Discovery and characterisation of the first menstruating rodent: The spiny mouse for use as an *in vivo* model of reproductive biology

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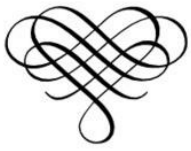
The reward of a thing well done is to have done it.

- Ralph Waldo Emerson

Dedicated to Simon James Pederick

More than life



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SUMMARY

Menstruation, the shedding of the functional layer of the endometrium at the cessation of an infertile reproductive cycle, is a strategy rarely adapted by mammals. Reproductive (oestrous) cycles in most mammalian species is under the control of external triggers, and the endometrium is resorbed rather than shed. As such, menstrual research has been confined to primarily humans and non-human primates, which has significantly hindered progression of medical breakthroughs in the identification and treatment for associated conditions. Specifically, knowledge of the underlying mechanisms of post-menstrual uterine repair, the development of pregnancy complications specific to menstruating species, and the causes of numerous endometrial conditions which have severe implications for future fertility, is limited. For example, over one hundred million women (around 1 in 8) worldwide are afflicted by endometriosis; an inflammatory disorder where endometrial lesions grow outside of the uterus, often causing painful menstruation and for which there is currently no cure. Menstrual species are also particularly vulnerable to diseases during pregnancy. The leading cause of maternal death worldwide - with a mortality rate of 10-15% - is pre-eclampsia, with evidence demonstrating that faulty decidualisation and inadequate blood vessel restructuring during the menstrual cycle is a key component of this potentially fatal disease. This condition and its associated mortality risk stems from our limited understanding of the pathophysiological mechanisms of such conditions. This thesis begins with the discovery of a menstruating rodent, the spiny mouse; the first recorded rodent to exhibit a natural menstrual cycle. The spiny mouse has a menstrual cycle which lasts approximately 9 days, with the relative timing of each of the uterine phases mimicking that of humans. Patterns of progesterone across the cycle similarly reflect those of menstruating species,

with levels peaking during the window of implantation with a rapid decline coinciding with the onset of menstrual bleeding.

This discovery perpetuated a series of studies with the primary aim of validating the spiny mouse as a species in which to conduct menstrual research. Comparison of uterine menstrual tissues from the spiny mouse, human and an artificially induced mouse model of menstruation confirmed natural spontaneous decidualisation and spiral arteriole formation as features shared between the spiny mouse and human but not with the mouse model. In addition, the spiny mouse demonstrated remarkable variation between individuals in the timing and degree of menstrual bleeding, with an inflammatory and repair profile which closely resembles that observed in naturally menstruating higher order primates.

Furthermore, the spiny mouse showed behavioural and metabolic changes associated with specific phases of their menstrual cycle. Cycling females had increased overall stress during the immediate pre-menstrual phase compared to females in their follicular phase, as evidenced by a preference for isolation, increased handling time when interacting with the investigator and a tendency for decreased exploratory behaviour. Females also fluctuated in weight, with corresponding increases in food consumption immediately prior to menstruation. The severity of premenstrual symptoms in the spiny mice was relative to individual subjects and the timing of these physiological changes suggests that spiny mice exhibit symptoms analogous to the premenstrual syndrome of higher order primates.

The evidence from these studies confirmed the spiny mouse as an appropriate model for both physiological and behavioural changes during menstruation and this was followed by a study that examined the potential of the spiny mouse as an alternative model for reproductive ageing. A menopausal transition was observed similar to that seen in women – i.e. gradual, as opposed to a sudden cessation of reproductive activity seen in most rodents. Females retained the capacity to

ovulate and menstruate up to 42 months of age, with the proportion of females cycling declining from 86% at 6 months to 44% at 42 months. However, antral follicles, uterine proliferation, ovarian and uterine weight in 42-month old females were reduced compared to 6-month old spiny mouse controls. Moreover, the total length of the menstrual cycle increased in aged females by approximately 3 days, with this being attributed to a prolonged follicular phase. These gradual changes to the menstrual cycle highlight the promise of the spiny mouse as a model for human menopause.

Finally, the reproductive biology of this species was assessed for similarities to other laboratory rodents in their response to pseudopregnancy induction and the Whitten Effect. Spiny mice did not exhibit an extended phase of receptivity to embryo implantation from progesterone injections, mechanical stimulation or sterile mating. In addition, females' menstrual cycles could not be synchronized or induced to immediate oestrus through exposure to male soiled bedding. These responses indicate the spiny mouse does not behave as a typical rodent, but rather emphasizes their comparability to higher order primates.

The data captured in this thesis provides overwhelming evidence in support of the spiny mouse as a novel animal model of menstruation. I have identified, for the first time, a rodent that exhibits physiological and behavioural features found only in menstruating species and demonstrated the need to account for individual variations in these features, as required in humans. I further emphasize the relevance of the spiny mouse as a valid model in which to study menstruation and associated conditions and advocate for this underutilized species to be recognized for its unique potential in translational studies for women's reproductive health.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institute 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: _[redacted]_ **PRINT NAME**: Nadia Bellofiore **DATE**: 27/08/2018

THESIS INCLUDING PUBLISHED WORKS DECLARATION

I hereby declare that this thesis 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.

This thesis includes 3 original papers published in peer reviewed journals and 1 unpublished publication. The core theme of the thesis is exploration of a newly discovered menstruating species, the spiny mouse, and the potential for translation to studying women's reproductive health. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Ritchie Centre, Hudson Institute of Medical Research, and the Department of Obstetrics and Gynaecology, Monash University under the supervision of Dr Jemma Evans, A. Prof Peter Temple-Smith and Dr Fiona Cousins).

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of *(1, 3, 4 and 5*) my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
1	*A missing piece: the spiny mouse and the puzzle of menstruating species*	*Published*	*85% Compilation of review, formulation of hypotheses, manuscript formulation*	*1) Fiona Cousins 2% - manuscript editing* *2) Peter Temple-Smith 3% - manuscript input* *3) Jemma Evans 10% - manuscript input, structuring review*	*No* *No* *No*
3	*First evidence of a menstruating rodent: the spiny mouse*	*Published*	*80% Initial hypothesis formulation, experimental design,*	*1) Stacey Ellery 2% - manuscript input* *2) Jared Mamrot 1% - manuscript editing*	*No* *Yes*

			laboratory/animal work, data analysis, manuscript formulation	*3) David Walker 2% - manuscript input* *4) Peter Temple-Smith 5% - manuscript input* *5) Hayley Dickinson 10% - manuscript input, project planning, financial support*	*No* *No* *No*
4	*Characterisation of human-like menstruation in the spiny mouse: Comparative studies with the human and induced mouse model*	*Published*	*80% Experimental design, laboratory/animal work, data analysis, manuscript formulation*	*1) Shreya Rana 3% - sample collection, manuscript editing* *2) Hayley Dickinson 2% - manuscript editing, financial support* *3) Peter Temple-Smith 5% - manuscript input* *4) Jemma Evans 10% - manuscript input, project planning*	*Yes* *No* *No* *No*
5	*Anxiety-like behaviour and food intake are increased in the spiny mouse before menstruation: a unique pre-clinical model for examining premenstrual syndrome*	*Submitted (Under review)*	*80% Experimental design, laboratory/animal work, data analysis, manuscript formulation*	*1) Fiona Cousins – manuscript input 5%* *2) Peter Temple-Smith 5% - manuscript input* 3) *Jemma Evans 10% - manuscript input, project planning*	*No* *No* *No* *No*

**If no co-authors, leave fields blank*

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date:** 27/08/2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date:** 27/08/2018

PUBLICATIONS

Bellofiore, N., Ellery, S.J., Mamrot, J., Walker, D.W., Temple-Smith, P. and Dickinson, H., 2017. First evidence of a menstruating rodent: the spiny mouse (Acomys cahirinus). *American Journal of Obstetrics & Gynecology*, *216* (1), pp.40-e1.

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Bellofiore, N, Cousins, F., Temple-Smith, P. and Evans, J. 2018. Pre-menstrual increases in anxiety-like behaviour and food intake in the menstruating spiny mouse: implications for studies of premenstrual syndrome. *Human Reproduction (submitted July 2018, currently under review).*

CONFERENCE PROCEEDINGS AND PRESENTATIONS

2018

Bellofiore N, Cousins, F, Temple-Smith P, Evans J "Pocket Primate? The spiny mouse as a model for menstrual research" 2017 Fetal and Neonatal Workshop of Australia and New Zealand, Queenstown, New Zealand, ***Oral***

2017

Bellofiore N, Evans J, Temple-Smith P, Dickinson H "Spiny mice exhibit metabolic and behavioural changes across the menstrual cycle reflective of women and indicative of premenstrual syndrome" 2017 4th World Congress of Reproductive Biology, Okinawa, Japan ***Poster***

Bellofiore N, Evans J, Temple-Smith P, Dickinson H "The spiny mouse: a model for menstruation and reproductive ageing" 2017 Perinatal Society of Australia and New Zealand Canberra, Australia ***Oral/Poster***

Bellofiore N, Evans J, Temple-Smith P, Dickinson H "Increased anxiety during the premenstrual phase in the spiny mouse" 2017 Endocrine Society of Australia/Society for Reproductive Biology Perth, Australia ***Poster***

Bellofiore N, Evans J, Temple-Smith P, Dickinson H "Increased anxiety during the premenstrual phase in the spiny mouse" 2017 Fetal and Neonatal Workshop of Australia and New Zealand Magnetic Island, Australia ***Oral***

2016

Bellofiore N, Ellery SJ, Mamrot J, Walker DW, Temple-Smith P, Dickinson H "First evidence of a menstruating rodent: the spiny mouse (*Acomys cahirinus*)" 2016 Society for Reproductive Investigation, Canada ***Poster***

Bellofiore N, Ellery SJ, Walker DW, Temple-Smith P, Dickinson H "Pre-menstrual mouse? The common spiny mouse as a model for PMS" 2016 Fetal and Neonatal Workshop of Australia and New Zealand Magnetic Island, Australia ***Oral***

Bellofiore N, Ellery SJ, Mamrot J, Walker DW, Temple-Smith P, Dickinson H "First evidence of a menstruating rodent: the spiny mouse (*Acomys cahirinus*)" 2016 Perinatal Society of Australia and New Zealand Townsville, Australia ***Oral***

Bellofiore N, Ellery SJ, Mamrot J, Walker DW, Temple-Smith P, Dickinson H "Magnificent menstruating mammals: The spiny mouse (*Acomys cahirinus*)" 2016 Australian Society for Medical Research, Melbourne, AUS ***Poster***

Bellofiore N, Ellery SJ, Mamrot J, Walker DW, Temple-Smith P, Dickinson H "First

evidence of a menstruating rodent: the spiny mouse (*Acomys cahirinus*)" 2016 Society for Reproduction and Fertility, United Kingdom ***Poster***

Bellofiore N, Ellery SJ, Mamrot J, Walker DW, Temple-Smith P, Dickinson H "Of mice and men: the spiny mouse as a natural model of menstruation" 2016 Fertility Society of Australia, Perth, Australia ***Oral***

Bellofiore N, Evan J, Walker DW, Temple-Smith P, Dickinson H "Endometrial morphology across the menstrual cycle in the spiny mouse" 2016 Endocrine Society of Australia/Society for Reproductive Biology, Gold Coast, Australia ***Oral*** (**New Investigator Award Finalist**)

2015

Bellofiore N, Mamrot J, Bernadette TB, Walker DW, Temple-Smith P, Dickinson H "Pseudopregnancy induction in the spiny mouse" 2015 Fetal and Neonatal Workshop of Australia and New Zealand, Melbourne, AUS ***Oral***

Bellofiore N, Mamrot J, Bernadette TB, Walker DW, Temple-Smith P, Dickinson H "Pseudopregnancy induction in the spiny mouse" 2015 Endocrine Society of Australia/Society for Reproductive Biology, Adelaide, AUS ***Poster***

AWARDS AND PRIZES

2018

Monash Post-Graduate Publication Award ($4,624)

Hudson Institute of Medical Research Travel Award ($2,500)

Monash University Research Training Program Scholarship ($27, 353 p.a.)

2017

Monash University Research Training Program Scholarship ($27, 353 p.a.)

Ritchie Centre Senior PhD Student Travel Award ($1270)

Monash University Postgraduate Travel Award ($1035)

Fetal Neonatal Workshop of Australia and New Zealand Award for Late PhD Best Presentation ($200)

Society for Reproductive Biology Student Travel Award ($500)

2016

Society for Reproductive Biology Student Travel Award ($200)

3 Minute Thesis Ritchie Centre Heats: First Place (Certificate)

Perinatal Society of Australia and New Zealand President's Award for Best Presentation ($1500)

Fetal Neonatal Workshop of Australia and New Zealand Award for Early PhD Best Presentation ($200)

Perinatal Society of Australia and New Zealand Early Career Researcher Travel Award ($500)

Monash University Departmental Scholarship ($10,000 p.a.)

2015

Ritchie Centre Junior PhD Student Travel Award ($300)

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This research was supported by an Australian Government Research Training Program (RTP) Scholarship.

I have a distinct memory of sitting in a lecture theatre amongst my fellow graduate diploma students way back when, and a PhD student was giving a talk about what it was like to be said PhD student. At the end, he asked if anyone was interested in pursuing a career in research. I glanced at my friends and we burst into a fit of giggles. The notion was absolutely ridiculous.

… Well, egg on my face. I somehow find myself now sitting here, writing a thesis about something I couldn't have imagined in my most nerd-tacular of dreams. And while it would be easy to sit back and bask in the praise of my non-scientific social circles, I cannot accept it. There are so many who deserve it so much more, whose patience, guidance and shoulders to cry on are unequivocal.

To **my supervisor Peter**, who ignited my newfound passion for research, who has been there with me from ground zero: I continue to be amazed at how much time and effort you have put into mentoring me and shaping my budding research career when you have an indiscernible number of other things going on! You have this incredible way of making me feel as if I were your only student, and nothing was ever too much to ask. I am humbled by your willingness to go the extra mile for not only me, but anyone lucky enough to work with

you, for those glowing last-minute letters of recommendation, and finally, for taking on so much and asking so little in return. You are the epitome of "above and beyond".

To **my supervisor Jemma**, who taught me that *gasp!* There IS a life outside of a PhD, I am truly grateful for all your advice, for your calm and coolheaded approach to problems, work-related or otherwise, and for a level of support I could not have dreamed of. Even when I felt I couldn't cut it, you made me see that I was competent and capable. You got me through till the end, and I don't think I would have without our coffee sessions and certainly not without your friendship. You have inspired me, not just through your career and all you have achieved, but with your constantly upbeat attitude and your ability to prioritise what's truly important in life.

To **my supervisor Fiona**, who kept me grounded and stable when I needed a down to earth opinion, thank you for taking me on so late in the game. I don't know how I would've managed without your super-efficient feedback and I know that I have only achieved this because of the help you have given me.

To my **uni buddies**, **Lara Rijkmans** and **Shreya Rana**, this trio is undoubtedly the best outcome of these past 3 years. I am indebted to Lara for coming and giving my dog so many cuddles when I wasn't able, and for her chocolate entrance fee to the house which kept me soldiering on with this thesis. Shreya, we are pancake buddies for life – that bond is unbreakable. I would never have made it even halfway without our "coffee" dates (which somehow spanned several hours at a time). To **Madi Paton,** desk buddies for ever! You made the nightmare of writing up so much more bearable.

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xx

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To **my family, Ma, Poolo, Laura** and **Cara**, your unwavering support, especially when I missed out on awards and scholarships, will never be forgotten. If I fail, I only keep trying because of your unrivalled faith in me. If I achieve anything in life, it is because of you.

Finally, **my fiancé Simon**. Firstly, thanks for proposing in my final year and genuinely surprising me; I honestly never thought you could pull it off, but alas, here we are! Secondly, I could write another entire thesis and never come close to touching on how many times you were my rock; how many hours were spent consoling me over the bad days, and rejoicing over the good; how many times you thought if you hugged me so intensely you might absorb my stress so I wouldn't have to feel it (P.S. it did not work)… not to mention how sick of hearing about the same thing over and over you must have been! There were so many times I wanted to express how much more this journey has meant because you were in it with me from the start. And now, as it finally comes to an end, I am struggling to find the words; at last the incessant chattering of my mind is clear… because my heart is full.

This is for us.

LIST OF ABBREVIATIONS

A	alpha
~	approximately
B	beta
°C	degrees Celsius
=	equals
>	greater than
≥	greater than or equal to
<	less than
≤	less than or equal to
-	minus
%	percent
+	plus
±	plus or minus
aSMA	alpha smooth muscle actin
ANOVA	analysis of variance
AMH	Anti-Müllerian Hormone
AB	antibody
BSA	bovine serum albumin
CD45	leukocyte common antigen
cm	centimetre/s
CFSG	cold fish skin gelatine
DSC	decidualised stromal cells
Di	diestrus

Endo	endometrium
ESF	endometrial stromal fibroblasts
ELISA	enzyme-linked immunosorbent assay
eCG	equine chorionic gonadatrophin
EtOH	ethanol
FSH	follicle-stimulating hormone
GEE	generalised estimating equations
GnRH	gonadotrophin releasing hormone
g	grams
HPLC	high-performance liquid chromatography
HMB	heavy menstrual bleeding
h	hour/s
Hu	human/s
hCG	human chorionic gonadotrophin
IF	immunofluorescence
IHC	immunohistochemistry
IL	Interleukin
Kg	kilograms
L	litres
LH	luteinising hormone
MIF	macrophage inhibitory factor
mens	menses
Met	metestrus
M	metre/s

mL	millilitre/s
Mm	millimetres
mM	millimolar
Min	minute/s
M	molar
MMoM	mouse model of menstruation
Myo	myometrium
NGAL	neutrophil gelatinase associated lipocalin
NDS	normal donkey serum
NGS	normal goat serum
NHS	normal horse serum
NRS	normal rabbit serum
E2	oestradiol
Oes	oestrus
OVX	ovariectomised
PBS	phosphate-buffered saline
Poly I:C	polyinosinic:polycytidylic acid
PMS	premenstrual syndrome
Pro	proestrus
P_4	progesterone
PRL	prolactin
PCNA	proliferating nuclear cell antigen
PGF	prostaglandin F
RPL	recurrent pregnancy loss

rpm	revolutions per minute
s/sec	second/s
SpM	spiny mouse/spiny mice
SD	standard deviation
SEM	standard error of mean
TBS	tris-buffered saline
Tris-EDTA	tris-ethylenediaminetetraacetic acid
uM	micrometre/s
VEGF	vascular endothelial growth factor

CHAPTER 1:
INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

1.1 Overview

Herein I describe my discovery of the only menstruating rodent, the common spiny mouse (*Acomys cahirinus*). A feature of true menstruating species includes an active luteal phase, in which a fully functional corpus luteum synthesises progesterone to prepare the endometrium for impending embryo implantation. A series of morphological changes is mediated by progesterone in this animal, including a spontaneous decidual reaction and spiral artery remodelling. Withdrawal of progesterone causes shedding of this differentiated endometrium, i.e. menstruation.

Until now, a rodent has never been reported to exhibit these features, and the recurring question of why this reproductive strategy may have evolved in this species alone is still largely a mystery. This chapter explores the possible links between menstruating species (chiefly, higher order primates) and how the spiny mouse may have evolved menstruation.

1.2 Overall Research Questions and Thesis Aims

Menstruation in a rodent is unprecedented and a completely unique and unexpected finding of this thesis. As such, no data currently exists in the female spiny mouse on the mechanisms of menstruation, key hormones involved or menstrual heterogeneity, behavioural or metabolic changes across the cycle, or how age impacts the menstrual cycle. Hence, the purpose of this thesis is to provide the first comprehensive analysis and characterise this phenomenon.

1.3 Review of Relevant Literature

Published (below):

REVIEW

A missing piece: the spiny mouse and the puzzle of menstruating species

Nadia Bellofiore[1,2], Fiona Cousins[1,2], Peter Temple-Smith[2], Hayley Dickinson[1,2] and Jemma Evans[3]

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Abstract

We recently discovered the first known menstruating rodent. With the exception of four bats and the elephant shrew, the common spiny mouse (*Acomys cahirinus*) is the only species outside the primate order to exhibit menses. There are few widely accepted theories on why menstruation developed as the preferred reproductive strategy of these select mammals, all of which reference the evolution of spontaneous decidualisation prior to menstrual shedding. Though menstruating species share several reproductive traits, there has been no identifiable feature unique to menstruating species. Such a feature might suggest why spontaneous decidualisation, and thus menstruation, evolved in these species. We propose that a ≥3-fold increase in progesterone during the luteal phase of the reproductive cycle is a unique characteristic linking menstruating species. We discuss spontaneous decidualisation as a consequence of high progesterone, and the potential role of prolactin in screening for defective embryos in these species to aid in minimising implantation of abnormal embryos. We further explore the possible impact of nutrition in selecting species to undergo spontaneous decidualisation and subsequent menstruation. We summarise the current knowledge of menstruation, discuss current pre-clinical models of menstruation and how the spiny mouse may benefit advancing our understanding of this rare biological phenomenon.

Key Words

- menstruation
- evolution
- decidualisation
- hormones

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Introduction

Menstruation occurs in a select number of mammals. The majority of menstruating species are higher order primates, including humans and Old World monkeys (Emera *et al*. 2012, O'Neil 2014, The International Union for Conservation of Nature and Natural Resources 2015); only the elephant shrew (Van der Horst & Gillman 1941) and four species of bats (Rasweiler 1991, Rasweiler & De Bonilla 1992, Zhang *et al*. 2007) have also been shown to menstruate. Evolutionary links between these menstruating species are ambiguous.

Shedding of the superficial layer of the endometrium (stratum functionalis) in the absence of embryonic implantation is the discerning feature separating menstruating and non-menstruating species. The limited number of menstruating species, particularly laboratory species, has restricted research into this poorly understood biological process. Specifically, knowledge of the underlying mechanisms of post-menstrual endometrial repair the development of pregnancy complications specific to menstruating species, and infertility of

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non-gonadal origin remain elusive (Salamonsen 2003, Brosens *et al.* 2009, Treloar *et al.* 2010).

We recently discovered the common or Egyptian spiny mouse (*Acomys cahirinus*), a rodent native to Africa and the Middle East, undergoes spontaneous decidualisation and subsequent menstruation, being the only known rodent to exhibit overt menses (Bellofiore *et al.* 2017). This discovery has the potential to supersede the current induced models of menses and aid in unmasking the mechanisms of menstrual associated disorders in women and provide a pre-clinical model for pharmacokinetic studies of drug targets for contraceptives.

This review will provide an update on what is currently known about the menstrual cycles of primate and non-primate species, with an emphasis on the spiny mouse. It will also highlight the potential use of the spiny mouse as a translational model for menstruation and menstrual disorders and propose a new hypothesis to explain why menstruation may have evolved.

Female mammalian reproductive cycles

Around half the world's population are women; billions of women therefore undergo menstruation each month for approximately a third of their lives. As humans experience menses, it is a common assumption that cyclical endometrial shedding at the conclusion of an infertile cycle is widely experienced throughout the animal kingdom. However, menstruation as a cyclical physiological phenomenon is surprisingly rare, with the majority of female reproduction in mammals characterised by oestrous cycles.

Common to both oestrous and menstruating species are the physiological changes to the reproductive tract in preparation for mating and pregnancy, which occur in response to ovarian steroidal hormones. Oestradiol-17B is the key hormone during the follicular phase of the ovarian cycle (proliferative phase of the uterine cycle) leading up to ovulation, while progesterone is essential during the luteal phase of the ovarian cycle (secretory phase of the uterine cycle) when females are most receptive to embryo implantation.

During the follicular phase of menstrual/oestrous cycles, final maturation of a dominant ovarian follicle occurs. A substantial increase in oestrogen production triggers a pre-ovulatory surge in luteinising hormone (LH) and subsequent ovulation. In species with an oestrous cycle, these ovulatory triggers are variable and dictated by the environment. Oestrous mammals generally remain under-influenced by factors such as nutrition, temperature, rainfall and light. In the case of light-mediated reproductive regulation, the pineal gland and melatonin secretion are particularly important (Downey 1980). Hence, in most of the mammalian kingdom, mating is restricted to a specified breeding season and/or relies on induction from male pheromones or vagino-cervical stimulation from coitus, within a very discrete window of female receptivity (oestrus/'heat') during the late follicular phase. In contrast, menstruating species exhibit cyclical ovulation, irrespective of light, temperature or pheromonal cues, and spontaneously transition into their active luteal phase (Witherspoon 1971, Schams *et al.* 1977, Downey 1980).

Some mammalian species do not exhibit a luteal phase at all unless ovulation is first triggered. Cats, ferrets, squirrels and rabbits are all reflex ovulators; only ovulating after a surge in LH resulting from the mechanical stimulation by male genitalia. In these species, an excitatory threshold during coitus is required in order to stimulate pituitary LH production of sufficient levels to induce follicular rupture, followed by formation of a functional, progesterone-secreting corpus luteum to initiate a luteal phase (Downey 1980). Figure 1 summarises key differences between menstruating and oestrous species.

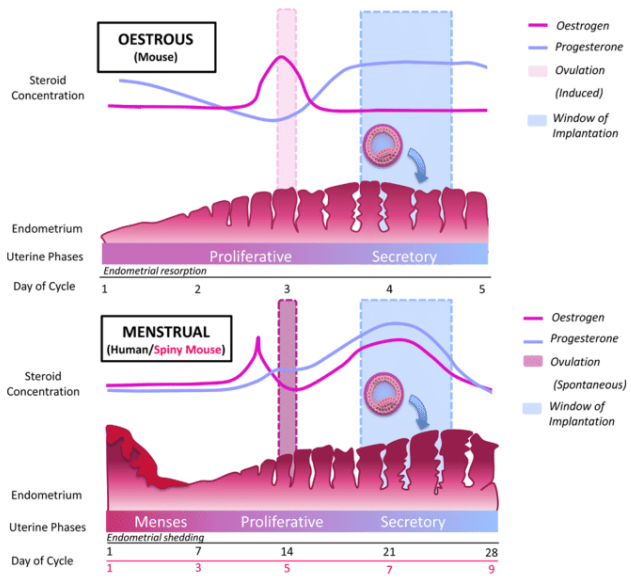

Figure 1
Comparative reproductive cycles of menstrual vs oestrous mammals. In oestrous species (e.g. wild mice), ovulation is triggered by seasonal cues, and a decline in progesterone leads to endometrial resorption. In menstruating species (e.g. the human and the captive spiny mouse), breeding is year-round and rapid withdrawal of progesterone leads to endometrial shedding i.e. menstruation.

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In the absence of pregnancy, luteolysis, functional degradation of the corpus luteum, is an active process in oestrous mammals that requires a complex endocrine interplay between the uterus and the ovary. Endometrial secretions of the luteolytic agent prostaglandin F2-alpha (PGF2A) are transferred from the uterine vein and to the ovarian artery. Here, PGF2A acts directly to induce the regression of the corpus luteum (McCracken *et al.* 1972, Auletta & Flint 1988). In women and other menstruating species, however, this process is a primarily ovarian-driven event (McCracken *et al.* 1999). The lack of a viable pregnancy and subsequent luteotrophic rescue signal from human chorionic gonadotrophin (hCG), results in the corpus luteum's inability to sustain progesterone production, which is followed by a cascade of endometrial events leading to menstruation and initiation of a new fertile cycle.

Endocrine regulation of the luteal phase and spontaneous decidualisation in menstruating species

In menstrual species, hormonal priming of the uterus prepares the endometrium for implantation, irrespective of the presence of a blastocyst. Dynamic changes to the endometrial stromal fibroblasts (ESFs) are stimulated through progesterone, regardless of exogenous signals from a developing embryo. Progesterone promotes the differentiation of ESFs into decidualised stromal cells (DSC); a terminal event in menstruating species (Brosens & Gellersen 2006). While progesterone withdrawal by the regressing corpus luteum occurs in all oestrous mammals, causing regression of the endometrium, breakdown and shedding of the decidualised endometrium is only observed after regression of the corpus luteum and subsequent progesterone withdrawal in menstruating species (Brosens *et al.* 2009). DSC have a significant upregulation of progesterone receptors (PR) which, when activated, result in the production of anti-abortive factors such as progesterone-induced blocking factor and to signal recruitment of leukocytes in preparation for implantation (Druckmann & Druckmann 2005). In the absence of successful embryo implantation, declining progesterone levels mediated by corpora lutea regression results in the withdrawal of luteal support from DSCs leading to an increase in production of inflammatory chemokines and cytokines known to mediate inflammatory leukocyte recruitment (Evans & Salamonsen 2014). Inflammatory leukocytes perpetuate the inflammatory loop and release potent protease enzymes to initiate tissue breakdown (Salamonsen 2003). Endometrial stromal cells can no longer maintain haemostasis, resulting in focal bleeding and commitment to apoptotic pathways. In non-menstruating species, which do not exhibit a spontaneous decidual response, and where endometrial stroma is therefore not terminally differentiated, the decline in progesterone results in endometrial resorption rather than shedding.

Acomys cahirinus: the first known menstruating rodent

The common spiny mouse (*A. cahirinus*) is a Middle Eastern rodent, already well established as unique in its physiology. Although not a common laboratory species, it has proved to be a useful animal model for biomedical research in diabetes mellitus and obesity – for which it has a notable susceptibility (Gonet *et al.* 1966, Strasser 1968), precocial fetal organ development (Oosterhuis *et al.* 1984), scar-free skin and hair follicle regeneration (Seifert *et al.* 2012) and non–human primate-like endocrinology (adrenal production of cortisol and dehydroepiandrosterone, which the common mouse and rat do not produce) (Lamers *et al.* 1986, van Weerden *et al.* 1992, Quinn *et al.* 2013).

We have confirmed the spiny mouse has a menstrual cycle ranging from 6 to 10 days (mean 8.7 ± 0.4 days) with a menstrual period lasting on average 3 days. This follows an active luteal phase with endometrial transformation of the stroma corresponding to a spontaneous decidual reaction. Although the spiny mouse menstrual cycle length is shorter than the 28-day cycle in women, the proportion of time spent menstruating accounts for 20–40% of total cycle length, which is relatively comparable to 15–35% seen in women (Bellofiore *et al.* 2017).

Our recent unexpected observation that the spiny mouse endometrium appears to demonstrate spontaneous decidualisation and subsequently menstruates, a feature of female reproduction that has never been reported in a rodent, is further evidence that suggests the unique place this species occupies in rodent phylogeny.

The evolution of menstruation remains a perplexing enigma, which is further complicated by this unexpected discovery of a novel non-primate species, which exhibits menstrual cycle qualities. The incidence of spontaneous decidualisation in the spiny mouse inarguably reiterates the necessity of this physiological process for resultant menstruation.

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Evolution of specific reproductive traits in mammalian species: the critical role of decidualisation

Mammalian systems of reproduction present an extraordinary array of evolutionary outcomes, particularly evident in pregnancy and birth. Gestation length spans from days (monotremes and marsupials) to months (humans) and even years (elephant); the mode of nutrient supply during *in utero* development varies from the oviparous monotremes hatched from leathery soft-shelled eggs to the placental, viviparous pouched metatherians in which milk acts as a 'post-birth' placenta (Guernsey *et al.* 2017) and placental eutherians. Using RNA sequencing on tissues at the feto–maternal interface, Lynch *et al.* (2015) identified uterine genes important in eutherian gestation and suggested that specific recruitment of genes rather than alterations in ancestrally expressed genes are essential for implantation and pregnancy phenotypes exhibited by menstruating species, specifically the recruited genes within DSCs. Many genes were recruited from neural systems, digestive systems and the haemolymphoid systems. They demonstrate that in menstrual species, genes recruited to the endometrium are significantly enriched for immune function, including leukocyte activation, uterine natural killer cell (uNK), T-cell and cytokine recruitment and cellular signalling. They also show a loss of endometrial ion transporters, which are known to play a crucial role in mineralisation of the egg-shell in birds, in favour of enhancement in metabolic processes. Finally, they established that recruited genes exhibit a higher degree of dynamic expression at different stages of the menstrual cycle, compared to ancestrally expressed genes. This pertains in particular to endometrial stromal cells, which are highly regulated through physiological reprogramming, elaborating the most extensive non-pregnant decidual phenotype. The regulation of recruited gene expression in the endometrium of menstruating species, particularly in regards to inflammatory genes, supports many theories that this reproductive strategy may indeed have evolved as an adaptive advantage, especially with respect to the inflammatory reaction in preparation for menstrual breakdown. However, there is no clear phylogenetic link connecting the relatively small number of diverse, menstruating species.

Species that undergo menstruation have evolved a combination of several important reproductive strategies (Table 1): cyclical ovulation, spontaneous decidualisation and extensive vascular remodelling of the endometrial stroma; aggressive trophoblast invasion; a haemochorial placenta, production of cortisol and low numbers of precocial offspring (Emera *et al.* 2012). To date, we have confirmed all of these features except method of trophoblast invasion in the spiny mouse (Quinn *et al.* 2013, Bellofiore *et al.* 2017). Notably, while the key reproductive and physiological features are common to menstruating species, none of these are *exclusive* to menstruating species. We discuss unexplored factors, which may have contributed to the evolution of menstruation later in this review.

Menstruation and spontaneous decidualisation in other non-primate species

Menstrual-like events, i.e. necrotic sloughing of endometrial tissue after failure to achieve pregnancy, have been documented in several non-primate species, but evidence of spontaneous decidualisation is inconsistent. The black mastiff bat (*Molossus rufus*) exhibits spontaneous decidualisation. However, in Pallas' long-tongued bat (*Glossophaga soricina*) and the short-tailed fruit bat (*Carollia perspicillata*), while post-ovulatory thickening of the endometrial stroma is documented to occur, decidual characteristics of these endometrial cells could not be confirmed (Hamlett 1934, Rasweiler & De Bonilla 1992). Furthermore, endometrial shedding in *G. soricina* is confined to a single bleeding event per annum (Hamlett 1934).

Vascular remodelling of the arterioles in the endometrial basalis in *G. soricina* does not embody the typical angiogenesis and diverge into the functionalis, as in primates (Rasweiler 1991). Rasweiler's study also reported that recently mated female *C. perspicillata* often exhibited endometrial shedding despite the oviducts containing early-stage pronuclear zygotes to 8-cell embryos; when in other menstruating species, the uterus would be in the early secretory phases at the time when an embryo was present in the oviducts. Rasweiler suggests menstruation would be a means of eliminating differentiated endometrium or defective embryos during the breeding season and permits females another chance to mate if a successful pregnancy was not achieved.

Similarly, menstruation in *Elephantulus* typically only occurs at the end of the breeding season (Jul–Jan). Preparation for implantation in the eastern rock elephant shrew (*Elephantulus myurus*) also differs from the typical spontaneous decidual reaction seen in primates. Rather than transformation of the functionalis in its entirety, a single polyp forms, stemming from oedematous endometrium at the restricted caudal site of implantation in

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Table 1 Reproductive and physiological characteristics of menstruating and non-menstruating mammalian pre-clinical models.

Species	Menstruation	Breeding season	Wild diet	Decidual reaction	Trophoblast invasion	Placentation	Offspring	Glucocorticoid
Spiny mouse[a]	Yes, continual	Continuous	Omnivorous	Spontaneous	Unknown	Haemochorial	Precocial	Cortisol
Human[a]	Yes, continual	Continuous	Omnivorous	Spontaneous	Aggressive	Haemochorial	Precocial	Cortisol
Rhesus monkey[a]	Yes, continual	Continuous	Omnivorous	Spontaneous	Moderate	Haemochorial	Precocial	Cortisol
Elephant shrew[a]	Yes, specified	Specified	Insectivorous, some fruit supplementation	Inconclusive evidence	Aggressive	Haemochorial, with epithelio-chorial yolk sac	Precocial	Cortisol
Black mastiff bat[a]	Yes, specified	Specified	Insectivorous	Spontaneous	Aggressive	Haemochorial	Precocial	Cortisol
Pallas' long-tongued bat[a], short-tailed fruit bat[a]	Yes, specified	Specified	Insectivorous, nectivorous	Inconclusive evidence	Aggressive	Haemochorial	Precocial	Cortisol
Mouse[b]	No	Continuous	Omnivorous	Induced	Shallow	Haemohorial	Altricial	Corticosterone
Guinea pig[b]	No	Continuous	Herbivorous	Induced	Aggressive	Haemoochorial	Precocial	Cortisol
Sheep[b], cow[b]	No	Specified	Herbivorous	Induced	Shallow	Epitheliochorial	Precocial	Cortisol

[a]Menstruating species; [b]non-menstruating species.

each uterine horn, to later give rise to an embryo chamber. Often in these instances, a decidual reaction is absent until a blastocyst is engulfed in the chamber itself, though not having yet implanted. (Van der Horst 1949). While elephant shrews exhibit haemochorial placentation, the embryo chamber later also gives rise to a yolk sac, which in fact has been suggested to be an epitheliochorial secondary placenta. If pregnancy is successful, this chamber envelopes the conceptus in a capsule-like structure, the decidua pseudocapsularis. If pregnancy is not achieved, the polyp is shed in a necrotic process akin to menstruation (Van der Horst 1949, Oduor-Okelo *et al.* 2004). Van der Horst and Gillman (1941) were unable to keep large cohorts of elephant shrews alive in captivity. They postulated females were polyoestrous during spring and summer, and ceased breeding in cooler months, but were unable to confirm accurate menstrual cycle lengths of these species.

The lack of continuous menstrual cycles throughout the year and inconclusive evidence of cyclical spontaneous decidualisation renders the elephant shrew and short-tailed fruit bat inappropriate translations for human menstrual studies.

Seasonal breeding and oestrous cycles in the spiny mouse

The spiny mouse is, to our knowledge, the only rodent with a natural, recurring menstrual cycle and may prove to be highly useful as a non-primate animal model. Interestingly, our data showing 9-day menstrual cycle (ranging 6–10 days) (Bellofiore *et al.* 2017) conflict with the previous report of an 11-day oestrous cycle (ranging from 6 to 18 days) in *A. cahirinus* (Peitz 1981). Female spiny mice in the previous cohort were fed a high-energy diet (sunflower seeds, rolled oats, Purina cat chow) and weighed 60–80 g, double the weight of reproductively aged females in our colony (30–40 g). There is evidence in human cohorts of significant increases in overall menstrual cycle length with increased female weight, and indeed amenorrhoea (absence of periods) has been observed on obese women (Harlow & Matanoski 1991, Wei *et al.* 2009, Seif *et al.* 2015). In addition, the age of females within the Peitz study was not specified. These spiny mice may not, therefore, have been cycling regularly due to either pre-/peri-pubertal or advanced age.

A substantial difference in environmental temperature between the two studies is also noted; our colony of *A. cahirinus* is maintained at 25–26°C with the previous study (Peitz 1981) maintained at 22.5°C. *A. cahirinus* is a desert species, and as such is acclimatised

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to warmer conditions, female spiny mice subjected to cooler temperatures may have experienced amenorrhea. South African spiny mice (*Acomys spinosissimus*), a related species, breed seasonally during spring (Sep–Jan), but are anovular and have reduced circulating progesterone levels and lactation between February and August, when temperatures decline below 20°C (Medger *et al.* 2010). There are very few studies on factors influencing female reproduction in wild *A. cahirinus* populations. A more recent study comparing the effects of photoperiod length on golden (*Acomys russatus*, xeric or dry desert species) and common (*A. cahirinus*, mesic or moist desert species) spiny mice by Wube *et al.* (2008) showed females of both species were unaffected by changes in photoperiod; both species maintained gonadal function under long and short photoperiods. Females of both species were of similar ages (3–4 months) to those in our study, but while vaginal lavages were conducted to monitor the cycle of females, they were only done so every 3 days, and cytology was analysed using methylene blue. It is also possible that by using methylene blue in lieu of haematoxylin and eosin, cytological signs of menstruation, comprising of dense, eosinophilic mucus, erythrocytes and cellular debris may have been overlooked. The authors of this study suggest that *A. cahirinus* could be an opportunistic breeder, capable of reproducing year-round (Wube *et al.* 2008). This suggests the common spiny mouse behaves like typical menstruating species and is unrestricted by seasonal breeding.

We can deduce that photoperiod is not the key driver of reproduction in this species of spiny mouse and also that either other *Acomys* do not menstruate at all or do not exhibit frank menses. Why then has only one species of spiny mouse evolved over menstruation? It may be that captive breeding over generations with removal of predators and stable nutrition allowed menstruation to evolve. It is a possibility that related cortisol-producing species who also have precocial young and a haemochorial placenta would adapt in the same way given the same opportunities. To better comprehend this concept, we must assess all species and theories regarding evolution of menstruation with the added knowledge of the biology of the common spiny mouse.

Leading theories for the evolution of menstruation

Menstruation for screening of defective embryos

Intercourse for menstruating species is not bound by restricted periods of heat. It remains to be confirmed whether wild *A. cahirinus* are also capable of year-round mating (as seen in our captive breeding colony), and whether copulation outside of breeding seasons are a unique trait to this species. For orangutans, chimpanzees and humans, mating can be initiated during any time of the menstrual cycle. However, full implantation of the embryo relies on appropriate epithelial changes and, importantly, decidualisation of endometrium. The decidual response is believed to serve a balance of two purposes in menstruating species: (1) to modulate the depth of trophoblast invasion as a means of maternal protection – negative regulation or anti-invasive and (2) to form a micro-environmental niche encouraging implantation and averting rejection – positive regulation or pro-invasive (Modi *et al.* 2012). This is facilitated by an influx of uterine natural killer (uNK) cells in response to secretion of chemoattractants from the decidualised endometrium (including IL-6, IL-11, IL-15, LIF and IGFBP-1), and possibly from endometrial synthesis of prolactin, in the secretory phase of the menstrual cycle (Modi *et al.* 2012, Robson *et al.* 2012). The uNK cells surround the spiral arterioles, which is the location of decidualisation initiation, to promote vessel remodelling through secretion of angiogenic factors (Ang-1 and -2, VEGF and MMPs), during early gestation and in non-pregnant decidualised cells during the secretory phase (Smith *et al.* 2009, Robson *et al.* 2012). The spiny mouse demonstrates many of these characteristics of decidualisation, including typical immune cell recruitment and secretion of hallmark pro-invasive factors, including prolactin (Bellofiore *et al.* 2017) and IL-11 (N Bellofiore, S Rana, F Cousins, H Dickinson, P Temple-Smith, J Evans, unpublished observations). This is opposed to oestrous species, which require a signal transmitted from a viable conceptus to initiate the decidual reaction when displacement of the luminal epithelial cells of the endometrium occur by the implanting trophoblast (Abrahamsohn & Zorn 1993, Clarke 1994, Emera *et al.* 2012).

Because the uterus is already primed for implantation, this results in an increased likelihood that a chromosomally abnormal embryo, produced by the union of potentially aged or reduced quality gametes, can in fact implant (Clarke 1994). The proportion of first trimester miscarriages in women due to genetic irregularities is 50–60% (Wasser & Barash 1983), and up to 90% of cleavage-stage human embryos from otherwise fertile young women, have mitotic chromosomal abnormalities.

In healthy females, the ESFs of women can discriminate embryo quality after differentiation into DSCs (Teklenburg *et al.* 2010). Salker and associates (2010)

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hypothesised that women with recurrent pregnancy loss (RPL), defined as three or more consecutive miscarriages, would likely have an impaired decidualised endometrium, unable to distinguish between healthy and developmentally defective embryos at the time of implantation. Salker *et al*. demonstrated through culturing of ESCs from patients presenting with RPL that upon standard *in vitro* decidualisation, RPL decidual cells had significantly reduced expression of prolactin (PRL), a classic decidual biomarker, but increased expression of prokineticin-1 (PROK1), imperative in embryonic reception and implantation. They went on to show treatment with hCG in RPL women had opposing effects seen in control patients, resulting in prolonged induction of PRL and PROK1 and enhanced uterine receptivity (Salker *et al*. 2010). This incited the theory of a prolonged 'window' of time during which an embryo could implant, resulting in a decreased selectivity for abnormal embryos.

Dysregulation of the endometrial decidua, therefore, significantly damages fetal–maternal interactions, and results in promotion of implantation, even of poor-quality embryos. Given the evidence of the dual roles of both pro- and anti-invasive regulation of the embryo by the decidualised cells, it may be that the risk of poor-quality embryo implantation is a fundamental driver for the evolution of menstruation. We could consider that menstrual shedding of terminally differentiated DSCs may provide a secondary 'fail-safe' mechanism, by which embryos incapable of establishing connections to the maternal blood supply, in a timely progression as would a viable embryo, could be removed with the decaying decidua.

Menstruation as a non-adaptive consequence of spontaneous decidualisation

The most current theory speculates that the answer lies within spontaneous decidualisation, which is thought to have emerged through genetic assimilation caused by conflict between the mother and foetus (Emera *et al*. 2012, Renfree 2012) and is a maternal-protective adaptation in the evolutionary 'tug-of-war' between the conflicting survival interests of each (Finn 1996, Emera *et al*. 2012, Gundling & Wildman 2015). Emera and colleagues further argue that menstruation is a non-adaptive consequence of spontaneous decidualisation. The shedding of the differentiated endometrial decidual cells results in breakdown of the tissue matrix, containing the increased number of blood vessels. With the structural integrity of the stroma surrounding these arterioles lost, the blood is expelled into the uterine lumen. This is the menstrual bleeding we observe; an adaptation of no specific benefit, but an indirect result of the evolution of spontaneous decidualisation.

The non-adaptive evolution of menstruation hypothesis is supported by observations in women, other catarrhine primates, the spiny mouse and the black mastiff bat (*Molossus rufus*) (Rasweiler 1991, Gellersen & Brosens 2014, Carter *et al*. 2015, Bellofiore *et al*. 2017), but sufficient evidence of a spontaneous decidual reaction is conflicting in the elephant shrew and short-tailed fruit bat (van der Horst 1949, Rasweiler & De Bonilla 1992).

Unravelling the mystery of menses with clues from the spiny mouse: decidualisation, progesterone and prolactin

We can say with certainty that progesterone decline is inherently important for initiation of endometrial breakdown in humans, higher order primates, the spiny mouse and the fulvous fruit bat (Brosens & Gellersen 2006, Zhang *et al*. 2007, Emera *et al*. 2012, Bellofiore *et al*. 2017), though hormonal data are not available in other bat species. When serum progesterone levels are compared across mammalian species (Table 2), we note distinctive patterns in the progesterone increases during the luteal phase (where data are available). Although variations exist in blood sample collection and detection methods, a distinct trend is evident: all menstruating species have a >3-fold increase in maximum circulating serum progesterone in the luteal phase compared to the maximal levels detected in the follicular phase. In contrast, non-menstruating species have <3-fold increase in maximum circulating serum progesterone compared to maximal levels detected in the follicular phase, and in some species (mouse, rat, guinea pig, sheep), no increase in serum progesterone is apparent. We suggest that the high ratio of luteal to follicular phase serum progesterone may represent a characteristic, which is unique to menstruating species.

The high levels progesterone necessary to induce a spontaneous decidual reaction has certainly influenced other aspects of menstruating species' hormonal profiles during the luteal phase. Where data are available, we note that some menstruating species have a noticeable secondary surge of prolactin during their late luteal phase – including the spiny mouse (refer to Table 3 and Supplementary data, see section on supplementary data given at the end of this article), as opposed to the mouse, rat and sheep, which do not (Table 3). Prolactin, a peptide

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Table 2 Approximate comparative range of plasma progesterone levels (ng/mL) in common mammals.

