The effects of diet and letrozole treatment on reproduction in the female spiny mouse (*Acomys cahirinus*)

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BSc. Science, Hons. Physiology.

A thesis submitted for the degree of Master of Reproductive Sciences at Monash University

2022

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Wordcount: 19,139

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Abstract

Background: The spiny mouse (SpM) is the only rodent, reported to have a menstrual cycle, rather than an oestrous cycle as previously reported (Peitz 1981). This discovery, in our Monash colony of SpM has led to subsequent research showing that the SpM is a new and useful model for research into aspects of women's health. Previous studies using SpM have shown that these animals fed a high fat, high carbohydrate (HFHC) diet resulting in an overall weight between 60-80g leading to reproductive dysfunction. In women, obesity and polycystic ovarian syndrome (PCOS) appear to be strongly linked: women who suffer from PCOS tend to be obese and obese women tend to have PCOS. Therefore, I conducted two studies to further explore this concept.

Objectives:

Study 1: To investigate how different diets impact on metabolic and reproductive function in the female SpM.

Study 2: To examine the effects of treatment with letrozole, on ovarian function and the menstrual cycle in SpM, and to induce PCOS.

Study design: All animals were virgin female aged 40 ±7 days.

Study 1: Three groups of SpM were established and fed the following diets, control (n=12), two high energy diets; Peitz diet (n=8) and HFHC diet (n=9) for 12 months. Vaginal lavages, to monitor the effect on the menstrual cycle, and glucose-tolerance testing procedures were performed at 2-month intervals during the 12-month study.

Study 2: Four groups of SpM aged 60 ±5 days were established, control (n=9) (placebo) or a subcutaneous 60-day slow-release implant of letrozole; 2mg (n=8), 4mg (n= 5) and 8mg(n=6). Vaginal

lavages were used to monitor and compare the effect of these treatments on the menstrual cycle on D60 following the surgery.

Results: Study 1 – No significant differences were observed in bodyweight between controls and diet treated groups after the 52-weeks, however, significance was found between Peitz SpM (36.617 ± 2.026g) and HFHC SpM (41.144 ± 2.606g; $p<0.0031$). No significance differences in fasted blood glucose levels, selected organ weights nor in the activity of the menstrual cycle after 52 weeks.

Study 2 – No significance differences in bodyweight, selected organ weights nor the menstrual cycle activity in the three letrozole treatment groups compare with controls. However, the number of large follicles in the ovaries of control SpM (0.889 ± 0.601) were significantly different ($p<0.0456$) from those of the 8mg letrozole group (1.833 ± 0.753). Dark bodies, that were identified as data revealed significant differences between the control SpM and 4mg as well as between the 2mg and 4mg SpM. Control SpM and 2mg had lower counts of dark bodies compared with the 4mg group.

Conclusion: SpM appear to have an adaptive physiology that protects them from gaining excess weight when placed on high energy diets. PCOS symptoms were unable to be produced using letrozole, however as SpM have a naturally occurring menstrual cycle, their usefulness as an animal model to investigate reproductive disorders, cannot be overlooked.

Keywords: SpM, diet, HFHC, menstruation, PCOS

Declarations

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

Print Name: Vanessa Di Bartolomeo

Date: 28/03/2022

Acknowledgements

Completing my Masters in the middle of the Covid-19 global pandemic has taught me to be more resilient and patient, as well as the importance to be surrounded by a good supportive network.

I would firstly like to acknowledge the outstanding amount of support I received from my ***main supervisor Peter.*** Thank you for always making time for me, even when you had a million other things to do, you always put in effort and were willing to help in any way that you could. Thank you for always providing kind constructive criticism and for always being positive, when I would be stressed out, I would always feel better after our meetings and had a renewed drive to keep pushing forward. I am so lucky to have had the opportunity to work with you, it has been an absolute pleasure; words cannot express how grateful I am for your help and support throughout my academic journey.

To my ***co-supervisors Karin and Beverley,*** thank you both for your continued support throughout my Masters. It has been an absolute delight working with you both, your positiveness and support from my initial milestones to now, has been very much appreciated. To the ***EPRD team***, everyone's excitement when we could have our in-person journal clubs, really sparked my enthusiasm for the research that we do and provided a supportive environment to troubleshoot problems, so thank you to you all! To ***Min and Emily,*** thank you for sharing your unpublished data with me and allowing me to include it in my thesis – your willingness to collaborate with me and your shared interest in how our studies make up a bigger picture, has been a very positive experience.

To ***my Mum and siblings, Anthony, and Marcie,*** thank you for your continued support and understanding when I have been too busy. Thank you for being positive, when I have had doubts and for giving me strength to carry on. Your unwavering support during this time has meant more to me than words can express, I love you all and am so grateful for you. To ***my Nonna Rosa,*** our daily phone calls in Italian give me such life and positivity. You truly put a smile on my face every time we speak, your love and support during this time has meant the world to me. To ***my Dad,*** it truly hurts me that you have not been able to be here on this journey, but I know in my heart, you are with me every step

of the way. You first sparked my interest in research during our car rides going to work together and since then, that spark has ignited and the work I have done as part of my Masters is the product, thank you for always believing in me.

To ***my friends,*** thank you for always understanding when I have been too busy and for always checking in. Your love and support through this journey has been greatly appreciated and I am so grateful to you all. Finally, to ***my fiancé Greg,*** your love and support during this time has been immense, I truly do not know where I would be if it weren't for you. Thank you for giving up your time and space for me when I have needed it, and for being so willing to do so, that I never had to ask, nor would you ever complain. Your genuine wish to make life easier for me, in any way that you can is so selfless, you are so generous in your love, understanding and support and I am truly lucky to have you in my life. Thank you for always lifting me up on my low days, and for being a constant source of comfort and positivity. I love you so much and I am eternally grateful for you; I cannot wait to spend a lifetime with you by my side.

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Abbreviations

2mg	2mg letrozole treated SpM
4mg	4mg letrozole treated SpM
8mg	8mg letrozole treated SpM
ANOVA	analysis of variance
DHEA	dehydroepiandrosterone
DI	diestrus
DHT	dihydrotestosterone
FSH	follicle-stimulating hormone
GnRH	gonadotrophin releasing hormone
HFHC	high fat high carbohydrate diet
LET	letrozole
LH	luteinising hormone
MENS	menses
MET	metestrus
mmol/L	millimoles of glucose per litre of blood
m	month/months
*NB	note well
EST	oestrous
PCOS	poly cystic ovary/ovarian syndrome
Peitz	SpM treated with diet based on Peitz (1981) (1)
PRO	proestrus
SpM	spiny mouse/spiny mice
SD	standard deviation
SEM	standard error of mean
VL	vaginal lavage

Chapter 1: Introduction and Literature Review

Background

Reproduction is an important evolutionary strategy to ensure offspring are produced and genes are passed on, to continue a species' existence (2). Reproduction occurs by external and internal fertilisation. External fertilisation occurs outside of the female, typically in an aquatic environment when male and female gametes are released into the water. Internal fertilisation occurs when the sperm fertilises the oocyte within the female animal. Methods of internal fertilisation include oviparity (egg outside of the female body), ovoviparity (egg held within the female) and viviparity (development within the female followed by a live birth) (3-6).

Mammals are endothermic chordates, that possess mammary glands that produce milk to nourish their young. Mammalian reproduction consists of diverse reproductive strategies within the three different types of mammals: monotremes, marsupials and, placental (3-7). Monotremes, produce a single egg in a pouch, that hatches after a few days and the young remains in the pouch which produces milk through pores for the offspring. Marsupials give birth to altricial young, which they carry in a pouch where they develop further. Eutherian (placental) mammals produce live young at varying developmental stages; altricial young require more postnatal care, whereas precocious young become independent of their parents relatively earlier. Most mammals have an oestrous cycle, whereas primates and a few other different species have a menstrual cycle (3-6, 8, 9). This chapter will explore mammalian reproduction and the reproductive cycle of the spiny mouse (*Acomys cahirinus*) was the chosen animal model described in this thesis, because of its suggested menstrual cycle.

Introduction

Female mammalian reproductive - oestrous and menstrual cycles

All female mammalian species experience a reproductive cycle that is physiologically controlled by hormones. For most of these species, female reproduction is based around an oestrous cycle (10, 11), but in a few species (higher order primates, elephant shrews and some bat species) (6, 8, 9) female reproduction involves a menstrual cycle. Differences in the features that distinguish these two cycles are stark (***Figure 1.1*** (12)). In mammals with an oestrous cycle the endometrium is reabsorbed at the end of an infertile cycle and replaced to commence a new cycle (2, 13, 14). Whereas in a menstrual cycle the superficial functional layer of the endometrium is not reabsorbed, it is shed in the absence of implantation, this is known as the menses phase of the menstrual cycle (6, 8, 12, 14-18)

In most mammals that have an oestrous cycle, differentiation of the endometrium (decidualisation) occurs in response to implantation. This initiates the site where foetal tissue invades the endometrium, and a series of reactions occur such as an influx of natural killer cells to the uterus and differentiation of endometrial stromal cells into decidual cells (6, 14, 19). These events are critical to establish the functional connection between maternal and foetal cells and, for the pregnancy to develop and thrive. In menstruating mammals decidualisation is spontaneous; it occurs irrespective of implantation. The main catalyst for spontaneous decidualisation in menstruating species, is progesterone rather than signals from the foetus as occurs in species with an oestrous cycle. Spontaneous decidualisation only occurs in menstrual species and is initiated during the luteal phase following ovulation and production of progesterone from the developing corpus luteum (6, 14, 19).

Oestradiol-17B is a vital hormone during the ovarian (follicular phase) and uterine cycles (proliferative phase), and especially the peak leading up to ovulation. During the luteal phase of the ovarian cycle, and the secretory phase of the uterine cycle, progesterone is essential in preparing the endometrium for implantation and for maintaining pregnancy (8, 11, 12, 15, 20).

The menstrual cycle and ovulation are regulated by the release of gonadotrophins and steroid hormones from the hypothalamic-pituitary-ovarian (HPO) axis. Gonadotropin releasing hormone (GnRH), synthesised, and released by the hypothalamus causes the pituitary gland to release luteinising and follicle stimulating hormone, (LH and FSH) to stimulate normal function (follicle development, oocyte maturation and ovulation) of the ovaries (12, 15, 21). During the proliferative phase, LH and FSH stimulate the theca interna and granulosa cells, respectively, within the leading pre-ovulatory follicles to synthesise and release oestradiol (22, 23). LH also supports oocyte maturation and ovulation and increasing levels of oestradiol stimulates proliferation of the endometrium. Granulosa cells that are not released with the oocyte after ovulation become luteinised and form an enlarged corpus luteum, a progesterone-secreting structure, which then supports early pregnancy if fertilisation and implantation occurs. If fertilisation does not occur, the corpus luteum begins to regress and degenerate to form a corpus albicans (12, 15, 21, 23).

Typically, rodents have an oestrous cycle and the spiny mouse has been previously described as having a typical oestrous cycle (1). However, a recent study made the surprising discovery that female spiny mice in our Monash University colony menstruate (12, 15, 16). The Egyptian, or common, spiny mouse (*Acomys cahirinus)*, a native to Africa and the Middle East, is the first rodent that shows spontaneous decidualisation and consequently menstruation, with a cycle length of only 9 ± 3 days (15, 17). SpM have been reported to show spontaneous decidualisation and terminal differentiation of the endometrial functionalis as well as an active luteal phase with progesterone support, a peak in prolactin secretion during the luteal phase. In the absence of pregnancy passive luteolysis and endometrial sloughing and bleeding occurs at the end of an infertile cycle (12, 16, 17, 19, 24, 25). The differences in reproductive cycles, specifically the events and timing between a laboratory rodent, human and SpM are described in ***Figure 1.1*** (12).

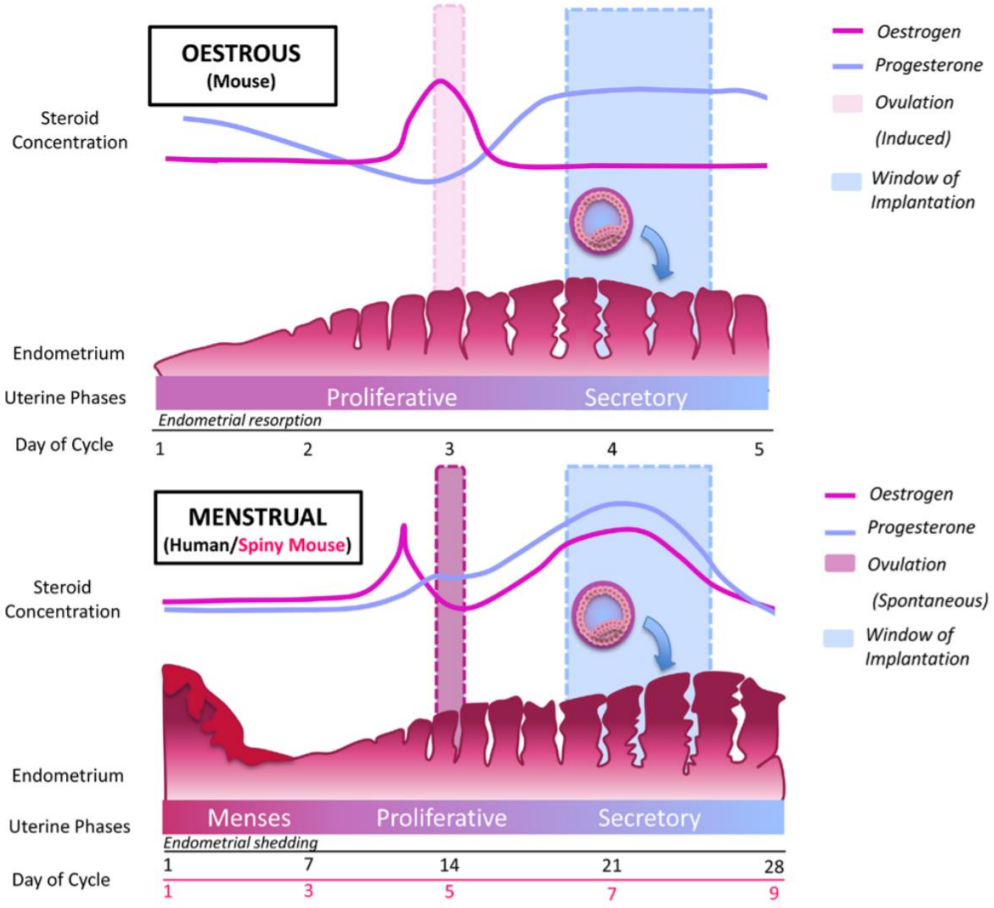


Figure 1.1 *Comparing a rodent (mouse) oestrous cycle with a menstrual cycle in humans and spiny mice.*

Common laboratory rodents can experience two types of cycles; in the absence of pregnancy a shorter oestrous cycle of 4-5 days occurs, the corpora lutea are not functional during this time. During pseudopregnancy, a longer oestrus cycle takes place during which the corpora lutea are actively secreting progesterone and consequently stimulating the endometrium (26, 27). As represented in ***Figure 1.1*** (12) a typical laboratory rodent has an oestrous cycle lasting 5 days which is typically triggered by seasonal cues. For example, mating with a male causes a peak in oestrogen at day 3, followed by a window of implantation on approximately day 4 of the cycle. On day 5, if a pregnancy has not occurred, the endometrium is reabsorbed, and a new cycle will commence. By contrast, in SpM ovulation is not triggered by seasonal cues, ovulation occurs spontaneously on day 5, as indicated

by a peak in oestrogen. If no pregnancy occurs following this, the endometrium is shed, resulting in menses and the start of a new cycle. The SpM menstrual cycle has similar elements to humans; it has been reported that the SpM menstrual cycle has the following stages: proestrus, oestrous, metestrus, diestrus, and menses. Although the SpM have the same stages present in their menstrual cycle, proliferative and secretory stages, the timing of these stages and the cycle length differs from the human menstrual cycle; the menstrual cycle is longer in humans, typically a 28-day cycle and SpM have a 9-day cycle. Spontaneous ovulation occurs in humans at day 14 of the cycle, and in the absence of pregnancy, the endometrium is shed, resulting in menses and the start of a new cycle. In SpM ovulation occurs at approximately day 5, and like humans, in SpM in the absence of pregnancy, the endometrium is shed and a new cycle occurs; the first day after menstruation is the first day of the new cycle (12, 15-17, 19, 24, 25, 28).

As the only small laboratory mammal that menstruates, the spiny mouse has great translational potential for human reproductive health studies, as its menstrual cycle is similar to humans.

The spiny mouse presents a new and innovative model for research into female reproduction and women's health, especially for developing new disease-related models. Previous rodent models have been used to explore various aspects of women's health, however in many of these previous studies the models relied on an artificial menstrual event created by sequential injections of hormones (20, 29, 30). Because menses occurs naturally in spiny mice, hormonal manipulation is not required, suggesting that results from these studies will be more representative, robust and relevant to human studies (12, 15, 17).

This natural phenomenon of menses in the spiny mouse has been exploited as the foundation for the studies presented in this thesis. Study 1 aimed to investigate the effect of different diets on SpM body weight, glucose metabolism and the menstrual cycle. Study 2 aimed to explore the effect of letrozole on the menstrual cycle, as well as the structure and function of the ovary. Letrozole has been shown

to induce PCOS-like symptoms in rodent models (31-35). My second study therefore investigated whether letrozole treatment had a similar effect in the SpM to gain translational information from a PCOS model in a small laboratory animal that has a menstrual cycle, similar to humans (12, 15, 17). Furthermore, this thesis aimed to add further knowledge about the biology of this interesting but poorly studied species (15, 16).

Literature review

Nutrition and reproductive health

Nutrition is defined by the food consumed and how the body processes it to grow and sustain life; it is the combination of different processes regulating the intake of nutrients and using them in the maintenance of body tissues as well as an energy source. Health is a holistic optimal state of physical, mental, and social well-being with a general absence of disease. Nutrition is the foundation for life and overall health (36, 37), and good nutrition is essential for overall optimal health and reproductive health (36, 37).

Obesity in women: implications for reproductive health

Levels of obesity are on the rise, leading to increased health complications and cost to health care systems. Factors contributing to obesity include educational and socioeconomic status, health behaviours such as diet and exercise. There are numerous causes of obesity in western society however, the most common is diet related; on average women consume 35% of fat in their diet although, it is recommended to consume between 20% and 30%. A chronic, high intake of fat leads to increased adiposity which causes metabolic dysfunction, negatively impacting upon fertility (37-39). Obese women are more likely to experience reproductive complications such as menstrual dysfunction, anovulation, infertility, miscarriage, and pregnancy complications. There is currently an obesity epidemic in western society with high proportions of older women living with obesity (37-39). Thus, the need to understand the complications arising from excess weight is critical.

Women with obesity have increased levels of circulating insulin, which stimulates the production of androgens from the ovaries (37, 40). Functional alterations in the hypothalamic-pituitary-ovarian (HPO) axis negatively impact upon reproductive potential: for example, excess androgens produced by the ovaries are aromatised into oestrogens causing an excess of oestrogens that negatively feedbacks to the HPO axis, disrupting the gonadotrophin production. The culmination of this is menstrual and ovulatory dysfunction, resulting in abnormalities that drive the pathogenesis of various

reproductive disorders. Excess insulin is a predominant feature leading to the development of polycystic ovarian syndrome; a common reproductive disorder comprising the following main symptoms: multiple ovarian cysts, hyperandrogenism and chronic anovulation (37, 41, 42). Obesity contributes to the development of insulin resistance, which worsens the overall symptoms of PCOS. Obese women tend to present with a more extreme PCOS phenotype, compared with lean women. The pathogenic deposition of visceral fat due to hyperandrogenism, leads to hyperinsulinemia and insulin resistance, which further stimulates ovarian production of androgens; this is a continuous cycle in PCOS, which progressively worsens the condition overtime. In the general population it is estimated between 10-20% of women with a normal BMI (18.5-24.9) suffer from PCOS. The incidence of PCOS in obese women (BMI ≥30) is higher, approximately 30%, an exact causative relationship between obesity and the development of PCOS has not yet been established (37, 41-43).

Effect of diet in rodent physiology

The SpM in the 1981 study published by Peitz (1) weighed 60-80g, double the weight of female SpM in our colony (30-40g). This observation led me to investigate why there was a dramatic difference in body weight between two colonies of the same species, and to explore the effect of diet in rodent studies.

