Young Adults reported that the high levels of oxidised low-density lipoproteins (oxLDL) correlate with development of MetS and it risk factors (Koenig *et al.*, 2011). Oxidised low-density lipoproteins have also been positively correlated with increased levels of CRP and inversely correlated with levels of adiponectin.

Free radical development is often described as a purely negative event however, moderate concentrations of the reactive species have been found to be vital in normal reproductive

physiology. Reactive oxygen and nitrogen species have been found to modulate oocyte maturation, ovarian steroidogenesis, corpus luteal function and ovum expulsion by various mechanisms (Agarwal *et al.*, 2005).

Nitric oxide (NO) is a RNS and is produced by the NO synthase (NOS) enzyme. These enzymes have been found to present in the theca cells, granulosa cells and the oocyte during follicular development. In a normal homeostatic environment this allows adequate localised formation of NO for normal physiological functioning (Agarwal *et al.*, 2005). However, research has indicated that inducible NOS is activated by inflammatory cytokines such as IL-1 and TNF-α producing toxic levels of NO (Ben-Shlomo *et al.*, 1994; Hung *et al.*, 2004).

2.2 The female reproductive system

The reproductive system consists of the ovaries, fallopian tubes, uterus, vagina and mammary glands. Oogenesis and follicle development occur in the ovary under the influence of a hormonal feedback from the hypothalamic-pituitary-gonadal (ovarian) (HPG) axis. Primary functions of the female reproductive system include the production and maturation of the female gamete known as the oocyte, the site of fertilisation, foetal development and foetal maturation.

2.2.1 Ovarian morphology

In humans the ovaries are ovoid in shape and located lateral to the uterus within the pelvic cavity (Moore, Dalley and Agur, 2014). Ovaries are secured in place by the ovarian ligament, suspensory ligament and broad ligament and consist of a capsule, outer cortex and medulla (Moore, Dalley and Agur, 2014).

The capsule of the ovary is comprised of two layers, the outer germinal epithelium which is continuous with the mesovarium and the tunica albuginea (TA) (Young *et al.*, 2006). Thickening of the tunica albuginea has been highly correlated with the development of PCOS (Amirikia *et al.*, 1986). Post puberty, the largest constituent of the ovary is the cortex, which comprises of stroma, numerous collagen fibres and the quiescent and developing follicles. The medulla is highly vascularised area which consists mainly of loose connective tissue (Young *et al.*, 2006).

2.2.2 Follicular morphology

Follicles have a varying morphology dependent on their current stage of maturation. General structures of the follicle include the oocyte surrounded by concentric layers of granulosa cells surrounded by two layers of theca cells. Pedersen and Peters (1968) were the initial researchers to propose a system to classify follicles based on their stage of development indicated by size, layers of granulosa cells and antrum formation. Their initial classifications

were highly stratified with three major groups and eight types excluding sub-types (Pedersen and Peters, 1968). Subsequently, many researchers choose to make use of a simplified classification system using five groups; primordial follicles, primary follicles, primary developing follicles, secondary developing (antral) follicles and mature (Graafian) follicles (Yoshida *et al.*, 2009).

Primordial follicles consist of an oocyte surrounded by a flatted layer of granulosa precursor cells. At the onset of follicular recruitment, various factors trigger several morphological changes in the follicle. The first observable change is the granulosa cell change from a flatted to a cuboidal cell shape, at which point the follicle is referred to as a primary follicle (McGee and Hsueh, 2000). Activated granulosa cells become proliferative, causing the formation of multiple granulosa cell layers as well as the first appearance of theca cells. At this stage the follicle is referred to as a primary developing follicle (Young *et al.*, 2006). Granulosa cells secrete follicular fluid into the intestinal space and cause the formation of an antrum, at which point the follicle is referred to as secondary developing or antral follicles (Hsueh *et al.*, 2015). A follicle is considered mature once the antrum has increased considerably in size and displays the formation a cumulus stalk (Figure 2.2) (Young *et al.*, 2006; Ross and Pawlina, 2011).

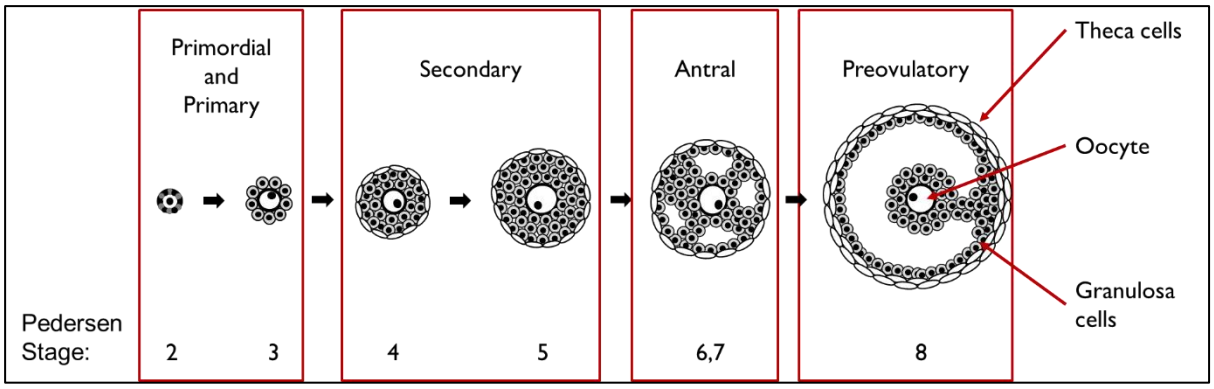

Figure 2.2: Stages of follicular development. Indicated below are the represented stages according to Pedersen and Peters. Modified from Edson, Nagaraja and Matzuk (2009).

2.2.3 Hormonal regulation of reproduction

Regulation of the reproduction is predominantly controlled by the hypothalamic mediated release of gonadotrophic hormones from the anterior pituitary (Sherwood, 2012). The relationship between the hypothalamus, anterior pituitary and the gonads is referred to as the hypothalamic-pituitary-gonadal axis (HPG). Modulation of the HPG axis is achieved through multiple integrated feedback loops, resulting from gonadotrophic hormone levels, gonadal steroidogenesis and hormones secreted by metabolically sensitive tissue (Pralong, 2010; Sherwood, 2012; Fontana and Della Torre, 2016).

2.2.3.1 Hypothalamic-pituitary-gonadal axis

Hypothalamic Kisspeptin 1 and gamma-amino butyric acid (GABA) are vital in initiating and modulating the secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus (Roseweir and Millar, 2009; Roa, Navarro and Tena-Sempere, 2011; Watanabe, Fukuda and Nabekura, 2014). This in turn triggers the production and secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary (Sherwood, 2012). In the ovary, FSH triggers and mediates follicular development, while LH is responsible for final stages of maturation and ovulation (McGee and Hsueh, 2000). Steroidogenesis occurs throughout follicular development and continues after ovulation via the corpus lutueum. Hormones produced by the ovary include oestrogen, progesterone and inhibin (Xu *et al.*, 2011; Sherwood, 2012). Gonadotrophic hormone variations occur cyclically on a conventional 28 day cycle in humans and can be separated into the follicular and luteal phases separated by the ovulation of an oocyte (McGee and Hsueh, 2000; Edson, Nagaraja and Matzuk, 2009).

Feedback control of the HPG axis, is achieved by the release of oestrogen, inhibin and progesterone from the granulosa cells and corpus luteum (McGee and Hsueh, 2000; Sherwood, 2012). The effect of oestrogen varies as it is dependent on its concentration (Young and Jaffe, 1976). During the follicular phase, low levels of oestrogen secreted by the developing follicle inhibit the release of LH and FSH. On approximately day 10 of the reproductive cycle, increased levels of oestrogen selectively promote the release of LH, triggering ovulation (Sherwood, 2012). The resultant corpus luteum maintains relatively high levels of oestrogen and secretes inhibin and progesterone. Inhibin acts at the level of the anterior pituitary by further selectively inhibiting the secretion of FSH (McGee *et al.*, 1997). Progesterone supresses the release of GnRH from the hypothalamus, effectively supressing gonadotropin hormone release. This cycle is repeated when the corpus luteum degenerates, and the levels of GnRH, and FSH can return to normal (Sherwood, 2012).

Metabolic influence on the HPG axis is provided by secretion of insulin and SHBG from the pancreas and liver respectively, in conjunction with the secretion of leptin and adiponectin from adipose tissue (Fontana and Della Torre, 2016). In normal physiological conditions, insulin serves as a co-gonadotropin and modulates the production of SHBG from the liver. Sex hormone binding globulin is chiefly responsible for the removal of excess androgens produced by the gonads (Diamanti-Kandarakis and Bergiele, 2001; Diamanti-Kandarakis and Dunaif, 2012). Leptin is chiefly responsible for indicating satiety in an individual, however, it has also been found to be a stimulus for GnRH release from the hypothalamus (Campos *et al.*, 2008). Adiponectin acts as an insulin sensitising hormone. Additionally, adiponectin exerts insulin-like functions in tissues and with a large constituent of adiponectin receptors in the reproductive tissues, this indicates a direct relationship with reproduction and lipid metabolism (Kadowaki and Yamauchi, 2005; Kim *et al.*, 2011). See Figure 2.3 for a summary of these actions.

Functions of insulin include stimulation of GnRH from the hypothalamus, as well as being an analogue for gonadotropins in the stimulation of steroid production in the ovary (Medina and Nestler, 1998). Dysregulation of the reproductive system will be discussed in the following section.

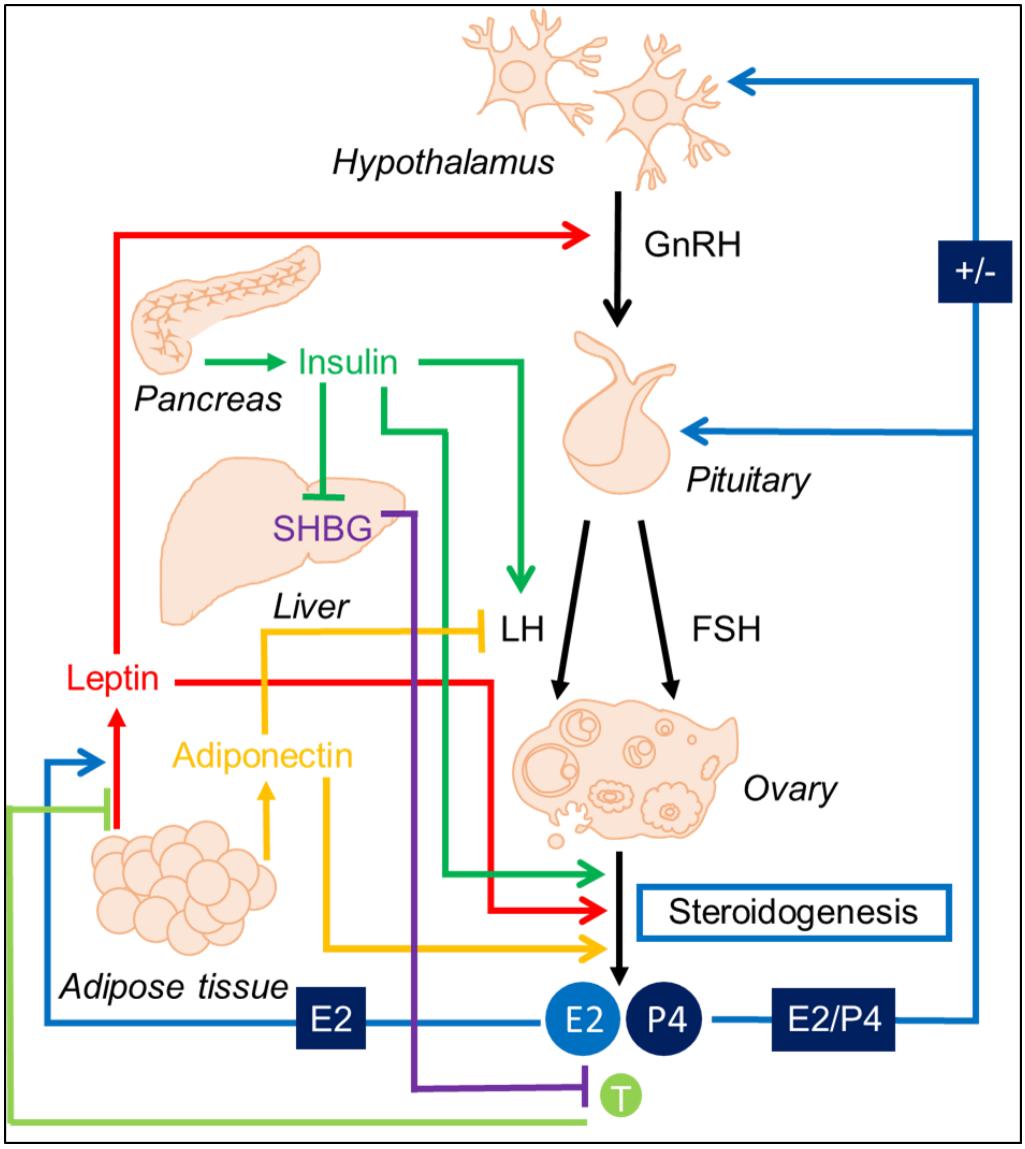

Figure 2.3: Hormonal control of the HPG axis. E2 = oestradiol, P4 = progesterone and T = testosterone. Adapted from Fontana and Della Torre, (2016).

2.2.3.2 Dysregulation of the HPG axis

Central factors in the metabolic syndrome include obesity, dyslipidaemia and IR (Huang, 2009). In this metabolically compromised state, increased levels of insulin and leptin are increased with a decrease in levels of adiponectin (Fontana and Della Torre, 2016). Leptin receptors are found on granulosa cells, theca cells and stromal cells in the ovary (Caprio *et al.*, 2001; Sirotkin, 2011). Studies in rats have shown that a medium to high dose of leptin, mimicking that of an obese individual, caused a decrease in steroidogenesis, with a marked

decrease in ovulated oocytes (Spicer and Francisco, 1997; Ghizzoni *et al.*, 2001; Kendall *et al.*, 2004).

Increased levels of insulin lead to increased inhibition of SHBG leading to increased levels of testosterone (Diamanti-Kandarakis and Dunaif, 2012). High testosterone levels result in irregular reproductive cycles and correlate strongly with the development of PCOS (Soto *et al.*, 2009; Sirotkin, 2011). Insulin resistance is found to correlate strongly with development of PCOS independent of other factors, which is hypothesised to be as result of the direct hyper-stimulating effect of insulin on steroidogenesis in the ovary (Evanthia *et al.*, 1999; Asuncion *et al.*, 2000).

Studies have shown that metabolic disturbances, and hyperglycaemia in particular, supress the expression of hypothalamic KiSS/kisspeptin, resulting in decreased reproductive potential. Roa *et al.* (2006, 2008) report that the administration of kisspeptin is sufficient to restore normal gonadotrophic hormone release in rats (Roa *et al.*, 2006, 2008). Similarly, the use of kisspeptin-10 has been used to improve the longstanding reproductive deficits of streptozotocin (STZ) induced diabetic rats (Castellano *et al.*, 2010). This strengthens the hypothesis of hypothalamic Kiss 1 neuron tone being fundamental in the regulation of the HPG axis.

2.2.4 Oogenesis and Folliculogenesis

Oogenesis is the formation of the female gamete within the ovaries. Prior to the birth of females, primordial germ cells in their ovaries undergo mitosis, resulting in the production of 6 to 7 million oogonia (Sherwood, 2012). Proliferation ceases by the fifth month of gestation whereby all oogonia enter a state of mitotic arrest until puberty and subsequent follicle recruitment (McGee and Hsueh, 2000). Post-early mitotic divisions and prior to the birth of an individual, oogonia are encapsulated by a flattened layer of granulosa cells at which point they are referred to as primordial follicles (Sherwood, 2012). Oogonia that have not been encapsulated will undergo programmed cell death, namely apoptosis. At the time of birth, it is reported that approximately 1 to 2 million viable primordial follicles remain (Oktem and Urman, 2010).

2.2.5 Follicle recruitment and selection

Recruitment of follicles can be separated into two main stages in the follicular life cycle, namely initial recruitment and cyclical recruitment (McGee and Hsueh, 2000). Initial recruitment occurs well before the onset of puberty, is constant and thought to be controlled by intraovarian growth factors and other unknown paracrine stimuli (Hsueh *et al.*, 2015). Follicles recruited at this stage are not capable of undergoing germinal vesical breakdown and thus are never released

from the ovary (Hirshfield, 1991). The majority of primordial follicles will remain in a state of quiescence until pubertal onset, and cyclic recruitment (Sherwood, 2012).

Cyclic recruitment of follicles occurs after the onset of puberty as the result of increased levels of circulating FSH (Rombauts *et al.*, 1998). Follicle stimulating hormone acts as a sparing hormone allowing cohorts of these follicles to progress to maturation (McGee *et al.*, 1997). Recruited follicles have developed antrums with the oocyte undergoing its final maturation processes. Oocytes increase in size, form a zona pellucida and undergo the final stages of meiosis. Multiple follicles are recruited, however, majority of these follicles will undergo apoptosis (atresia) owing to the process of selection (Hsueh, Billing and Tsafriri, 1994).

Cyclic recruitment results in the production of multiple devolving pre-ovulatory follicles, however, it can be seen that among this group a single follicle will have a higher rate of growth (Figure 2.4). Follicle selection is most easily identified in the premenstrual phase of the reproductive cycle, whereby approximately 10 antral follicles are recruited, with a single follicle showing an increased rate of growth (McGee and Hsueh, 2000). The exact mechanisms of follicle selection are unclear. Some authors theorise that increased numbers of FSH and LH receptors, and / or size mediated increase in the production of oestrogens and inhibin's, may lead to the selection of specific follicles (Yoshida *et al.*, 1997; Rombauts *et al.*, 1998) .

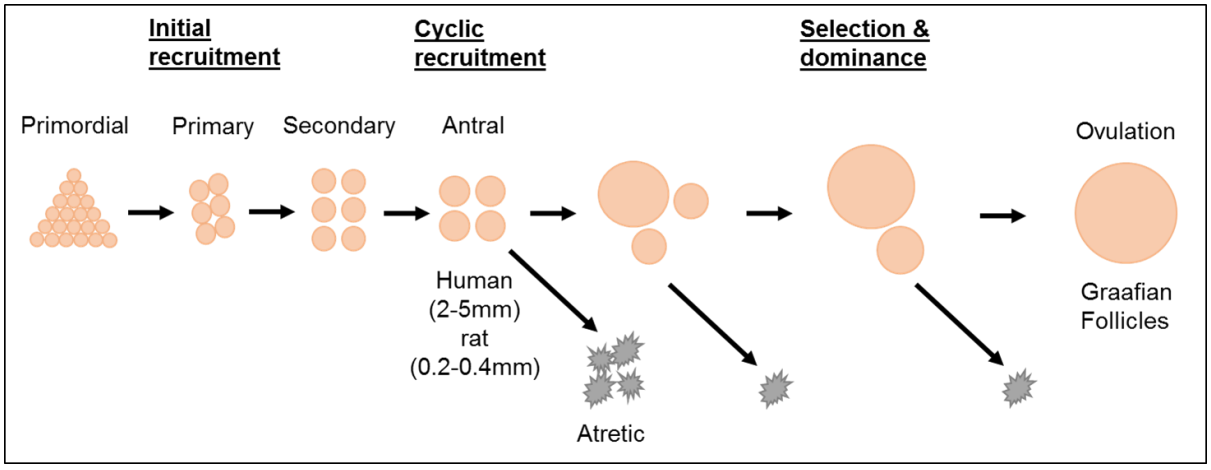

Figure 2.4: Stages of follicular recruitment. Adapted from McGee and Hsueh, (2000).

Investigating initial follicle recruitment is a challenging procedure, as this process occurs over a protracted period whereby a considerable number of primordial follicles develop into small follicles (McGee and Hsueh, 2000). Many studies have taken the approach of enumerating and classifying follicles, however this method has limitations when identifying abnormalities if initial follicle recruitment is of relevance to the researcher (Myers *et al.*, 2004).

Quiescent pools of primordial follicles are hypothesised to be maintained by the tonic release of systemic and intraovarian inhibitory factors (Nilsson and Skinner, 2001). The initial recruitment of primordial follicles has largely been considered to be independent of circulating

levels of FSH, due to the low levels of FSH receptors located on the oocytes of primordial follicles. Studies on hypophysectomised mice showed decreased numbers of pre-antral follicles with many atretic follicles, supporting this hypothesis (McGee *et al.*, 1997). However, when mice were treated with FSH, an increased rate of follicular development was observed (Abel *et al.*, 2000), which suggests that FSH may not be directly responsible for initiating follicular recruitment but rather has a complementary role.