Species (reference)	**Blood collection**	**Detection method**	**Early follicular phase**	**Late luteal phase**	**Relative fold increase**
Spiny mouse (Bellofiore *et al.* 2017)[a]	Cardiac puncture (S)	ELISA	30–64	70–199	3.1
Human (Sherman & Korenman 1975)[a]	Venipuncture (R)	RIA	1–5	12–25	5.0
Rhesus monkey (Monroe *et al.* 1970)[a]	Femoral (R)	CPB	0–1	2–6	6.0
Baboon (Su *et al.* 1980)[a]	Saphenous/antecubita (R)	RIA	0–1	3–6	6.0
Fulvous fruit bat (Zhang *et al.* 2007)[a]	Unspecified (R)	RIA	0–1	1–3	3.0
Mouse (Nelson *et al.* 1981)[b]	Retro-orbital puncture (S)	RIA	2–3	1–2	–
Guinea pig (Croix & Franchimont 1975)[b]	Decapitation (S)	RIA	1–9	2–7	–
Sheep (Thorburn *et al.* 1969)[b]	Jugular (R)	CPB	0–2	1–3	1.5
Rat (Butcher *et al.* 1974)[b]	Decapitation (S)	CPB	5–53	2–27	–

Collections are Single (S) or Repeated (R) samples from individual subjects. Early follicular and late luteal phases in menstruating species correspond to proestrus and diestrus in non-menstruating species, respectively. Relative fold increase refers to fold increase in progesterone maximum values in late luteal phase compared to maximum values in early follicular phase. For ease of comparison, values have been rounded to nearest whole number. Values are approximated based on original data available.

[a]Non-menstruating species; [b]menstruating species.

CPB, competitive protein–binding assay; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay.

hormone long thought to be primarily synthesised in the pituitary and involved in mammary gland development and lactogenesis, can also be synthesised in other organs including the uterus and placenta and is known to play key roles in corpus luteum maintenance and immune surveillance.

In the human endometrium, prolactin synthesis coincides with decidualisation during the secretory/luteal phase and is essential for progesterone synthesis during early pregnancy in most mammals. The role of prolactin in maintaining the corpus luteum differs between species and type of reproductive cycles. In some species, prolactin exerts a luteotrophic effect. Prolactin sustains progesterone production by the thecal- and granulosa-lutein cells by inhibiting the catalysis of biologically inactive progesterone by enzyme 20-alpha-hydroxysteroid dehydrogenase (Rothchild 2013). However, prolactin can also be luteolytic, such as in the guinea pig, and in the rat, prolactin can exert both luteotrophic and luteolytic properties. The contradictory actions of prolactin have been further explored in terms of angiogenesis, whereby intact human prolactin exerts an angiogenic effect, but

Table 3 Approximate comparative ranges of plasma prolactin levels in common mammals.

	Blood collection	**Detection method**	**Proestrus/early follicular**	**Oestrus/late follicular**	**Metestrus/early luteal**	**Diestrus/late luteal**
Spiny mouse*	Cardiac puncture (S)	DXI immunoassay	3.3 ± 4.1	10.0 ± 7.9	5.0 ± 5.0	11.7 ± 2.6[c]
Human (Halbreich *et al.* 1976)[a]	Venipuncture (R)	RIA	11–13	17–28	14–22	24–34
Rhesus monkey* (Jahan *et al.* 2007)[a]	Saphenous (R)	EIA	151–176	213–239	273–285	365–485
Mouse (Sinha *et al.* 1975)[b]	Decapitation (S)	RIA	78–90	95–115	75–91	89–99
Sheep (Kann & Denamur 1974)[b]	Jugular (R)	RIA	200–300	500–600	7–40	10–40
Rat (Butcher *et al.* 1974)[b]	Decapitation (S)	RIA	12–291	12–330	12–35	12–76

Values are ng/mL excluding (*) where values are IU/L. Values are approximated based on original data available, except for the spiny mouse where values are mean ± S.D. based on raw data. Full details of DXI Immunoassay can be found in supplementary information. Collections are Single (S) or Repeated (R) samples from individual subjects. For ease of comparison, values have been rounded to nearest whole number.

[a]Menstruating species; [b]non-menstruating species; [c]denotes $P < 0.05$ compared to early follicular phase (ANOVA).

EIA, enzyme immunoassay; RIA, radioimmunoassay.

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N-terminal fragments are antiangiogenic (Struman *et al.* 1999). The potential role of prolactin in delaying corpora lutea regression and perpetuating the active luteal phase in menstruating species requires further exploration.

There is increasing evidence that prolactin is important in immune system regulation. Gubbay and associates identified novel targets in the prolactin signalling pathway; prolactin receptor–positive cells were present on uterine natural killer cells (Gubbay *et al.* 2002), employing mitogenic actions. Uterine natural killer cells are the primary leukocyte recruited during the secretory phase of the menstrual cycle.

Decidual prolactin secretion likely plays a role in immune surveillance during the menstrual cycle and supports the theory that spontaneous decidualisation evolved to screen for embryo viability. Menstruating species all possess high maternal investment in their offspring, as well as invasive placentation. For these species, any mating that occurs outside oestrus (even if only by a couple of days) could increase the probability of poorer quality gametes meeting. Given the high demands of nutrient and blood supply from the offspring, devoting energy and resources in inferior offspring is a substantial maternal risk. Prolactin could circumvent this from occurring in the following ways: (1) upregulating immunosurveillance by increasing recruitment of natural killer cells to the endometrium in preparation for the impending allogeneic pregnancy, and for these natural killer cells to then aid in adequate spiral arteriole remodelling and (2) to increase angiogenesis in preparation for aggressive trophoblast invasion.

Why would spontaneous decidualisation evolve in some species but not others?

We can reasonably infer so far that cyclical menstruation and spontaneous decidualisation are not mutually exclusive events. We can also postulate that spontaneous decidualisation evolved in species that produce cortisol to protect against implantation of lower quality embryos arising from unrestricted breeding year-round.

But why would mating evolve independent of season in these precocial species? To shed some light on one of biology's greatest peculiarities, we must examine every aspect of conceivable relevance. At first, there are seemingly few clues linking the chain of menstruating species together, which explain why, in this handful of geographically diverse mammals, menstrual cycles evolved in lieu of oestrous cycles. It is remarkably difficult finding common ground in the biology and/or ecology of menstruating species that is in fact, unique to only menstruating species. The authors here scrutinised as many potential areas of interest as possible and found little connection in the way of mating and parental care (e.g. hierarchical, monogamous, communal), male penile anatomy (e.g. spindled in the spiny mouse vs smooth in humans), female size (ranging from a 35 g spiny mouse to a 75 kg human), female uterine anatomy (e.g. the bicornuate horns of the spiny mouse vs. the fundal body of higher order primates) or average litter size (e.g. spiny mouse 2–3 vs primates, bats 1–2). However, one key factor requiring further exploration is diet.

A link between the natural diets of these species is initially unclear. Baboons, chimpanzees, rhesus monkeys and orangutans derive a majority of their energy from fruits, though foods range from seeds and flowers, to invertebrates and on occasion, small vertebrates. However, others are primarily nectar and fruit based (fruit bats and Pallas' long-tongued bat) or insectivorous, such as the elephant shrew and the black mastiff bat, surviving on insects and worms, with occasional fruit and plant matter (The International Union for Conservation of Nature and Natural Resources 2015). In the human's case, almost nothing is off limits. To complicate the mix, we now add the common spiny mouse who feasts on grubs, insects and dates (Encyclopaedia Britannica 2017). But perhaps this variation in diet provides a clue? Is it coincidence that no solely herbivorous or carnivorous menstruating species have been found? It is possible that it is not the type of diet of the mammals as such, but the availability and continual supply of energy, which may have enabled spontaneous decidualisation to evolve in precocial species.

Fruit, a major dietary component of primates and bats, and supplementary to the diets of shrews and spiny mice, are high in sucrose and fructose, and also in fibre, slowing its passage through the gastrointestinal tract and allowing more time to breakdown sucrose into glucose. In addition, fructose is metabolised to glucose in the liver, without the need for an insulin response (Lehninger *et al.* 2008). This ultimately means that the glucose from ingestion of fruit can be utilised for energy longer than other carbohydrate sources, such as starch, which would provoke an insulin response upon digestion and encourage the conversion and storage of glucose to glycogen. Fruit is not only a superior source of energy, but also is a food source available year-round in the natural habitats of the aforementioned species. Similarly, insects are not merely a source of high protein, but also moderate to high in unsaturated fats and essential fatty acids, particularly larvae (Van Huis *et al.* 2013). Most menstruating species supplement their

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diet with insects, minimising the risk of starvation or fasting should fruit and flowers be unavailable. However, perhaps a highly variable omnivorous diet could in fact become detrimental for reproduction in the ancestors of menstruating species. Nutrition is a critical element in determining breeding seasons in oestrous cycle species, as the timing of birth needs to coincide with periods of abundant food to support a lactating mother and growing neonate (Downey 1980). With the continual supply of energy, it could be that stability in nutrition throughout seasons lead to an absence of an external trigger in these species; that is, without any change in food availability, females were not environmentally stimulated to commence breeding. So, rather than these species never receiving the signal to mate, they are able to do the opposite: mate continuously throughout the year. Hence, spontaneous rather than induced ovulation evolved, but so too did the risk of mating outside of oestrus. With the potential for pregnancy to occur from the formation of poorer quality gametes, these species required a mechanism to protect maternal resources from investing in nonviable offspring, i.e. spontaneous decidualisation. The persistent cyclical differentiation and maintenance of decidualised endometrium is, however, energetically costly. But with a considerably adaptable diet, obtaining adequate nutrients to do maintain a decidual reaction is achievable. This may aid in explaining why some mammals that share many characteristics of menstruating species (e.g. guinea pig) but do not exhibit spontaneous decidualisation: the high metabolic demands of maintaining such a reaction is not satisfied by their diet, due to unpredictable or limited seasonal food availability. It may also explain why some primarily insectivorous menstruating species are still dictated by breeding seasons, as a lack in variable dietary intake may not allow for constant cyclic sustention of decidua. Figure 2 summarises our hypothesis on the evolution spontaneous decidualisation and subsequent menstruation taking into consideration dietary preferences and endocrinological profiles of menstruating species.

Decidualisation requires increased energy. Reproductive anthropologists have speculated that menstrual cycles have evolved because cyclical shedding and renewal are less energetically costly than maintaining differentiated and metabolically demanding endometrium required for implantation. Food consumption in menstruating primates has been shown to peak during the luteal phase, but not in oestrous species such as rodents (Dye & Blundell 1997). The most extreme examples of this are observed in menstruating species where 6 days' worth of food requirements can be saved over 4 cycles during

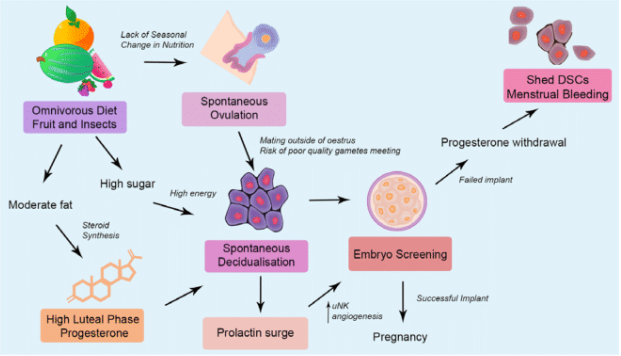

Figure 2
Proposed role of nutrition in the evolution of spontaneous decidualisation and menstruation. uNK, uterine natural killer cells; DSCs, decidualised stromal cells.

the follicular phase (Strassmann 1996). A supporting study showed a 2% increase in fat consumption in women during the late luteal phase compared to the pre-ovulatory follicular phase (Johnson *et al.* 1994). Based on the high levels of progesterone required to maintain a decidual reaction, an increase in fat consumption is likely due to the body's increasing demand for cholesterol and steroidogenesis. This, in conjunction with the dietary preferences of mammals which terminally differentiate their endometrium, coincides with reports of women experiencing increased severity of food cravings and carbohydrate intake during their premenstrual phase, with particular reference to those experiencing symptoms of premenstrual depression (Wurtman *et al.* 1989).

Current pre-clinical models for the study of menstruation and menstrual disorders

Reproductive complications exclusively associated with menstruation are pronounced in humans abnormal uterine bleeding (AUB), which includes heavy menstrual bleeding (>80 mL blood loss and/or more than 7 days of bleeding), are experienced by up to 30% of women (Liu *et al.* 2007, Knight *et al.* 2009), with reports in the United States of America of over 63% of these patients electing to undergo surgical treatment such as hysterectomy (Jensen *et al.* 2012), and an estimated annual indirect cost of $12 billion (Liu *et al.* 2007). In addition, Liu *et al.* (2007) demonstrated AUB patients received scores below the 25th percentile for health-related quality of life in categories such as pain, mental health, physical and social function. For these often silent sufferers, diagnosis and treatments are frequently reduced to invasive surgery, ineffective medications or else go untreated. This stems

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from our limited understanding of the pathophysiological mechanisms of these conditions.

Studies of true, natural menstruation and related conditions are restricted to women and higher order primates and both present with ethical, cost and resource concerns. The lack of a large scale, cost-effective and translational animal model has slowed our understanding of menstruation and menstrual-associated disorders and limited progression of therapeutic developments in this field.

To overcome these issues, researchers have developed artificial models of menstruation. Exogenous administration of reproductive hormones, in an attempt to mimic the hormonal cycling in humans, have been used in mice which, under specific conditions can be induced to undergo a menstruation-like event (Brasted *et al.* 2003, Rudolph *et al.* 2012). The first report by Finn and Pope of an induced mouse model of menstruation described the induction of decidualisation and menstrual-like breakdown of the endometrium after cessation of exogenous hormonal administration (Finn & Pope 1984). This model was later refined with administration of daily oestradiol-17B for 3 days after ovariectomy, followed by insertion of a progesterone-releasing implant and induction of decidualisation via oil injection into the uterine horn. Rapid withdrawal of progesterone support after implant removal led to a menses-like event, with a loss of tissue integrity from 4 to 8 h post progesterone removal followed by extensive shedding of endometrial tissue between 16 and 36 h (Brasted *et al.* 2003, Wang *et al.* 2013, Cousins *et al.* 2014). This development of a mouse model of menstruation overcame the ethical issues and high cost of husbandry and resources of primate models and provided a significant advance in women's reproductive research. While the substantial increase in progesterone is mimicked in the induced mouse model of menstruation, these species have not evolved an accompanying behavioural response to increase their energy intake to support such a reaction, as menstruating species do (Johnson *et al.* 1994, Strassmann 1996, Dye & Blundell 1997). This is likely one of the key contributing factors as to the necessity for artificial stimulation of the uterine decidual reaction. Moreover, the use of an ovariectomised model eliminates other important endocrine interactions, which are vital to our understanding of this complex physiological process. A lack of ovarian structures, including a corpus luteum, limits the translation of this model. For example, progesterone has been suggested to be autoregulatory, in that its secretion from the corpus luteum in fact perpetuates further synthesis (Rothchild 1981); mechanisms that cannot be mirrored in a model without lutein cells. Such hormonal pathways are still largely unexplored and ill understood, and therefore, present limitations for our reliance on induced models.

The mouse model has enabled a new understanding of the events of menstrual breakdown, and the key roles of steroidogenic input (Wang *et al.* 2013, Cousins *et al.* 2016). Efforts have been made to further refine this model and adapt it for studies of endometriosis by intraperitoneal injection of mouse or human menstrual tissue suspension (Bruner-Tran *et al.* 2009, Greaves *et al.* 2014). Endometriosis is a disorder of inflammation and endocrinology, which affects around 1 in 8 women of reproductive age worldwide. It has also been observed with similar clinical pathologies in non-human primates, including rhesus monkeys and baboons (Merrill 1968, McCann & Myers 1970, Folse & Stout 1978), and in up to 25% of individuals in captive baboon populations (D'Hooghe *et al.* 1991). Although the pathophysiology of endometriosis is not completely understood, one of the leading theories suggests it is caused by retrograde menstruation. Retrograde menstruation is the process wherein shed menstrual debris may be forced up the reproductive tract, through the fallopian tubes and into the peritoneal cavity (Sampson 1927). Here, endometrial tissue fragments seed onto organs leading to formation of endometriotic lesions, which may even bleed at the time of menstruation (Laux-Biehlmann *et al.* 2015). By definition, only menstruating species naturally develop this disorder.

Development of a small animal model, which exhibits ectopic endometrial lesions comparable phenotypically and histologically to those in women and demonstrating similar inflammatory reactions significantly advance our ability to study the pathophysiology of a poorly understood, yet highly prevalent disorder. However, despite the initial success of the induced mouse menstrual endometrium, it is not a physiologically appropriate representation of normal human menstrual events. Collection of menstrual debris through scraping of the inner uterine horn inevitably increases the probability that not only the functionalis (the superficial endometrial layer, which is shed during menstruation in humans) will be collected, but also underlying basalis, which is not shed but aids in endometrial repair, and likely myometrial tissue as well. Given that quantity of basalis and myometrial cells cannot be controlled using this method and are not shed during true menstruation, this model is not ideal physiological mimic of natural menstruation in the human.

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Additionally, although it is possible to induce a menstruation event in the mouse model, the lack of recurrent menstrual cycling ultimately means not only can the individual animals not be assessed over multiple cycles, but the model will not have natural uterine pre-conditioning, nor the diseases arising from impaired decidualisation. The mouse model also does not show vascular remodelling and hence is not able to model diseases such as pre-eclampsia, placenta accreta or recurrent miscarriage due to inadequate angiogenesis of the spiral arterioles. This emphasises the important research and clinical translation benefits of the newly discovered spiny mouse small animal model of true menstruation.

Non-human primates are ideal in terms of translation of menstrual mechanistic and disorder research to humans, due to their similarity in timing of menstrual cycles (ranging from 21 to 40 days) and the anatomy of the reproductive tract; a simplex uterus with one fundal body as opposed to the rodent duplex uterus (including the spiny mouse) with separate uterine horns. However, in research, a duplex uterus may also have an advantage for experimental purposes in providing a control and treated uterus in the same individual.

Primates have been used to study menstruation and menstrual disorders, including premenstrual syndrome (PMS). PMS is experienced by up to 75% of women and can disrupt daily function and maintenance of interpersonal relationships. In extreme cases, 10% of women exhibit symptoms of premenstrual dysphoric disorder (PMDD) and may experience recurring clinical depression and psychological distress around the time of menstruation (Reid 1991). PMS-like symptoms closely mimicking those seen in humans were first observed in baboons in captive females (Hausfater & Skoblick 1985). They confirmed female baboons displaying social withdrawal, increased solitude and increased eating prior to menstrual onset in wild populations in Kenya; the first observation of PMS in a non-human species. This important observation was among the first scientific evidence to affirm that PMS was not merely a psychological incidence, but stemmed from hormonal fluctuations, leading to greater awareness of PMS as a genuine condition. However, ethical requirements and substantial financial costs involved in the maintenance and care of research populations of non-human primates have limited the number of animals in captivity and restricted progress in these studies. Wild population studies while less invasive for the animals are complicated in terms of long-term research. The study in Kenya, reported on a large group of females, but long-term assessment of individual female behaviour to determine whether symptoms are recurring or similar in magnitude for each baboon was not possible. Here, the spiny mouse could serve as an invaluable research commodity for repeated measure, long-term studies on individual females across their menstrual cycles.

The spiny mouse and the future of menstrual research

Previous work surrounding numerous aspects of reproduction and pregnancy in *A. cahirinus* has demonstrated the capacity for this species to be invaluable for translational research. Initially established in 1986 by Lamers and colleagues, the spiny mouse produces cortisol as its circulating glucocorticoid, as opposed to murine relatives synthesising corticosterone. The resultant changes in the timing of organogenesis in the spiny mouse, in which offspring maturity on the day of birth equates to that of the rat during its second to third postnatal week, appoints the spiny mouse as a preferred rodent model of precocial development analogous to the human gestational condition (Lamers *et al.* 1986). The benefits of using a species in which postconceptual maturation is delayed in mid gestation, by contrast to altricial species, has been highlighted with particular reference to mechanisms that occur during development of the fetal brain (Brunjes 1989), kidney (Dickinson *et al.* 2005) and adrenal gland (with synthesis of DHEA in the fetal adrenal glands confirmed) (Quinn *et al.* 2013). Similarities between the placenta of the spiny mouse and primates have been established, with continual growth of the placenta throughout gestation, an increased ratio of labyrinth to spongy zone, and notable sex differences in genetic regulation of the former (O'Connell *et al.* 2013). Together, these findings have highlighted the underlying advantages of using the spiny mouse as a model to study placental and perinatal development and injury.

The newly discovered knowledge of a naturally occurring menstrual cycle in the spiny mouse in conjunction with its other shared features of primate reproduction has the potential for this species to be a powerful research tool for studying reproductive disorders. In addition to the disorders directly associated with regular tissue shedding, menstruating species are particularly vulnerable to diseases during pregnancy. The leading cause of maternal death worldwide (with a mortality rate of 10–15%) is pre-eclampsia (Duley 2009), characterised by hypertension, poor angiogenesis and shallow invasion of the trophoblast into the uterine vasculature (Huppertz

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2014), with delivery of the placenta the only current treatment of this life-threatening disease.

While these diseases are referred to as human-specific conditions, correlations between abnormalities in the menstrual cycle and such disorders are likely to exist. Brosens *et al.* (2009) emphasises that the risk of pre-eclampsia and other obstetric complications is vastly increased in young (particularly adolescent), primiparous women. They suggest that this may be due to insufficient uterine and menstrual pre-conditioning. Uterine pre-conditioning is defined here as repeated exposure to cyclical breakdown and regeneration of the uterine tissues in tolerable measures, allowing for physiological preparation for the extreme invasiveness, oxidative stress, angiogenesis and inflammatory reactions of haemochorial placentation (Brosens *et al.* 2009). Spiral arteriole remodelling is significantly compromised in pre-eclamptic women, with impaired extravillous trophoblast invasion, resulting in a highly resistant, restricted blood flow to the placental tissues, presenting clinically as hypertension (Dekker & Sibai 1998). DSCs are thought to be important through chemokine secretion in structural destruction of these arteries and the displacement of smooth muscle media to enable adequate penetration of the trophoblast cells. An inadequate decidual response or insufficient exposure to repeated shedding and repair in preparation for the extensive physiological toll of a highly demanding pregnancy is therefore hypothesised to be a key component of complications such as pre-eclampsia, and as such it is imperative we understand what constitutes an adequate decidual response during the menstrual cycle.

The possibilities of exploiting the natural menstrual cycle of a small, captive rodent species to study menstrual health and arising complications are unparalleled. Long-term studies analysing the changes to the menstrual cycle could give rise to a novel model of menopause, overcoming the current pitfalls of conventional laboratory species, such as rats and mice. These rodents experience reproductive ageing in quite a different manner to menstruating species, and cessation has a very sudden onset. In humans, the complex perimenopause transition is constituted by a gradual shortening and irregularity in menstrual cycles with trending hyper-oestrogenic ovulations in many women, accelerated loss of oocytes and endocrinological changes unable to be experimentally mimicked in rodents, such as elevated DHEA sulphate (Finch 2014). The spiny mouse, being capable of synthesising DHEA and derivatives, may provide a more genuine model of steroid and adrenal androgen interactions during reproductive senescence.

The diversity of menstrual bleeding, cycle lengths, phases of the menstrual cycle and behaviour of the spiny mouse is prominent between individual females (Bellofiore *et al.* 2018). This natural variety reinforces the promise of the spiny mouse as a comparable imitation of the human menstrual cycle, and potentially through selective breeding, we could isolate females demonstrating extreme phenotypes and isolate models of HMB, oligomenorrhea, ovulatory dysfunction and potentially, PMS. The spiny mouse also appears to exhibit spiral arteriole remodelling with decidualisation initiating from these regions in the endometrium (Fig. 3), similar to humans, suggesting the possibility of spontaneous or induced model for pre-eclampsia during spiral artery remodelling.

The novelty of the spiny mouse, however, presents a variety of obstacles which need to be addressed before wide spread use of this relatively unexplored species is a reality. To date, our captive colony has not seen an introduction of new genetic material since first establishment in 2002, and the implications of this are not yet known. Genetic studies in the spiny mouse have, in the past, been a hindrance, with significantly less data available than frequently used species. Subsequently, specific reagents for research using common laboratory techniques relying on genetic sequencing, or immune-based reactions are lacking, which in turn may produce suboptimal results. Despite these temporary setbacks, we encourage the recognition and future incorporation of the spiny mouse as an invaluable asset in the field of women's reproductive health.

Whether or not the discovery of menstruation among a captive colony of spiny mice is reflective of a natural reproductive strategy in these species or if it has somehow evolved only in said colony, requires further investigation. Regardless, we find ourselves in an extraordinary position to manipulate these circumstances to our advantage and progress our understanding of menstruation.

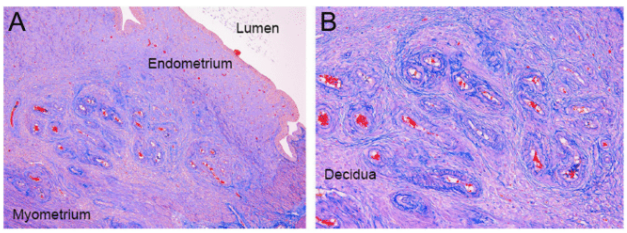

Figure 3
(A) Spiral arteriole remodelling (arrows) in the endometrium of the spiny mouse. ×100 (scale bar 200 microns) and (B) decidualisation initiating around the formation of these spiral blood vessels (arrow). ×200 (scale bar 100 microns).

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Conclusion

The first report of a small rodent model of menstruation provides an exciting prospect and important new direction for future research concerning women's reproductive health and fertility. Investigations are currently being conducted to determine the appropriateness of the common spiny mouse as a pre-clinical model for menstrual-associated diseases and pregnancy disorders. Further evidence is still required to support the leading theories of the evolution of menstruation as a non-adaptive consequence. We have determined the only discernible unique features specific to perennial menstruating species only, to our knowledge, are a high ratio of luteal:follicular phase progesterone required to maintain a spontaneous decidual reaction, and sustainable nutrition to compensate for the high energy demands of premenstrual decidua. We propose that spontaneous decidualisation has evolved in cortisol-producing species, including the spiny mouse, primarily as a means of embryo screening. We hope to attain more clues to the secrets of menstruation through the spiny mouse and its unique position as a humanesque rodent.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/JME-17-0278.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

Nadia Bellofiore: Generation of content, review of literature, formation of ideas and hypotheses, building of figures, manuscript drafting and editing. Fiona Cousins: Content refinement, manuscript editing. Peter Temple-Smith: Content refinement, formation of ideas, manuscript editing. Hayley Dickinson: Manuscript editing. Jemma Evans: Generation of content, review of literature, manuscript drafting and editing.

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CHAPTER 2: METHODS

2.1 Overview

A majority of methods in this thesis have been published or submitted for journal review. However, these published methods required extensive optimisation as I found that many standard protocols used in conventional laboratory mouse studies were not applicable to the spiny mouse. Therefore, this chapter provides details of how the various techniques used in this thesis were optimised. Additionally, the methods used in two chapters (Chapter 6: Reproductive ageing in the spiny mouse and Chapter 7: Pseudopregnancy cannot be induced using conventional methods in the spiny mouse) are presented as unpublished studies. Prior to my discovery of menstruation in the spiny mouse, the original research plan was to study pseudopregnancy in this species and an extensive effort was initially directed towards inducing pseudopregnancy in the spiny mouse for breeding purposes. Identification of menstruation in these animals explains the failure of my efforts to induce pseudopregnancy; however, the details of these studies have also been presented as evidence of the work I performed in trying to establish pseudopregnancy. Where optimisations are presented in table format, the final method used in published studies is highlighted in purple, if applicable.

The methods related to each chapter are presented in order, with the methods used in the pseudopregnancy studies presented at the end (chapter titles below, for reference).

Chapter 3: First evidence of a menstruating rodent

Chapter 4: Characterisation of human-like menstruation in the spiny mouse: comparative studies with the human and induced mouse model

Chapter 5: Anxiety-like behaviour and food intake are increased in the spiny mouse before menstruation: a unique pre-clinical model for examining premenstrual syndrome

Chapter 6: Reproductive ageing in the menstruating spiny mouse

Chapter 7: Pseudopregnancy cannot be induced using conventional methods in the spiny mouse

2.1.1 Vaginal Lavage (All Chapters)

The husbandry and maintenance of spiny mice have been previously described (1). The fragility and susceptibility to frequent tearing of the skin or inadvertent removal of spines from *Acomys cahirinus* renders the simple act of restraining the spiny mice for routine procedures a more difficult task than in commonly used laboratory rodents. The optimal method of restraining an adult spiny mouse requires a small hand towel to prevent injury to the animal. The original technique previously performed in our laboratory and the modifications I made to this protocol are described stepwise in the Table 1 below:

TABLE 2.1: OPTIMISING THE RESTRAINT AND VAGINAL LAVAGE PROTOCOL IN THE COMMON SPINY MOUSE

Timepoint	Capture of individual	Immobilisation	Restraint	Lubrication	Pipette and Lavage	Slide	Comments
Jul 2014-Jan 2015 (Original Method)	Personal preference: scoop with both hands or herd into container	Towel atop of container, hand over towel, scruff through the towel	Supine	Non-scented, water-based lubricant (Lifestyles, Ansell, Melbourne, Australia)	1mL plastic transfer pipette Squeeze 3-5 times 50-100uL saline	1 smear/slide	Efficient, but anxious females difficult to a. pick up via scooping b. grasp first go through towel 3-5 times palpating unnecessary-lose too much saline Slide wastage
Jan 2015 - Dec 2015	Herd into container	Towel atop of container, hand over towel, use fingers in corners to push towel down and surround female BEFORE scruffing	Supine, and removal of container while female parallel to floor	Non-scented, water-based lubricant (Lifestyles, Ansell, Melbourne, Australia)	1mL plastic transfer pipette, Palpate 1-3 times 50uL saline max.	3 smears/slide	Container herding in cage means lid does not have to be completely removed and more secure of other animals Manipulating towel before scruffing female reduces her movement/stress Holding container when female on back enables test of scruff before commencing procedure All sample recovered in less saline with more room on slide Ethics suggested numbing gel for lavage process
Dec 2015-May2016	Herd into container	Towel atop of container, hand over towel, use fingers in corners to push towel down and surround female BEFORE scruffing	Supine	2% Xylocaine gel	1mL plastic transfer pipette, Palpate 1-3 times 50uL saline max.	3-9 smears/slide	During behaviour documenting, stressed females tending to lick lower abdomen repeatedly. Gel also can cause excessive haematoxylin staining and difficulty staging 9 samples/slide run into each other
May 2016 - present	Herd into container	Towel atop of container, hand over towel, use fingers in corners to push towel down and surround female BEFORE scruffing	Supine	No lubrication	1mL plastic transfer pipette, Palpate 1-3 times 50uL saline max.	3-7 smears/slide	No licking observed even in extremely anxious animals and no interference with staining. No signs of pain during or after lavaging process i.e. lubrication not needed. Approve by the Ethics Committee

2.1.2 Protocol for Restraint and Vaginal Lavage of Female Spiny Mice

1. Pre-label a slide in grey lead pencil with animal ID(s), and date. Prepare a petri dish containing sterile 0.9% saline solution and draw up 25-50uL saline into a 1mL plastic transfer pipette.

2. Lift the lid of the cage with one hand and with the other, place a transparent, rectangular plastic container (roughly hand-sized) onto the cage floor with the opening facing the animals.

3. Shepherd the selected spiny mouse toward the opening of the container with your free hand. Once a female has entered/been directed into the container, quickly but gently flip the container so the opening now faces upwards.

4. Cover the container with your hand to prevent the spiny mouse escaping and remove the container with the animal. Replace the lid of the cage, ensuring no other animals have their heads or limbs caught between the wire.

5. Place a small hand towel on top of the opening of the container, either single or double folded (NB: this is personal preference when scruffing the animal - both are acceptable as long as there is a barrier between your hands and the spiny mouse to protect the skin from tearing)

6. Spread your hand over the towel with fingers in each of the four corners of the container. Use your fingers to push the towel down into each corner, taking care not to squash the spiny mouse. In this way, the female should be centred and secured by surrounding towel.

7. Scruff the female through the towel using your thumb on one side and fingers on the other side of the spiny mouse, gathering up the fur from their shoulder blades and the nape

of the neck. During this manoeuvre hold the container about eye level, to enable visualisation of the spiny mouse

8. When confident the female is secured, tilt the container and your hand holding the spiny mouse backwards so its parallel to the bench top

9. Remove the container and stroke your finger down the abdomen of the spiny mouse. An insufficiently restrained animal will wriggle to avoid this. The spiny mouse should be straight with all limbs free, no vocalisations, and breathing movements should not be restricted.

10. Insert pipette containing saline into the vaginal opening. If met with resistance, gently twist the pipette in circular motions back and forth while slowly pushing the pipette inwards. Gently squeeze the saline into canal and draw the solution back into the pipette.

11. Replace the spiny mouse into the cage by gently placing her on her feet (no need to put into container again).

12. Smear the fluid from the vaginal lavage onto the slide. Air dry at 27 degrees and fix with spray fix (Surgipath Medical Industries, Australia), Slides can be stored at room temperature for up to 14 days before staining.

2.2 Immunohistochemistry

(Chapter 3: First evidence of a menstruating rodent;

Chapter 4: Characterisation of human-like menstruation in the spiny mouse: comparative studies with the human and induced mouse model;

Chapter 6: Reproductive ageing in the menstruating spiny mouse)

As the spiny mouse is still a novel laboratory species, there are no specific antibodies commercially produced to perform immunohistochemistry in this species, unlike mice, rats and humans. For this reason, extensive optimisation of all immunohistochemistry procedures was necessary, often requiring different methods of antibody retrieval/antibody dilutions for the target tissues i.e. the degree of staining in the uterus differed from staining in the ovaries using the same method. Furthermore, immunostaining of protein in the spiny mouse tissues required antibodies derived for different species, i.e. contrary to expectations anti-mouse antibodies did not generally result in optimal immunohistochemical staining for the spiny mouse samples. For this study, optimal immunohistochemical staining of uterine tissues was achieved using anti-human (rather than anti-mouse) antibodies, as anti-mouse antibodies often produced cross-reactivity and non-specific background staining of the endometrial stromal matrix. This issue was also apparent when using anti-human antibodies raised in mouse; specific immunohistochemical staining was generally observed using goat anti-human or rabbit anti-human. These complications required extensive optimisation of immunohistochemical staining for even quite routine antibodies, such as the pan-leukocyte marker CD45 which, in human endometrial tissues stains without antigen retrieval. However, other tissues such as the brain respond sufficiently to anti-rat and anti-mouse

antibodies. The tables below detail the extensive optimisation protocols trialled in the spiny mouse.

Table key:

No AR = no antigen retrieval. Slides dewaxed, rehydrated and straight into peroxide blocking step.

Citrate 1 = simmer on medium-high in citrate buffer (pH = 6.0) for 5 min in microwave, then hot buffer retrieval for 20 mins.

Citrate 2 = boil for 9 min on high in citrate buffer (pH = 6.0), simmer for 7 min, and then hot buffer retrieval for 40 mins.

Tris-EDTA = heat buffer in water bath for 30 mins at 95-100 degrees, then hot buffer retrieval for 20 mins.

Pressure Retrieval = 5 mins in rice pressure cooker in buffer at maximum power, 40 mins in hot buffer.

TABLE 2.2.1: OPTIMISATION TRIALS OF IMMUNOHISTOCHEMISTRY PROTOCOLS FOR ABCAM RABBIT ANTI-HUMAN CD45* (#AB10558) IN THE SPINY MOUSE

Antigen Retrieval	Buffer wash	Blocking Solution	Other Steps	Antibody/Dilution	Result
No AR	3x5 min intervals ALL TBS-Tween20 0.02%	10% NGS		Rabbit anti-human CD45 1:250 1:500 1:1000	No AR = no staining
Citrate 1	3x5 min intervals ALL TBS-Tween20 0.02%	10% NGS		Rabbit anti-human CD45 1:250 1:500 1:1000	All too much background non-specific staining
Citrate 2	3x5 min intervals ALL TBS-Tween20 0.02%	10% NGS		Rabbit anti-human CD45 1:250 1:500 1:1000	All too much background non-specific staining
Citrate 1	3x5 min intervals ALL TBS-Tween20 0.02%	10% NGS 2% HuS		Rabbit anti-human CD45 1:500 1:1000 1:2000	All too much background non-specific staining
Citrate 1	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS		Rabbit anti-human CD45 1:1500 1:2000 1:3000 1:4000	Background reduced but too faint staining in 1:4000, 1:3000 1:1500 best specific staining but still too much background
Citrate 1	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS	Immediately freshly cut blocks	Rabbit anti-human CD45 1:1500 1:2000 1:3000 1:4000	No change, staining still too faint
Citrate 1	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS	Additional glycine blocking step after peroxidase step 1 hr RT Vs none	Rabbit anti-human CD45 1:1500 1:2000	Glycine no effect

Citrate 1	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	20% NGS 4%BSA 0.1% CFSG 0.1% Triton X-100 0.05% Tween 20	Antibody diluent solution: 1%BSA 2% NGS 0.1% CFSG 0.1% Triton X-100	Rabbit anti-human CD45 1:1500 1:2000	Much clearer differentiation between positive cells and background but not as dark as other specific stains of leukocytes (e.g. NGAL). 1:2000 best
DAKO PT Link in 1 x DAKO Target Retrieval Solution (Cat# S1699) at 98◦C for 30 min	DAKO EnVision Flex Wash Buffer (Cat# K8000)	DAKO Real Peroxidase Blocking Solution (Cat# S2023) for 10 min	Additional blocking: DAKO Protein Block (Cat# X0909) Secondary AB: DAKO EnVision+ System-HRP Labelled Polymer Anti Rabbit (Cat# K4003)	Rabbit anti-human CD45 1:2000 1:3000 1:4000 1:5000	Slightly better than manual staining, 1:4000 best reduction of background

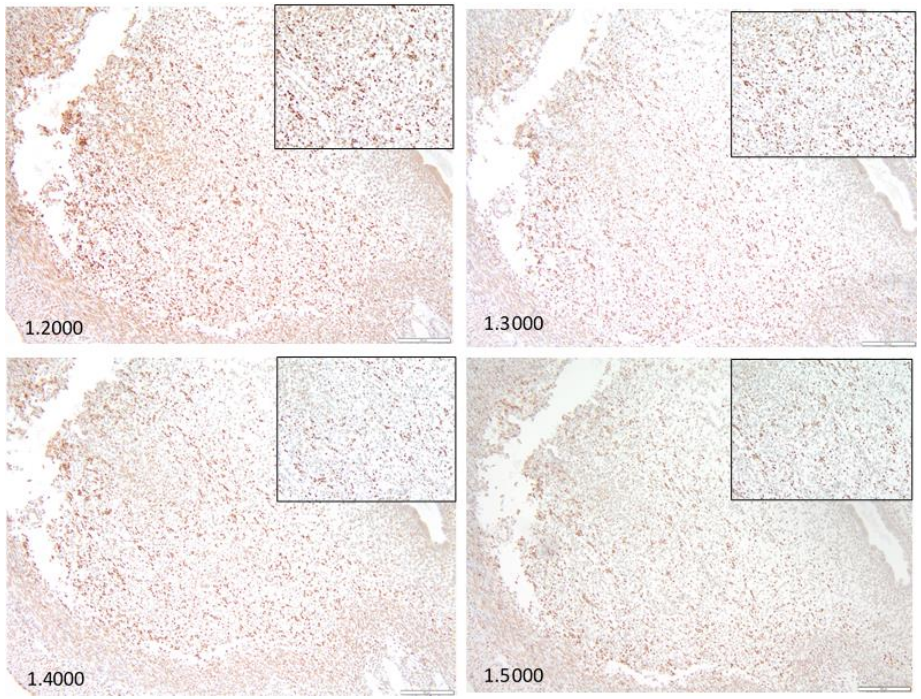


Figure 2.2.1 (Left): Optimisation of DAKO Autostainer CD45 immunohistochemistry in the endometrium of the spiny mouse. Immunopositive leukocytes are stained in brown.

*NB: CD45 data not presented due to staining inconsistency and highly variable quantification

TABLE 2.2.3: OPTIMISATION TRIALS OF IMMUNOHISTOCHEMISTRY PROTOCOLS FOR SANTA CRUZ RABBIT ANTI-HUMAN IL-11 (#SC-7924) IN THE SPINY MOUSE

Antigen Retrieval	Buffer wash	Blocking Solution	Other Steps	Antibody/Dilution	Result
No AR	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS		Rabbit anti-human IL-11 1:50 1:100 1:200	No staining
Citrate 1	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS		Rabbit anti-human IL-11 1:50 1:100 1:200	Staining very faint, even at 1:50
Citrate 2	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS		Rabbit anti-human IL-11 1:50 1:100 1:200	More staining, but still faint
Tris-EDTA	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS		Rabbit anti-human IL-11 1:50 1:100 1:200	Both 1:50 and 1:100 acceptable, but slightly non-specific in surrounding stroma.
Tris-EDTA	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	20% NGS 4%BSA 0.1% CFSG 0.1% Triton X-100 0.05% Tween 20	Antibody diluent solution: 1%BSA 2% NGS 0.1% CFSG 0.1% Triton X-100	Rabbit anti-human IL-11 1:50 1:100	No difference from above

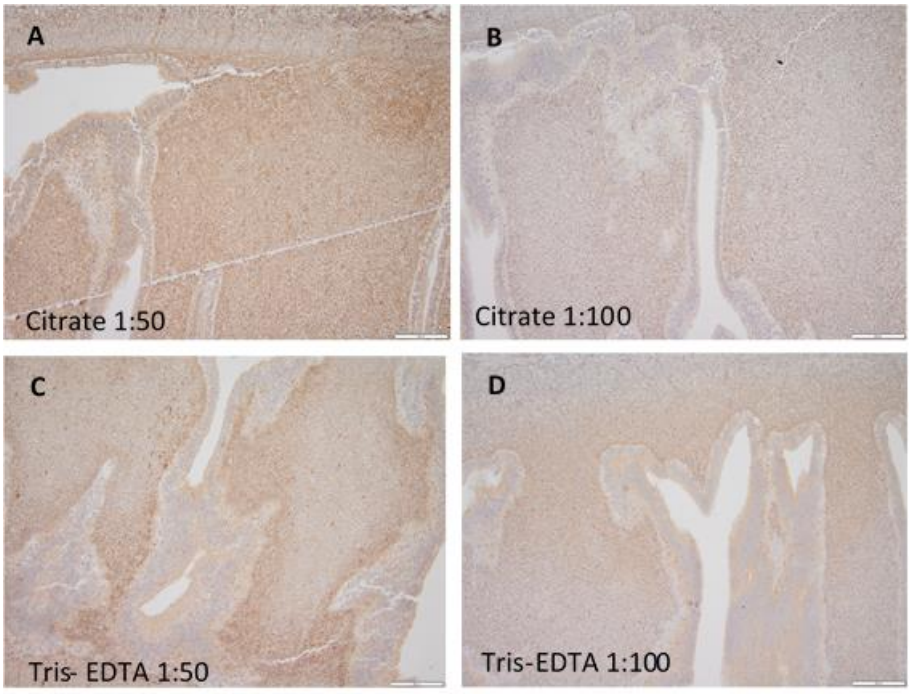


Figure 2.2.2: Optimisation of IL-11 immunohistochemistry in the endometrium of the spiny mouse. Immunopositive decidual regions are stained in brown.

TABLE 2.2.4: OPTIMISATION TRIALS OF IMMUNOHISTOCHEMISTRY PROTOCOLS FOR R&D SYSTEMS GOAT ANTI-HUMAN NGAL (#AF1757) IN THE SPINY MOUSE

Antigen Retrieval	Buffer wash	Blocking Solution	Antibody/Dilution		Result
No AR	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS	Goat NGAL 1:300 1:500 1:1000	anti-human	No staining
Citrate 1	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS	Goat NGAL 1:300 1:500 1:1000	anti-human	Staining too faint for all
Citrate 2	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NRS 2% HuS	Goat NGAL 1:300 1:500 1:1000	anti-human	Staining more prominent with longer citrate retrieval, but still too faint compared to positive controls
Citrate 2	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NRS 2% HuS	Goat NGAL 1:50 1:75 1:100 1:150 1:200	anti-human	1:50/1:75 too much background 1:100 slightly too much background 1:150/200 too faint
Citrate 2	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NRS 2% HuS	Goat NGAL 1:110	anti-human	Clear specific stains

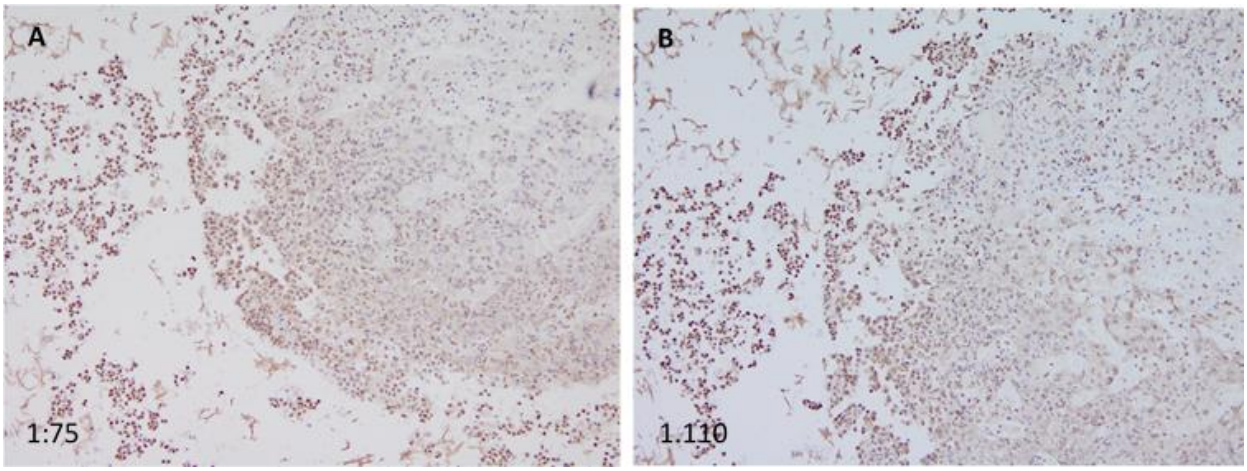


Figure 2.2.3: Optimisation of NGAL immunohistochemistry in the endometrium of the spiny mouse. Immunopositive neutrophils are stained in brown.

TABLE 2.2.5: OPTIMISATION TRIALS OF IMMUNOHISTOCHEMISTRY PROTOCOLS FOR SANTA CRUZ RABBIT ANTI-HUMAN PAN-CYTOKERATIN (#sc-15367) IN THE SPINY MOUSE

Antigen Retrieval	Buffer wash	Blocking Solution	Other Steps	Antibody/Dilution	Result
Citrate 1	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS		Rabbit anti-mouse pan-cytokeratin 1:500	Staining on IgG negative too dark and too fast. Need to bring back a little
Citrate 1	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NGS 2% HuS		Rabbit anti-mouse pan-cytokeratin 1:750 1:1000	1:1000 too faint 1:750 perfect

TABLE 2.2.6: OPTIMISATION TRIALS OF IMMUNOHISTOCHEMISTRY PROTOCOLS FOR R&D SYSTEMS GOAT ANTI-HUMAN IL-8* (#AF-208-NA) IN THE SPINY MOUSE

Antigen Retrieval	Buffer wash	Blocking Solution	Other Steps	Antibody/Dilution	Result
No AR	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NRS 2% HuS		Goat anti-human IL8 1:100 1:250 1:500	No Staining
Citrate 1	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NRS 2% HuS		Goat anti-human IL8 1:100 1:250 1:500	No Staining
Citrate 2	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NRS 2% HuS		Goat anti-human IL8 1:100 1:250 1:500	Staining only in blood vessels Some non-specific throughout endometrium and myometrium
Tris-EDTA	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NRS 2% HuS		Goat anti-human IL8 1:100 1:250 1:500	Non-specific staining
Pressure Retrieval – Citrate	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NRS 2% HuS		Goat anti-human IL8 1:100 1:250 1:500	Non-specific staining
Formic Acid	Day 1 2x TBS-Tween20 0.02%, 1X TBS Day 2 3x TBS	10% NRS 2% HuS		Goat anti-human IL8 1:100 1:250 1:500	No staining

*NB - Il-8 could not be optimised through IHC but was detectable through western blot (see Chapter 4).