High caloric diets are known to cause obesity in conventional laboratory rodents (44): diets with a percentage of 15-20% fat have been shown to increase body weight significantly, and cause metabolic disturbances such as insulin resistance, elevated blood glucose and hepatic stenosis (45, 46). SpM placed on high fat diets for 12 months have been shown to weigh 76.3 ± 1.8g and to experience elevated glucose levels (47). Obese SpM tend to develop metabolic dysfunction such as pancreas abnormalities, insulin resistance and glucose intolerance (47, 48). Based on previous literature documenting the effect of high caloric intake in rodents, I aimed to investigate the effect of diet in SpM by using a diet based on the Peitz 1981 study (1) and a scientifically formulated high fat and high

carbohydrate diet in the SpM, to mimic the typical western diet driving the obesity epidemic in humans.

Poly cystic Ovarian Syndrome (PCOS)

Polycystic ovarian syndrome (PCOS) is a reproductive disorder currently characterised by two or more of the following symptoms: polycystic ovaries, androgen excess and oligomenorrhea. The specific diagnosis depends on which criteria are applied (***Table 1.1*** (49)). The pathophysiology of PCOS also involves a degree of metabolic dysfunction, typically increased adiposity, abnormal adipocytes, insulin resistance, glucose intolerance and an increased risk of developing type II diabetes (37, 40). Worldwide, approximately, 10% of females are afflicted with this condition, with limited treatment strategies available to them. PCOS is a complex condition that has many metabolic abnormalities, typically referred to as metabolic syndrome. It is estimated that women who suffer from PCOS are twice as likely to have metabolic syndrome and one and a half more times more likely to be classed as obese as other women (40, 49, 50). PCOS is also associated with an increased incidence of type II diabetes, non-alcoholic fatty liver disease, hyperinsulinemia, and dyslipidaemia (40, 49, 50). Compared with the general population of women, PCOS sufferers also have a higher prevalence of cardiovascular disease such as, hypertension, coronary artery disease and myocardial infarctions (42, 49, 51).

Table 1.1 Criteria for Diagnosis of PCOS.

Criteria for Diagnosis of PCOS

CLINICAL FINDING	*NATIONAL INSTITUTES OF HEALTH CRITERIA, 1990 (MUST HAVE BOTH OF THE FINDINGS MARKED BELOW)*	*ROTTERDAM CRITERIA, 2003 (MUST HAVE ANY TWO OF THE FINDINGS MARKED BELOW)*	*ANDROGEN EXCESS AND PCOS SOCIETY, 2009 (MUST HAVE A PLUS EITHER B OR C)*
Hyperandrogenism*	X	X	A
Oligomenorrhea	X	X	B
Polycystic ovaries		X	C

Current treatments for PCOS include metformin, hormonal contraception, and lifestyle modifications such as diet and exercise. The treatment is specific to the individual sufferer, however as this condition impacts a range of organs the treatment is often limited (40, 49, 50). The few treatment options for these women is largely attributed to the lack of an appropriate animal model in which to better understand the processes involved in the development of PCOS and to trial specific treatments. The discovery of a human-like menstrual cycle in the SpM, presents a unique opportunity to investigate the possibility of establishing a small animal model for various reproductive health problems in a species with a naturally occurring menstrual cycle similar to humans (12, 15, 17, 19). As such, results obtained from studies in SpM could have high translational value for human studies and provide opportunities to develop new treatment strategies reproductive disorders such as PCOS.

Animal models of PCOS

Different interventions have been used over the years to induce PCOS in animals such as chronic treatment with dehydroepiandrosterone (DHEA) or dihydrotestosterone (DHT) (52, 53). Hyperandrogenism is a predominant symptom of PCOS and chronic exposure of androgens in animal models has therefore been a common practice to induce PCOS. Chronic exposure (daily injections) of DHEA, in either mice or rats for approximately 30 days results in animals becoming acyclic and anovulatory (53, 54). Typically, this treatment leads to reproductive abnormalities such as, the development of cystic ovaries, increased serum levels of testosterone; estrogen; follicular stimulating hormone (FSH) and luteinising hormone (LH). Increased fasting glucose levels have also been reported after chronic treatment with DHEA (40, 54). Chronic exposure to DHT has also been used to induce PCOS symptoms. Typically, after 13 weeks abnormalities in the ovarian cycle and polycystic ovaries are present in rodents treated with DHT compared with the controls. In this particular model, estrogen and testosterone are not elevated, which does not correspond with the PCOS phenotype. The metabolic disturbances from chronic exposure to DHT include decreased sensitivity to insulin, increased body weight and increased leptin levels (35). These particular models are unable to produce

all of the reproductive and metabolic dysfunctions associated with PCOS, however they are able to produce a combination of the symptoms, allowing certain aspects of the condition to be investigated. Currently, chronic exposure of letrozole in rodents has been successful in producing most of the reproductive and metabolic symptoms of PCOS (52, 55). Letrozole is a non-steroidal aromatase inhibitor; aromatase converts androgens to estrogens, and disruption in this activity leads to an accumulation of androgens (hyperandrogenism) which is a significant clinical symptom of PCOS and a requirement in the diagnosis of PCOS (***Table 1.1,*** (34, 35, 40). Prolonged exposure to letrozole in rodents, typically 5 weeks or more, produces reproductive and metabolic disruptions, resulting in the development of PCOS (31, 33, 34). Typically, rodents treated with letrozole have polycystic ovaries, elevated levels of testosterone and LH, while FSH serum levels are decreased (31, 33-35, 52, 56, 57). Rodent models treated with letrozole have reduced uterine and increased ovarian weights and have disrupted oestrous cycles with more time spent in diestrus than the other phases of the cycle (33, 34, 52).

Letrozole treated rodents tend to have increased body weights, generally have a higher percentage of fat and abnormal adipocyte deposition and morphology (29, 31, 32). Consequently, letrozole treated rodents develop glucose intolerance, insulin resistance and increased inflammatory markers, all of which are present in PCOS (34, 35, 40). Letrozole treated rodents become infertile: they are unable to produce a litter when mated, however this is not a permeant side-effect from the chronic letrozole exposure. This infertility is reversible once the letrozole treatment has ceased over a period of approximately 5-10 weeks (34). In summary letrozole exposure appears to be a novel and successful way to induce PCOS symptoms in animal models as it causes not only reproductive disturbances seen in PCOS but metabolic complications too, unlike previous interventions (52, 53). This review suggested that exposing SpM to letrozole, known to cause both reproductive and metabolic disturbances in other rodent models, would provide a unique and unexplored opportunity to model PCOS and may lead to a better understanding of the cause of PCOS and, eventually, to improved treatment strategies.

SpM offers a unique avenue for scientific research in obesity and female fertility, because of its natural menstrual cycle. The research hypothesis for Study 1 was that exposure to a high energy diet will cause diet induced obesity (DIO) and demonstrate that obesity in SpM causes reproductive dysfunction which disrupts menstruation and may explain the doubling of body weights observed in SpM in the Peitz 1981 study (1). I also used the SpM model in Study 2 to investigate the effect of letrozole on reproduction in female SpM to determine if this letrozole exposure induces PCOS. These two studies were intended to examine in more depth the relationship between diet and obesity and to provide new information that may lead to the development of new translational treatments for female reproductive health disorders, such as PCOS.

In Chapter 2 and Chapter 3, I describe the two studies I conducted detailing the hypotheses, aims, results and conclusions. I also describe a specialised blood sampling method for glucose testing specifically developed for SpM (see Chapter 2) that I developed in collaboration with my research team because the use of the conventional tail vein methods used in other laboratory rodents could not be used in SpM (16).

Chapter 2: Investigating the effect of diet on reproduction in female spiny mice

Introduction

The spiny mouse has been identified as demonstrating many similar characteristics in their menstrual cycle with other menstruating primates and presents an exciting new opportunity to investigate the impact of obesity upon fertility (15). An earlier study in 1981 by Peitz using SpM described an oestrous cycle for SpM in her colony, not menstruation, even though her SpM were the same species as in the Monash University SpM colony (1) and reported that body weights of her females as twice as large (60-80g) as our females (30-40g). We hypothesised that the animals in the Peitz study were obese, and that this may have disrupted the normal cycle resulting in the possible misreporting or misidentification of the female reproductive cycle. Alternatively, it was possible that the Peitz SpM were in fact a different species of spiny mouse to ours, which may have accounted for the differences observed in the female reproductive cycle between Peitz's colony and ours.

Hypothesis and aims

The overarching hypothesis tested in this study was: that a high energy diet, similar to Peitz (1) or a commercially produced high fat/high carbohydrate diet (HFHC) will increase body weight and interrupt the normal menstrual cycle of female SpM in our colony.

The aims were:

1) to investigate the impact of two high energy diets on the Monash University female SpM colony
2) to examine why an oestrous cycle, rather than a menstrual cycle, was identified and describe the Peitz colony (1)
3) to establish a new model for diet and reproductive health

Rational and Study design

As discussed in Chapter 1, there is a relationship between Western style high energy diets, obesity and reproductive disorders, such as PCOS and these diets are driving an obesity epidemic in humans. The experiments in this study were designed to test the effects of two diets in female SpM against females fed our standard colony control diet. Study 1 was a 12 month longitudinal study, in which I monitored each animal in their respective study groups by weighing SpM to assess the effects of these high energy diets on carbohydrate metabolism and the menstrual cycle using glucose testing and cytological assessments of vaginal lavages respectively.

Materials and Methods

Baseline glucose tests were done prior to starting each group on their diet and at 2-month intervals from baseline resulting in a total of 7 glucose tests over the 12-month duration of the study. We performed the first vaginal lavage at 20 days of animals on the diet, as animals were too young to receive this procedure prior, as vaginal opening occurs at 60 days of age. However, we did not want to confound the data by giving SpM control diets until this time point, and therefore deemed it appropriate to have an adjusted baseline for vaginal lavage procedures. The vaginal lavage procedures occurred on the same day, animals were aged between 60 and 67 days of age with a mean of 63 days old. Glucose testing occurred at the start of the month and vaginal lavage in the middle, to avoid stress in SpM by having too many procedures performed in a short time frame.

Ethics

All experiments were conducted at Monash Medical Centre as a research component in the degree of Masters of Reproductive Sciences. All experiments were approved by the Monash University Animal Ethics Committee under application "MMCB_2018-10 - Does obesity disrupt normal menstruation in the spiny mouse?" prior to commencement. All studies were conducted in accordance with the Australian National Health and Medical Research Council (NHMRC) code of practice for the care and use of animals for scientific purposes.

Animals

Twenty-nine virgin female SpM aged 40 ±7 days were allocated to this study and fed their specific diets for the duration of 12 months. Control SpM (n=12) were fed standard rat and mouse cubes (Speciality Feeds Glen Forrest, WA) supplemented with carrot or celery on a weekly basis. Peitz SpM (n=8) were given a diet based on the 1981 Peitz study (1), which consisted of sunflower seeds, shelled corn, rolled toasted barley and cat chow (Purina Cat Chow Adult Salmon). The high fat high carbohydrate diet group (HFHC, n=9) was fed scientifically formulated high fat and high carbohydrate

pellets, (SF04-001 diet, Speciality Feeds Glen Forrest, WA). The control SpM diet contains 4.6% fat, Peitz diet (Purina chow) contains 14% fat and HFHC SpM diet contains 23.5% fat. All food and water were provided ad libitum and animals were maintained on a 12:12 light/dark cycle.

Body weight measurements

All SpM were weighed to 1 decimal places on a weekly basis for 52 weeks using a Entris II Laboratory Balance (Sartorius).

Glucose testing

Baseline blood glucose levels were recorded at the start of the experiment and monitored every 2 months for 12 months. Animals were fasted overnight (7pm-7am); all food was removed, and animals were placed into clean, individual cages with water overnight and blood samples were taken the following morning. As the spiny mice have extremely fragile tails, it is not possible to take blood samples from the tail vein, as in other laboratory rodents, for measuring glucose levels. To collect blood samples for this purpose, I developed a new ear pinna sampling technique, specifically tailored to SpM. Each female was initially anaesthetised using 4% isoflurane in an induction box and then removed from the induction box and maintained on a nose cone with 1.5% isoflurane on a heating pad for the duration of the sampling procedure and using a cold light to illuminate blood vessels in the pinna. Warm water was applied to the pinna to cause vasodilation if required. When the vessels were sufficiently visible, a suitable vessel was identified, and micro-scissors were used to make a small incision through the upper layer of dermis into the vessel and blood drops that formed were then transferred to a glucometer strip attached to a glucometer (Accu Chek Performa, Roche) to provide a blood glucose reading. Following this, the incision site was gently cleaned with iodine and the animal was taken off anaesthetic, allowed to recover, and then placed back into its individual cage, food and

water were provided and the animal was closely monitored until fully recovered. All SpM were then returned to their original group cages 2 days after the procedure.

Vaginal lavage

The vaginal lavage procedures occurred on the same day, animals were aged between 60 and 67 days of age with a mean of 63 days old, this occurred at 2-month intervals, 7 times during the study. An initial lavage was taken from each female when they had been on their selected diet for 20 days. Lavages were performed at 2-month intervals, to reduce any potential stress from excessive handling from the procedure. This procedure involved gently scruffing and immobilising each female using a hand towel. Once the animal was in a suitable supine position, a transfer pipette was used to flush a small amount of saline into the vaginal canal and recollect the sample for cytological analysis. This was performed every second day for a period of 14 days (15). The sample was placed onto a microscope slide, dried at room temperature, fixed using a cytology fixative spray (Surgipath Medical Industries, Australia), stained using haematoxylin and eosin (H&E) and the sample viewed under a microscope to determine the stage and activity of the menstrual cycle. The 14-day sampling period every 2 months was done to ensure that it covered the reported length of a spiny mouse menstrual cycle. This has been described as 8.7 ± 0.4 days but has also been reported to extend by up to 3 days in older females (9±3 days) (15, 16).

Staging SpM menstrual cycle

SpM vaginal cytology is demonstrated in ***Figure 2.0.*** The early follicular phase (proestrus) was identified by the presence of nucleated epithelial cells as the dominant cell type in the vaginal lavage sample and the late follicular phase (oestrous) by the presence of cornified epithelial cells. During the early luteal phase (metestrus) the dominant cell types were cornified epithelial cells and leukocytes and during the late luteal phase (diestrus) leukocytes were the prominent cell type. The menstrual

phase was identified by the presence of leukocytes and erythrocytes as the dominant cell types, as described by Bellofiore *et al.* (15). Half days are noted when an animal is transitioning between stages***.***

The early follicular phase represents proestrus and late follicular phase represents oestrus. The early luteal phase represents metestrus and late luteal phase represents diestrus.

This vaginal cytology procedure and staging of the SpM menstrual cycle was the same for the studies 1 and 2, an example is provided in ***Table 2.1*** (15)***.***

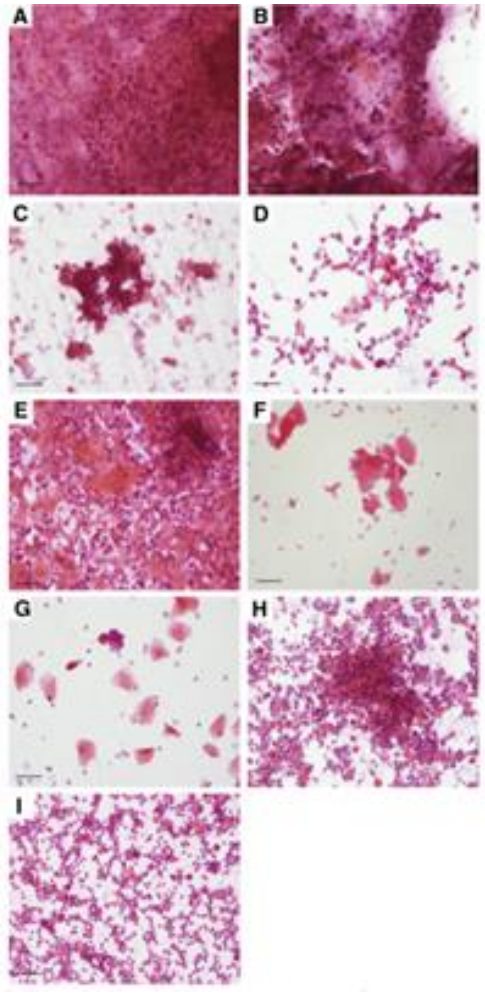


Figure 2.0 Cytology from a female SpM A and B, showing vaginal cytology in a female spiny mouse with 9-day cycle. Early menses at conclusion of previous infertile cycle. C and D, Proestrus, beginning of follicular phase, containing nucleated epithelial cells. E, Transition to estrus. F, Estrus, characterized by cornified epithelial cells. G, Metestrus; transitioning to luteal phase. H and I, Diestrus, luteal phase, containing high leukocytic infiltration. Menses will follow within 24–48 hours. Scale bars = 50 µm. Magnification ×200X. Hematoxylin-eosin stain Bellofiore *et al.* 2017 (15).

Please note: Figure included with permission from the authors

Table 2.1: *Menstrual cycle stage data from vaginal lavage (VL) performed on: SB - cage 945*

Days spent in each stage: PRO = 1.5, EST =0.5, MET = 2, DI =2, MENS = 2.5

PRO – proestrus, EST – oestrous, MET – Metestrus, DI – diestrus, MENS – menses

Animal ID ***SB - cage 945***	**Date**	**Day of cycle**	**Cycling stage**	**Days spent in stage**
VL performed	**10/4/18**		MENS	
	11/4/18			
VL performed	**12/4/18**		MENS	
	13/4/18			
	14/4/18			
VL performed	**15/4/18**	*Cycle starts 1*	PRO	1
	16/4/18	2	OEST *0.5 given as the next sample showed transition*	0.5 PRO; 0.5 OEST
VL performed	**17/4/18**	3	MET	1
	18/4/18	4		1
VL performed	**19/4/18**	5	DI	1
	20/4/18	6		1
VL performed	**21/4/18**	7	MENS	1
	22/4/18	8		1
	23/4/18	*Cycle ends 9* 8.5-day cycle	*0.5 given as the next sample showed transition*	0.5
VL performed	**24/4/18**	*New cycle starts:*	L MENS - PRO	

Tissue collection and weighing

After 12 months, the animals were humanely killed with an overdose of isoflurane and the following tissues were collected: ovaries, reproductive tract, liver, kidneys, and spleen. These organs from each female were weighed fresh using a Entris II Laboratory Balance (Sartorius), fixed in 10% neutral buffered formalin (NBF) and stored in individual specimen jars in NBF for further analysis if required.

Statistical analysis

All data were analysed using GraphPad Prism (9.1.1). All data were tested for normality using Anderson-Darling or Shapiro-Wilk test and are presented as either mean ± SD, specified in each data set. Significance ($p<0.05$) was determined by using ANOVA and Tukey's multiple comparisons, specified in each data set.

Results

Body weights measurements

No significant differences were observed in baseline (initial) body weights for the three groups: control SpM (26.1 ± 1.4g), Peitz (26.5 ± 1.9g) and HFHC groups (25.3 ± 1.4g). There was a significant difference at 10 weeks between the body weights of the control (31.2 ± 1.9g), and Peitz groups (33.6 ±1.7g), and the control and HFHC groups (33.6 ± 1.9g; ***Figure 2.1a*****).** This pattern of significant difference continued until 14 weeks. At 15 weeks all 3 study groups were significantly different from each other (***Figure 2.1a***). Control SpM were (31.4 ± 2.5g), Peitz were (33.9 ± 1.8g) and HFHC were (36.9 ± 2.4g). At 16 weeks control and Peitz groups were not significantly different, whereas control and HFHC, and Peitz and HFHC remained significantly different and continued at this level of significance through to 30 weeks.

At 30 weeks the body weight growth curve of the HFHC group began to flatten out whereas those of the control and Peitz groups continued to trend upwards. At this point body weights of the control (35.5 ± 2.4g) and Peitz group (35.5 ± 1.8g) body weights were similar, whereas significant differences continued between the HFHC group (40.1 ± 2.6 g) and the control ($p<0.005$) and Peitz group ($p<0.0005$).

At the end of the study (52 weeks) body weights of control group (38.2 ± 3.3g) were not significantly different from either the Peitz (36.6 ± 2.0g) or HFHC groups (41.1 ± 2.6g), but the Peitz and HFHC group body weights remained significantly different ($p<0.005$; ***Figure 2.1a*** and **b**).

