MONASH University

Early Pregnancy and Assisted Reproduction in the Spiny Mouse, *Acomys cahirinus*

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BSc, GradDipRepSci

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This one's for me.

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Summary

The Egyptian spiny mouse (*Acomys cahirinus*), a small rodent species native to northern Africa and the middle east, has recently been shown to exhibit true menstruation; the first of any rodent species. I consider from this, and some other human-like characteristics, that this species will be a more appropriate small animal model for human reproductive studies than current models. However, while the later stages of pregnancy have been investigated in this species, the events leading up to pregnancy including mating behaviour, ovulation, embryogenesis and embryo implantation have not yet been characterized. Additionally, a paucity of information regarding assisted reproductive techniques (ARTs) is available in spiny mice and there is therefore a need to develop specific laboratory-based techniques to make this model more relevant.

I have conducted four studies during my candidature to add to our understanding of spiny mouse reproductive biology. Firstly, I describe the effect of menstrual cycle stage on virgin spiny mouse mating behaviour and pregnancy and provided a reliable and more effective protocol for estimating gestational age without the need for postpartum dams. I have also investigated the novel finding of postpartum ovulation (PPO) in the menstruating spiny mouse and successfully characterised the postpartum changes in endocrinology, uterine and ovarian morphology and uterine receptivity to embryo implantation. Lastly, I have defined a protocol for cryopreservation of mature spiny mouse epididymal spermatozoa and outlined a human-based superovulation protocol for the collection of mature spiny mouse oocytes and two cell embryos.

This thesis further highlights the unique reproductive physiology of spiny mice and their role as a novel, small animal model of female reproductive function. My project has

increased our understanding of spiny mouse reproductive biology by conducting an in-depth analysis of mating behaviour and postpartum reproductive tissues to define more clearly spiny mouse ovulation, embryo implantation and early pregnancy, whilst providing the ARTs and protocols for future reproductive research in *A. cahirinus*.

Declaration

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.

Signature: 

Print Name: Jarrod McKenna

Date: 18/1/2021

Thesis including published works

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 2 original papers published in peer reviewed journals and *2* submitted publications. The core theme of the thesis is reproductive biology. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the department of Obstetrics & Gynaecology under the supervision of A/Prof Peter Temple-Smith, Dr. Sally Catt, Dr. Mulyoto Pangestu and Dr. Nadia Bellofiore.

(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 *chapters 2, 3, 4 and 5*, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Student contribution percentage	Co-author's name(s) and % contribution of co-authors
2	Good things come to those who mate: Analysis of the mating behaviour in the menstruating rodent, Acomys cahirinus	Manuscript submitted (under review)	85% Hypothesis formulation, study design, animal/lab work, data analysis, manuscript formulation	1. Peter Temple-Smith (10%) study design and manuscript editing 2. Nadia Bellofiore (5%) manuscript editing and technical training
3	Postpartum ovulation and early pregnancy in the spiny mouse, *Acomys cahirinus*	Manuscript submitted (under review)	75% Hypothesis formulation, study design, animal/lab work, data analysis, manuscript formulation	1. Nadia Bellofiore (10%) study design, data collection/analysis, manuscript formulation 2. Eva Dimitriadis (5%) study design and manuscript editing 3. Peter Temple-Smith (10%) study design

				and manuscript editing
4	Technical report: Successful sperm cryopreservation in Egyptian spiny mice (Acomys cahirinus)	Published manuscript	80% Hypothesis formulation, study design, animal/lab work, data analysis, manuscript formulation	1. Peter Temple-Smith (10%) study design and manuscript editing 2. Sally Catt (5%) study design and manuscript editing 3. Mulyoto Pangestu (5%) study design and manuscript editing
5	A human-based assisted reproduction protocol for the menstruating spiny mouse, *Acomys cahirinus*	Published manuscript	40% Hypothesis formulation, study design, manuscript formulation	1. Nadia Bellofiore (40%) Hypothesis formulation, study design, manuscript formulation 2. Peter Temple-Smith (10%) study design and manuscript editing 3. Sally Catt (5%) study design

				and manuscript editing 4. Mulyoto Pangestu (5%) study design and manuscript editing

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

Student signature: [redacted] **Date:** 18/1/2021

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.