TABLE 2.2.7: OPTIMISATION TRIALS OF IMMUNOHISTOCHEMISTRY PROTOCOLS FOR ABCAM RABBIT ANTI-HUMAN PCNA (#AB18197) IN THE SPINY MOUSE

Antigen Retrieval	Buffer wash	Blocking Solution	Other Steps	Antibody/Dilution	Result
No AR	Day 1	10% NGS		Rabbit anti-human PCNA	Limited staining, epithelium good but little in stroma
	2x TBS-Tween20 0.02%, 1X TBS	2% HuS		1:250	1:250 best
				1:500	
	Day 2			1:1000	
	3x TBS				
Citrate 1	Day 1	10% NGS		Rabbit anti-human PCNA	1:1000 a little too faint
	2x TBS-Tween20 0.02%, 1X TBS	2% HuS		1:250	1:250 far too strong
				1:500	1:750 best
	Day 2			1:750	
	3x TBS			1:1000	

2.3 Behavioural and Metabolic studies

(Chapter 5: Anxiety-like behaviour and food intake are increased in the spiny mouse before menstruation: a unique pre-clinical model for examining premenstrual syndrome)

2.3.1 Ovariectomised Control Females

In order to determine what, if any, influence of circulating steroid sex hormones has on the adaptation to the behavioural arenas or metabolic changes, we ovariectomised age-matched female spiny mice to use as a pilot for non-hormonally influenced controls. Spiny mice (n=5) received 5mg/kg subcutaneous Carprofen at the time of anaesthetic induction with ~5% isoflurane in room air. Depth of consciousness was monitored with the pedal reflex prior to maintenance level between 1.5-2.5% throughout the surgery. Females were placed in the prone position on warming pad. The incision site of approximately 1cm x 1.5cm was shaved and disinfected with 70% v/v ethanol. A 1cm dorsal midline incision was made adjacent to hips and the muscle layer and skin were separated gently using blunt dissection. A second 0.5cm incision was made through musculature over the left ovarian fat pad to access the peritoneal cavity. The left uterine horn was gently pulled to the opening, the ovarian fat pad clamped and organs positioned under dissecting microscope. The oviduct and ovarian artery were ligated using two Maxon® dissolvable sutures (5/0, 15 mm). The ovary was then gently removed using fine forceps and the uterine horn eased back into the body cavity. The peritoneal incision was sutured and the process repeated on the right side of the reproductive tract. The skin incision was flushed with 0.2mL topical analgesic (Bupivacaine) and the skin closed with silk Dynek® Sutures (Silk Dysilk 5/0). Females were monitored through visual

observation every 2h for 8h post-surgery, then bi-daily for the first 48 hours and daily thereafter. Spiny mice were weighed 3 days after surgery to confirm no acute loss of body weight. Post-operative meloxicam (1mg/kg) was given at 12 and 24h post-surgery. The spiny mice were allowed a minimum of 2 weeks to recover before exposure to researcher handling.

2.3.2 Metabolic Cage Recording

Standard operating procedures to collect metabolic data in the spiny mouse have previously been optimised, however, the previous studies spiny mice were typically exposed only once (after a 4-6h training period of isolation in the cage) experimentally, or if exposed multiple times, only up to three times per animal (2). An established protocol was lacking in a number of ways for the current study as the standard protocol did not take into consideration differences between male and females. This was a major issue, since the discovery that female spiny mice have a menstrual cycle highlights the potential for cycle associated weight changes which had previously been attributed to stress. To examine menstrual cycle related changes in body weight the method for metabolic cage experiments had to be refined. All optimisation studies were conducted using virgin female spiny mice aged 3-6 months. Training period refers to amount of time female acclimated to cage BEFORE 24h induction. Females would have a training period 24h prior to actual experiment.

TABLE 2.3.1: OPTIMISATION TRIALS OF METABOLIC CAGE RECORDING IN THE SPINY MOUSE IN OVARIECTOMISED (OVX) AND CYCLING FEMALES

Female ID	Training Period	Date/ Time In	Date/ Time Out	Vaginal Lavage	% Weight Change	%Food Consumed	% Water Consumed	% Urine	%Faeces	Comments
HD648 BLUE (OVX)	None (i.e. straight into met cage for 24h)	16/09/2016 10:44	17/09/2016 10:35	Prior to going in and as soon as removal from met cage	-10.01	0.00	+3.94	+1.44	+1.52	Huge weight loss, definitely requires training for both ovariectomised and cycling females.
HD684 RED (Cycling - Early Luteal)	None	19/12/16 14:15	20/12/16 14:51	Prior to going in and as soon as removal from met cage	-7.89	+1.94	+0.58	+0.85	+1.37	
HD648 Black (OVX)	4h 5/12/16 12:30-16:30	6/12/16 12:24	7/12/16 12:10	Prior to going in and as soon as removal from met cage	-4.54	+2.27	+5.17	+0.86	+1.12	
HD648 REDGREEN (OVX)	4h 6/12/16 13:15-17:03	7/12/16 14:36	8/12/16 14:25	Prior to going in and as soon as removal from met cage	-2.01	+5.14	+4.55	0.00	+1.34	Original protocol says 4-6hr sufficient, Overall still weight loss, but acceptable range for the expected stress of isolation.
HD648 GREENRED (OVX)	4h 5/12/16 10:00-14:00	6/12/16 12:28	7/12/16 12:21	Prior to going in and as soon as removal from met cage	-4.73	+4.02	+3.74	+1.25	+1.61	
HD657 Black (Cycling - Early Luteal)	4h 19/09/2016 14:23-16:30	20/9/16 09:50	21/9/16 09:47	Prior to going in and as soon as removal from met cage	-4.30	+4.53	+5.60	+0.09	+1.27	Maximum weight loss outside of met cage = ~7% during early luteal phase. 4h sufficient to keep within this range in cycling females. In theory, all other stages should be fine. Large variation in urine excretion.

HD657 REDGREEN (Cycling - Early Luteal)	4h 17/9/16 10:30 - 14:30	18/9/16 10:25	18/9/16 10:25	Prior to going in and as soon as removal from met cage	-0.20	+5.71	+2.58	+4.15	1.58	
HD657 BLACKRED (Cycling - Early Luteal)	4h 17/9/16 10:30 - 14:30	18/9/16 10:27	19/9/17 10:27	Prior to going in and as soon as removal from met cage	-1.35	+7.81	+5.52	+1.38	+1.20	
HD657 BLACKGREEN (Cycling – Late Follicular)	4h 17/9/16 10:30 – 14:30	18/9/16 10:29	18/9/16 10:30	Prior to going in only	-1.13	+4.91	+6.63	+1.38	+3.43	Reduces handling of the spiny mouse, unnecessary to get stages in and out as rare for a single stage to last significantly less than 24h.
HD668 REDBLUE (Cycling – Late Follicular)	4h 17/9/16 10:30 – 14:30	18/9/16 09:56	18/9/16 09:51	Prior to going in only	-0.78	+9.57	+12.41	+2.60	+3.81	

2.3.3 Repeated Metabolic Cages

As highlighted in Table 2.3.1, females, whether cycling or ovariectomized, must be acclimatised to the metabolic cage through a 4h training period before collecting data to minimise the rapid weight loss induced by isolation stress and familiarise the spiny mice with the water and food access. These trials demonstrated notable variation between food/water consumption and weight fluctuations. I established that the most accurate method of recording metabolic cage data across the menstrual cycle of the spiny mouse would be to map each individual animal across each of their six cycle phases (Early and late: follicular, luteal and menses). Ideally, metabolic cage recording should be blinded, i.e. without knowing the stage of menstrual cycle of the female. It is not possible to leave each animal in a metabolic cage for the duration of one menstrual cycle as ethics approval allowed a maximum period of 24h in the metabolic cage before each female must be returned to its home cage for a minimum of 24h. In addition, the investigator should be blind to stages going in to the metabolic cage. Thus, based on an average 9-day menstrual cycle for each spiny mouse, I devised a system of metabolic cage readings as follows to cover all cycle stages with a minimum of stress to each animal as to not confound the data. In this way, blinded data can be collected from 3 of the 6 menstrual cycle stages during one menstrual cycle (approximately 9-day period), assuming females continue to cycle during experiment. The data could be collected as follows:

TABLE 2.3.2: OPTIMISATION TRIALS OF REPEATED METABOLIC CAGE RECORDING IN THE CYCLING SPINY MOUSE

Day of Cycle	Stage (Rodent Terminology)	Stage (Human Terminology)	METABOLIC CAGE ACTION
Day 1	Pro	Early Follicular	IN
Day 2	Pro-Oes	Late Follicular	OUT
Day 3	Oes	Late follicular	BREAK
Day 4	Met	Early Luteal	IN
Day 5	Di	Early Luteal	OUT
Day 6	Di	Late Luteal	BREAK
Day 7	Menses	Early Menstrual	IN
Day 8	Menses	Late Menstrual	OUT
Day 9	Menses	Late Menstrual	BREAK
Day 10	Pro	Early Follicular	BREAK
Day 11	Pro-Oes	Late Follicular	IN
Day 12	Oes	Late follicular	OUT
Day 13	Met	Early Luteal	BREAK
Day 14	Di	Early Luteal	IN
Day 15	Di	Late Luteal	OUT
Day 16	Menses	Early Menstrual	BREAK
Day 17	Menses	Late Menstrual	IN
Day 18	Menses	Late Menstrual	OUT

This method can collect data from each of the stages irrespective of what stage the females starts in. However, this protocol does have some limitations. This protocol relies on every spiny mouse having a menstrual cycle of 9 days which is not the case; menstrual cycles are observed to range from 7-12 days. This protocol will not work in females that become stressed; in these animals the cycle is extended >12 days based on previously collected vaginal cytology and subsequent exclusion of subjects after experimentation. Consequently, stages may be inadvertently repeated or missed, as the examples below demonstrate:

TABLE 2.3.3: EXAMPLES OF COMPLETELY BLIND METABOLIC CAGE RECORDINGS IN THE SPINY MOUSE

ID:	Date:	Cycle Stage (SpM):	Human Equivalent
657 GR	7/12/16	Oes	LATE FOLLICULAR
	10/12/16	early di	EARLY LUTEAL
	13/12/16	late di	LATE LUTEAL
	17/12/16	early pro	EARLY FOLLICULAR
	20/12/16	early di	EARLY LUTEAL
	23/12/16	late mens-pro	LATE MENSTRUAL
	14/01/16	early mens	EARLY MENSTRUAL
657 BK	6/12/16	Early di	EARLY FOLLICULAR
	9/12/16	Late di	LATE FOLLICULAR
	12/12/16	Early Mens	EARLY MENSTRUAL
	16/12/16	Late mens	LATE MENSTRUAL
	19/12/16	Non-cycle	-
	22/12/16	Non-cycle	-

The only way to overcome these pitfalls would be to use a second investigator to either a) stage the vaginal lavages of the females and dictate which dates each female undergoes metabolic cage experiments to capture all cycle stages; or b) perform the metabolic cage experiments while the primary researcher conducts the cycle stage analysis. However, there are major issues associated with both these points including:

a. Subtlety of changes in spiny mouse vaginal cytology: these are difficult to ascertain without extensive training. From my previous experience, lack of confidence in staging the females can lead to misidentification of menstrual cycle stages and inaccurate data collection. This can be overcome with long term practice; however, given the timeline of these experiments this was not a feasible option.

b. Sensitivity of spiny mice to outside influences: the spiny mice are extremely sensitive to new scents, sounds and handlers. It is imperative that a new investigator not compromise the experiment by causing excessive stress to these animals and introducing potential confounding factors. In addition, the lavage process, order of experimental steps and handling of the spiny mouse should be consistent to eliminate inter-investigator bias.

In order to reduce excessive experimentation and exposure to the metabolic cages, data can be recorded blind to 5/6 stages. However, to avoid repeated or missed stages and without the introduction of a new investigator, one of these stages must be predicted/confirmed as in the example below:

TABLE 2.3.4: OPTIMAL METHOD OF METABOLIC CAGE RECORDINGS IN THE SPINY MOUSE

ID:	Date:	Cycle Stage (SpM)	Human Equivalent
668 BR	5/1/17	Pro	EARLY FOLL
	8/1/17	Di	LATE LUT
	10/01/17	Early mens	EALY MENS
	14/1/17	Late mens	LATE MENS
	17/01/17	Met	EARLY LUT
	22/01/2017	Oes	LATE FOLL

Due to the lack of cycle synchronicity in the spiny mouse, each female will start metabolic cage experimentation at a different stage. Hence, I do not consider the final stage being unblinded to be a significant confounding factor to the data analysis.

2.3.4 Behavioural Testing

For initial behavioural test protocol design, OVX (rather than cycling) spiny mice were assessed for behavioural changes using TopScan Lite® to determine whether females showed any indication of an adaptive response to repeated exposure to the arena after consecutive days. Females were subjected to vaginal lavage prior to placing in the tests in the following order:

AM:

Open Field (OF) (Exploratory Behaviour)

- Spiny mouse placed in 50x50cm arena (walls 60 cm high)
- Movement recorded by video camera for 10 min
- Topscan® software used to measure distance travelled by the spiny mouse, number of times spiny mouse rears or jumps, and duration spent in the inner or outer regions of the arena.

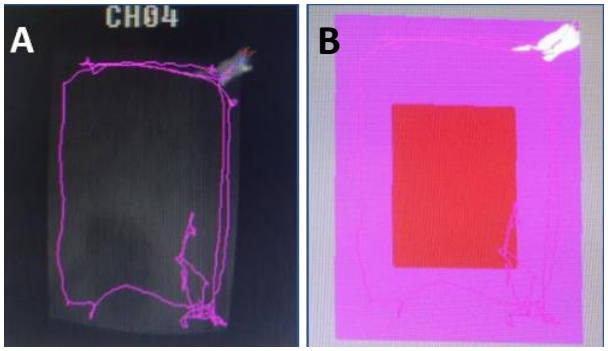


Figure 2.3.1: A) open field chamber and B) arena regions of spiny mice undergoing TopScan tracking

Novel Object Recognition Test Part 1 (NORT 1) (Exploratory Behaviour, Spatial Memory)

- Immediately after the open field test, 2 similar objects (e.g. clear plastic bottles) are placed in opposite corners of the open field.

- The time spent by the spiny mouse exploring each object are recorded over 10 min.
- Spiny mice are then returned to their home cage for 1 hour.

PM:

Novel Object Recognition Test Part 2 (NORT 2)

- One of the objects is replaced by a dissimilar object (e.g. green glass bottles) before the spiny mouse is reintroduced into the open field for 10 min.
- The periods of time spent exploring each of the different objects are recorded for comparison to those spent exploring the each of the objects.

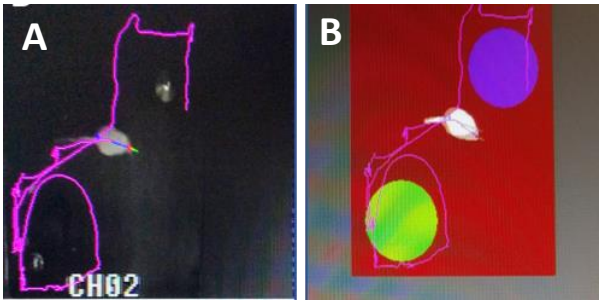


Figure 2.3.2: A) NORT chamber and B) arena regions of spiny mice undergoing TopScan tracking

Social Interaction Test (SI) (Sociality)

- Spiny mouse placed into central chamber of a rectangular box divided into 3 chambers
- Spaces in the dividing walls to allow spiny mice to move between chambers.
- The spiny mouse allowed 5 minutes to acclimatise, during which movement is recorded

- The spiny mouse is then removed and a ‘stranger’ spiny mouse (of the same age and sex, OVX) is placed in one of the small holding boxes
- The spiny mouse undergoing testing is then reintroduced into the middle chamber of the box and is allowed to freely explore for 5 minutes. After this time the test subject and ‘stranger’ will be replaced in their home cages. Topscan® software will be used to measure the period of time spent exploring the ‘stranger’ mouse.

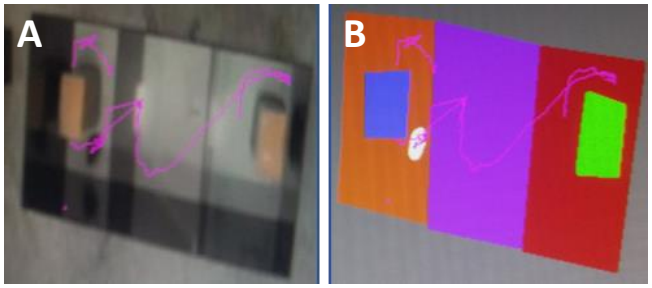


Figure 2.3.3: A) SI chamber and B) arena regions of spiny mice undergoing TopScan tracking

Elevated Plus Maze Test (EPM) (Anxiety behaviour, based on spiny mouse’s aversion to open spaces)

- The spiny mouse is placed at the intersection of the 4 arms of an elevated cross; (2 arms are open and 2 are enclosed by walls)
- Movement is recorded by video camera for a period of 5 min

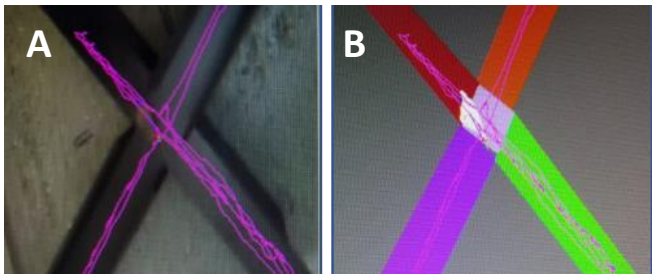


Figure 2.3.4: A) EPM chamber and B) arena regions of spiny mice undergoing TopScan tracking

Discrimination Index (measurement of preference of spiny mouse to explore particular arena):

1. OF: -1 = Inner, 0 = no preference, +1 = outer
2. NORT: -1 = Known, 0 = no preference, +1 = Unknown
3. SI: -1 = Known, 0 = no preference, +1 = Stranger
4. EPM: -1 = Open arms, 0 = no preference, +1 = Closed arms

OVX females repeated these tests on 6 consecutive days in the same order. Figures 2.3.5 and 2.3.6 show response of OVX females to OF and NORT, respectively, across consecutive days tested (timepoint). Testing in the OVX highlights that female spiny mice were capable of rapid acclimatisation to the behavioural tests and testing in cycling females needed to be conducted to minimise this. Results indicate that females retain memory of the arena and/or test order and show significant changes between first exposure and later exposures. These are:

1. OF: increase in exploration (distance travelled in, and preference for OF inner zone)
2. NORT2: decrease in discrimination index i.e. less time spent exploring unknown object

3. EPM: decrease in distance travelled in open arms

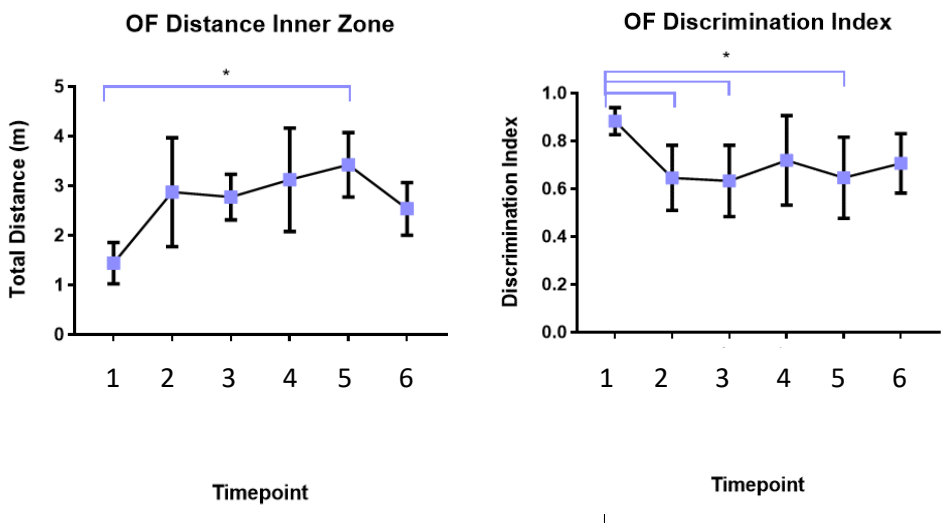


Figure 2.3.5: Open field results from ovariectomised spiny mice during optimisation of testing (data presented as mean ± SD).

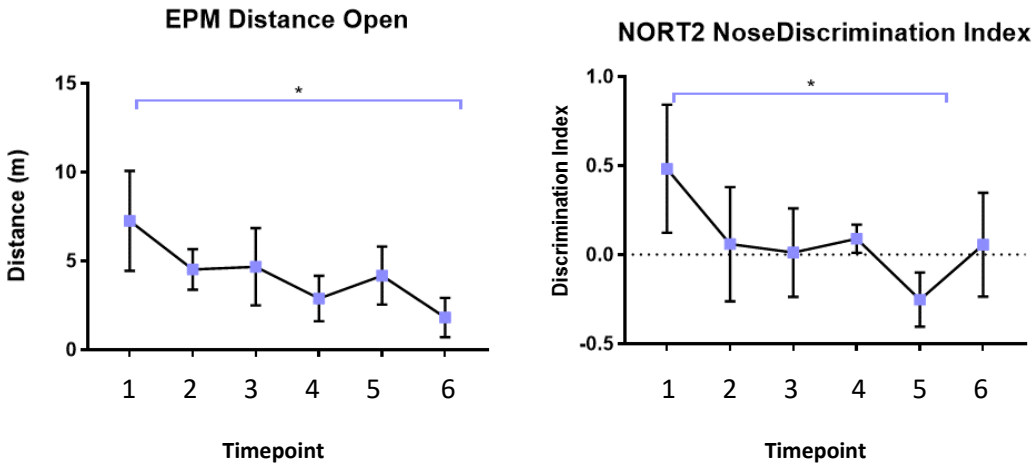
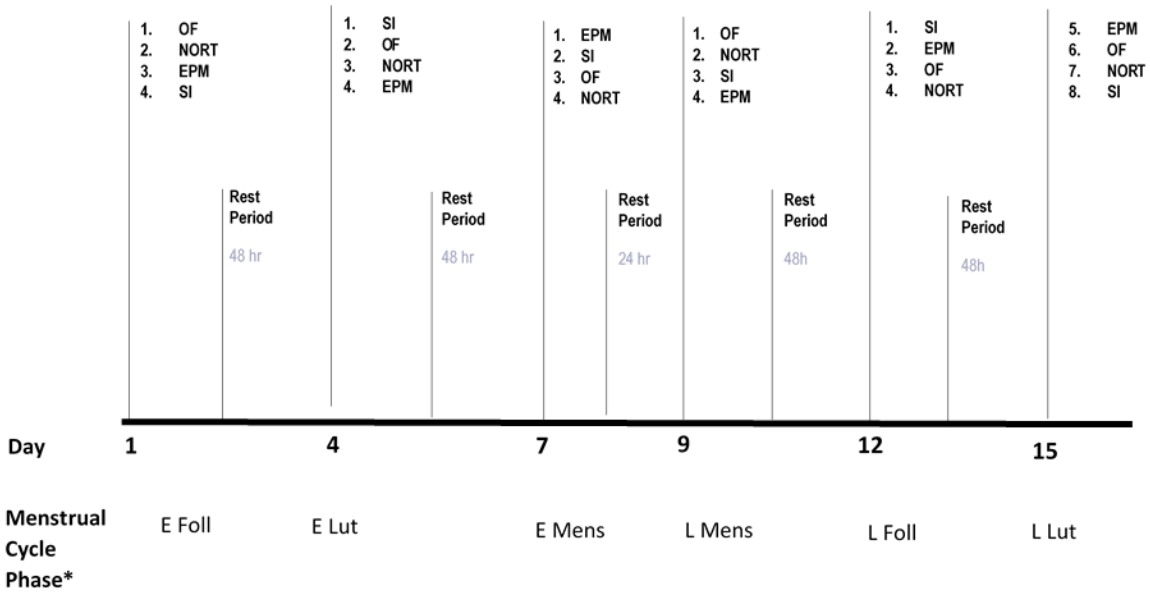


Figure 2.3.6: EPM and NORT results from ovariectomised spiny mice during optimisation of testing (data presented as mean ± SD).

The increase of time spent in the OF inner zone suggests more willingness to explore/travel through an exposed space, as rodents tend to cluster to the surrounding edges of the OF area

if particularly anxious. However, I observe a direct contradiction in exploration tendencies when paired with the EPM (designed to complement the findings of OF) with a decrease in exploration in the open arms. In addition, NORT results from OVX spiny mice indicate females are less interested in the unknown object after consecutive testing. This could be a result of boredom, rather than anxiety, as females may have tired of attempting to escape the OF (visualised as excessive scurrying and increased distance travelled in the Outer Zone) once realising they could not climb the walls and that they would be left alone for a duration (i.e. the 5-minute testing period), so were not fearful of the exposed inner zone. Two types of bottles were used in the novel object tests and, although alternated on each day, may not have been different enough to elicit an exploratory response with repeated exposure in this species, resulting in the decreased preference for the unknown in NORT2 (Figure 2.3.6).

Testing in cycling female spiny mice was performed in a similar manner as metabolic cages. Across 2 cycles on alternating days with 24-48h breaks between and each female commencing behavioural testing during differing stages of her menstrual cycle, to capture behavioural changes within individual females. In addition, the order of tests was swapped every day to avoid routine (NB: OF always comes before NORT 1, and NORT 1 always comes before NORT 2).



*This protocol allows blind analysis if females have a 9 menstrual cycle. However due to natural variation in cycle length, it is necessary to perform a lavage after day 12 to estimate when the female will enter the final phase (e.g. late luteal) to ensure phases aren't repeated or missed. This may extend timeframe by up to 7 additional days, depending how quickly the female is cycling.

Figure 2.3.7: Optimised protocol of behavioural assessment in spiny mice.

2.3.5 Qualitative Behavioural Scoring

Based on years of experience in the restraint and handling of the spiny mouse females, I developed the following score sheet for use in semi-quantitative behavioural assessment. This protocol has been successfully used in three separate studies, two of which are external to the ones presented in this PhD. It is adaptable for other investigators, but only if they are sufficiently confident in handling as time of capture and number of attempts to restrain may be influenced by inexperience of the researcher rather than as a measure of stress of the animal.

TABLE 2.3.5: QUALITATIVE BEHAVIOUR SCORE SHEET DURING VAGINAL LAVAGE IN THE SPINY MOUSE

SMEARING	0 - Normal	1 - Minor	2 – Moderate	3 – High
Initial Position in Cage	Nesting with littermates With majority of group	Hiding in/under enrichment with 1-2 other litter mates	Hiding in/under enrichment alone OR Moderate Cage Climbing	Isolated, in cage corner farthest away from group OR Continual cage climbing
Ease of Capture	Immediate Approaches container without aid ~5 sec	Some mild herding required Easily coaxed into container ~10 sec	Extensive herding required Intentionally avoids handling, Frequent climbing on roof/walls and or flipping 15-20 sec	Difficult and takes multiple attempts Animal uncooperative; Multiple "close captures" in and out of container Animal constantly flipping, jumping and/or climbing 20 sec +
Response to Weighing	Still and placid	Initial movement (i.e. animal turns around 1-4 times) then placid	Animal constantly turning and/or attempts to escape	Animal escapes 1 or more times
Response to towel	Placid, no excessive movement	1-2 repositions (i.e. animal will turn 180 degrees) Minor wriggling and/or foot scurrying	3-4 repositions Repeated wriggling and/or foot scurrying Head/neck frequently exposed and tilted around container edges	Animal escapes from container one or more times Constant struggling/wriggling/scurrying Head/neck and torso frequently exposed
Ease of Scuffing	1 attempt	2 attempts	3-4 attempts	5+ attempts
Attitude toward restraint	Placid, easily subdued, easily kept straight	Exhibits slight apprehension to restraint, body may be at minor angle	Animal struggles, with claws stuck in towel fibres (hind or fore), minor kicking or head movement	Animal appears stressed, attempts to bite self or handler and repositioning does not alleviate
Lubrication	Not bothered	Minor Flinch on one side (unilateral)	Noticeable flinching, minor vocalisation on one or both sides (uni or bilateral)	Attempt to bite, noticeable vocalisation
Smearing	Not bothered	Some reaction, leg reflex at palpation	Intense hind body reaction, accompanying vocalisation	Total body reaction, all limbs flinch, torso movement, and/or animal attempts escape
Belly Stroke	Not bothered	Minor Flinch on one side (unilateral) LHS	Noticeable flinching, minor vocalisation on one or both sides (uni or bilateral)	Attempt to bite, noticeable vocalisation
Vocalisation	None	Rare 1-2	Frequent 3-5	Constant 6+
Escaping	None	1 attempt (from container or cage) OR 1 escape from cage	2 or more attempts (from container and/or cage) OR 1 escape from container and 1 or more escapes from cage	Multiple attempts from container and cage Escape upon returning to cage Escape on smearing
Reaction to Cage Return	Crawls out of restraint slowly, placid	Small jump out of restraint, some initial hurried scurrying	Leap out of restraint, backflipping and cage climbing OR licking of lower abdomen	Flip out of restraint, recapture necessary, constant backflipping and climbing on return +/- licking

2.4 Embryo Transfer in the Spiny Mouse

2.4.1 Overview

The original project was conceived on the basis of optimising the breeding potential of the in-house colony of spiny mice at Monash University. The project had two overall main aims: 1) to establish a method of pseudopregnancy induction in *Acomys cahirinus* and 2) to perform live embryo transfers, with or without the aforementioned protocol. It was during this original project that the unanticipated discovery that females were bleeding from the vaginal canal, and menstruation in the spiny mouse was confirmed. However, significant energy was put into both aims above before this observation was made. During the initial project, pseudopregnancy was thought to have been successfully induced using a single subcutaneous injection of progesterone (P_4) 2mg based on persistent leukocytic vaginal cytology, as seen in persistent diestrus, therefore all recipients for embryo transfer received P_4 during oestrous, and embryo transfer occurred either 48h following injection (oviduct transfers) or 96h following injection (uterine transfers). However, it was later determined that many of these diestrus samples contained shed endometrial debris and erythrocytes, and were in fact menstrual, following the discovery of menstruation in the spiny mouse and re-evaluation of all data. The studies on pseudopregnancy induction in the spiny mouse are presented in Chapter 7. Prior to the discovery however, several attempts to perform live embryo transfers were made, and the methods are outlined below.

2.4.1 Embryo Donors

Superovulation donors

Spiny mice underwent superovulation protocols of 2x 10 IU eCG (9h apart) followed by 20 IU hCG 60h later.

Natural ovulation donors

Spiny mice smeared to confirm oestrus and paired with males for 48h.

SHAM:

Affi-Gel® Blue Gel 100-200 mesh (BioRad, #153-7302) were substituted instead of embryos

2.4.2 Oviduct Embryo Transfer

1. Donor female is culled via cervical dislocation at 72h post-pairing with a male, which is anticipated to produce a fertilised embryo of 1-2 cell stage (NB: spiny mouse does not produce external copulatory plug at the vaginal opening like conventional mice, so timed mating is used. For oviduct, this is 48h post-mating).
2. The ovary, oviduct and first third of the uterus are dissected out and placed in a dish containing pre-warmed KSOM media (37°C) under dissecting microscope. Embryos are expelled through flushing of the oviduct with media and collected in a glass pasteur pipette containing intermittent air-media pattern as follows: air-media-air-media-air-embryos in media.
3. Recipient females received paracetamol orally (3mL/100mL in drinking water) for 3 days prior to surgery, and 3 days following (6 consecutive total). Females were placed in the prone position on warming pad. The incision site of approx. 1cmX1.5cm was shaved and disinfected with 70% v/v ethanol. A 1cm midline incision was made adjacent to hips along the dorsal midline and the muscle layer and skin separated gently using blunt dissection. A

second 0.5cm incision was made through musculature over the left ovarian fat pad to access the internal cavity. The left uterine horn was gently pulled to the opening, the ovarian fat pad clamped and organs positioned under dissecting microscope.

4. Using watchmaker forceps, a small incision in the bursa was made to expose the opening of the infundibulum. The preloaded pipette containing the embryos was gently inserted into the oviduct cavity via the infundibulum and embryos transferred via mouth-pipette until air bubbles were seen filling oviduct (indicating successful placement of pipette tip)

5. The reproductive tract was eased back into the peritoneal cavity and peritoneal incision was sutured. The skin incision was flushed with topical analgesic (Bupivacaine 0.2%) and the skin closed with Michel clips. Females were monitored every 2h for 8h post-surgery, then bi-daily for the first 48 hours and daily thereafter. The spiny mice were weighed daily for 2 weeks from day 3 of recovery to determine any increases in weight indicating pregnancy.

2.4.3 Uterine Embryo Transfer

1. Donor female is culled via cervical dislocation at 120h post-pairing with a male, which is anticipated to produce a fertilised embryo of blastocyst cell stage (96h post-mating). Embryos are collected as per oviduct protocol above.

2. Recipient females received the same protocol as oviduct above, and the ovarian fat pad clamped to expose the upper third of the uterine horn.

3. The uterus was pulled taught and a hole in the uterine cavity was produced by penetration of the uterus with a 26-gauge needle.

4. The pipette tip containing embryos (NB: air-media pattern) was inserted and media containing embryos ONLY expelled.

5. After transfer, recipients were sutured, monitored as above.

TABLE 2.4.1: TRIALLED EMBRYO TRANSFERS IN SPINY MICE

Female ID	Date of Surgery	Method	Embryos	Outcome
547 RDGN	14/04/2015	Unilateral Oviduct Progesterone 2mg	9x Super ovulated; 1-2 cell	Failed implantation/ pregnancy
539 BL	18/05/2015	Unilateral Uterine Progesterone 2mg	2x Natural, 4-5 cell	Failed implantation/ pregnancy
549 BK	20/05/2015	Unilateral oviduct Progesterone 2mg	3x SHAM	N/A
549 BKRD	20/05/2015	Unilateral oviduct Progesterone 2mg	3x SHAM	N/A

None of the trials showed any signs of implantation (weight gain in the female, or implantation scarring/resorbed embryos in the uterus at post mortem) after 3 weeks of monitoring. During the course of these trials, the discovery of menstruation in the spiny mouse was made and it was later determined that the pseudopregnancy method (P_4) was not in fact, successful, and the likely cause of failure of implantation could have been attributed to the onset of menstruation and transfer of the embryos into a non-receptive endometrium.

CHAPTER 3:

FIRST EVIDENCE OF A MENSTRUATING RODENT: THE SPINY MOUSE (*ACOMYS CAHIRINUS*)

GYNECOLOGY

First evidence of a menstruating rodent: the spiny mouse (*Acomys cahirinus*)

Nadia Bellofiore, BSc; Stacey J. Ellery, PhD; Jared Mamrot, BSc; David W. Walker, PhD, DSc; Peter Temple-Smith, PhD; Hayley Dickinson, PhD

BACKGROUND: Advances in research relating to menstruation and associated disorders (eg, endometriosis and premenstrual syndrome) have been hindered by the lack of an appropriate animal model. Menstruation, the cyclical shedding of the decidualized endometrium in the absence of pregnancy, is believed to be limited to 78 higher-order primates (human beings and Old World monkeys), 4 species of bat, and the elephant shrew. This represents only 1.5% of the known 5502 mammalian species and <0.09% of these are nonprimates. Thus, many aspects of menstruation remain poorly understood, limiting the development of effective treatments for women with menstrual disorders. Menstruation occurs as a consequence of progesterone priming of the endometrial stroma and a spontaneous decidual reaction. At the end of each infertile cycle as progesterone levels decline the uterus is unable to maintain this terminally differentiated stroma and the superficial endometrium is shed. True menstruation has never been reported in rodents.

OBJECTIVE: Here we describe the first observation of menstruation in a rodent, the spiny mouse (*Acomys cahirinus*).

STUDY DESIGN: Virgin female spiny mice (n = 14) aged 12–16 weeks were sampled through daily vaginal lavage for 2 complete reproductive cycles. Stage-specific collection of reproductive tissue and plasma was used for histology, prolactin immunohistochemistry, and enzyme-linked immunosorbent assay of progesterone (n = 4–5/stage of the menstrual cycle). Normally distributed data are reported as the mean ± SE and significant differences calculated using a 1-way analysis of variance. Nonnormal data are displayed as the median values of replicates (with interquartile range) and significant differences calculated using Kruskal-Wallis test.

RESULTS: Mean menstrual cycle length was 8.7 ± 0.4 days with red blood cells observed in the lavages over 3.0 ± 0.2 days. Cyclic endometrial shedding and blood in the vaginal canal concluding with each infertile cycle was confirmed in all virgin females. The endometrium was thickest during the luteal phase at 322.6 μm (254.8, 512.2), when plasma progesterone peaked at 102.1 ng/mL (70.1, 198.6) and the optical density for prolactin immunoreactivity was strongest (0.071 ± 0.01 arbitrary units).

CONCLUSION: The spiny mouse undergoes spontaneous decidualization, demonstrating for the first time menstruation in a rodent. The spiny mouse provides a readily accessible nonprimate model to study the mechanisms of menstrual shedding and repair, and may therefore be useful in furthering studies of human menstrual and pregnancy-associated disorders.

Key words: endometrium, menstruation, progesterone, spontaneous decidualization

Introduction

Menstruation, the cyclical shedding of the decidualized endometrium in the absence of pregnancy, is believed to be limited to 78 higher-order primates (human beings and Old World monkeys), 4 species of bat,[1,2] and the elephant shrew.[1,3,4] This represents only 1.51% of the known 5502 mammalian species[5]; <0.09% of menstruating species are nonprimates. Common to these species, and inherent to the process of menstruation, is spontaneous decidualization of the endometrial stroma without initiation from an implanting embryo. Under the control of progesterone from the ovary, the decidual reaction occurs in unison with a series of intricate structural changes to the uterine stratum functionalis, including extensive angiogenesis of maternal vasculature into spiral arterioles.[6] In the absence of pregnancy, degeneration of the corpus luteum results in progesterone withdrawal and endometrial shedding that, due to extensive vascularization of the endometrium, is accompanied by bleeding into the uterine cavity.[1] In nonmenstruating species, decidualization of the endometrium does not eventuate unless fertilization occurs and the process is signaled from the conceptus.

The spontaneous nature of the morphological changes that result in decidualization of the endometrial stroma are considered a preparatory maternal response to the impending invasion of the trophoblast and to aid in the adhesion of the attaching embryo.[1,7,8] The extent of trophoblastic invasion is greater in menstruating species; reaching as far as the inner third of the myometrium in women.[9] For successful implantation and pregnancy to occur, the maternal decidual reaction involves extensive remodeling of the myometrial and endometrial vascular beds, success of which ensures support of the invading trophoblast and development of the placenta.[1] Clinical diseases such as preeclampsia, currently one of the leading causes of fetal-maternal morbidity and mortality, are thought to be due to inadequate vascular remodeling and an impaired decidual reaction, resulting in shallow trophoblastic invasion and placental hypoxia.[10] Alternatively, if the trophoblast invades too deeply, women may experience placenta accreta, with abnormal placental-uterine adhesion. In extreme cases, this may only be able to be treated with peripartum

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0002-9378/$36.00

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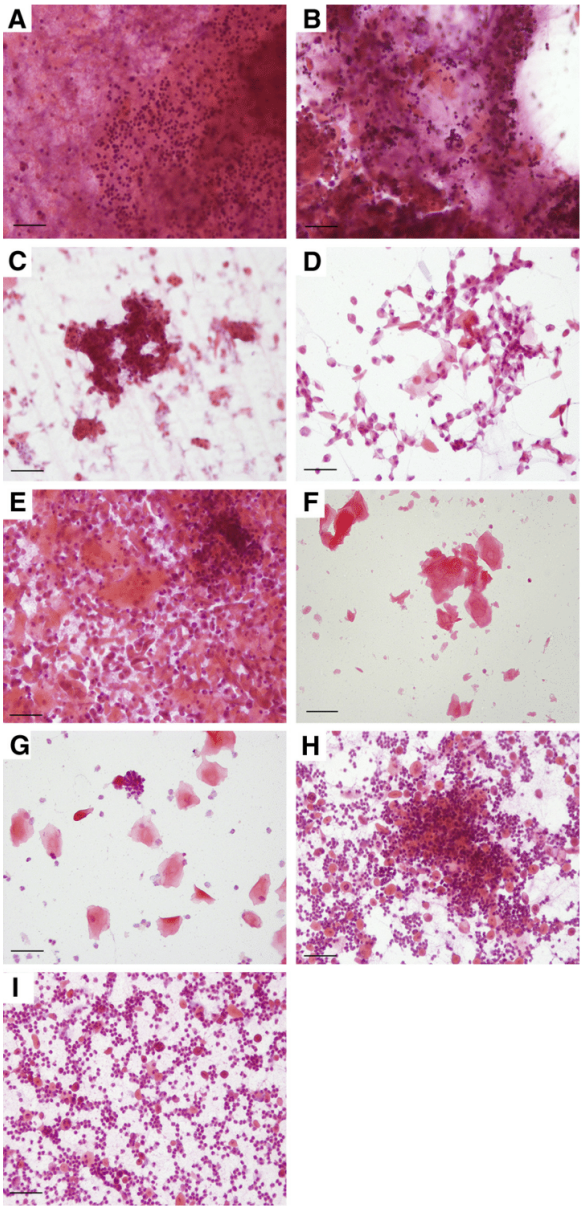
FIGURE 1
Vaginal cytology of female spiny mouse



A and **B**, Cytology showing 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.

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hysterectomy.[11] Endometriosis, resulting from the presence of endometrial tissue outside the uterine cavity, affects up to 10% of women with symptoms such as dysmenorrhea, chronic pelvic pain, dyspareunia, and infertility. As in preeclampsia and placenta accreta, the etiology of endometriosis is unknown and there is no cure, with treatments only targeting symptoms and not the underlying causes.

Although mouse models of artificial menstruation exist,[12,13] the limited research into menstruation and its related disorders is largely due to the absence of a cost-effective and practical laboratory model of natural menstruation. This study describes the first report of menstruation in a rodent: the common or Cairo spiny mouse (*Acomys cahirinus*), which is native to Northern Africa. The spiny mouse produces small litters (typically 2–3) of precocial pups, with most organogenesis completed in utero during the relatively long (for rodents) gestation of 39 days.[14] Observations of blood at the vaginal opening of nonpregnant female spiny mice in our breeding colony led to an investigation of the changes of endometrial structure and physiology during the reproductive cycle.

Materials and Methods

Animal care

All experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and all experiments approved by the Monash University/Monash Medical Centre Animal Ethics Committee. These animals were sourced from our own research colony, where temperature is maintained at 25–27°C, humidity 30–40%, with a 12-hour light-dark cycle (lights on 7:00 AM).[14] Virgin female spiny mice (n = 18) aged 12–16 weeks, were housed in groups of 5–6 per cage. Cages were lined in wooden shavings and enrichment provided in forms of cardboard tunnels and climbing apparatuses. Food (rat and mouse cubes, Specialty Feeds, Glen Forrest, WA) and water were provided ad libitum and supplemental fresh vegetables (carrots and celery, up to 50 g per

TABLE
Characteristics of reproductive cycle of spiny mouse

	Follicular phase		Luteal phase		
	Proestrus	Estrus	Metestrus	Diestrus	Menses
Length of cycle stage, d[a]	1.80 ± 0.2	1.30 ± 0.2	0.71 ± 0.1	1.80 ± 0.3	3.00 ± 0.2
Uterine wet weight, g[b]	0.26 (0.22, 0.29)	0.32 (0.27, 0.33)[a,b]	0.15 (0.13, 0.16)	0.09 (0.07, 0.11)[a]	0.12 (0.09, 0.16)[b]
Endometrial thickness, μm[b]	64.7 (53.49, 144.3)[a]	112.7 (73.60, 181.4)[b]	225.9 (180.6, 375.7)[a]	322.6 (254.8, 512.2)[a,b]	213.3 (171.3, 300.1)[a]
Diameter of uterine lumen, μm[b]	671.1 (437.9, 1378.0)[a]	1334.0 (1158.0, 1868.0)[b]	345.9 (131.6,772.9)[b]	127.0 (110.3, 192.3)[a,b]	614.9 (228.4, 840.1)[b]
Endometrial optical density, arbitrary units (prolactin)[a]	0.029 ± 0.01[a]	0.046 ± 0.01	0.047 ± 0.01	0.071 ± 0.01[a]	0.061 ± 0.01
Plasma progesterone, ng/mL[b]	46.6 (29.4, 63.9)[a]	65.38 (32.9, 82.7)	42.9 (37.2, 54.6)	102.1 (70.9, 198.6)[a]	50.5 (30.6, 65.4)

Superscript letters correspond to groups significantly different from each other. Data subjected to normality tests. For normally distributed data, mean ± SE is reported.

[a] Median (25th percentile, 75th percentile) is reported for all other values; [b] Significant differences corresponded to *P* value of <.05 (1-way analysis of variance and Dunnett post-hoc for normally distributed data and Kruskal-Wallis and Dunn post-hoc for all other data).

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cage) were provided weekly. Animals were checked at least twice daily and cages were cleaned weekly. Female weights were monitored daily throughout the experiment.