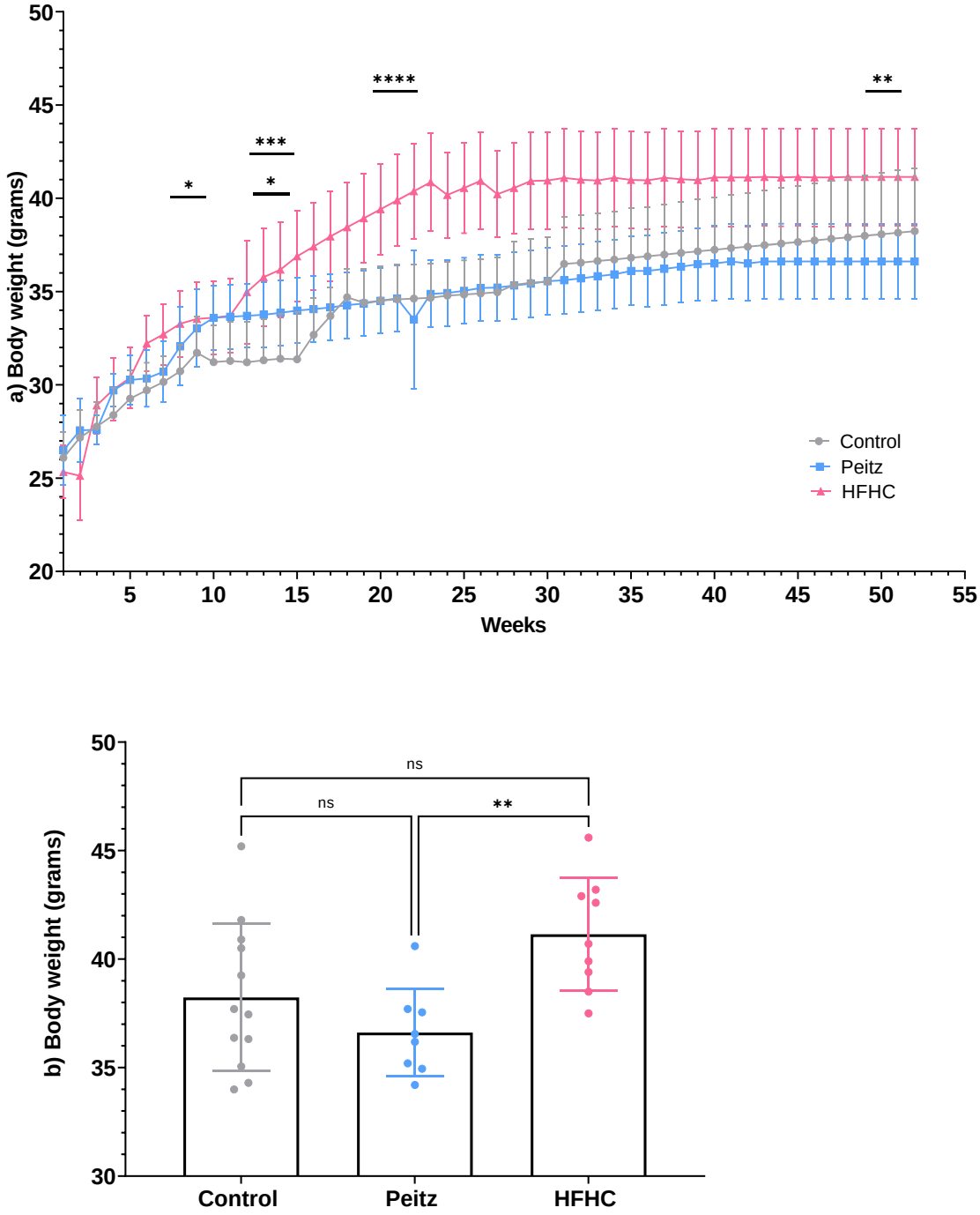


Figure 2.1: Body weights from control SpM vs diet treated SpM. a) *Body weights ± SD in control vs diet treated SpM from 0 - 52 weeks on the diet. Two-way ANOVA with Tukey's multiple comparisons.* ***b)*** *Body weight data from end point (52 weeks). One-way ANOVA with Tukey's multiple comparisons. Significant difference denoted by * ($p<0.05$) or ** ($p<0.005$) or *** ($p<0.0005$) or **** ($p< 0.0001$) and non-significant difference by ns on the figure. Data were presented as mean ± SD for all groups: Control (n=12), Peitz (n= 8) and HFHC (n=9).*

Fasted glucose levels

There were no significant differences between groups at any time point during the study (***Figure 2.2*****a** and ***b*****).** Baseline readings at 0 months on the diet were control 3.8 ± 0.4 mmol/L, Peitz 3.7 ± 0.3 mmol/L and HFHC 3.7 ± 0.3 mmol/L. At the 12-month end point control and Peitz were lower than HFHC, but not significantly, fasted blood glucose levels in control SpM were 4.3± 0.2 mmol/L, Peitz 4.3 ± 0.2 mmol/L and HFHC as 4.4± 0.2 mmol/L seen in ***Figure 2.2 a*** and ***b.***

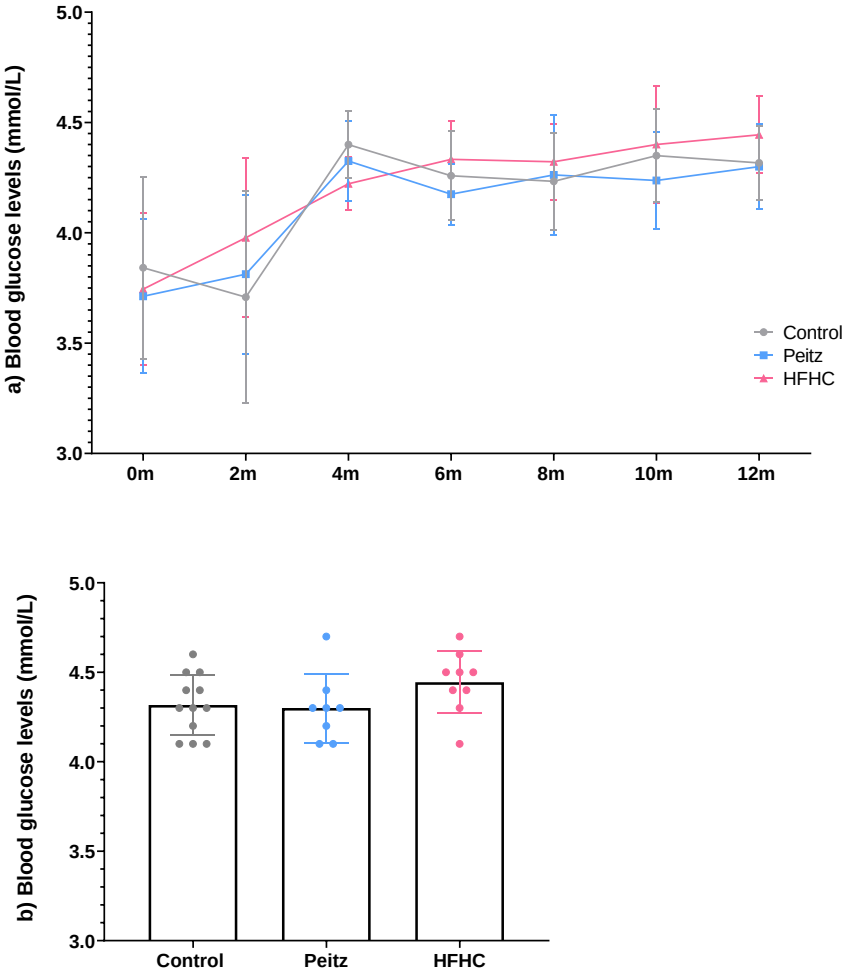


Figure 2.2: Fasted blood glucose from control SpM vs diet treated SpM. a) *timeline from 0-12 months.* ***b)*** *endpoint at 12 months. One-way ANOVA with Tukey's multiple comparisons did not reveal any significant differences between groups. Significance was indicated by a value of p< 0.05. Data were presented as mean ± SD, in Control (n=12), Peitz (n= 8) and HFHC (n=9).*

Reproductive cycling

Stages of the menstrual cycle

All data were obtained from the vaginal lavage procedures, showed no statistical differences in control SpM vs diet treated SpM, between stages in the menstrual cycle or the total length of the menstrual cycle during the study. As the data were similar across all groups during the study, data from the 6-month time point has been provided as a representative as seen in ***Figure 2.3a-f***.

In the early follicular phase (proestrus) no significant differences were observed across study groups. The duration of time spent in the early follicular phase (proestrus) in each group were recorded as, control SpM 2.0 ± 1.0 days, Peitz 2.4 ± 0.9 days and HFHC as 2.2 ± 1.0 days as shown in ***Figure 2.3a***. During the late follicular phase (oestrous), no significant differences were observed, the time spent was recorded in control SpM as 1.5 ± 0.8 days, Peitz as 1.1 ± 0.8 days and HFHC as 1.7 ± 0.7 days as seen in ***Figure 2.3b***.

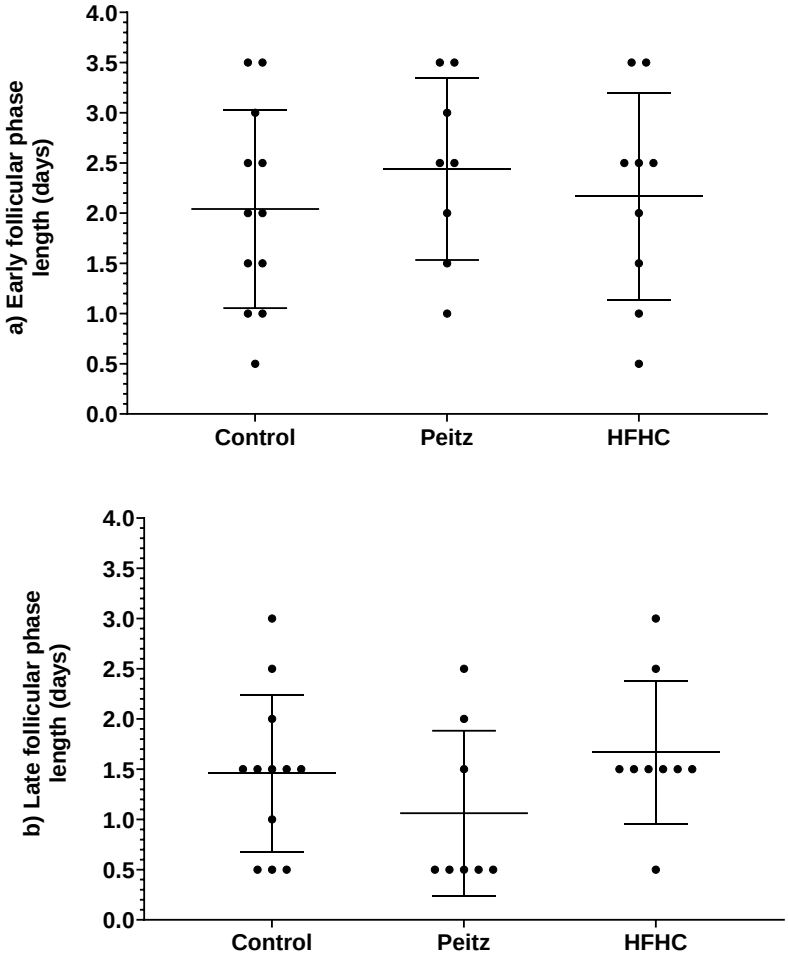


Figure 2.3: Follicular phase in control SpM vs diet treated SpM at 6 months. a) *Early follicular phase.* ***b)*** *Late follicular phase. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all groups Control (n)=12, Peitz (n)= 8 and HFHC (n)=9.*

No significant differences were observed between study groups in the early luteal (metestrus) as shown in ***Figure 2.3c***, during this time control SpM spent 1.8± 1.0 days, Peitz 1.4 ± 0.9 days and HFHC 1.5 ± 0.7 days in the early luteal phase. In the late luteal phase (diestrus), no significant differences were observed across study groups, as shown in ***Figure 2.3d***.

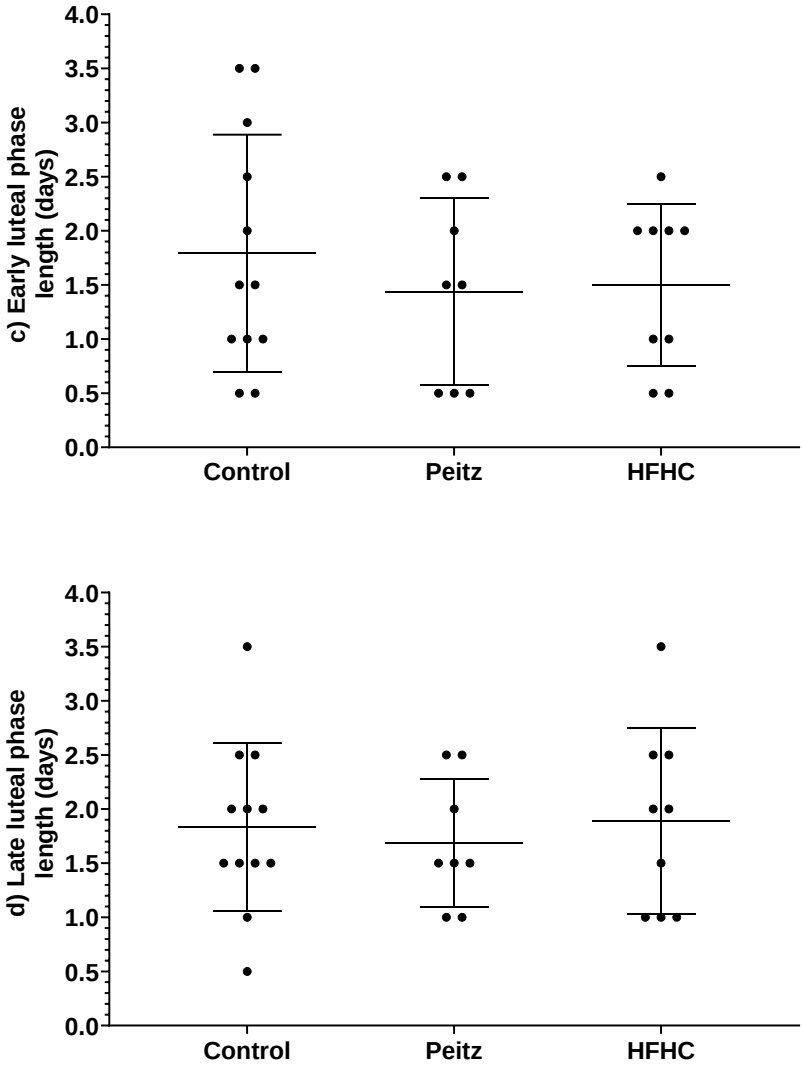


Figure 2.3 Luteal phase in control SpM vs diet treated SpM at 6 months. c) *Early luteal phase.* ***d)*** *Late luteal phase. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all groups Control (n)=12, Peitz (n)= 8 and HFHC (n)=9.*

No significant differences were observed in the menses phase, as shown in ***Figure 2.3e***. No significant differences were observed in the total menstrual cycle length at the 6-month time point, as seen in ***Figure 2.3f,*** control SpM had a menstrual cycle of 8.9 ± 0.2 days, Peitz 8.9 ± 0.2 days and HFHC 8.9 ± 0.2 days.

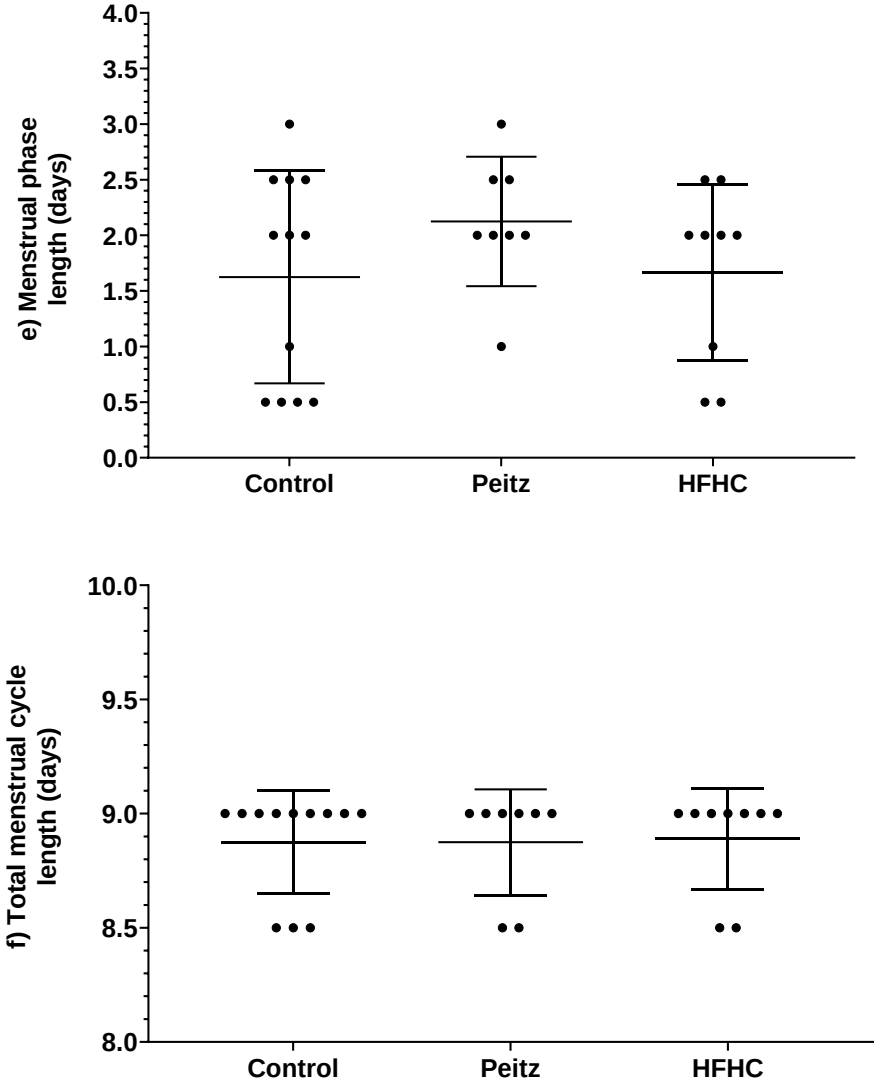


Figure 2.3 Menstrual phase and total menstrual cycle length in control SpM vs diet treated SpM at 6 months. *Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all groups Control (n)=12, Peitz (n)= 8 and HFHC (n)=9.*

Total menstrual cycle lengths

At baseline no significant differences were observed between study groups, control SpM had a menstrual cycle length of length (9.0 ± 0.5 days), Peitz (9.3 ± 0.8 days) and HFHC (9.3 ± 0.9 days) as shown in ***Figure 2.4a.*** At the 2-month time point menstrual cycle length was insignificantly different, with control SpM recorded as (8.9 ± 0.2 days) Peitz (9.1 ±0.8 days) and HFHC (9.0 ± 0.6 days) as seen in ***Figure 2.4b.***