[redacted]

Main Supervisor signature: **Date:** 18/1/2021

Conference proceedings and participation

2020

Mckenna J, "Reproductive biology in captive environments. What can we learn?" 2020 Wildlife Appreciation Group, **Online Oral**

Mckenna J, Catt S, Pangestu P, Temple-Smith P "Cryopreservation of Spermatozoa in the Spiny Mouse, Acomys Cahirinus" 2020 International Congress on Animal Reproduction, Bologna, Italy, **Oral ***

2018

Mckenna J, Catt S, Pangestu P, Temple-Smith P "Sperm Cryopreservation in the Spiny Mouse, Acomys cahirinus" 2018 Scientists in Reproductive Technologies, Hunter Valley, NSW, Australia **Oral**

Mckenna J, Catt S, Pangestu P, Temple-Smith P "Cryopreservation of Spiny Mouse Epididymal Spermatozoa" 2018 Cryobiology, Madrid, Spain

Oral

2017

Mckenna J, Catt S, Pangestu P, Temple-Smith P "Gene-banking and the role of gamete cryopreservation in species conservation" 2017 Australasian Wlidlife Management Society, Blue Mountains, NSW, Australia, **Oral**

*

Conference cancelled due to COVID-19 global pandemic

Acknowledgements

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

For as long as I can remember, I've wanted to become a veterinarian. When I didn't get into any vet course in the country, I vividly remember talking to a university councillor about my options (Vet nursing, ecology, zoology), most of which I scoffed at. It was vet or nothing in my naïve, 20-year-old eyes. Nevertheless, I enrolled in a Bachelor of Science at the University of Melbourne, where, in my final year, I came across a field I would eventually fall in love with: Reproductive biology. Speaking more with the lecturers about repro-bio, the proposal of starting an honours or masters, instead of applying for vet again, seemed ridiculous. Talking my parents, we joked – "Wow, not many people would want to dedicate their life to research! I can't imagine you doing that…"

…Oops

Here I am, writing a thesis about a menstruating mouse and *in-vitro* fertilisation; not exactly what 20-year-old Jarrod had in mind. While it wasn't my first choice, I'm so glad I've done it. You can read all about what doing a PhD is like, but nobody can truly prepare you for it. Sure, I knew I'd come away with some fancy science skills and know-how, but I didn't realise the pressures I'd be put under. Experiments not working, imposter syndrome, research during a global pandemic, manuscript rejections and redrafting work you spent months/years building. A PhD is a true test of your mental stamina and boy am I tired.

The resolve and mental strength I've developed during my PhD is one of the most important thing I can take away from my candidature, and for that, I have so many people to thank.

To my **supervisor Peter**, there are not many people I hold in higher regard than you. You are the one who convinced me to do a PhD and opened my eyes to the possibility of becoming a conservationist-embryologist (as niche as it is). The amount of time and effort you put into every student (and every animal for that matter) is incredible. Whether that's sending me an edited manuscript at 2am, buying a $70 cheesecake and bottle of Mumm for passing a PhD review, or letting your (me), completely (not) sober, student bludgeon their way through Proud Mary at the Christmas party, it's all so appreciated, and it doesn't go unnoticed. You breathe encouragement and support and it's admirable.

To my **supervisor Sally**, who helped ignite my passion for embryology. I'm so grateful for your calm and cheerful approach to work, and life in general! I never imagined I would meet someone who swam the English Channel, let alone that person also teach me how to do IVF. It's a testament to your mental and physical strength and it rubs off on your students, which I will be forever grateful for.