Vaginal lavage

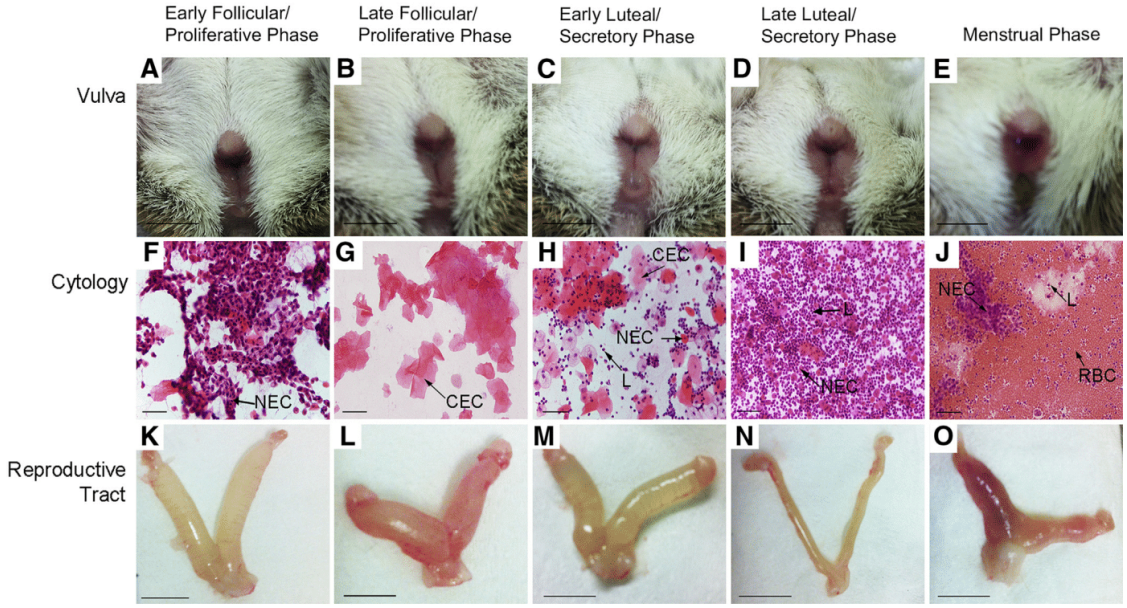
Vaginal lavages were performed daily (n = 18 females) between 12:30 PM and 2:30 PM using sterile saline solution (0.9% wt/vol), and the time of sampling recorded each day. Female spiny mice were restrained in the supine position with a small hand towel by gently scruffing behind the neck and shoulders (a hand towel is necessary due to the fragility of spiny mouse connective tissues and the propensity for skin to easily tear). The vulva was then lubricated with nonscented, water-based lubricant (Lifestyles, Ansell, Melbourne, Australia). A 1-mL plastic transfer pipette was used to draw up ~50 μL of saline, before gentle insertion into the vaginal canal. The length of the spiny mouse vaginal canal is 16.3 ± 1.1 mm based on measurements in our laboratory (unpublished). Hence, we inserted the pipette no deeper than 8 mm, and well before contact with the cervix to avoid possible stimulation of mechanoreceptors, which can cause alteration to the cycle stages in other rodents. The saline was flushed into the vaginal cavity twice, the solution redrawn into the pipette, the pipette removed and the sample expelled onto a glass histological slides (Menzel-Gläser Superfrost, Thermo-Scientific, Braunschweig, Germany). Samples were dried at 27°C for 5 minutes then sprayed with cytology fixative (Spray Fix, Surgipath Medical Industries, Melbourne, Australia). Smearing occurred consecutively for 18 days. Cycle stages were distinguished based on the dominant cell type(s) present in the smear.[15,16] If the smear appeared to be in a transitional phase, ie, between 2 consecutive stages, each of the stages was designated a time of 0.5 days to the total length. Otherwise, when a smear type was observed, it was designated a period of 1 day to the total length for that stage. Of the n = 18 females subjected to daily lavaging, 4 were omitted from cytological analysis due to non-cycling. All cytological data are based on data from n = 14 females.

For technical comparison, F_1 (C57BL/6J X CBA/J) hybrid mice (n = 5) sourced from the Monash Animal Research Platform weighing 17–20 g (40–60 days old) were smeared daily for 12 consecutive days. All aspects of animal handling, vaginal sample collection, and smearing technique were the same as described for the spiny mouse above, except use of a protective hand towel was not necessary.

Cytology staining

Slides were stained with hematoxylin-eosin (Harris hematoxylin and 1% aqueous eosin; Amber Scientific, Midvale, Australia). Briefly, slides were rehydrated in running tap water for 30 seconds –1 minute, prior to hematoxylin staining for 5–6 minutes. Slides were then washed 3 times in tap water to remove excess stain and differentiated in 5% acid ethanol. Following rinsing, slides were submerged repeatedly in a 5% ammonia solution to develop blue coloration. Slides were counterstained with eosin for 3 minutes. Slides were progressively dehydrated in graded ethanol solutions and underwent 3

FIGURE 2
Changes in reproductive tract morphology and cytology across spiny mouse menstrual cycle



Vulva prior to smearing showed **A** and **B**, gaping opening in follicular phase, cellular debris transitioning to **C**, luteal phase before **D**, closure. **E**, Bloody discharge was observed on vulva of 29% of females at time of smearing. Scale bar = 1 cm. Vaginal cytology (hematoxylin-eosin) consisted of **F**, nucleated epithelial cells (NEC), **G**, cornified epithelial cells (CEC), and **H** and **I**, leukocytes (L). **J**, Heavy onset of bleeding during first day of menses shows high numbers of red blood cells (RBC), some L and NEC. Scale bar = 50 μm. Uteri during proliferative phase were **K** and **L**, heavily distended with fluid before **M**, resorption and **N**, complete absence of fluid in secretory phase. **O**, Blood was clearly visible in uterine horns during menses. Scale bar = 1 cm.

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successive changes of clearing xylene for 2 minutes each. Each slide was coverslipped with DPX mounting medium.

Postmortem analysis and tissue collection

Female spiny mice (n = 20) were killed humanely at each distinct stage of the menstrual cycle (n = 4/cycle stage). Each female was heavily sedated using isoflurane before a cardiac puncture was performed for blood collection, followed immediately by cervical dislocation. Whole blood (0.3–1.2 mL per animal) was collected in a heparin-lined tube and plasma obtained after centrifugation (3000 RPM at 4°C for 10 minutes), which was then stored at −20°C for later analysis. The whole, intact uterus with both ovaries and the cervix attached was trimmed of fat and removed. Ovaries were then separated, and the uterus weighed (wet weight) before fixation in 10% buffered formalin for 48 hours, followed by immersion in 70% ethanol for 24–72 hours. Samples were processed to paraffin wax using a Leica ASP-300 processer and embedded in Paraplast paraffin medium; then 5 μm-thick sections were obtained, adhered to slides, and baked at 60°C for 20–30 minutes.

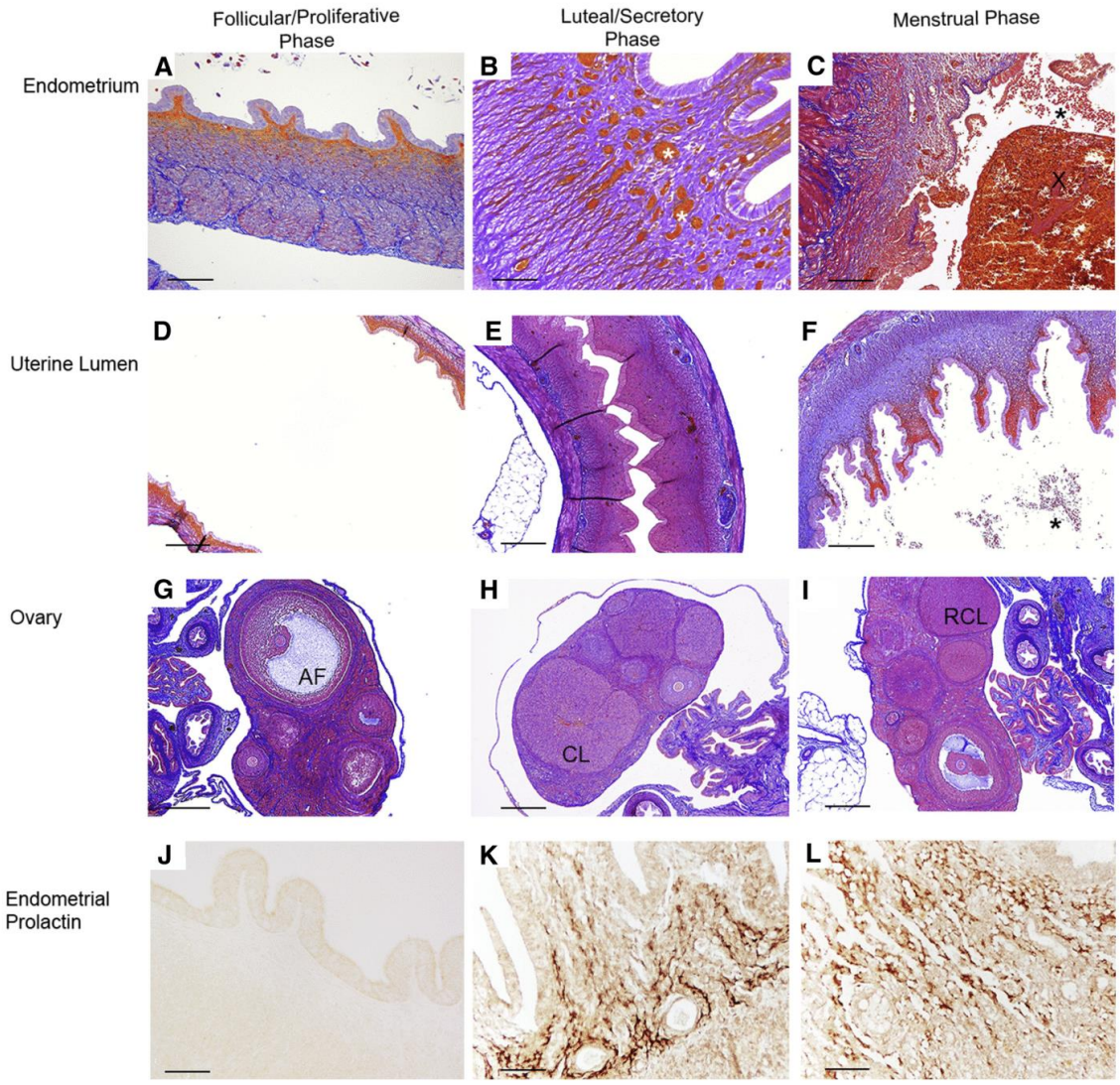
Histological staining

Samples were dewaxed through successive xylene changes, cleared in graded ethanol, and rehydrated in tap water. To visualize reproductive tissues, slides were stained with Mallory's trichrome. Briefly, sections underwent a secondary fixation in Bouin's solution at 60°C for 60 minutes prior to dewaxing. Following this, tissues were stained with acid fuchsin 1 g/100 mL dH_2O (distilled water) for 2 minutes, rinsed thoroughly in distilled water, and stained with phosphomolybdic acid 1 g/100 mL dH_2O for 2 minutes. Slides were rinsed before staining with orange G 2 g, methyl blue 0.5 g, and oxalic acid 2 g/100 mL dH_2O for 15 minutes. Following thorough rinsing, tissues were dehydrated and differentiated as for hematoxylin-eosin above. Red staining was indicative of nuclei and muscle, blue staining indicated collagen, and orange staining indicated anucleate cells (eg, erythrocytes).[17]

Progesterone assay

Plasma progesterone (P4) concentration was measured using a commercially available mouse/rat enzyme-linked immunosorbent assay kit (ALPCO, no. 55-PROMS-E01). All samples (n = 5 females per cycle stage) were measured

FIGURE 3
Histological changes of spiny mouse reproductive tract across menstrual cycle



Structural changes to endometrium (scale bar 100 μm), uterine lumen (scale bar 200 μm), and ovary (scale bar 200 μm). Endometrium during **A**, proliferative phase is thin before increasing in **B**, secretory phase, with presence of new blood vessels and vascular remodeling (white asterisks). During **C**, menses, endometrium is shed, visible in peeling of uterine epithelium, detachment of masses of old endometrial tissue (x), and **F**, red blood cell infiltration in uterine lumen (black asterisk). Uterine lumen is wide in diameter during **D**, proliferative phase and significantly reduced during **E**, secretory phase. Antral follicles (AF) are prominent in ovaries during **G**, follicular phase before collapse into **H**, corpora lutea (CL) and **I**, regressing copora lutea (RCL). Immunopositive endometrial prolactin is absent during **J**, proliferative phase, but secreted from decidualized stromal cells during **K**, luteal phase surrounding newly formed blood vessels before **L**, decidual cells are shed during menses.

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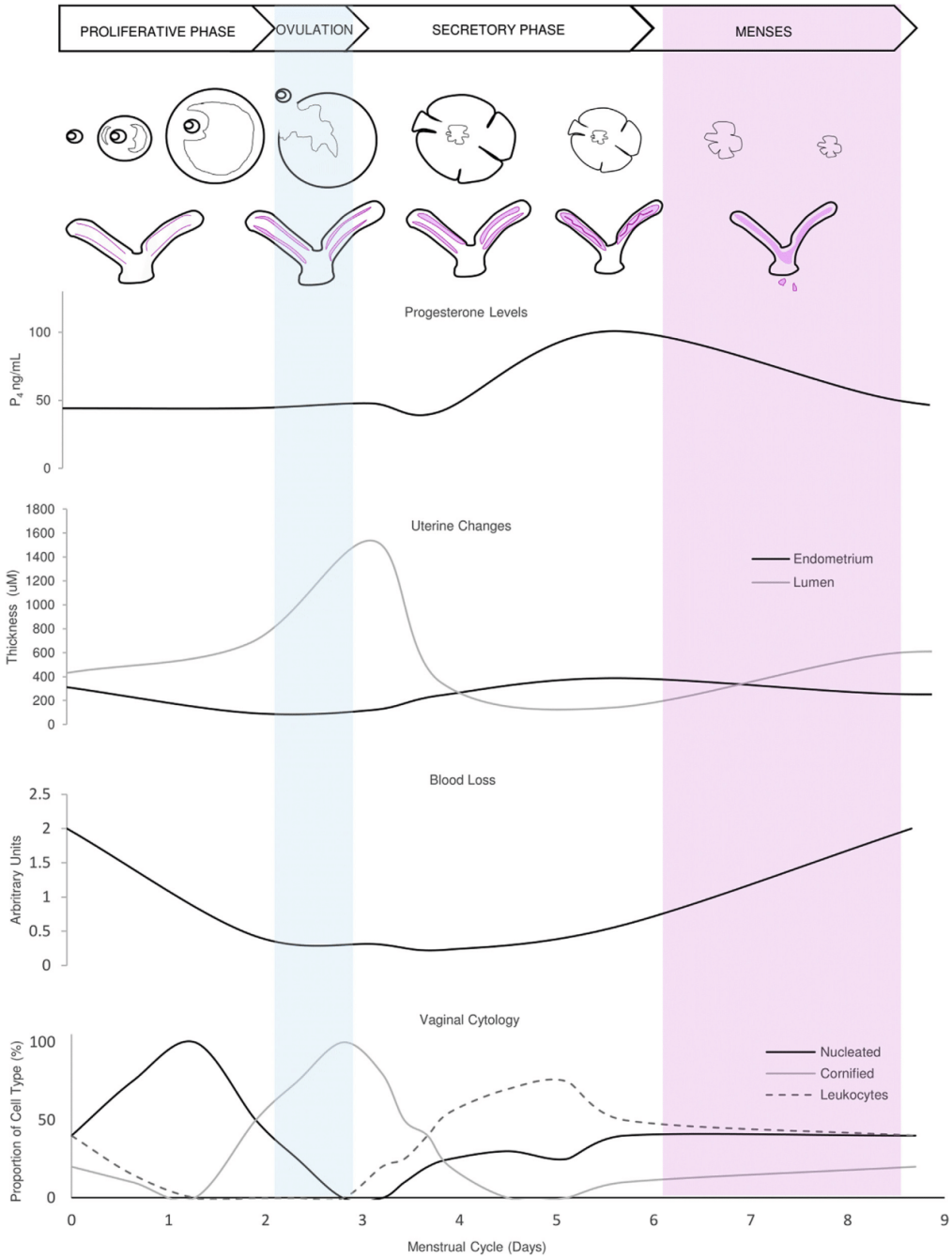
in duplicate and the median value reported. The validity of the assay for spiny mouse plasma was tested by spike and recovery and linearity of dilution procedures following the manufacturer's protocols. Selected samples from individual animals were spiked with 0, low (10 ng/mL), medium (25 ng/mL), or high (50 ng/mL) analyte (progesterone

FIGURE 4
Summary of spiny mouse menstrual cycle



Cyclical shedding of endometrium correlates with regression of corpus luteum and falling progesterone levels.

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powder, Sigma-Aldrich, Castle-Hill, Australia) and 1 sample diluted by a factor of 4, 8, and 16, and the expected vs observed recovery measured.

Immunohistochemistry

Fixed tissues were subjected to immunohistochemistry to detect presence of decidualization using prolactin as biomarker.[18,19] Samples were dewaxed through multiple changes of histolene, and rehydrated through decreasing concentrations of ethanol until submerged in running tap water for 1 minutes. All washing steps occurred in TBS-Tween 20 (0.1%) buffer solution. Slides were first washed for 3 × 5 minutes before exogenous peroxidase activity was blocked by applying 1% H_2O_2 solution for 20 minutes. Slides were washed 3 times in buffer and serum block was applied for 30 minutes at room temperature (X090930-2, Dako, Botany, Australia). Slides were incubated in primary polyclonal rabbit antihuman prolactin (A0569, Dako) at 1:400 dilution in 10% normal goat serum at 4°C overnight or 10% normal goat serum as a negative control. Slides were thrice washed and incubated in secondary biotinylated goat antirabbit IgG (Vector Laboratories, CA) at 1:200 dilution for 60 minutes at room temperature. Following washing, slides were incubated in avidin-biotin complex at room temperature for 60 minutes, then washed in buffer solution. 3,3'-Diaminobenzidine was then applied for 6 minutes for visualization. Slides were washed in buffer 3 times and washed again in running tap water before dehydration in increasing graded ethanol. Slides were cleaned and coverslipped as in the hematoxylin-eosin protocol outlined above. Uterine tissue from an early gestation (7 days postconception) pregnant spiny mouse was used as a positive control for prolactin staining (not shown).

Image acquisition

Images of whole organs were captured using an iPhone 4 (iPhone 4, Apple, Cupertino, CA). All histological images were captured using a Leitz Diaplan imaging bright-field microscope (Diaplan, Leitz, Baden-Württemberg, Germany), with light settings kept constant and analyzed with the programs Axio-Vision version 4.6.3 (Zeiss, Oberkochen, Germany) and Image Pro-Plus version 6 (Media Cybernetics, Tokyo, Japan). Images of the vaginal cytology of both the spiny mouse and F_1 mouse were taken at ×200 magnification. Histological images of the cervix and ovaries of the spiny mouse were taken at ×40 magnification, and the sections of the uterine horns taken at ×100 and ×200 magnification. Using Axio-Vision, the thickness of the endometrium and myometrium were measured, as was the diameter of the uterine lumen (n = 4 animals per stage receiving 3 random recordings per uterus to give n = 12 measurements per stage; μM), across all stages of the reproductive cycle in the spiny mouse. The degree of immunopositive staining was determined using Image Pro-Plus, as described by Atik[20] in 2014. Calibration of the light and dark fields was implemented by the use of a control image containing both incident light and infinite optical density; brought about by use of a scalpel blade placed on the stage to partially obscure the light. All images were captured in 1 session, with all program settings and light exposure kept exact for each section. Images were converted to grayscale and the mean optical density of positive prolactin-stained areas of the endometrium and myometrium was measured. Mean values were calculated for each section, with 3 fields of view at ×200 magnification per structure of interest (endometrium or myometrium) per animal. Regions containing background staining were measured for each image and subtracted from the mean optical density of each sample to adjust for nonspecific staining. The average optical density was calculated for each group. The investigators conducting measurements of the reproductive tract and optical density were blinded to the treatment groups at the time of analysis.

Image alterations

After completion of analysis, images were subjected to minor aesthetic alterations using Adobe Photoshop CC (Adobe Systems, San Jose, CA). Images were sharpened, and backgrounds brightened if required. Due to the variability in staining, color balancing was performed on some histological images to match specific sections of tissues for easier comparison.

Statistical analysis

All statistical analyses were conducted using SPSS (Version 22) (IBM, New York, NY) and GraphPad Prism (Version 6.01) (Graphpad Software, La Jolla, CA). Normality of the data was tested before statistical analysis. Uterine weight, endometrial and myometrial thickness, uterine lumen diameter, and plasma progesterone concentrations (n = 5 samples per stage) are displayed as the median values of replicates (with 25 and 75 percentile values) and significant differences were calculated using Kruskal-Wallis. Optical density and menstrual cycle length are reported as the mean ± SEM and significant differences (corresponding to a P value of $< .05$) were calculated using a 1-way analysis of variance. Tukey multiple comparisons test was used for comparing parameters between stages of the reproductive cycle during post-hoc analysis.

Results

We examined cytology of daily vaginal lavages from cycling virgin females (n = 14) and found an overall cycle length ranging from 6−10 days, with an average of 8.7 ± 0.4 days. Cytology showed all of the expected stages of a rodent estrous cycle: a follicular phase comprising proestrus and estrus and a luteal phase denoted by metestrus and diestrus. However, an additional stage was present in the spiny mouse, characterized by the presence of large numbers of red blood cells (Figure 1) over a period of 3.0 ± 0.2 days, consistent with menses. Blood was present in the vaginal lavage of all females (14/14) during the transition from the luteal to the follicular phase in both of the cycles studied. Blood was visible macroscopically on the external genitalia in 4 subjects (29%) during the lavage process. Reproductive tract dissections revealed significant differences in uterine weight during the cycle (Table), with

blood present in the uterine lumen corresponding to the time it was detected in the smears (Figure 2). To ensure that bleeding was not caused by vaginal lavage the technique was used in F_1 (C57BL/6J X CBA/J) hybrid mice. We found no evidence for the presence of red blood cells during any stage of the mouse estrous cycle (Supplemental Figure 1) and therefore concluded that the cyclical changes and uterine bleeding seen in the spiny mouse vaginal cytology were a natural phenomenon and not caused by trauma from the lavage technique.

Mallory trichrome stain was used to visualize histological changes in the reproductive tract (Figure 3). During the follicular phase of the spiny mouse cycle, the endometrial layer of the uterus was thin (64.77 [53.49, 144.3] μm) (Table) and the ovaries contained antral follicles, before rupture, and development of corpora lutea (Figure 3). During the luteal phase, angiogenesis and decidualization were observed in the uterus, endometrial thickness increased 4−5 fold (322.6 [252.8, 512.2] μm), and the diameter of the uterine lumen decreased (Table). Degeneration of the corpus luteum followed, coinciding with shedding of the endometrium (Figure 3 and Table).

Decidualization of the endometrium was confirmed with immunohistochemical staining for prolactin[18,21] (Figure 3). Endometrial prolactin staining was absent during ovulation. Immunopositive decidual cells in the endometrium were abundant during the luteal phase (Figure 3, A and B). To quantify prolactin immunostaining, we measured optical density. The strongest staining in the endometrium was during the luteal phase, which was followed approximately 2 days later by shedding of the uterine lining (Table).

Plasma progesterone was measured with an enzyme-linked immunosorbent assay kit (Supplemental Tables 1 and 2) to determine if histological and immunohistochemical findings were correlated with hormonal changes consistent with the process of menstruation. There was a significant increase in plasma progesterone concentrations to 102.1 (70.1, 198.6) ng/mL during the luteal phase compared to the follicular phase 46.6 (29.4, 63.9) ng/mL (Table).

Comment

The timing and recurrence of uterine bleeding is evidence for menstruation in the spiny mouse; indeed the onset of the cytological and hemorrhagic changes commenced at the time plasma progesterone concentrations begin to fall, a hallmark of spontaneous decidualization.[1,22]

The spiny mouse reproductive cycle is divided into uterine and ovarian phases consistent with other menstruating species (Figure 4), although this is the first report of such observations in a rodent. Recent hypotheses suggest that menstruation is a consequence of spontaneous decidualization, and that spontaneous decidualization evolved in those species with highly invasive trophoblast, deep penetration of the established placenta into the endometrium, and a high rate of embryonic chromosomal abnormality.[1,8]

Because the spiny mouse shares many reproductive features with other menstruating mammals, such as hemochorial placentation, spontaneous ovulation, and few, well-developed offspring,[16,23] this suggests that spontaneous decidualization and menstruation may be an example of parallel evolution of these traits.

The unprecedented discovery of menstruation in a rodent species suggests that this phenomenon was overlooked by previous investigators[16] because of the current dogma that rodents are not menstruating mammals. Studies are now required to determine if menstruation occurs in other species of spiny mice and in the related genera of *Uranomys*, *Deomys*, and *Lophuromys* and perhaps *Meriones*, *Gerbillus*, and *Tatera*.[24-26] The peripheral position of the *Acomyinae* in most rodent phylogenies[24,27] suggests that it may be useful and appropriate, in the light of these unusual characteristics of spiny mice, to broaden phylogenetic analysis of this and related genera.

Previous studies have established the spiny mouse as a more useful laboratory species for studies of various aspects of human reproduction and neonatal development than other rodents. The hormonal profile and precocial offspring greatly enhance the utility of this species as a research tool.[28,29] The spiny mouse should also now be regarded as an accessible and cost-effective laboratory model for research into menstruation and menstrual disorders of women, including premenstrual tension and endometriosis.[30] ■

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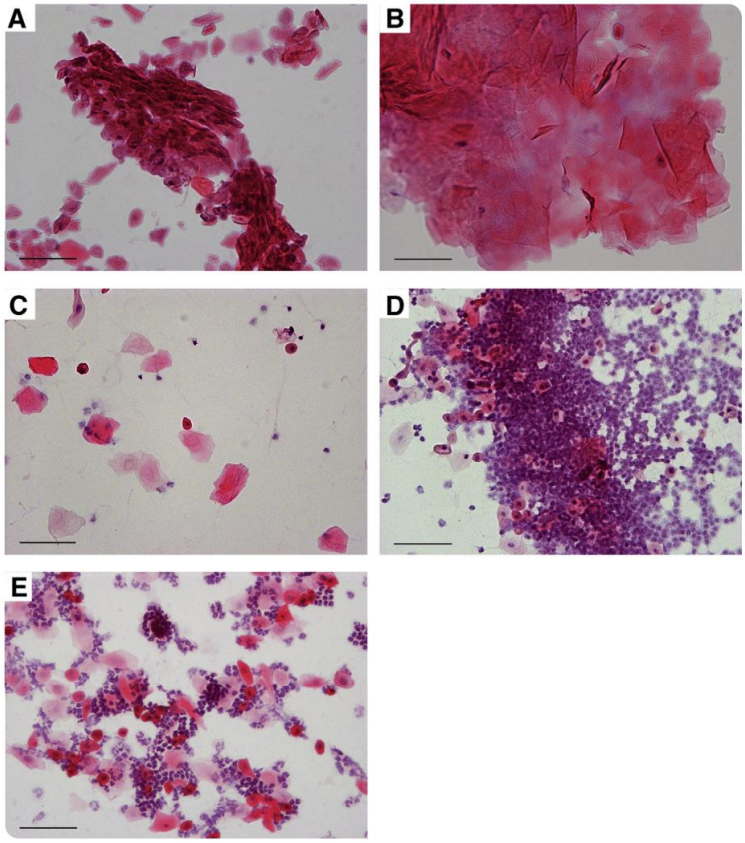
SUPPLEMENTAL FIGURE 1
Vaginal cytology of female F_1 mouse at ×200 magnification



A, Early follicular phase; transition from proestrus to estrus, containing both nucleated and cornified epithelial cells. **B**, Estrus characterized by cornified epithelial cells. **C**, Metestrus, transition to luteal phase. **D**, Early diestrus with large leukocytic infiltration. **E**, Transition to new fertile cycle. No red blood cells are observed during mouse estrous cycle. Scale bar = 50 μm.

Bellofiore et al. Menstruation in spiny mouse. Am J Obstet Gynecol 2017.

SUPPLEMENTAL TABLE 1
Progesterone spike and recovery for spiny mouse plasma

Sample, n	Level of analyte spike	Expected	Observed	Recovery, %
Plasma (4)	Low	16.7	18.8	112.5
	Medium	22.9	20.9	91.1
	High	70.4	103.7	147.4
Mean recovery				117.0

Recovery of >80% indicates sufficient detection of progesterone in spiny mouse plasma to consider derived values valid for analysis.

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SUPPLEMENTAL TABLE 2
Progesterone linearity of dilution for spiny mouse plasma

Sample	Dilution	Expected (neat)	Observed × dilution factor	Recovery, %
	Neat		26.6	
High P_4	1/4	26.6	46.6	174.8
Plasma	1/8		69.5	261.1
	1/16		28.9	108.3

Bellofiore et al. Menstruation in spiny mouse. Am J Obstet Gynecol 2017.

CHAPTER 4:

CHARACTERISATION OF HUMAN-LIKE MENSTRUATION IN THE SPINY MOUSE: COMPARATIVE STUDIES WITH HUMANS AND THE INDUCED MOUSE MODEL

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human reproduction

ORIGINAL ARTICLE *Reproductive biology*

Characterization of human-like menstruation in the spiny mouse: comparative studies with the human and induced mouse model

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STUDY QUESTION: Is the newly discovered menstruating rodent, the spiny mouse, a valid model for studying endometrial morphology and menstruation?

SUMMARY ANSWER: Our study is the first to demonstrate a primate-like pattern of natural menstruation in a rodent, with decidualization, spiral arteriole remodeling and piece-meal endometrial shedding.

WHAT IS KNOWN ALREADY: The spiny mouse has a naturally occurring menstrual cycle. This unique feature has the potential to reduce the heavy reliance on primates and provide a more appropriate small animal model for menstrual physiology research.

STUDY DESIGN, SIZE, DURATION: This study compares morphological changes in the endometrium during early, mid and late menstruation of the spiny mouse ($n = 39$), human ($n = 9$) and the induced mouse model of menstruation ($n = 17$).

PARTICIPANTS/MATERIALS, SETTING, METHODS: We assessed tissue morphology with hematoxylin and eosin and erythrocyte patterns with Mallory's trichrome. We conducted staining for neutrophil gelatinase associated lipocalin (NGAL), cytokeratin and interleukin-11 (IL-11) in all species. We used double immunofluorescence staining for vascular endothelial growth factor and alpha-smooth muscle actin to detect vasculature remodeling and western immunoblot to detect interleukin-8 (IL-8) and macrophage migration inhibitory factor (MIF) in the menstrual fluid of spiny mice.

MAIN RESULTS AND THE ROLE OF CHANCE: Menstruation occurs in the spiny mouse over a 72-h period, with heaviest menstrual breakdown occurring 24 h after initial observation of red blood cells in the vaginal cytology. During menstruation, the endometrium of the spiny mouse appeared to resemble human menstrual shedding with focal epithelial breakdown observed in conjunction with lysis of underlying stroma and detection of IL-8 and MIF in menstrual fluid. The mouse exhibits extensive decidualization prior to induced menses, with transformation of the entire uterine horn and cytokeratin expression absent until initiation of repair. Decidualization occurred spontaneously and was less marked in the spiny mouse, where epithelial integrity remained intact. In all species, the decidua was positive for IL-11 secretion and neutrophil recruitment was similar in each. Spiral arteriole formation was confirmed in the spiny mouse.

LARGE SCALE DATA: N/A

LIMITATIONS, REASONS FOR CAUTION: This is a descriptive study comparing primarily morphological traits between the spiny mouse, the mouse and the human. Reagents specific to the spiny mouse were limited and resources for global use of this novel species are few.

WIDER IMPLICATIONS OF THE FINDINGS: Our work supports the spiny mouse as a viable model, sharing many attributes of physiological menstruation with humans. The strength of a natural as opposed to an artificial model is validated through the striking similarities observed between the spiny mouse and human in uterine breakdown. The spiny mouse may be highly useful in large-scale investigations of menstruation and menstrual disorders.

STUDY FUNDING/COMPETING INTEREST(S): N.B. and S.R. are each recipients of a Research Training Program scholarship supported by Monash University. This work was supported by the Victorian Government Operational Infrastructure and laboratory funds to H. D. The authors declare no competing interests.

Key words: menstruation / spiny mouse / decidualization / animal model / inflammation

Introduction

True menstruation, the cyclical shedding of the endometrial functionalis resulting from rapid progesterone withdrawal, is a rare biological phenomenon. Menstruation occurs almost exclusively in higher order primates, including women, with several species of bats and the elephant shrew being the exceptions as the only non-primate species to menstruate (Van der Horst and Gillman, 1941; Rasweiler, 1991; Rasweiler and De Bonilla, 1992; Zhang *et al.*, 2007).

It was widely accepted that the order Rodentia does not naturally menstruate, with experimental intervention necessary to induce menstrual-like changes in laboratory rodents. The laboratory mouse (*Mus musculus*) exhibits an estrous cycle and, failing viable pregnancy, naturally resorbs endometrial tissue. In contrast, menstruating species shed the functionalis at the end of an infertile cycle. This is largely attributable to the fact that menstruating species have a spontaneous decidual reaction, in which decidualization of endometrial stromal cells occurs under the influence of high circulating levels of progesterone. This reaction occurs irrespective of signals from an implanting blastocyst, which is required for decidualization in non-menstruating species. These decidual cells cannot be de-differentiated and therefore, in the absence of luteotropic chorionic gonadotropin, luteolysis proceeds. A rapid decline of progesterone results in production of chemokines, infiltration of immune cells, protease activation and endometrial sloughing of decidua during menstruation (Evans and Salamonsen, 2014).

Menstruation induction in the mouse requires the females to be ovariectomized and the uterus to be hormonally primed using sequential estrogen injections and progesterone implants. Decidualization must then be mechanically induced. Only through artificial decidualization in conjunction with progesterone withdrawal by implant removal can the common mouse exhibit menstrual-like shedding.

We recently discovered that the common spiny mouse (*Acomys cahirinus)* undergoes cyclic endometrial shedding with frank menses observed. To our knowledge, this is the only rodent to have a natural menstrual cycle, with a new cycle initiated every 8–9 days (Bellofiore *et al.*, 2017). We propose that the spiny mouse presents a suitable small animal model of menstruation. This study directly compares the timing and nature of epithelial and stromal endometrial breakdown and repair in the induced mouse model, the spiny mouse and women to validate our assertions.

Materials and Methods

Ethics

Human ethics

All tissue collections were approved from Institutional Ethics Committees at Monash Health and Monash Surgical Private Hospital (Ethics 03066B & 06014 C).

Endometrial biopsies were collected by curettage from normally cycling women following informed consent. By this method, the functionalis and a small amount of basalis endometrium are usually sampled. All women had regular menstrual cycles (28–32 days), were under 40 years of age and had not received steroid hormone therapy in the last 6 months. All women had morphologically normal endometrium as assessed by an experienced pathologist. Menstrual cycle staging was assessed using the Noyes criteria. Tissues obtained during menstruation were used in the current study, during early (Day 1, $n = 3$), mid (Day 2, $n = 3$) or late (Day 5–7, $n = 3$) menstruation, and during the proliferative phase ($n = 3$).

Animal ethics

All experimental procedures adhered with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All research was approved in advance by the Monash University/Monash Medical Centre Animal Ethics Committee (MMCA 2016/40).

Husbandry: spiny mice

Virgin female spiny mice, (SpM, a total $n = 39$) aged 12–16 weeks and weighing 29–35 g, were used from our in-house colony and maintained as previously described (Bellofiore *et al.*, 2017).

Mouse model of menstruation (MMoM)

Female C57BL/6 mice (a total $n = 17$), aged 8–12 weeks, were maintained as previously described (Brasted *et al.*, 2003).

Induction of menstruation and tissue collection

Uterine tissues from the induced mouse model of menstruation (MMoM) were sourced from previous studies (Brasted *et al.*, 2003; Kaitu'u-Lino, 2007). Ovariectomized females received a subcutaneous injection of estradiol-17B (E_2) 100 ng for 3 days. After 3 days rest, a progesterone implant was inserted subcutaneously with injections of 5 ng E_2 continued. The lumen of the uterine horn was injected with sesame oil (20 µl) to stimulate decidualization. Progesterone implants were removed 48 h after decidualization induction, and mice were euthanized by carbon dioxide inhalation: at the time of implant removal ($n = 3$); after 24 h ($n = 7$) and after 48 h ($n = 7$). Uterine horns were fixed in 10% buffered formalin for 48 h, and dehydrated in 70% ethanol for 24 h, before processing to paraffin wax; tissues were embedded transversely and sectioned at 5 µm.

Spiny mouse post-mortem analysis and timed tissue collection

Female spiny mice were euthanized by isoflurane overdose at following menstrual cycle stages; proliferative ($n = 6$), secretory ($n = 7$) and

menses. Menstrual collection occurred at stages determined from the time of first sign of erythrocytes in the vaginal cytology (obtained through vaginal lavage, encompassing early (0–12 h, $n = 9$), mid (24–36 h, $n = 9$) and late menstruation (48–72 h, $n = 10$), based on our previous data of a 3-day menstrual bleed. One uterine horn was fixed transversely, and one longitudinally; both were processed and sectioned as described above. Additional spiny mice ($n = 4$) were culled during mid menstruation (24 h) to collect blood and debris via saline flush into the uterine horn for western immunoblot analysis.

Staining

Tissues were stained with hematoxylin and eosin (H and E) for overall morphological comparisons and Mallory's trichrome to identify vasculature containing erythrocytes, as per previously described protocols (Bellofiore *et al.*, 2017).

Immunohistochemistry

To assess epithelial integrity and neutrophil infiltration denoting inflammatory processes across menstruation, tissues were stained for cytokeratin and neutrophil gelatinase-associated lipocalin (NGAL), respectively.

Tissue samples were dewaxed through three changes of xylene for 5 min each, rehydrated through graded ethanol for 2 min each and then submerged in dH_2O for 5 min. If relevant, antigen retrieval was then performed (Supplementary Table I). Slides were washed in Tris-Buffered Saline with Tween-20 (0.05%) (TBS-T, 2×5 min), followed by one 5 min wash in TBS, and blocked with 3% hydrogen peroxide solution for 20 min occurred prior to washing as above. Serum block was applied for 30 min prior to primary antibody incubation overnight (4°C). Negative controls substituted host species IgG for the primary antibody. Sections were washed (3×5 min) in TBS before being incubated in secondary biotinylated IgG (Vector Laboratories, CA) at 1:200 dilution for 1 h. Following washing (three times 5 min in TBS), sections were incubated in avidin-biotin complex for 45 min, then washed. 3, 3′-Diaminobenzidine (DAB) was applied for 1–3 min. Tissues were rinsed in dH_2O before counterstaining in 10% Harris hematoxylin for 3 min. Slides were dehydrated and coverslipped as above.

Immunofluorescence

To assess the changes to vasculature across the menstrual cycle in the spiny mouse, tissues were subjected to double immunofluorescent staining for vascular endothelial growth factor (VEGF) and alpha smooth muscle actin (aSMA) (Supplementary Table I).

Western immunoblots

Western immunoblot was used to determine the presence of inflammation and repair factors in the menstrual fluid of spiny mice during mid menses (24 h). Menstrual flushings, containing sloughed endometrial tissue, were homogenized in radioimmunoprecipitation assay (RIPA) buffer prior to refrigerated centrifugation for 10 min. Supernatant was retained and 4× SDS loading buffer containing B-mercaptoethanol was added. Lysates were denatured at 95°C for 5 min with 15 µl of sample run on a 10% SDS polyacrylamide gel. Membranes were blocked in 5% skim milk for 90 min prior incubation in primary antibodies, interleukin-8 and macrophage migration inhibitory factor (IL-8, R&D Systems #AF-208-NA and MIF, R&D systems, #AF-289-PB), overnight at 4°C. Immunoblots were washed prior to incubation for 90 min in anti-rabbit horseradish peroxidase conjugated antibody (1:1000, Dako #D0487), followed by development using Clarity western enhanced chemiluminescence substrate (BioRad, #1705061), and visualized using the BioRad ChemiDoc MP Imaging System. Membranes were stripped and reprobed for B-actin loading control (1:10,000) as above.

Image acquisition

Immunofluorescent images were captured using a Nikon C1 Confocal microscope, and all other images using an Olympus BX41 bright-field microscope. To analyze DAB stained tissues, three randomly selected fields of view (FOV) per uterine sample were taken at ×200 magnification. These images were blindly analyzed using ImageJ software (National Institutes of Health) that provided an automated analysis of area coverage (%) of immunopositive cells (denoted by brown/black staining). The average of these FOV were calculated to give a mean value per sample which were then compared across species and stage of menstrual cycle. Samples were analyzed as follows: For Hu, $n = 3$/group for all stains; SpM, $n = 6–9$/group (NGAL and cytokeratin) and $n = 3$/group for IL-11; MMoM $n = 3–7$/group for all stains.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism Version 6.01 (GraphPad Prism Software Inc. CA). Non-normally distributed data is presented as median (25th percentile, 75th percentile) and normally distributed data is presented as mean ± standard deviation (SD). Significant differences were calculated using Kruskal–Wallis test or ANOVA for non-normal or normal data, respectively.

Results

Timing of menstrual breakdown in the spiny mouse

Overall, mean body and uterine weight of female spiny mice were 32.38 ± 2.68 g and 0.074 ± 0.029 g, respectively, with no differences observed at any timepoint during menstruation at the time of post-mortem. A positive correlation between female body weight and uterine weight was observed ($r = 0.42$, $P = 0.03$, Pearson correlation). We found no evidence that female weight in this cohort had any impact on whether we observed frank menses, and no significant relationship was observed between body weight and length of menstrual period (Fig. 1A,B). From the histology, 54% of spiny mice fell within the expected timing of menstruation based on our previous data of an average 3-day menstrual bleed. Slight deviations within 24 h of expected menstruation were noted. A total of 21% exhibited endometrial breakdown slightly prior to (7%) or slightly later than (14%) expected, while 25% exhibited substantially longer (7%) or substantially shorter (18%) bleeding times (>24 h discrepancy) than previously recorded (Fig. 1C).

Median body weight of females that had noticeably shorter menses was less than those with longer menses (Fig. 1C). However, neither

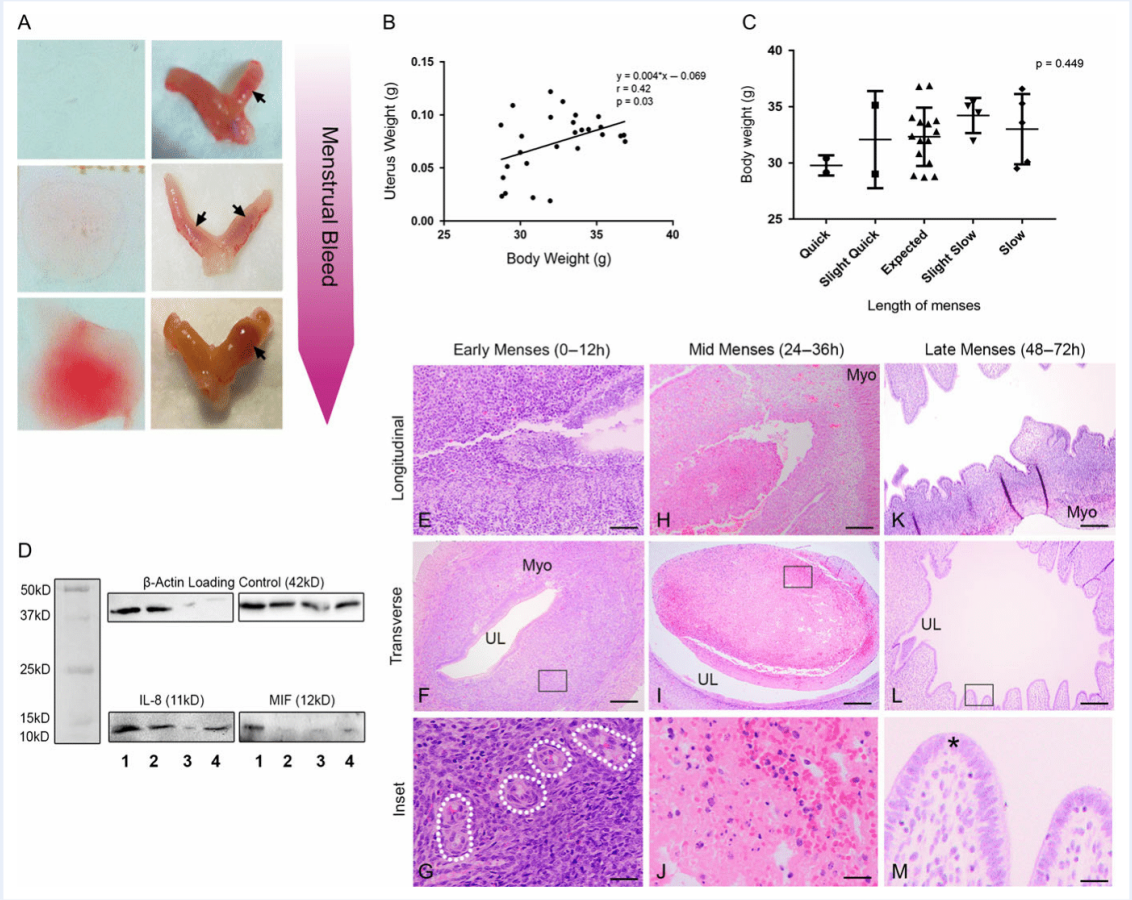

Figure 1 Menstrual breakdown in the spiny mouse. Myo = myometrium, L = luminal space (**A**) Variation in the degree of bleeding between individual animals is observed, ranging from light to heavy, visible in both vaginal lavage and corresponding uterine dissection. (**B**) Uterine weight is positively correlated with body weight (linear regression, $P = 0.03$). (**C**) Correlation between body weight and expected length of menstruation, where 'slight quick/slow' are within 24 h of expected histological point of menstruation, and 'quick/slow' are more than 24 h of histological deviation from expected point of menstruation. No significant relationship is observed. (**D**) Western blot of individual, heavily bleeding spiny mice (1–4) during mid menses (24 h). Inflammation markers interleukin-8 (IL-8) and macrophage migration inhibitory factor (MIF) are shown to varying degrees in all samples, however are most prominently detected in 3/4 and 2/4 female spiny mice respectively. (**E**) At 0–12 h following initial bleeding, the stroma of the decidualized endometrium begins to break away. The thickness of the differentiated endometrium renders the luminal space relatively small (**F**). Spiral arterioles can be identified at higher magnification (**G**, dotted lines). Between 24–36 h in both longitudinal and transverse sections, endometrial sloughing (**H**, **I**) of decidua (**J**) peaks, with menstrual debris in the lumen (L). Menstrual breakdown nears completion 48–72 h after initiation of bleeding, with the majority of endometrium shed, a clear and larger lumen (**K**, **L**), and regenerated luminal epithelium (*) (**M**). Scale bars (E, F, H, I, K, L) = 200 μM and (G, J, M) = 50 μM.

body nor uterine weights were a significant contributing factor for the differences observed.

Western blot analysis confirmed the presence of both IL-8 and MIF protein in the menstrual debris of heavily bleeding females at variable levels. IL-8 was most obvious in 3 of 4 samples, and MIF was in 2 of 4 samples (Fig. 1D).

Up to 12 h after first evidence of red blood cells in vaginal cytology, the beginning of the degradation of decidualized endometrial stroma containing spiral arterioles (Fig. 1E–G) was observed. Transverse sections showed that decidualization does not cause a complete closure of the uterine lumen. During mid-menstruation (Fig. 1H–J), 24–36 h after initial observation of cytological bleeding, large masses of decidualized tissue are shed in discrete regions of the uterine horn. The later stages of menstruation (Fig. 1K–M), between 48 and 72 h, shows near completion of the shedding and regeneration process, with a visibly larger luminal space and reduced thickness of the endometrium. The luminal epithelium has regenerated (*) with the cessation of peeling, and fewer fragments of old epithelial debris are present in the lumen.