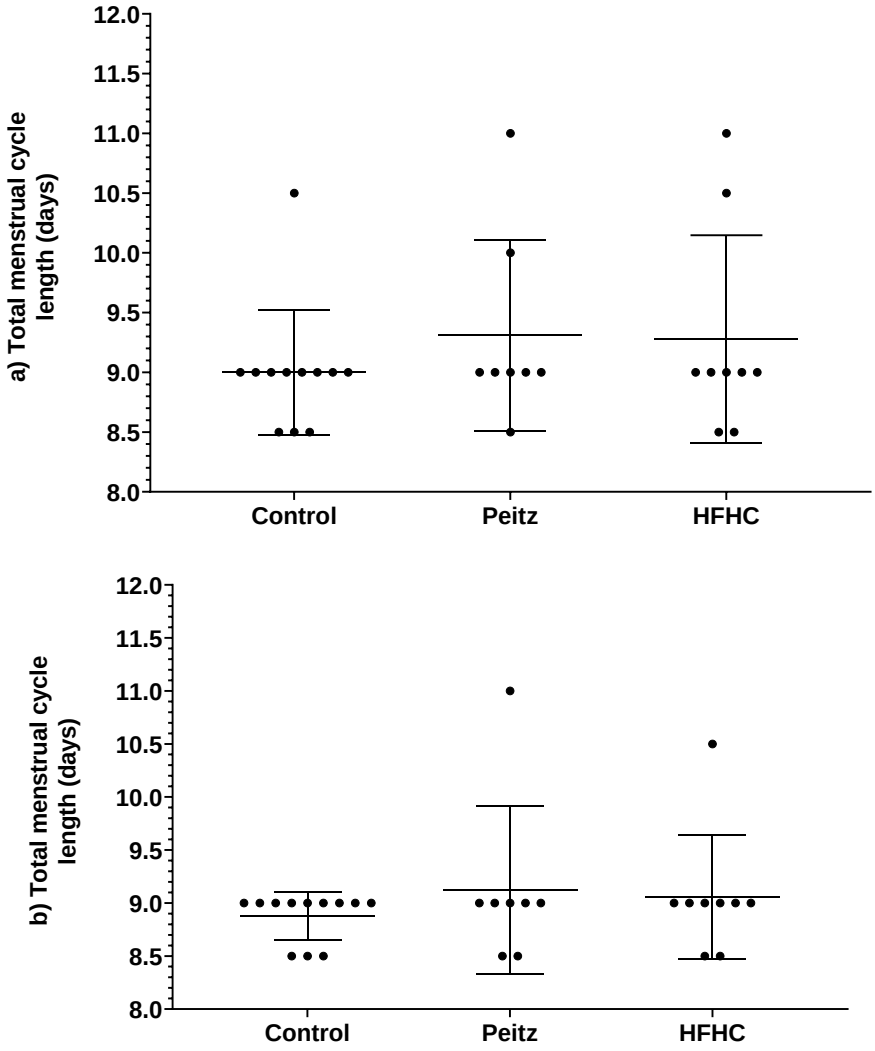


Figure 2.4 Menstrual cycle length in control SpM vs diet treated SpM. a) *baseline and* ***b)*** *2 months. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all groups Control (n)=12, Peitz (n)= 8 and HFHC (n)=9.*

At the 4-month time point menstrual cycles were insignificantly different, control SpM had a cycle length of (8.8 ± 0.2 days), Peitz (8.8 ± 0.3 days) and HFHC (8.9 ± 0.2 days), as seen in ***Figure 2.4c.***

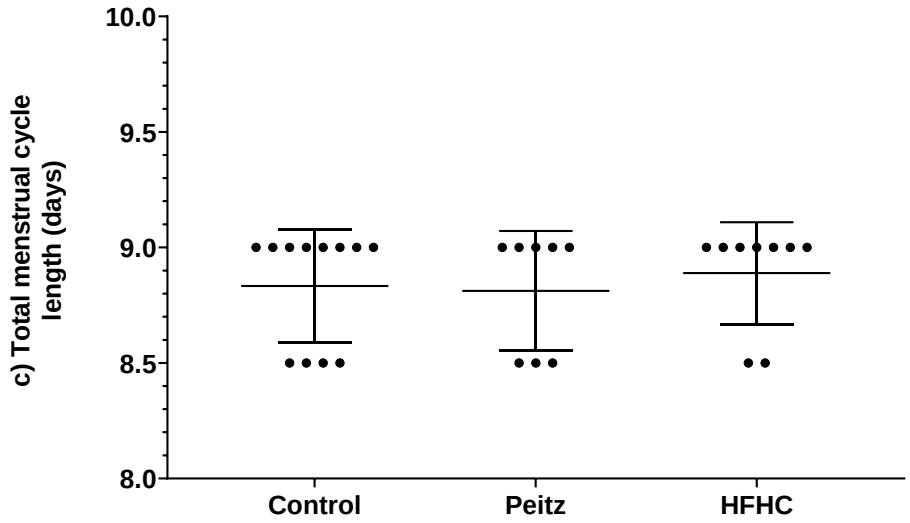


Figure 2.4 Menstrual cycle length in control SpM vs diet treated SpM. c) *4 months. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all groups Control (n)=12, Peitz (n)= 8 and HFHC (n)=9.*

No significant differences were established at the 8-month menstrual cycle, as shown in ***Figure 2.4d.*** During this time control SpM were reported as having an (8.9 ± 0.2 days) cycle length, Peitz as (8.9 ± 0.2 days) and HFHC as (8.9 ± 0.2 days). At the 10-month menstrual cycle, insignificant differences were found between groups, control SpM (9.1 ±0.6 days), Peitz (9.1 ± 0.6 days) and (HFHC 9.1 ± 0.6 days), as seen in ***Figure 2.4e.***

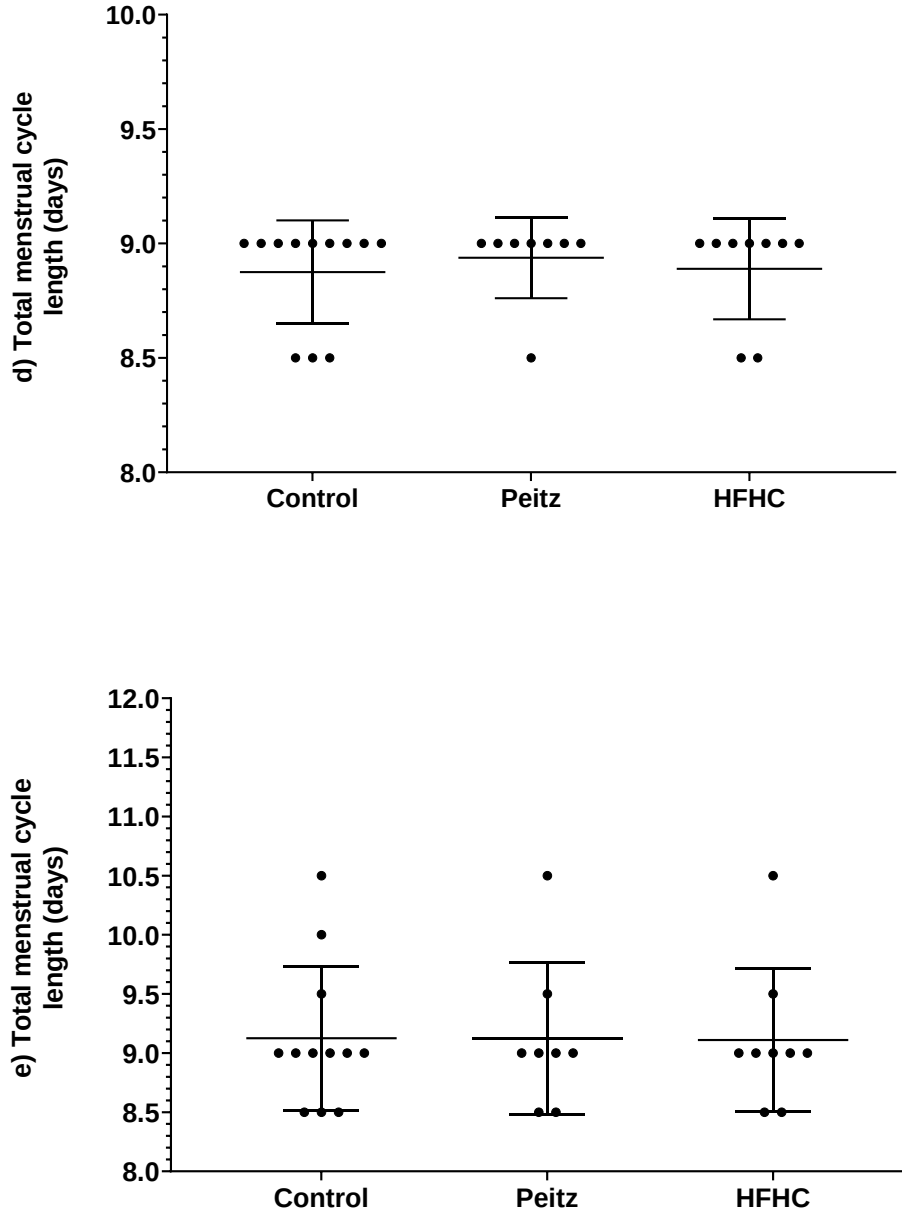


Figure 2.4 Menstrual cycle length in control SpM vs diet treated SpM. d) *8 months and* ***e)*** *10 months. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all groups Control (n)=12, Peitz (n)= 8 and HFHC (n)=9.*

Insignificant differences were observed in the 12-month menstrual cycle, control SpM had a cycle length of (9.0 ± 0.6 days) Peitz (9.2 ± 0.7 days) and HFHC (9.2 ± 0.6 days) as shown in ***Figure 2.4f***.

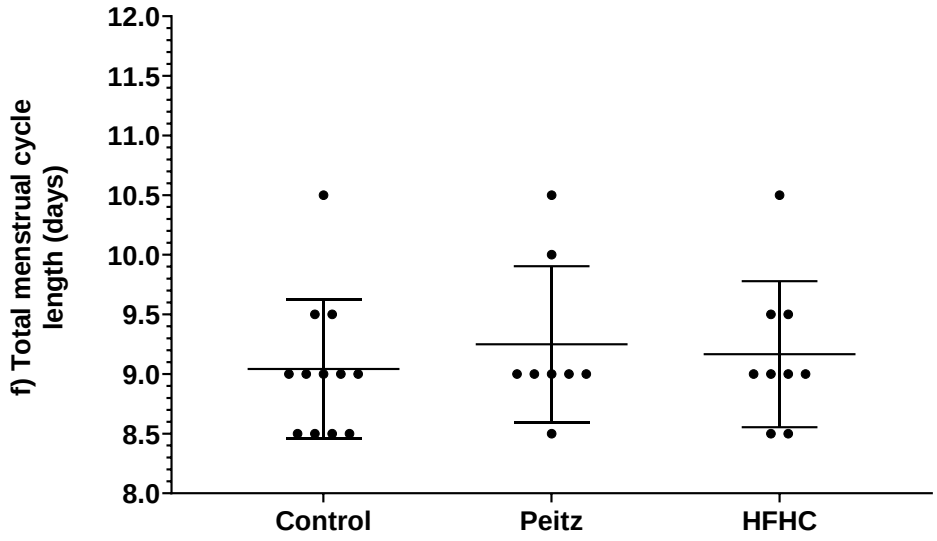


Figure 2.4 Menstrual cycle length in control SpM vs diet treated SpM f) *At 12 months. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all groups Control (n)=12, Peitz (n)= 8 and HFHC (n)=9.*

Organ weights

There were no significant differences in ovarian weights between control SpM (0.005 ± 0.001g), Peitz (0.005 ± 0.001g) and HFHC (0.006 ± 0.001g) as seen in ***Figure 2.5a.*** Insignificant differences were also observed in the uterine weight as shown in ***Figure 2.5b*** Control SpM uterine weights were 0.189 ± 0.024g, Peitz were (0.190 ± 0.021 g) and HFHC were (0.186 ± 0.022g).

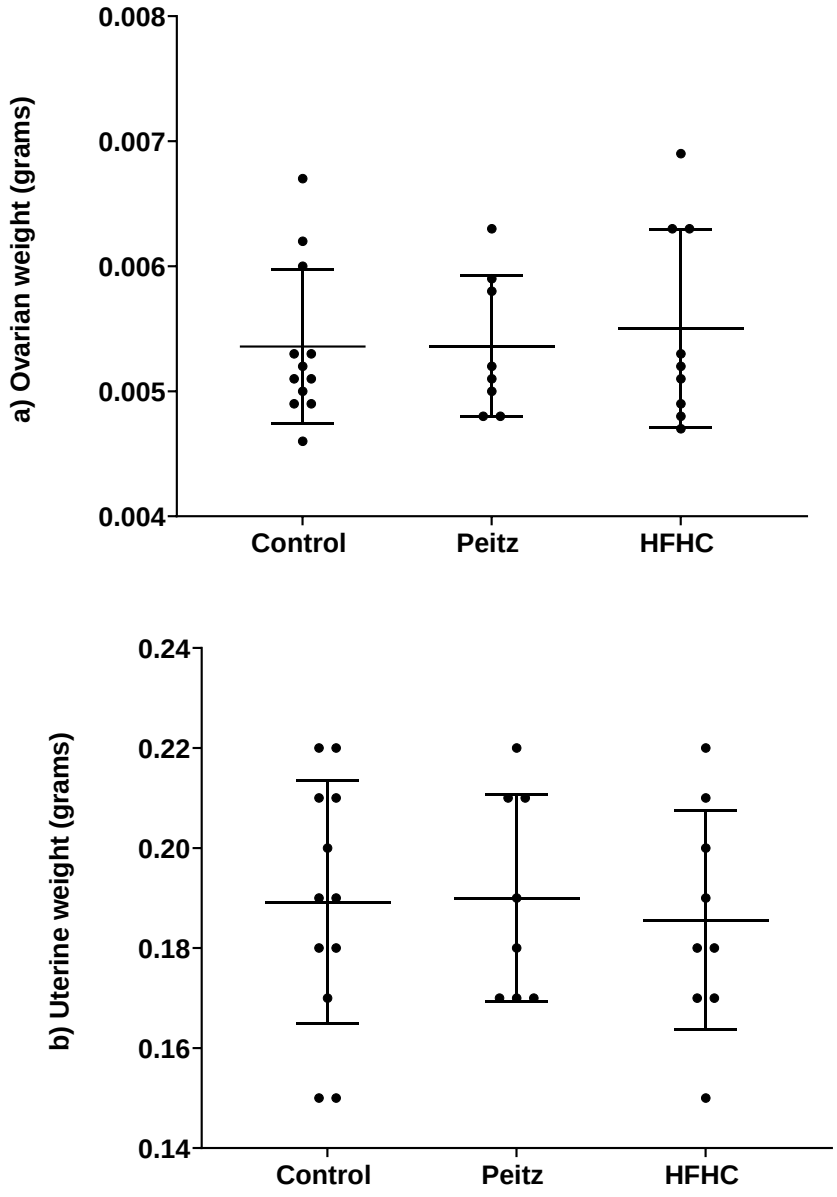
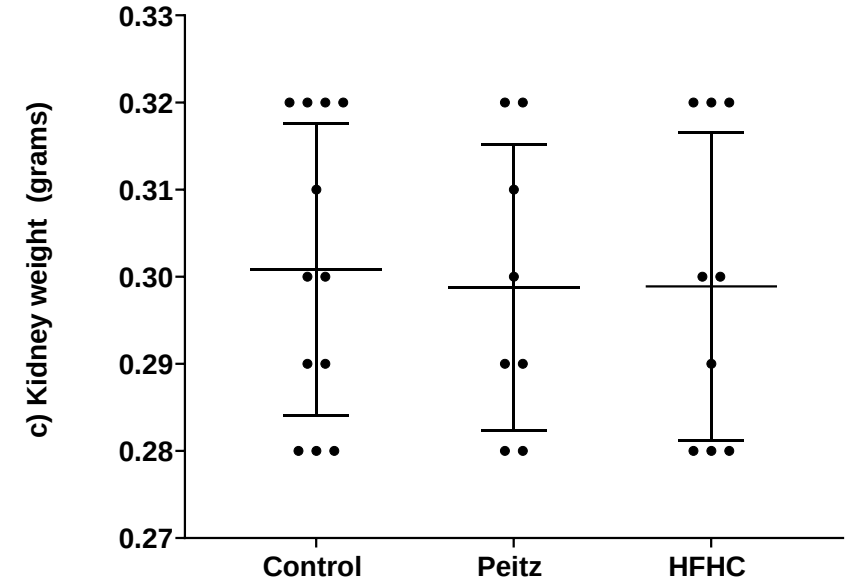


Figure 2.5: Organ weights in control SpM vs diet treated SpM at 12 months. a) *Ovarian weights.* ***b)*** *Uterine weights. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences*

across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all groups Control (n)=12, Peitz (n)= 8 and HFHC (n)=9.

No significant differences were observed in kidney weights across groups, control SpM were recorded as (0.301 ±0.017g), Peitz as (0.299 ± 0.016g) and HFHC as (0.299 ± 0.018g), as seen in ***Figure 2.5c.*** Insignificant differences between liver weights were observed amongst groups, control SpM were (1.203 ± 0.019g), Peitz were (1.206 ± 0.023g) and HFHC (1.202 ± 0.022g), as shown in ***Figure 2.5d.***



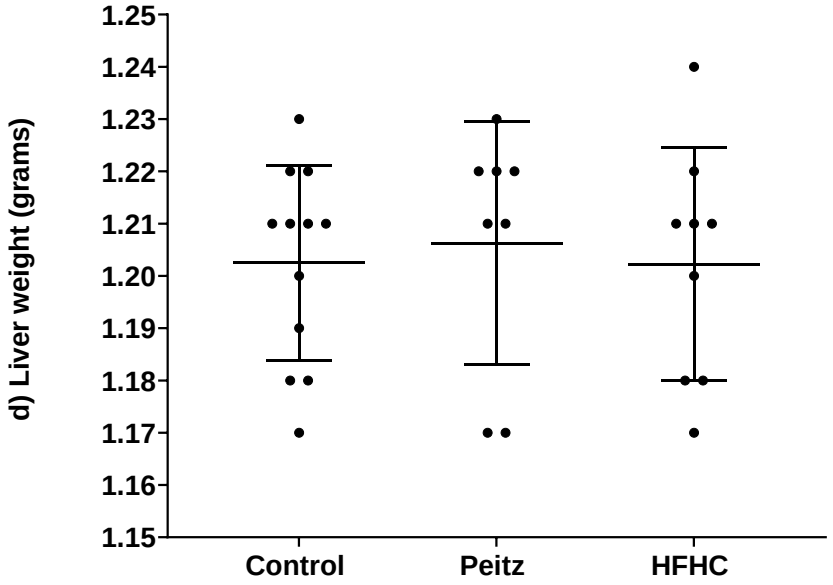


Figure 2.5: Organ weights in control SpM vs diet treated SpM at 12 months. c) *Kidney weights.* ***d)*** *Liver weights. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all groups Control (n)=12, Peitz (n)= 8 and HFHC (n)=9.*

Insignificant differences were observed in spleen weights across groups, control SpM were recorded as (0.072 ± 0.002g), Peitz as (0.072 ± 0.002) and HFHC as (0.072 ± 0.002g) as seen in ***Figure 2.5e.***

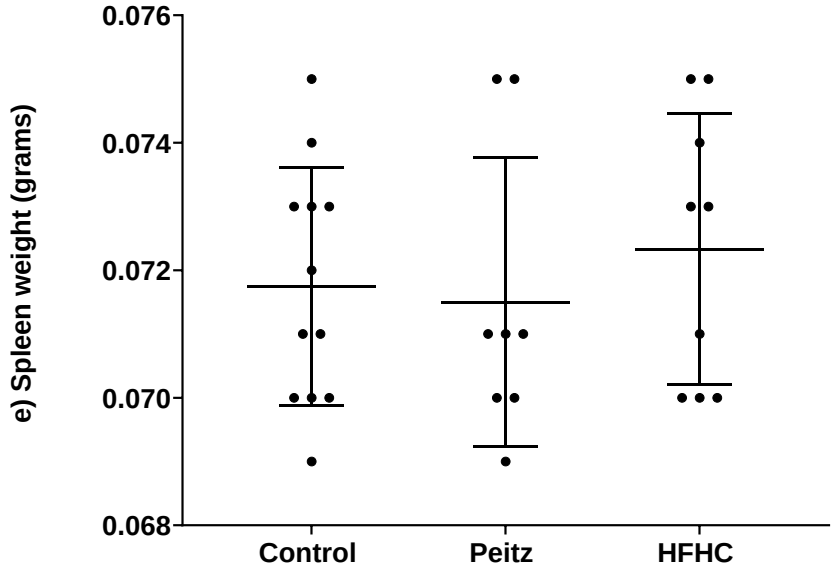


Figure 2.5: Organ weights in control SpM vs diet treated SpM at 12 months. e) *Spleen weights. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all groups Control (n)=12, Peitz (n)= 8 and HFHC (n)=9.*

Discussion

I have demonstrated that SpM fed a HFHC weigh significantly more than our Peitz SpM whilst Peitz SpM are not significantly different than control SpM at the 52-week endpoint, as shown in ***Figure 2.1a*** and ***b.*** I did observe a significant difference between the control SpM and Peitz and control SpM and HFHC at 10 weeks, that continued to 14 weeks. This may be attributed to an initial growth spurt, as SpM mature and the different diets allowed this to occur at different rates; the control SpM diet contains 4.6% fat, Peitz diet (Purina chow) contains 14% fat and HFHC SpM diet contains 23.5% fat. At 15 weeks all SpM were significantly different to each other, however this did not follow through to the 52-week endpoint.