To my **supervisor Mulyoto**, the man with the best laugh in Clayton. Your constant upbeat, never-say-die attitude is so contagious. Having a bad day? Go see Mulyoto. Your experiments didn't work? Mulyoto will know why. How do you slow-cook beef tongue? Mulyoto has got the recipe. Working with you the past few years has made all the failings, or tough times during my PhD so much more bearable. You're not just fun, but so incredibly knowledgeable and you've helped out in so many different aspects of my PhD, whether you intended to or not.

To my **supervisor Nadia**, or 'buddeh': The person who brought the spiny mouse into my life - I haven't decided if I'm happy about that or not yet. You've been my big sister, post-doc, supervisor and friend all in the span of 3 years. You've been through everything I was going through and got out of it unscathed (or maybe a little bit scathed). Subtle family guy and south park jokes here and there kept me sane in the dark times and you've taught me so much about perseverance, patience and immunohistochemistry – the latter also being something I haven't decided if I'm happy about or not. I can't thank you enough for your help the past few years and hopefully we'll both end up as a dynamic duo vet team helping all the Billies.

To **my PhD buddies**, Molly and Nicola. Our friendship is probably the best thing to come out of my PhD and I genuinely would not have been able to cope or get anywhere close to submitting a thesis without you by my side. PhD's are a mental marathon and having people to lean on, complain with, rejoice with make the hard times easier and the good times great. Crisis croissants, baked goods, the occasional dress up and many, many drinks, we've found ways to cope together and get through it together. For that, I'm forever grateful for your friendship: Long live the gang!

To the **MCE crew (Penny, Duc and Emma)**, the supervisors that weren't! I have a vivid memory of all 4 of us in the lab with 4 oviducts each searching for COCs about 15 minutes before an MCE prac was about to begin. Unwavering loyalty and support for the EPRD students is a trait you all possess and it's what makes the teaching at EPRD so amazing. All of you have been so incredibly helpful and I think you all so much.

To **Angela Vais** and MHTP Histology, my unofficial supervisors! I can't express how helpful you all have been from showing me basic embedding and sectioning techniques or committing two full days to teaching me immunohistochemistry whilst I show you photos of my 100 pets as payment. Thank you all so much for your dedication and patience with me;

I know I asked a lot, but you gave me nothing but positivity and support, and I can't thank you enough.

To my partner **Ash**, you've been there through the best and worst times of my PhD and given nothing but support and encouragement. Sitting through my lectures/rants about the peer-review process, sperm cryopreservation or how embryos implant probably (and rightfully) flew over your head, but you were there nonetheless and provided the loving support I needed.

To the best boy **Gandalf, and the cherished members of my home zoo: Kyra, Willow and Lola.** Thank you so much for your loving energy, night-time cuddles and belly rubs when I needed them most and for bringing me joy every single day.

To **my parents and my brother Brad**, thank you all for constantly believing in me and giving me the encouragement that I needed to keep going. **Brad**, you've been through it all before, and it was extremely helpful having a big brother to lean on in tough times and remind me that things do get better; you've just got to PUSH. **Mum and dad**, I promise I'll try and leave you alone now and finally become a real adult! Hopefully we can all find a beautiful villa in Italy where Brad and I can do some research whilst you two sit back, relax and finally enjoy your retirement.

List of abbreviations

~	Approximately
ºC	Degrees Celsius
=	Equals/equal to
>	Greater than
<	Less than
≥	Greater than equal to
≤	Less than equal to
-	Minus
+	Plus
±	Plus or minus
%	Percent
10R5SM	10% raffinose 5% skim milk
10R10SM	10% raffinose 10% skim milk
15R3SM	15% raffinose 3% skim milk
15R5SM	15% raffinose 5% skim milk
15R10SM	15% raffinose 10% skim milk
18R3SM	18% raffinose 3% skim milk