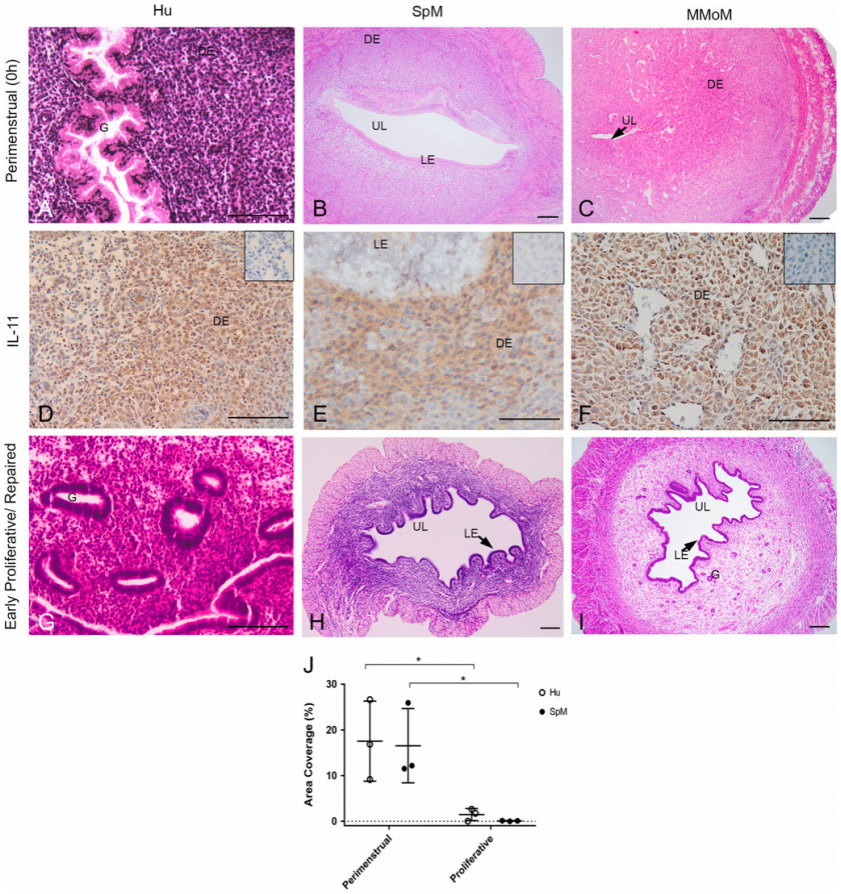

Figure 2 Comparative decidual reaction and resultant morphology of the human (Hu), spiny mouse (SpM) and mouse model of menstruation (MMoM). Myo = myometrium, UL = uterine lumen, LE = luminal epithelium and DE = decidualized endometrium. Early menstrual sections of the uterine horn (~0 h) stained with H and E in the human (functionalis only) (**A**), SpM and MMoM (**B**, **C** respectively, whole uterus, transverse plane). Tubular secretory glands (G) are prominent in the human. MMoM exhibits an extreme decidualized endometrium which has caused almost complete loss of the lumen and luminal epithelium. Myometrial structures are condensed at the periphery with histological artefacts (tearing/holes) in the musculature. SpM maintains the lumen, epithelium, and an intact bilayer of the myometrium. All species exhibit immunopositive brown staining for IL-11 (**D**–**F**) in the decidualized endometrium. Samples of corresponding negative controls are depicted in inset squares. Proliferative endometrium shows new glands in the proliferative human endometrium and repaired (48 h) MMoM with uterine lumen now evident in MMoM and proliferative SpM tissues (**G**–**I**). Area (%) of decidualized endometrium is significantly decreased after shedding in Hu and SpM (**J**). Scale bar = 100 μM.

Comparative endometrial morphology between species

All species undergo a decidual reaction (Fig. 2A–C). Secretory glands are pronounced in human early menstrual endometrium. Transverse sections of MMoM at the time of implant removal (0 h), show an extensive decidual reaction encompassing most of the endometrium, and obstruction of the lumen by highly differentiated stroma. The myometrial structures are separating and friable (Myo). In contrast, the spiny mouse exhibits a less extensive decidual response, with the lumen and luminal epithelium (LE) intact and readily observable. A distinguishable myometrial bi-layer is clear in SpM, but is not in MMoM.

Decidualized stroma in all species express IL-11 (2D–F), with staining most pronounced during the latest stages of the secretory phase, immediately prior to initiation of menstrual breakdown. Decidualization is significantly reduced ($P < 0.01$, Two-way ANOVA) after entering the proliferative phase where all decidual tissue has been shed in human and SpM tissues, nor is it evident in the repaired (48 h) endometrium of MMoM (Fig. 2G–J, Supplementary Fig. 1). Invariably, the decidualized endometrium in MMoM is entirely transformed at the time of implant removal (0 h).

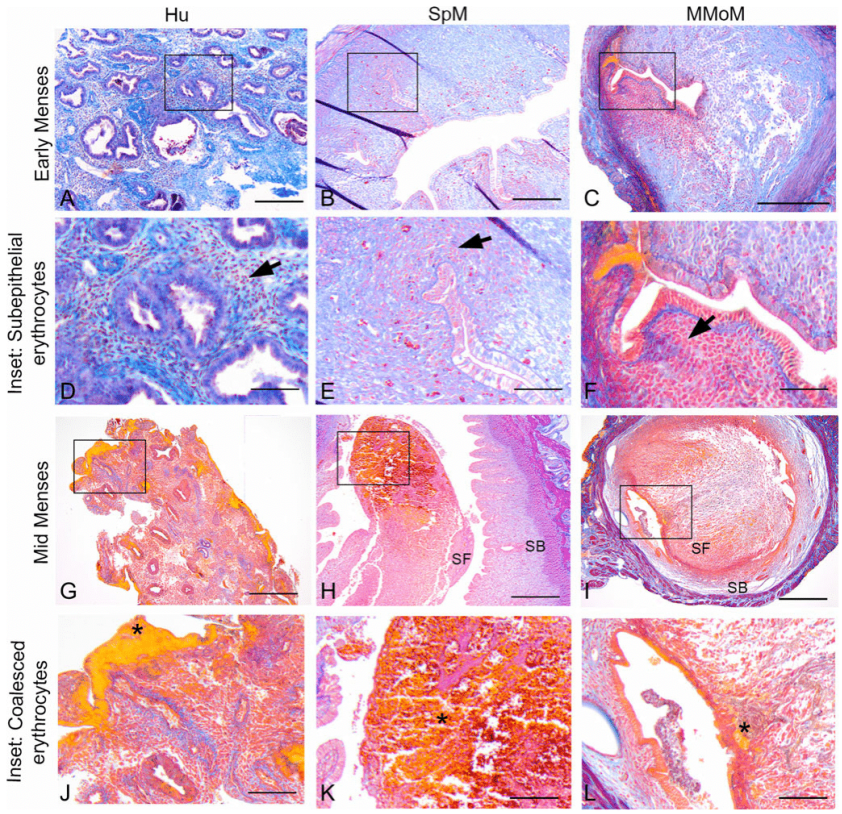

Figure 3 Comparative early and mid-menstrual phase endometrium in the human (Hu), spiny mouse (SpM) and mouse model of menstruation (MMoM). Mallory's trichrome stain. In all three species, sub-epithelial bleeding occurs during the early initiation of stromal breakdown (0–12 h) (**A**–**C**), denoted by regions of erythrocyte infiltration (arrows) (**D**–**F**). Coalescing of erythrocytes is pronounced during mid menstruation (Hu 24–48 h, MMoM and SpM 24 h) (**G**–**I**). Pools of red blood cells (**J**–**L**) are easily recognized in the human and spiny mouse and, to a lesser extent, in MMoM (*). Shedding persists into the stratum basalis (SB) layer in MMoM until the myometrium is reached, but not in SpM, which is restricted to the stratum functionalis (SF). Scale bar (A–C, G–I) = 200 μM and (D–F, J–L) = 50 μM.

During early menstruation in all three species (Fig. 3A–F), patches of sub-epithelial red blood cell infiltration (arrows) are distinguishable. During mid-menses (3G–L), bright orange pools of erythrocytes are notable in all species (*). The integrity of the stratum basalis (SB) in MMoM is compromised during the shedding process, as evidenced by fragmentation and tearing away of the SB from the stratum functionalis (SF). In SpM, shedding is limited to the SF.

Endometrial inflammation, breakdown and repair during menstruation

Cytokeratin positive staining in the early menstrual stages in luminal endometrial epithelium is absent in MMoM. The luminal space is swallowed by extensive decidualization, and epithelial structures are not readily identified. In contrast, both the human and spiny mouse show notable cytokeratin staining in the luminal epithelium (Fig. 4A–C). Progressive menstrual breakdown reduces (but does not abolish) this signal in Hu and SpM (Fig. 4D–F, arrows), with little expression in MMoM until repair initiates. The return of a strong cytokeratin signal coincides with epithelial repair during the later phases of menstruation across all three species (Fig. 4G–I). MMoM demonstrate active menstrual shedding by 24 h with centralized cellular debris present in the lumen (Fig. 4F), although variation exists between females. Some demonstrate continued closure of the lumen with the majority decidua remaining intact and the beginning of SF tearing away (Fig. 3I) at 24 h. SpM shows higher area fraction of cytokeratin positive endometrium during repair than both Hu and MMoM (J-K, Two-way ANOVA, $P < 0.05$). SpM exhibits distinctive regions of focal shedding (*), whereby unshed regions of decidua, distinguished from newly generated stroma by its compact and cobblestone appearance of tightly adhered cells (#), lie adjacent to regions of matrix degradation and epithelial peeling.

NGAL expression in the human and spiny mouse endometrial glands at the initiation of menstruation is similar (Fig. 5). Neutrophils are present in the endometrial stroma, but are mostly discernible in the myometrial

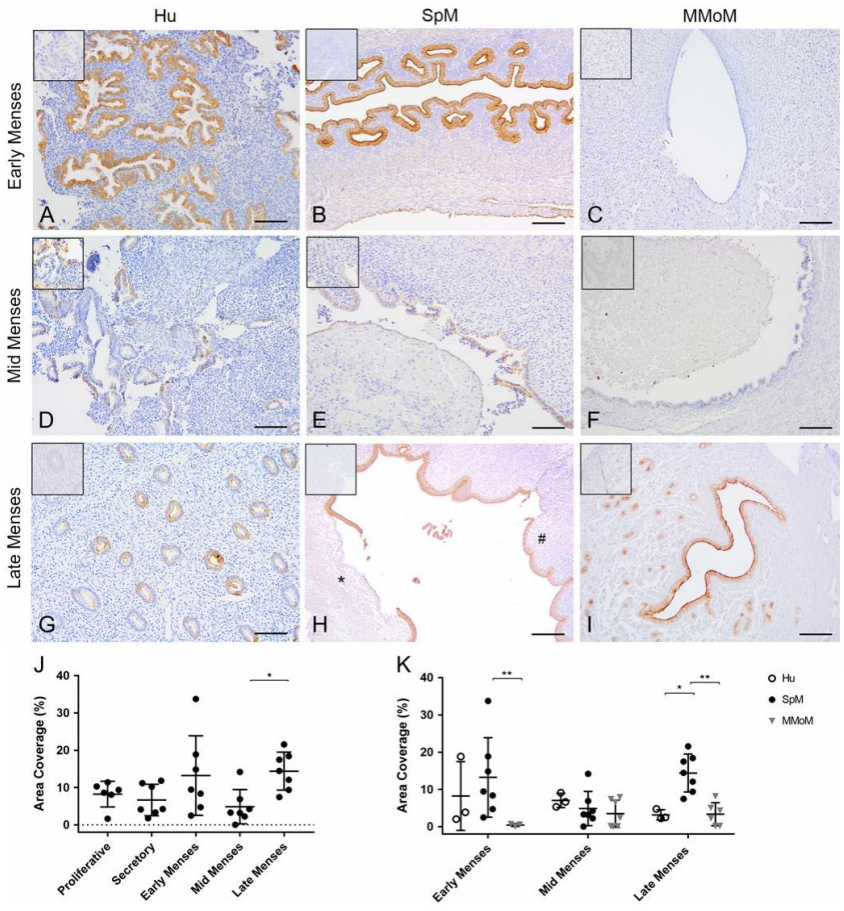

Figure 4 Comparative epithelial changes during menstruation in women (Hu), the spiny mouse (SpM) and the mouse model of menstruation (MMoM). During early menses, stromal and luminal glands are relatively intact in both Hu and SpM (**A**, **B**), but no cytokeratin positive staining is apparent in MMoM (**C**). Cytokeratin signals are minimal during sloughing in all species (**D**–**F**) during mid-menses. As endometrial repair occurs, a strong cytokeratin signal returns in the final phases of menstruation across all species (**G**–**I**). Distinct regions of focal shedding (*) are observed underneath peeling luminal epithelium, opposite to yet unshed regions of compacted, decidualized stroma (#). Scale bars = 100 μM. Samples of corresponding negative controls are depicted in inset squares. A significant decrease in cytokeratin expression is demonstrated in the spiny mouse during mid menses (breakdown) compared to late menses (repair) (**J**, ANOVA, $P < 0.05$). Compared to SpM, cytokeratin expression in MMoM is significantly reduced in early and late menses, and significantly reduced in Hu during late menses (**K**, Two-way ANOVA, $P < 0.001$).

tissue of the spiny mouse. Sporadic clusters of neutrophils are evident in the mouse in the endometrium bordering the myometrium during early menstrual breakdown. Few neutrophils are evident in Hu during early menses (Fig. 5A–C). As menstruation progresses, neutrophil infiltration peaks across all three species (Fig. 5D–F) prior to a marked reduction nearing the end of menstrual breakdown (Fig. 5G–I). A significant increase ($P = 0.01$, ANOVA) in mean area coverage of NGAL immunopositive staining is seen in the functionalis of the spiny mouse uterus during mid menses compared to the proliferative phase (Fig. 5K), and compared to early menses in Hu and MMoM (Fig. 5J–K).

Spiral arteriole remodeling in the spiny mouse endometrium

During the late secretory phase of the spiny mouse menstrual cycle (Fig. 6), spiral artery assemblies in the endometrium (Endo, *) are immunopositive for aSMA (green), while the endothelium and surrounding stroma show positive expression of VEGF (red, arrows, Fig. 6A–F). VEGF staining localizes to the luminal epithelial cells and uterine fluid, with reduced staining in the endometrium during the proliferative phase (arrows, Fig. 6G–I). VEGF is present in the myometrium (Myo) in both phases.

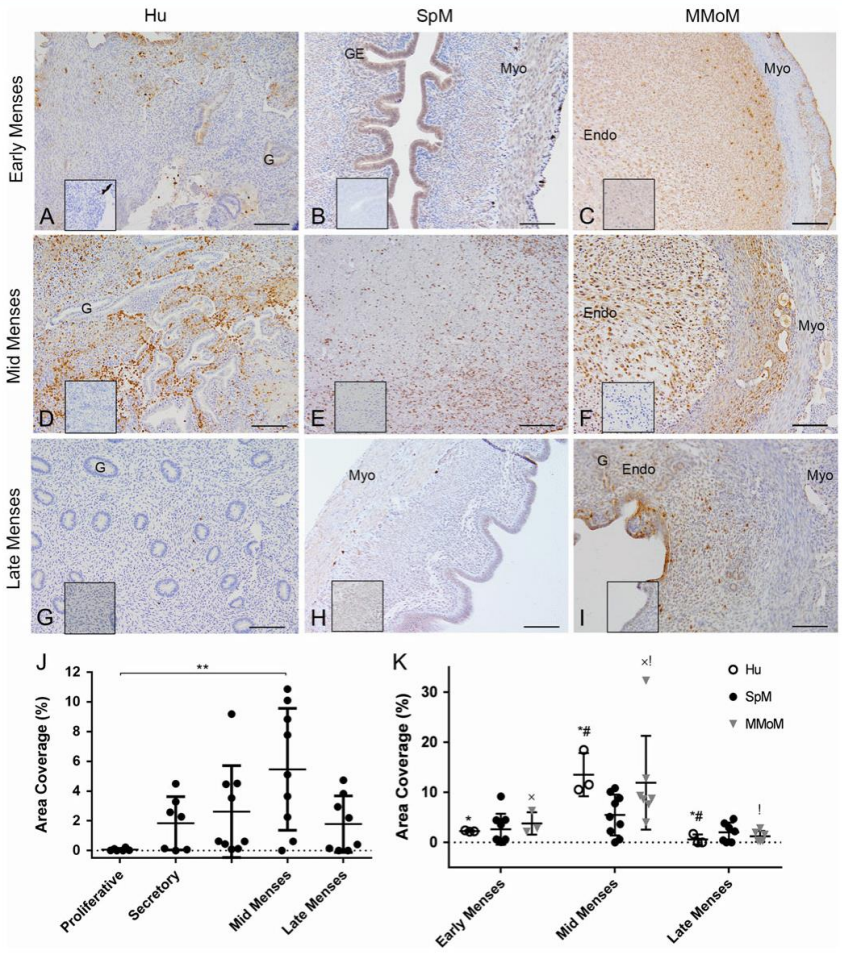

Figure 5 Comparative changes to NGAL expression and neutrophil recruitment during menstruation in women (Hu), the spiny mouse (SpM) and the mouse model of menstruation (MMoM). Neutrophils have not yet obviously infiltrated the endometrial stroma. Glandular epithelial NGAL staining is seen in Hu and SpM. In SpM, neutrophils appear clustered in the myometrium. (**A–C**). During mid menses, neutrophilic influx is at its greatest in all species (**D**–**F**). During the final stages of menstruation, neutrophils are distinctly reduced (**G**–**I**). A significant increase in NGAL in the functionalis is seen during the mid menstrual phase compared to the proliferative phase in the spiny mouse (J, $P < 0.01$, ANOVA). No differences in NGAL expression are seen between species, however all show an increase during mid menses (K, $P < 0.001$, Two-way ANOVA). Like-symbols denote groups which are significantly different from each other within same species (*, # and!). Scale bar = 200 μM. Samples of corresponding negative controls are depicted in inset squares.

A comparison of features of menstruation between the human, mouse and spiny mouse is summarized in Table I.

Discussion

Our data agrees with our initial observation of an average 3-day menstrual bleed in the spiny mouse, with 75% of females examined menstruating according to the range of time previously described (Bellofiore *et al.* 2017). In the spiny mouse, the heaviest endometrial degradation occurs ~24 h after initial observation of red blood cells in the vaginal cytology. The process of menstrual shedding in this species is less predictable than that observed in MMoM, which exhibits menstrual-like bleeding and resolution of repair within 48 h after removal of progesterone (Kaitu'u-Lino *et al.*, 2007). However, this menstruation event is controlled by the removal of exogenous hormone mediated support rather than endogenous reduction in hormone levels upon corpus luteum demise, which will clearly lead to variation in onset of bleeding. The importance of the spiny mouse menstrual model is that it exhibits natural variation between individuals in the overall length of the menstrual cycle. The application of the spiny

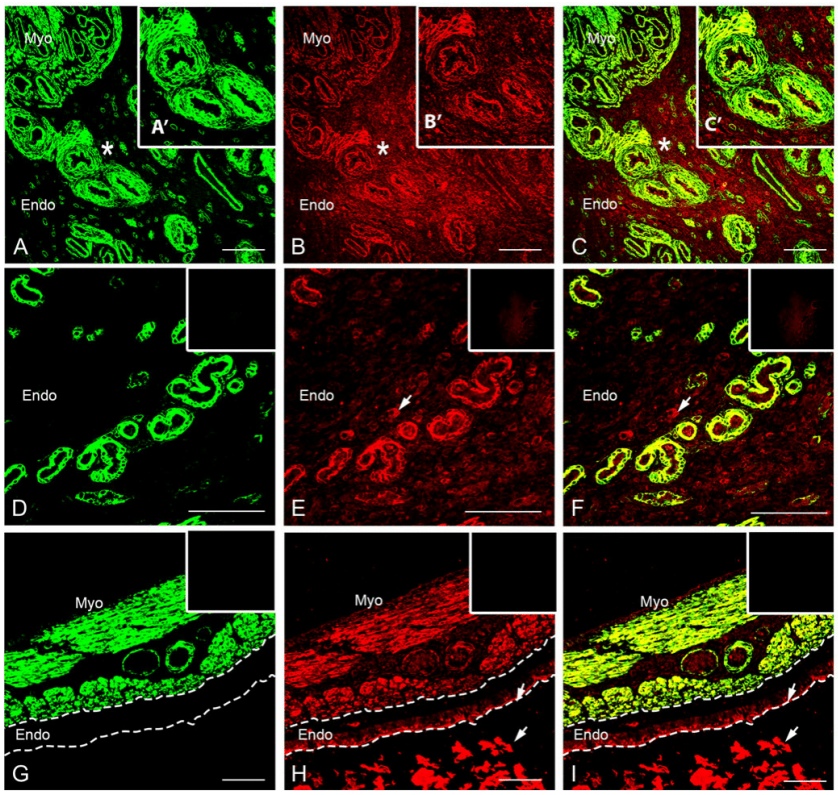

Figure 6 Spiral arteriole formation in the endometrium of the spiny mouse. Green = aSMA, red = VEGF and yellow = merge. **A–F**: Late secretory phase. Clusters of aSMA positive spiral arteries with VEGF positive endothelium (*) stemming from the myometrial base (Myo) of the uterus into the endometrium (Endo) (**A–C**). Inset square shows magnified spiral arteries (**A', B', C'**). aSMA (**D**), VEGF (**E**) and merge (**F**) positive spiral arterioles penetrating endometrium (Endo) at higher magnification. VEGF also stains positively in endometrium stromal cells (**E–F**, arrows). **G–I**: Mid proliferative phase of the spiny mouse menstrual cycle. aSMA positive spirals are not present in the endometrium (**G**). VEGF is positive in the myometrium (Myo), endometrial luminal epithelium and uterine fluid (arrows) (**H**).

mouse as a model for abnormal uterine bleeding, including infrequent and heavy menstrual bleeding, presents an attractive opportunity for further research.

While the uterus of the spiny mouse anatomically corresponds to the Rodentia order in possessing a bicornuate uterus, the physiological likeness to humans of the spontaneous cyclic decidualization observed in the spiny mouse overcomes the pitfalls of using an artificially induced animal model. Although the human uterine samples demonstrate only functionalis as opposed to MMoM and SpM, the extensive and uncontrolled endometrial decidualization observed in MMoM does not recapitulate the human endometrium prior to menses. The extent of this decidual reaction forces not only the endometrial glands and myometrial bilayer to the periphery of the uterine horn but also compresses the lumen and luminal epithelium, and is resolved only after repair is complete. In contrast, the spiny mouse shows natural thickening and receding of the endometrium, while the structural integrity of the organ in its entirety is not compromised. A lumen lined by an intact epithelium is evident overlying decidualized stromal cells.

IL-11 has been proven a classical marker of decidua in humans during the peri-implantation phase (Dimitriadis *et al*., 2000) and presence of IL-11 immunoreactivity in the decidualized spiny mouse uterus reinforces this is a true decidual event.

The spiny mouse endometrium lacks distinctive remodeling of the glands from elongated (proliferative phase) to coiled (secretory phase), as we see in human endometrial tissue. We do observe invaginations of the luminal epithelium, and a few glands protruding from this region, but not to the same degree as seen in women. It may be possible that in the spiny mouse, luminal epithelial glands alone can facilitate the necessary secretions during the secretory phase of the menstrual cycle. Further studies are required in this area.

Humans and spiny mice exhibit similar timing and degree of bleeding relative to the length of the menstrual cycle in each species. All three species are similar in the initiation of menstruation, with focal regions of degrading stromal matrix and infiltration of erythrocytes, and are again comparable in terms of their heaviest day of menstrual. However, the unusual extent of shedding, likely to be due to the

Table I Summary comparison of menstruation in the human (hu), spiny mouse (SpM) and Mouse model of menstruation (MMoM).

	Hu	SpM	MMoM
Reproductive tract	Simplex uterus with fundal body Fallopian tubes	Bicornuate uterus with uterine horns Oviducts	Bicornuate uterus with uterine horns Oviducts
Menstrual cycle	Natural	Natural	Artificially induced
Shedding	Menstrual waves	Menstrual waves	Menstrual waves
Length of menses	5–7 days	2–4 days	1–3 days
Decidualization (Peak % area of endometrium)	Spontaneous IL-11 positive 17.54 ± 8.77	Spontaneous IL-11 positive 16.54 ± 8.13	Artificially induced IL-11 positive 11.75 ± 1.30
Cytokeratin signal in luminal epithelium (% Area of endometrium at early menses)	Present throughout 8.25 + 9.27	Present throughout 13.23 + 10.67	Minimal until menstrual repair initiates 0.45 ± 0.36
Peak neutrophil influx (% Area of endometrium)	Mid menses (48 h) 13.52 ± 4.31	Mid menses (24 h) 5.47 ± 4.10	Mid menses (24 h) 11.91 ± 9.37
Peak erythrocyte infiltration	Mid menses (48 h)	Mid menses (24 h)	Mid menses (24 h)
Spiral artery remodeling	Yes	Yes	No
Glands	Both epithelial and intrauterine	Mostly epithelial	Both epithelial and intrauterine

extensive mass of the decidua that develops in the induced model, appears to cause regions of the stratum basilis to undergo shedding in conjunction with the functionalis. In contrast, the basalis in the spiny mouse remains intact, and is more reflective of menstruation in women, in which only the upper two-thirds of the endometrium is shed (Ferenczy, 1980).

We demonstrated further structural similarities between the spiny mouse and human through analysis of epithelial changes across menstruation. The persistence of cytokeratin staining throughout the menstrual process in the spiny mouse is comparable to the re-epithelialization and repair which occurs in women prior to the cessation of menstrual breakdown, ensuring the breakdown and repair process is simultaneous (Ferenczy, 1980; Nair and Taylor, 2010). In this study, the luminal epithelium of the MMoM was not immunopositive for cytokeratin during early menses. This is in contrast to previous studies which identified new proliferating luminal epithelial cells lining the residual basal stroma within 8 h of bleeding onset (Cousins, *et al.*, 2014). In many of the MMoM sections, early menses tissues contained little to no uterine lumen. This may be the consequence of luminal damage during administration of the decidual stimulus (oil), however, more likely reflects the extensive decidual reaction which appears to severely compress the luminal epithelial cells, and consequently shows significantly less cytokeratin staining than SpM. Possibly due to both (a) small sample size and (b) the wave like nature of menstruation, and thus, random sampling of incomplete repaired tissue, we did not observe a significant increase in cytokeratin expression during late menses in Hu or MMoM.

Transverse sections of the uterus are traditionally used for histological analysis in reproductive studies of rodents. However, we demonstrate that longitudinal sections during menstruation show focal regions of simultaneous matrix degradation and epithelial shedding adjacent to unshed decidua in SpM. This pattern of smaller localized shedding and neighboring areas of endometrium yet to undergo breakdown supports the menstrual waves theory demonstrated in both the MMoM and women (Kaitu'u-Lino, 2007; Garry *et al.*, 2009), where not all endometrial tissue is shed at once, but rather in fragmentary ripples to avoid mass tissue and blood loss, and allow for the repair process to take place. This method of collection likely accounts for the variability seen at 24 h in MMoM, where some sections demonstrate a total degradation of stromal matrix with shedding decidua and prominent blood loss, and others have most of the functionalis intact with less erythrocyte infiltration into the lumen.

The detection of IL-8 in menstrual debris of the spiny mouse demonstrates a typical inflammatory response during uterine shedding. This observation, in conjunction with NGAL analysis, also supports, as previous finding have in human tissues, that IL-8 is involved in attracting and activating neutrophils during menstruation (Arici *et al.*, 1998). The maximum area of coverage of neutrophils during the inflammatory process of menstruation in the spiny mouse is less than that in humans and MMoM, but not significantly so. Our findings confirm the role of neutrophils in stromal matrix destruction seen in both humans and the mouse model (Armstrong *et al.*, 2017). The presence of the pro-inflammatory and antibacterial cytokine MIF in the spiny mouse menstrual debris suggests macrophage activation, and supports recent findings that macrophages play a crucial role in both the degradation and regeneration processes of menstruation (Calandra and Thierry, 2003; Garry *et al.*, 2010; Evans *et al.*, 2016). The variation in MIF and IL-8 proteins as identified by western immunoblotting likely reflects the different amount of menstrual debris recovered from the endometrial lumen of each individual mouse. As highlighted in Fig. 1, the timing and degree of shedding at 24 h varies between spiny mice, as observed in humans, therefore the amount of shed material also varies, again, as in humans (Evans *et al.*, 2014). However, we do demonstrate that proteins identified in human menstrual fluid are also readily detectable in menstrual debris obtained from the spiny mouse, reinforcing the utility of this model as a human like model of menstruation.

An additional advantage of the spiny mouse as a natural model of menstruation is that spiral arteriole remodeling during the pre-menstrual phase is characteristic of menstruating primates, and cannot

be induced in MMoM. Epithelial VEGF expression during the mid-proliferative phase concurs with previous findings that VEGF plays an essential role in post-menstrual re-epithelialization and repair, and detection in the stromal cells during the secretory phase is consistent with past studies of the regulative role in endometrial vascularization (Shifren *et al.*, 1996; Fan *et al.*, 2008). This suggests that the spiny mouse is a may be used to investigate disorders of poor angiogenesis and how disruption of the remodeling of this vasculature impacts pregnancy outcomes (e.g. pre-eclampsia).

We acknowledge two primary methods of euthanasia used in this study in the rodents: carbon dioxide (mouse) and isoflurane overdose (spiny mouse). Standard operating protocols for our spiny mouse colony preference isoflurane overdose given its more immediate effects due to the fragile nature of the skin (Seifert *et al.*, 2012) and highly active behavior of this species, minimizing the potential stress (Wong *et al.*, 2013) and subsequent risk of the spiny mice of sustaining injury. While mice produce corticosterone, spiny mice, like primates, secrete cortisol as their stress hormone. For the purposes of this study, we therefore maintain that the method of euthanasia could have potential implications in comparing inflammation reactions between the rodent species, but unlikely between the spiny mouse and human. Variable stress levels between the spiny mice and mouse may have altered corticosteroid production and subsequent levels, but are unlikely to significantly affect endometrial expression of immune marker NGAL given the abrupt exposure to hypoxic environment.

High throughput uses and the ability to analyze entire organs histologically are undeniably advantageous using small, non-primate species for menstrual research. Our research demonstrates the assets of the spiny mouse, in particular for vascular studies, decidualization, stromal breakdown and epithelial shedding. However, MMoM demonstrates a closer immune profile in neutrophilic recruitment and may be preferable when examining inflammatory processes and initiation of repair.

While our study is the first to demonstrate primate-like qualities of the menstrual cycle in a rodent species, and highlights the advantages of the spiny mouse for menstrual research, we acknowledge the current limitations of this model. The uniqueness of the physiological profile of the spiny mouse limits use of commercially available anti-mouse antibodies for immediate successful immunohistochemical staining. Indeed, we determined that anti-human antibodies generally provided positive immunohistochemical staining within the spiny mouse endometrium, although this may not be the case in other tissues. However, many anti-human antibodies are raised in mice, and often lead to substantial cross-reactivity in the spiny mouse. For these reasons in the current study, we were unable to optimize several markers of immune cells within the endometrium of the spiny mouse, including CD45 (total leukocytic recruitment) and CD68 (macrophages), due to either non-specific staining in the stromal matrix, and therefore inaccurate quantification, or no identifiable staining. Our colony of spiny mice may have a unique CD profile, or immune antigens which require non-standard fixation methods and/or use of other media (e.g. paraformaldehyde). Researchers must bear in mind the current lack of widespread use of spiny mice and difficulties implementing common techniques on reproductive tissues.

Our findings of the nature of menstruation in the spiny mouse, including controlled spontaneous decidualization, presence of spiral arteriole formation and, importantly, the natural variation and degree of bleeding are encouraging. We continue to support the spiny mouse as a future potential alternative, and readily available animal model, to study menstrual health and disorders.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

N.B. and J.E. conceived the project, performed experiments and analyzed data. S.R. performed experiments. H.D. and P.T-S. provided critical revisions of project design and data and manuscript editing.

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Conflict of interest

The authors declare no competing interests.

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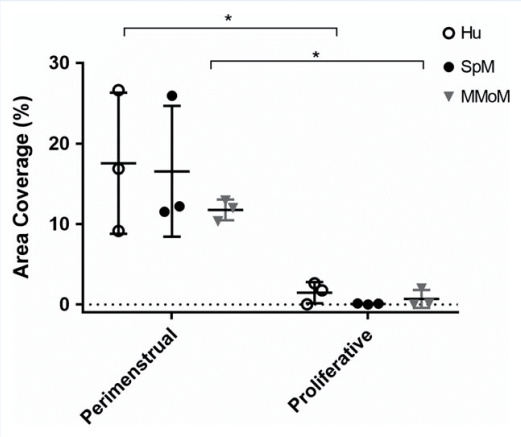

Supplementary Figure S1 Area (%) of endometrial functionalis immunopositive for IL-11 during early menstrual (~0 h) and proliferative phases in the human (open circle) and spiny mouse (closed circle). The mouse model of menstruation (triangle) is ovariectomized and hence does not demonstrate a proliferative phase. For within species analysis of decidualization, early menstrual endometrium (0 h) has been compared to fully repaired (48 h) endometrium at the cessation of menstruation.

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SUPPLEMENTARY DATA

Supplementary Table SI Antibodies and DAB and immunofluorescent immunohistochemistry protocols for paraffin embedded uterine tissues in the spiny mouse (SpM), mouse model of menstruation (MMoM) and the human.

Primary antibody	Manufacturer/ Catalog number	Purpose	SpM		MMoM		Human		Blocking solution	Secondary antibody	Negative control
			Dilution	Retrieval	Dilution	Retrieval	Dilution	Retrieval			
Rabbit polyclonal anti-PCNA	Abcam #AB152112	Proliferation	1:1000	Citrate buffer	1:1000	Citrate buffer	1:1000	Citrate buffer	10% NGS 2% HuS in TBS-T	Goat α Rabbit Vector (#BA-1000)	Rabbit IgG (Dako, #X093602)
Rabbit polyclonal anti-Cytokeratin	Santa Cruz #sc-15367	Epithelial changes	1:750	Citrate buffer	1:500	Citrate buffer	1:500	Citrate buffer	10% NGS 2% HuS in TBS-T	Goat α Rabbit Vector (#BA-1000)	Rabbit IgG (Dako, #X093602)
Goat polyclonal anti-NGAL	R&D Systems #AF1757	Neutrophil recruitment	1:110	Citrate buffer*	1:110	Citrate buffer	1:500	Citrate buffer	20% NRS 4% BSA 0.01% CFSG 0.1% Triton-X 100 0.05% Tween20 In TBS	Rabbit α Goat Vector (#BA-5000)	Goat IgG (R&D systems, #AB-108-C)
Rabbit polyclonal anti-IL11	Santa Cruz # SC-7924	Decidual cell formation	1:50	Tris-EDTA	1:100	Tris-EDTA	1:100	Citrate buffer	10% NGS 2% HuS in TBS-T	Goat α Rabbit Vector (#BA-1000)	Rabbit IgG (Dako, #X093602)
Mouse monoclonal anti-aSMA	Dako (#M0851)	Vasculature identification	1:250	Citrate buffer*					20% NGS 4%BSA 0.01% CFSG 0.1% Triton-X 100 0.05% Tween20 In PBS	Alexa Fluor 488 (Invitrogen, #A11029)	Blocking solution
Goat polyclonal anti-VEGF	R&D Systems (#AF-293-NA)	Vasculature identification	1:500	Citrate buffer*					20% NDS 4%BSA 0.01% CFSG 0.1% Triton-X 100 0.05% Tween20 In PBS	Alexa Fluor 594 (Invitrogen, #A11058)	Blocking solution

Microwave citrate buffer antigen retrieval (pH 6.0) was used on med-high setting for 5 min, and tissues sat in hot citrate buffer for 20 min, except for tissues marked with * where tissues were boiled for on high for 9 min and simmered on med-low for 7 mins, followed by sitting in hot buffer for 40 min, or ** where tissues were boiled at 98°C for 30 min. Tris-EDTA retrieved tissues were heated in a Tris-EDTA (pH X) in a water bath for 30 min at 95–100°C, before removal and sat in hot Tris-EDTA buffer for 20 min. Following antigen retrieval, slides were then washed twice in PBS or TBS-Tween 20 (0.02%) and once in PBS or TBS. Serum blocks are Normal Goat Serum (NGS), Human Serum (HuS), Bovine Serum Albumin (BSA), Normal Horse Serum (NHS), Normal Donkey Serum (NDS) and Normal Rabbit Serum (NRS). aSMA and VEGF tissues were subjected to a 2-day incubation, each antibody overnight at 4°C.

CHAPTER 5:

ANXIETY-LIKE BEHAVIOUR AND FOOD INTAKE ARE INCREASED IN THE SPINY MOUSE BEFORE MENSTRUATION: A UNIQUE PRE-CLINICAL MODEL FOR EXAMINING PREMENSTRUAL SYNDROME

Chapter 5 of this thesis comprises of the following manuscript, submitted to Human Reproduction (12 July 2018; currently under review):

Anxiety-like behaviour and food intake are increased in the spiny mouse before menstruation: a unique pre-clinical model for examining premenstrual syndrome

Running Title: Premenstrual syndrome in the spiny mouse

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ABSTRACT

Study Question: Does the newly discovered menstruating spiny mouse exhibit behavioural and metabolic changes in correlation with premenstrual phases of the menstrual cycle?

Summary answer: This is the first report of cycle variability in the exploratory and interactive behaviour, and food consumption in menstruating spiny mice, and demonstrates that physiological changes are also dependent on within-subject variation.

What is known already: Premenstrual syndrome (PMS) is a prominent cyclic disorder that affects millions of women worldwide. Up to 90% of women endure symptoms of impending menstruation, such as bloating, abdominal cramping and nausea. Consequently, over 75% of women experience recurrent physical and emotional symptoms which are extreme enough to seek intervention. Due to a lack of an appropriate animal model, the mechanisms underlying PMS are poorly understood, and subsequently, effective treatments are limited.

Study design, size, duration: This study analyses the changes in behavioural responses to the investigator during vaginal lavage (1, n=14), exploratory behaviour (2, n=11) and metabolism (3, n =20) across the menstrual cycle in the spiny mouse.

Participants/materials, setting, methods: We performed vaginal lavages on virgin spiny mice (6-8 months of age) and subjected each cohort of females to repeated measures for (1), (2) and (3) above. Stages of the menstrual cycle were designated as early follicular, late follicular, early luteal, late luteal, early menstrual and late menstrual, with the late luteal and early menstrual phases considered as pre-menstrual phases and analysed using generalised estimating equations. (**1**) The behavioural responses to researcher handling were scored on an increasing scale of severity during the lavage process (e.g. restraint, frequency of vocalisations, total handling time). (**2**) Exploration, memory and sociability were assessed

through subjection to Open Field (OF), Novel Object Recognition (NORT), Social Interaction (SI) and Elevated Plus Maze (EPM) tests. (**3**) Physiological changes were measured over a 24-hour period in metabolic cages. Results are mean ± SD with statistical significance set to $p<0.05$.

Main results and the role of chance: 1) During pre-menstrual phases, cycling females had significantly increased probability of: manifesting difficulties during restraint (4x), vocalising (8x) and exhibiting isolation in the cage (40x). We saw significant increases in handling time during the pre-menstrual phase (76 ± 16 secs) compared to controls (55 ± 7 secs). **2**) Cycling females in their early menstrual phase travelled significantly less distance in the outer zone of the OF arena (13.3 ± 9.0 m) than females in their early luteal phase (22.3 ± 9.9m) and at significantly reduced velocities (40.2 ± 10.5mm/s and 78.8 ± 31.0mm/s, respectively). These females also travelled less distance in the EPM open arms (3.2 ± 2.8m versus 7.0 ± 5.5m, respectively). No differences were observed in NORT or SI across the cycle. **3**) Spiny mice demonstrated a significant increase in food consumption (percentage of body weight) during the early follicular and late luteal phases (3.9 ± 2.4%, 3.8 ± 2.1%, respectively) compared to the late follicular phase (2.3 ± 2.6%).

Large scale data: N/A

Limitations, reasons for caution: This is an observational study to determine fundamental changes in behaviour and metabolism in a novel species which lacks commercially available reagents for common laboratory techniques.

Wider implications of the findings: The timing of these behavioural and physiological changes suggests that spiny mice exhibit symptoms analogous to premenstrual syndrome in higher order primates, thus providing a pre-clinical model for testing novel interventions to

alleviate pre-menstrual symptoms overcoming many limitations associated with this research area.

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Keywords: Menstruation, spiny mouse, premenstrual syndrome, behaviour, metabolism

INTRODUCTION

The commencement of the menstrual cycle is identified by shedding of decidualised endometrium and accompanying overt vaginal bleeding in in a non-conception cycle. Without the prolonged luteotrophic support provided by an embryo, a cascade of inflammatory events is mediated by the degeneration of the progesterone-secreting corpus luteum. Progesterone withdrawal positively regulates chemokine and proteolytic enzymes production, including interleukin-8 (Kelly, et al., 1994), matrix metalloproteases -3 and -9 (Salamonsen, 2003, Salamonsen and Woolley, 1999), and prostaglandins (Mannix, 2008, Martin, et al., 2014).

The intricacies of endometrial degradation and simultaneous repair are not entirely dissimilar to that of wound-healing (Salamonsen, 2003), involving complex interplay between ovarian and endometrial tissue, and the onset of menstruation is typically viewed as an inflammatory reaction. Recent evidence has demonstrated a link between inflammation marker high sensitivity C-reactive protein and symptoms of premenstrual syndrome (Gold, et al., 2016).

Significant alterations in mood and behaviour during the pre-menstrual phase have been clinically recognized since ancient times. However, the acceptance of premenstrual syndrome (PMS) as a physiological, rather than a solely psychological, condition is relatively recent among both the scientific and general community, and formal criteria for specific diagnoses were only proposed 40 years ago (Endicott, 2000). Reports of fluctuations in eating behaviour, sexual, and social behaviour in wild yellow baboons (Hausfater and Skoblick, 1985) and captive vervet monkeys (Rapkin, et al., 1995) sparked newfound interest in the notion that there were potential biological and perhaps even evolutionary underlying foundations for the syndrome in not only humans but other higher order primates. Classical

symptoms of PMS frequently culminating in the 48 hours leading to menstruation are commonly linked with discernible behavioural changes; most notably, anxiety, anger/irritability and social withdrawal, with anger often manifesting earlier than other emotional symptoms (Meaden, et al., 2005, Yonkers, et al., 2008, Zaka and Mahmood, 2012). However, the myriad of not only emotional but also physical symptoms immediately prior to, or in the early stages of menses, are experienced to some degree by an estimated >70% of sufferers worldwide (Borenstein, et al., 2005, Mishell Jr, 2005, Reid, 1991), and up to 8% severely enough to disrupt functions of daily life (Yonkers, O'Brien and Eriksson, 2008). Accompanying somatic symptoms may also include mastalgia, increase in carbohydrate intake, hypertension, nausea, fatigue, sleep deprivation and abdominal pain.

The severity and subtypes of PMS are subject to individual variation, but studies suggest that around half of these women's symptoms are extreme enough to seek intervention due to disruption of day to day life, especially impacting inter-personal relationships within the workplace and home life. In addition, PMS inflicts a high economic burden, with an estimated that 15% reduction of work productivity in the USA attributable to menstrual related problems, and indirect associated costs of up to $4333 USD per patient (Borenstein, Chiou, Dean, Wong and Wade, 2005).

Despite many advances in identification and treatment of other menstrual conditions, PMS continues to lack widespread and efficacious treatment options for women. This is primarily due to the limitations imposed by the restricted number of menstruating species available for long-term research, having been confined to studies in humans and non-human primates. However, the discovery of a novel menstruating species belonging to the order Rodentia may play a key role in the future of menstrual disorder research. Our team identified a human-like

menstrual cycle (lasting approximately 9 days) in the Egyptian spiny mouse (Acomys cahirinus), with similarities in pre-implantation decidualisation, progesterone profile and endometrial breakdown (Bellofiore, et al., 2017).

Whilst we have shown the similarities in physiological aspects of menstruation between spiny mice, including early menstrual inflammation (Bellofiore et al., 2017, Bellofiore et al., 2018), behavioural changes corresponding with late luteal and early menstrual phases e in this species have not yet been investigated. This is essential in determining the suitability of the spiny mouse as a complete and robust model of human-like menstrual biology. This study will examine whether behavioural and metabolic changes occur across the spiny mouse menstrual cycle and propose the existence of anxiety-like behaviours during premenstrual phases (i.e. the late luteal and early menstrual phases).

METHODS

Ethics

All research was approved in advance by the Monash University/Monash Medical Centre Animal Ethics Committee (#MMCB-2016/40), and all experimental procedures adhered with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Spiny mouse husbandry

The spiny mouse used in this study were sourced from our in-house research colony (Monash University, Clayton, Australia). Virgin females aged 6-8 months and weighing between 28-38g were maintained as previously described (Bellofiore et al., 2017). Due to the fragility of spiny mouse skin, our animals are captured using a transparent, plastic container and are restrained with a small handtowel, as demonstrated in Figure 1. Stages of the menstrual cycle were assigned as early follicular, late follicular, early luteal, late luteal, early menstrual and late menstrual, with the late luteal and early menstrual phases designated as being premenstrual. The average length of the menstrual phase is 3 days (Bellofiore et al., 2017), hence early menstrual was defined as within the first 24h of blood in the vaginal cytology. Due to the susceptibility of spiny mice to demonstrate alterations in menstrual cycle length, and increased anxiety-like behaviour when introduced to foreign scents (i.e. unfamiliar people), a single investigator conducted all experimental procedures to reduce the risk of confounding the data. Prior to data collection, each cohort of females were acclimatised to the tests, which is specific to each and described in detail below.

1) Qualitative assessment of spiny mouse behaviour

To assess the changes in interaction between spiny mice and the researcher during routine vaginal lavage sampling, n=14 females were scored on an increasing scale of severity of response (0 = None, 1 = Mild, 2 = Moderate, 3 = Severe) to 10 parameters: 1) initial position in the cage prior to handling; 2) weighing; 3) towel; 4) scruffing; 5) restraint; 6) lavage; 7) abdominal palpation;8) vocalisations escapes and; 10) return to cage (listed in detail in Table 1). Females were acclimated to the investigator, handling and behavioural suite for four days prior to collection of behavioural data. This is based on our own observations and previous determination of optimal acclimatisation periods (unpublished observations). Spiny mice were subjected to daily vaginal lavage and behavioural response scoring for 10 consecutive days (which we have previously proven to be well tolerated) to account for individual subject variation. During this assessment, the investigator was blind to menstrual cycle stage until completion of data collection for each female. At the end of every lavage process, a total stress score was calculated for each spiny mouse by combining the 10 scores for each above parameter, with the maximal total stress score being 30. At the end of 10 days, the scores were standardised for each individual by subtracting the lowest total stress score value from all other scores e.g. if a female's lowest total stress score recorded was "7", and her highest total stress score was "23", her standardised total stress score would be "16". In this way, changes in behaviour were relative to the individual female's baseline of normal behaviour. Other parameters measured included body weight (g), time taken to capture and overall procedure time (s). Data was analysed using RM-ANOVA. Data was analysed using Generalised Estimating Equations (GEE) in SPSS (IBM, Version 22) by categorising the spiny mice as having a "Mild" (0 or 1) or "Severe" (2 or 3) response. For GEE analysis, the

early follicular phase served as the "control" stage of the menstrual cycle, and data deemed significant if changes $p < 0.05$ observed versus this control phase.

2) Quantitative assessment of spiny mouse behaviour

To quantify behavioural characteristics across the menstrual cycle, n=11 females were subjected to previously validated tests (Ratnayake, et al., 2012). Spiny mice underwent blinded vaginal lavage immediately prior to testing. To ensure blind capture of each menstrual cycle stage, each female performed 4 tests within a 24h period, with intermittent breaks across a 2-week period (Figure 2A). In addition, the order of the tests was altered each time to minimise the risk of familiarisation with the arenas. This protocol allows blinded capture of at least 5/6 menstrual cycle stages by a single investigator (necessary to minimize handling variation as outlined). After 5 sets of tests were completed, vaginal cytology was analysed to determine when a subject would enter the single remaining un-tested cycle phase to complete testing and ensure cycle stages were not repeated and/or missed. As cycle synchronisation upon co-housing does not occur in this species, the menstrual cycle stage of each female was variable. Hence, we consider the effect of order of tests taken/unblinding for only one stage to be negligible on data extraction. This sampling method was optimised previously in our laboratory (unpublished) as sufficient to remain blinded without evoking excess handling stress in the spiny mice. Spiny mice were acclimated to the behavioural suite for a minimum of 30 mins prior to testing. All behavioural tests were recorded using Topscan Lite™ 2.0 (CleverSys), with GraphPad Prism 7.02 used to analysis statistically significant differences of $p < 0.05$. (Refer to supplemental Figure S1 for arena fields of each test).