At the 15-week time point the HFHC SpM gained weight more rapidly than the control or Peitz SpM, this may be due to the higher percentage of fat in the diet, causing them to put on weight at a faster rate. Interestingly, the Peitz SpM tend to weigh more than the control SpM up to week 16, after this point control SpM tend to weigh more than the Peitz SpM. Perhaps during this time, a growth spurt occurred in the Peitz SpM as they were maturing at a higher rate, I hypothesised this was due to their diet.

My findings regarding the Peitz SpM weights conflicts with the earlier Peitz 1981 study (1) as SpM in this colony were reported as weighing 60-80g. The Peitz SpM weighed approximately half the size as the Peitz 1981 (1) SpM, this may be due to a difference in the food, as this study occurred 40 years ago in America, perhaps the manufacturer of the cat biscuits used in the 1981 Peitz study had a higher fat content or were easily digested and absorbed by SpM (1). Unfortunately, an exact comparison regarding the nutritional content of the cat biscuits cannot be made as few details regarding the type of cat biscuits are provided in the Peitz 1981 study (1). It is not certain that our SpM and the Peitz 1981 SpM (1) are the same species or how old the SpM were at the time of this study, these factors may alter the way food is processed by the body and consequently weight gain.

I also speculate that the Peitz SpM may not have been able to fully absorb the total calories from their diet of cat biscuits, corn, oats and sunflower seeds, which caused them to tend to weigh less than the controls and significantly less than the HFHC SpM at the 52-week endpoint.

It is possible that the Peitz SpM weight at 52 weeks may be due to the Peitz SpM eating less or not absorbing the diet fully, compared with the Peitz 1981 study SpM (1), and that this may be manifesting as an adaptive physiology similar to how the HFHC SpM weight plateaued. As growth occurred at a slower rate in the Peitz SpM, than the HFHC SpM I hypothesise this may be because the SpM body had more time to adjust to the body weight growth, and to consequently start to adapt and not gain excess weight. These responses may be responsible for the significant difference in weight between HFHC and Peitz SpM at the 52-week endpoint.

The control SpM at the 52-week endpoint, were not significantly lighter than the HFHC, although the HFHC SpM did tend to weigh more than control, and significantly more than Peitz as seen in ***Figure 2.1a*** and ***b***. Interestingly, the HFHC SpM body weights appear to plateau after 35 weeks, whilst the control SpM continue to rise and Peitz plateau after 45 weeks. I hypothesised that the HFHC SpM are experiencing an adaptive physiology, which is inhibiting them from gaining weight to excess which may impair other physiological functions. Perhaps after the 35-week timepoint they may be eating less food, as they are mature SpM and do not require more to sustain rapid growth, as seen when they were younger. The control SpM continue to increase in body weight slightly, as they tended to weigh less than HFHC I hypothesise that they may not have reached a critical body weight and physiologically can continue to gain weight to this degree, within their normal control weight range. Interestingly, a metabolic protective mechanism preventing excessive weight gain in SpM has been described previously, attributing it to how energy is processed in SpM and a dissipation of energy inhibiting weight gain (58, 59). This may be why I observed a plateau in weight gain in our SpM. Further metabolic studies would need to be conducted to investigate this.

Even though the HFHC SpM experienced a significant increase in weight during our 12-month study, the end weight of animals seen in other diet induced obesity models is higher, at approximately 76g, (47) whereas our SpM were only approximately 41g. I hypothesised that based on our SpM not achieving this level of obesity, that the diet treatment was not optimal to induce metabolic dysfunction. This is supported by the blood glucose data I obtained, as seen in ***Figure 2.2a*** and ***b.*** Although not significant, HFHC SpM tended to have higher blood glucose levels compared with control SpM. I concluded that due to the diet also being high carbohydrate 20.1 % this may contribute to why I saw a lower-than-expected end weight in our SpM, as their end weight was similar to other SpM (approximately 45g) on a high sucrose diet (47). This in conjunction with a potential adaptive physiological mechanism in SpM, particularly when high sucrose diets are implemented (59) and based on the HFHC SpM, which tended to have higher blood glucose levels, I concluded that a diet with a fat content more than 23.5% and reduced sucrose content may cause SpM to achieve a higher level of obesity, and to subsequently have elevated glucose levels.

Interestingly, unpublished data obtained from a member in our research team (appendix 2.1), using the same HFHC pellets but in conventional laboratory male mice (C57BL6/J males) reported an average weight of 50g after 24 weeks on this diet. Our SpM and the C57BL6/J mice do appear to put on weight at a faster rate than their respective controls. The overall bodyweight in the C57BL6/J males is higher than the final average bodyweight in the HFHC SpM, which at 52 weeks of being on the HFHC diet, was approximately 41g. The HFHC SpM at 24 weeks weighed on average 40g, which is less than the C57BL6/J male mice at 24 weeks. I hypothesised that perhaps this is because of the potential adaptive physiology at play in SpM as mentioned. This may also be because the C57BL6/J mice are male, which tend to have a naturally higher bodyweight. To investigate these differences further I propose to have the animals on the same diet and using both female SpM and C57BL6/J mice to eliminate any weight differences caused by sex.

I found that the diet treatment did not impact upon reproductive cycling in SpM as shown in ***Figure 2.3a-f.*** All SpM spent appropriate time in each of the stages of the cycle and all experienced a menstrual cycle during the study as shown in ***Figure 2.4a-f.*** I did observe slightly elongated cycles at baseline and at 2 months (***Figure 2.4a*** and ***2.4b***) however, this was not significant, and I believed this was within normal cycling of SpM, that the SpM were adjusting to reproductive age and this elongation is a result of that. Similarly, I observed an elongated cycle across all groups as shown in ***Figure 2.4e*** and ***Figure 2.4f*** I attribute this to aging SpM, as older female SpM have been described to experience longer cycles (15, 16). As the diet treatment did not significantly impact upon the physiology, the organ data as shown in ***Figure 2.5a-e*** corresponds with this, as no significant differences were observed between control SpM, and diet treated SpM.

My findings add to the described literature that SpM do not increase dramatically in weight when on high fat and sucrose diets but do respond more on high fat diets (47, 58-60). This may be due to a protective physiological adaption, as previously described (59). To investigate this further, I propose that in future studies, SpM be given a diet with increased fat and reduced carbohydrate content, to increase obesity and to investigate the consequent metabolic and reproductive outcomes. The SpM remains a unique model for reproductive science, as its menstrual cycle is similar to humans, thus, to investigate the effects of obesity caused by high fat diet and consequent related reproductive illness, the SpM provides a model to do this with the hope to obtain translational data that may help women suffering from obesity and related reproductive dysfunction.

Chapter 3: Investigating the effect of letrozole on reproduction in female spiny mice

Introduction

PCOS is a commonly occurring condition, with limited treatment options. This has been largely attributed to the lack of an appropriate animal model in which to better understand the pathophysiology of this disease. The discovery of a human-like menstrual cycle in the SpM, presents a unique opportunity to establish a small animal model for various women's health problems in a species with a naturally occurring menstrual cycle similar to humans (12, 15, 17, 19). Different interventions have been used over the years to induce PCOS in animals such as chronic treatment with DHEA or DHT (52, 53). Typically, this treatment causes cystic ovaries, increased serum levels of testosterone; estrogen; follicular stimulating hormone (FSH) and luteinising hormone (LH). These models are unable to produce all of the reproductive and metabolic dysfunctions associated with PCOS, however, chronic exposure of letrozole has been successful in encapsulating the majority of both reproductive and metabolic symptoms of PCOS (52, 55). Letrozole (LET) is a non-steroidal aromatase inhibitor; aromatase converts androgens to estrogens, and disruption in this activity leads to an accumulation of androgens. Prolonged exposure to letrozole, typically 5 weeks or more, produces reproductive and metabolic disruptions, resulting in the development of PCOS (31, 33, 34). Typically, rodents treated with letrozole have polycystic ovaries, elevated levels of testosterone and LH, while FSH serum levels are decreased (31, 33-35, 52, 56, 57). Rodent models treated with letrozole have reduced uterine and increased ovarian weights and have disrupted oestrous cycles with more time spent in diestrus than the other phases of the cycle (33, 34, 52).

Study 2 was proposed with the hope to obtain new information that may lead to the development of new translational treatments for female reproductive health disorders, such as PCOS by using letrozole and SpM model.

Hypotheses and Aims

The overarching hypothesis tested in study 2 is that letrozole exposure will induce PCOS in the SpM.

The main aim of this study is to investigate the effect of letrozole in the SpM along with establishing and characterising a PCOS model in the SpM. I aimed to identify the optimum dosage and length of time to induce PCOS in the SpM through using a 60-day continuous release pellets with 2mg, 4mg and 8mg letrozole. Through this, I aimed to cause SpM to become acyclic, an increased number of cystic follicles and metabolic disturbances such as increased body weight, fat content and increased testosterone levels.

Rationale and study design

It was decided to opt for implant insertion surgery over daily injections as the SpM are prone to stress, which may impact their menstrual cycle and their skin is easily torn (15, 61).

I decided to perform a single series of vaginal lavages at the end of the 60-day continuous release pellet treatment, as we wanted to avoid compounding variables. This was deemed suitable as prior literature had demonstrated the long-lasting effects of letrozole treatment for at least 5 weeks after cessation (34).

Materials and Methods

Ethics

All experiments were conducted at Monash Medical centre as a research component in the degree of Masters of Reproductive Sciences. All experiments were approved by the Monash University Animal Ethics Committee under application "MMCB/2018/38: Letrozole-induced polycystic ovary syndrome (PCOS) in the spiny mouse", prior to commencement. All studies were conducted in accordance with the Australian National Health and Medical Research Council (NHMRC) code of practice for the care and use of animals for scientific purposes.

Animals

Thirty-three virgin female SpM aged 40 ±5 days were allocated to the following groups: control (n=9), 2mg letrozole (n=8), 4mg letrozole (n=8) and 8mg letrozole (n=8). All animals were fed standard rat and mouse cubes, (Speciality Feeds Glen Forrest, WA) supplemented on a weekly basis with carrot or celery, and water was provided ad libitum. Animals were maintained on a 12:12 light/dark cycle.

Body weight measurements

All SpM from each of the four groups were weighed on a weekly basis using Sartorius Entris II Laboratory Balance, starting from 40 ±5 days of age until the end of the study

Letrozole implant surgery

At age 60 ± 5 days, female SpM in each group underwent subcutaneous implant surgery using letrozole or placebo implants sourced from Innovative Research of America (Sarasota, Florida 34236 USA). Anaesthesia was induced in each female using 4.5% isoflurane in a perspex induction box and then maintained throughout the surgical procedure with 1.5-2.5% isoflurane delivered through a nose

cone. During the procedure each female was placed in a prone position, a 5mg/kg subcutaneous injection of Carprofen was given pre-operatively, and a suprascapular cutaneous site was identified, shaved and cleaned with 70% v/v ethanol. A 4mm inter-scapular incision was made through the skin and a 2cm x 1cm pocket formed in the natural space between the scapulae by blunt dissection. The subcutaneous pocket was flushed with topical analgesic (Bupivicaine) and a 3mm diameter implant containing either letrozole (2mg, 4mg or 8mg) or placebo (empty implant) was inserted into the pocket and a 6/0 Maxon® dissolvable suture was used to close the incision. Nails on the front paws were clipped to prevent females from opening the incision during grooming. Meloxicam (1mg/kg/24h) was provided in drinking water as post-operative analgesia for 72h after surgery. The animals were housed separately for 2 weeks whilst they healed from the surgery, to avoid them pulling out other animals' sutures and tearing their skin. 60 days was chosen as the length of the study based on previous studies utilising letrozole (31, 33, 34), during the study, females were weighed each week but were given no other intervention to allow the effects of letrozole to impact upon the animal's physiology, without any additional confounding factors, especially a possible effect of stress from handling. Details of monitoring during and post-surgery are provided as part of the appendices.

Vaginal lavage

A single vaginal lavage procedure was performed at the end of the study 60 days after insertion of the implant; a lavage was performed every second day for a period of 14 days using the procedure describe in Chapter 2, and previously (15). Lavage samples were placed onto a microscope slide, dried at room temperature, fixed using a cytology fixative spray, stained using a standard H&E protocol, and then viewed at 10X and 40X with a Nikon transmitted light microscope to establish the stages of the menstrual cycle during the 14 days of this part of the study. I then compared the length of the menstrual cycle in females from each of the 4 groups with the published SpM menstrual cycle length of 9±3 day (15, 16).

Staging SpM Menstrual cycle

This vaginal cytology used to stage the menstrual cycle was the same as previously described in the Material and Methods in Chapter 2.

Tissue collection and weighing

On the last day of the 14-day vaginal lavage procedure each female was killed humanely by an isoflurane overdose and the following tissues collected: ovaries, reproductive tract, spleen, kidneys, and liver. The organs for each animal were placed in an individual specimen jar filled with fixative solution of 10% neutral buffered formalin (NBF). All organs were later weighed in milligrams to two decimal places using an Entris II Laboratory Balance (Sartorius).

Image acquisition and follicle counting

The NBF-fixed ovaries from all female SpM in each study group were isolated by fine dissection and viewed under 20X magnification using a dissecting microscope (Stereomicroscope system SZX7 Olympus) connected to a computer screen. The ovaries were briefly placed in a petri dish containing clean, distilled water and photographs taken of the “front” and “back” longitudinal views to visualise with transmitted light the structural features, including developing follicles, of each: a total of 4 images were taken of each pair of ovaries and stored onto an SD card. During this process a log was made of each group to track the ovaries of each female in each of the four study groups. These 4 images per female were placed into individual folders and then blinded and randomised for assessment. The follicles were counted based on ***Figure 3.1.*** Images were viewed on the desktop screen using the following criteria: large follicles were based on a diameter range of 2.5cm-3.5cm, medium follicles 1.5cm-2.5cm and small follicles ≤1.5cm. (*NB: an ovary was damaged in the 4mg letrozole study group (n=5) and has been excluded from the follicle data and ovarian weights, leaving a sample size of 4 females for the data set in this group).

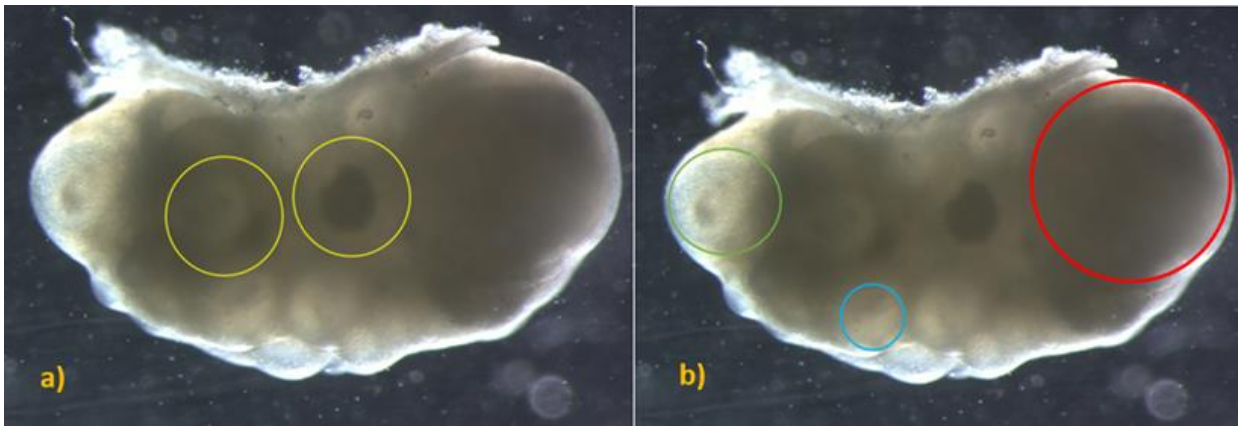


Figure 3.1 Ovary from a control SpM. a) *yellow circles indicate dark bodies.* ***b)*** *red circles indicate large follicles, green circles indicate medium, and blue indicate small follicles.*

Statistical analysis

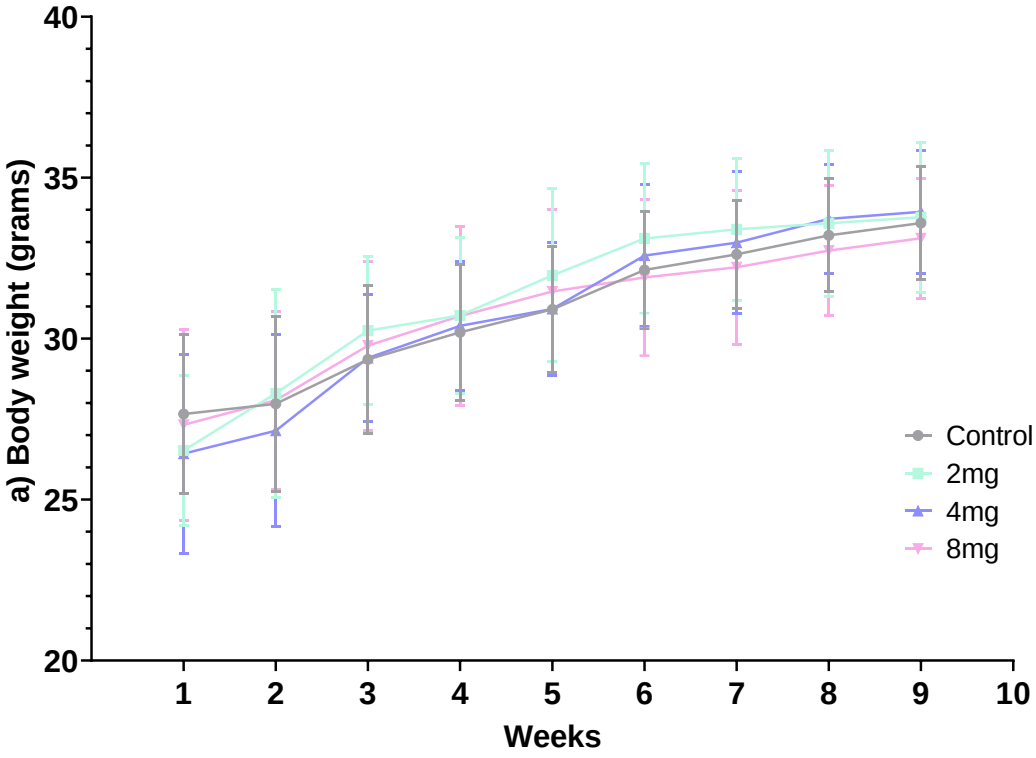
All data were analysed using GraphPad Prism (9.1.1). All data were tested for normality using Anderson-Darling test and are presented as mean ±SD, specified in each data set. Significance was determined by ($p < 0.05$) using ANOVA and Tukey's multiple comparisons, specified in each data set.

Results

Unfortunately, following pellet implantation surgery there were SpM deaths, which reduced the original sample sizes from: control (n=9), 2mg letrozole (n=8), 4mg letrozole (n=8) and 8mg letrozole (n=8), to: control (n=9), 2mg letrozole (n=8), 4mg letrozole (n= 5) and 8mg letrozole (n=6), (*NB in the follicle data 4mg letrozole is (n=4) due to a damaged ovary).

Body weight measurements

No significant differences were found in baseline (1 week) body weights prior to implant insertion surgery, nor those taken at the midpoint of the study or at the end of the study (***Figure 3.2a*** *and* ***b***).