Intraovarian factors that are hypothesised to influence the recruitment of follicles include factors expressed by the oocyte granulosa cells and the theca cells. It has been suggested that by unknown mechanisms the morphological change (flattened cells to cuboidal) in the granulosa cells is as a result of the factors expressed by the developing oocyte (Hsueh *et al.*, 2015). Oocyte-granulosa cell communication has been demonstrated to be of paramount importance in follicle recruitment (Barnes and Sirard, 2000).

Stem cell factor (SCF), also known as kit ligand or the Steel factor is expressed by granulosa cells in the developing follicles (Manova *et al.*, 1993). Receptors for SCF form part of the platelet derived growth factor receptor family and can be found on the oocyte as well as the theca cells (Manova *et al.*, 1993). Studies have indicated that the cessation in production of soluble SCF results in follicular development not progressing past the primary stages (Kuroda *et al.*, 1988; Huang *et al.*, 1993; Bedell *et al.*, 1995). Reduction in the quantity of soluble SCF produced has been shown to promote a small number of follicles to the antral stages of development. Animals with low levels of soluble SCF display irregular reproductive cycles with a reduction in fecundity (Yoshida *et al.*, 1997). This indicates that the functioning of SCF may extend further than the initial recruitment of follicles.

Growth differentiating factor 9 (GFD-9) and connexin 37 have been identified as key intraovarian factors, being expressed by the oocyte and granulosa cells respectively (Simon *et al.*, 1997; Elvin *et al.*, 1999). Functions of GFD-9 are not fully understood, however, irregular levels result in the failure of follicles to progress past the primary stage of development (Dong *et al.*, 1996). Expression of GFD-9 has been shown to be dependent of levels SCF present (Nilsson and Skinner, 2002). Connexin 37 has been shown to be vital in oocyte-granulosa cell communication (Teilmann, 2005). Individuals with irregular connexin 37 have normal follicular development until antrum development, after which further development ceases (Simon *et al.*, 1997).

2.3 Sucrose diet effect on reproduction

Many studies have explored the deleterious effects of sucrose on the body, with increased fervour in recent years owing to the rise of MetS and its related risk factors (Coulston *et al.*, 1987; Douard *et al.*, 2013; King *et al.*, 2013). However, few studies have addressed sucrose in isolation (Kendig *et al.*, 2015) with many studies using high-fat and sugar in combination to

mimic a western diet (Volk *et al.*, 2017). Fewer studies yet have commented on the effects of sucrose on reproduction, especially when foetal programming is considered (Kendig *et al.*, 2015). To the best knowledge of the author, no literature exists where the maternal and transgenerational effects of a sucrose diet has been investigated on ovarian morphology.

Sucrose is a common form of sugar and is used widely in society. Sucrose is a dimer formed by a glucose monomer bound to a fructose monomer. Due to the fructose content, the glycaemic insult is less than that of pure glucose. However, it has been found in small animal studies that sucrose consumption, matching that of the levels of sucrose found in commercial soft drinks, are sufficient to cause metabolic changes in the body that lead to deleterious consequence (Kendig *et al.*, 2015).

2.3.1 Maternal effects of a sucrose diet

Sucrose diets display sexually dimorphic results on males and females in small animal studies. In males, sucrose diets of four weeks and longer lead to significant weight gain and increase of plasma glucose levels (Fuente-Martín *et al.*, 2012). In females, mixed reports have indicated normal plasma glucose levels with no abnormal weight gain (Sánchez-Lozada *et al.*, 2010). However, long term exposure to sucrose diet has been shown to lead to the development of fatty liver, increased abdominal adiposity and impaired glucose tolerance (Sánchez-Lozada *et al.*, 2010). Other relevant effects and actions of diet on the reproductive system have been discussed in section 2.2.

2.3.2 Transgenerational effects of a maternal sucrose diet

It is well accepted that poor diet during pregnancy can lead to difficulties during pregnancy as well as poor pregnancy outcomes, for both mother and child (Manova *et al.*, 1993; Torre *et al.*, 2014; Al Awlaqi, Alkhayat and Hammadeh, 2016). It is common practice for pregnant individuals to alter their diets in order to insure correct nutritional balance to support the growing foetus. However, acceptance of the hypothesis of transgenerational or foetal programming has only recently becoming widely accepted (Aiken and Ozanne, 2014).

Transgenerational programming can be described as an individual having an increased likelihood of developing a disorder due to prenatal exposure to the disorder. The individual may not be born with the disorder but is likely to develop it later on in life. Disorders for which transgenerational programming is best described include IR (Martin-Gronert and Ozanne, 2012) and obesity (Cottrell and Ozanne, 2008) as confirmed by animal studies and epidemiological studies.

Mechanisms by which maternal programming occurs are not well understood with two main theories proposed; DNA methylation and poor maternal uterine environment (Aiken and

Ozanne, 2014). The ‘thrifty gene hypothesis’ proposed by Hales and Barker (2013) suggest that poor foetal nutrition causes alterations in the epigenetics of the foetus, predisposing individuals for poor metabolic health later in life. Epigenetic alterations have been shown to be as result of low circulating levels of methionine, vitamins $B_{6,}$ B_{12} and folate (Brunaud *et al.*, 2003; Li *et al.*, 2018). Additionally, the uterine environment has been shown to lead to transgenerational programming. Gill-Randall *et al.* (2004) demonstrated this by transferring wild-type rat embryos into a hyperglycaemic uterine environment, with the offspring developing hyperglycaemia in later life.

Chapter 3: Materials and Methods

3.1 Ethical consideration

This study makes use of animals resulting from a current PhD study, for which, ethical clearance from the Stellenbosch University Research Ethics Committee: Animal Care and Use (REC: ACU) has been obtained. Ethics number SU-ACUD16-00074. The author of this study is a listed co-worker of the overarching PhD study and has permission to make use of metabolic data and ovaries from animals indicated below.

3.2 Study groups and animal care

Twenty-one-day old female albino Wistar rats (*Rattus norvegicus)* (n=28), were used in the present study. These animals were provided by, and housed in the Stellenbosch University Animal unit, Faculty of Medicine and Health Sciences, Tygerberg campus. Initially only 18 female albino Wistar rats were assigned and were randomly selected and divided into two groups namely an experimental group 1: High-Sucrose feed 1 (HSF1, n=9) and a control group 1: Control feed (CF1, n=9). Due to requirements of the overarching PhD study, an additional 10 female albino Wistar rats with identical feed and housed under identical conditions were added to the study and formed experimental group 2: High-sucrose feed 2 (HSF2, n=10)

Female rats were mated with unexposed males (n=28), with resultant female offspring of each group being included into the study. Female offspring from the HSF dams were divided into two groups; HSF/HSF (pups from a HSF dam, and maintained on high-sucrose feed) (n=6) and HSF/CF (pups from a HSF dam, and maintained on control feed) (n=4). Female offspring from the CF dams were maintained on control feed and labelled CF/CF (n=6). See Figure 3.1 for a visual depiction of study groups.

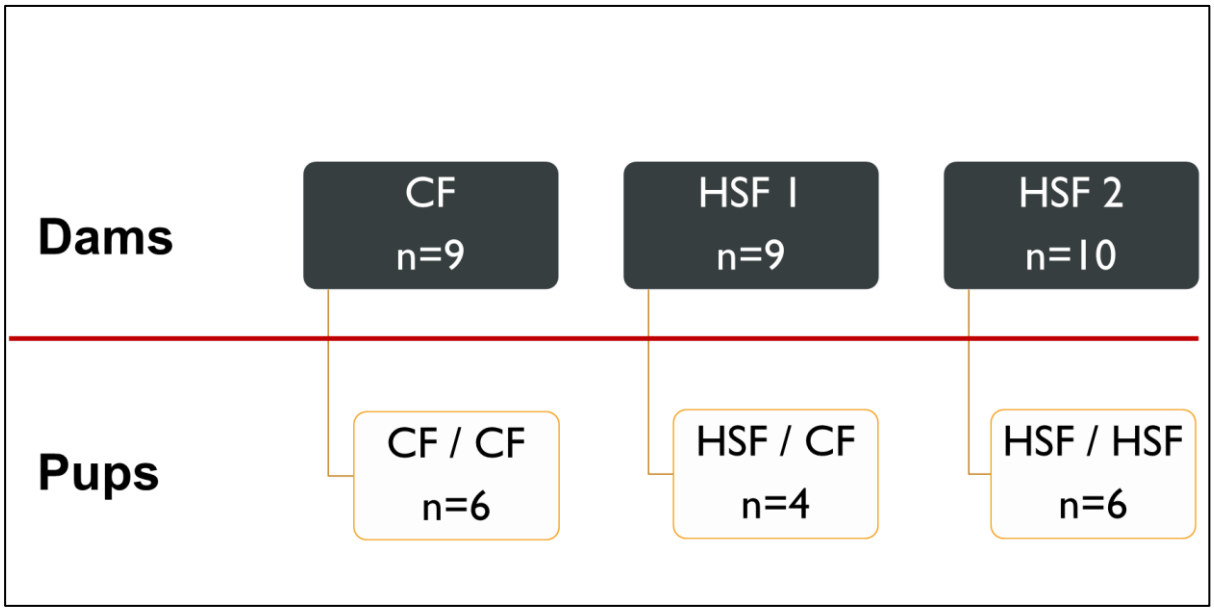

Figure 3.1: Research animal number design showing animal numbers in maternal and pup groups.

All animals in this study were cared for in abidance to the regulations stipulated by the REC: ACU in accordance with the South African National Standard (SANS) document. Rats were monitored twice a day with the use of an animal wellness sheet, observing for signs of stress, illness, pain or injury for the duration of the study.

All animals were housed in standard rat cages according to group, in an isolated temperature and humidity controlled room following a 12-hour reverse light cycle. During this study, all animals had unrestricted access to water and their respective feed. Food was only withheld when rats underwent a fasting blood glucose test (FBGL) or oral glucose tolerance test (OGTT). All mating and technical procedures will be detailed in the following sections.

3.3 Diets

Two diets were used in this study and sourced from *Research Diets incorporated, Open Source diets (New Jersey, USA)* and produced by *Nutritionhub (PTY) LTD (Stellenbosch, South Africa)*. This study made use of the D11708 diet as the control feed, and the D10001 diet as the high-sucrose feed. Detailed composition of both diets can be found in Appendix A.

Diet compositions of both the high-sucrose feed and control feed were equal with regards to macro- and micronutrient quantities. Both diets consist of 20% protein, 68% carbohydrate, 5% fat. The high sucrose diet used sucrose and corn starch as a carbohydrate source, while the control feed diet used corn starch exclusively as a carbohydrate source.

3.4 Experimental design

The experimental portion of this study consisted of three different phases; maternal feeding, mating and offspring feeding. Throughout all phases dietary groups of all animals were maintained.

3.4.1 Maternal feeding

During this phase all maternal groups were maintained on their respective feeds for a period of four weeks. This is the equivalent time it would require a male Albino Wistar rat to become insulin resistant on a diet with a large sucrose component. (Fuente-Martín *et al.*, 2012).

During this period, all animals were weighed, and their FBGL tested weekly.

3.4.2 Mating

All maternal groups were individually placed into small format cages with a single unexposed male. During this phase both the male and female consumed the diet assigned for the female in the cage. All handling and weekly tests were suspended with minimal handling taking place. Males remained in cages with the females until the first female gave birth ± 21-days after union, subsequently males were then removed from all cages. Number of offspring, sex of pups and body mass were recorded in all cases.

Pups remained with their respective dam for 21-days, corresponding with normal weaning times for rats. Dams continued on the respective feed for their group. During this time no interventions were performed on the dams or pups, other than routine handling for animal wellness observations. Animals failing to give birth, with no visible signs of pregnancy 21-days after the removal of the male was considered a sterile mating and underwent further investigation by means of vaginal smear analysis (Goldman *et al*., 2007).

At the end of the 21-day weaning period, pups were removed from their dams. Dams remained in solitary cages for approximately five days. During this period, weight measurements, and fasting blood glucose level measurements were performed. At the end of this period dams underwent deep sedation using 60 mg/kg sodium pentobarbitone *(Kyron Laboratories, Johannesburg, South Africa)* in combination with 0.1 ml of heparin intraperitoneal injection, and euthanised by transcardial perfusion.

3.4.3 Offspring feeding

Once removed, offspring were housed in large cages according to sex and their respective dietary group. Baseline weights and FBGL were recorded at the commencement of this phase for all animals. Animals were maintained for a further 10 weeks on their respective feed, whilst undergoing weekly weight and FBGL assessments. Animal wellness observations were

continued with special emphasis on secondary sex characteristics development, vaginal opening and prominent nipple formation.

At the conclusion of this phase all animals were sedated and euthanised in the same manner as the maternal animals.

See Figure 3.2 for a visual depiction of the phases of this study.

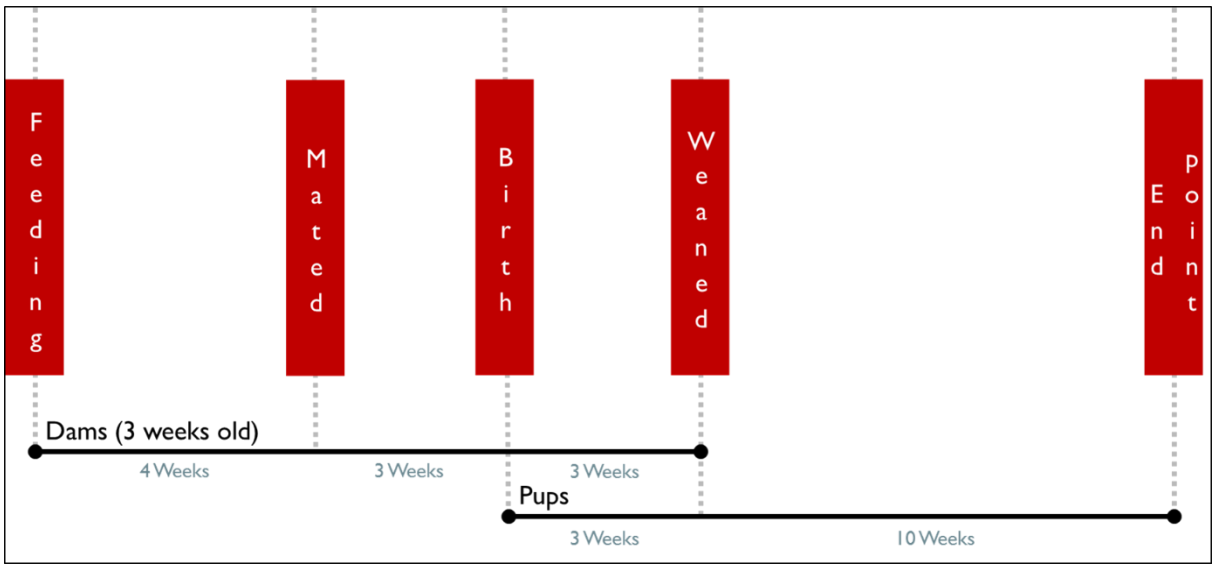

Figure 3.2: Phases of the present study.

3.5 Technical procedures

3.5.1 Weighing

Animals are all weighed with use of an automated scale fitted with a hemispherical container.

3.5.2 Blood glucose testing

Blood used for the FBGL testing was obtained using of the standard tail vein prick method (Van Herck *et al.*, 2001). A vein was identified in the tail of the rat and pierced approximately 2 cm from the tip of the tail. Blood was allowed to pool into a droplet which was then transferred to a BGL test strip *(On Call® Plus, Acon®, United States)* and inserted into a glucometer *(On Call® Plus, Acon®, United States)*. Readings were reported in mmol (glucose) / L(blood).

Animals underwent fasting prior to BGL testing. Fasting time was dependent on the weight of the animals undergoing the procedure. Fasting time varied between 4 hours (for animals weighing less than 50 g) and 12 hours (for animals exceeding 150 g). Specific weight to fast length ratios can be found in Appendix B.

3.5.3 Euthanasia

Prior to euthanasia the body mass of all animals were recorded to be used for anaesthetic dosage calculations. Rats were initially anesthetised using sodium pentobarbitone at a dosage of 60 mg/kg, with addition of 0.1 ml heparin to avoid blood clots. Once animals appeared to be sedated, both pedal and corneal reflexes were assessed. When unresponsive, non-recoverable surgery could commence. All procedures were conducted by an individual authorised by the South African Veterinary Council in accordance with Section 21 of the Veterinary and Para-veterinary Act 19 of 1982.

3.5.4 Perfusion of animals

An incision was made to expose the superior portion of the abdominal cavity. The incision was continued through the lateral aspect of the thoracic cage to expose the thoracic cavity. An 18-gauge needle, at the end of the hose of a perfusion device was used to pierce the left ventricle of the heart whilst the right atrium of the heart was nicked to allow outflow of blood. Perfusion was initiated by clearing the rat with approximately 200 ml of physiological saline at a rate of 167ml/min using the Leica Perfusion One *(Leica Biosystems, Wetzlar, Germany)* perfusion device. Subsequently, the perfusion device was switched to dispense 10% neutral buffered formalin (NBF) at the same quantity and rate.

Signs of adequate clearing included clear saline flowing from the nose, clearing of the liver and inflation of the lungs (Gage, Kipke and Shain, 2012).

Upon completion of perfusion, the abdominal cavity was exposed fully and flushed out with formalin. Whole perfused rats were post-fixed by emersion in 10% NBF for 24 hours, after which these were transferred into 50% ethanol for short-term storage. Noteworthy anomalies were photographed and recorded.

3.5.5 Tissue harvesting

All animals from the principal PhD study were dissected to form part of a biorepository of material and therefore a standardised dissection procedure was followed to preserve tissue for future studies (Morawietz *et al.*, 2004).

The gastrointestinal tract was initially removed, including accessory organs. Subsequently, the uterine horns were located, followed inferiorly to its point of union where it was severed in close proximity to the vaginal canal. The uterine horns were traced superiorly to identify the ovaries embedded in the paranephric fat pads, and carefully loosened. Uteri and ovaries were removed together and intact for later separation. Further dissection took place to remove the kidneys and bladder.

Uteri and ovaries were separated by dissection using forceps and a dissecting magnifying glass, when needed. Minimal contact was made with either structure by handling the surrounding connective tissue in order to prevent any damage of tissues. Noteworthy anomalies were photographed and recorded.

3.6 Macroscopic analysis

3.6.1 Photography

High quality standardised photographs were captured of uteri and ovaries using a digital camera *(Nikon, Tokyo, Japan)* and a fixed tripod. Photographs were used for subjective analysis of fat deposition and additional morphological changes.

3.6.2 Ovarian mass

Both ovaries of all animals were weighed with the use of an anatomical mass balance. Ovaries were cleaned of all excess fat and uterine tissue before weighing.

3.7 Histological techniques

3.7.1 Processing, embedding and sectioning

Formalin-fixed and paraffin-embedded ovaries were processed in a Leica ASP 6025 automated tissue processor *(Leica Biosystems, Wetzlar, Germany)* using a modified 12 hour xylene program (See Appendix C for processing schedule). Both ovaries from each animal were embedded together using the Leica HistoCore Arcadia H tissue embedder *(Leica Biosystems, Wetzlar, Germany)*. The method of tissue allocation on slides is illustrated in Figure 3.3. All samples were sectioned at 4 µm using a Leica RM 2125 RT microtome *(Leica Biosystems, Wetzlar, Germany)*. Standard uncharged glass slides were used to mount sections for Haematoxylin and Eosin (H&E) and positively charged glass slides were used for mounting sections selected for immunohistochemical analysis.