Open Field (OF). Females were placed in the centre of a 50x50cm square box with walls 60cm high. Movement was recorded by a video camera for 5 minutes. Parameters assessed were time/discrimination index, distance and velocity in the inner and outer zones.

Novel Object Recognition Test (NORT). This test was performed in two parts (NORT 1 and NORT2) with OF always preceding NORT1 and 2. In NORT1, two similar objects were placed in opposite corners of the OF arena (in this case, clear plastic bottles). The spiny mouse was then introduced and movement recorded for 10 mins. The female was returned to her home cage for a minimum of 1 hour before NORT2 commenced. One of the bottles was replaced by a slightly dissimilar object (e.g. coloured glass bottles) before the spiny mouse was reintroduced into the arena for a further 10 min. The distance, velocity and periods of time spent exploring each of the objects was recorded.

Social Interaction Test (SI). The spiny mouse was placed into central chamber of a rectangular box which was divided into 3 chambers with spaces in the dividing walls to allow spiny mice to move between them. Females were allowed 5 minutes acclimatisation to the box before she was removed. In each of the small holding boxes opposite ends of the testing arena, either a "stranger" spiny mouse (of the same age and sex, but ovariectomized – see supplemental data 1) or a "known" cage mate (in early follicular phase) was placed. The test subject was then reintroduced into the middle chamber of the box, and left to explore for 5 min, with the exploration of each of the three chambers recorded.

Elevated Plus Maze Test (EPM). The spiny mouse was placed at the intersection (central zone) of an elevated cross with 4 arms; 2 arms were open and 2 enclosed by walls. The female's time spent in the open, closed or central zones, with distance and velocity travelled, were recorded for 5 minutes.

Metabolic Cages

Females were placed in glass cages for 24 hours to measure food and water intake, and faeces and urine excretion (Supplemental Figure S2). All spiny mice were trained and acclimatised to the cages for a period of 4 hours the day before the experiment commenced. Semi-blind metabolic cage experiments were conducted in the time frame described in Figure 2B. While similar to the behavioural testing timeline, metabolic cage isolation can increase the stress of the females if overly prolonged and subsequently alter females' menstrual cycles. Hence, this method of exposure to metabolic cage analysis has been optimised to account for adequate acclimatisation to the cages and also provide sufficient rest periods without the need to re-train the females. The spiny mice were weighed and a vaginal lavage sample taken prior to isolation, and also weighed upon removal from the metabolic cage.

RESULTS

Anxiety response in the spiny mouse across the menstrual cycle

Standardised behavioural scoring of female spiny mice demonstrated significant changes in response to multiple aspects of the vaginal lavage procedure during different phases of the menstrual cycle (Figure 3, Table 2). We demonstrate that compared to the early follicular (EF) phase, early menstrual (EM) females were approximately 40 times more likely to be isolated ($p = 0.041$, Table 2) and 3 times more likely to resist restraint ($p = 0.012$, Table 2). Spiny mice in the late luteal (LL) phase were 8 times more likely to frequently vocalise ($p<0.01$), and 8 times more likely to respond severely to the handtowel holding during the late luteal phase ($p<0.01$), compared to the EF phase. The likelihood of females scoring severely upon return to the cage was increased 7-fold in early luteal (EL) females ($p<0.01$), and 2-fold in late menstrual (LM) females ($p = 0.037$).

The overall stress score exhibited, established by combined responses to each parameter (initial position, weighing, towel, scruffing, restraint, lavage, abdominal palpation, vocalisations, escapes and return to cage) was increased in the LL phase (8.35) compared to the early luteal (EL) phase (5.25) (Figure 3A). The total handling time of the spiny mouse significantly increased from 62.5 ± 9.9s in the EF phase to 75.7 ± 15.8s in the LL phase (3B) reflecting the previously described stress/anxiety responses (3A). During the EM phase, females travelled significantly less distance in the OF outer zone (13.3 ± 9.0 m) than females in their EL phase (22.3 ± 9.9m) and at significantly reduced velocities (40.2 ± 10.5 (EM) versus 78.8 ± 31.0 mm/s (EL), respectively) (Figure 4A-B). This behaviour was recapitulated in the EPM test, whereby EM females travelled less distance in the open arms versus EL phase mice (3.2 ± 2.8 versus 7.0 ± 5.5 m, respectively), at significantly reduced velocity

through the central zone (42.5 ± 22.6 (EM) versus 84.9 ± 50.0 mm/s (EL)) with a trend for reduced distance through the central zone (3.9 ± 1.8 versus 2.4 ± 1.7 m, p = 0.059) (Figure 5A-C). No preference for the known or unknown object were observed in NORT2 between stages of the menstrual cycle. However, in both NORT 1 and 2, a significant effect of matching was observed in both distance and velocity (Figure 6A-E). No differences in social interaction were apparent across the menstrual cycle, although spiny mice showed preference to explore either of the other animals over the neutral zone in all phases (Figure 6F).

Metabolic changes in the spiny mouse across the menstrual cycle

Spiny mice demonstrated cyclical changes in food consumption (Figure 7A), with a bimodal pattern exhibited. The period of greatest food intake occurs during the EF phase at 3.9 ± 2.5%; prior to ovulation. The secondary peak occurs during the LL phase at 3.8 ± 2. %, preceding the onset of menstruation. No changes were observed between phases of the menstrual cycle for water consumption and urine excretion (Figure 7B and C, respectively), although within-subject variation is a significant contributor to both results obtained.

Despite substantial increases in food intake, no significant changes in percentage of body weight upon removal from the metabolic cage were recorded (Figure 7D). This is and contrary to significant cyclical fluctuations in body weight of spiny mice maintained in conventional cages (Figure 7E), peaking during the LF phase (1.4 ± 1.4) and troughing during the EL phase (-1.5 ± 2.0).

DISCUSSION

Our study is the first to report both behavioural and metabolic alterations across the menstrual cycle of a rodent species which reflect those observed in human females. This further reinforces the similarity of spiny mouse reproductive physiology and associated behaviours to that of menstruating humans and non-human primates. We also advance our understanding of the biology of this captive species as an emerging research model.

The selective advantages of PMS symptoms, including alterations in mood is still not understood, though it is thought to be remnant of oestrous cycles, particularly in increased activity and energy uptake during ovulation(Yonkers, O'Brien and Eriksson, 2008). Severe premenstrual symptoms as currently experienced by modern society is hypothesized to have arisen as result of conflicting cultural versus biological evolution, whereby women have more frequent menstrual cycles (as opposed to lactational amenorrhea) but still retain the genetic blueprint of our hunter-gatherer ancestors(Gillings, 2014). It may also be that some psychological and somatic symptoms experienced in premenstrual syndrome is necessary to ward off males and minimize the risk of mating during times of subfertility, which after generations has been exacerbated by lifestyle factors such as diet and stress. Understanding the underlying biological cues for PMS and the characterization of a suitable model to study and manipulate the manifestations of this syndrome are important in the development of therapeutics. This is particularly true in the treatment of PMDD, which can seriously impact sufferer's mental health and is associated with suicidal attempts highlighting the need for appropriate models.

. Of note, the menstrual cycles of female spiny mice do not develop spontaneous pseudopregnancy when housed with several cage mates, and do not synchronise based on

cage mates or the influence of an alpha-females as demonstrated by other rodents and non-human primates, respectively (Parkes and Bruce, 1961, Rapkin, Pollack, Raleigh, Stone and McGuire, 1995, Van der Lee and Boot, 1955). Therefore, each animal used in the current study was carefully staged, with cage mates presenting with various menstrual cycle stages. This staging is critical for interpretation of study data and is an essential finding for use of this unique model in future studies of menstrual physiology.

Behavioural scoring of individual females' interactions with the investigator demonstrated a reduced tolerance to the lavage process during pre-menstrual and early menstrual phases, as evidenced by a striking preference for isolation, increased frequency of vocalisations and resistance to the handtowel restraint, increase in overall handling time and standardised stress scores. By accounting for the variation in individual subject behaviour, we are able to demonstrate relative changes not only in overall phases of menstrual cycle, but within the subject herself compared to her own baseline of 'normal'. Again, the careful mapping of each female is critical for understanding overall behavioural changes. As with humans, the response of each spiny mouse is unique and great care must be taken not to ascribe generalized changes to a study cohort. Primate studies demonstrate a preference for perimenstrual female baboons to eat in treetops and avoid interaction with others (Hausfater and Skoblick, 1985) with initiation of avoidance and submission in vervet monkeys suggestive of behavioural characteristics analogous to premenstrual syndrome in humans (Rapkin, Pollack, Raleigh, Stone and McGuire, 1995). Despite spiny mice living as social animals demonstrating a tendency to huddle together, our observation of high preference for isolation during premenstrual phases suggests a similar preference for social withdrawal, further highlighting parallels with other menstruating species. Furthermore, this preference

for isolation during the early menstrual phase corresponds with previously recorded progesterone withdrawal in the spiny mouse (Bellofiore et al., 2017), supporting the notion that progesterone withdrawal is important in inducing premenstrual dysphoria, as demonstrated in rat models using exogenous hormonal manipulations (Li, et al., 2012).

The initial semi-quantitative cyclical changes in behaviour were validated by open field and elevated plus maze tests; both designed to test anxiety and exploratory behaviour. The spiny mice showed peak exploration, as depicted by distance travelled and velocity, during the early luteal phase, and a nadir in exploration during the early menstrual phase correlating with the time of social isolation described above. In rats, females are typically most active during oestrous (Anderson, 1940, Birke and Archer, 1975), when oestrogen levels are highest and ovulation occurs. Our expectation was peak exploration would occur during the late follicular phase, when vaginal cytology is prominently cornified epithelial cells. However, the very final stages of oestrous may overlap with infiltration of leukocytes and there is a small possibility of cytology being deemed metestrous/early luteal phase thus there is a possibility in the current study that late follicular samples may have been misclassified as early luteal. Nonetheless, the activity data herein appear to correlate largely with that observed in the human, with altered physical activity during the later cycle stages(Julian, et al., 2017) . It is thought that the endocrinology of the spiny mouse likely mimics menstruating species more closely than oestrous species, with oestrogen levels rising in unison with progesterone during the luteal phase to support decidual cell proliferation. Hormone assays within the spiny mouse in conjunction with cytology would enable more accurate cycle staging. However, the precise levels of oestrogen and fluctuations throughout the menstrual cycle have not yet been determined in spiny mice due to the immense difficulty in validating

methods of measurement, the high likelihood of cross reactivity when using commercially derived antibodies or ELISA kits, and the fact the spiny mice often behaves as both human and rodent when seeking to determine gene or protein expression (Bellofiore, et al., 2018). These obstacles are currently being investigated (Bellofiore et al., 2017, Bellofiore et al. 2018)

Interestingly, despite the above described behavioural changes, no differences were observed when testing for memory or sociability between phases of the menstrual cycle. Our data showed the spiny mice did not show a strong preference to explore the stranger mouse in any phase. However, our social interaction test only looked at a) females, and; b) ovariectomised (stranger) or early follicular phase (known) animals. Given the menstrual cycle of females housed in group cages do not synchronize and therefore, each animal was in a different phase of their cycle, there is a possibility that those spiny mice which were isolated in the premenstrual phases may have been avoiding: interaction with other premenstrual females, more inquisitive females during other phases of their menstrual cycles, or the investigator only. In addition, females may respond differently to males, as humans have been observed to do so. Gillings (2014) speculates that PMS may evolved as a means of dissolving pair bonds among infertile partners (i.e.: relationships without resulting in pregnancy). This hypothesis is based on the (in part) hereditary nature of PMS, preferential direction of animosity towards partners and increases in risky and competitive behaviours during the late luteal phase to aid in seeking a new fertile mate (Gillings, 2014). The spiny mouse may prove useful in assessing premenstrual male-female interactions and further studies may expose altered behaviour in the luteal phase.

Further similarities in the menstrual cycle between spiny mice and primates are demonstrated in the fluctuations in weight and food consumption. Studies exploring the relationship between eating habits and the menstrual cycle are numerous, with the consensus indicating an increase in appetite, food cravings, basal metabolic rate and energy requirements during the premenstrual phase in women (Dye and Blundell, 1997, Johnson, et al., 1994, Solomon, et al., 1982, Strassmann, 1996). The spiny mouse housed in group cages peaks in relative weight gain during the late follicular phase (ovulation), and this likely reflects the increased food consumption in the early follicular phase; a pattern observed in many rodents. However, females show a secondary almost equivalent peak in weight and food consumption during the premenstrual late luteal phase, a trait exemplified by primates(Dye and Blundell, 1997), likely due to the high metabolic demand of spontaneous decidualization (Bellofiore, et al., 2018, Strassmann, 1996) and may indicate behaviour analogous with carbohydrate cravings experienced by premenstrual women (Wurtman, et al., 1989). Increased feeding is also seen in premenstrual baboons in conjunction with increased isolation (Hausfater and Skoblick, 1985). No inter-phase differences in water consumption, urine or faeces excretion was observed, although for both these parameters a significant effect of subject matching was noted.

The effect of repeated measures in the spiny mouse supports that the severity of premenstrual syndrome symptoms stem from natural variability of physiological parameters. With numerous variants of premenstrual symptoms, there is overwhelming evidence to support that within subject biological variation is a key component in assessing the severity of said symptoms, and the intensity of symptoms (behavioural in particular) is related to steroidal imbalances, including high estrogen during the luteal phase (Reid, 1991, Wang, et al., 1996),

gene polymorphisms in estrogen receptors (Huo, et al., 2007) and dysregulation of serotonin uptake (Rapkin, 1992). The spiny mouse may enable in depth analysis of behavioural, metabolic, environmental, genetic and endocrinological interplay in the development and exacerbation of PMS, and potentially, PMDD.

We have confirmed the physiological aspects of the menstrual cycle in the spiny mouse and investigated the timing, degree and repair of menstruation (Bellofiore et al., 2017, Bellofiore et al., 2018). The similarities expressed in the biology of this species with our own strongly suggests correlations in behaviour between the spiny mouse and the human during the pre-menstrual/early menstrual phases. Identification of anxiety-like behaviour through reduced exploration suggests a state akin to that of premenstrual syndrome in women and may provide the opportunity to further explore extreme cases of fear and/or withdrawal. These mice with extreme reactions/behavioural manifestations could in turn, prove highly useful for studies relating to premenstrual dysphoric disorder. As in humans, spiny mice appear to display a spectrum of PMS responses, these data further highlight the importance of careful behavioural characterization of each mouse to prevent heterogeneity of data in this model.

We provide unique evidence that spiny mice indeed demonstrate cyclical changes in both metabolism and behaviour in different phases of their menstrual cycle; further highlighting the utility of this model for use in multiple aspects of menstrual related research. This study demonstrates a crucial necessity to account for individual variations in both physiological and psychological measurements, as required in humans, further emphasizing the relevance of the spiny mouse as a species in which to study menstruation and associated premenstrual behavioural and metabolic conditions.

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AUTHORS' ROLES

N.B. designed and performed experiments and analysed the data, F.C. and P.T-S. provided critical reading of the manuscript, J.E. assisted in interpretation of the data and manuscript drafting.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1: Capture (A) and handling (B) of spiny mice using a container and handtowel. A placid female will lie flat in the supine position with limbs parallel to body (C); a more stressed female will try to twist head and body, limbs may be angled and spiny mouse may urinate or defecate (D).

Figure 2: Timing of (A) quantitative behavioural tests and (B) metabolic cage experiments conducted on female spiny mice. E = Early, L = Late, Foll = Follicular, Lut = Luteal phases of menstrual cycle. These protocols allow blind analysis if females exhibit a 9-day menstrual cycle exactly. However, due to natural variation in cycle length and phases, it is necessary to perform a lavage after (A) day 12 and (B) day 14 to estimate when the female will enter the final phase: (A) late luteal and (B) late menstrual. This is to ensure phases are not unnecessarily repeated or missed entirely. This may extend the time frame by up to 7 additional days depending on the length of the female's individual cycle.

Figure 3: (A) Relative overall stress score and (B) overall handling time during vaginal lavage procedure of female spiny mice across the menstrual cycle. Significant results shown $p < 0.05$ (*), RM-ANOVA.

Figure 4: Open Field (OF) analysis across the menstrual cycle in female spiny mice. Distance (A) and velocity (B) travelled in OF outer zone. Significant results shown $p < 0.05$ (*), RM-ANOVA.

Figure 5: Elevated Plus Maze (EPM) analysis across the menstrual cycle in female spiny mice. Distance travelled in EPM open arms (A), velocity in EPM central zone (B) and distance travelled in EPM central zone (C). Significant results shown $p < 0.05$ (*), RM-ANOVA. Significant effect of subject matching present in (A-C).

Figure 6: Novel Object Recognition Test (NORT) and Social Interaction (SI) analyses in across the menstrual cycle in female spiny mice. NORT1 (A,B) and NORT2 (C,D) show significant effect of subject matching in distance and velocity, respectively, but neither demonstrate differences between cycle phases. SI discrimination index showed females preferred exploring other spiny mice (SpM) compared to the neutral zone (E), but no differences were seen across the menstrual cycle, or preference to explore either the stranger or known subjects (F).

Figure 7: Metabolic changes across the menstrual cycle in spiny mice. Females show significant peaks in food consumption during the early follicular and late luteal phases compared to the late follicular phase (*). Water consumption (B) and urine excretion (C) demonstrate a significant effect of subject variation. Changes in body weight after 24h metabolic cage isolation were negative overall, with no significant variations between menstrual cycle phase but significant effect of subject matching (D). Changes in body weight in conventional housing demonstrate significant fluctuations in each of the phases (E).

TABLES

TABLE 1: SEMI-QUANTITATIVE BEHAVIOURAL SCORING OF FEMALE SPINY MICE DURING VAGINAL LAVAGE*

SMEARING	0 - Normal	1 - Minor	2 - Moderate	3 - High
Initial Position in Cage	Nesting with littermates With majority of group	Hiding in/under enrichment with 1-2 other litter mates	Hiding in/under enrichment alone OR Moderate Cage Climbing	Isolated, in cage corner farthest away from group OR Continual Cage Climbing
Response to Weighing	Still and placid	Initial movement (i.e. animal turns around 1-4 times) then placid	Animal constantly turning and/or attempts to escape	Animal escapes 1 or more times
Response to towel	Placid, no excessive movement	1-2 repositions (i.e. animal will turn 180 degrees) Minor wriggling and/or foot scurrying	3-4 repositions Repeated wriggling and/or foot scurrying Head/neck frequently exposed and tilted around container edges	Animal escapes from container one or more times Constant struggling/wriggling/scurrying Head/neck and torso frequently exposed
Ease of Scuffing	1 attempt	2 attempts	3-4 attempts	5+ attempts
Attitude toward restraint	Placid, easily subdued, easily kept straight	Exhibits slight apprehension to restraint, body may be at minor angle	Animal struggles, with claws stuck in towel fibres (hind or fore), minor kicking or head movement	Animal appears stressed, attempts to bite self or handler and repositioning does not alleviate
Lavaging	Not bothered	Some reaction, leg reflex at palpation	Intense hind body reaction, accompanying vocalisation	Total body reaction, all limbs flinch, torso movement, and/or animal attempts escape
Abdomen palpation	Not bothered	Minor Flinch on one side (unilateral) LHS	Noticeable flinching, minor vocalisation on one or both sides (uni or bilateral)	Attempt to bite, noticeable vocalisation
Vocalisation	None	Rare 1-2	Frequent 3-5	Constant 6+
Escaping	None	1 attempt (from container or cage) OR 1 escape from cage	2 or more attempts (from container and/or cage) OR 1 escape from container and 1 or more escapes from cage	Multiple attempts from container and cage Escape upon returning to cage Escape on smearing
Reaction to Cage Return	Crawls out of restraint slowly, placid	Small jump out of restraint, some initial hurried scurrying	Leap out of restraint, backflipping and cage climbing OR licking of lower abdomen	Flip out of restraint, recapture necessary, constant backflipping and climbing on return +/- licking

*Females scoring a 0 or1 were designated as having a "Mild" response. Females scoring a 2 or 3 were designated as having a "Severe" response.

TABLE 2: SEMI-QUANTITATIVE BEHAVIOURAL SCORING ANALYSIS OF FEMALE SPINY MICE

Parameter	Cycle Stage	Odds Ratio	p Value (* = significant)	Parameter	Cycle Stage	Odds Ratio	p Value (* = significant)
Initial Position	Early Follicular (Control)	1.0	-	Weighing	Early Follicular (Control)	1.0	-
	Late Follicular	7.3	0.932		Late Follicular	.640	.587
	Early Luteal	19.1	0.200		Early Luteal	.267	.165
	Late Luteal	9.4	0.388		Late Luteal	.889	.883
	Early Menstrual	40.7	0.041*		Early Menstrual	.436	.338
	Late Menstrual	10.5	0.736		Late Menstrual	1.200	.816
Towel	Early Follicular (Control)	1.0	-	Scruffing	Early Follicular (Control)	1.0	-
	Late Follicular	4.4	0.03*		Late Follicular	0.82	0.954
	Early Luteal	1.3	0.02*		Early Luteal	0.81	0.908
	Late Luteal	8.4	<0.01*		Late Luteal	0.25	0.224
	Early Menstrual	4.4	<0.01*		Early Menstrual	0.53	0.063
	Late Menstrual	1.3	0.968		Late Menstrual	0.53	0.623
Restraint	Early Follicular (Control)	1.0	-	Lavage	Early Follicular (Control)	1.0	-
	Late Follicular	0.9	0.931		Late Follicular	.000	.999
	Early Luteal	1.1	0.637		Early Luteal	.554	.647
	Late Luteal	3.6	<0.01*		Late Luteal	.428	.508
	Early Menstrual	3.6	0.063		Early Menstrual	.554	.647
	Late Menstrual	4.4	0.043*		Late Menstrual	.463	.549
Abdominal Palpation	Early Follicular (Control)	1.0	-	Vocalisations	Early Follicular (Control)	1.0	-
	Late Follicular	.525	.395		Late Follicular	4.4	0.03*
	Early Luteal	.525	.395		Early Luteal	1.3	0.02*
	Late Luteal	.525	.395		Late Luteal	8.4	<0.01*
	Early Menstrual	.525	.395		Early Menstrual	4.4	<0.01*
	Late Menstrual	1.801	.390		Late Menstrual	1.3	0.968
Escapes	Early Follicular (Control)	1.0	-	Return	Early Follicular (Control)	1.0	-
	Late Follicular	.267	.165		Late Follicular	1.2	0.861
	Early Luteal	.889	.883		Early Luteal	7.0	<0.01*
	Late Luteal	1.200	.816		Late Luteal	1.2	0.824
	Early Menstrual	.267	.165		Early Menstrual	1.7	0.634
	Late Menstrual	.267	.165		Late Menstrual	1.9	0.037*

FIGURES

Figure 1

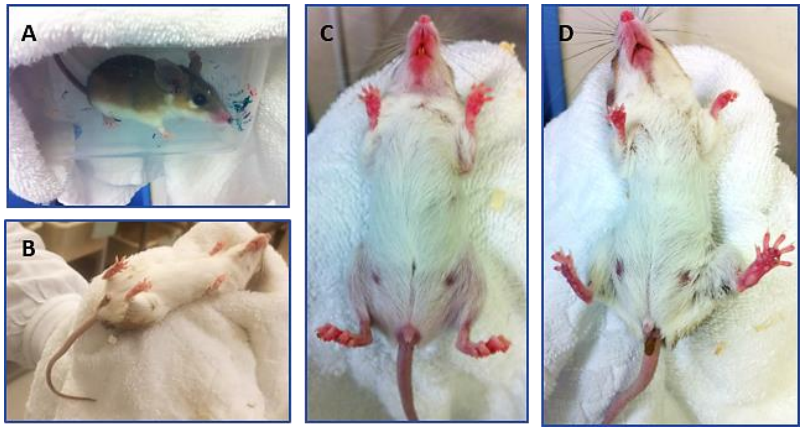


Figure 2

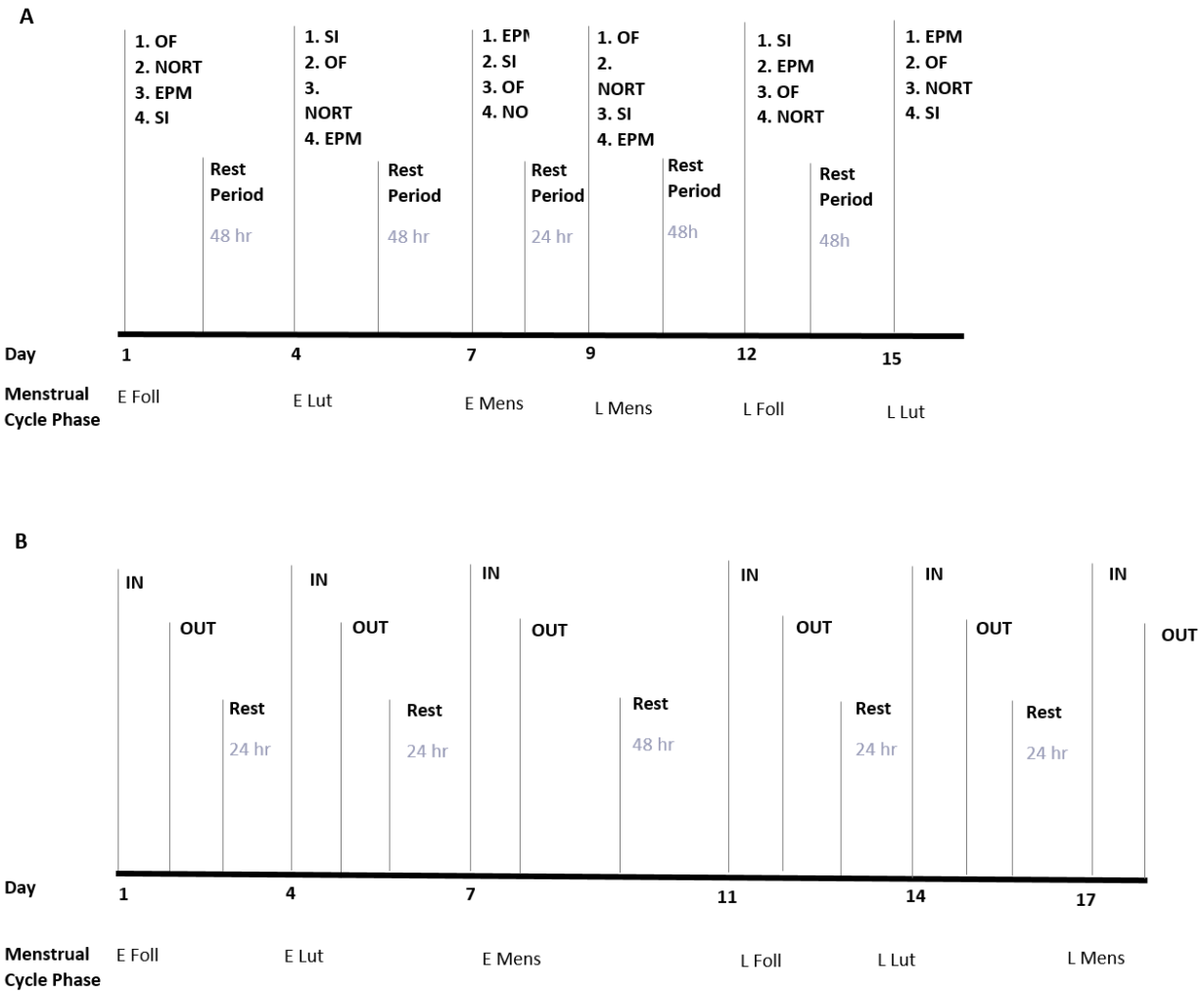

A
1. OF
2. NORT
3. EPM
4. SI
Rest Period
48 hr
1. SI
2. OF
3. NORT
4. EPM
Rest Period
48 hr
1. EPM
2. SI
3. OF
4. NO
Rest Period
24 hr
1. OF
2. NORT
3. SI
4. EPM
Rest Period
48h
1. SI
2. EPM
3. OF
4. NORT
Rest Period
48h
1. EPM
2. OF
3. NORT
4. SI
Day
1
4
7
9
12
15
Menstrual Cycle Phase
E Foll
E Lut
E Mens
L Mens
L Foll
L Lut
B
IN
OUT
Rest
24 hr
IN
OUT
Rest
24 hr
IN
OUT
Rest
48 hr
IN
OUT
Rest
24 hr
IN
OUT
Rest
24 hr
IN
OUT
Day
1
4
7
11
14
17
Menstrual Cycle Phase
E Foll
E Lut
E Mens
L Foll
L Lut
L Mens

Figure 3

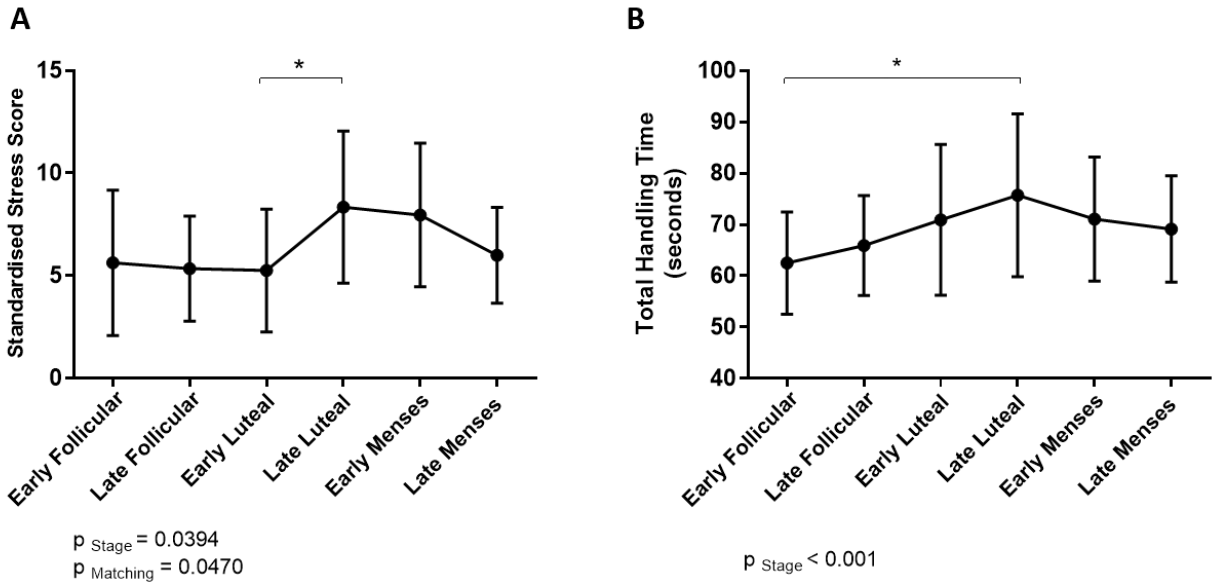

A
Standardised Stress Score
15
10
5
0
*
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Stage = 0.0394
p Matching = 0.0470
B
Total Handling Time (seconds)
100
90
80
70
60
50
40
*
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Stage < 0.001

Figure 4

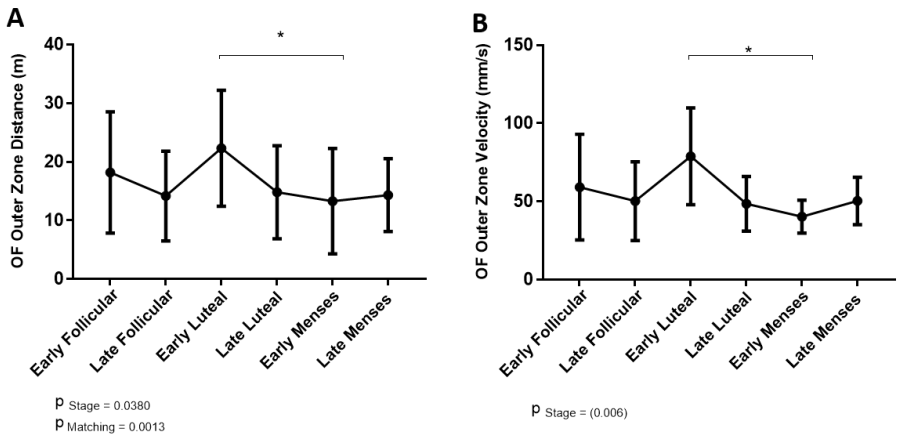

A
OF Outer Zone Distance (m)
40
30
20
10
0
*
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Stage = 0.0380
p Matching = 0.0013
B
OF Outer Zone Velocity (mm/s)
150
100
50
0
*
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Stage = (0.006)

Figure 5

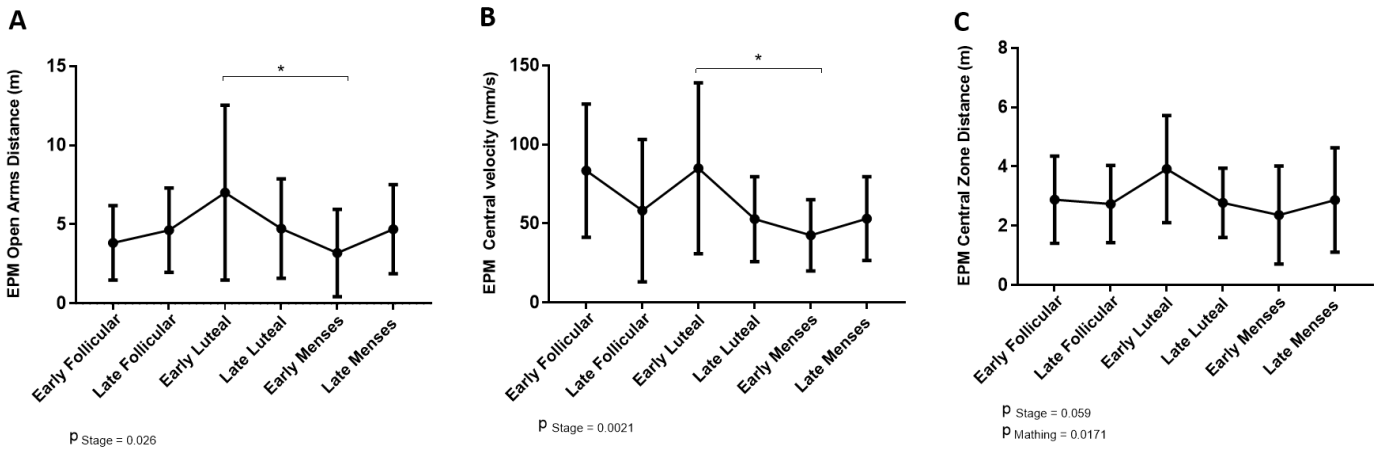

A
EPM Open Arms Distance (m)
15
10
5
0
*
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Stage = 0.026
B
EPM Central velocity (mm/s)
150
100
50
0
*
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Stage = 0.0021
C
EPM Central Zone Distance (m)
8
6
4
2
0
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Stage = 0.059
p Mathing = 0.0171

Figure 6

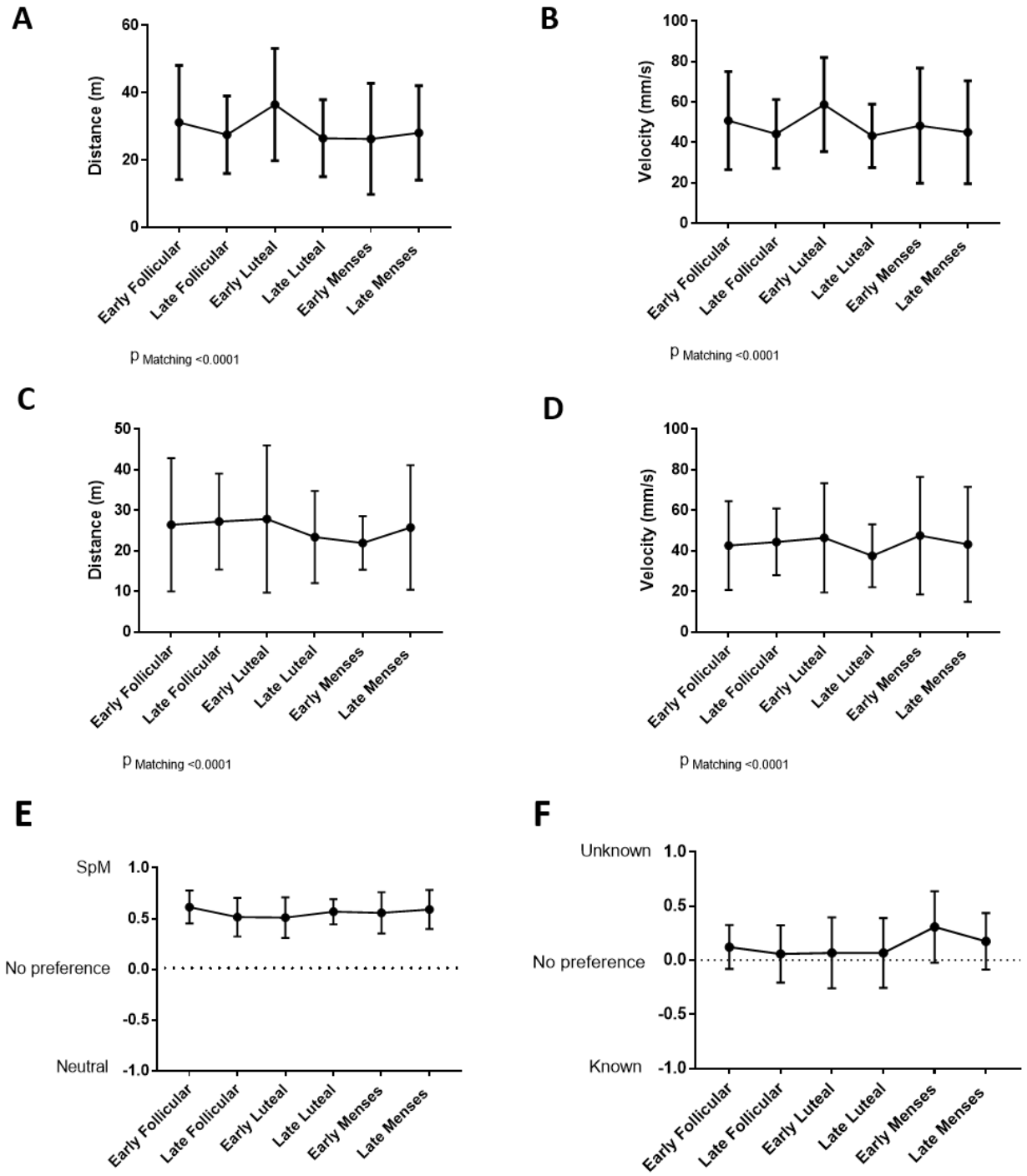
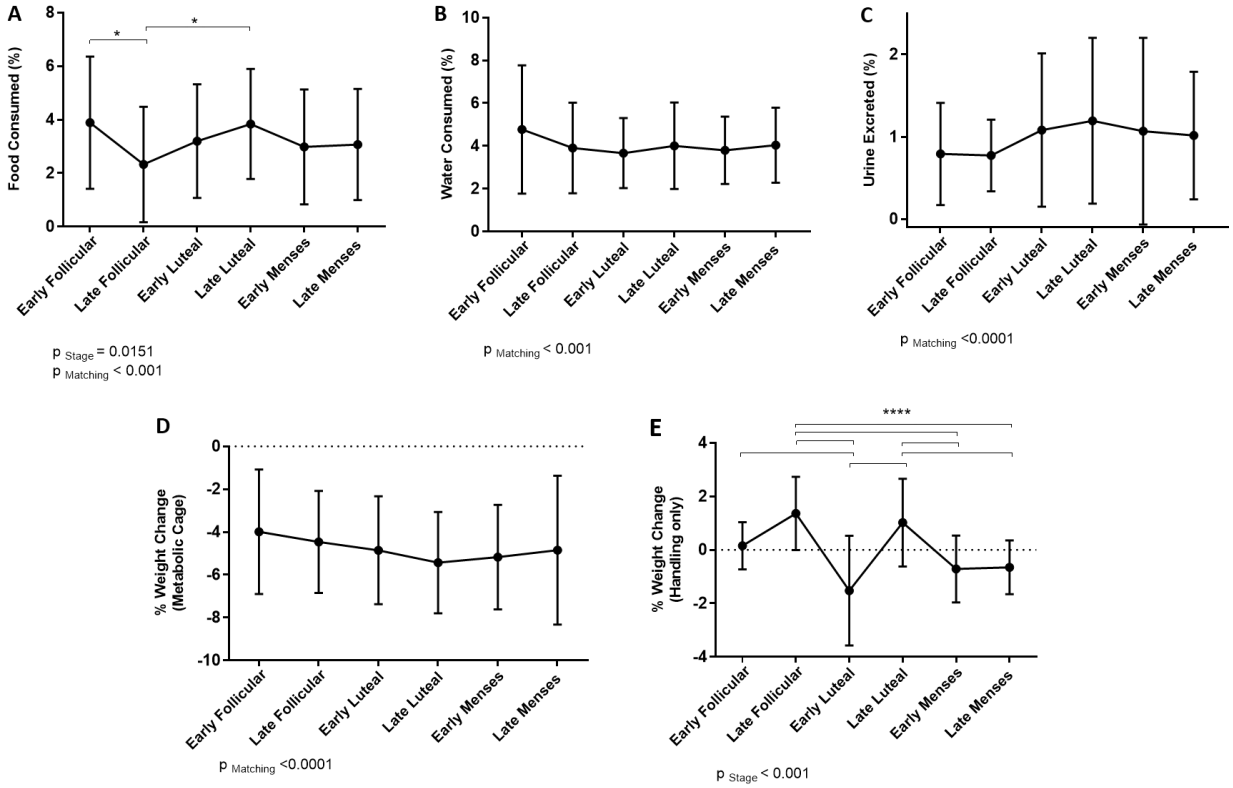

A
Distance (m)
60
40
20
0
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Matching <0.0001
B
Velocity (mm/s)
100
80
60
40
20
0
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Matching <0.0001
C
Distance (m)
50
40
30
20
10
0
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Matching <0.0001
D
Velocity (mm/s)
100
80
60
40
20
0
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Matching <0.0001
E
SpM
1.0
0.5
No preference
0.0
-0.5
Neutral
-1.0
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
F
Unknown
1.0
0.5
No preference
0.0
-0.5
Known
-1.0
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses

FIGURE 7

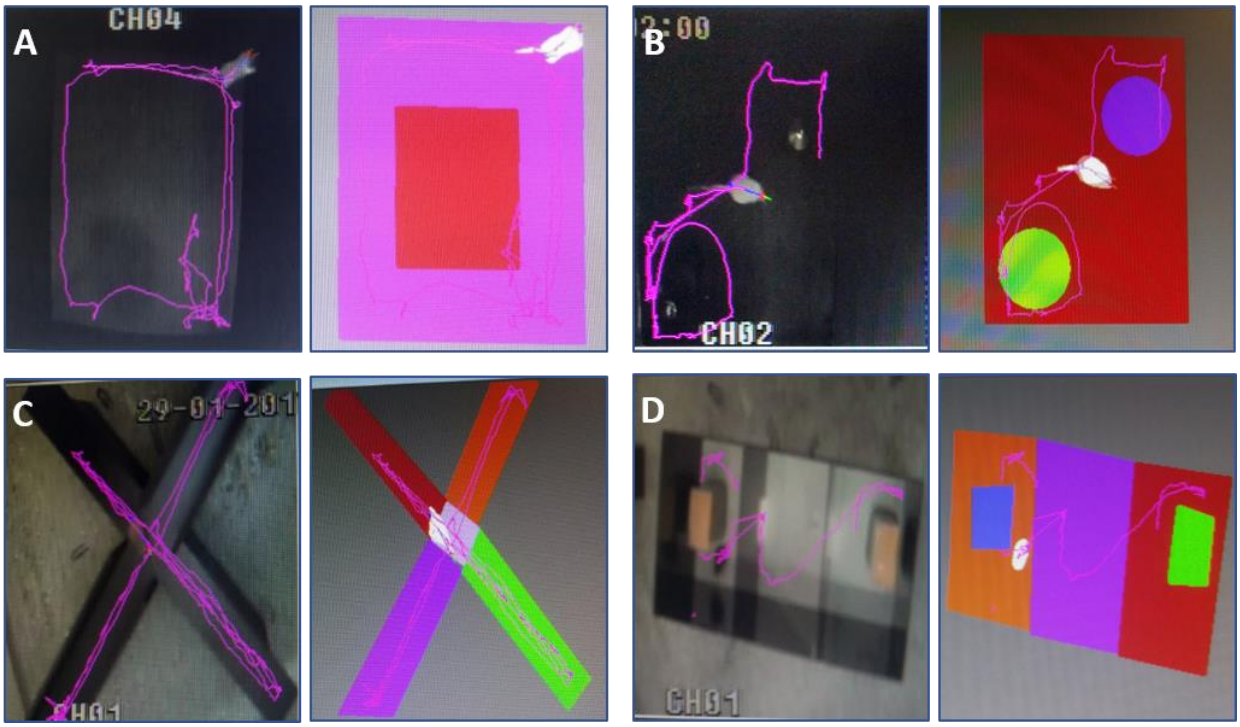

A
Food Consumed (%)
8
6
4
2
0
*
*
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Stage = 0.0151
p Matching < 0.001
B
Water Consumed (%)
10
8
6
4
2
0
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Matching < 0.001
C
Urine Excreted (%)
2
1
0
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Matching <0.0001
D
% Weight Change (Metabolic Cage)
0
-2
-4
-6
-8
-10
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Matching <0.0001
E
% Weight Change (Handling only)
****
4
2
0
-2
-4
Early Follicular
Late Follicular
Early Luteal
Late Luteal
Early Menses
Late Menses
p Stage < 0.001

SUPPLEMENTAL DATA

Ovariectomy Procedure

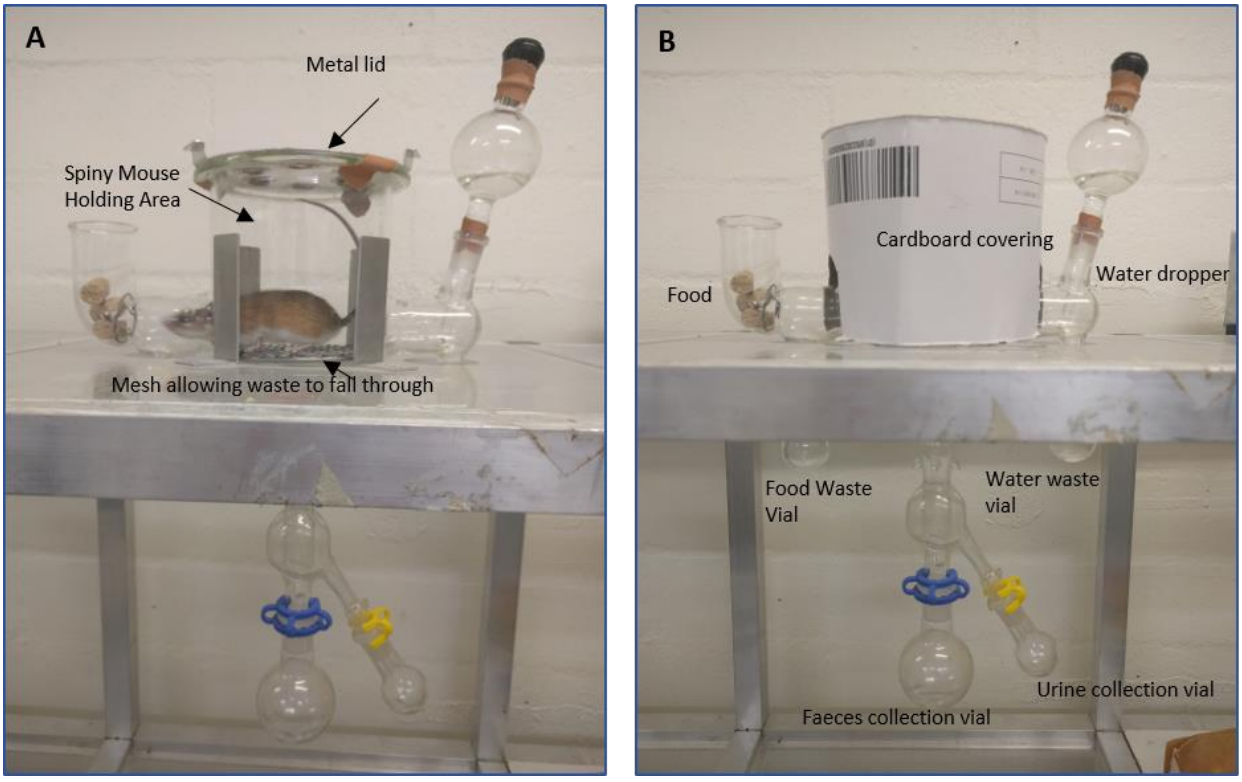
Spiny mice received 5mg/kg subcutaneous Carprofen at the time of anaesthetic induction with ~5% isoflurane in room air. Depth of consciousness was monitored with the pedal reflex prior to maintenance level between 1.5-2.5% throughout the surgery. Females were placed in the prone position on warming pad. The incision site of approximately 1cmX1.5cm was shaved and disinfected with 70% v/v ethanol. A 1cm midline incision was made adjacent to hips along the midline of the dorsal side and the muscle layer and skin separated gently using blunt dissection. A second 0.5cm incision was made through musculature over the left ovarian fat pad to access the internal cavity. The left uterine horn was gently pulled to the opening, the ovarian fat pad clamped and organs positioned under dissecting microscope. Two knots using Maxon® dissolvable suture material (5/0, 15 mm) were tied to ligate the oviduct and ovarian artery. The ovary was gently removed using forceps and the uterine horn eased back into the body cavity. The peritoneal incision was sutured and the process repeated on the right side of the reproductive tract. The skin incision was flushed with topical analgesic (Lignocaine 2%) and the skin closed with silk Dynek® Sutures (Silk Dysilk 5/0).9. Females were monitored every 2h for 8h post-surgery, then bi-daily for the first 48 hours and bi-daily thereafter. Post-operative meloxicam (1mg/kg) was given at 12 and 24h post-surgery. The spiny mice were allowed a minimum of 2 weeks to recover before exposure to researcher handling.