18R3SM+GLU	18% raffinose 3% skim milk 100mmol L-Glutamine
2C	Two-cell embryo
4C	Four-cell embryo
aSMA	Alpha smooth muscle actin
ADT	Androstenedione
AFs	Antral follicles
AMA	Advanced maternal age
ANOVA	Analysis of variance
AOA	Assisted oocyte activation
ARTs	Assisted reproductive techniques
AMH	Anti-Mullerian hormone
AB	Antibody
ASMA	Alpha smooth muscle actin
COC	Cumulus oocyte complex
COS	Controlled ovarian stimulation
CL	Corpus luteum
CLs	Corpora lutea
CPA	Cryoprotective agent

CYTO	Cytokeratin
DAB	3, 3′-Diaminobenzidine
DAPI	4’ ,6’ -diamidino-2- phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2	Oestradiol-17b
EG	Ethylene glycol
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
EJI	Ejaculation interval
EJF	Ejaculation frequency
EJL	Ejaculation latency
EM	Embryo
END	Endometrium
ET	Embryo transfer
FOV	Field of view
FOX01	Forkhead box 01
FSH	Follicle stimulating hormone

g	Gram(s)
GFP	Green fluorescent protein
GnRH	Gonadotrophin releasing hormone
GV	Germinal vesicle
H33342	Hoechst 33342
H&E	Hematoxylin and eosin
hCG	Human chorionic gonadotrophin
HMG	Human menopausal gonadotrophin
HPG axis	Hypothalamic pituitary gonadal axis
ICM	Inner cell mass
IF	Intromission frequency
IF	Immunofluorescence
IHC	Immunohistochemistry
IL	Intromission latency
IL-11	Interleukin 11
IL-11R	Interleukin 11 receptor
IVF	*In-vitro* fertilisation
IVC	*In-vitro* culture

JC-1	5,5' ,6,6' -tetrachloro- 1,1'3,3'-tetraethylbenzimi-dazolylcarbocyanine iodide
K-10	Kisspeptin-10
K-54	Kisspeptin-54
L	Litre(s)
LC-MS	Liquid chromatography mass spectrometry
LE	Luminal epithelium
LECs	Luminal epithelial cells
LH	Luteinizing hormone
LU	Uterine lumen
MI	Metaphase 1 oocyte
MII	Metaphase 2 oocyte
MII+B	Metaphase 2 + bulge oocyte
MMP	Mitochondrial membrane potential
mL	Millilitre(s)
ML	Mount latency
mRNA	Messenger ribonucleic acid
MYO	Myometrium
ng	Nanogram(s)

OHSS	Ovarian hyperstimulation syndrome
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween-20
pg	Picogram(s)
PI	Propidium iodide
PLC ζ	Phospholipase C ζ
PMSG	Pregnant mare serum gonadotrophin
PNA	Peanut agglutinin
PNA-FITC	Peanut agglutinin with fluorescein isothiocyanate
PPO	Postpartum ovulation
PGs	Prostaglandins
rFSH	Recombinant follicle stimulating hormone
RIA	Radioimmunoassay
P4	Progesterone
pp	Postpartum
PRL	Prolactin
PVA	Polyvinyl alcohol
RFP	Red fluorescent protein

ROS	Reactive oxygen species
SD	Spontaneous decidualisation
SUZI	Subzonal insemination
TFF	Total fertilisation failure
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween-20
TUNEL	Terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP– digoxigenin nick end-labelling
uFSH	Urinary follicle stimulating hormone
um	Micrometre(s)
VEGF	Vascular endothelial growth factor
VP	Vascular permeability
ZP	Zona pellucida

Chapter 1: Introduction and Literature Review

1.1 Introduction

Herein, I describe my investigation into the menstruating Egyptian spiny mouse (*Acomys cahirinus*) as a novel animal model of early pregnancy and assisted reproduction in humans. Features of menstrual species include cyclical ovulation, spontaneous decidualisation, aggressive trophoblast invasion and formation of a haemochorial placenta. While the later stages of pregnancy have been well studied in this species, events of early pregnancy including ovulation, embryogenesis and spiral artery remodelling have not been characterised. Until now, a species with true, human-like menstruation has not been reported to exhibit these features whilst also presenting with postpartum ovulation. Therefore, I believe, that this species will be a more appropriate small animal model for natural and assisted human reproduction than current models.