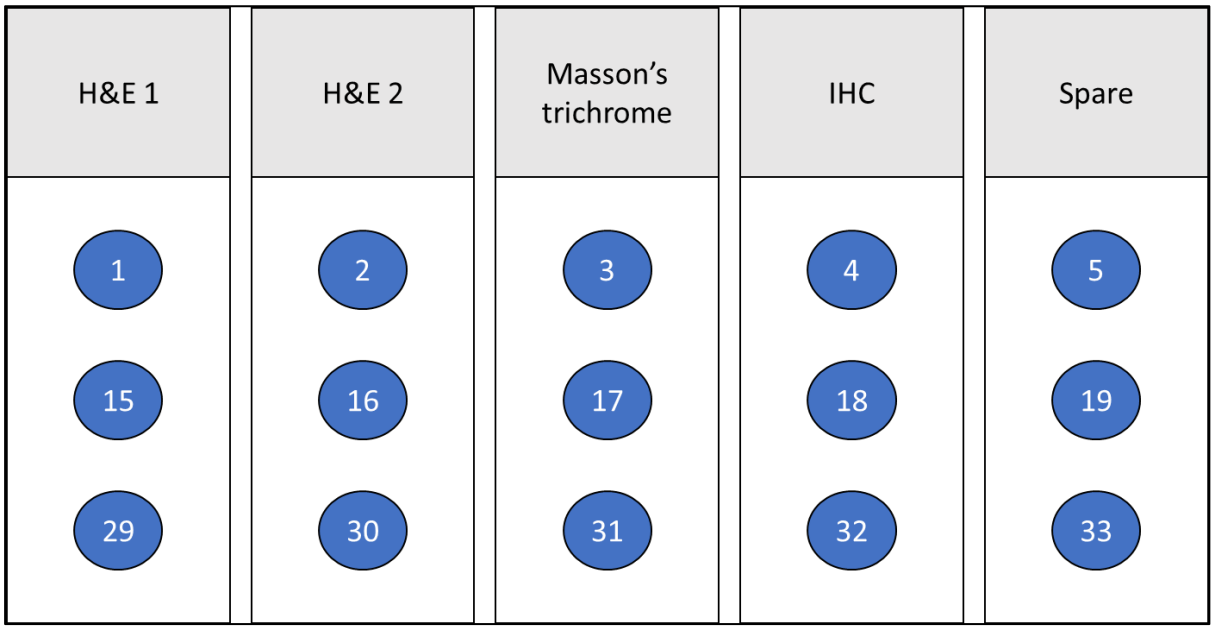

Figure 3.3: Distribution of serial sections on respective slides. Five slides with 3 sections each were obtained from each animal for various stains, slide 1: haematoxylin and eosin (H&E), slide 2: H&E, slide 3: potential Masson's trichrome stain, Side 4: immunohistochemical (IHC) analysis and slide 5: spare. Serial sections were obtained and distributed between the slides, with a minimum of 10 sections being discarded before the following set of serial sections was taken.

3.7.2 Tinctorial staining

Haematoxylin and eosin staining

Five random sections from each tissue block were deparaffinised and hydrated to be used for routine H&E staining by means of the Leica Auto stainer XL *(Leica Biosystems, Wetzlar, Germany)*.

Haematoxylin was used to stain the basophilic tissue, such as the nucleic acid in the nuclei, with eosin used as counter stain for acidophilic tissue, such as cytoplasm. See Appendix D for H&E staining protocol. All slides were mounted using a resinous mounting medium.

3.7.3 Immunohistochemistry

Samples were stained using the Leica BondMax™ auto stainer in conjunction with the Bond™ Polymer Refine detection kit *(Leica Biosystems, Wetzlar, Germany).* This detection kit uses a two stage indirect staining method with horseradish peroxidase and dextran polymer (Poly-HRP) and 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) as the chromogen. Use of an automated platform allows for consistent staining of tissue as it strictly controls all volumes of reagent dispensed.

After deparaffinisation and hydration, endogenous peroxidase activity was blocked using hydrogen peroxide and the primary antibody applied. This was to reduce artefactual

background staining, due to the reaction of DAB and the endogenous peroxide. Subsequently, epitope retrieval was conducted to unmask antigen binding sites. The affinity of antigen binding sites to bind, or the antigenicity of a tissue, is affected by factors relating to fixation of the tissue. Included in this, is the time before fixation occurred, type of fixative used, concentration thereof and duration of fixation.

Using the Bond™ Polymer Refine detection kit required the use on an immunoglobulin G (IgG) mouse anti-rat primary antibody to be used. Successive binding of the rabbit anti-mouse post primary and the anti-rabbit Poly-HRP. The introduction of DAB with hydrogen peroxide causes an oxidation reaction that is catalysed by HRP which causes a brown percipient to form in the region of the antigen.

Stem Cell Factor staining

Stem cell factor ligand (SCF) was labelled with the use anti-SCF polyclonal affinity purified antibody *(ab64677, Abcam®, United Kingdom)*. After endogenous peroxidase quenching, slides underwent epitope retrieval (ER) using ethylenediaminetetraacetic acid at a pH 9 at 100 °C, referred to as ER 2 when using the BondMax™ platform, for 20 minutes. As can be seen in Appendix E.

Primary antibody was incubated at a dilution of 1:500, for 40 minutes at room temperature. Slides were counterstained with haematoxylin to provide sufficient contrast for visualisation.

After staining all sections were dehydrated through graded ethanol and mounted using a resinous mounting agent. All tissue presenting with well localised, granular staining, owing to the DAB, was considered to be positive for SCF ligand. Stem cell factor can be free floating, bound to the granulosa cells theca cells oocytes.

3.7.4 Morphometry and morphology

All light microscopy was conducted with the use of a Zeiss Axioskop 2 *(Carl Zeiss Microscopy, Oberkochen)*. Micrographs were captured using the Zeiss Axiocam 105 colour camera *(Carl Zeiss Microscopy, Oberkochen)* with image analysis being conducted using Zen10 Blue 2012 *(Carl Zeiss Microscopy, Oberkochen)*.

Follicle classification and numeration

Follicles were classified by methods adapted from Pedersen and Peters (1968). Initial parameters were primarily identified for application in mice, however, the use of these parameters in the rat and hamster has been recommended and applied in various studies to date (Yoshida *et al.*, 2009; Gaytan *et al.*, 2015; Picut *et al.*, 2015). In Pedersen and Peters (1968), oocyte / follicle maturation was stratified into three main groups and further into eight

types. Stratification is based on the number of surrounding granulosa cells (follicle cells used in original article) and antrum development as can be seen in Appendix F.

In the present study, follicles were similarly classified with regard to quantity of granulosa cells and the level of antral development. Categories in the present study were; primordial / primary (Type 1), primary developing (Type 2), secondary developing (Type 3), mature / Graafian follicles (Type 4). Oocytes that have a single layer of granulosa cells or less are labelled as Type 1. Oocytes with multiple layers of granulosa cells and oocytes with multiples layers granulosa cells and with antral development are labelled Type 2 and Type 3 follicles respectively. Follicles consisting of an oocyte, multiple layers granulosa cells and a large antrum development with the presence of a cumulus stalk have been considered as a Type 4 follicle. Figure 3.4 is a schematic representation of these types. Table 3.1 depicts the relationship with the Pedersen and Peters (1968) method.

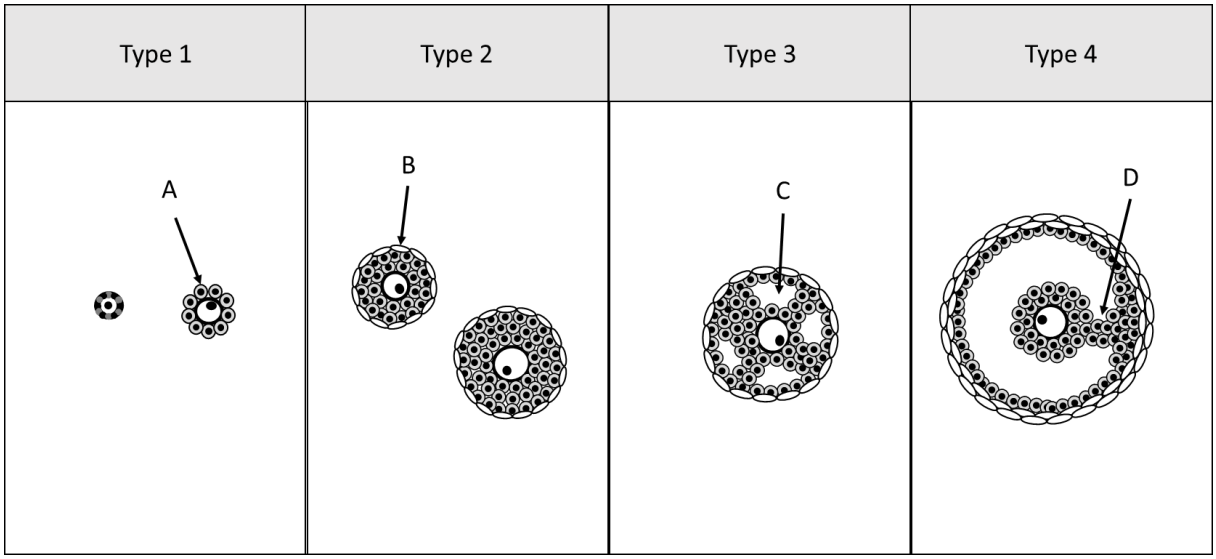

Figure 3.4: Classification of follicle types. A = Granulosa cells, B = Theca cells, C = Antrum formation and D = cumulus stalk.

Follicles were enumerated by counting the follicles present in five random sections from each animal, as was considered adequate by Smith, Plowchalk, Sipes, & Mattison (1991). Follicles were only considered complete and counted if the oocyte was visible. Follicles were counted at 100x magnification.

Enumeration of both corpora lutea and atretic follicles were conducted. Follicles were considered atretic according to guidelines stipulated by Devine *et al.*, (2000). Atretic follicles were identified by the visualisation of apoptotic markers in granulosa cells or a hyalinised scar formed from the degeneration of the oocyte with accompanying cell debris. Corpora lutea were identified as large congregations of luteal cells. For the purpose of this study, secretory and non-secretory corpora lutea were not distinguished.

Table 3.1: Comparison of classification criteria

Description		Pedersen & Peters (1968)		Present study
Number of granulosa cells	Antral development	Type	Group	Group
0	-	Type 1	Small follicles	Type 1 (Primordial / Primary follicles)
Incomplete ring surrounding oocyte	-	Type 2		
Complete ring surrounding oocyte (≤ 20)	-	Type 3a		
21 – 60	-	Type 3b	Medium follicles	Type 2 (Primary developing follicle)
61 – 100	-	Type 4		
101 – 200	-	Type 5a		
201 – 400	-	Type 5b	Large follicles	
401 – 600	Initial growth of antrum	Type 6		Type 3 (Secondary developing follicle)
> 600	Large antrum but lacks cumulus oophorus	Type 7		
> 600	Large antrum with cumulus stalk present	Type 8		Type 4 (Mature / Graafian follicle)

Morphology evaluation

Haematoxylin and eosin stained slides were used of for morphology evaluation. All morphological examinations were conducted with the observer being blinded to the groups examined.

A minimum of five sections were examined for various histological changes:

1. Inflammation
2. Oedema
3. Fibrotic changes
4. Tunica albuginea thickening
5. Follicular cysts

Inflammation is a response by vascularised tissues to attenuate infections, and repair affected tissues by recruiting immune cells and inflammatory mediators. Morphological changes in the tissue due to inflammation can be identified as increased numbers of white blood cells. These cells, polymorphonuclear (PMN) white blood cells (WBC) and mononuclear WBCs were identified using slides stained with H&E. Polymorphonuclear cells include: eosinophils, neutrophils and basophils, which all present with multi-lobulated nuclei and granular cytoplasm. Mononuclear WBC's include: B and T lymphocytes (not distinguishable using H&E staining) and monocytes, which present with single, mostly round nuclei.

Normal follicular rupturing is said to be assisted by inflammatory-like functions (Oakley *et al.*, 2010), therefore diffuse inflammatory cells were expected. All tissue was examined for any inflammatory foci.

Oedema is characterised by the excess build-up of fluid in tissues and can be as result of various causes. Fluid accumulation can occur either within cells, or more commonly within the interstitial space. This can be characterised by a distortion of normal cellular morphology by large fluid filled spaces in the interstitial tissue, often accompanied by highly congested surrounding blood vessels. Oedema was scored as follows: 0 = none and 1 = present.

Fibrosis is defined by the excessive deposition of extracellular matrix and it constituents which commonly occurs in the case of injury and chronic inflammation. In sections stained with H&E, fibrosis was visualised by a highly eosinophilic area, but cannot always be easily visualised. The present study displayed no overt fibrotic changes in the ovaries and therefore a Masson's trichrome stain was omitted. Collagen deposition was scored as follows: 0 = none or 1 = present.

The TA is a dense regular connective tissue layer rich in connective tissue fibres and found on the surface of the ovary. Thickness of the tunica albuginea was measured in two regions per section for all groups, for a minimum of 3 sections. Randomisation of sampling sites was conducted by measuring nine potential sites in each section. Every second digit in a random number table (Appendix G) (which was not a repeat of any previously selected digit for that sample) were used to select the measurement site.

Follicular cysts appeared as thin-walled follicles filled with acidophilic residue and often the debris of degraded oocytes. These cysts usually occurred with approximately four layers of flattened to cuboidal un-luteinised granulosa cells, lined with a thin layer of fibrous connective tissue. Follicular cysts were scored as: 0 = absent and 1 = present, with the number of follicular cysts being tallied (Camargo *et al.*, 2014).

Stem Cell Factor staining interpretation

Stem cell factor is a ligand that can be either free floating, bound to granulosa cells, bound to theca cells or the oocyte itself. Scoring of SCF staining was conducted using a modified oestrogen receptor scoring tool (Detre, Saccani Jotti and Dowsett, 1995).

Scoring was stratified for all cases as can be seen in Table 3.2. In the case of scoring free floating SCF, the area of tissue stained, as well as intensity of staining was evaluated.

Table 3.2 SCF staining score

Location	Scoring
Free floating ligand proportion stained	0 = Absent 1 = Slight (< 33% of tissue stained) 2 = Moderate (34% - 65% of tissue stained) 3 = Extensive (66% - 100% of tissue stained)
Free floating ligand intensity staining	0 = None 1 = Weak 2 = Moderate 3 = Strong
Theca bound	0 = Absent 1 = Present in less than 50% of cells 2 = Present in more than 50% of cells
Granulosa bound	0 = Absent 1 = Present in less than 50% of cells 2 = Present in more than 50% of cells
Oocyte bound	0 = Absent 1 = Present in less than 50% of cells 2 = Present in more than 50% of cells

3.8 Statistical analysis

Statistical analysis was conducted with the assistance of a biostatistician from the Centre for Statistical Consultation at Stellenbosch University. Analysis was conducted using Tibco® Statistica™ version 13.3 *(Palo Alto, USA)*.

Descriptive statistics were used to examine all data to produce means values, standard deviation and standard error values. Raw data has been analysed with the use of a theoretical linear model in order to identify and exclude outliers. Data was represented as graphs and other visual aids at a confidence interval of 95% (p= 0.05).

An analysis of variance was conducted between the sample groups. This test uses the variance of normally distributed samples groups and compares them by assuming that the variance in each sample group is the same. If analyses result in a p-value ≤ 0.05, it indicates that there is significant difference between the sample groups, and the null hypothesis must be rejected. Validity of this test was tested with the use of Levene's test at significance of 0.01%. In this event, a Welch test would be conducted to consider weighted means with the Games-Howell test being used to determine the least significant difference.

Chapter 4: Results

This chapter aims to provide a descriptive, graphical and statistical evidence of the effect of a high sucrose diet on ovarian morphology in parental and F1 generation age-matched female albino Wistar rats. Metabolic status, mating outcome, macroscopic and microscopic changes have been presented. Note in all sections, except for mating outcomes, both parental and F1 generation results have been reported together. Parental groups consist of: Control feed (CF), High sucrose feed group 1 (HSF1) and High sucrose feed group 2 (HSF2), whilst F1 generations consist of: Control feed / Control feed (CF/CF) (pups from a CF dam and maintained on a control feed diet), High-sucrose feed /High-sucrose feed (HSF/HSF) (pups from a HSF dam and maintained on a high sucrose diet) and High-sucrose feed / Control feed pups (HSF/CF) (pups from a HSF dam and maintained on a control feed diet).

An analysis of variance was conducted, with a significance being defined by p-value less than 0.05. Trends are defined by a non-significant f-test value, with a Fischer's least significant difference value less than 0.05.

4.1 Metabolic findings

4.1.2 Body mass

Significant differences ($p<0.01$) in mean body mass (at time of death) were in all animals were identified among various groups. Control feed dams were found to weigh significantly more than all other groups. A detailed description of all significant differences can be seen in the caption of Figure 4.1. All means and standard deviations can be seen in Appendix H.

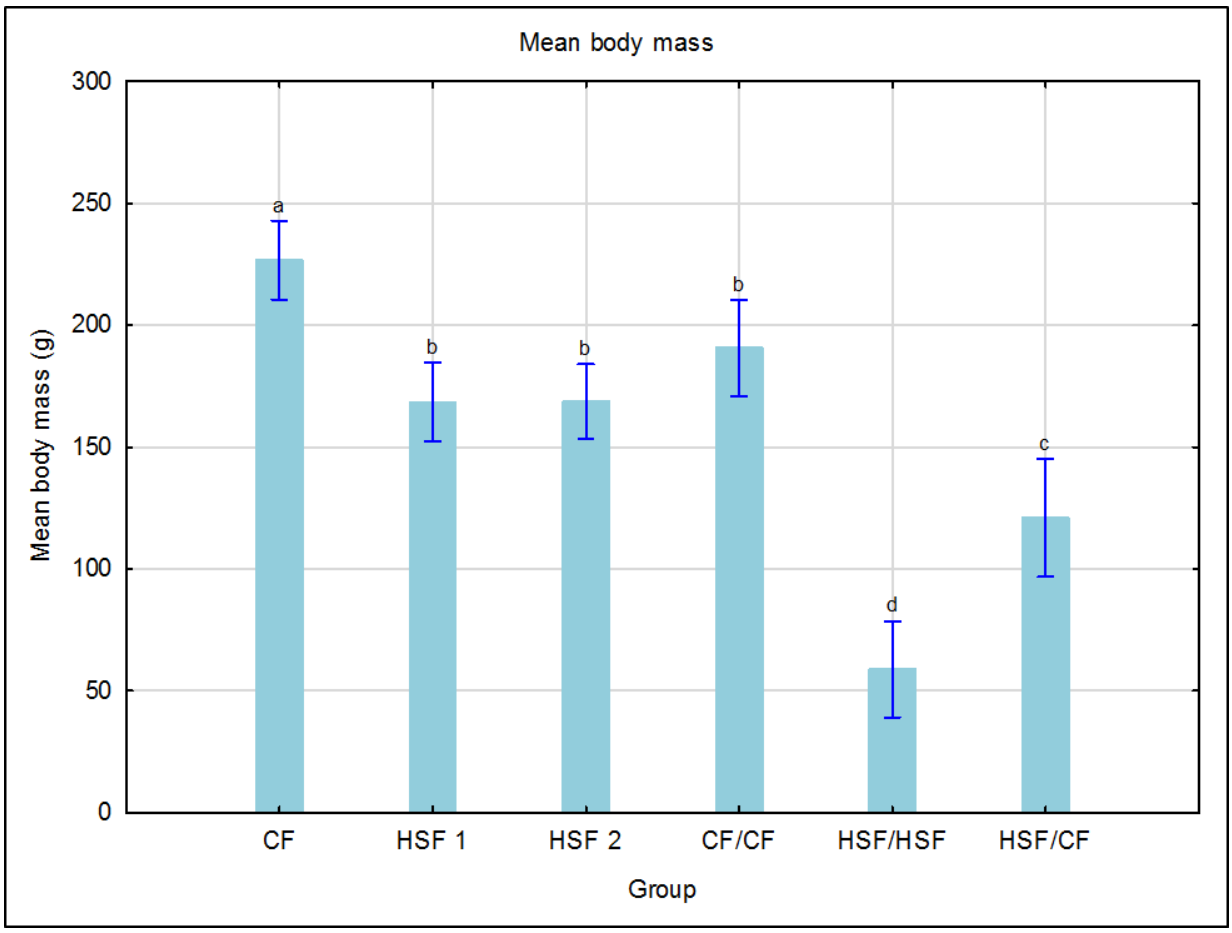

Figure 4.1: Mean body mass comparison. CF dams were found to be significantly ($p<0.01$) heavier than all other groups. HSF1, HSF2 and CF/CF groups were found to be significantly lighter than the CF group, but significantly heavier than the HSF/HSF and HSF/CF groups. The HSF/HSF group was found to be significantly lighter than all other groups. All differences occurred with LSD values less than 0.01. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Differing letters indicate significant differences. Vertical bars denote 0.95 confidence interval.