Fig. S1:



Supplementary Figure 1: Recorded images of spiny mice and tracking analysis of spiny mice undertaking (A) Open Field, (B) Novel Object Recognition, (C) Elevated Plus Maze and (D) Social Interaction tests.

Fig. S2



Supplementary Figure 2: 24-h glass metabolic cage set-up for spiny mice. (A) Adequate space allows the spiny mice to access food and water while the metal cage lid prevents discourages excessive chewing. Food scraps, water spillage, faeces and urine can be collected and measured. (B) Carboard coverings enable added security for the spiny mice.

CHAPTER 6:

REPRODUCTIVE AGEING IN THE MENSTRUATING SPINY MOUSE

6.1 Introduction

While oocyte depletion and exponential decline in primary follicular reserve is a process all mammalian species undergo with age, humans are unique in their transition to reproductive senescence. Menopause, or the menopausal transition, is the permanent cessation of menstruation following the failure of ovarian function (3). Even among other menstruating primates, women are the only documented species to experience a substantially increased post-menopausal phase of life following the arrest of active reproduction (~40-50% of total lifespan), attributable to our equally increased longevity in developed countries (4).

Menopause is characterised by considerable changes in endocrinology. As the follicular pool depletes, the ovary becomes gonadotrophin insensitive, and subsequently, circulating levels of luteinising hormone (LH), follicle stimulating hormone (FSH) increase, while inhibin B and Anti-Müllerian Hormone (AMH) levels fall (3). This leads hypoestrogenism and menstrual cycle irregularity with prolonged periods of amenorrhea (Figure 6.1.1).

During the perimenopause in humans, oestrogen levels become virtually undetectable. Non-human primates such as rhesus monkeys may be used as intact, natural models for reproductive ageing given their similarities in menstrual cycle physiology, but the significant cost involved in using primates for research is a limiting factor. Aside from the cost, the menopausal transition in other higher order primates differs in a) timing; non-human primates enter menopause much later and spend only ~20% of life post-menopausal as opposed to ~50% in humans (5); and b) onset of menopause; this is gradual in humans spanning up to 4 years, but sudden in old world monkeys (6).

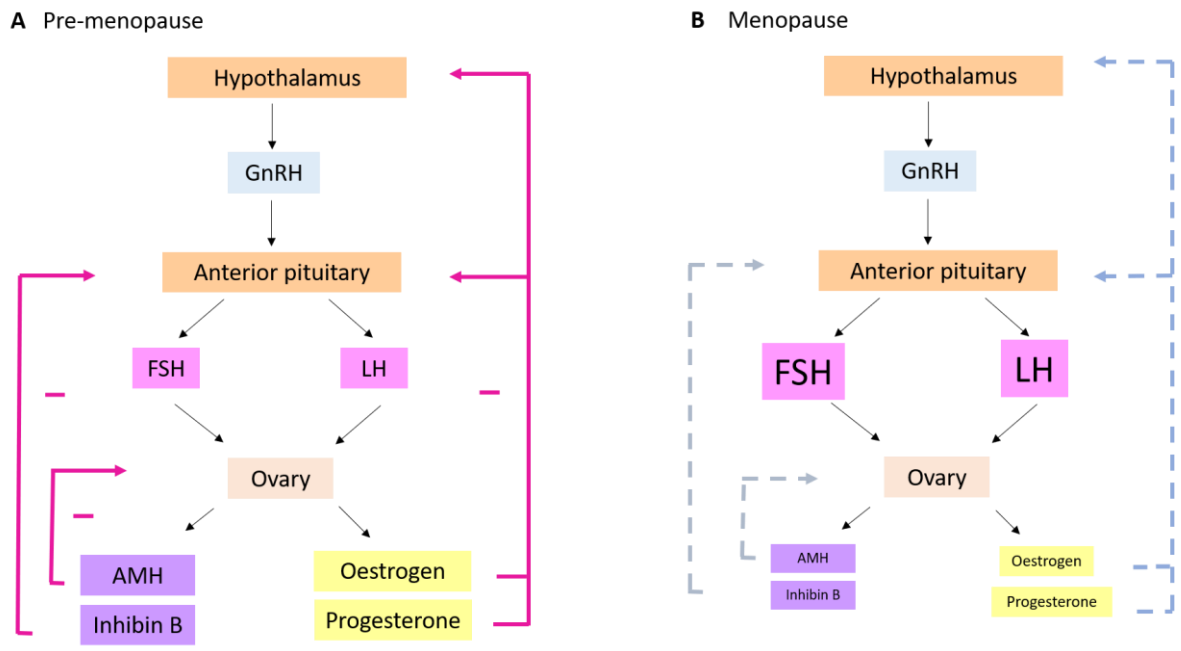


Figure 6.1.1 Hypothalamic-pituitary-ovarian axis during (A) pre-menopause and (B) post-menopause in women. During normal menstrual cycles (A), the hypothalamus secretes GnRH which signals production of FSH and LH from the anterior pituitary. These act on the ovaries to synthesis steroid sex hormones progesterone and oestrogen, and peptide hormones AMH and inhibin B. Oestrogen and progesterone negatively feedback on the hypothalamus and pituitary to halt gonadotrophin production, as inhibin B and AMH similarly inhibits FSH production through negative feedback on the pituitary. During menopause (B), a decline in the number of follicles in the ovary results in hypoestrogenism, reduced inhibin, reduced AMH and consequently signals to the hypothalamus and pituitary to elevate gonadotrophin levels.

Natural mouse and rat models also exhibit an age-related decline in reproductive function. However, although they share some features of ovarian senescence with humans, the human endocrine profiles are not mimicked. The complicating factor in these comparisons is that rats and mice have oestrous cycles, as opposed to menstrual cycles in primates, and the transition to reproductive senescence in rodents is sometimes referred to as “estropause”. Rats show an increase in FSH prior to the onset of estropause, as in human menopause, but a moderate production of oestrogen continues due to retention of a larger pool of primary follicles (6, 7), and in ageing female mice oestrogen secretion is significantly reduced but is not ablated (8). For these reasons, an ovariectomised (OVX) model of menopause may be

preferable to study the effects of complete oestrogen abolishment. However, not only is oestrogen depletion sudden in rodents, rather than gradual as in humans, it is not the only hormone affected through ovariectomy. Researchers must consider the effects of altering the neuroendocrine pathways, and the interplay of other endocrines. In particular, humans have demonstrated a decline in DHEA and increase in DHEA-sulphate production from the ovaries (4, 9). OVX rodents therefore are not authentic models for the effects of menopause as they lack ovaries and have naturally low plasma DHEA production.

The potential for a more refined rodent model of menopause with better translation to the human may lie with our recently discovered naturally menstruating spiny mouse (*Acomys cahirinus*). The spiny mouse is a desert-dwelling species with few captive research colonies worldwide, and is primarily recognised for its potential application in skin regenerative research (10), organogenesis (11, 12) and primate-like secretion of DHEA and cortisol (13). We have confirmed similarities with the human in morphological characteristics of menstrual shedding and repair in sexually mature females in this species (14), however we have not explored the effects of age on menstruation and reproductive potential. My aim in this chapter is to assess functional and histological changes to the menstrual cycle of the ageing spiny mouse, and to determine whether a perimenopause transition analogous to that in women occurs in this rodent.

Our hypothesis was that the reproductive tract of female spiny mice that were acyclic would demonstrate uterine qualities similar to those seen during the very early follicular/proliferative phase, prior to oestrogenic proliferation, resulting in thin endometrium and reduced uterine weight.

6.2 Materials and Methods

Ethics

All experimental procedures adhered with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All research was approved by the Monash University/Monash Medical Centre Animal Ethics Committee (# MMCB 2015/47).

Spiny mouse husbandry

Virgin female spiny mice were sourced from our in-house research colony and maintained as previously described (1).

Vaginal Lavage and assessment of menstrual cycle

Based on previous findings in our colony, the spiny mouse (aged 3-6 months) has a menstrual cycle averaging 9 days (range: 6-10 days) (1, 14). Therefore, females aged 6 (n=14), 12 (n=7), 24 (n=13) and 36 (n=9) months were subjected to blinded daily vaginal lavage for 14 consecutive days to ensure the capture of at least one full menstrual cycle, with 36 months being the oldest females in the colony. Lavage samples were prepared and stained with haematoxylin and eosin as previously described, and cytology assessed for stage of menstrual cycle ("proestrus", "oestrus", "metestrus", "diestrus" or "menses") after all samples had been obtained (1). Spiny mice were weighed every three days to obtain an average body weight. Females were deemed to be actively "cycling" if they exhibited at least one ovulation (designated by a vaginal lavage containing >90% cornified epithelial cells) and one menstruation (lavage containing primarily leukocytes, erythrocytes and endometrial stromal debris). After analysis, 36-month old females showed no evidence of health related or cytological issues, and therefore these 9 females were set aside to age an additional 6 months

(total of 42 months old), and undergo cycle monitoring as described above. For the purpose of this thesis, 36-month old cycle and weight data is compared separately to 6-month old control data, and all tissue analysis was conducted on 42-month old females.

Post mortem tissue collection

Spiny mice were euthanized by isoflurane overdose, independent of menstrual cycle stage at 6, 12, 24 and 42 months. We have previously demonstrated that during the late follicular/proliferative phases when ovulation takes place, the uterus is significantly heavier than other phases of the menstrual cycle, owing to the accumulation of uterine fluid (1). However, the menstrual cycle of co-housed spiny mice does not naturally synchronise. Therefore, the likelihood of inadvertently collecting an entire cohort of uterine tissue during the same phase was extremely unlikely, and hence we do not consider this to be a confounding effect in the study.

For each group of females, the reproductive tract was dissected out and the uterus and ovaries were weighed. The uterus and left ovary were fixed in 10% neutral buffered formalin, processed to paraffin, sectioned at 5 uM and stained with H&E for morphological assessment. The right ovary* was fixed in Bouin's fluid and processed to resin for stereological analysis (*NB: stereological data is not shown in this thesis as it comprises part of a separate study by another PhD student, Miss Evgenia Pereleshina). Any health or severe reproductive tract abnormalities were recorded, but the cytology and weight data of these females were excluded from analysis as our study aims to analyses healthy, aged reproductive tissues.

Immunohistochemistry

To assess changes in uterine proliferation, tissues were stained for proliferating cell nuclear antigen (PCNA). Samples were dewaxed (3x 5 min in xylene), rehydrated through graded ethanol and then washed in dH_20 for 5 minutes. Citrate antigen retrieval was performed by microwaving slides in citrate buffer (pH 6.0) on medium-high for 5 min, followed by 20 min hot buffer retrieval. Slides were washed in Tris-Buffered Saline with Tween-20 (0.05%) solution (TBS-T) twice for 5 min each, followed by one 5 min wash in TBS. A 3% hydrogen peroxide solution was applied for 10 min to block endogenous peroxidase activity. Slides were thrice washed in buffer as above and a block (10% normal goat serum, 2% Human serum in 0.02% TBS-Tween20) applied for 30 min at room temperature. Rabbit anti-human PCNA primary antibody (Abcam, #ab152112) was applied at 1:750 dilution overnight at 4°C, or rabbit IgG (Dako, #X093602) as a negative control in lieu of primary antibody. Slides were thrice washed in TBS and incubated in biotinylated secondary goat anti-rabbit (Vector Laboratories, #BA-1000) at 1:200 dilution for 60 min at room temperature. Following buffer washing thrice in TBS, avidin-biotin complex was applied to slides at room temperature for 45 minutes, slides were thrice washed in TBS, and 3, 3'-Diaminobenzidine (DAB) applied for 2-3 minutes for visualisation. Tissues were rinsed in dH_20 before counterstaining in 10% Harris haematoxylin for 3 minutes, blued in running tap water for 5 min, dehydrated, cleared and coverslipped. Endometrial tissues were then assessed for immunopositive proliferation (% area coverage) by region specific tracing using ImageJ software as previously described (14).

Statistical analysis

All data was analysed using GraphPad Prism (Ver 6.01). Normally distributed data is presented as mean ± SD, and non-normal data as median (25^{th} percentile, 75^{th} percentile). Significance ($p < 0.05$) was determined using a one-way ANOVA or Kruskal-Wallis test, respectively.

6.3 Results

The proportion of females actively cycling were: 86% (12/14) at 6 months, 71% (5/7) at 12 months, 69% (9/13) at 24 months, 56% (5/9) at 36 months and 44% (4/9) at 42 months. Of the original 9 females assigned to the 42-month old cohort, only 5 were included for final cytological comparisons, as 4 presented with health issues. These included reproductive tract deformities (3) and mastitis (1) (Figure 6.3.1).

Overall, the total length of the menstrual cycle was significantly increased in 42-month old females (13.0 ± 1.4 days) compared to 6 (10.3 ± 1.3 days) and 12 (10.3 ± 1.8 days) months, and a trend (p=0.08) between 36 months (12.0 ± 2.9 days) and 6 months. This increased cycle length was attributed to an extended proliferative/follicular phase by ~2-3 days compared to 12 and 24-month old females, with a trend (p = 0.07) compared to 6 months (Table 6.3.1).

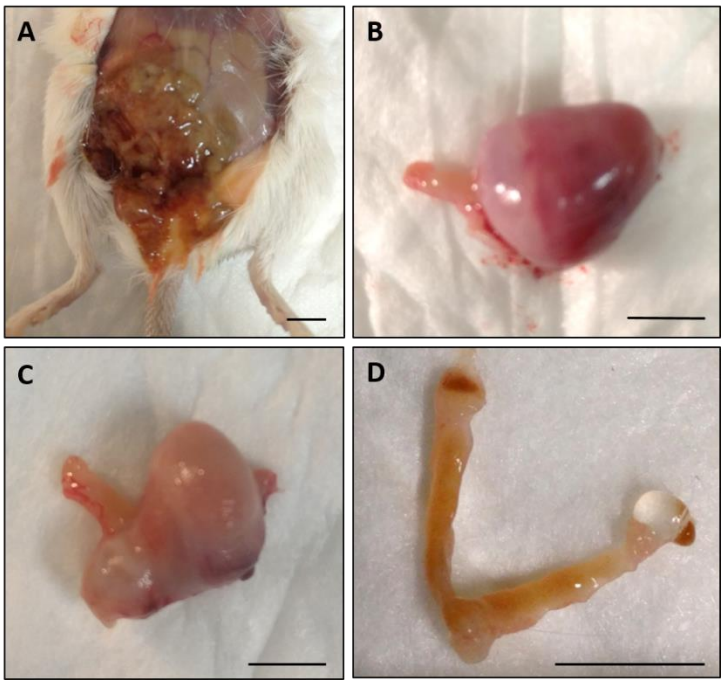


Figure 6.3.1: Reproductive tract abnormalities in the spiny mouse at 42 months of age. (A) Abscess beneath mammary gland and within peritoneal layer indicative of mastitis. (B) Large, dark red, vascularised mass in the uterine horn. Dissection revealed necrosis, and indicative of tumour. (C)

Solid, pale coloured uterine masses. Dissection revealed characteristics of fibroids. (D) Protruding ovarian oedema. Scale bar = 1 cm.

TABLE 6.3.1: MENSTRUAL CYCLE COMPARISON ACROSS AGEING SPINY MOUSE FEMALES

Length (days)	6	12	24	36[#]	42	Significance
Total Cycle	10.3± 1.3[a,c]	10.8 ± 1.3	9.6 ± 1.7 [b]	12.0 ± 2.9[c]	13.0± 1.4[a,b]	a*, b** c: p =0.08
Proliferative/Follicular	3.3 ± 0.5[d]	2.1 ± 0.7[a]	2.3 ± 1.1[b]	3.7 ± 2.2	4.9 ± 1.8[a,b,d]	a,b** d: *p=0.07*
Secretory/Luteal	3.6 ± 0.8	3.8 ± 0.9	3.3 ± 1.0	4.0 ± 1.7	3.9 ± 1.9	ns
Menstrual	3.2 ± 1.6	3.8 ± 1.3	3.7 ± 0.9	3.9 ± 0.7	4.4 ± 1.6	ns

Superscript letters denote groups which are significantly different from each other (One-way ANOVA (total cycle) and Two-way ANOVA (cycle stages))
*$p < 0.05$
**$p<0.01$
ns = non-significant
[#]36-month compared to 6-month controls only

Mean body weight of 6-month old females was 34.5 ± 1.8g. This significantly increased during ageing in all groups compared to females 6 months of age (Figure 6.3.2A-B), peaking in 24-month old females at 41.8 ± 3.8g (~20% increase in body weight). Uterine weight declined in 42-month old spiny mice (0.03 ± 0.02g) compared to both 6 and 12-month old females (0.10 ± 0.05g and 0.09 ± 0.02g, respectively), while ovarian weight was reduced in both 24 and 42 -month old females (0.001±0.001g and 0.002±0.002g respectively) compared to 6 -month old females (0.004±0.002g; Figure 2C-D).

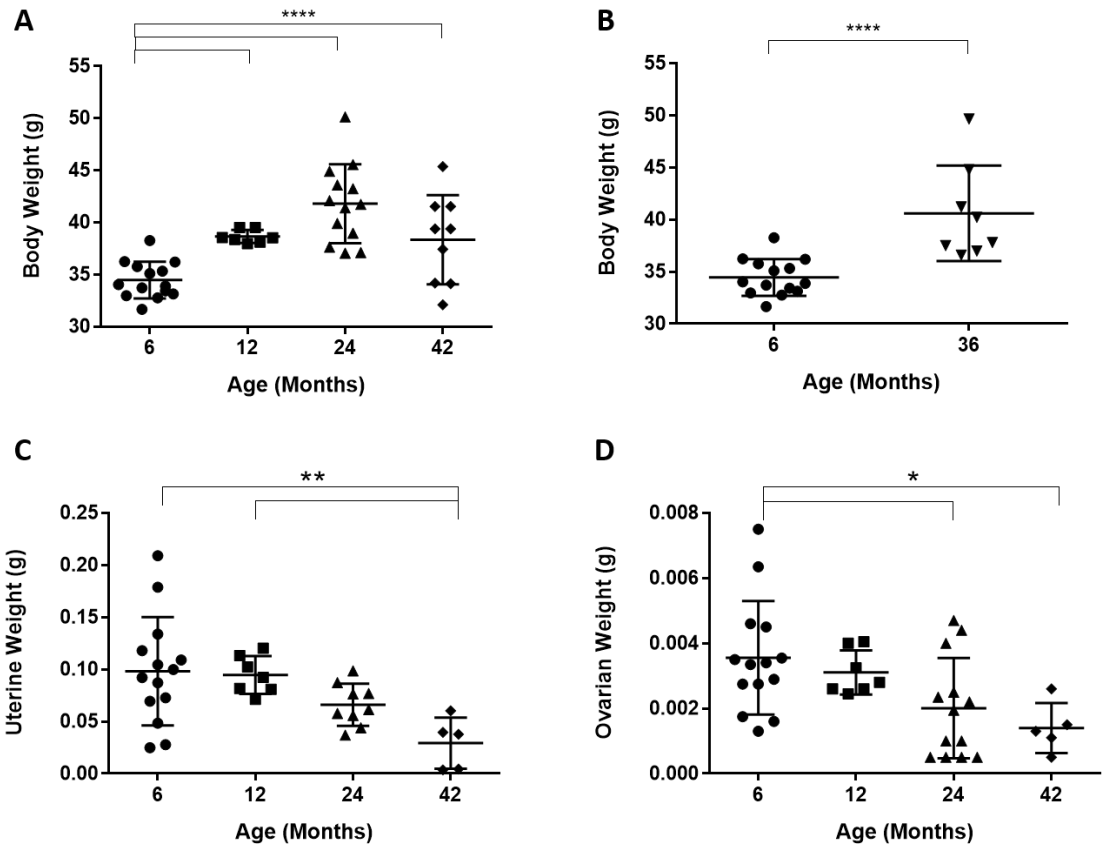


Figure 6.3.2: (A) Changes in body weight during ageing in female spiny mice at 6, 12, 24 and 42 months. (B) Weight of 36-month old females compared to controls. Comparative weight of (C) uterus and (D) ovaries in 6, 12, 24 and 42-month old female spiny mice. Student's t-test (B) or One-way ANOVA (A, C and D) *p<0.05, **p<0.01 and ****p<0.0001.

The cytology of cycling 42-month old females demonstrates oestrous stage smears within the 14-day period of assessment, designated by samples containing only cornified epithelial cells and corresponding to the ovulatory phase (Figure 6.3.3). Non-cycling females (in all age groups) demonstrated persistent vaginal cytology similar to that of a late menstrual smear, without red blood cells, i.e.: leukocytes, some cornified epithelial cells and sporadic clusters of nucleated epithelial cells which often precede an early follicular phase lavage. In acyclic

females, vaginal cytology does not progress to the next fertile cycle and the follicular phase, denoted by dominance of smear with nucleated epithelial cells, is not initiated (Figure 6.3.4).

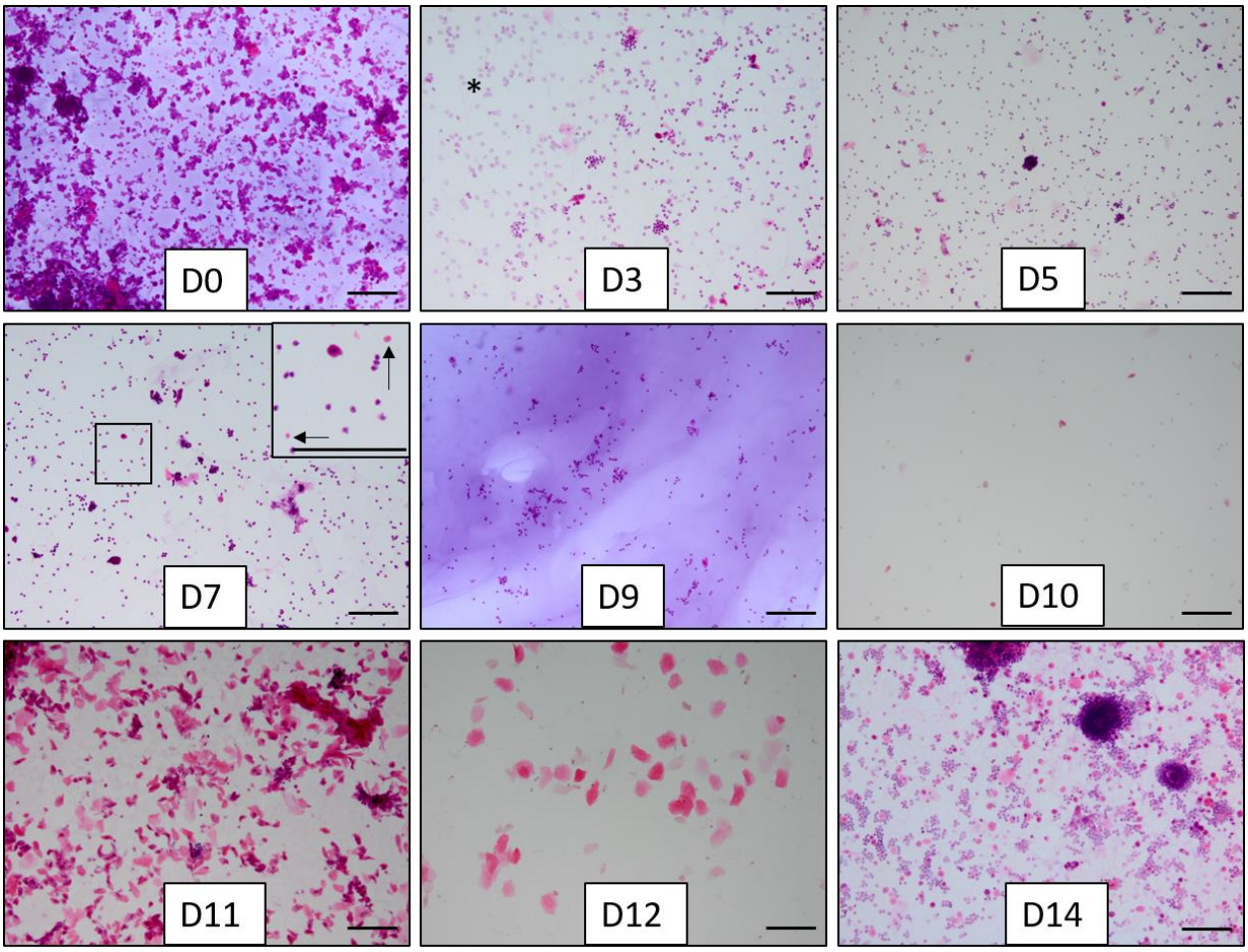


Figure 6.3.3: Cytology of a 42-month old cycling female spiny mouse across 14 days. Female is in luteal phase on Day (D) 0, with late luteal phase characterised by sparse coverage of lysed, pale leukocytes (*, D3). Menstrual phase lasts from D5-D9, with extremely light bleeding indicated by low densities of red blood cells (D7, arrows). Mucus in prominent in later stages of menstrual phase (D9), leading in to early follicular phase (D10-11). Vaginal cornification indicative of "oestrus"/ovulation is present on D12, with initial luteal phase cytology reached again on D14. Scale bars = 100 um.

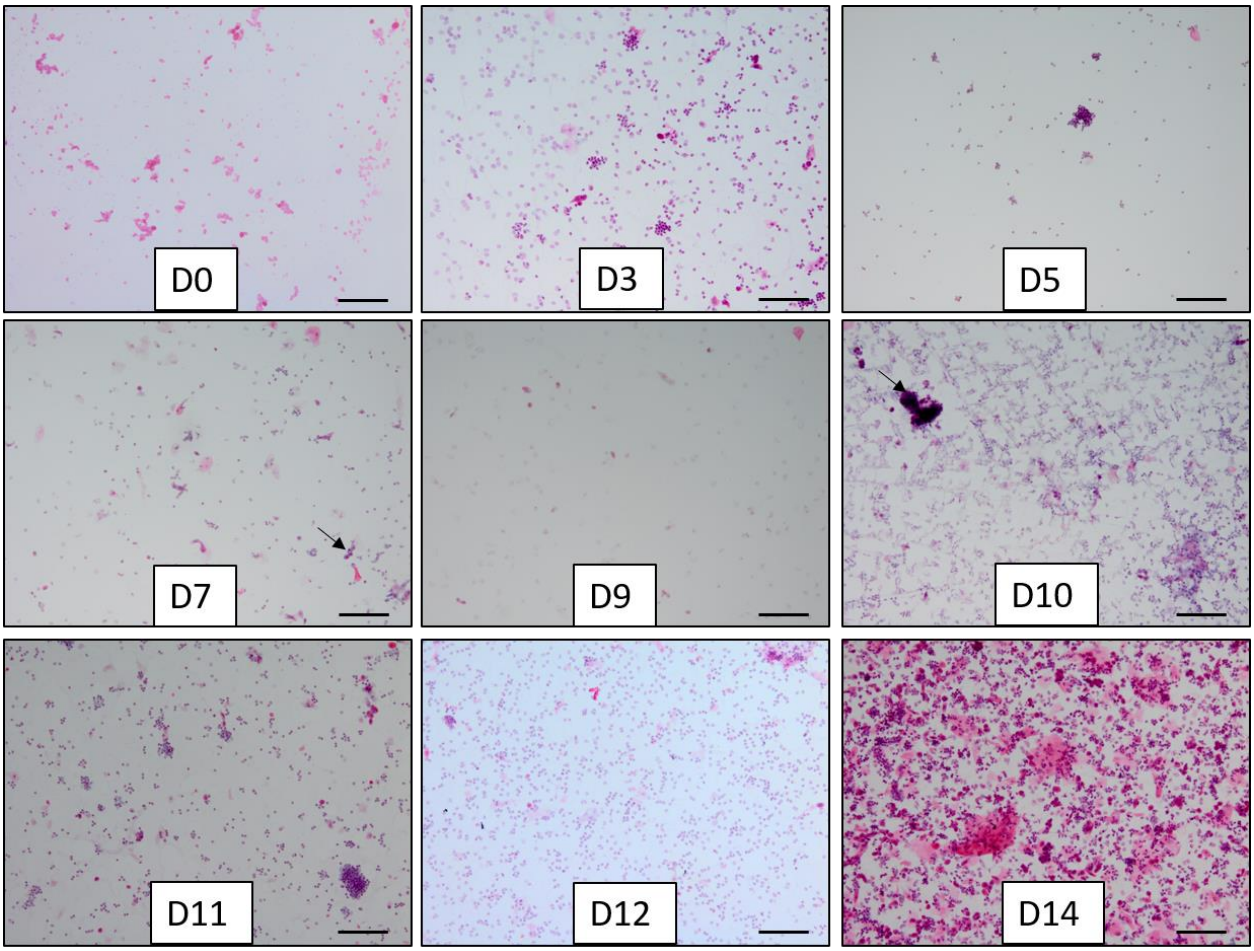
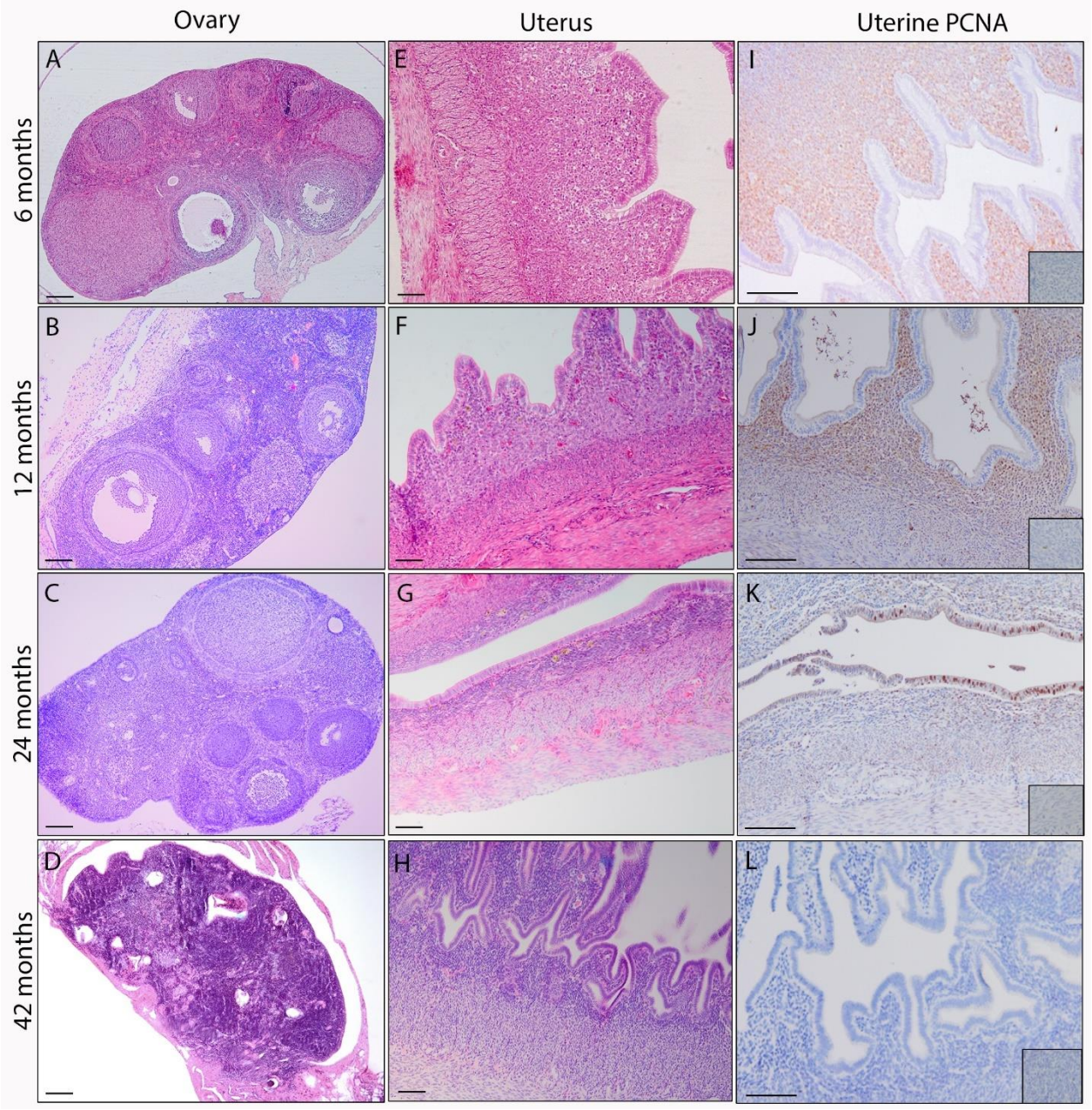


Figure 6.3.4: Cytology of a 42-month old acyclic female spiny mouse across 14 days. Female shows cytology containing anucleated epithelial cells (D0), followed by extended periods of non-specific cytology reminiscent of late menstrual phases, with leukocytes, some cornified and nucleated (arrows) epithelial cells (D3-12). However, this cytology does not progress to the early follicular ("proestrus") phase and a new cycle was not initiated. D14 shows cytology containing higher proportions of cornified epithelial cells, but ovulation was not observed.

Changes in ovarian weights are mirrored in histological assessment, whereby mature antral follicles and corpora lutea are easily distinguished in all ages except females aged 42 months (Figure 6.3.4A-D).

Uterine proliferation is evident in the analysis of haematoxylin and eosin stained sections(Figure 6.3.5E-H) and correlation of PCNA immunohistochemistry comparing cell proliferation in the endometrial functionalis (Figure 6.3.5I-L). 6-month old females have the

highest median percentage of area coverage (10.91% (range? 5.78 to 26.40)) compared to all other groups. 42-month old female spiny mice do not show prominent staining for PCNA in the endometrium (0.02% (range 0.00 to 0.22)) and which was significantly reduced compared to 6-month old controls (Figure 6.3.5M, $p<0.01$, Kruskal-Wallis). The luminal epithelium demonstrated specific PCNA localisation when undergoing shedding and simultaneous repair during menstruation, which still occurred up to 24 months of age in the spiny mouse.


Ovary
Uterus
Uterine PCNA
6 months
12 months
24 months
42 months
A
B
C
D
E
F
G
H
I
J
K
L

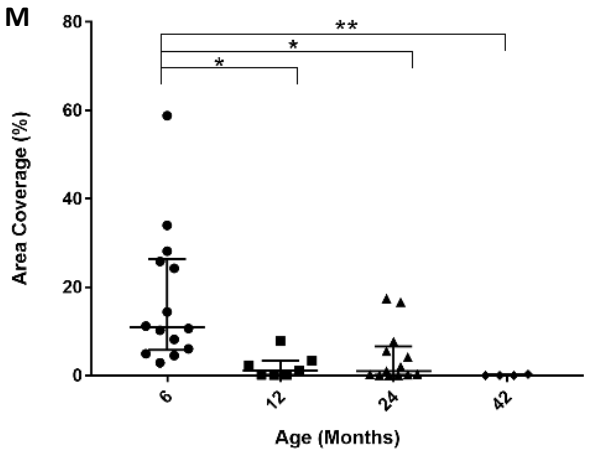

M
Area Coverage (%)
80
60
40
20
0
**
*
*
6
12
24
42
Age (Months)

Figure 6.3.5: Morphological changes to the reproductive tract in ageing female spiny mice. Scale bars = 200 um. Antral follicles and corpora lutea are readily identifiable in the ovaries of 6 (A), 12 (B) and 24 (C) month old females, but not in 42 (D) month old spiny mice. The endometrium in 6 and 12-month aged females (E,F) shows characteristic decidualisation and epithelial invaginations. The thickness of the endometrial functionalis appears reduced from 24 months onwards (G,H) with less marked epithelial invaginations. The functionalis is notably PCNA immunopositive up to 12 months (I,J), with localised staining in the repairing luminal epithelium following menstruation in 24-month aged females (K) but no staining at 42 months (L). Inset squares show negative controls. (M) significant decline in median area coverage of PCNA positive endometrium across all groups compared to 6 months (One-way ANOVA, *$p<0.05$, **$p<0.01$).

6.4 Discussion

We have shown for the first time, a transition to reproductive senescence in the spiny mouse which is unlike that of any other rodent. Importantly, this transition appears gradual, rather than abrupt, as approximately two thirds of females in our captive colony retained the ability to ovulate through demonstration of oestrus stage lavages and the histological presence of antral follicles at 24 months of age, and still nearly half of females at 42 months of age. Reproductive senescence in spiny mice is considerably longer than in the standard mouse which shows signs of estropause from approximately 9-15 months of age (4).

The cytology of only 4 females could be extrapolated from the original cohort of 9 at 42 months of age, because illness or detection of reproductive anomalies at post-mortem required their exclusion from this analysis. In our colony, tumours do not appear to discriminate against sex or age of parity, however these tumours are generally located external to the reproductive tract, most commonly on the limbs or neck. The findings in this study suggest that the spiny mouse may also be a candidate for studies of endometrial cancers and potentially uterine fibroids and the impact of these conditions on the menstrual cycle, as aged virgin females appear to have a predisposition to such health issues. Unlike oestrous species, no female spiny mice presented with uterine pus consistent with pyometra. In canines, for example, persistent stimulation with oestrogen at oestrus and subsequent periods of progesterone dominance causes endometrial proliferation, glandular secretion and immune suppression. Repeated oestrous cycles have a cumulative effect, leaving the uterus susceptible to bacterial infection (15). The spiny mouse does not behave as an oestrous cycling species under these conditions.

Interestingly, although the follicular phase showed an overall increase in length at 42 months compared to 6 months in cycling animals, we did not observe persistent vaginal cornification (PVC) in this cohort of spiny mice. Acyclic females instead demonstrate anestrus-like smears seen in mice, with decreased cornified cells and increases in leukocytic recruitment (16). Some metestrus-like samples were also observed, with higher densities of cornified epithelial cells, but these samples did not follow oestrus phase cytology, and therefore is likely to reflect remnant oestrogen production as the follicular pool is depleted, or possibly due to the increased storage of fat in older females. In addition, the degree of erythrocyte infiltration during menses in the 42-month old cohort appeared less than that of previously observed young menstruating females (see Chapter 3, (1)), which is likely to be due to declining peripheral oestrogen, and an inability to support a proliferative endometrium, resulting in lighter bleeding.

Anestrus cytology is not uncommon in anxious females in our colony, and we have observed these types of lavages in spiny mice subjected to high stress, such as >20 consecutive days of lavaging. Indeed, stress may account for up to 25% of irregular cycling in female spiny mice under experiment (unpublished) At post-mortem no reproductive tract abnormalities were found in 6-month old controls suggesting that stress was likely the cause of cessation of menstruation and ovulation in 2/14 females assessed. As highlighted in Chapter 5, spiny mice demonstrate a spectrum of anxiety in relation to PMS-like symptoms and these animals may therefore be generally more susceptible to stressors. Direct transition to anestrus is less frequently observed in mice, which instead show a preference for PVC or repetitive pseudopregnancy (RPP) pathways to estropause (4, 16). RPP is unlikely to be the cause of leukocytic dominant lavages in the spiny mouse, for several reasons. Firstly, mice and rats

require pseudopregnancy induction to maintain the functionality of the corpus luteum and sustain an active luteal phase to support deciduomata formation (17-20); in contrast, spiny mice exhibit an active luteal phase, functional corpus luteum and consequently, spontaneous decidualisation irrespective of pseudopregnancy (1). Secondly, pseudopregnant spiny mice should have functional corpora lutea in the ovaries, but these were not identified histologically. Third, the endometrium upon examination in 42-month old females did not mirror decidualised or proliferating endometrium seen in younger spiny mice, and was negative for PCNA altogether, suggesting the equivalent of uterine atrophy seen in anestrus rodents (4). Finally, pseudopregnancy is not readily induced in spiny mice through mechano-receptor stimulation (see Chapter 7).

While at 12 and 24 months of age PCNA staining is present, it is significantly reduced compared to 6-month old females. Based on both the cytological and histological assessments, this was unexpected. Low sample size may account for this in the 12-month age group (n=7), as there may not have been enough females during the late proliferative phase of the menstrual cycle at the time of post mortem, or repair during the late menstrual phase may have been largely complete prior to a new fertile cycle and before sufficient ovarian oestrogen from recruited follicles could induce endometrial growth.

42-month old females were the oldest animals available during this study. Initially, we conducted the study by examining most of the females from the 42-month cohort at 36-months and found a very gradual decline in proportion of cycling females, with over half (56%) still exhibiting the capacity for ovulation. From this observation we decided to allow these females to age an additional 6 months and revaluate their reproductive activity. The cost of prolonged housing/husbandry and the increased incidence of health issues ultimately

restricted our ability to continue assessing female reproductive changes beyond 42-months of age. Our observations showed that a menopausal transition was underway by 42 months, and we estimate that a menopausal state is likely to be complete in female spiny mice by ~ 4-year old (48 months). In the wild, the lifespan of *Acomys* is estimated to be around 3 years, and up to 5 years in captivity (21). The most recent published observation of the lifespan of the spiny mouse in our colony was made some years ago, and prior to the discovery of menstruation (22), with adults surviving up to 4 years. Based on previous findings in conjunction with our data at this point in time, the spiny mouse appears to have late onset menopause, and may spend as little as 20% of its life postmenopausal, like chimpanzees(5). However, the previous cohort of animals were maintained in slightly different conditions, and spiny mice weighed up to 60g at >2 years of age (22), compared to the heaviest female in the current study; 50.1g at 24 months. This may contribute to obesity-associated health problems and earlier deaths. Therefore, the natural life expectancy of our captive colony maintained under recently optimized conditions requires further investigation.

Determination of the suitability of the spiny mouse as a model for human menopause will also require further studies, particularly in regard to changing endocrinological profiles with age. Ideally, this research would demonstrate increased gonadotrophin secretions, and corresponding changes in oestrogen levels. The spiny mouse has shown remarkable heterogeneity in menstrual physiology, and likely repeated blood sampling is required for accurate hormonal analysis. This is problematic due to the fragility of their skin and ethical concerns of injury during multiple sampling using conventional methods i.e. the tail vein in mice. De-sheathing of the tail is a defence mechanism in nature for this species and restricts our ability to utilise the tail vasculature for needle insertion as is done in laboratory rats and

mice. Similarly, jugular sampling is also precarious due to the likelihood of skin tearing. In addition, the cost of optimising valid methods of steroid detection in this relatively novel species is high, and there are also potential challenges in ascertaining true readings in a species which behaves as neither mouse nor human(23).

The prospect for the spiny mouse as a model for research into menopause is currently hindered by a lack of widespread usage of this unique rodent, but nonetheless our data suggests continued exploration is warranted. A gradual rather than sudden menopausal transition suggests that the spiny mouse is an appropriate and useful perimenopausal model to examine the neuroendocrine pathways which encompass all hormonal interactions in the hypothalamic-pituitary-gonadal axis, as opposed to the current OVX rodent models. Finally, the logistic and economic advantages of such a model may reduce our reliance on old world monkeys and enable more thorough and invasive investigation than is possible in humans. Therefore, we continue to advocate for the spiny mouse as an important, useful and relevant model of menstruation and reproductive ageing.

CHAPTER 7:

PSEUDOPREGNANCY CANNOT BE INDUCED USING CONVENTIONAL METHODS IN THE SPINY MOUSE

7.1 Introduction

The spiny mouse (*Acomys cahirinus*) is the only rodent species to undergo a menstrual cycle (1). This, in addition to what is already known about the reproductive biology of this species, has sparked recent interest in this desert-dwelling species as an alternative model for human-specific conditions. The spiny mouse in many ways is more similar to humans than other rodent models such as the laboratory mouse (*Mus musculinis*) and rat (*Rattus novegicus*). This is especially exemplified in the number of pups (typically 2-3), gestation length (39 days), placentation (24), endocrinology (13, 25) and the developmental profile of offspring (12, 26, 27). In particular, the spiny mouse demonstrates natural variation in their menstrual bleeding, analogous inflammatory and repair profiles during menstruation and demonstration of spiral arterioles during the late secretory phase (23) .

The spiny mouse shares many integral characteristics of true menstruating species, including an active luteal phase providing progesterone support, a peak in luteal-phase prolactin secretion, spontaneous decidualisation and terminal differentiation of the endometrial functionalis, passive luteolysis in the absence of pregnancy and, finally, endometrial sloughing and bleeding at the end of an infertile cycle (1, 23). The discovery of menstruation in a small, non-primate species greatly expands the possibilities of future research in women's reproductive health, in particular, to develop a better understanding of fundamental menstrual physiology, including the mechanisms of uterine breakdown and repair, and the contributing factors which may predispose women to disorders of menstruation and subsequent pregnancy (23).