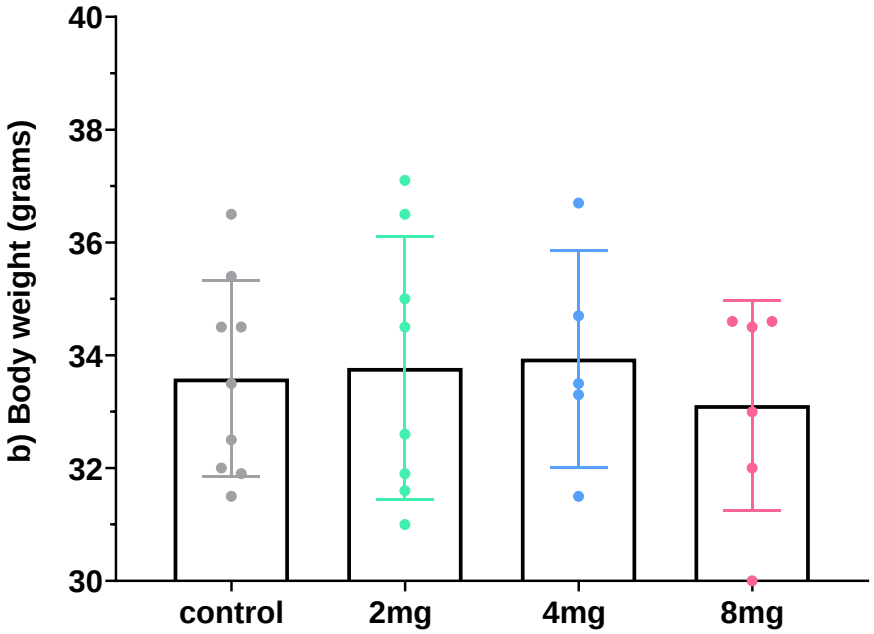


Figure 3.2: Body weights in control SpM female compared with those treated with letrozole treated SpM. a) *Body weights from the baseline without treatment (Week 1), following 60-days of letrozole treatment (week 2 – week 9)* ***b)*** *Endpoint data obtained at week 9, following 60-days of letrozole treatment. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, in all four groups: control (n=9), 2mg letrozole (n=8), 4mg letrozole (n=5) and 8mg letrozole (n=6).*

Effects of treatments on the duration of the menstrual cycle

During the early follicular phase (proestrus) insignificant differences were found between groups, as seen in ***Figure 3.3a.*** In the late follicular phase (oestrus) no significant differences were established between groups, as shown in ***Figure 3.3b.***

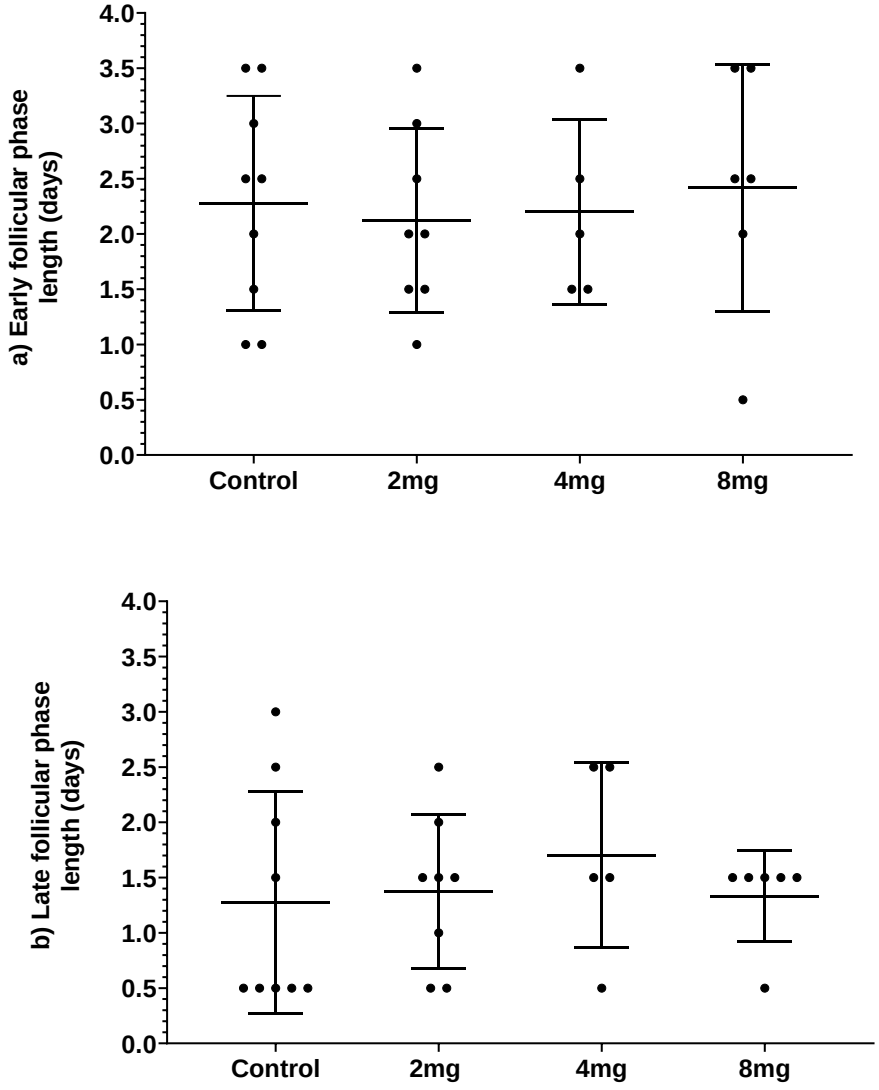


Figure 3.3: Menstrual cycle in control SpM vs letrozole treated SpM. a) *Early follicular phase.* ***b)*** *Late follicular phase. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD for each of the 4 groups: control (n=9), 2mg letrozole (n=8), 4mg letrozole (n=5) and 8mg letrozole (n=6).*

During the early luteal phase (metestrus) and the late luteal phase (diestrus) insignificant differences were observed between groups, as seen in ***Figure 3.3c*** and ***Figure 3.3d.***

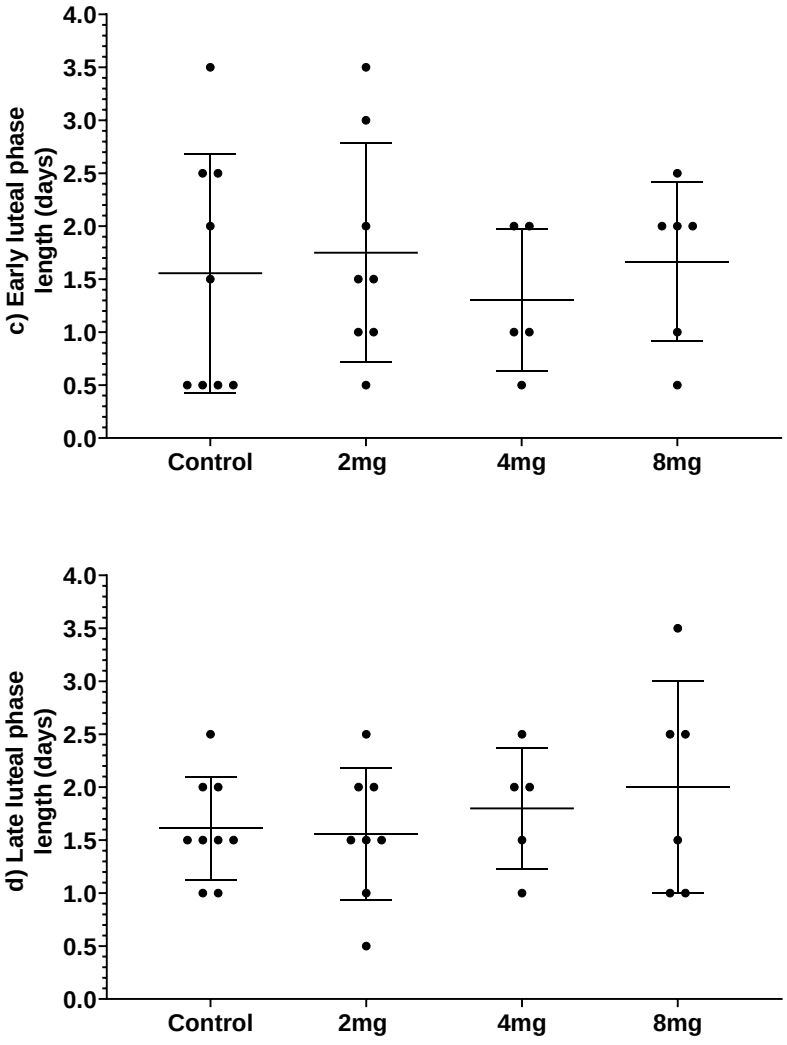


Figure 3.3: Menstrual cycle in control SpM vs letrozole treated SpM. c) *Early luteal phase.* ***d)*** *Late luteal phase. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean ± SD, for each of the 4 groups: control (n=9), 2mg letrozole (n=8), 4mg letrozole (n=5) and 8mg letrozole (n=6).*

No significant differences were found between study groups, during the menstrual phase (menses) as seen in ***Figure 3.3e.*** The menstrual cycle length did not reveal any significant differences between control SpM as shown in ***Figure 3.3f.***

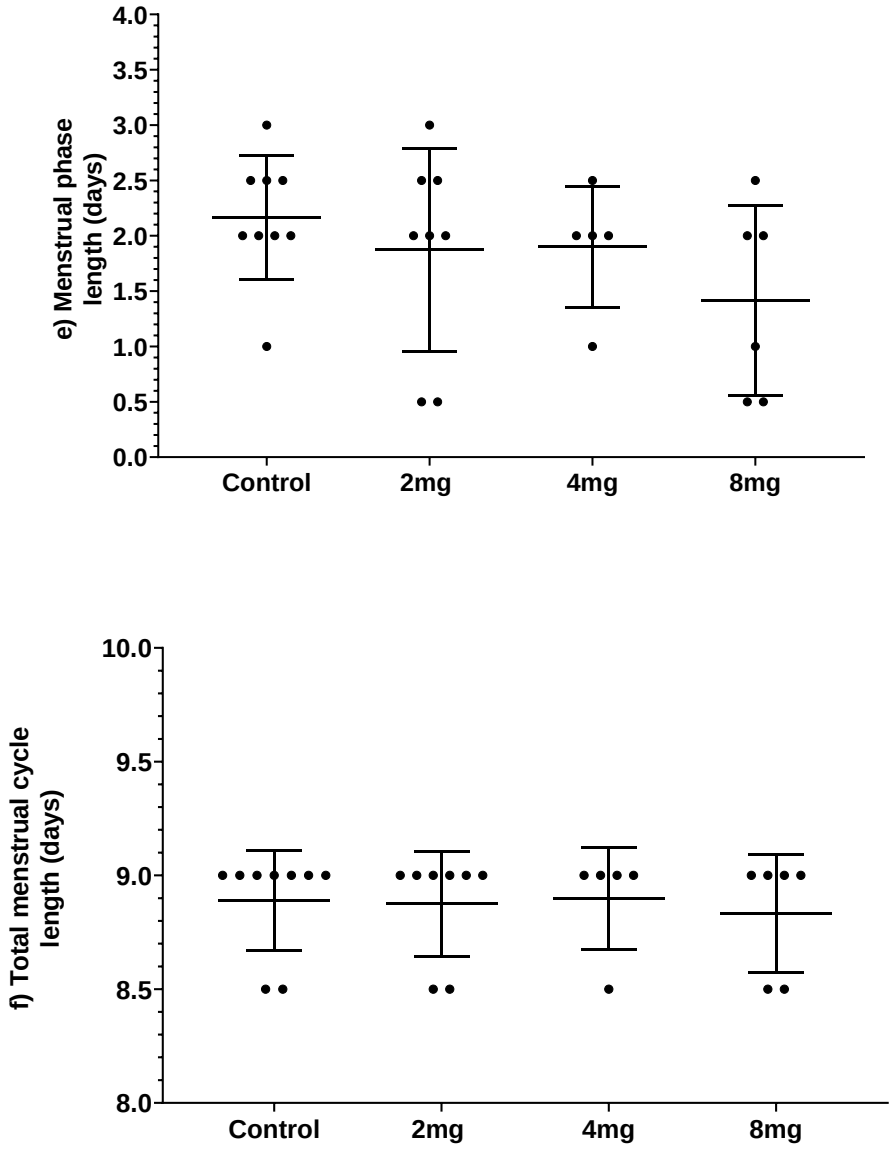
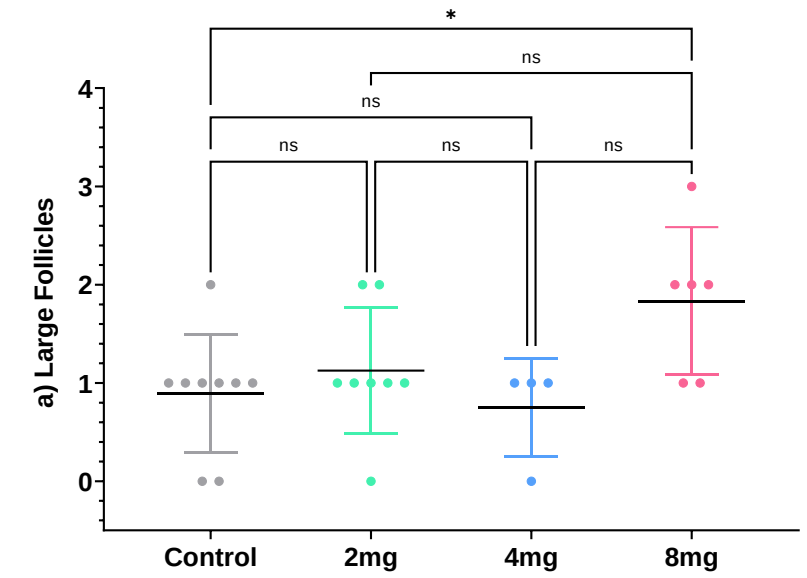


Figure 3.3: Menstrual cycle in control SpM vs letrozole treated SpM. e) *Menstrual phase.* ***f)*** *Total menstrual cycle length. Two-way ANOVA with Tukey's multiple comparisons revealed insignificant differences across all groups. Significant difference measured by p< 0.05. Data were presented as mean*

± SD, for each of the 4 groups: control (n=9), 2mg letrozole (n=8), 4mg letrozole (n=5) and 8mg letrozole (n=6).

Follicle counts

The large follicle data between control SpM (0.889 ± 0.601) and 8mg (1.833 ± 0.753) was significantly different (***Figure 3.4a*).** The medium follicle data did not reveal significant differences in the numbers of follicles recorded between control SpM (2.778 ± 1.641) and letrozole treated groups 2mg (2.125 ± 0.991), 4mg (3.250 ± 1.258) or 8mg (2.000 ± 0.894) **(*Figure 3.4b*).**



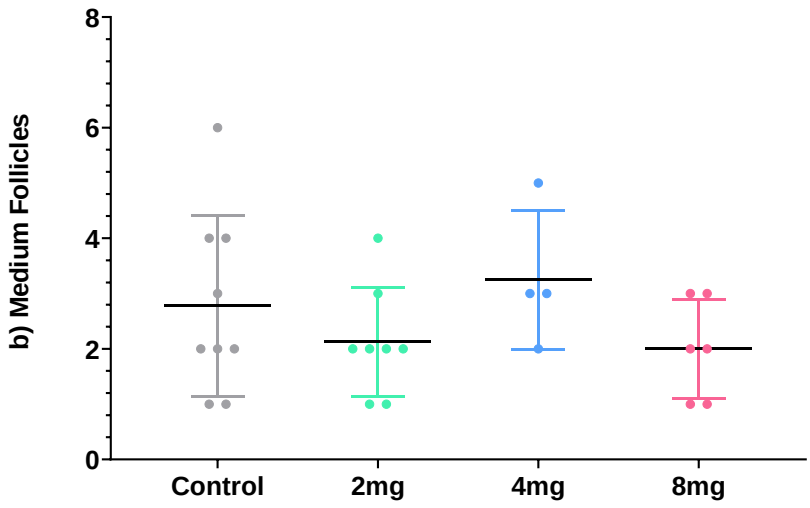
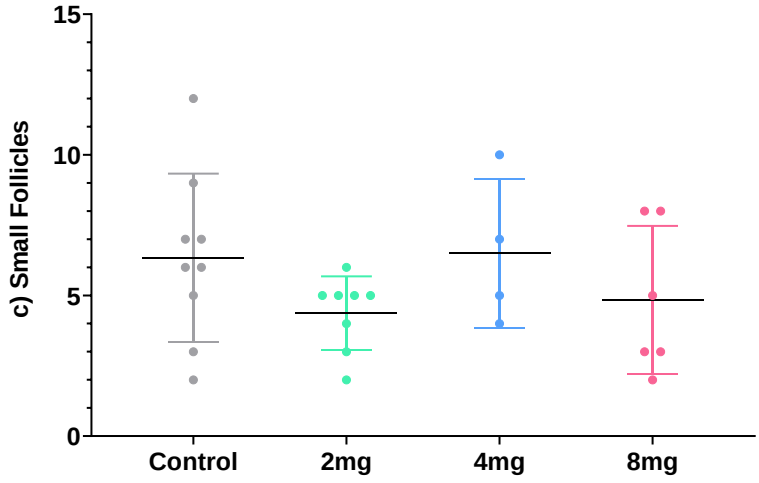


Figure 3.4: Follicle counts in control SpM vs letrozole treated SpM. a) *Large follicles - Two-way ANOVA with Tukey's multiple comparisons revealed significant differences.* ***b)*** *Medium follicles – Two-way ANOVA with Tukey's multiple comparisons, revealed no significant differences in follicle counts. Significant difference p< 0.05 denoted by * and non-significant difference by ns. Data were presented as mean ± SD, for each of the 4 groups: Control (n=9) and letrozole 2mg (n=8), 4mg (n=4) and 8mg (n=6).*

No significant differences were found between treatment groups in the small follicle as seen in ***Figure 3.4c.*** The dark bodies data revealed significant differences (p<0.0098) between the control SpM and 4mg as shown in ***Figure 3.4d.*** Significance was revealed (p<0.0036) between the 2mg and 4mg SpM, photos of control, 2mg and 4 mg ovaries are shown in ***Figure 3.4d.i.***



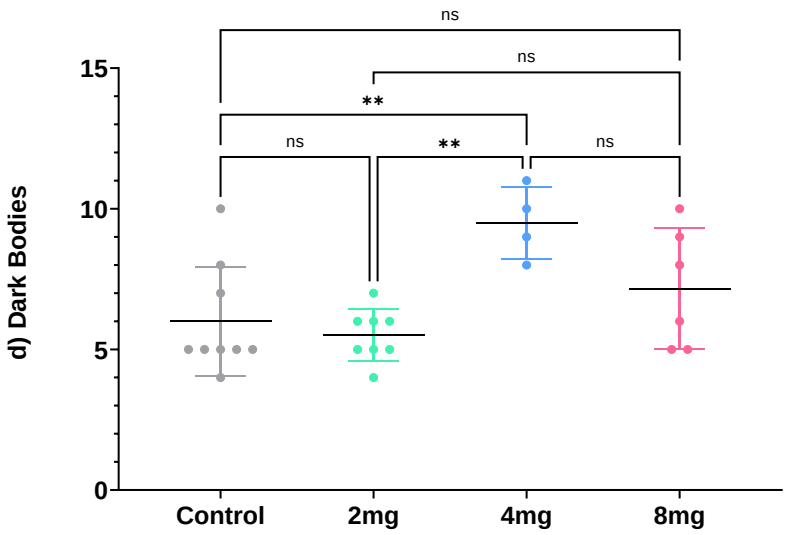


Figure 3.4 follicle count in control SpM vs letrozole treated SpM. c) *Small follicles - One-way ANOVA with Tukey's multiple comparisons revealed no differences across all groups.* ***d)*** *Dark bodies. One-way ANOVA with Tukey's multiple comparisons revealed significant difference. Significant difference of p< 0.001 denoted by ** and non-significant difference by ns on the figure. Data were presented as mean ± SD, for each of the 4 groups: Control (n=9) and Letrozole 2mg (n=8), 4mg (n=4) and 8mg (n=6).*

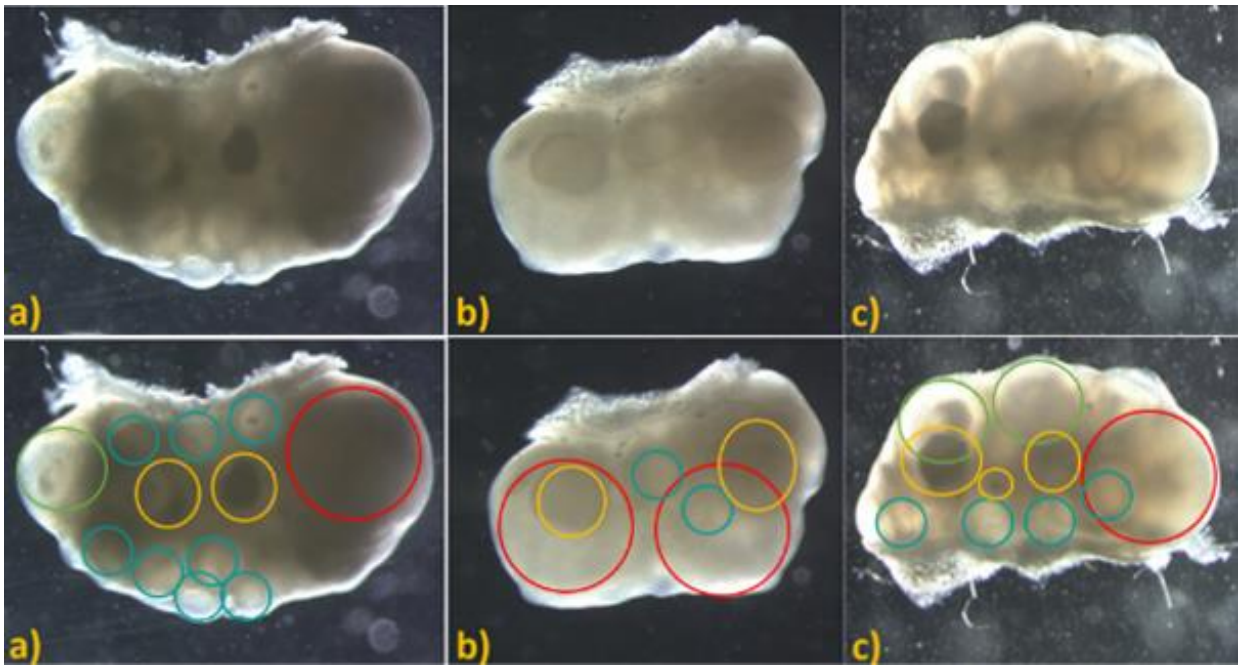


Figure 3.4d.i Ovary from a) control SpM, b) 2mg letrozole treated SpM and c) 4mg letrozole treated SpM showing small, medium and large follicles, and dark bodies. Note: yellow circles indicate dark bodies. Red circles indicate large follicles, green circles indicate medium follicles, and blue circles indicate small follicles.