4.1.3 Fasting blood glucose levels.

Fasting blood glucose levels (FBGL) were found to vary significantly ($p<0.01$) among groups as was confirmed by a Welch test ($p<0.01$) owing to a lack of homogeneity of variance. A Games-Howell post hoc test was conducted to calculate LSD values. A detailed description of all significant differences can be seen in Figure 4.2. Means and standard deviations can be found in Appendix H.

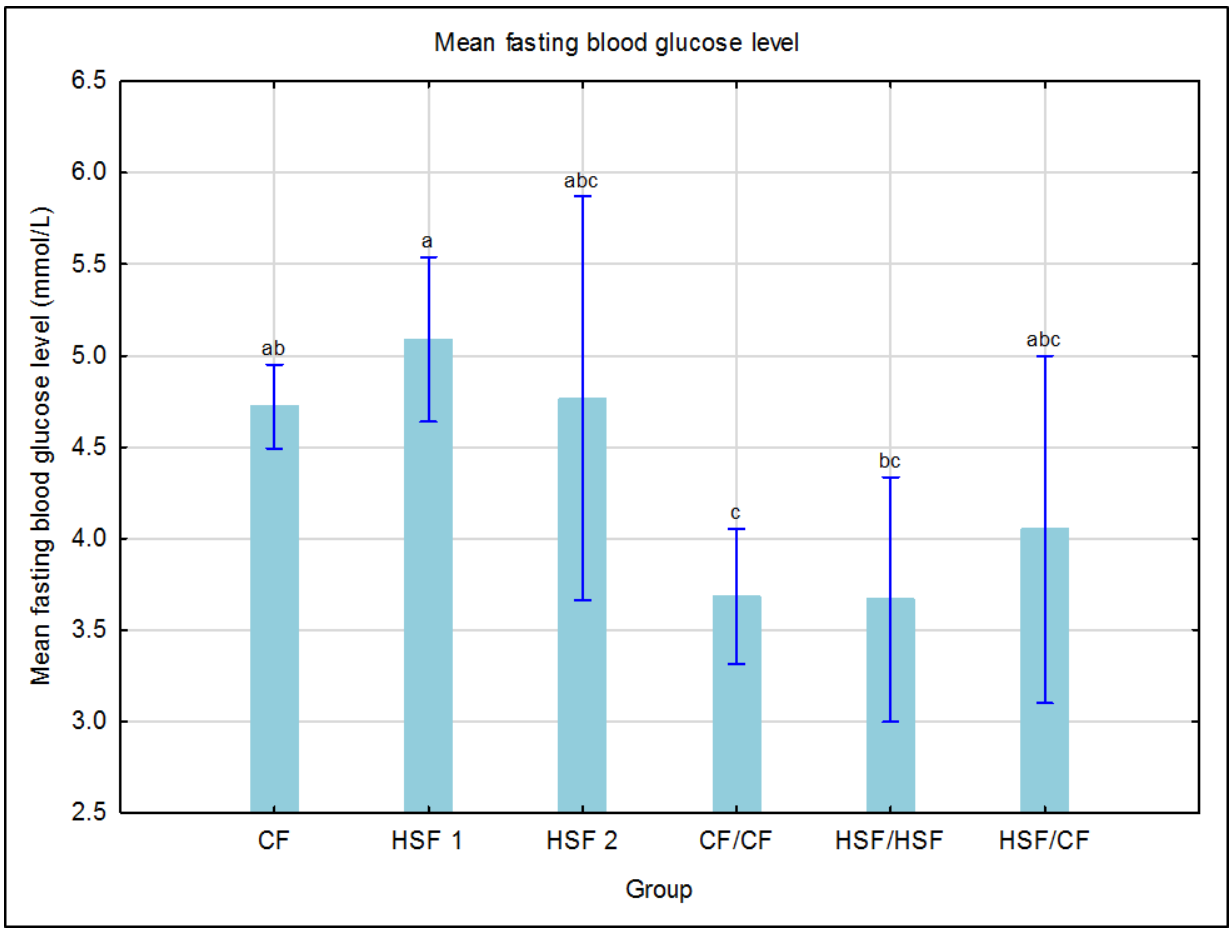

Figure 4.2: Mean fasting blood glucose levels. Fasting blood glucose levels (FBGL) in the HSF1 were found to be significantly (F test, p<0.01 & Welch test, P<0.01) higher than those of the CF/CF (Games-Howell, LSD<0.01) and HSF/HSF (Games-Howell, LSD=0.01) groups. Additionally, CF/CF pups were found to have a significantly lower FBGL than that of the CF (Games-Howell, LSD<0.01.) Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Differing letters indicate significant differences. Vertical bars denote 0.95 confidence interval.

4.2 The effect of diet on mating outcomes

Mating outcomes recorded were total pups birthed, sex of pups and wean weights.

4.2.1 Litter sizes and sexes

No significant differences in mean litter sizes were identified between different feeding groups (p=0.14). However, the LSD post hoc test demonstrated a trend where the HSF2 group litter sizes were smaller than that of the CF group (LSD=0.049) (Figure 4.3 A). Results displayed no significant differences in male pup numbers between groups (p=0.83) (Figure 4.3 B), however, a significant decrease (p=0.03) in female pup numbers were found in the HSF2 group when compared to the CF group (p=0.03 and LSD=0.001) (Figure 4.3 C). Means and standard deviations can be seen in Appendix H.

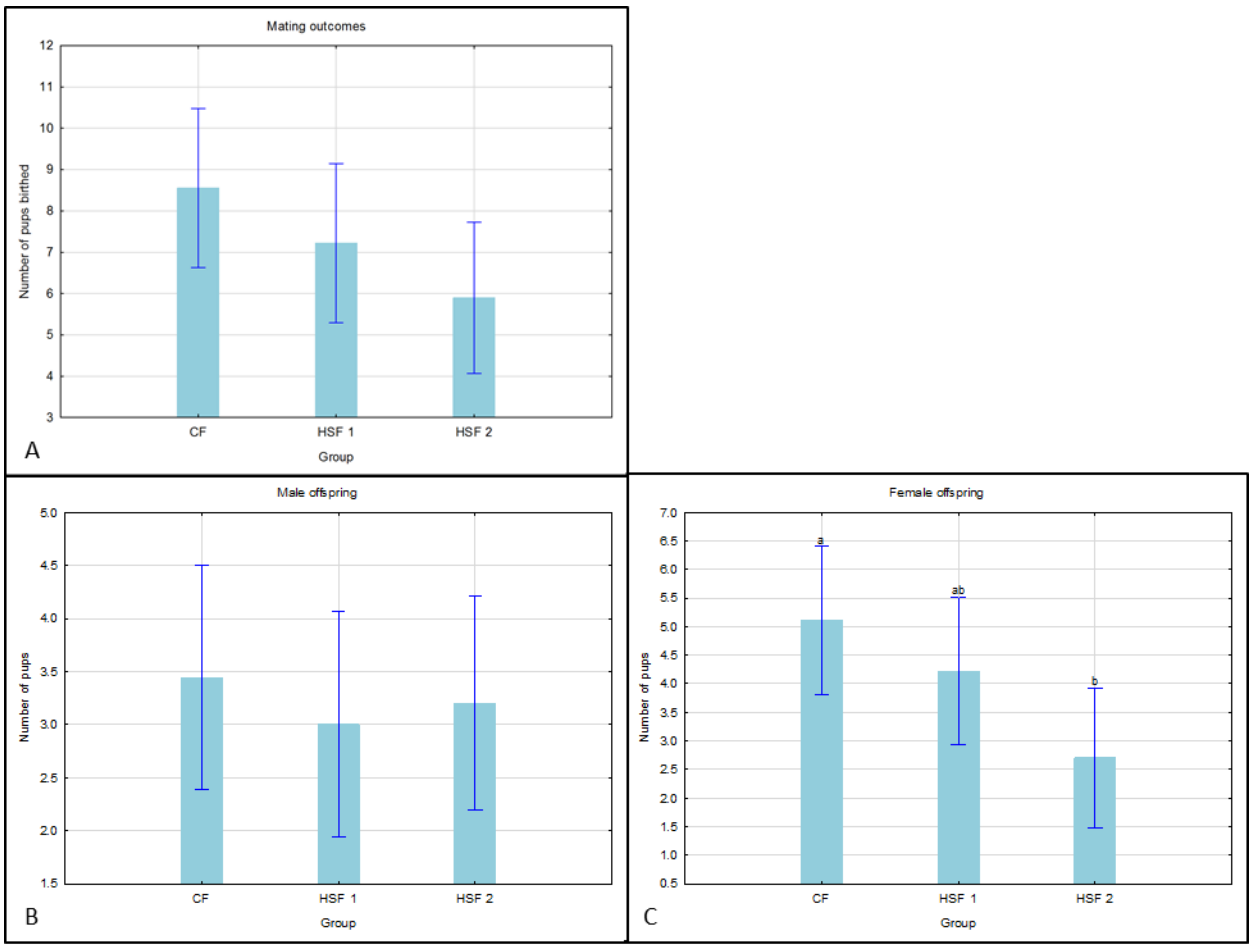

Figure 4.3: Mating outcomes. A) No significant differences were identified (p=0.14) in litter size. A trend was observed in the HSF2 group (LSD=0.049) in comparison to the CF group. B) No significant differences were found between number of males per litter (p=0.83). C) Significantly less females were born in HSF2 group in comparison to the CF group (p=0.03 and LSD=0.01). Control feed (CF), High-sucrose feed 1 (HSF1) and High-sucrose feed 2 (HSF2). Differing letters indicate significant differences. Vertical bars denote 0.95 confidence interval.

4.2.2 Pup body mass

At time of weaning both male and female pup body mass were significantly decreased by the high sucrose diet as can be seen in Figure 4.4 A (p=<0.01) and Figure 4.4 B (p=<0.01). Male pup weight was found to be significantly different across all groups, with all LSD values less than 0.01. Female CF pup weights were found to be significantly different to those of the HSF1 (LSD<0.01) and HSF2 (LSD<0.01). No significant difference in body mass was found between the HSF1 and HSF2 female pups. Means and standard deviations can be seen in Appendix H.

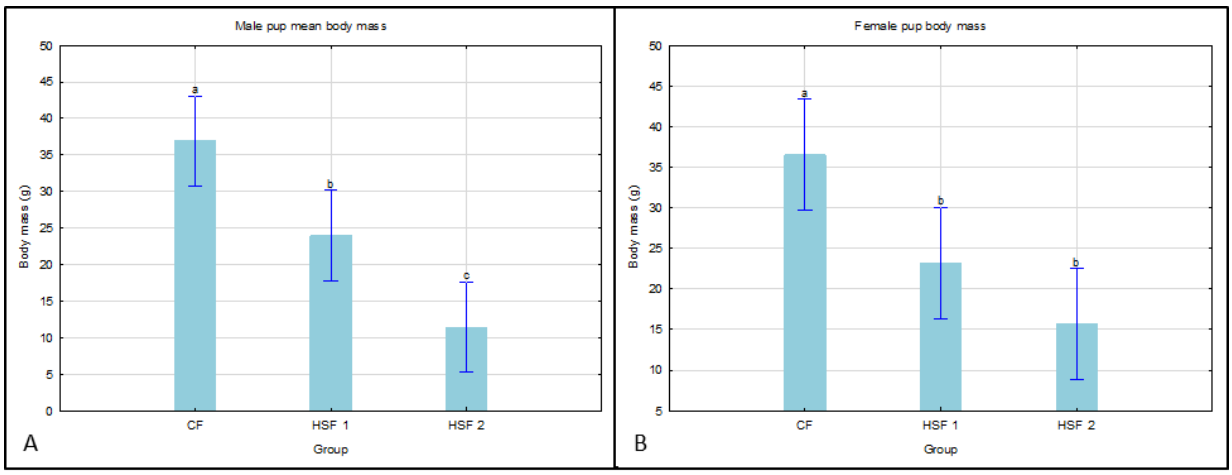

Figure 4.4: Mean pup body mass. A) Significant differences in male pup body mass were found between all groups ($p<0.01$), with LSD values less than 0.01. B) In females, CF pup body mass was found to be significantly different ($p<0.01$) to HSF1 ($LSD<0.01$) and HSF2 ($LSD<0.01$). Control feed (CF), High-sucrose feed 1 (HSF1) and High-sucrose feed 2 (HSF2). Differing letters indicate significant differences. Vertical bars denote 0.95 confidence interval.

4.3 Macroscopic findings

Macroscopic findings are defined by qualitative dissection descriptions and ovarian organ mass results.

4.3.1 Dissection findings

Specific areas that were noted include general visceral adiposity in the abdominal cavity, uterus bound and ovarian bound fat. Figure 4.5 demonstrates the difference in ovarian and para-uterine fat between a uterus and ovaries harvested from a CF animal (Figure 4.5 A) and those from HSF2 and HSF/HSF group.

In dissection, subjective comments concluded that overall size and fat deposition was decreased in the all groups that were maintained on a sucrose diet (HSF1, HSF2 and HSF/HSF). However, in the CF CF/CF feeding condition, groups presented with normal fat deposition whilst the HSF/CF group responded as an intermediate between these groups.

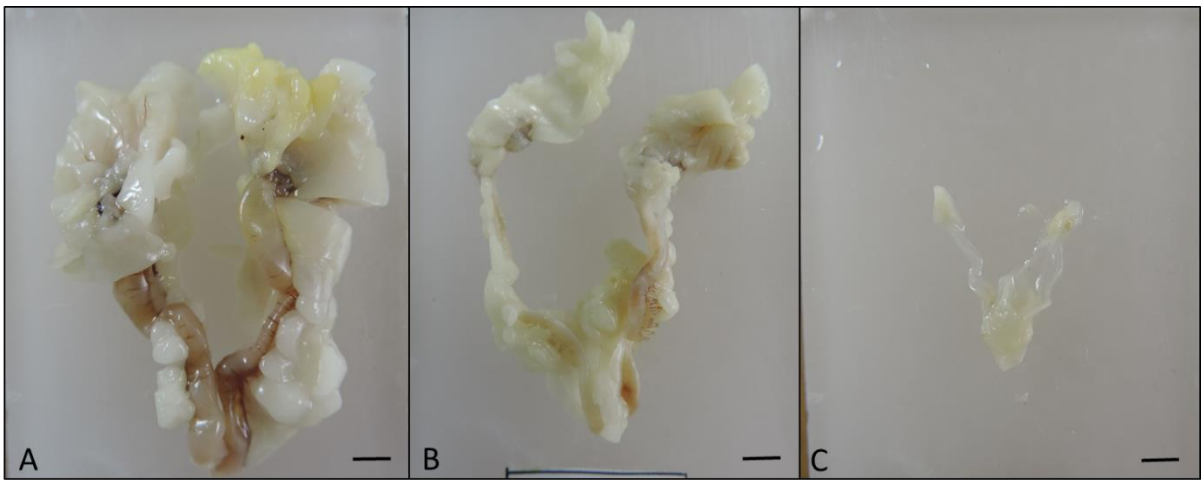

Figure 4.5: Differences in para-uterine fat deposition. A) Uterus of a CF dam. B) Uterus of a HSF2 dam. C) Uterus of an HSF/HSF pup. Control feed (CF), High-sucrose feed 1 (HSF1) and High-sucrose feed 2 (HSF2). Scale bars = 0.5cm.

4.3.2 Ovarian mass

Multiple significant differences in ovarian mass were identified among groups ($p<0.01$) (Figure 4.6). A detailed description of all significant differences can be seen in the caption of Figure 4.6. All means and standard deviations can be found in Appendix H.

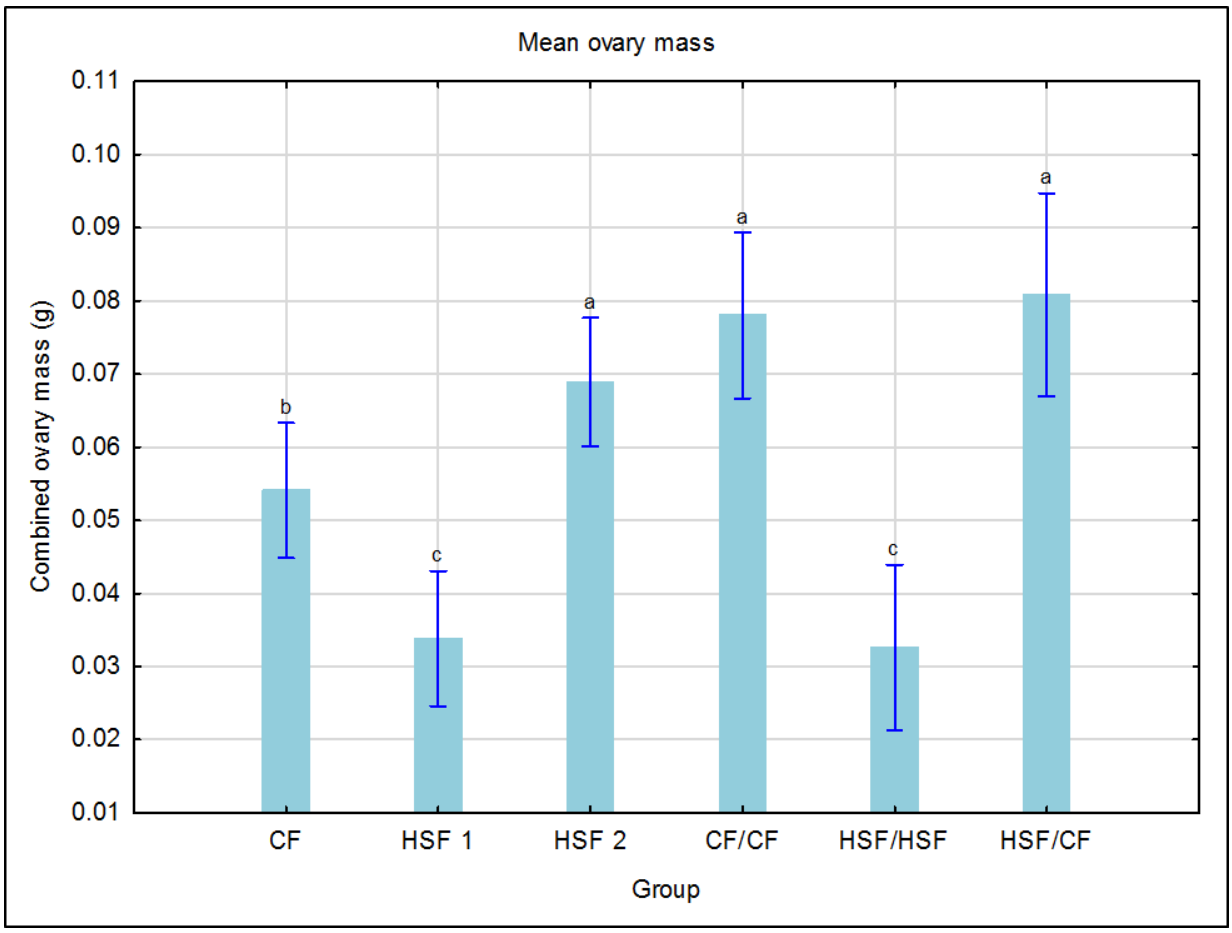

Figure 4.6: Mean ovarian mass. Multiple significant differences were identified in ovarian mass ($p<0.01$). The HSF1 and HSF/HSF groups were found to be significantly lighter than all other groups. The HSF2, CF/CF and HSF/CF groups were found to be significantly heavier than all other groups. CF animals were found to be significantly different to all other groups. All LSD values are <0.01 except when HSF2 and CF (LSD=0.02) groups are compared. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Differing letters indicate significant differences. Vertical bars denote 0.95 confidence interval.

4.4 Microscopic findings

4.4.1 Follicle numbers

No significant differences were observed when number of Type 1 follicles were compared. Initial f-test results ($p<0.01$) and LSD values indicated multiple significant differences. However, after conducting Levene's Test for Homogeneity of Variances, the Games-Howell post hoc test was conducted and these differences were found to be non-significant (Figure 4.7 A).

The number of Type 2 follicle in the HSF2 group were found to be significantly less ($p=0.01$) when compared to CF (LSD<0.01), HSF1 (LSD=0.03) and CF/CF (LSD<0.01) groups. Additional significant differences were observed between CF/CF and HSF/HSF (LSD=0.01) group, as well as the CF and HSF1 (LSD<0.01) group (Figure 4.7 B).