While it is clear the spiny mouse shares many attributes akin to primate reproductive biology, it has yet to be established whether pseudopregnancy can be induced in this species as is

commonly used for many studies in conventional rodents. Pseudopregnancy is a progestational state in which the female mimics endometrial and endocrinological changes of early pregnancy and lactation, but in the absence of a fertilised embryo (28). In spontaneously ovulating rodents, the oestrous cycle is short (4-5 days) with an inactive luteal phase and a corpus luteum which is only semi-functional. Progesterone secretion is brief, and halts almost immediately without a pregnancy rescue signal. However, the abrupt lifespan of corpora lutea may be substantially prolonged, and consequently the luteal phase in mice and rats is increased over a course of ~12 days, by taking advantage of these species' neuroendocrinology.

Neuroendocrine reflexes to vagino-cervical stimulation through coital stimulation, or non-coital triggers such as extended periods of concaveation (20), causes a long-term bi-daily surges of pituitary prolactin into peripheral circulation; these pulsatile secretions sustain the life and functionality of the corpus luteum (18-20). Only then do oestrous cycle species exhibit an active luteal phase with corresponding rises in progesterone production resulting in a predisposition to deciduoma formation in preparation for pregnancy(17). Coitally-induced pseudopregnancy may be advantageous in induced ovulators, such as cats, after mating with sub- or infertile males as it allows females to return to oestrous and breed faster than the length of a full-term pregnancy. However, non-coital pseudopregnancy in spontaneous ovulators, such as rats, is more readily observed in nature, and is likely to aid in cohabitation and fostering of pups (20).

Both coital and non-coital pseudopregnancy induction continue to be useful tools in a modern laboratory setting, and particularly advantageous for researchers performing high throughput experiments, whereby cycle synchronisation of large cohorts is required for timed mating

and embryo transfers. Common successful methods in rats, mice, hamsters and gerbils use cervical stimulation (electric or mechanical) (29-31), sterile mating (32, 33) or hormonal induction, with progesterone being particularly effective (29, 32, 34, 35).

The potential applications of pseudopregnancy induction in the spiny mouse would enable further investigation into fundamental reproductive processes in this species, transgenic manipulation (gene insertions and gene knock outs) and chimera productions; an ever-growing need due to the remarkable biological profile of this curious species. However, the spiny mouse is a relatively novel model of female reproduction and relatively little is understood regarding their fundamental biological behaviour, compared with their murine relatives. For example, I have observed in our colony that cohabitation in all female cages does not induce either pseudopregnancy or cycle synchronisation (unpublished observation), unlike in female mice (20). Other common reproductive phenomena in mice such as the Whitten effect (oestrous cycle synchronisation in response to male pheromones) (36) have not been explored in the spiny mouse. Specifically, the degree of rodent/oestrous cycle versus primate/menstrual cycle characteristics are largely unknown in our menstruating captive colony. Hence, my aims are to determine whether 1) pseudopregnancy and 2) menstrual cycle synchronicity can be achieved using conventional rodent protocols in the spiny mouse.

7.2 Materials and Methods

Animal Care

All experiments were approved in advance by Monash University, Monash Medical Centre Animal Ethics Committee, and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (SOBSA/2008/78). The spiny mice used for this study were obtained from our own laboratory colony, with a 12 hr light cycle (lights on 07:00) and maintained as previously described (1).

Controls: Experiments 1 and 2

Virgin females aged 90-150 days and weighing between 30-38g were allocated to one of several treatment groups, summarised in Table 1. All females were subjected to vaginal lavage between 13:00 and 15:00 and cytological assessment for cycle stage identification as previously described (1). Lavage stages are herein referred to as proestrus (early follicular (EF)), oestrus (late follicular (LF)), metestrus (early luteal (EL)), diestrus (late luteal (LL)) or menses. Controls (Group 1) were subjected to daily vaginal lavage only, and no other manipulation was conducted.

Experiment 1

Induction of Pseudopregnancy

For all other groups, treatments (described in detail below) were administered within the window of ovulation (late follicular (LF) phase, rodent equivalent "oestrus") when females presented with pink, slightly engorged vulvas, little resistance upon insertion of pipettes during lavage and produced smears containing >80% cornified epithelial cells, as previous

work in rats demonstrated a higher rate of pseudopregnancy success if treatment was administered during oestrus (29). After administration, subjects were lavaged either daily from the day of treatment (D0) or daily from day 3 (D3) following treatment to determine whether the success rates of pseudopregnancy would increase with females left undisturbed immediately after treatment. Spiny mice were lavaged until females reached their next ovulation.

Pseudopregnancy induction in standard laboratory rodents is typically deemed successful if deciduomata formation is observed after application of uterine trauma following method of induction (17, 29). However, the spiny mouse spontaneously decidualises the endometrial stroma, and therefore these criteria are not applicable. Persistence of late luteal phase cytology was used as the measure of pseudopregnancy. Diestrus (LL phase) in the spiny mouse lasts 1.8 ± 0.3 days, with predominantly leukocytic cytology ranging from 2-4 days (1). We therefore considered the persistence of leukocytic cytology for >12 days as confirmation of pseudopregnancy, based on observations in mice and rats (20). Three conventional methods of pseudopregnancy induction were trialled: i) progesterone administration; ii) mechanical stimulation and; iii) sterile mating as described below and outlined in Table 7.2.1.

i) *Progesterone in Oil by Subcutaneous Injection*

Progesterone powder (Sigma-Aldrich, NSW) in sesame oil was administered in a single subcutaneous injection (0.14 mL) to towel-restrained female spiny mice in the supine position through subcutaneous injection. Females received either a high (4mg) or low (2mg) dose.

ii) *Mechanical Stimulation*

Mechanical rods for cervical stimulation were produced by pulling glass pasteur pipettes under application of heat. Elongated pipette tips were rounded and closed to ball point. Glass rods have been successful in cervical stimulation and induction of pseudopregnancy in rats (37). Females were restrained using a towel and positioned in the prone position, with their hind quarters then manipulated to mimic lordosis behaviour (Figure 7.2.1). Mechanical stimulation consisted of two sets of 15 intromissions, each lasting approximately 0.5 seconds, with 60 seconds intermission between sets, based on a previous study of spiny mouse copulation (38).

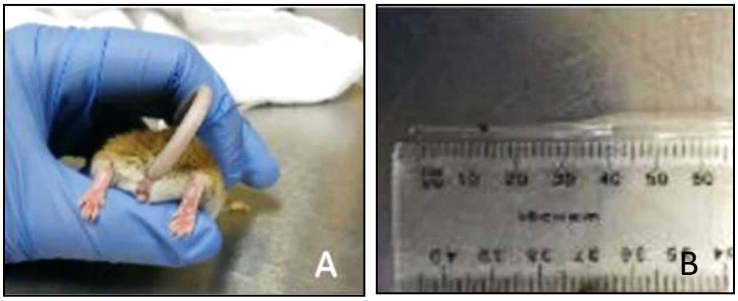


Figure 7.2.1: Mechanical stimulation as a method of pseudopregnancy induction in the spiny mouse. A) Female in prone position with exposed vulva and back curved to mimic lordosis. B) 15mm marked glass rod used for vagino-cervical stimulation.

iii) *Sterile Mating*

Male spiny mice 12 weeks of age (n=7) weighing between 33-41g were anaesthetised with 5% isoflurane in air and maintained at 2.5% for throughout the duration of the surgery. A single mid-ventral incision was made on the lower abdominal skin to expose the connective

tissue beneath. Two separate incisions either side of the linea alba were made to locate testicles and vas deferens. An approximate 10mm section of vas was excised using a cauterising pen and the testicle carefully replaced into the scrotum. The peritoneal wall was sutured with interrupted Maxon® dissolvable sutures (5/0, 15 mm) and the skin incision repaired with interrupted Dysilk® surgical sutures (6/0, 12mm). The testes remained intact, as we did not wish to eliminate testosterone secretion. Males were allowed a minimum of 3 weeks recovery before mating overnight with females and removed after 24h.

TABLE 7.2.1: TREATMENT GROUPS FOR PSEUDOPREGNANCY INDUCTION IN FEMALE SPINY MICE

Group	Treatment	N	Lavage
1	Control	8	Daily
2	P4 (4mg)	4	D0
3	P4 (4mg)	5	D3
4	P4 (2mg)	4	D3
5	Sterile Mating	4	D0
6	Sterile Mating	7	D3
7	Mechanical Stimulation	3	D0

Post Mortem and Histological Analysis

Control females (Group 1, n=8) underwent one complete cycle of vaginal lavage. During their second cycle, animals were killed by isoflurane overdose during each cycle phase for histological comparisons of ovaries, uterus and cervix (n=1-2 per cycle phase). Treated females (Group 2-7) were exposed to pseudopregnancy induction and lavaged until the

following oestrous was reached. An additional n=3 females per group were selected as histological representatives for examination of pseudopregnancy induction. These representative females were killed on day 7 post manipulation, which we expected would be midway through pseudopregnancy (if induction successful).

Samples were fixed in formalin for 24-48 hours before being submerged in 70% ethanol for at least 24 hours. Tissues were then processed to paraffin and sectioned at 5 uM. Slides were stained with haematoxylin and eosin as described (1).

Experiment 2

To examine whether the cycle synchronicity through the Whitten effect could be induced in spiny mice, paper tissues were placed in the cage of an aged-matched fertile male spiny mouse for 5 days. The soiled tissues, cardboard apparatuses and soiled sawdust bedding was then introduced to a cage of regularly cycling females (n=6) for 72 hours, after which, the females were lavaged daily for 20 days (at least two full menstrual cycles).

Statistical Analysis

Normally distributed data are displayed as Mean ± Standard Deviation (SD), and non-normal data as median (25^{th} percentile, 75^{th} percentile). Differences between treatment groups were analysed using ANOVA or Kruskal-Wallis, respectively. GraphPad PRISM (Version 6.0) was used to conduct analyses, with a $p < 0.05$ considered significant.

7.3 Results

Experiment 1

Pseudopregnancy induction

The menstrual cycle of control female spiny mice ranged from 7-13 days (mean = 9.6 ± 2.1 days), with a median diestrus (late luteal) phase of 1.3 (1.0, 2.0) days, and median menstrual phases lasting 2.0 (1.6, 2.8) days, similar to previous findings (1). The late luteal phase was not significantly prolonged in any of the treatment groups compared to control (Figure 7.3.1A, p = 0.237, Kruskal-Wallis). The median length of the menstrual phase, however, did significantly increase in groups 4-7 (Figure 7.3.1B, p = 0.0008, Kruskal-Wallis), extending by a minimum of 3 days (Group 6) and maximum of 5 days (Group 4). An example of vaginal cytology from a progesterone induced female (Group 3) is presented in Figure 7.3.2. Menses lasted for 5 days in this female, with the next oestrus cytology reached by day 10 post-injection.

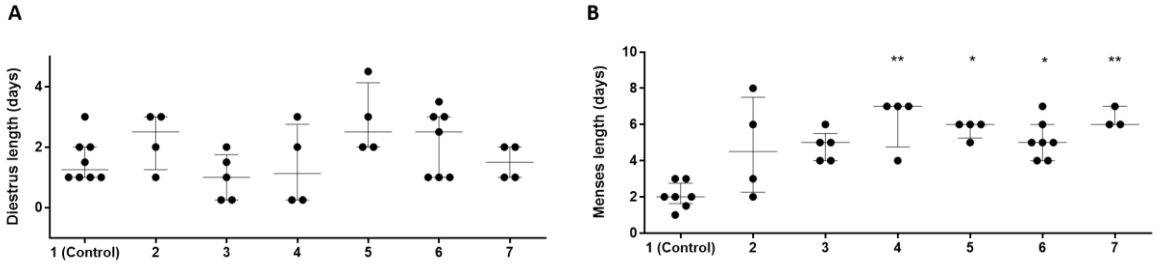


Figure 7.3.1: Comparative length of A) diestrus and B) menses following treatments for pseudopregnancy induction in the spiny mouse. No significant increases in diestrus were observed in any treatment group but significant increases in the menstrual phase of groups 4-7 were noted (Kruskal-Wallis, *p<0.05, **p<0.01).

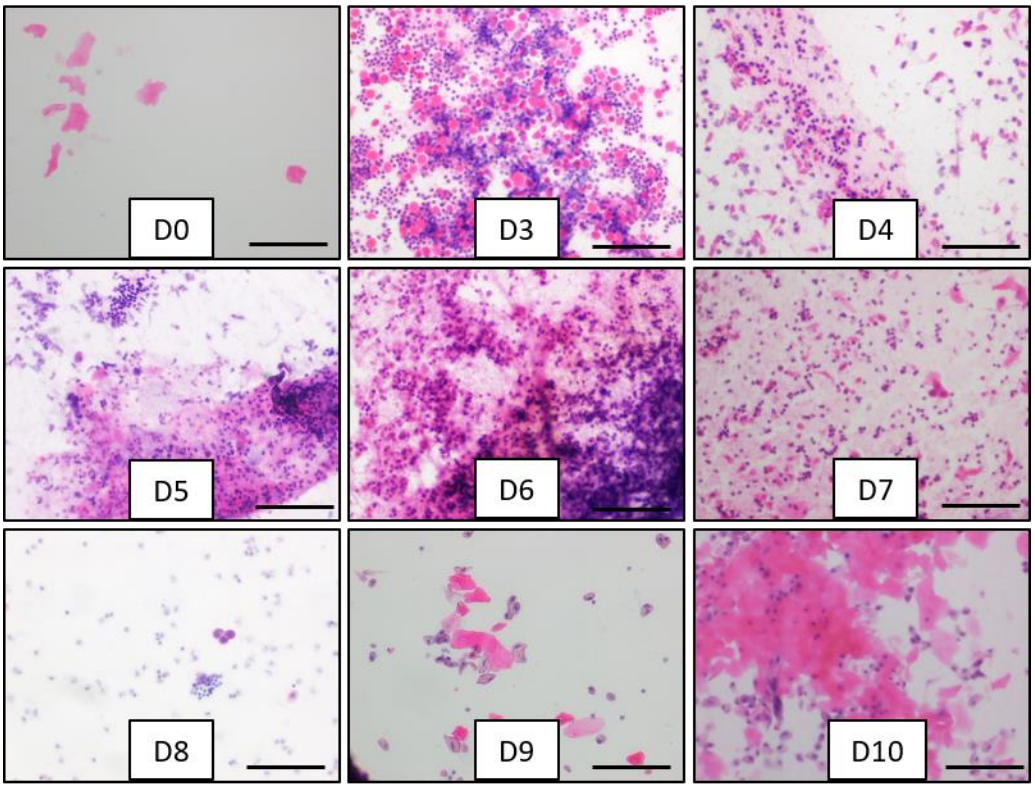


Figure 7.3.2: Vaginal cytology of female spiny mice injected with progesterone (4mg) on Day (D) 0 at during late follicular phase (rodent equivalent "oestrus"). Haematoxylin and eosin. Scale bar = 200 um. Female was rested for 3 days before consecutive lavaging until next "oestrus" (D10). Menses occurred over 5-day period (D4-D8), with red blood cell infiltration, mucus and stromal debris peaking on D6 post injection. Pseudopregnancy, classified as prolonged luteal phase cytology as seen on D3, was not achieved.

Vaginal cytology and reproductive tract morphology

Reproductive tract stained with haematoxylin and eosin is shown in Figure 7.3.4. Ovarian and uterine changes across the menstrual cycle are in agreement with previous observations (1) of follicular recruitment and dominant follicle maturation in the EF and LF phases, respectively, corpora lutea formation and maintenance in the EL and LL phases and regression of the corpus luteum with initiation of new follicular recruitment during menses. Cyclical proliferation, differentiation and shedding of the endometrium was also confirmed in the uterus. Analysis of the cervices during each phase of the menstrual cycle demonstrated cellular debris similar to that seen in the vaginal lavage cytology. These observations correlated with changes in the ovaries and uterus e.g. nucleated and cornified epithelial cells were most prominent in the cervico-vaginal junction during the follicular phases, while leukocytes infiltrated during the luteal phase, and finally some mucus and blood smears observed during menses.

The post-mortem observations of representative histological replicates of potential pseudopregnant females (Figure 7.3.5) revealed ovarian and uterine reproductive tract morphology that corresponded with observed cytology data showing that no females demonstrated diestrus phase endometrial or ovarian characteristics. In groups 2 and 4, females progressed to a new fertile cycle and exhibited proestrus (EF) or oestrus (LF) cytology, a thin endometrium and antral follicular recruitment. In groups 3,5,6 and 7, some corpora lutea were identified but endometrial and cytological analysis indicated these were either newly formed in the metestrus (EL) phase (groups 5-6) or regressing at the initiation of early menstrual shedding

(groups 3 and 7). No reproductive tract abnormalities were macroscopically observed in any group.

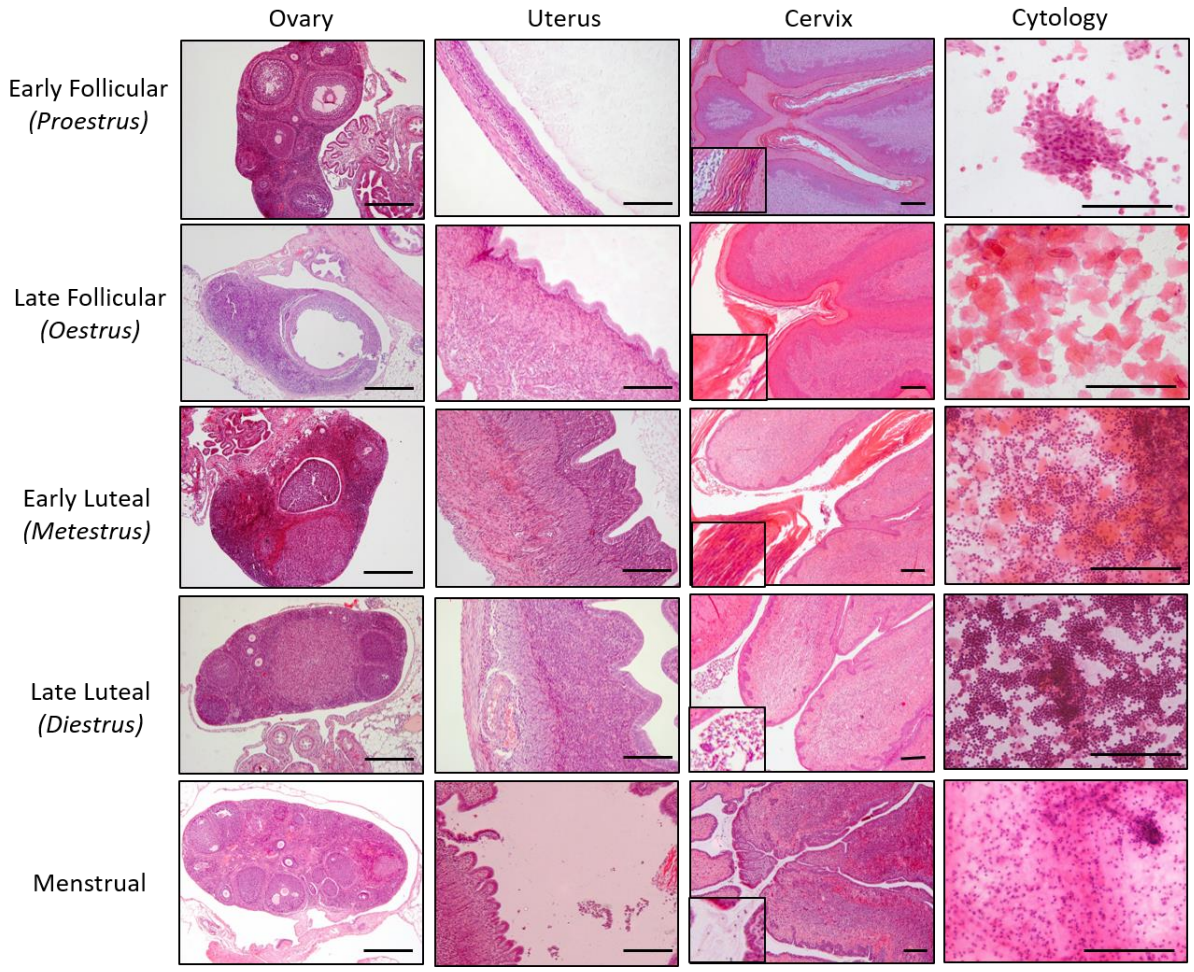


Figure 7.3.4: Comparative histology and cytology of reproductive tract of female spiny mouse at five stages of the menstrual cycle. Rodent equivalent cycle stage shown in italics. Haematoxylin and eosin. Scale bar = 200 um. *Ovary:* During early follicular (EF) phase, antral follicles are recruited with dominant follicle maturation occurring during late follicular (LF) phase. Luteal phase is active, with formation of corpora lutea during early luteal (EL) phase and maintenance during the late luteal (LL) phase. Corpora lutea have regressed and the beginning of a new follicular recruitment occurs during menses. *Uterus:* EF and LF stages show relatively narrow uterine endometrium, which undergoes proliferation, endometrial thickening and spontaneous decidualisation during EL and LL phases. During menses, differentiated stroma is shed, with cellular debris and blood in the uterine lumen. *Cervix:* Combinations of nucleated and cornified epithelial cells infiltrate the vaginal canal at the cervico-vaginal junction during EF and LF phases, with leukocytic infiltration during EL and LL phases. At menses, mucus, leukocytes and erythrocytes enter the vaginal canal. These cell types are

magnified in inset squares. *Cytology:* the cell types reflected in the vaginal lavage mirror those identified in cervices.

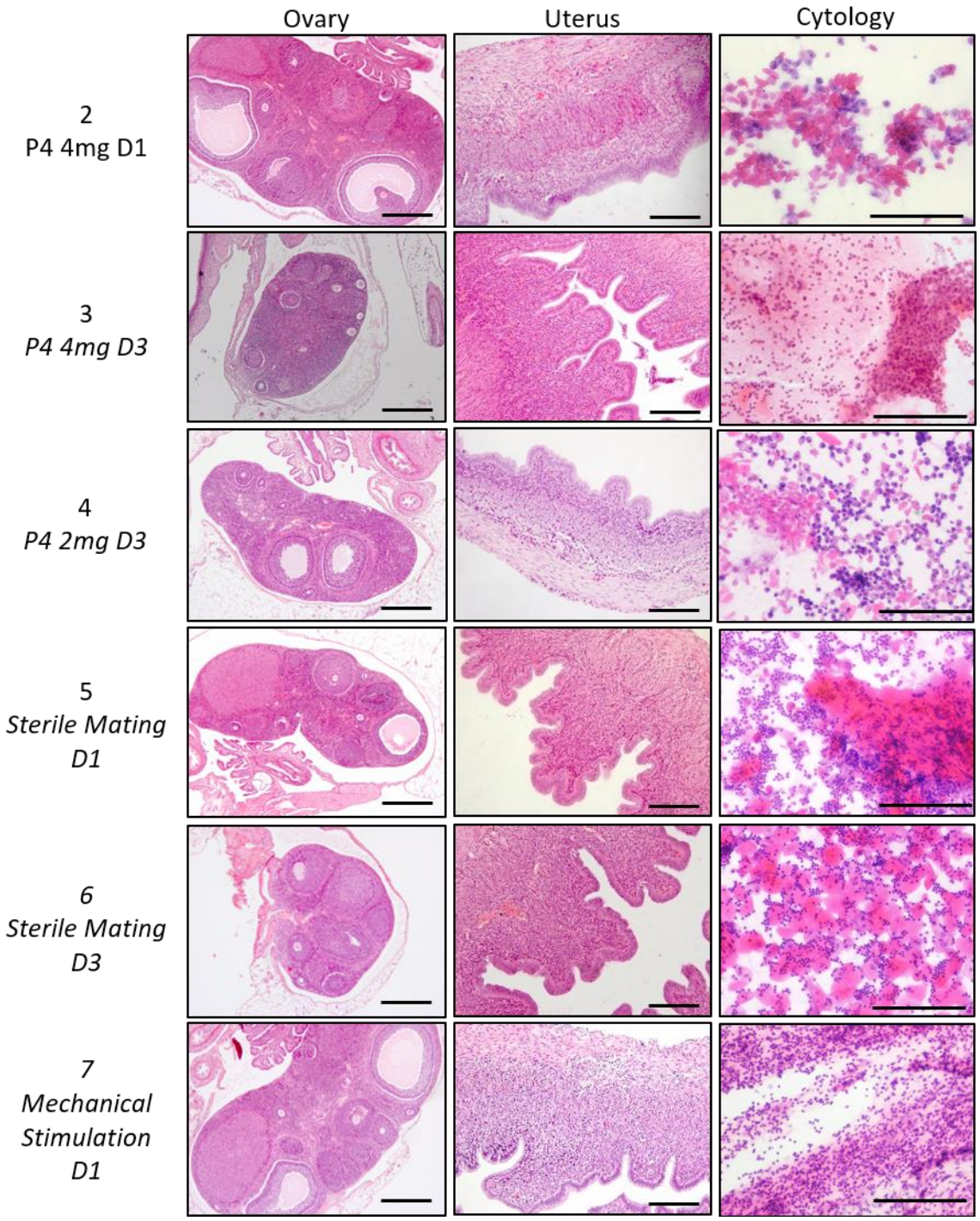


Figure 7.3.5: Comparative histology and cytology of reproductive tract of females treated for induction of pseudopregnancy at 7-day post-treatment. Haematoxylin and eosin. Scale bars = 200 um. No groups successfully induced. Ovaries, uteri and cytology are

representative of early follicular (EF) phase (Groups 2 and 4), early luteal (EL) phase (Groups 5 and 6) or early menstrual (EM) phases (Groups 3 and 7).

Experiment 2

The Whitten effect was not observed in this cohort of spiny mice, and exposure to male soiled bedding neither induced oestrus nor synchronised the cycle of females (Table 7.3.1). Additionally, it was established through assessment of the menstrual cycles that male pheromones had no significant impact on the length of any stages, nor on the total length of the menstrual cycle (Figure 7.3.6).

TABLE 7.3.1: MENSTRUAL CYCLE AFTER WHITTEN EFFECT INDUCTION IN FEMALE SPINY MICE

Day	A	B	C	D	E	F
1	P	Mens	D	P	Mens	P
2	P-O	Mens- P	P	O-M	Mens	D
3	O	P	O	E D	Mens	Mens
4	M	P	O - M	D	Mens	Mens
5	M – i	P	M	D	Mens	Mens
6	P	P-O	M	Mens	P	Mens – P
7	P – O	O	D	Mens	P	P
8	O	O	D	Mens	O	P
9	O – M	M	D – Mens	Mens – P	O	O
10	M – D	D	Mens	P	M	O
11	D	D	Mens	P	D	M
12	D – Mens	D	P	P – O	D	M – D
13	Mens	Mens	P	O	D	D
14	Mens – P	Mens	M	O	Mens	D – Mens
15	P – O	P	D	M	Mens	Mens
16	O – M	P	D	D	Mens – P	Mens
17	M	O	Mens	D	O	Mens
18	M	O	Mens	D	O – M	P
19	D	M	Mens	D	M	P – O
20	D	D	Mens –Pro	D	Di	O – M

Spiny mice were lavage daily for 20 consecutive days beginning 48h post exposure to male soiled bedding (D0). A-F represents individual female subjects. P = proestrus (early follicular phase), O = oestrus (late follicular phase), M = metestrus (early luteal phase), D = diestrus (late luteal phase), Mens = menstrual phase, – indicate transition between two phases.

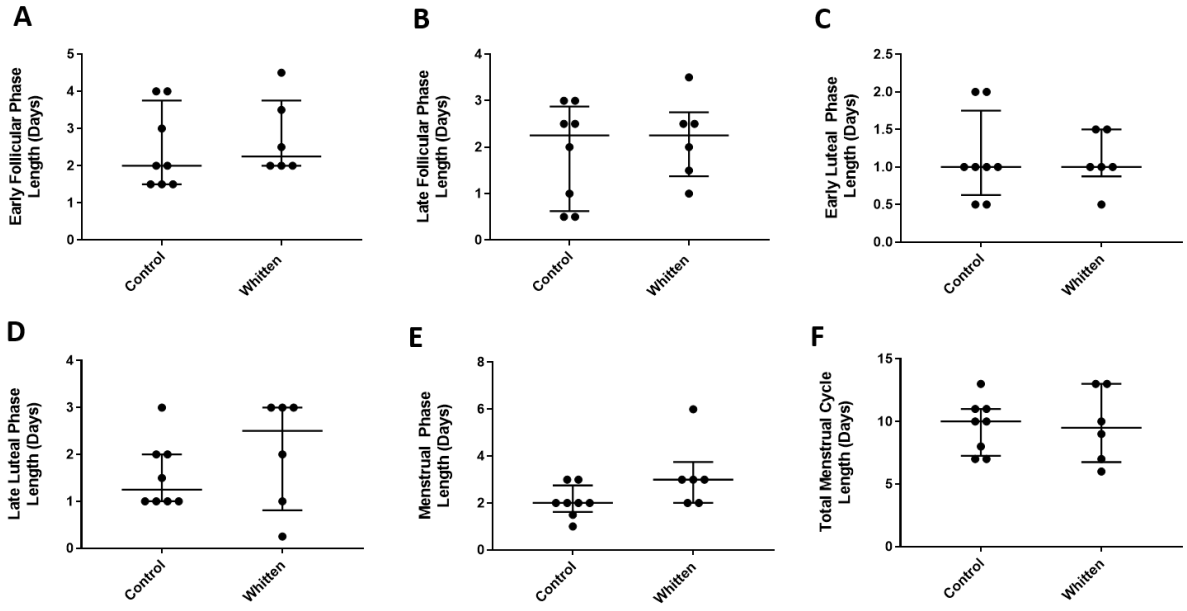


Figure 7.3.6: Comparative length of menstrual cycle phases in Whitten effect treated female spiny mice. No significant differences in any stages (A-E) or total cycle (F) were observed between exposed (Whitten) and unexposed (Control) females.

7.4 Discussion

The previous finding of menstruation in our captive colony demonstrated that the process of reproduction in our spiny mice was at odds with the central dogma of rodent reproductive biology. Here, we continue to demonstrate the unique and unexpected features of spiny mice in their lack of expected response to common manipulations applied to most other laboratory species. Although we failed to 1) induce pseudopregnancy using progesterone, sterile mating or mechano-receptor stimulation, and 2) induce oestrus and synchronise the cycles of females, we view this study as a great success in verifying another example of the unusual primate-like nature of this species, in that their follicular and luteal phases cannot be influenced in the same way as their murine relatives.

There is a possibility that pseudopregnancy induction was not successful in this study due to insufficient mechanical stimulation and inability to adequately mimic natural copulatory behaviour (38), or to the effect of stress from the restraint method. In addition, spiny mice in our colony do not produce an external vaginal plug like their mouse relatives, and therefore visual confirmation of copulation with the vasectomised males was not possible. However, previous studies have explored the nature of pheromone mediation in the mouse and showed that females are receptive to pseudopregnancy induction even without physical contact with males or their urine, but merely exposure to close proximity, showing that in mice volatile pheromones secreted in the urine are sufficient to induce pseudopregnancy (39). The spiny mice did not respond to male proximity during exposure to vasectomised males, despite intact testes and presumable normal testosterone production, nor did they respond to soiled bedding from fertile males. While the effect of female group housing has been documented to

suppress oestrus and extend the diestrus phase in mice (20, 39, 40), we did not observe any evidence of cycle synchronisation in our females.

The absence of the Whitten effect or group housing induced pseudopregnancy in spiny mice was not unexpected; in fact these results are further validation that the spiny mouse is a true menstruating species. Spiny mice in our colony are housed in sex specific cages and each female is at a different stage of her menstrual cycle, despite male and female cages being housed in close proximity. Male cages are within 15-20 cm of females, and volatile urine excretion does not appear to have any positive or negative effect on the female menstrual cycle in spiny mice.

Injection of a single sub-cutaneous high dose of progesterone during oestrus is sufficient to induce pseudopregnancy in rats (34, 41, 42). Based on a successful induction dose of 10mg in 230-280g Wistar rats, we calculated our minimal dose required in a 38g spiny mouse to be 1.4 mg. We chose to assess both low (2mg) and high (4mg) doses and neither were effective. The spiny mouse may require multiple low doses (41), or may only respond to progesterone during the morning of oestrus (43). Due to time, and ethical and cost constraints, this experimental group, plus an additional control group in which females were injected with a sesame oil vehicle, were not able to be further explored.

It is more likely, given the behaviour of the spiny mouse reproductive cycle and its similarities to humans, that continued administration of exogenous hCG after ovulation may induce a pseudopregnant state by prolonging the life of the corpus luteum and endometrial decidua, delaying menstrual onset and simulating the circulating hCG levels in early gestation (44, 45). Other avenues which could be explored to suppress ovulation and provide luteal phase support may be graduated progesterone/progestin administration over several

days, or insertion of long-acting subcutaneous progestin releasing implants, which have been shown in mice to mimic the effects of progestogen-only implants (46). The development of a similar *in vitro* fertilisation protocol in this human-like rodent remains an exciting prospect. Further investigation as to the response of spiny mice to the combined oral contraceptive pill and exogenous steroidal administration is paramount for future menstrual cycle and embryology-based studies.

Spiny mice in are particularly susceptible to investigator-induced stress which may alter the length of their menstrual cycle (unpublished observation, see Chapters 2 and 8), therefore additional groups commencing lavages from D3 were investigated. Females showed an increase in the length of the menstrual phase compared to our controls in most methods of induction, irrespective of D0 or D3 lavaging, and also failed to show a dose-response relationship with progesterone treatment. Excessive handling may be a contributing factor, as there is some evidence in women for a role of stress in increased menstrual bleeding, although amenorrhea is typically more common in menstruating species (47). In this context, prolonged menstrual bleeding observed may potentially be induced ovulatory dysfunctional uterine bleeding (48) as a result of direct (progesterone) or indirect (sterile mating, mechanical stimulation) disruption to the hypothalamic-pituitary-ovarian axis.

At present the evolution of the unique and unusual mode of reproduction in the spiny mouse largely remains a mystery. The results of this research into pseudopregnancy provide yet another example of how atypical spiny mouse reproduction is to other rodents and emphasise again their similarities to human reproduction, in that commonly used methods of pseudopregnancy induction in other rodent species such as sterile mating, mechanical stimulation and single progesterone injections, have no impact on the follicular or luteal

phase of the spiny mouse menstrual cycle. In this regard, the spiny mouse continues to demonstrate surprising attributes that link it more closely to primate, rather than rodent, reproduction.

CHAPTER 8:

GENERAL DISCUSSION

8.1 Overall Findings and Significance

8.1.1 Unbelievable… Literally

After the realisation that the blood I observed during vaginal lavage of the spiny mice was not in fact due to injury, I put my hypothesis forward to my (at the time) research group. My previous supervisor, unsurprisingly, did not believe the spiny mice were having a menstrual period. It isn't difficult to understand why, given dozens of scientists, including students, research assistants, post-doctoral researchers and the head of the laboratory herself, had worked with the spiny mice for nearly a decade and the accepted dogma; rodents don't menstruate. How could no one have seen it before? The answer is quite simple: nobody looked for something that (supposedly) did not exist. Menstruation in a rodent was unheard of, and therefore any sightings of unexplained blood in female cages were thought to have arisen from fighting wounds. Generally, menstrual blood that is heavy enough to leave traces in the cage would be in very small amounts on the sawdust bedding or the cardboard enrichment. I then questioned a) how could it be assumed these were fight wounds if no actual wounds were identified and b) didn't it seem odd that there were only small amounts of blood, localised to the bottom of the cage when typically, fight wounds severe enough to draw blood would also stain the sides and wire tops of the cage? A reasonable response ensued; spiny mice have scar free wound healing, therefore the wound had closed before a researcher could find it. Once again, this didn't quite add up. Fresh blood is easily recognisable, and despite the spiny mouse's ability to self-heal, it wasn't instantaneous. So, I persisted. And here we are.

8.1.2 Challenging Dogma and Breaking the Status Quo

As discussed in Chapter 1, vaginal lavages have been previously conducted on captive *Acomys cahirinus* (49). Peitz (1981) did report an active luteal phase with a decidual reaction maintained 24h after uterine trauma (through endometrial scratching with a needle around the ampulla) applied during diestrus. More interestingly, the investigator states: "Decidual tissue examined on the 7th day of diestrus showed some pycnotic cells and there was bleeding into the lumen of the uterus…Red blood cells were seen in vaginal smears at the end of diestrus in 3 of the 5 animals with uterine trauma." So, it is very possible that Peitz was in fact observing a natural decidual reaction and sloughing of necrotic endometrium is some of her animals, but rather than describing the bleeding as a menstrual response she instead linked it to the endometrial scratch, because of course rodents don't menstruate. This clearly begs the question of whether other users of captive spiny mice have also observed such menstrual-like anomalies in vaginal smears and disregarded the observation due to the accepted belief that rodents have oestrous cycles.

8.1.3 Menstruating Mouse or Pocket Primate?

The ability of our spiny mouse colony to menstruate continually throughout the year, rather than a singular event (50) or following pseudopregnancy (51) renders it unlike any other rodent. Indeed, a distinctive spontaneous decidual reaction and vascular remodelling during the late secretory phase mirroring that observed in humans affirms the rather surprising extent of how primate-like is this "mouse". Certainly, initial interest in obtaining spiny mice for perinatal research in our laboratory was founded on their eccentric, human-like qualities, particularly their developmental profile *in utero* (see Chapter 1 for details).

Previous genetic analysis conducted on the Cairo spiny mouse (the same species as our own colony) implies a closer phylogenetic relationship to the Mongolian gerbil (*Meriones unguiculatus*) than to the mouse (*Mus musculus*), suggesting that the spiny mouse should perhaps be considered a member of the Gerbellinae rather than the Murinae sub-family to which mice and rats belong (52). This, combined with their abilities to synthesis the steroid hormones cortisol and DHEA and menstruate, characteristics never-before seen in any other rodent, provides reasons to consider re-classification of *Acomys cahirinus,* first suggested by Chevret et al. (1993), or even to rename the animal itself to break the distant ties with Old world mice completely.

8.2 Limitations for Menstruation Research

First and foremost, the spiny mouse, regardless of its primate-like menstruation detailed in Chapters 3-5, is a rodent and because of this there are substantial implications for experimental design and cost efficiency in research using this species. Namely, the effect of prolonged studies and the potential influence on the menstrual cycle need to be meticulously considered prior to data collection, analysis and interpretation. The history in our laboratory and my own personal experience has emphasised the impact of stress on the reproduction in these animals. For example, with daily vaginal lavage, stress from investigator handling may result in as much as 35% of females presenting with anestrus (non-cycling) cytology after >20 days of lavage alone, or >14 days of lavage plus other manipulation where extended handling is required (e.g.: semi-quantitative behavioural testing). Timelines of experiments and the intrusive nature of some research protocols initially did not account for this and some females in our earlier investigations therefore had to be excluded from studies or allowed a

minimum of 14 days rest before continuing with vaginal lavages to provide relatively stress-free cycle time to recuperate.

The impact of stress on the regularity of women's menstrual cycles is well established and stress may, depending on the type and/or severity of the stressor, suppress the HPG axis resulting in functional hypothalamic amenorrhea (47, 53, 54). Stressful experiments with multiple investigators or prolonged handling may have contributed to a lack of observation and missed identification of menses in spiny mice in our colony. My observations on the sensitivity of the spiny mouse menstrual cycle to stress suggests it will be a useful future model for studying the neuroendocrinology of exogenous stress and its impact on menstruation.

In addition to behavioural considerations, the anatomy of the spiny mouse reproductive system corresponds to that expected in rodents; it retains two cervices, uterine horns and oviducts and each ovary is enclosed in a bursal membrane. This may explain why I have not observed any natural signs of endometriosis in the spiny mouse. Retrograde menstruation may be inhibited or limited by the presence of two cervical barriers. It may also be less powerful because menstrual debris is isolated in two separate horns rather than one fundal body and is contained within the uterus, and perhaps the oviducts, where access to the peritoneal cavity is blocked by the ovarian bursae.

The current lack of research colonies has also restricted the growth in users of the spiny mouse. In particular, our captive colony in Australia is currently the only research colony in the Southern hemisphere. Established from 5 breeding pairs in 2002, imported from a colony at the University of Amsterdam (originally established in 1981 from 4 breeder pairs from the University of Geneva) it has clearly gone through at least two bottlenecks and detailed

genetic studies are likely to show that the colony has a high inbreeding coefficient, like many of the standard laboratory mouse strains. To our knowledge and based on collaborative responses from colleagues in the United States, menstruation is unique to our spiny mice. There are notable differences between these two colonies, other than geographical location of the laboratories. The United States colony has females of greater body weight than ours, and weight may be a contributing factor to amenorrhea, as discussed in Chapter 4. These animals are also kept on a natural, rather than artificially timed, light cycle, allowing the colony to experience the effects of seasonal changes in light and temperature unlike our colony which is under controlled temperature year-round and a 12/12 light/dark cycle. Finally, the housing conditions may be more stressful for the spiny mouse in the United States as, through personal communication with colleagues who have visited and handled these other animals, the spiny mice have their tails bitten off during adolescence by cage mates. In our colony, cage fighting is a very prominent cause of stress and acyclicity among our females. Despite these differences in husbandry, it is unclear whether frank menses in our females is a by-product of random selection of few individuals during breeding colony establishment (founder effect), and whether wild types may also exhibit this reproductive trait to some degree. It's possible that the wildtype spiny mice due to greater nutritional availability (as they are a moist desert species) and preference for nesting in rocky, sheltered areas which could have seen a reduction in predation and greater safety for gestating dams and neonates, could have selected for greater placental investment in offspring during pregnancy, and thus a genetic mutation resulting in spontaneous decidualisation may have evolved. As discussed in depth in Chapter 1, menstruation is thought to have evolved as a

side effect of spontaneous decidualisation. Selective breeding of heavily menstruating females and environmental vs. genetic input require further analysis.

8.3 Future Directions

This discovery of a menstruating rodent holds exciting prospects for the future of menstrual research. The presence of spiral artery remodelling in the spiny mouse encourages continued scrutiny as to their purpose during early gestation. Although late placentation has been described previously in this species with pregnant dams exhibiting haematrichorial placenta as conventional mice (24), there is a necessity to explore embryo attachment, early implantation (interstitial as primates or eccentric as rodents) and importantly, depth of trophoblast invasion, as this is integral in the theory of menstrual evolution (51, 55).

Menopause in the spiny mouse requires further examination, with blood samples collected from females ranging from 6 months to 42 months for steroid hormone and gonadotrophin analysis. Previous work with the spiny mouse has proven difficult to measure oestrogens, FSH, AMH and LH in plasma and serum samples, with conflicting results from radioimmunoassay, and little funding to allow complete validation of commercially available ELISA methods. Optimal methods for measurement likely require high sensitivity, such as mass spectrometry or HPLC. Stereological analysis of the ovarian reserve using tissues I collected from young and aged spiny mice is currently being performed by another student, as part of a PhD, and the data will likely reflect the histological changes described in Chapter 6. If so, the spiny mouse could serve to replace conventional laboratory rodents as a more appropriate model of menopause, and aid in our understanding of premature menopause,

development of treatments for associated symptoms of oestrogen decline, and possibly premature ovarian failure.

Correlations between heavy menstrual bleeding and both behavioural and physiological symptoms have yet to be examined, nor has the genetic influence on menstrual bleeding been explored in the spiny mouse. Here lies potential for selective breeding of varying degrees of menstruation or PMS symptoms, enabling further understanding of how menstruation can impact quality of life and allow thorough examination of the changes to underlying pathology.

8.4 Conclusions

This thesis demonstrates a direct contradiction to a long-held, adamant perception that rodents do not naturally menstruate. I have provided evidence to the contrary, with the initial discovery of a spontaneous decidual response and subsequent menstrual shedding in the common spiny mouse. The spiny mouse is also a distinctive species, in both its physiological and behavioural likeness to primates but its anatomical correspondence to rodents.

The data and interpretations presented within this thesis stem from a singular observation, and branch into detailed examination of a potentially overlooked asset to the field of women's reproductive health. The spiny mouse not only retains the ability to shed differentiated endometrium, but also exhibits corresponding mood and appetite fluctuations across the menstrual cycle and a gradual decline in fecundity spanning several years. None of these observations have previously been made in a rodent species.

To go forward, it may now be necessary to go backward, and revaluate the conclusions drawn from previous studies knowing what we know now about menstruation in the spiny mouse.

This will be particularly important for behavioural studies. The effect of poly I:C on pups exposed during pregnancy pooled male and female data of exploration using OF, NORT and EPM behavioural tests (presented in Chapter 5), for example, uses females 9 weeks of age (63 days) (56). While we have not confirmed the age of menarche in the spiny mouse, we do know that vaginal opening occurs between at ~55 days (27). Hence, menstrual onset and stage of the menstrual cycle may be confounding factors. Additionally, a study examining the effects of dietary creatine supplementation on pregnant spiny mice uses virgin females as controls for metabolic cage data extraction (2) – another procedure I have shown to demonstrate significant changes in across varying stages of the menstrual cycle (Chapter 5). Stage of menstrual cycle will need to be controlled for in future experimental design, or at least, be factored in to analyses and interpretation.

The importance of this data cannot be overstated. We are now fortunate enough to have a non-primate, convenient and economic small animal model which, if implemented worldwide, will provide pivotal clues to unlocking the long-held secrets of impaired uterine function, abnormal bleeding and pregnancy-associated disorders. Armed with this new evidence and a novel small animal model of menstruation, the spiny mouse has the capacity to reduce the invasiveness of experimental procedures and drug trials for women, vastly improving the speed of pharmaceutical developments, and lead the field of menstruation into a new era.

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APPENDIX

Stock Solutions for immunohistochemistry

PBS (1L Stock Phosphate Buffered Saline)

80g of NaCl (Sodium Chloride)

2g of KCl (Potassium Chloride)

14.4g of Na_2HPO_4 anhydrous (Disodium Phosphate)

2.4g of KH_2PO_4 (Monopotassium Phosphate)

1. dissolve all in 800 mL deionised water (dH_2O)

2. Adjust pH to 7.4 with either HCL (hydrochloric acid) or NaOH (Sodium Hydroxide)

3. Add dH_2O until 1L mark

4. To use, dilute 1:10 with dH_2O

TBS (1L Stock Tris-Buffered Saline)

24.2g Trizma Base ($C_4H_{11}NO_5$)

80g NaCl

1. Dissolve in 800 mL dH_2O
2. Adjust pH to 7.6 with HCl
3. Make up to 1L with dH_2O
4. To use, dilute 1:10 with dH_2O

TBS + Tween20 (0.002% TBS-Tween)

1. Follow above protocol and add 2mL Tween20 to 1L stock solution
2. For use, dilute 1:10 (wash buffer = 0.0002%)

Citrate Buffer

Citric Acid

- Dissolve 4.2g Citric Acid in 20mL dH_2O

1M Citrate Solution

- Dissolve 2.94g of Tris-sodium citrate in 800mL dH_2O
- Adjust to pH 6.0 with citric acid
- Add dH_2O to 1L mark

Tris-EDTA

1.21g Trizma Base

0.37g EDTA

- Dissolve trizma base and EDTA in 800 mL dH_20.
- Adjust to pH 9.0
- Add 0.5 mL Tween20

Glycine Block

- Dissolve 7.5g glycine in 800 ml dH_2O
- Adjust pH to 2.7 and bring volume to 1 L with dH_2O.

Serum Blocking Solutions:

E.g. 10% NGS = 1 mL normal goat serum in 9 mL wash buffer specific to protocol i.e. if protocol requires TBS, use 9 mL TBS.