There was no significant effect of letrozole treatment on the overall follicle count (large, medium, and small follicles) when compared with control ovaries (***Figure 3.4e***).

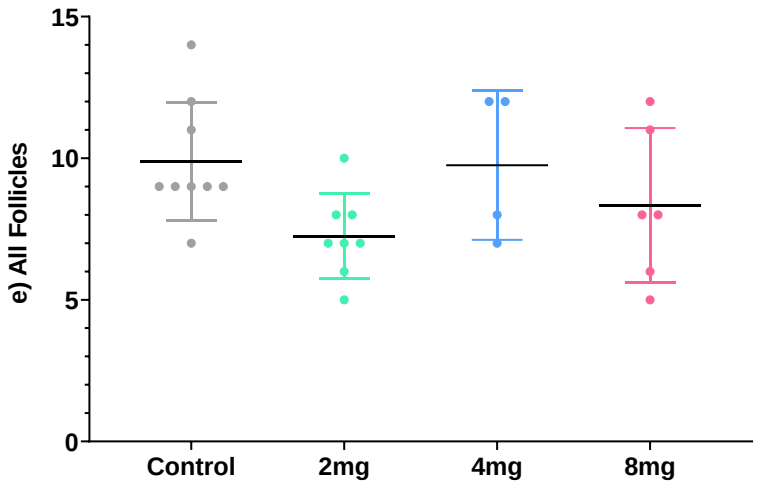


Figure 3.4: Follicle counts for the control SpM vs letrozole treated SpM. e) *Overall follicles. Two-way ANOVA with Tukey's multiple comparisons revealed no difference in total follicle counts between an of the 4 groups. Significant difference indicated by $p < 0.05$. Data were presented as mean ± SD, for each if the 4 groups Control (n=9) and Letrozole 2mg (n=8), 4mg (n=4) and 8mg (n=6).*

Organ weights

No significant differences were established between study groups in the ovarian weight data as shown in ***Figure 3.5a***. The uterine weight revealed no significant differences, as seen in ***Figure 3.5b.***

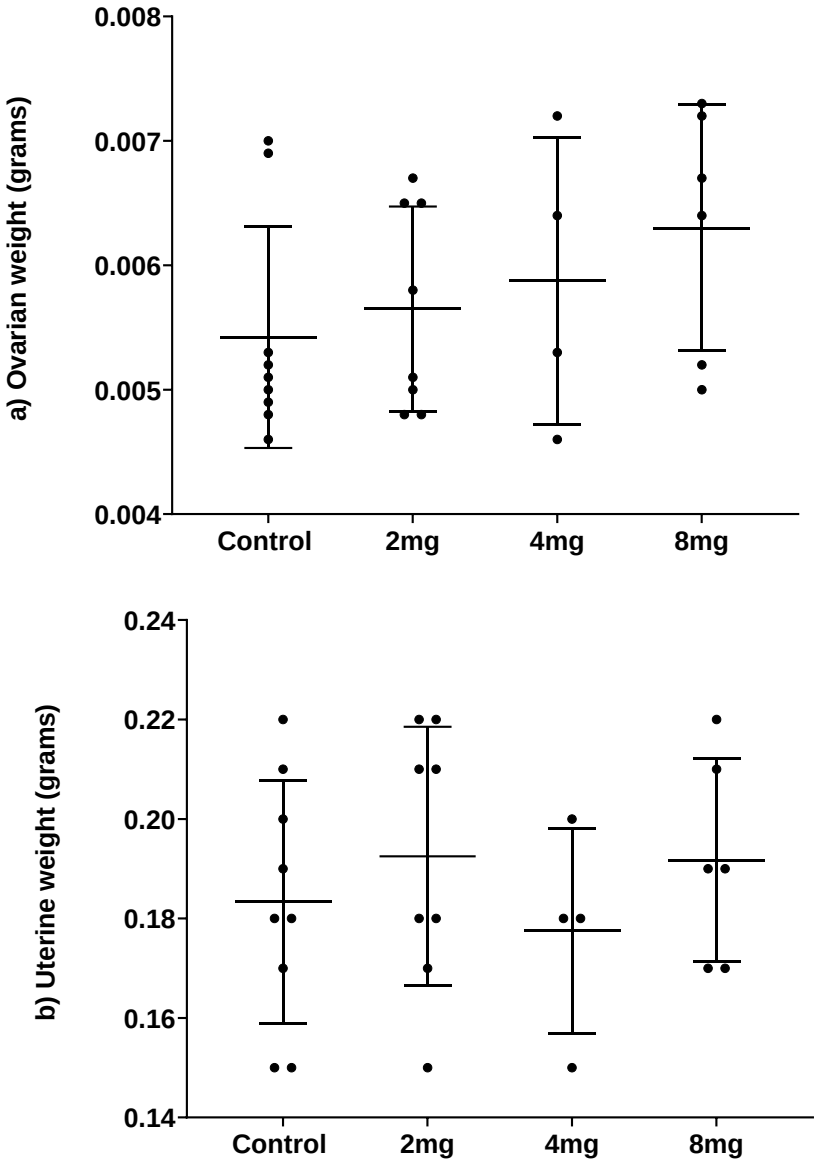


Figure 3.5: Ovarian a) and uterine weights b in the control SpM vs letrozole treated SpM taken at the end of the study. *Two-way ANOVA with Tukey's multiple comparisons revealed no significant differences across all groups. Significant difference indicated by p< 0.05. Data were presented as mean ± SD, for each of the 4 groups Control (n=9) and letrozole 2mg (n=8), 4mg (n=4) and 8mg (n=6).*

The kidney weights and liver weights were not significantly different as shown in ***Figure 3.5c*** and ***Figure 3.5d,*** respectfully***.***

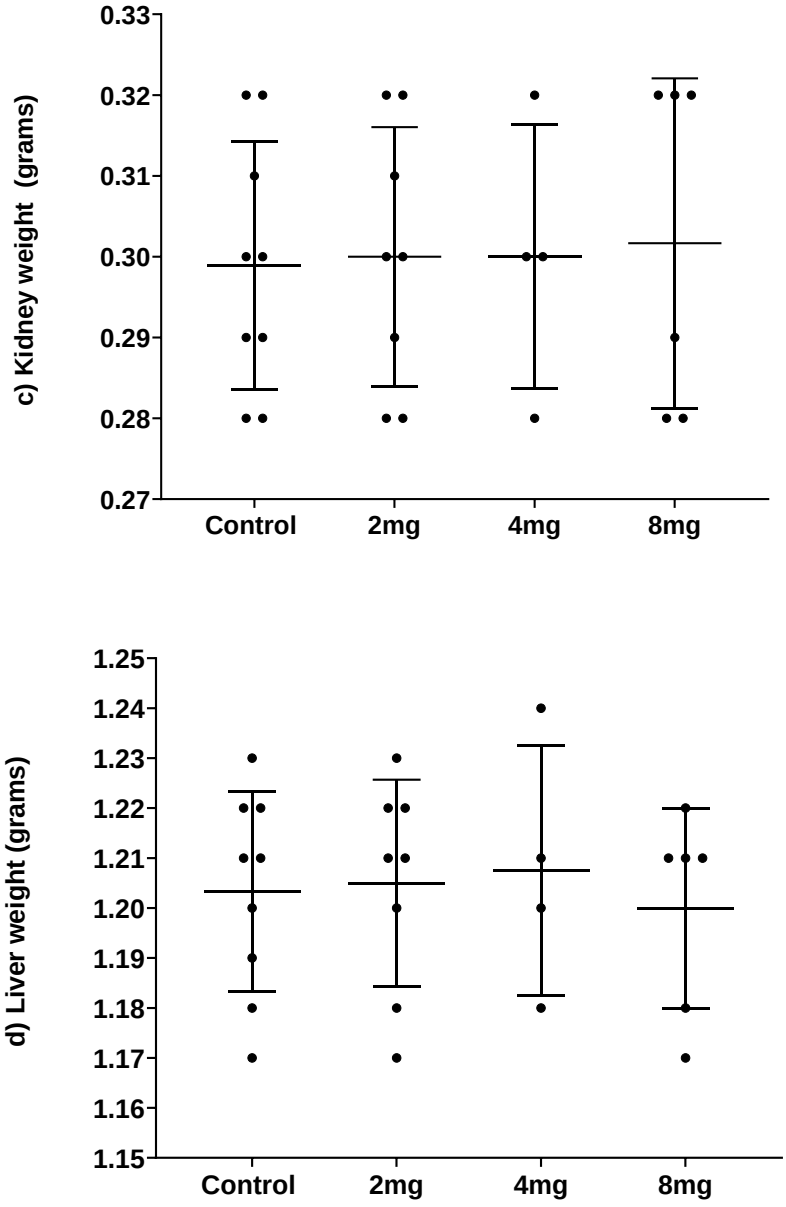


Figure 3.5: a) Kidney and b) liver weights in control SpM vs letrozole treated SpM. *Two-way ANOVA with Tukey's multiple comparisons revealed no significant differences across all groups. Significant difference indicated by p< 0.05. Data were presented as mean ± SD, for each of the 4 groups Control (n=9) and Letrozole 2mg (n=8), 4mg (n=4) and 8mg (n=6).*

Spleen weights were insignificantly different across all treatment groups, as seen in ***Figure 3.5e.***

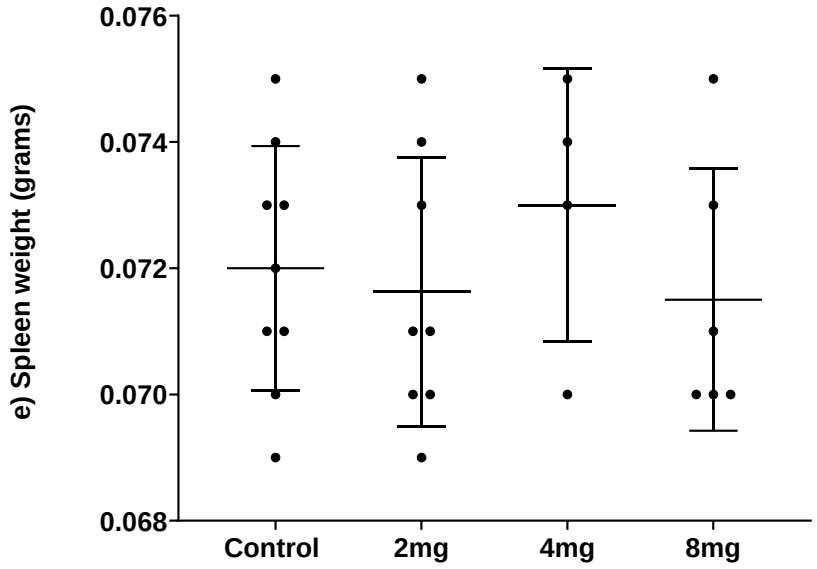


Figure 3.5: e) Spleen weights in control SpM vs letrozole treated SpM. *Two-way ANOVA with Tukey's multiple comparisons revealed no significant differences in spleen weight across all groups. Significant difference indicated by p< 0.05. Data were presented as mean ± SD, for each if the 4 groups Control (n=9) and letrozole 2mg (n=8), 4mg (n=4) and 8mg (n=6).*

Discussion

Unlike previous studies on other conventional laboratory rodents (31-35, 52, 55), SpM were not affected by letrozole exposure in my study. Although my results did not support my original hypotheses, they pose the interesting question of why female SpM did not respond to letrozole treatment. This study has added further interesting and, perhaps, controversial data to the growing scientific knowledge on this unique menstruating rodent.

Letrozole is a non-steroidal inhibitor that works by partially mimicking androstenedione, a naturally occurring steroid and enzyme substrate, leading to the inhibition of aromatase activity and associated conversion of androgens to estrogens, which leads to high levels of androgens, a major symptom of PCOS which can cause reproductive dysfunction such as poly cystic follicles and infertility (34, 55, 62). Failure of female SpM to provide any obvious response to the letrozole implants, when compare with the placebo, suggests a number of possible explanations, in particular how they process letrozole and possible resistance by females to produce PCOS and if they are resistant to the levels of letrozole treatments that produce PCOS in other laboratory rodents.

No significant differences in body weight between control SpM and letrozole treated SpM nor in the activity or duration of the menstrual cycle: females in the placebo and 3 letrozole treatment groups had normal menstrual cycles (9±3 days), which corresponds with control SpM previously described in the literature (12, 15, 16). No significant differences were also observed in the duration of each stage of the menstrual cycle in the 4 groups. However, 8mg SpM tended to spend a longer time in late luteal phase (diestrus). Previously data from a study on female mice (*Mus musculus*) showed that when treated with letrozole they became acyclic and spent more time arrested in diestrus (34). This opens the possibility that SpM metabolise letrozole differently from other rodents and they perhaps require a dosage higher than the 8mg implant dose in this study to produce the letrozole induced PCOS, and other metabolic and reproductive disturbances, reported previously (31-35, 63).

There were significant differences in the numbers of large follicles between the placebo control and 8mg letrozole-treated SpM ovaries, but not in the overall follicle count**.** This suggests that these large follicles are unlikely to be cysts because of the observed normal cycling in SpM rather than a PCOS-like disease process but more likely to be enlarging follicles in preparation for ovulation. However, as suggested for the ovarian weight observation in the 8mg letrozole-treated group, this may be an indication of a developing sensitivity to letrozole and that a 12 or 16 mg implant may produce a PCOS-like condition in the ovaries.

The dark bodies data revealed significant differences between control and 4mg SpM, and between 2mg and 4mg SpM as shown in ***Figure 3.4d***. I believe that the dark bodies are either atretic follicles or corpora haemorrhagic; a temporary blood-filled structure formed after ovulation. However further investigation in this and another unpublished study, suggests that these structures are atretic follicles containing large numbers of infiltrating leucocytes. Follicle atresia is a natural part of ovarian function in removing developing follicles that fail to reach ovulation (Mazi M (2021). Studies in infertility: i) assessing the appropriate use of IVF, a global perspective; ii) testing a human-based superovulation protocol in the spiny mouse. Honours thesis, Bachelor of Biomedical Science, Department of Obstetrics and Gynaecology, Monash University).

At this stage as it is difficult to explain the significant differences in the effect of letrozole treatment on dark bodies in the ovaries between the control and 4mg SpM and also between the 2mg SpM and 4mg SpM. However it is possible that this is a random error in the statistical analysis caused by the reduced sample size in the 4mg letrozole treatment group (n=4), compared with the control (n=9), 2mg (n=8) and 8mg (n=6) (64).

As with most of the other data collected during this study, organ weights showed no significant differences between the placebo and the 3 letrozole groups, again supporting the current conclusion that SpM metabolise letrozole differently from rodents in other studies. However, a possible trend for higher ovarian weights in the 8mg letrozole treatment group compared with placebo controls, again

suggests that treatment of SpM with a higher dose of letrozole, or a longer duration of treatment, or both, than those used in the study may result in a polycystic state in SpM ovaries leading to increased ovarian weight as described in a previous report (23).

Despite letrozole treatment in this SpM study failing to cause the PCOS-like state reported in ovaries of other rodents (31, 33, 34, 55), it is an interesting outcome because the SpM differ in various other ways from conventional rodents, such as menstruating and producing precocial young (15), as well as differences in their response to diets as seen in chapter 2 and previous literature (21, 47, 58, 59). I have hypothesised the reasons for these observations may be that SpM metabolise letrozole differently and they may have a protective mechanism against developing cystic follicles and/or gaining excess weight.

As stated earlier in this chapter, I opted to perform the vaginal lavage at the end of the letrozole treatment, to allow the full effects of letrozole to manifest without any additional variables that may have influenced the effects of the treatment. As I did not see any significant differences in any aspects of the data collected during this study, except for dark bodies in the ovaries, there is a minor, and perhaps unlikely, possibility that any effects of the letrozole treatment had been reversed, during the 14 days at the end of the study when the menstrual cycle was assessed, and this possibility would need to be considered in any future studies. Regarding the dark bodies, further investigation is needed, and I recommend pursing histological studies to determine exactly what these structures are and if any cellular changes occurred, unfortunately due to impacts resulting from the Covid-19 pandemic, this was not possible for my study. Currently not enough is known about the biology of SpM to make definitive conclusions, however speculation and curiosity may lead to further scientific enquiry which may bring us closer to advancing our knowledge of these unique animals and how research findings using these animals, may have translational potential for human studies in reproductive health.

Chapter 4: General discussion

Discussion

My findings in this study were surprising and unexpected. The evidence from previous studies suggested a high possibility that SpM would respond to high fat and high carbohydrate diets with significant increases in body weight and glucose intolerance over the time period these diets were provided to my study animals. Despite my hypotheses on how SpM would be expected to respond to the diets provided, the results have provided a new understanding about the physiological responses to high energy diets in these unique animals and have added to the body of knowledge of SpM. This chapter provides a general discussion of the final outcomes from my projects with regard to my overall findings, the limitations of the research, and the overall conclusions and provides suggestions for possible future directions from these studies.

Despite my original hypothesis that female SpM fed the two high energy diets will show significant increases in body weight and interrupted reproductive cycles, based on previous evidence from the Peitz 1981 study (1) and a concurrent study within our research team, using the same HFHC diet in mice (*Mus musculus*), no effects were observed from either diet over a year of exposure. Why there was no increase in body weight over controls could not be determined from my studies but there are some possible explanations that have operated singly or in combination to inhibit a significant response. I have suggested that a difference in the nutritional composition of cat biscuits from the 1981 study compared with those used in my study, may be a possible explanation.

Possible reasons: differences in the energy content of the diets – the Peitz study occurred many years ago and from the little details that were provided in the article, it is difficult to ascertain the nutritional content of the diet. Potentially a difference in species – the species used in the 1981 study may have been different to our colony, the fact they were double the weight of our SpM and that they presented with an oestrous cycle, leads us to speculate they may not be the same species, as previously thought. However, the exact species in Peitz study was not recorded and the colony is long gone, and no data

or tissue samples are available for genetic comparison – current concerns that not all *Acomys* colonies around the world are *A. cahirinus*, for example Professor Ashley Seifert's SpM colony in the University of Kentucky may be *A. dimidiatus* (with an apparent menstrual cycle, personal communication (65)). Furthermore, the exact ages of the SpM used in the Peitz study was not provided the 1981 study (1), as this may impact on weight gain and reproductive results from that study.