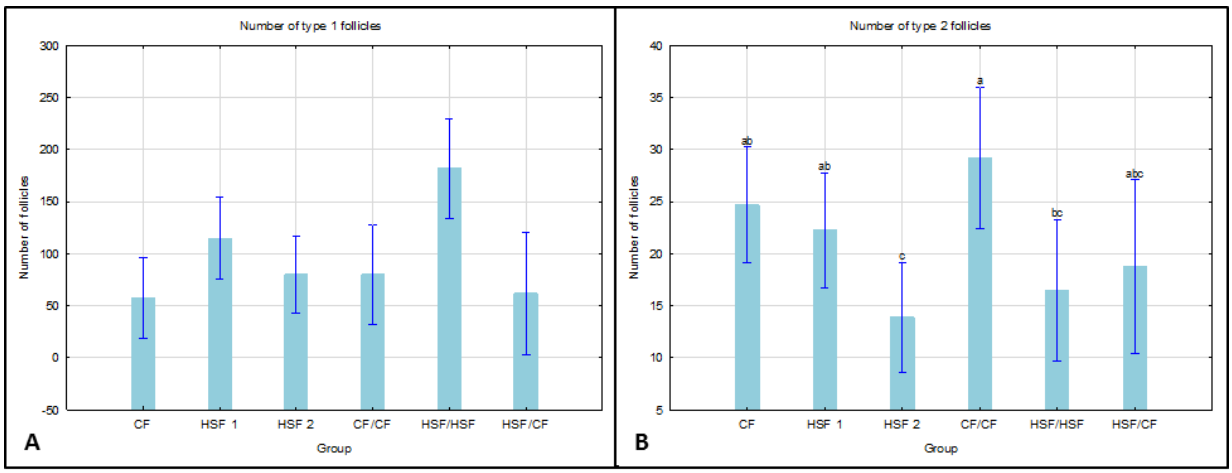

Figure 4.7: Number of Type 1 and Type 2 follicles. A) Number of Type 1 follicles did not vary significantly between groups (as confirmed by Games-Howell post hoc test). B) Significantly fewer Type 2 follicles were found in the HSF when compared to the CF (LSD<0.01), HSF1 (LSD=0.03) and CF/CF groups. Additional significant differences were observed between CF/CF and HSF/HSF (LSD=0.01) groups, as well as between CF and HSF1 (LSD<0.01) groups. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Differing letters indicate significant differences. Vertical bars denote 0.95 confidence interval.

The number of Type 3 follicles did not vary significantly (p=0.12) among groups. However, various trends were observed and are noted in the caption of Figure 4.8 A. Type 4 follicle distribution displayed no significant differences between groups (p=0.22) (Figure 4.8 B). Means and standard deviations can be found in Appendix H.

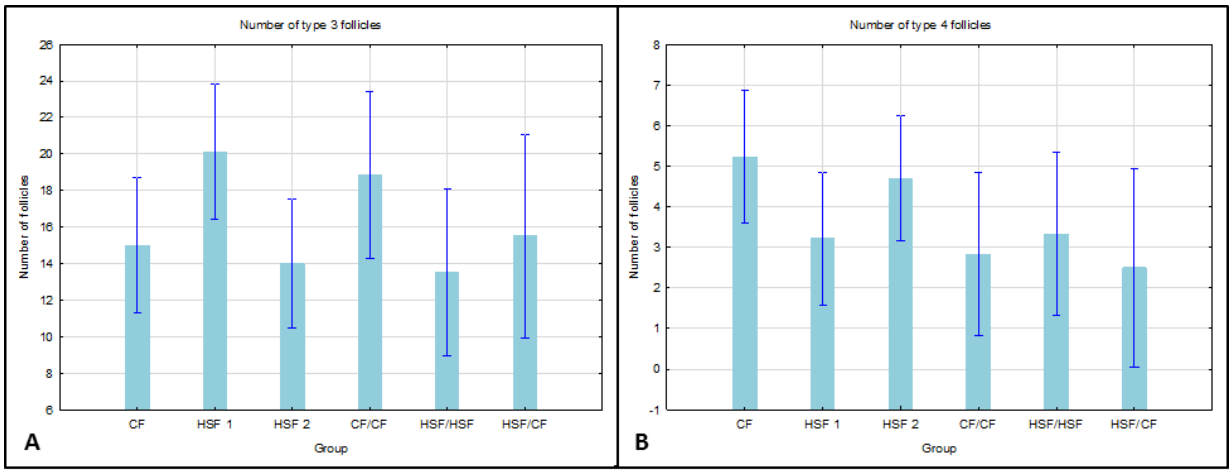

Figure 4.8: Number of Type 3 and Type 4 follicles. A) No significant differences (p=0.12) were observed in the number of Type 3 follicles among groups. A trend was observed with the HSF1 group having a greater number of Type 3 follicle than the HSF2 (LSD=0.02) and HSF/HSF (LSD=0.03) groups. B) No significant differences (p=0.22) were observed in the number of Type 4 follicles between groups. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Vertical bars denote 0.95 confidence interval.

4.4.2 Atretic and cystic follicles

Multiple significant differences (p=0.01) were observed in the number of atretic follicles among groups and are detailed in the caption of Figure 4.9 A.

All differences in the number of cystic follicles among groups was deemed to be non-significant (p=0.30). Levene's test for homogeneity was violated (p=0.002) and prompted a Welch test which remained again reported no significant differences (p=0.54) in the number of cystic follicles between groups (Figure 4.9 B). Means and standard deviations can be found in Appendix H.

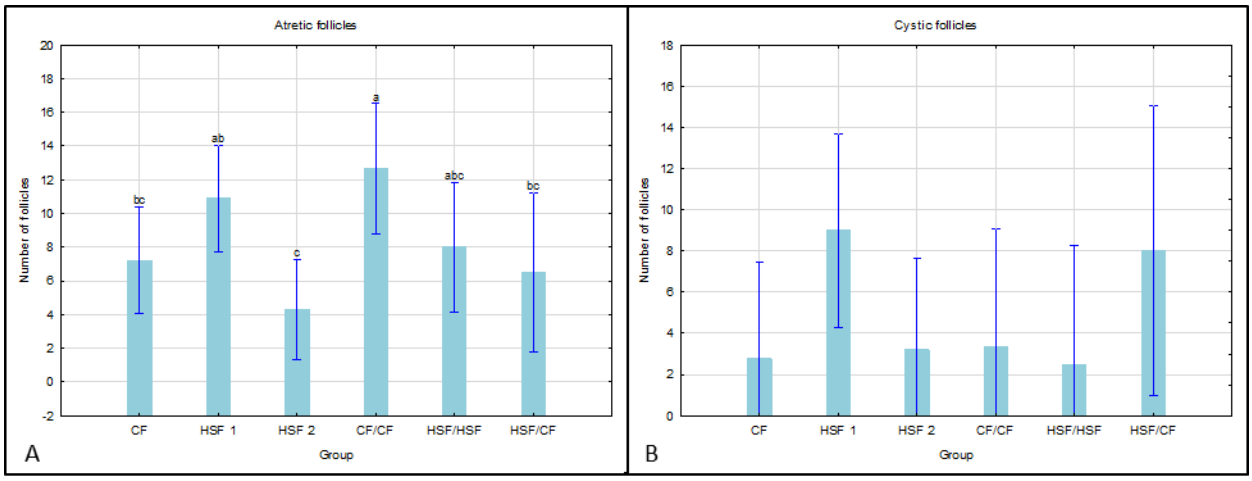

Figure 4.9: Atretic and cystic follicles. A) The number of atretic follicles in HSF2 was found to be significantly (p=0.01) lower than that of the HSF1 (LSD<0.01) and CF/CF (LSD<0.01) groups. Additional significant difference were observed between CF/CF and CF (LSD=0.03) as well as CF/CF and HSF/CF (LSD=0.047). B) No significant differences (p=0.30) in the number of cystic follicles were identified. Due to heterogeneous variance in the data a Welch test conducted to confirm no significant differences between groups (P=0.54). Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Differing letters indicate significant differences. Vertical bars denote 0.95 confidence interval.

4.4.3 Stem cell factor staining

Stem cell factor (SCF) staining was evaluated by assessing the SCF staining of the oocyte, granulosa cells, theca cells and stroma and stratified according to follicle type where applicable. Score were as follow: 0 = no staining, 1 = Less than 50% of cells structures stained and 2 = more than 50% of structures stained.

4.4.3.1 Oocyte staining

No significant differences were identified in the SCF staining in the oocytes of Type 1 follicles as evaluated by the f-test (p=0.05) (Figure 4.10 A). Trends were initially indicated using conventional LSD tests, however, these were deemed false by a Games-Howell post hoc test (conducted due to heterogeneous variation in data). Similarly, differences in staining of oocytes

in Type 2 (p=0.19) (Figure 4.10 B) and Type 3 (p=0.26) (Figure 4.10 C) follicles were found to be non-significant with no trends indicated by the Games-Howell post hoc test (conducted due to heterogeneous variation in data). All oocytes of Type 4 follicles were found to stain positively regardless of group.

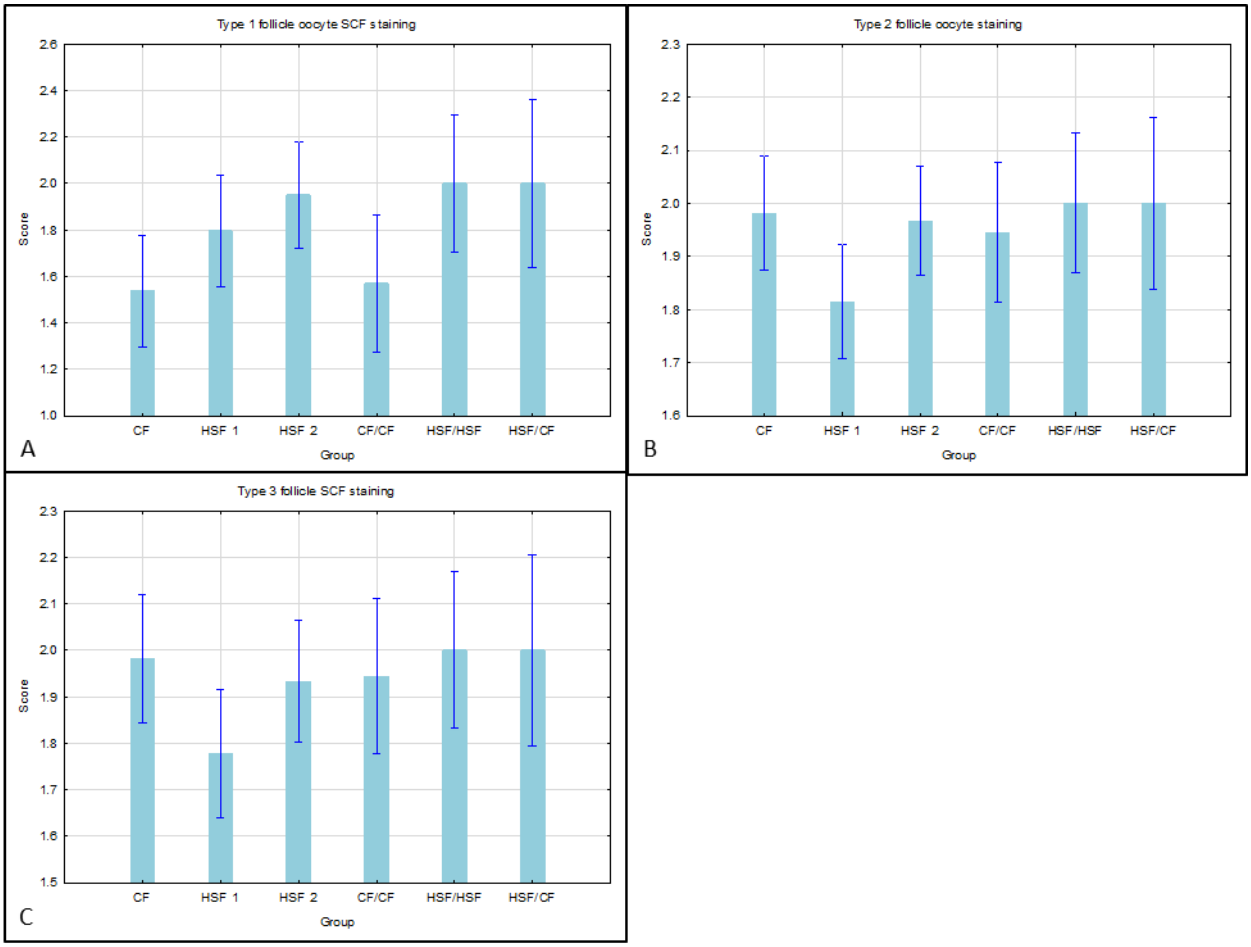

Figure 4.10: Stem cell factor oocyte staining. No significant differences were identified in the staining of oocytes for Type 1 (A, p=0.05), Type 2 (B, p=0.19) or Type 3 follicles (C, p=0.26). Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Vertical bars denote 0.95 confidence interval.

4.4.3.2 Granulosa cell staining

No granulosa cells of the Type 1 follicles were found to stain positively. Type 2 follicle granulosa cells were found to be significantly different (p<0.01) (Figure 4.11 A), with heterogeneous variation. Significance was confirmed with a Welch test (p<0.01) and LSD values determined using the Games-Howell post hoc test. The CF group scored significantly lower than the HSF2, CF/CF, HSF/HSF and HSF/CF groups, with LSD values less than 0.01. Additionally, the HSF1 group was found to score significantly lower than all other groups. (CF; LSD=0.03, HSF2; LSD<0.01, CF/CF; LSD<0.01, HSF/HSF; LSD<0.01, HSF/CF; LSD<0.01)

Similarly, differences (p<0.01) (Figure 4.11 B) in the staining of granulosa cells of Type 3 follicles were found to be significant, with heterogeneous variation. Significance was confirmed

with a Welch test ($p<0.01$) and the Games-Howell post hoc test used to determine LSD values. CF and HSF scored significantly lower than that of the HSF2, CF/CF, HSF/HSF and HSF/CF groups.

Stem cell factor granulosa staining of the Type 4 follicle was found to be significantly ($p<0.1$) (Figure 4.11 C) lower in the CF and HSF1 groups than all other groups. All LSD values are less than 0.01 except for when CF and CF/CF (LSD=0.02) groups are compared

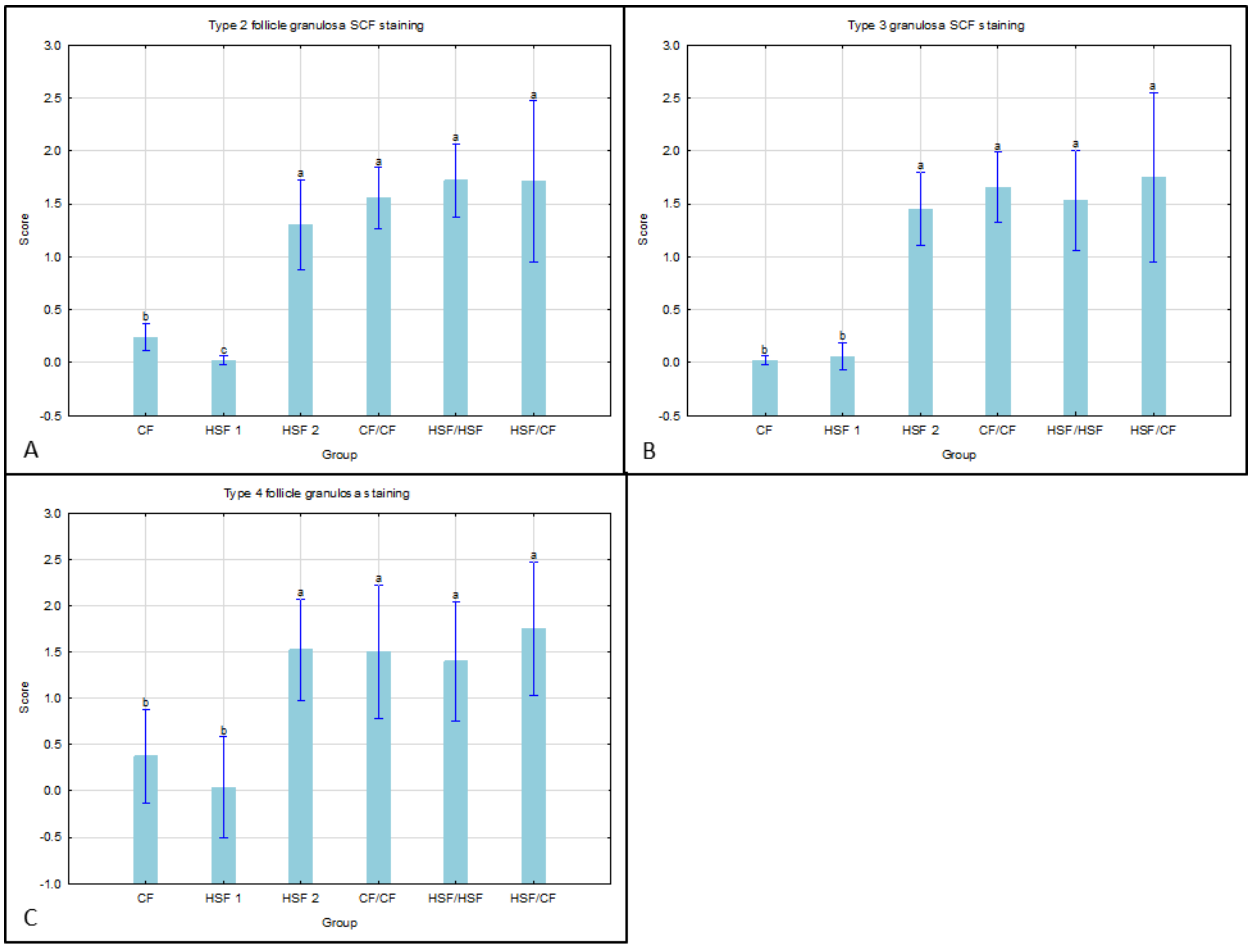

Figure 4.11: Stem cell factor granulosa staining. A) Staining of Type 1 granulosa cells in CF and HSF1 groups were found to score significantly ($p<0.01$) less than all other groups with LSD values less than 0.01. Additionally, the HSF1 group was found to score significantly lower than the CF group (LSD=0.3). B) Type 3 follicle granulosa cells of the CF and HSF1 groups were found to score significantly ($p<0.01$) lower than all other group (All LSD values less than 0.01). C) Similarly, staining of the Type 4 follicle granulosa cells in the CF and HSF1 staining scored significantly ($p<0.01$) lower than all other groups (All LSD values less than 0.01). Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Differing letters indicate significant differences. Vertical bars denote 0.95 confidence interval.

4.4.3.3 Theca cell staining

Theca cell staining was only observed in Type 3 and 4 Type follicles across all groups. No significant ($p=0.14$) (Figure 4.12 A) differences in theca cell staining was identified between

groups for Type 3 follicles however trends were present. The CF group tended to score higher than the HSF1 (LSD=0.04), HSF2 (LSD=0.03) and HSF/HSF (LSD=0.01) groups. Similarly, staining of theca cells in Type 4 follicles did not vary significantly (p=0.18) (Figure 4.12 B). However, the HSF2 group tended to score higher than the CF/CF (LSD= 0.03) group.

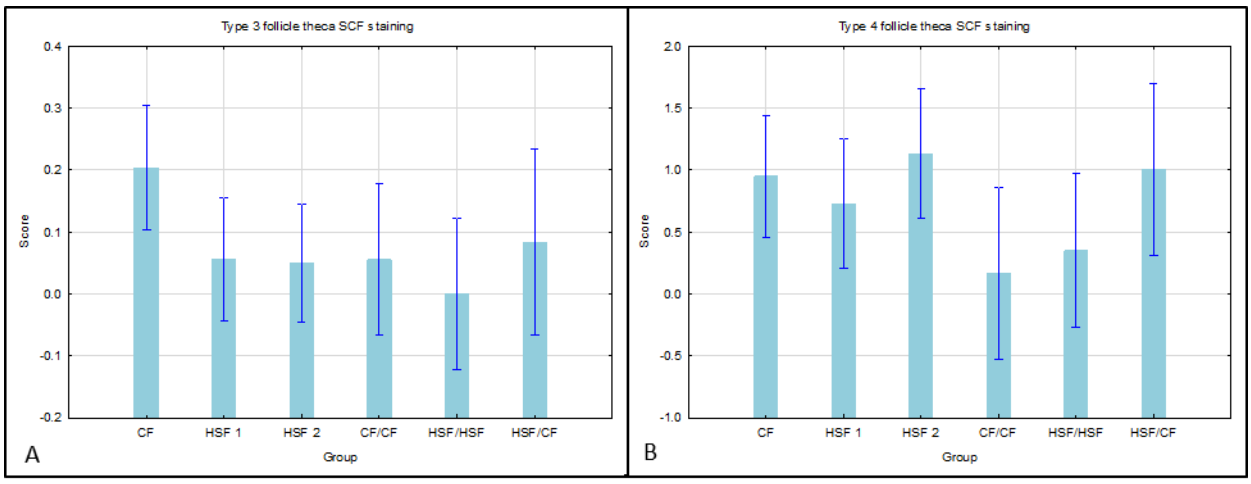

Figure 4.12: Stem cell factor theca staining. A) No significant differences were observed in the staining of Type 3 follicle theca cells. However, the CF tended to score higher than the HSF1 (LSD=0.04), HSF2 (LSD=0.3) and HSF/HSF (LSD 0.01) groups. B) No significant differences were found in the staining of Type 4 follicle theca cells. However, the HSF2 tended to score higher than the CF/CF (LSD=0.03) group. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Vertical bars denote 0.95 confidence interval.