1.2 Overall research question and thesis aims

Being a comparatively recent laboratory species, limited information is available regarding early pregnancy and assisted reproduction in *A. cahirinus*. Thus, the purpose of this thesis is to provide the first comprehensive analysis and characterisation of the mating behaviour and postpartum reproductive tract, whilst defining novel ARTs to make the spiny mouse a more relevant and accessible model to biomedical research.

Therefore, this opening chapter explores the nature and impact of reproductive research in farm and laboratory species in advancing our understanding of natural and assisted human reproduction, whilst introducing the spiny mouse as a novel animal model.

1.3 Literature review

1.3.1 Introduction

Assisted reproductive techniques (ARTs) involve the manipulation of gametes *ex-vivo* to achieve fertilisation and subsequent development and transfer of embryos with the aim of achieving a pregnancy. The history of assisted reproduction is now more than 120 years old and began with the transfer of rabbit embryos (Heape, 1890). ARTs in humans have made great advances over this period, and over 8 million children have been born from ARTs globally (Fauser, 2019). Today, it is estimated that 8-12% of couples of reproductive age suffer from infertility and require various interventions and technologies to conceive (Veiga et al., 2020).

Much of the early, ground-breaking work on *in-vitro* fertilisation (IVF) and embryo culture was performed in rodents and cattle (Bavister, 1995; Schatten & Constantinescu, 2017). However, due to differences in reproductive physiology or complex colony and animal welfare requirements, the ideal laboratory animal model for human reproduction has yet to be identified. Even great apes, our closest relatives, are a suboptimal option, largely due to the complexities of their animal welfare requirements, high running costs and logistical challenges in using them for experimentation (Schatten & Constantinescu, 2017). Although great advances have been made in understanding human reproduction due to the work in animals, various aspects are still poorly understood. In particular, ovarian folliculogenesis, mechanisms of embryo implantation, and fetal and placental development. This research would benefit from a suitable menstruating laboratory animal model similar to humans, but as most mammals present with an oestrous cycle, including the widely used laboratory mouse, this has been a challenge until recently.

Menstruation occurs in <2% of mammals and is primarily found in higher-order primates such as gorillas and orang-utans (Emera et al., 2012). The Egyptian spiny mouse (*Acomys cahirinus*), native to the deserts of Northern Africa, is a rodent species which exhibits several human-like reproductive characteristics that are not found in most other rodents. These include precocial young (Brunjes, 1990), a relatively long gestational period (Dickinson & Walker, 2007) and, most recently, a menstrual cycle (Bellofiore et al., 2017); all important features as a model for translational studies in women's reproductive health . These unique reproductive traits in *A. cahirinus*, now an established, but rarely used, laboratory animal, places a strong focus on it as an important small animal model for studying human reproductive physiology and the development of ART.

This review will describe the use and impact of research undertaken in farm and laboratory species on natural and assisted human reproduction with the focus on folliculogenesis, controlled ovarian stimulation (COS), IVF and embryo implantation. Lastly, a detailed review of literature describing the reproductive biology of *A. cahirinus* will be presented and the potential role of the spiny mouse as a novel animal model of natural and assisted human reproduction will be discussed.

1.3.2 Menstrual and oestrus cycles

Among mammals, the complex, cyclical process menstruation is an exceptionally rare reproductive strategy that is found almost exclusively in humans and higher-order primates. Common to both oestrus and menstruating species are the structural and physiological changes to the reproductive tract in preparation for embryo implantation and pregnancy. However, key events regulating menstruation do not occur in oestrous species. Most importantly, the spontaneous, terminal differentiation of endometrial stromal cells (spontaneous decidualisation; SD) (Gellersen & Brosens, 2014) and the shedding, rather than reabsorption, of the endometrial lining in the absence of pregnancy (menses) occurs uniquely in menstruating species.