An interesting observation from my study was that the cohort of SpM fed the HFHC diet showed a significant increase in body mass over the controls and the Peitz SpM at 15 weeks, but this increase did not continue nor was it maintained in the subsequent 37 weeks of the study. This could be rapid growth as SpM were maturing and beyond this, I hypothesised that the HFHC SpM had an adaptive physiology that prevented them from gaining excess weight, as discussed in chapter 2 and seen in previous studies, when a high fat diet and high carbohydrate diet has been used (47, 58, 59). Interestingly, unpublished data (appendix 4.1) from a member within our research team has found that SpM fed the same HFHC pellets (SF04-001 diet, Speciality Feeds Glen Forrest, WA) with an additional carbohydrate component (high fat high sugar, HFHS) in female SpM for 16 weeks, are not significantly higher in body weight compared with their respective controls. Their SpM weights are tending to be higher than controls but at this stage, this is not significant. Our research colleague's SpM treated with the HFHS diet, seem to not put on weight as rapidly as our SpM treated with HFHC diet, during the same time period (baseline to 10 weeks). Because their HFHS diet has a higher carbohydrate percentage, this could be because of the adaptive physiology as mentioned in chapter 2, these HFHS SpM put on weight at a slower rate and are not significantly different from their controls. At this 10-week time point, this corresponds with our data and with other studies, that have noted SpM fed a diet higher in carbohydrate tend to put on less weight, when compared with diets that have solely a higher fat content (47, 58, 59). Interestingly, the fasting blood glucose obtained from our research colleague's unpublished data, shows at 8 weeks of being on the HFHS diet, the HFHS SpM tended to have slightly elevated fasted blood glucose levels, compared with controls as seen in ***Appendix 4.1 – figure 4.2***. Our HFHC SpM tended to have elevated fasted blood glucose levels, but

this was not significant, suggesting that the additional carbohydrate component in the HFHS diet may be optimal to cause the fasted blood glucose levels in these SpM to be significantly different to controls. This is interesting, as SpM do have a tendency to become diabetic as seen in other studies (47, 48, 58, 59) however, the reason I did not see significantly elevated fasted blood glucose levels was due to the adaptive physiology discussed in chapter 2, (47, 58, 59). Although, with the additional carbohydrate component in the HFHS diet, this may be optimal to cause significantly elevated blood glucose levels, leading to diabetes, with or without significant weight gain.

As discussed in chapter 1 there is a relationship between diet and health, and specifically between obesity and reproductive dysfunction, such as with PCOS; obese women (BMI ≥30) tend to suffer from PCOS and PCOS sufferers tend to be obese (37-41, 50, 66, 67). Broadly, based on this we decided to formulate the two studies, in chapter 2 and 3: I formulated study 1, to investigate if PCOS could be induced by diet and to investigate the metabolic and reproductive dysfunction, resulting from diet induced obesity; study 2, to induce PCOS chemically by using letrozole to investigate the effects from letrozole exposure, on metabolic and reproductive health, and to compare the 2 studies together, focusing on how PCOS could be induced and the resulting dysfunctions for the two different induction methods. I expected that the findings from these two studies would provide a foundation to develop a new more appropriate and effective animal model that menstruates and there would provide new avenues for testing novel treatments for PCOS sufferers.

The second study in this project used a standard experimental protocol with letrozole to induce PCOS-like changes in ovarian function. However as with the diets studies, my expectation that this treatment would result in PCOS-like changes in the letrozole-treated SpM ovary did not eventuate; no negative effects were observed between control SpM and letrozole treated or diet treated SpM. This corresponds with the bodyweight data: SpM did not reach the expected level of obesity based on the Peitz study (1). Although the HFHC SpM were significantly heavier than the Peitz diet SpM after the 52-weeks of treatment, no reproductive dysfunction was observed and female SpM from all treated

cohorts continued to cycle normally. I have suggested previously that this was because, although the HFHC and Peitz SpM were heavier than their controls in study 1, the increase in body mass was only 12% and 8% respectively, rather than the 100% or more expected from the HFHC or Peitz diet which we expected to cause SpM to be acyclic. It was also interesting that there were no significant differences in selected organ weights between control and diet treated SpM nor was there an obvious effect of the two diets on blood glucose levels and no indication of glucose intolerance despite previous studies showing SpM have a predisposition to diabetes. Study 2 (Chapter 3) also showed unexpected results with 3 different release concentrations of letrozole unable to produce the PCOS symptoms in SpM ovaries seen in other rodent studies (31-35, 52, 55, 56) including no significant differences in body weight, reproductive cycling or organ weight. Although unable to induce PCOS in SpM with letrozole the study has provided new insights into the physiological responses of SpM. Although letrozole in this study failed to induce PCOS, other methods should be considered in future research (53, 68-73); for example, simply increasing the dose of letrozole or treating females directly with androgen such as 5a-dihydrotestosterone may result in the a PCOS-like response. Despite this negative result, SpM still present a unique opportunity to study female reproductive health, as they have a naturally occurring menstrual cycle similar to humans, increasing the potential translational value to humans (12, 15-17, 19, 25, 28).

The unexpected negative outcomes from both studies in this thesis raise some interesting and, as yet unanswered questions about the nature of the SpM in our colony. For example, are they a different species from the SpM used in other studies, such as in the Peitz 1981 study? Has “bottle-necking” of the founders of the Monash colony and subsequent inbreeding resulted in genetic changes that might explain the unexpected responses? These and other related questions outlined in this thesis should be considered in the planning of future studies.

Limitations

Experimental timelines had been planned to allow time between procedures to reduce potential stress responses, as it has been reported from previous work by our research team that SpM can become stressed if excessive handling takes place or multiple procedures occur without sufficient recuperation time resulting in changes to the duration of SpM menstrual cycle. Based on this information I opted for lavages to be performed every second day rather than daily and for the glucose testing procedures to occur separately from the vaginal lavages, as detailed in chapter 2, to minimise potential negative impacts on SpM caused by stress. This protocol was clearly successful because in both studies that form this thesis no changes to the normal duration of the menstrual cycles were observed. As discussed in chapter 2, I was limited in the comparative investigation of the animals and diet used in the 1981 Peitz study (1) as few details such as age of animals and cat biscuit composition were provided in the article.

The Covid-19 Pandemic has also been a limiting factor in my research because, at times, it was difficult to gain access to samples during the extended lockdowns. Consequent backlogs ensued and made it challenging to process post-mortem tissue samples and blood samples for hormonal analysis. I also experienced processing delays with the ovarian tissue samples which limited a more detailed analysis of ovarian histology, especially investigations into the derivation and structure of the ovarian dark bodies, as discussed in chapter 3. Due to COVID-related backlogs in processing of clinical samples by the Monash Health Pathology Department, the planned analyses of steroid hormones in the blood samples obtained in study 1 and study 2 have not been done to provide these data for the thesis.

Future Directions

The data discussed in chapter 2, in conjunction with other studies (47, 48, 58, 59), suggest that a diet with a higher fat, and reduced carbohydrate, composition should be tried to see if this can induce obesity in SpM, and cause menstrual dysfunction and other metabolic disturbances. I suggest use of a scientifically formulated diet such as SF02-006 (Speciality Feeds Glen Forrest, WA) as it contains 60%

fat and minimal carbohydrate component or SF10-053 (Speciality Feeds Glen Forrest, WA) which contains 69% fat, with reduced carbohydrate. Using these diets may counteract the apparent adaptive physiology in SpM, when fed a high fat and high sugar diet as describe in previous studies (47, 48, 58, 59) and confirmed in the data from study 1. Based on our results and results from other studies, it appears the SpM may have a protective physiology that safeguards them from excessive weight gain from a typical western diet that is high in both sugar and fat. There could be potential research into investigating the exact mechanism further, as a potential avenue into treatment strategies regarding the obesity epidemic (37, 39, 47, 58, 59, 66, 74, 75). It would also be interesting to investigate if a variety of other seeds, such as pumpkin seeds, are able to increase weight in SpM and cause associated diet induced dysfunction in reproductive and metabolic health (47, 58, 59). As discussed in chapter 3, the letrozole treatment did not yield the expected results perhaps because SpM metabolise letrozole differently compared with other conventional laboratory rodents (31, 33-35). Based on this a future study could investigate optimising the dosage of letrozole, perhaps a higher dose of 12mg or higher may be tolerated by SpM and be required to produce PCOS symptoms observed in other rodents. Letrozole exposure could be extended to 90 days and vaginal lavages performed after 60 days of exposure, ensuring letrozole is circulating whilst reproductive cycling samples are taken, and to eliminate the possibility of rapid reversal of the effects of letrozole, as discussed in chapter 3. Alternatively slow-release pellets could be commissioned using androgens directly - DHEA or DHT - instead of relying on the anti-aromatase actions of letrozole to investigate if some PCOS symptoms can be produced in SpM (13, 72, 76-78). Irrespective of PCOS symptoms, following on from the exposure treatment of either letrozole, DHT or DHEA the animals could be mated, and the effects of the chemical exposure could be investigated and evaluate fertility by examining if SpM are able to mate successfully and produce viable young.

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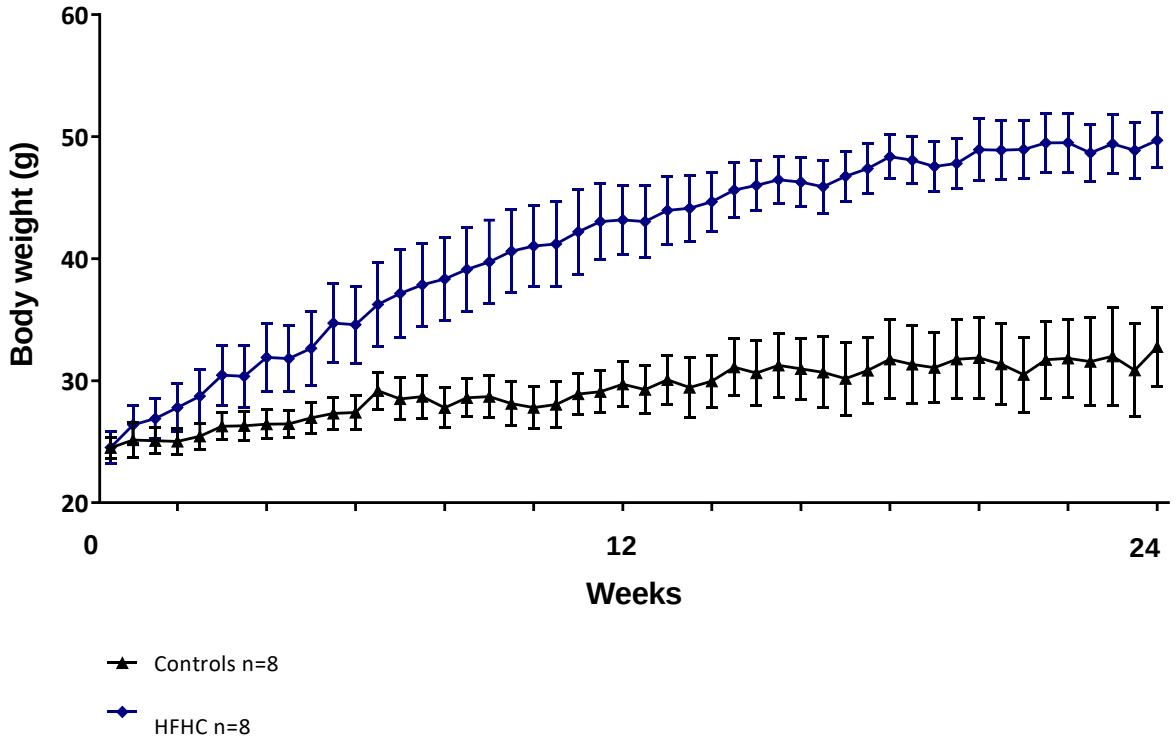
Appendices

Chapter 2

Appendix 2.1
Discussion – bodyweight data



Appendix 2.1 – figure 2.1: Unpublished bodyweight data obtained from a member of our research team using the same HFHC pellets (SF04-001 diet, Speciality Feeds Glen Forrest, WA) in C57BL6/J male mice, for 24 weeks.

Chapter 3

Appendix 3.1

Materials and methods – Letrozole implant surgery

During surgery

All animals are continuously monitored for breathing during the procedure, the isoflurane level is adjusted based on the animal's response detailed below. The approved range for induction is 5% isoflurane; maintenance on the nose cone is 1-2.5% isoflurane.

Breathing:

0 – normal: continue to monitor.

1 – shallow: reduce isoflurane level, increase monitoring until a score of 0 is reached.

2 – very shallow: remove animal and flush with oxygen and use gentle palpations, increase monitoring until a score of 0 is reached.

OR

0 – normal: continue to monitor.

1 – fast breathing, check reflexes (pedal and eye) if there is a response, slightly increase isoflurane. Monitor breathing closely.

2 – No previous isoflurane increase: very fast breathing, check reflexes (pedal and eye) if there is a response, slightly increase isoflurane. Monitor breathing very closely. If this is the second increase of isoflurane be extremely cautious, only increase if the animal still has reflexes and fast breathing after 1 minute since the previous increase.

Reflexes:

0 – normal: no response to pedal and eye stimulation

1 – slight response to pedal and eye stimulation. Check breathing, if increased, very cautiously increase maintenance isoflurane.

2 – No previous isoflurane increase: increased response to pedal and eye stimulation. Check breathing, if increased, very cautiously increase maintenance isoflurane. If the isoflurane has previously been increased, wait at least 1 minute, closely monitor breathing and reflexes before cautious increasing isoflurane within approved range.

isoflurane: maintenance on the nose cone is 1-2.5% isoflurane.

Appendix 3.1 – Table 3.1: *Monitoring of SpM study groups during letrozole implant surgery.*

Control SpM	0-2 mins of surgery	2-5 mins of surgery	5-8 mins of surgery	8-10 mins of surgery	10-15 mins of surgery
Breathing	0	0	0	0	0
Reflexes (pedal)	0	1	0	0	0
Reflexes (eyes)	0	0	0	0	0

2mg SpM	0-2 mins of surgery	2-5 mins of surgery	5-8 mins of surgery	8-10 mins of surgery	10-15 mins of surgery
Breathing	1	0	0	0	0
Reflexes (pedal)	0	0	0	0	0
Reflexes (eyes)	0	0	0	0	0

4mg SpM	0-2 mins of surgery	2-5 mins of surgery	5-8 mins of surgery	8-10 mins of surgery	10-15 mins of surgery
Breathing	1	0	0	0	0
Reflexes (pedal)	1	0	0	0	0
Reflexes (eyes)	1	0	0	0	0

8mg SpM	0-2 mins of surgery	2-5 mins of surgery	5-8 mins of surgery	8-10 mins of surgery	10-15 mins of surgery
Breathing	0	0	0	0	0
Reflexes (pedal)	0	1	0	0	0
Reflexes (eyes)	0	0	0	0	0

Post-surgery

All animals are continuously monitored post-surgery as detailed below. Closely monitor breathing in animals once off isoflurane. Surgery on the next animal will not commence until the previous animal is awake and displaying normal alertness.

Breathing:

0 – normal: continue to monitor.

1 – shallow: flush with oxygen, increase monitoring until a score of 0 is reached.

2 – very shallow: flush with oxygen and use gentle palpations, increase monitoring until a score of 0 is reached.

Awake:

0 – Normal: sniffing, gentle movements, becoming gradually more alert.

1 – Slow: flush with oxygen, gently caress animal.

2 – Very slow: flush with oxygen, gently caress animal and watch breathing very closely.

Alert

0 – normal: slowly moving around in the cage, gradually normalising. Responds normally to stimuli.

1 – slow: still slowly moving around cage / slight incoordination. Decreased response to stimuli. Increase monitoring.

2 – very slow: if slow movement is still observed, watch for signs of fitting / shallow breathing (gently flush with oxygen if this occurs), increase monitoring.

Movement:

0 – Normal gait, active climbing on wire, looping, steady movement. May huddle or hid in red tube.

1 – slight incoordination, not very active. Increase monitoring to ensure normalisation of movement.

2 – very uncoordinated / abnormal balance. Increase monitoring to ensure normalisation of movement.

Appendix 3.1 – Table 3.2: *Monitoring of SpM study groups post letrozole implant surgery.*

Control SpM	0-2 mins post-surgery	2-5 mins post-surgery	5-8 mins post-surgery	8-10 mins post-surgery	>10 mins post-surgery
Breathing	1	0	0	0	0
Awake	1	0	0	0	0
Alert	1	0	0	0	0
Movement	1	0	0	0	0

2mg SpM	0-2 mins post-surgery	2-5 mins post-surgery	5-8 mins post-surgery	8-10 mins post-surgery	>10 mins post-surgery
Breathing	1	0	0	0	0
Awake	1	0	0	0	0

Alert	1	0	0	0	0
Movement	1	0	0	0	0

4mg SpM	0-2 mins post-surgery	2-5 mins post-surgery	5-8 mins post-surgery	8-10 mins post-surgery	>10 mins post-surgery
Breathing	0	0	0	0	0
Awake	0	0	0	0	0
Alert	0	0	0	0	0
Movement	0	0	0	0	0

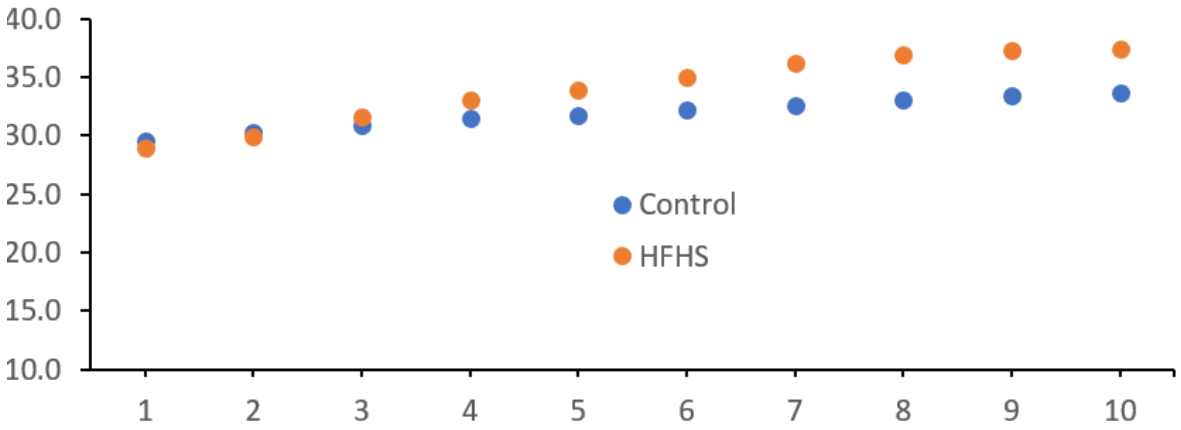
8mg SpM	0-2 mins post-surgery	2-5 mins post-surgery	5-8 mins post-surgery	8-10 mins post-surgery	>10 mins post-surgery
Breathing	0	0	0	0	0
Awake	0	0	0	0	0
Alert	0	0	0	0	0
Movement	0	0	0	0	0

Note: *These monitoring data sets are a typical representation for all groups. Unfortunately, the SpM that died during this study did not show any outward signs of distress and therefore nothing could be done to prevent their deaths. It was hypothesised that some SpM did not seem to tolerate the isoflurane as well as others, but did not visibly show this, prior to death.*

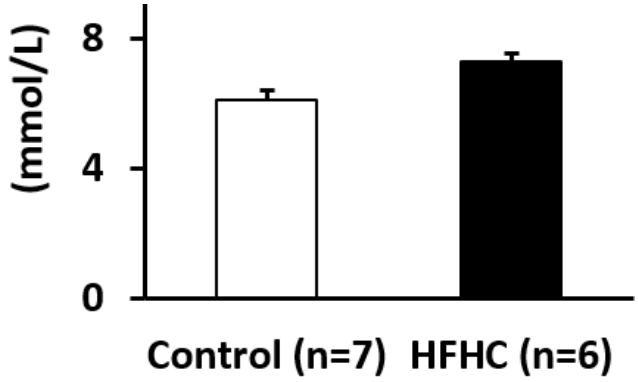
Chapter 4

Appendix 4.1

General discussion – bodyweight and fasted glucose data



Appendix 4.1 – figure 4.1: Unpublished bodyweight data obtained from a member of our research team using the same HFHC pellets (SF04-001 diet, Speciality Feeds Glen Forrest, WA) with an additional carbohydrate component in female SpM, for 10 weeks, noted as HFHS (high fat high sugar). Comparing female control SpM with HFHS diet treated female SpM. Body weight in grams and time in weeks.



Appendix 4.1 – figure 4.2: Unpublished fasted blood glucose data obtained from a member of our research team using the same HFHC pellets (SF04-001 diet, Speciality Feeds Glen Forrest, WA) with an additional carbohydrate component in female SpM, noted as HFHS (high fat high sugar). Comparing female control SpM with HFHS diet treated female SpM. Fasted blood glucose levels shown in mmol/L, data obtained at 8 weeks on the diet treatment.