4.4.3.4 Stromal staining

No significant differences were identified in the proportion stained (P=0.09) (Figure 4.13 A) or intensity (p= 0.14) (Figure 4.13 B) of stromal staining across all groups, however various trends were identified. The CF/CF (LSD=0.048) and HSF/HSF (LSD<0.01) group tended to have a higher proportion of stromal staining in comparison to the CF group. Intensity of stromal staining tended to score higher in the HSF2 (LSD=0.01) and HSF/HSF (LSD=0.03) groups when compared to the HSF1 group.

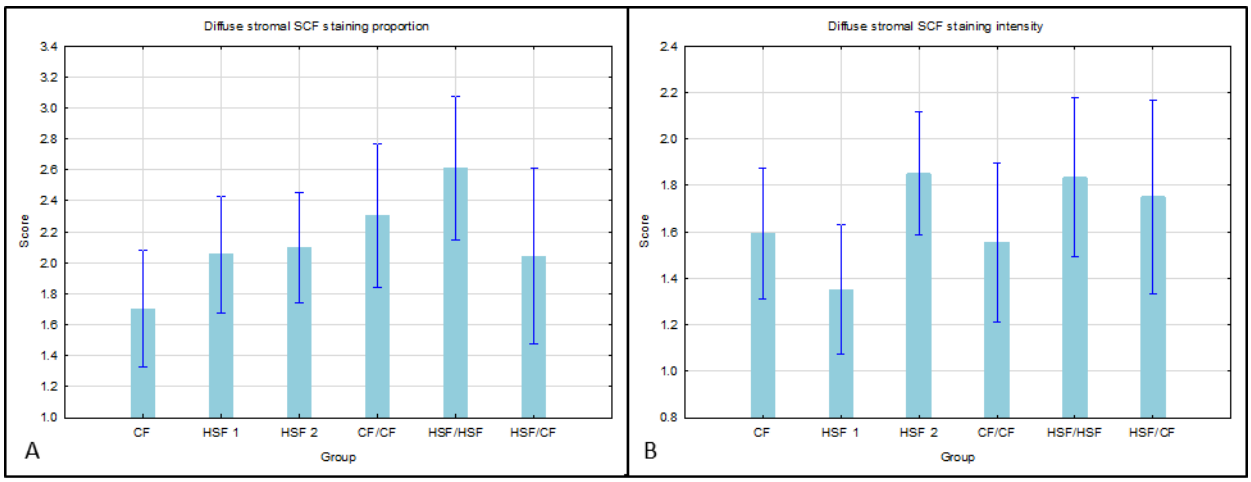

Figure 4.13: Stem cell factor stromal staining. A) No significant (p=0.09) differences were observed between the proportion of stroma stained among groups. However, the CF/CF (LSD=0.047) and HSF/HSF (LSD<0.01) groups did tend to have increased stromal staining when compared to the CF group. B) No significant (p=0.14) differences were identified in the intensity of stromal staining. However, it was noted that staining intensity in the HSF2 (LSD=0.01) and HSF/HSF (LSD=0.03) group tended to score higher than the HSF1 group. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Vertical bars denote 0.95 confidence interval.

4.4.4 Descriptive morphological changes

4.4.4.1 Inflammatory changes

Ovarian tissue contains a basal level of inflammatory cells as this organ is highly regenerative with constant cycles of follicle development, atresia and corpus luteum breakdown. Therefore, in all cases regardless of group, polymorphonuclear and mononuclear inflammatory cells were identified. None of the animals, regardless of group, presented with any inflammatory foci, thus it was concluded that no acute inflammatory processes were underway (Figure 4.15 & 4.16).

4.4.4.2 Oedema

Oedema characterised by large fluid filled spaces within the interstitium were not identified in any group. However, congestion, a precursor and hallmark of oedema was identified in various groups as indicated in Table 4.1. Congestion found to be prominent mostly in the HSF1 (Figure 4.15, C & D) and even more so in the HSF2 (Figure 4.15, E &F) group. Additionally, the HSF/CF group presented with cases of relatively mild congestion.

Table 4.1: Number of cases presenting with congestion.

	CF	HSF 1	HSF 2	CF/CF	HSF/HSF	HSF/CF
N	9	9	10	6	6	4
CONGESTED	1	6	9	1	2	2

Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF).

4.4.4.3 Fibrotic changes

No overt abnormal collagen depositions were observed in any of the study groups.

4.4.4.4 Tunica albuginea thickness

Significant ($p<0.01$) differences in TA thickening was observed between all groups except HSF1 and HSF/CF. The HSF1, HSF2 and HSF/CF were found to have the thickest TA with the HSF/HSF group having the thinnest TA. All comparisons presented with an LSD value less than 0.01, except for CF and CF/CF (LSD=0.02).

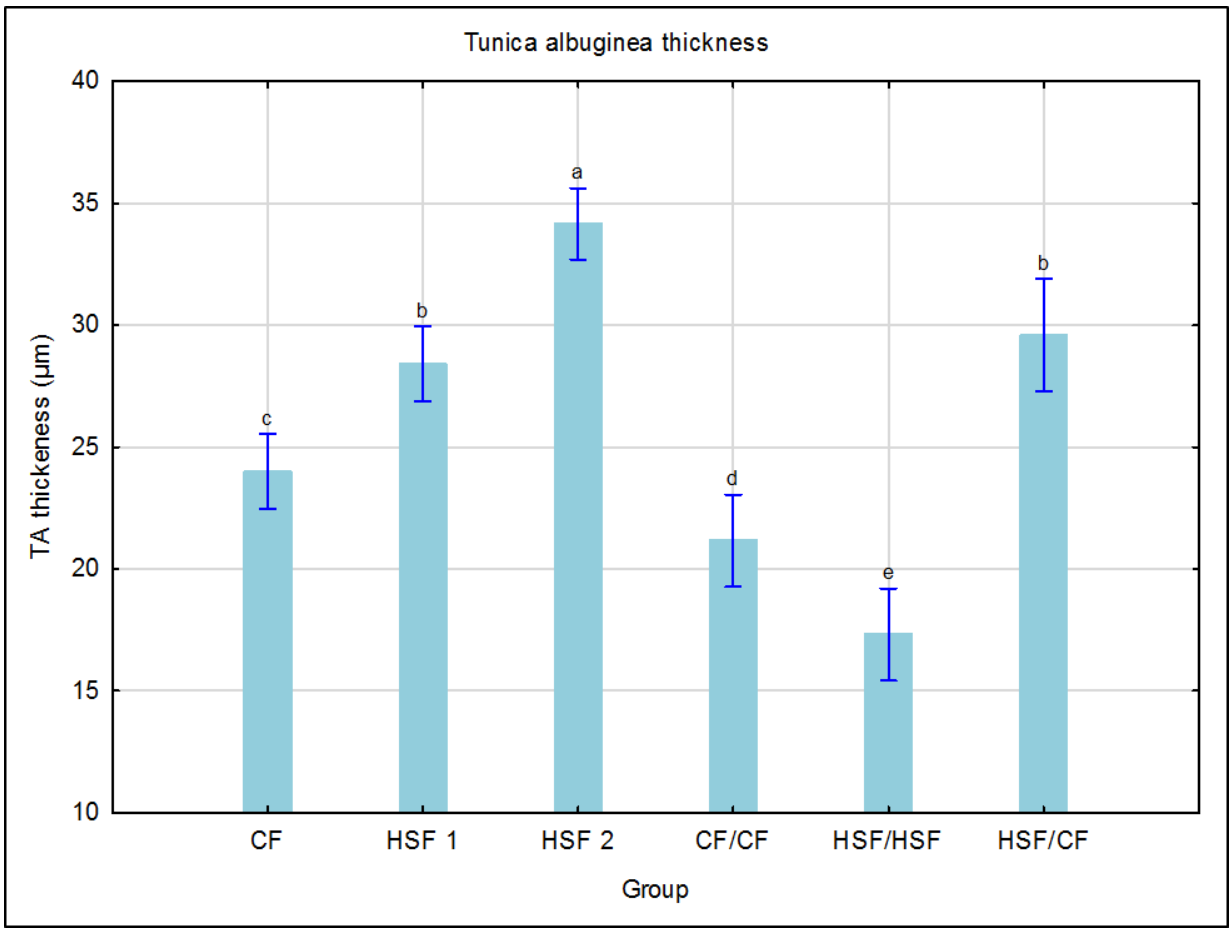

Figure 4.14: Tunica albuginea thickness in micrometres. Significant differences ($p<0.01$) were observed among all groups except between the HSF and HSF/CF group. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Differing letters indicate significant differences. Vertical bars denote 0.95 confidence interval.

4.4.4.5 Additional morphological changes

In addition to the predicted morphological changes, additional morphological changes were observed.

Control feed dams

In a single case within this group, two oocytes were found surrounded by granulosa cells forming a polyovular follicle. This is a normal variation that can occur at a low frequency within rat ovaries. No additional changes were identified (Figure 4.15, A & B).

High sucrose feed 1

In addition to congested blood vessels cases (6/9) from this group were found to have distinct changes to both their corpus luteum and stroma (Figure 4.15, E & F). Morphology of approximately a third of all corpora lutea presented cells with microvesicular and macrovesicular vacuolation and observable pyknotic nuclei. Within the same region, contracted cells, and additional vesicles containing cell debris were identified. (Figure 4.17 A).

This is indicative of an active apoptotic process. Likewise, the stroma also displays micro and macrovesicular vacuolation (Figure 4.17 B).

High sucrose feed 2

Nine out of ten cases from this group presented with notably more extensive congestion than all other groups (Figure 4.15 E & F). Multiple corpora luteua were found to have areas of eosinophilic cells with faded or non-staining nuclei. Peripheral nuclei were found to be pyknotic (Figure 4.17 C). Stroma presented with multiple congested blood vessels and increased levels of micro and specifically macrovesicular vacuolation (Figure 4.17 D).

Control feed / Control feed pups

All cases from this group were found to display normal morphology with physiological levels of macro and micro- vesicular vacuolation present.

High sucrose feed / High sucrose feed pups

Variable morphology was observed throughout this group. Most cases presented with increased macro and micro vesicular vacuolation, spindle-shaped and pyknotic nuclei (Figure 4.17 E). In 3/6 cases macrovesicular vacuolation caused severe distortion of stroma (Figure 4.17 F). Overall ovaries were found to contain fewer corpora lutea, with 2/6 cases presenting no observable corpus luteum. Most sections were found to have dense clusters of Type 1 follicles along the cortical periphery of the ovary.

In all cases, luteal cysts were identified surrounded by granulosa cells. Contents of the cysts appeared to be proteinaceous in nature (but remains undetermined) and was well circumscribed by flattened endothelial cells (Figure 4.18).

High sucrose feed / Control feed pups

All cases from this group presented with physiological to moderate levels of micro and macrovesicular vacuolation within the corpus luteum and physiological levels in the stroma.

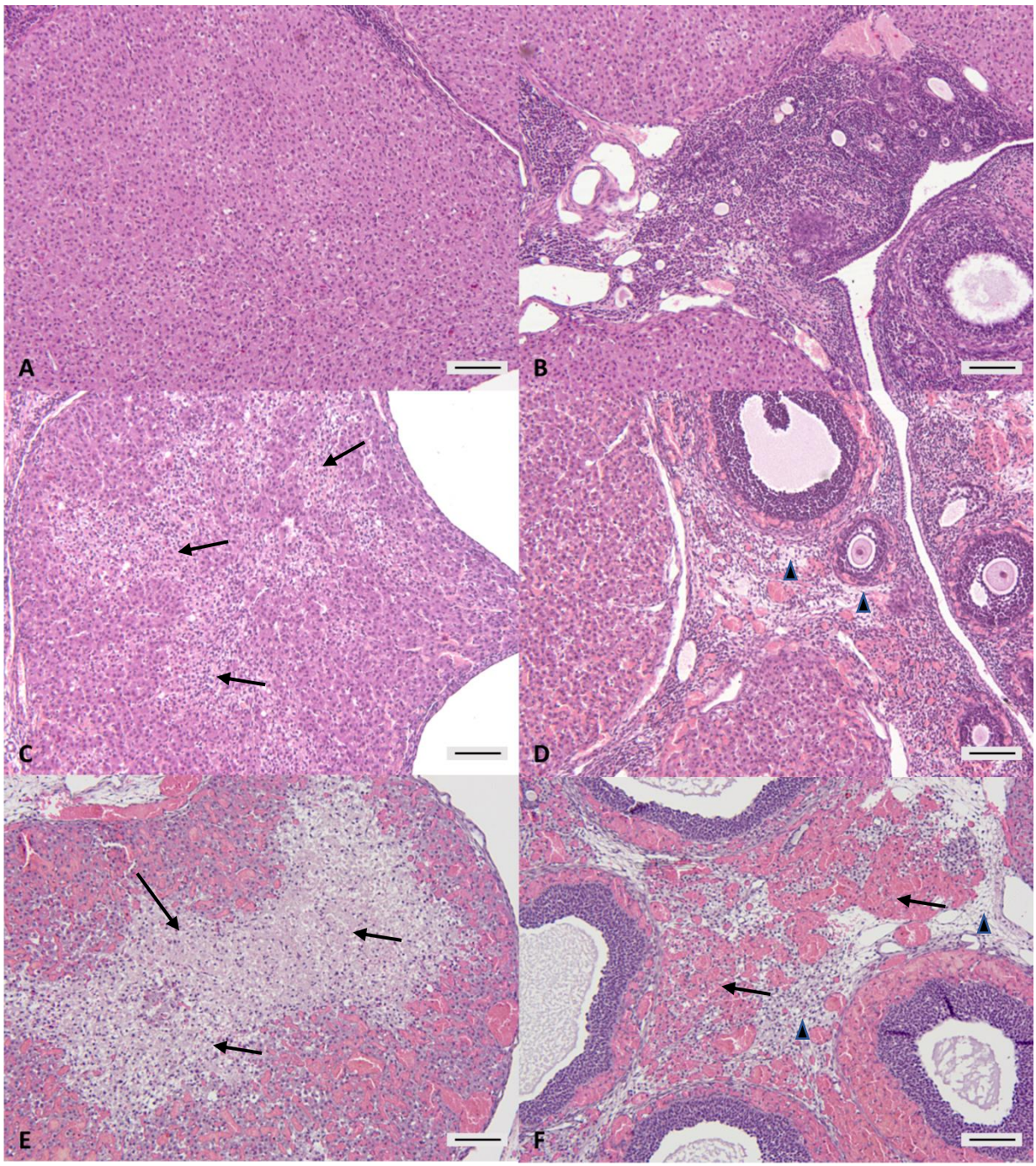

Figure 4.15: Overview of ovarian morphology in dams. A) Corpus luteum of a CF dam displaying typical morphology. B) Stroma of CF dam displaying typical morphology. C) Corpus luteum of HSF1 dam displaying morphological characteristics of an apoptotic process (represented by arrows). D) Congested stroma of a HSF1 dam presenting with micro- and macrovesicular vacuolation (Represented by arrow heads). E) Corpus luteum of a HSF2 dam, with observable congestion and necrotic lesion (represented by arrows). F) Extensive congestion of stroma (indicated with arrows) with increased micro- and macrovesicular vacuolation (Represented by arrow heads). Control feed (CF), High-sucrose feed 1 (HSF1) and High-sucrose feed 2 (HSF2). Haematoxylin and eosin preparations at 50x magnification (Scale bar = 100µm).

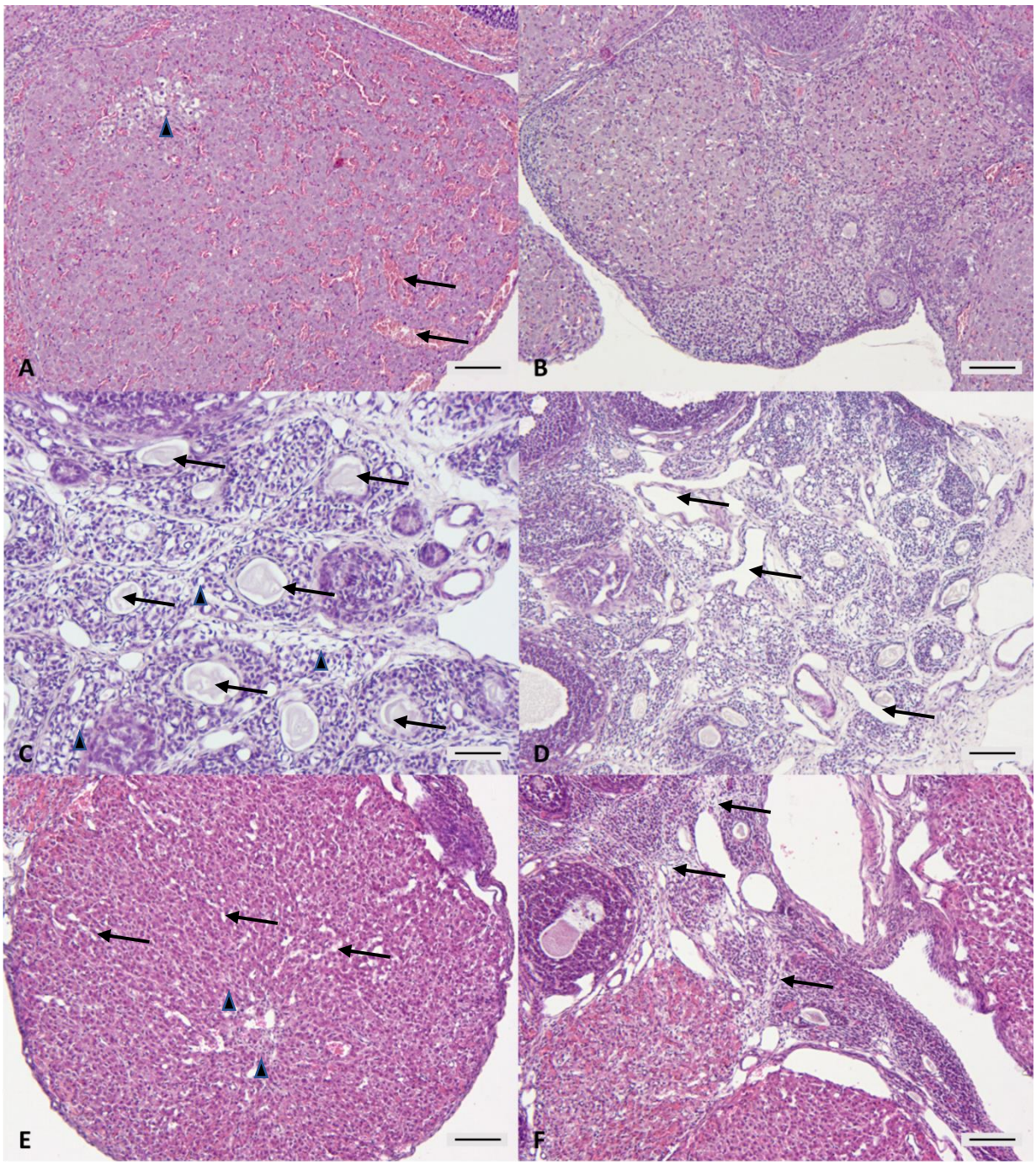

Figure 4.16: Overview of ovarian morphology in pups. A) Corpus luteum of a CF/CF pup displaying slight congestion (indicated by arrow), and physiological levels of micro- and macrovesicular vacuolation (indicated with arrow heads). B) Stroma of CF/CF pup displaying typical morphology. C) Stroma of a HSF/HSF pup displaying increased levels of micro- and macrovesicular vacuolation (indicated by arrow heads) including with scattered luteal cysts (indicated by arrows). D) Stroma of a HSF/HSF with multiple dilated blood vessels (indicated with arrows). E) Corpus luteum of a HSF/CF pup, with dilated sinusoids (indicated with arrows) and displaying physiological levels of micro- and macrovesicular vacuolation (indicated by arrow heads). F) Mildly congested stroma of a HSF/CF pup, with increased micro- and macrovesicular vacuolation (indicated by arrows). Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Haematoxylin and eosin preparations at 50x magnification (Scale bar = 100µm).