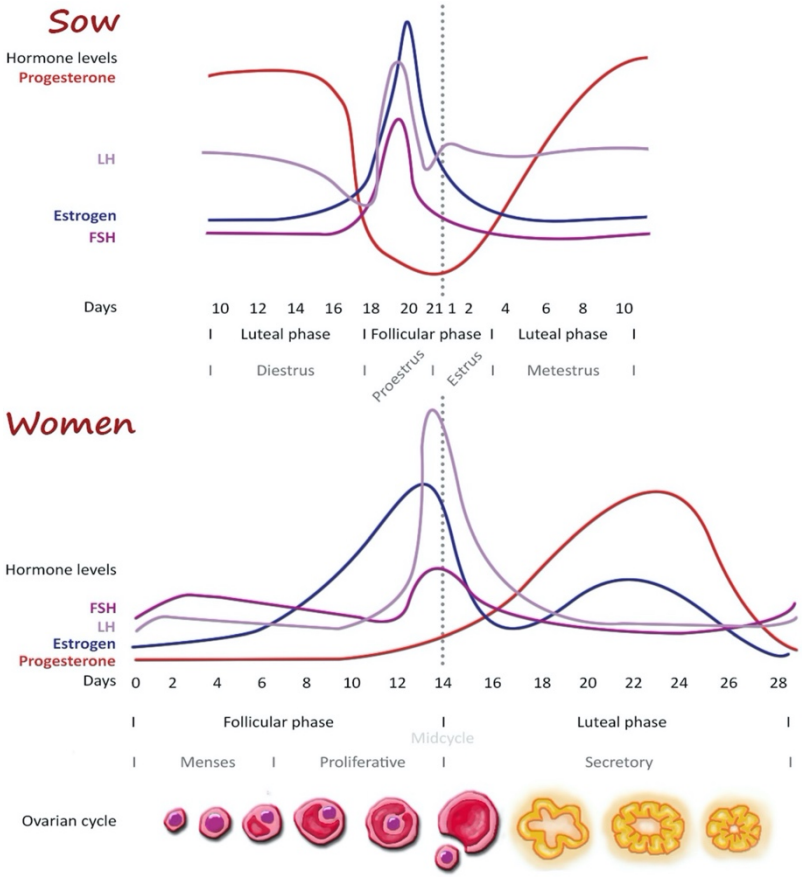

Figure 1 Comparative reproductive cycles in mammals. *Both oestrus (sow) and menstrual (women) cycles exhibit a period of endometrial proliferation governed by ovarian oestrogens followed by a secretory phase controlled by progesterone secretion from functional corpora lutea. In the absence of pregnancy, the endometrium is shed in menstrual species (menses). Image sourced from (Lorenzen et al., 2015).*

Although the underlying endocrinology of the female reproduction cycle is conserved in oestrus and menstruating species, the regulation and temporal release of ovarian and pituitary hormones varies (Figure 1; (Lorenzen et al., 2015)). Both reproductive cycles experience simultaneous periods of follicular growth and stromal cell proliferation, followed

by ovulation and formation of a corpus luteum (CL) or corpora lutea (CLs). However, in menstruating species, both ovulation and the decidual reaction occur spontaneously in response to a surge of luteinizing hormone (LH). Contrastingly, ovulation in the oestrous cycle is generally triggered by external factors such as copulation, photoperiod and/or nutrition, and the decidual reaction occurs only in response to an implanting blastocyst (Downey, 1980).