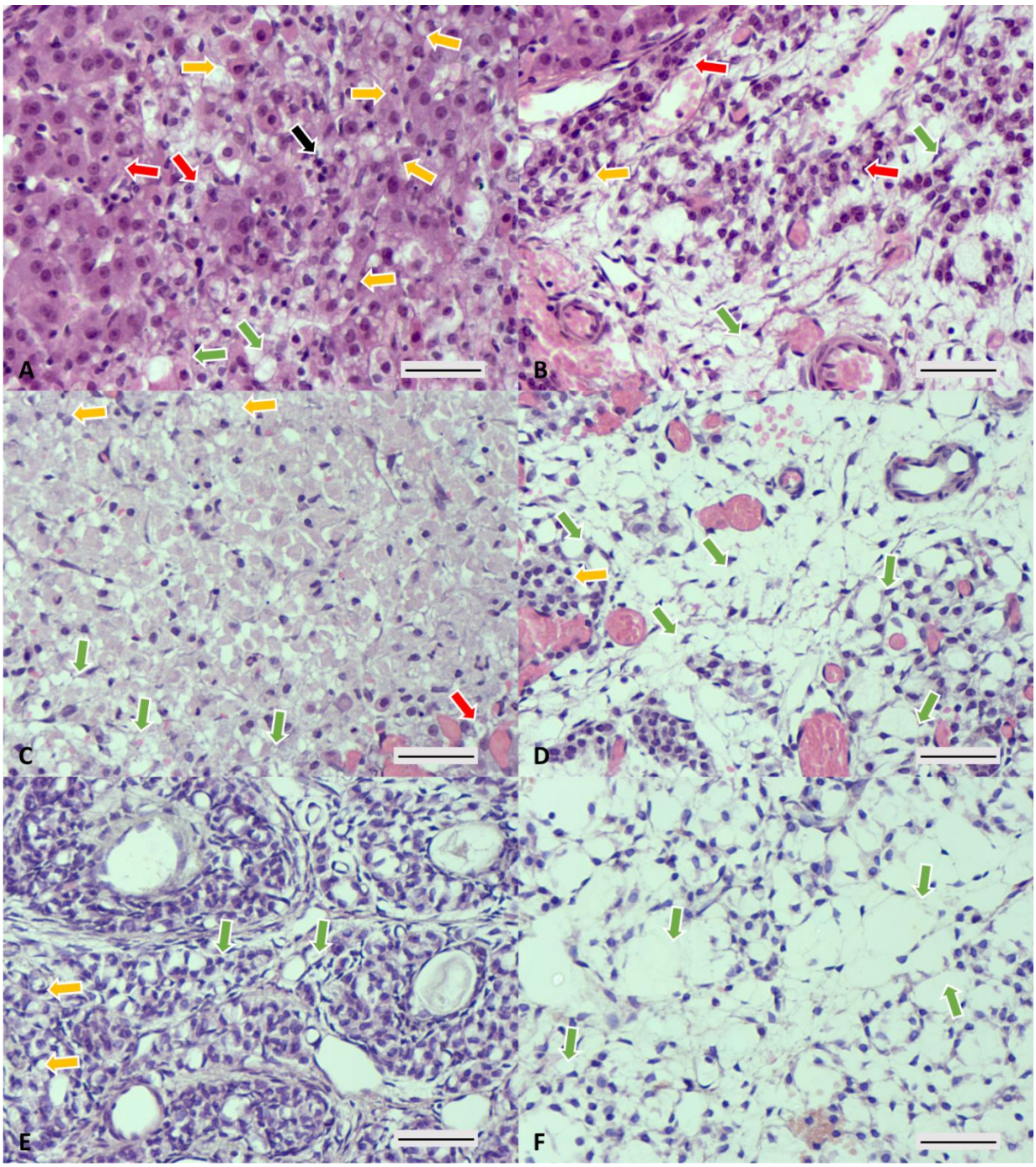

Figure 4.17: Overview of morphological changes. A) Corpus luteum of a HSF1 dam observable micro- and macrovesicular vacuolation, pyknotic nuclei and apoptotic body formation. B) Stroma of a HSF1 dam with observable micro- and macrovesicular vacuolation, and pyknotic nuclei. C) Necrotic lesion within the corpus luteum of a HSF2 dam. Additional micro- and macrovesicular vacuolation, and pyknotic nuclei can be observed. D) Stroma of a HSF2 dam presenting with macrovesicular vacuolation and wide spread macrovesicular vacuolation. Additionally, numerous congested blood vessels can be observed. E) Stroma of a HSF/HSF pup with observable micro- and macrovesicular vacuolation. Additionally, two well defined endothelial circumscribed luteal cysts can be observed (top right and middle right of the image). F) Extreme macrovesicular vacuolation, distorting normal cell morphology. Yellow arrows = Microvesicular vacuolation, Green arrows = Macrovesicular vacuolation, Red arrows = Pyknotic nuclei and Black arrows = Apoptotic bodies. High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2) and High-sucrose feed / High-sucrose feed (HSF/HSF). Haematoxylin and eosin preparations at 200x magnification (Scale bar = 20µm).

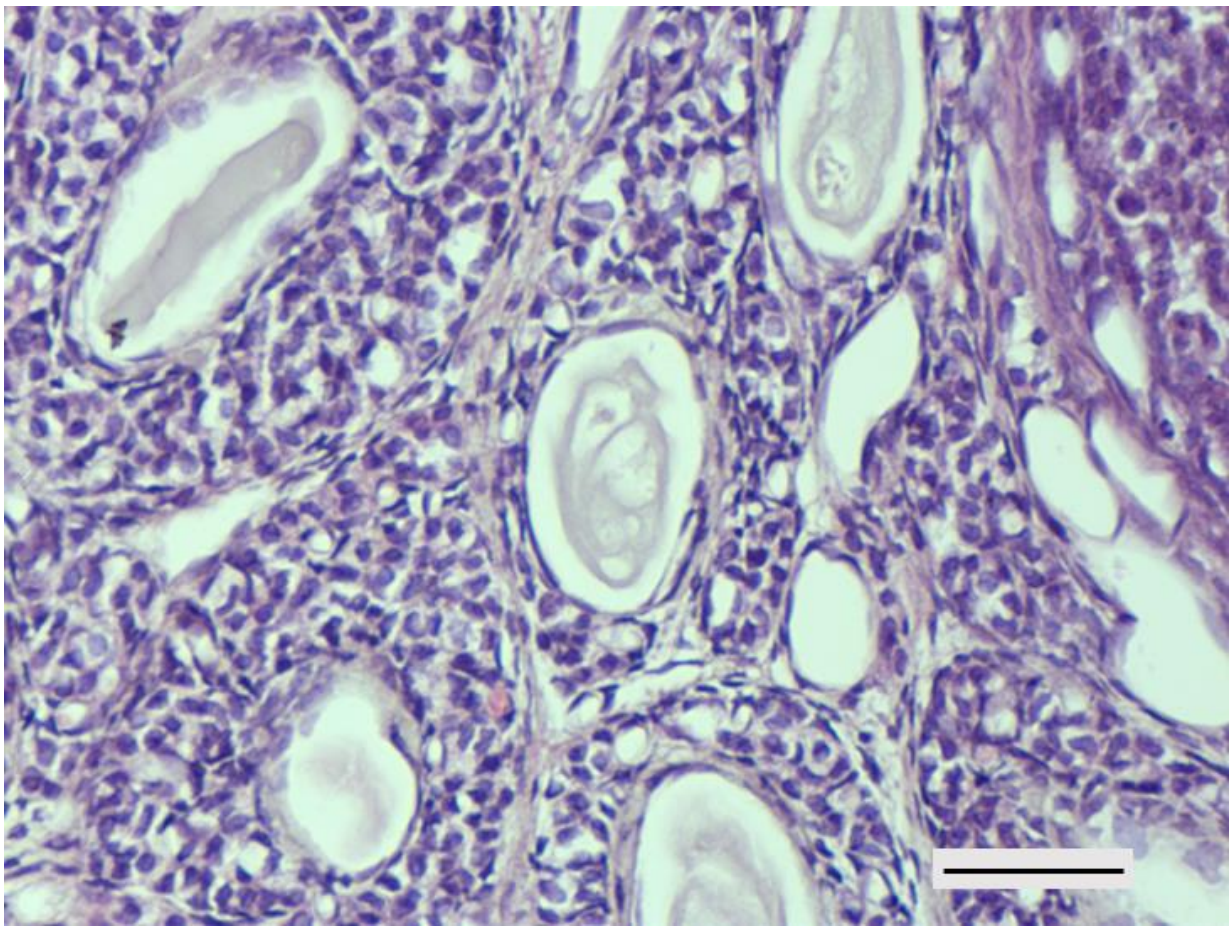

Figure 4.18: Abnormal cysts. Well defined, endothelial circumscribed cysts, surrounded by granulosa cells with an unknown proteinaceous content found in ovaries from the HSF/HSF group. (H&E preparation at 200x magnification) (Scale bar = 20µm).

Chapter 5: Discussion

5.1 Effects of a high sucrose diet on metabolic status

Results from the present study has shown that rats on a high-sucrose diet, had a reduced mean body mass (MBM), while maintaining a normal fasting blood glucose level (FBGL) when compared to animals maintained on a corn-starch control diet. This is contrary to the increase in body mass and FBGL which is considered a normal physiologic response to a high sugar diet (Romeo, Lee and Shoelson, 2012). Adaptations in hepatic fructose metabolism (Bizeau and Pagliassotti, 2005) and sexual dimorphism (Galipeau, Verma and McNeill, 2002) normally seen at the onset of metabolic syndrome are potentially responsible for these abnormal results. The reason is that in the postprandial state the liver consumes approximately 30% of glucose intake, however this is altered depending on carbohydrate source (Bizeau and Pagliassotti, 2005). In the present study sucrose constituted 68% of the high sugar diet and is a molecule consisting of a single glucose and fructose monomer. Glucose is absorbed and used as an insulin mediated energy source for most cells in the body, however up to 70% of all fructose is metabolised by the liver (Mayes, 1993). Increased levels of hepatic fructose has been found to increase the glucose requirements of the organ as well as increase the rate of fatty change in the liver, due to increased levels of energy storage (Bizeau and Pagliassotti, 2005). These changes in the liver could potentially lead to the development of a non-alcoholic fatty liver disease state, which would result in an immediate MetS diagnosis. This diagnosis will be made regardless of all other MetS risk factors (Alessandro *et al.*, 2012). Additionally, diets with increased fructose components could potentially result in a reduction in nutrient absorption, however the mechanisms by which this occurs remains unknown (Alessandro *et al.*, 2012). Therefore, in the present study the decrease in nutritional absorptive capacity in the liver may account for decreased BM in the HSF maintained groups. In addition, sexual dimorphism in the development of metabolic disturbances may be primarily or partly responsible for the unconventional results obtained in this study (Galipeau, Verma and McNeill, 2002).

In a study conducted on female Wistar rats using the same diet as the present study, no significant differences in MBM or FBGL were detected between the control or high sucrose fed groups (Horton *et al.*, 1997). The same high sucrose diet caused significant increases in weight and FBGLs after two weeks of feeding in males (Horton *et al.*, 1997). The present study agrees with findings of FBGLs, however differs when body mass is considered, with the HSF groups presenting with significantly lowered MBM.

Differences in MBM between Horton *et al.*, (1997) and the present study may be as result of the indicated study using a 7-week old rats, in contrast to the present study which initiated experimental feeding at 21-day old rats. This difference in findings may indicate that sucrose feeding has a significant effect on pre-pubertal developmental phases in rats. This hypothesis is supported by the HSF/HSF pup group weighing approximately a third of the HSF groups

and a quarter of the CF group. The HSF/HSF group was weaned by an HSF dam as well as maintained on a high sucrose diet and euthanised at the same chronological age as the dams.

A common hypothesis widely accepted in literature is that the increased levels of oestrogen and lack of androgens in female rats act as a protective factor against the development of metabolic syndrome (D'Eon *et al.*, 2005; Xu *et al.*, 2011; Stubbins *et al.*, 2012). This was demonstrated by Busserolles *et al.* (1990) where control, ovariectomised and oestrodial-treated ovariectomised rats were fed a high sucrose diet, and their oxidative stress status measured. All experimental groups presented with raised TG and NO levels like those of males. Lipid peroxidation on homogenised tissues indicated a significant increase in thiobarbituric acid-reactive substances (TBARS) in ovariectomised animals. Thiobarbituric acid-reactive substances are used to identify levels of oxidative stress and correlates with the development of non-alcoholic fatty liver disease. This was found to be reversed in the oestrodial treated ovariectomised group. Although oestrogen levels were not directly measured in the present study, it can be said that there was some degree of protection afforded to maternal animals on HSF, as they did not display an overt metabolic disorder. Additionally Busserolles *et al.*, (1990) found that the anti-oxidant super oxide dismutase was found to be increased in all groups except the ovariectomised group. Therefore, it is hypothesised that oestrogen acts as a protective agent against the OS and its adverse effects, not as an anti-oxidant, but rather increasing anti-oxidant potential.

The protective of effects of oestrogen cannot be confirmed in the present study due to the significant differences in metabolic status and morphological changes to the ovary. However, it must be noted that feeding was initiated at 21 days old, prior to the onset of puberty and thus changes could have been caused prior to the increase in oestrogen during puberty as was seen by Hilakivi-Clarke *et al.*, (1998) using a high fat diet in a mouse model. Increased severity of changes in metabolic status and ovarian morphology observed in the HSF/HSF pup group may also be as result of pre-pubertal exposure to the high sucrose feed and potentially comprised milk during weaning. Potential foetal programming may also be responsible for these results and will be discussed below (Mulder *et al.*, 2002).

5.2 Metabolic status and mating outcomes

Mating outcomes are determined by multiple factors which include the metabolic status and genetics of the parental generation (Aiken and Ozanne, 2014; Aiken, Tarry-Adkins and Ozanne, 2016). The metabolic status of the female during pregnancy has been known to be responsible for providing an adequate intrauterine environment (Aiken and Ozanne, 2014). It is well documented that, a compromise in the intrauterine environment can have immediate and / or predisposing effects on the foetus leading to the development of adulthood metabolic disturbances (Martin-Gronert and Ozanne, 2012). In the present study no significant

differences in litter sizes were identified, however the HSF2 group tended to produce fewer pups than the CF groups. Additionally, it was noted that all pups born to HSF dams were significantly lighter than that of pups birthed to CF dams at the time of weaning. Differences in the number of pups and the mass of the pups may been as result of a dysfunctional hypothalamic-gonadotropic hormone release and poor intrauterine environment respectively as was observed in rat studies by Fontana and Della Torre, (2016) and Aiken, Tarry-Adkins and Ozanne, (2013) respectively.

Underweight individuals have been shown to have disrupted reproductive cycles and altered ovarian steroidogenesis, resulting in significant conception difficulties (Leyendecker and Wildt, 1984; Devlin *et al.*, 1989; Xu *et al.*, 2011). Results of the present study did not record any significant differences in time of conception for HSF groups in comparison to other groups. However, a single dam from the HSF2 group was found to be a sterile mating. Vaginal smears determined that the animal was amenorrhoeic. If cohorts were enlarged and standard reproductive cycle monitoring conducted, this parameter may have become a significant finding in the present study.

Significantly fewer females were birthed to HSF dams than to CF dams. This disparity is potentially an indication of excess of nutrition as hypothesised by Trivers and Willard (1989). The hypothesis of Trivers and Willard (1989) suggests that when maternal nutrition is compromised, female offspring are naturally selected, but when excess nutrition is available, male offspring are favoured (Trivers and Willard, 1989). Mechanisms by which this occur, remain uncertain, with male placental sensitivity being identified as a potential key factor (Mao *et al.*, 2010).

Munetsuna et al (2018) found that using a diet consisting of 18% fructose caused dysregulation in oestradiol regulation in offspring, as indicated by the attenuation of oestrogen receptor alpha. However, in the indicated study as well as the present study, it is not possible to adequately identify whether the deleterious phenotypic effects seen in pups have been as result of direct effects of diet on the foetus during gestation and weaning or as result of an epigenetic modification. The latter hypothesis would result in the offspring predisposed to developing metabolic disturbances which was seen in the present study.

The HSF/CF group of the present study served as a crossover control diet. Pups in this group were birthed and weaned by a dam maintained on high sucrose feed. Body mass results at the time of death of this group were shown to be significantly higher than that of the HSF/HSF pups, yet still significantly lower than the CF/CF pups. These differences promote the hypothesis that changes identified in the HSF/HSF pup group are not purely result of their direct feeding after weaning. Mean body mass in the HSF/CF groups displayed some degree of reversal but failed to match that of the CF/CF pups. Therefore, it can be extrapolated that

potential semi-permanent changes were made to the metabolic and hormone homeostasis of the pups in utero or during weaning (Aiken, Tarry-Adkins and Ozanne, 2016; Munetsuna *et al.*, 2018).

5.3 Effects of metabolic status on the ovary

Ovarian mass was significantly decreased in groups maintained on high sucrose feed with exception of the HSF2 group in the present study. In most cases ovarian mass corresponded with a decrease in overall body mass except for in the HSF/HSF pup, in which follicle distribution and morphology was indicative of atrophy. Animals in this group displayed significantly increased numbers of Type 1 follicles, few corpora lutea and highly vacuolised stroma which is indicative of atrophy as result of immaturity as was described in rats (Dixon *et al.*, 2014).

Ovarian follicle development is a complex and integrative process governed by multiple factors which include: intraovarian factors, hypothalamic gonadotropic control and metabolic status (McGee and Hsueh, 2000; Webb *et al.*, 2004; Fontana and Della Torre, 2016). In the present study the only significant differences were found in Type 1 follicles as stated above, and significantly less Type 2 follicles were found in the HSF2 groups. This reduction of preantral follicles is potentially a result of dysregulation in the intrauterine follicle development (Manova *et al.*, 1993; McGee and Hsueh, 2000), as follicles at this stage of development are predominantly unresponsive to the control of gonadotrophic hormones. Similar non-significant decreases in Type 2 and Type 3 follicles were observed for the HSF2 group and indicate a decrease in reproductive potential and is confirmed by significantly fewer atretic follicles. Atresia is considered as an adequate indicator of ovarian activity (Hsueh, Billig and Tsafriri, 1994).

An interesting and unexpected finding was differences observed between HSF1 and HSF2 groups. Despite undergoing the same experimental measures, and presenting with identical metabolic profiles (with exception of a non-significant increase in FBGL of the HSF1 group) significant differences were observed in follicle numbers, atresia and cyst formation.

Increased numbers of Type 1, Type 2 and Type 3 follicles in addition to increased levels of atresia indicate that ovaries of the HSF1 group were potentially more active than that of the HSF2 group. In addition, increased numbers of follicular cysts were observed in this group with a non-significant increase in FBGL was observed for this group. It may therefore be that in the present study the developmental and cystic increase is potentially due to the analogous stimulatory effect of insulin in follicular development (Diamanti-Kandarakis and Dunaif, 2012).

Positive SCF staining is predominantly found in the oocyte and theca cells of developing follicles and is hypothesised to assist in the promotion follicles to the antral stage (Manova *et al.*, 1993). Staining of oocytes and theca cells indicated no significant differences in staining

pattern or quantity in the present study. Differences in the number of follicles found contradict the results of the SCF staining, indicating that SCF may not responsible for the changes in follicle number.

In the assessment of diffuse stromal staining it was found that the HSF/HSF pup group was found to have increased staining proportion and intensity in contrast to variable decreased follicle production. Although this finding is contradictory to literature it must be noted that SCF has additional proto-oncogenic functioning (Huang *et al.*, 1990; Manova *et al.*, 1993). This is an increased likelihood when the potential for oxidative stress, due to abnormal hepatic metabolism is considered.