Functional studies of menstruating females are restricted to women and the great apes, which present ethical, financial and practical challenges with experimentation. In an effort to overcome these issues, mice have been artificially induced to exhibit menstrual-like events under specific conditions (Brasted et al., 2003; Rudolph et al., 2012). Although these models have unveiled important characteristics of menstruation such as gene regulation and the role of steroidogenic input (reviewed by (Bellofiore, Cousins, et al., 2018)) they lack the true, causal mechanisms regulating SD and menstruation. In order to study and manage human reproductive function more effectively, a more appropriate, animal model of female reproduction is required. However, despite the difference in reproductive strategy, this does not discredit oestrous species as models for human reproduction entirely. Oestrus animals have explained many crucial aspects of human reproduction and our extensive understanding of folliculogenesis can be attributed to the large volume of work conducted in farm and laboratory species over the past century.

1.3.3 Folliculogenesis

In the mammalian ovary, follicular growth is a continuous process in which the follicle, and the oocyte within, undergo several key developmental milestones and processes to acquire the capacity for fertilisation (Campbell et al., 2003). Research in humans is

hampered by the restricted use and access to reproductive tissues due to ethical and legal restraints. However, the exploration of genetic traits and diseases using rodent and rabbit models and the increased demand for cattle and sheep breeding technologies simultaneously led to rapid elucidation of many aspects of mammalian reproduction previously unstudied. In the last century, a vast understanding of folliculogenesis and ovarian function has been gained from farm and laboratory species (Schatten & Constantinescu, 2017).Timing of follicle development, influence of pituitary and ovarian hormones as well as environmental factors on follicle growth and ovulation have been well documented and subsequently investigated in human tissues (Blanc et al., 2001; Schatten & Constantinescu, 2017).

However, substantial biological and physiological differences exist between humans and model species (Taft, 2008). Therefore, this section will review the historical findings of cattle, sheep, rodent, rabbit and non-human primate folliculogenesis and ovarian function and compare their similarities to the human events. Specifically, the endocrine control of follicle development, recruitment of follicles from the ovarian reserve, and environmental influences of reproductive function will be discussed.

Endocrine control of folliculogenesis

Many of the key aspects of mammalian folliculogenesis, particularly endocrine control of the ovary by both ovarian and pituitary secretions were uncovered in animal species (Schatten & Constantinescu, 2017). Two seminal papers in the early 20th century highlighted the effect of ovarian estrogens (Corner, 1938) and pituitary gonadotrophins (Evans et al., 1941) on ovarian function in rodents; the latter reporting both follicular growth and ovary luteinization following injection with pituitary extracts from sheep and

rats. Interestingly, the control and release of ovarian steroids showed a similar pattern in menstrual (Clark et al., 1979; Knobil, 1974) and oestrous species (Baird, 1978; Hori et al., 1968; Setty & Mills, 1987; Wettemann et al., 1972) (Figure 2). In all species, early folliculogenesis was shown to be largely influenced by FSH secretion and, as the follicles mature, estrogen levels rise. During the later stages of growth, however, this period of increased estrogen secretion feeds back on the pituitary to simultaneously decrease the release of FSH whilst increasing the release of LH (Bäckström et al., 1982; Baird, 1978; Knobil, 1974; Spies et al., 1969). Despite similarities during the follicular phase, marked differences in ovarian function during the luteal phase were observed between domestic and primate species.