5.4 Morphological changes in the ovary

The microscopic morphology of the ovary is of high importance, as morphological changes are potentially indicative of hormonal and metabolic homeostasis. The corpus luteum and the stroma were selected as regions of interest in this study. Ovaries undergo normal cyclical change that correlates with the reproductive stage. It is therefore important to compare ovarian morphology at similar stages of their reproductive cycle when assessing morphology (Dixon *et al.*, 2014).

No morphological changes above normal cyclical change were observed in any of the groups maintained on control feed. Morphological changes observed in high sucrose feed maintained groups indicated varying abnormal degrees of micro- and macrovesicular vacuolation, apoptosis and necrosis. Excessive micro- and macrovesicular vacuolation has been shown to occur when steroidogenic activity is inhibited and leads to lipid accumulation in the cells (Towns *et al.*, 1999). Additionally, vacuolation has been found to be indicative of phospholipidosis, an indication of potential metabolic dysregulation (Towns *et al.*, 1999; Dixon *et al.*, 2014). Apoptosis and small necrotic lesions are also occasionally seen in the metoestrus phase, however excess apoptotic activity and large necrotic lesions as seen in high sucrose maintained animals in the present study, indicate a possible disruption in steroid homeostasis (Dixon *et al.*, 2014). Potential changes in steroidogenesis may be as result of an end point insult due to oxidative stress, or dysregulation of the HPG axis (Fontana and Della Torre, 2016; Munetsuna *et al.*, 2018).

Metabolic effects on the HPG axis have been hypothesised to be due to glucotoxicity in the hypothalamus and or the effects of adipose tissue metabolites and insulin on gonadotropic hormones and endpoint steroidogenesis (Roa *et al.*, 2006; Castellano *et al.*, 2009; Roa, Navarro and Tena-Sempere, 2011). The present study indicated no evidence of excess blood glucose or hyperinsulinemia and thus do not consider any of the effects to be as result of glucotoxicity in the hypothalamus, however, any oxidative process in the hypothalamus cannot be discredited.

All animals maintained on high sucrose feed were found to be significantly lighter than control fed groups. In addition, dissection findings also indicated a reduction in fat and muscle deposition. Although fat quantity was reduced, it was not possible to assess the metabolic profile of the adipose tissue and assess if it was sufficient to cause hyperleptinemia and effect the gonadotropin hormone levels (Kendall *et al.*, 2004; Stubbins *et al.*, 2012; Comninos, Jayasena and Dhillo, 2014). It must also be noted that potential fatty change occurring in the liver in MetS may also effect the secretion of sex hormone binding globulin (SHBG), leading to increased plasma testosterone levels (Torre *et al.*, 2014; Fontana and Della Torre, 2016). Increased testosterone levels are highly correlated with the development polycystic ovarian syndrome (PCOS) (Vom Saal, Even and Quadagno, 1991; Steckler *et al.*, 2005). In contrast significant production of follicular cysts were observed in the present study. However, significant increases in tunica albuginea thickness, like those found in the HSF dam groups are consistent with PCOS (Stener-Victorin *et al.*, 2005).

5.5 Transgenerational effects of a high sucrose diet

Foetal programming and its effects are increasingly studied using various maternal diets (Martin-Gronert and Ozanne, 2012; Perrone *et al.*, 2016; Khanal and Nielsen, 2017). However, few studies have evaluated the potential foetal programming as a result of the isolated effects of maternal sucrose feeding (Kendig *et al.*, 2015). The present study makes no attempt at identifying the origin, if by uterine environment and or epigenetic modification, but rather suggests that maternal high sucrose feeding causes a potential predisposition for metabolic instability.

Significant differences in the metabolic status of the HSF/HSF pups were identified. However, in this group it is not possible to delineate the effects of foetal programming and the postnatal feeding. The HSF/CF pup group, which was born and weaned of a HSF dam, and then fed a post-natal control diet failed to recover a normalised metabolic profile at adulthood. Therefore it can be concluded that some form of foetal programming has occurred in the offspring of animals birthed to HSF dams (Aiken and Ozanne, 2014). In a similar study conducted using only male offspring, sucrose feeding was found to alter glucose homeostasis and hepatic lipid metabolism. This led to the development of insulin resistance and metabolic disorders in adulthood regardless of postnatal feed (Alessandro *et al.*, 2012).

In the present study no morphological or follicle number differences were identified despite the change in metabolic profile. This is in contrast to the morphological changes found in the males of the previously mentioned study. The lack of morphological change could potentially be as result of the ameliorating effects of oestrogen with regards to oxidative stress (Busserolles *et al.*, 1990; Riant *et al.*, 2009; Stubbins *et al.*, 2012).

Chapter 6: Conclusion

In conclusion, the consumption of high sucrose diet has been shown to affect the metabolic profile of female Wistar rats even though the results were non-significant. Metabolic changes were marked, however, not severe enough to suggest the development of insulin resistance. These results are in keeping with theories of metabolic robustness of female rats with regards to the maintenance of glucose homeostasis and protection against oxidative stress. Mean body mass values were found to be decreased in rats fed on a high sucrose feed, and is hypothesised to be due to the early onset of experimental feeding.

Sucrose feeding was found to decrease overall ovarian mass, as well as fat deposits surrounding the uterus and ovaries as determined by subjective evaluation. High sucrose feeding had varying effects on numbers of follicles within sucrose fed groups. Potential differences may be linked to the effects of insulin on the ovary. Stem cell factor staining showed no differences in intra-ovarian follicle stimulation, indicating that differences observed in follicle numbers were as result of extra-ovarian factors. Apoptosis, necrosis, congestion and vacuolation observed in ovarian morphology of stroma and corpora lutea were indicative of alterations in gonadotropin hormone release. Changes in the metabolic profile are hypothesised to be responsible for the potential changes in hypothalamic pituitary gonadal axis and gonadotropic hormones.

Additionally, it was found that animals born of HSF dams were found to have a low mean body mass, regardless of postnatal feed. Animals converted to control feed after weaning displayed some normalisation in metabolic profile, however remained significantly different to CF dam birthed and control fed pup. These results allowed the author to say with certainty that some degree of foetal programming has taken place in the present study within the HSF/HSF and HSF/CF groups.

Thus, answering the research questions, a high sucrose feed causes marked changes in the morphology of the ovary, which are compounded in the F1 generation. This study demonstrates that metabolic changes can influence the reproductive potential of parental and F1 generations, despite the lack of overt metabolic dysregulation.

Limitations and future studies

Specimens obtained for the present study were harvested from animals used in an unrelated PhD study in the Division of Clinical Anatomy, in an effort to maximise the information that could be attained from the feeding model. However, parameters of the main study limited potential additional investigations. Limitations were ameliorated to the best of the author's ability.

Monitoring of reproductive cycles of all animals were constrained by the ethical consideration of the overarching protocol of the study. In this case ovarian morphology was evaluated taking into consideration potential morphological differences. Additionally, molecular and advanced immunohistochemical investigations were limited due to financial restraints. These would have provided further insight into potential reasoning behind the phenotypic changes seen in this study

Future studies could investigate potential cyclical changes due to high sucrose feeding, as was highlighted by the changes in ovarian morphology. Additionally, the effect of pre-and post-natal high sucrose on oestrogen production in maternal animals should be assessed to further delineate the protective effects of oestrogen in female rats.

Duration of the feeding model was governed by the overarching study. This was not a significant limitation in the present study, however it may be of value to study the progressive effects over extended time periods. This is of particular interest when the foetal programming effects of high sucrose feed are considered.

In summary, this study could have benefited from reproductive cycle monitoring and additional molecular testing. This would give additional insights into delineating the direct effects of metabolic status on ovarian morphology and the indirect effects via dysregulation of the hypothalamic-pituitary-gonad axis.

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Appendices

Appendix A: Diet compositions

D10001, D11707, and D11708

Formulated by:
Research Diets, Inc.

Product #	D10001		D11707R		D11708	
	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*
Protein	20	*21*	20	*21*	20	*21*
Carbohydrate	66	*68*	66	*68*	66	*68*
Fat	5	*12*	5	*12*	5	*12*
Total		*100*		*100*		*100*
kcal/gm	3.9		3.9		3.9	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*	200	*800*
DL-Methionine	3	*12*	3	*12*	3	*12*
Corn Starch	150	*600*	0	*0*	650	*2600*
Sucrose	500	*2000*	0	*0*	0	*0*
Fructose	0	*0*	650	*2600*	0	*0*
Cellulose, BW200	50	*0*	50	*0*	50	*0*
Corn Oil	50	*450*	50	*450*	50	*450*
Mineral Mix S10001	35	*0*	35	*0*	35	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*
FD&C Blue Dye #1	0	*0*	0	*0*	0.2	*0*
FD&C Red Dye #40	0	*0*	0.2	*0*	0	*0*
FD&C Yellow Dye #5	0	*0*	0	*0*	0	*0*
Total	**1000**	***3902***	**1000.2**	***3902***	**1000.2**	***3902***

Research Diets, Inc.
20 Jules Lane
New Brunswick, NJ 08901 USA
info@researchdiets.com

D11707 and D11708.for.xls

RESEARCH DIETS INC.
www.ResearchDiets.com

Appendix B: Animal fasting times

Table 6.1: Fasting time per weight for fasted blood glucose level assessment

Animal weight (Grams)	Fasting time (hours)
0-49	4
50-99	6
100-149	8
150+	12

Appendix C: Tissue processing schedule

Table 6.2: Processing schedule for ovaries

Reagent	Temperature (°C)	Vacuum	Time (min)
Ethanol (70%)	45	No	30
Ethanol (80%)	45	No	30
Ethanol (95%)	45	No	30
Ethanol (100%)	45	No	30
Ethanol (100%)	45	No	60
Ethanol (100%)	45	No	90
Xylene	Ambient	No	45
Xylene	Ambient	No	45
Xylene	45	No	90
Paraffin wax	65	Yes	60
Paraffin wax	65	Yes	60
Paraffin wax	65	Yes	60

Appendix D: Haematoxylin and eosin staining protocol

Method:

1. Load slides into auto-stainer.
2. Run program as presented in Table 6.3.
3. Subsequently, mount slides using a resinous mounting agent.

Table 6.3: Automated staining protocol

Staining step	Reagent	Time
1	Xylene	10 min
2	Xylene	10 min
3	99% Ethanol	2 min
4	96% Ethanol	2 min
5	70% Ethanol	2 min
6	Distilled water	5 sec
7	Meyers Haematoxylin	8 min
8	Running water	5 min
9	Eosin	45 sec
10	96% Ethanol	10 dips
11	96% Ethanol	10 dips
12	70% Ethanol	10 dips
13	70% Ethanol	10 dips
14	Xylene	5 min

Appendix E: Stem cell factor

Method:

1. Titrate SCF antibody *(ab64677, Abcam®, United Kingdom)* to 1:500 concentration using antibody diluent. Load titrated antibody and Bond™ Polymer Refine detection kit *(Leica Biosystems, Wetzlar, Germany) into the* Bond Max™ platform.
2. Run the protocol as stated in Table 6.4.
3. Subsequently, remove slides, dehydrate and mount according to routine practice.

Table 6.4: Automated staining protocol

Staining step	Reagent	Time
1	Peroxide Block	5 min
2	ER 2 (antigen retrieval)	20 min
2	Bond wash	0
3	Primary antibody	40 min
4	Bond wash	0
5	Secondary antibody	8 min
6	Bond wash	0
7	Polymer	8 min
8	DAB	10 min
9	Deionized water	0
10	Haematoxylin	2 min
11	Deionized water	0
12	Bond wash	0

Appendix F: Pederson and Peters follicle classification

Table 6.5: Follicle classification according to Pederson and Peters (1968)

Group	Type	Number of granulosa cells	Follicle antrum development
Small follicles	Type 1	0	-
	Type 2	Incomplete ring surrounding oocyte	-
	Type 3a	Complete ring surrounding oocyte (≤ 20)	-
Medium follicles	Type 3b	21 – 60	-
	Type 4	61 – 100	-
	Type 5a	101 – 200	-
Large follicles	Type 5b	201 – 400	-
	Type 6	401 – 600	Initial growth of antrum
	Type 7	> 600	Large antrum but lacks cumulus oophorus
	Type 8	> 600	Large antrum with cumulus stalk present

Appendix G: Random numbers table

TABLE 1 - RANDOM DIGITS

11164	36318	75061	37674	26320	75100	10431	20418	19228	91792
21215	91791	76831	58678	87054	31687	93205	43685	19732	08468
10438	44482	66558	37649	08882	90870	12462	41810	01806	02977
36792	26236	33266	66583	60881	97395	20461	36742	02852	50564
73944	04773	12032	51414	82384	38370	00249	80709	72605	67497
49563	12872	14063	93104	78483	72717	68714	18048	25005	04151
64208	48237	41701	73117	33242	42314	83049	21933	92813	04763
51486	72875	38605	29341	80749	80151	33835	52602	79147	08868
99756	26360	64516	17971	48478	09610	04638	17141	09227	10606
71325	55217	13015	72907	00431	45117	33827	92873	02953	85474
65285	97198	12138	53010	94601	15838	16805	61004	43516	17020
17264	57327	38224	29301	31381	38109	34976	65692	98566	29550
95639	99754	31199	92558	68368	04985	51092	37780	40261	14479
61555	76404	86210	11808	12841	45147	97438	60022	12645	62000
78137	98768	04689	87130	79225	08153	84967	64539	79493	74917
62490	99215	84987	28759	19177	14733	24550	28067	68894	38490
24216	63444	21283	07044	92729	37284	13211	37485	10415	36457
16975	95428	33226	55903	31605	43817	22250	03918	46999	98501
59138	39542	71168	57609	91510	77904	74244	50940	31553	62562
29478	59652	50414	31966	87912	87154	12944	49862	96566	48825
96155	95009	27429	72918	08457	78134	48407	26061	58754	05326
29621	66583	62966	12468	20245	14015	04014	35713	03980	03024
12639	75291	71020	17265	41598	64074	64629	63293	53307	48766
14544	37134	54714	02401	63228	26831	19386	15457	17999	18306
83403	88827	09834	11333	68431	31706	26652	04711	34593	22561
67642	05204	30697	44806	96989	68403	85621	45556	35434	09532
64041	99011	14610	40273	09482	62864	01573	82274	81446	32477
17048	94523	97444	59904	16936	39384	97551	09620	63932	03091
93039	89416	52795	10631	09728	68202	20963	02477	55494	39563
82244	34392	96607	17220	51984	10753	76272	50985	97593	34320
96990	55244	70693	25255	40029	23289	48819	07159	60172	81697
09119	74803	97303	88701	51380	73143	98251	78635	27556	20712
57666	41204	47589	78364	38266	94393	70713	53388	79865	92069
46492	61594	26729	58272	81754	14648	77210	12923	53712	87771
08433	19172	08320	20839	13715	10597	17234	39355	74816	03363
10011	75004	86054	41190	10061	19660	03500	68412	57812	57929
92420	65431	16530	05547	10683	88102	30176	84750	10115	69220
35542	55865	07304	47010	43233	57022	52161	82976	47981	46588
86595	26247	18552	29491	33712	32285	64844	69395	41387	87195
72115	34985	58036	99137	47482	06204	24138	24272	16196	04393
07428	58863	96023	88936	51343	70958	96768	74317	27176	29600
35379	27922	28906	55013	26937	48174	04197	36074	65315	12537
10982	22807	10920	26299	23593	64629	57801	10437	43965	15344
90127	33341	77806	12446	15444	49244	47277	11346	15884	28131
63002	12990	23510	68774	48983	20481	59815	67248	17076	78910
40779	86382	48454	65269	91239	45989	45389	54847	77919	41105
43216	12608	18167	84631	94058	82458	15139	76856	86019	47928
96167	64375	74108	93643	09204	98855	59051	56492	11933	64958
70975	62693	35684	72607	23026	37004	32989	24843	01128	74658
85812	61875	23570	75754	29090	40264	80399	47254	40135	69916

Appendix H: Means and Standard deviations

Table 6.6: Mean body mass comparison (±Std. Dev.)

	CF	HSF 1	HSF 2	CF/CF	HSF/HSF	HSF/CF
N	9	9	10	6	6	4
MASS (G)	226.7 ± 16.4[a]	168.3 ± 16.2[b]	168.7 ± 38.2[b]	190.7 ± 20.2[b]	58.8 ± 21.0[d]	121.0 ± 5.5[c]

Differing letters indicate significant differences. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF).

Table 6.7: Mean fasting blood glucose levels (±Std. Dev.)

	CF	HSF 1	HSF 2	CF/CF	HSF/HSF	HSF/CF
N	9	9	6	6	6	4
FBGL (Mmol/L)	4.7±0.3[ab]	5.1±0.6[a]	4.8±1.1[abc]	3.7±0.4[c]	3.7±0.6[bc]	4.1±0.6[abc]

Differing letters indicate significant differences. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF). Fasting blood glucose level (FBGL).

Table 6.8: Mean litter size (±Std. Dev.)

	CF	HSF 1	HSF 2
N	9	9	6
MALE	3 ±1	3 ±1	3 ±2
FEMALE	5 ±2[a]	4 ±2[ab]	3 ±2[b]
TOTAL	9 ±3	7 ±2	6 ±3*

Differing letters indicate significant differences. * Indicates trends observed with an LSD<0.05. Control feed (CF), High-sucrose feed 1 (HSF1) and High sucrose feed 2 (HSF2).

Table 6.9 Mean pup weights (±Std. Dev.)

	CF	HSF 1	HSF 2
N	9	9	6
MALE	36.9±10.4[a]	23.9±9.8[b]	11.4±5.9[c]
FEMALE	36.5±10.5[a]	23.2±9.4[b]	15.7±9.8[b]

Differing letters indicate significant differences. Control feed (CF), High-sucrose feed 1 (HSF1) and High-sucrose feed 2 (HSF2).

Table 6.10: Mean ovarian mass (±Std. Dev.)

	CF	HSF 1	HSF 2	CF/CF	HSF/HSF	HSF/CF
N	9	9	6	6	6	4
OVARY 1	0.046 ±0.004	0.0274 ±0.006	0.064 ±0.017	0.067 ±0.015	0.025 ±0.010	0.067 ±0.011
OVARY 1	0.046 ±0.004	0.0274 ±0.006	0.064 ±0.017	0.067 ±0.015	0.025 ±0.010	0.067 ±0.011
OVARY 2	0.061 ±0.012	0.040 ±0.010	0.074 ±0.020	0.089 ±0.026	0.039 ±0.022	0.095 ±0.025
MEAN	0.054 ±0.007[b]	0.034 ±0.008[c]	0.069 ±0.017[a]	0.078 ±0.020[a]	0.033 ±0.011[c]	0.081 ±0.018[a]

Differing letters indicate significant differences. Control feed (CF), High-sucrose feed 1 (HSF1) and High-sucrose feed 2 (HSF2).

Table 6.11: Mean of follicle distribution (±Std. Dev.)

FOLLICLE TYPE	CF	HSF 1	HSF 2	CF/CF	HSF/HSF	HSF/CF

N	9	9	6	6	6	4
TYPE 1	57.4±29.1	114.6±47.8	80.0±35.0	79.8±78.4	181.8±110.3	61.8±17.6
TYPE 2	24.7±6.9^{ab}	22.2±7.2^{aab}	13.9±4.7^{c}	29.2±13.5^{a}	16.5±8^{bc}	18.8±10.7^{abc}
TYPE 3	15.0±3.6	20.1±4.8*	14.0±7.3	18.8±5.2	13.5±6.1	15.5±4.8
TYPE 4	5.2±2.5	3.2±1.3	4.7±3.5	2.8±1.9	3.3±2.1	2.5±1.3

Differing letters indicate significant differences. * Indicates trends observed with a LSD<0.05. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF).

Table 6.12 Means number of atretic and cystic follicles (±Std. Dev.)

	CF	HSF 1	HSF 2	CF/CF	HSF/HSF	HSF/CF
N	9	9	6	6	6	4
ATRETIC	7,2±3,2^{a}	10,9±5,6^{a}	4,3±3,4^{a}	12,7±7,2^{b}	8,0±4,7^{a}	6,5±2,9^{a}
CYSTS	2,8±3,8	9,0±12,2	3,2±3,3	3,3±2,1	2,5±3,1	8,0±10,9

Differing letters indicate significant differences. * Indicates trends observed with a LSD<0.05. Control feed (CF), High-sucrose feed 1 (HSF1), High-sucrose feed 2 (HSF2), Control feed / Control feed (CF/CF), High-sucrose feed / High-sucrose feed (HSF/HSF) and High-sucrose feed / Control feed (HSF/CF).