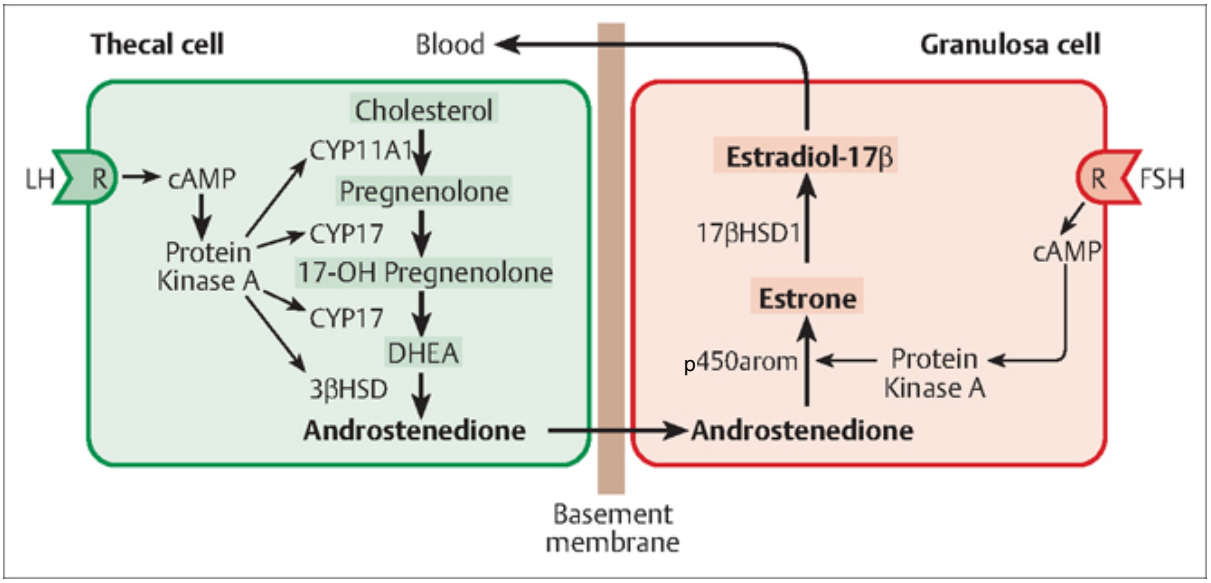

***Figure 2 Schematic displaying the secretions of the two cells of the follicle**: Theca cells (green, left) and granulosa cells (red, right). LH stimulates theca cells to produce androgens (notably androstenedione; ADT), which are then transported across the basement membrane to granulosa cells. Under the influence of FSH, granulosa cells produce estrogens (notably estradiol-17β) from ADT which are then secreted into the blood. Image sourced from (Wiznitzer, 2016)*

During the luteal phase in ewes (Baird, 1983) and heifers (Ireland et al., 1979), in contrast to primates (Dizerega & Hodgen, 1980), development of antral follicles continues. The relatively unchanged FSH and estrogen concentrations observed during the luteal phase in sheep and cattle highlighted the different responses in the hypothalamic-pituitary-

gonadal (HPG) axis compared to that of primates. However, this is not a result of decreased sensitivity to gonadotrophins in the primate ovary but rather due to decreased circulating levels. In the early eighties, Dizerega and Hodgen (1980) demonstrated how follicular growth can be stimulated in the luteal phase of cynomolgus monkeys following injections of an FSH analogue (human menopausal gonadotrophin; hMG). Similar findings have also been reported in women during infertility treatment where poor responders to ovarian stimulation in the follicular phase have been shown to experience ovarian stimulation from an FSH analogue given during the luteal phase (Llácer et al., 2020; Zhang et al., 2018). Together, these data indicate a shared sensitivity to gonadotrophins between mammalian species, even during a period of natural follicular quiescence.

However, although follicular growth and endocrine control are broadly similar between species, the mechanisms governing follicular recruitment from the ovarian reserve in humans remains elusive.

Follicular recruitment and the ovarian reserve

In most mammalian species, females are born with all the primordial follicles they will have in their lifetime (the ovarian reserve). Recruitment of these follicles and future growth generally occurs during postnatal life (Oktem & Oktay, 2008) and the total time from follicular activation to ovulation is similar in cows, ewes and women (roughly 6 months) (Cahill & Mauleon, 1980; Lussier et al., 1987; Trounson et al., 2013). However, experiments in sheep (Dufour et al., 1979), hamsters (Moore & Greenwald, 1974) and rabbits (Hutt et al., 2006) indicate that, unlike later stage follicles, primordial follicle activation occurs independently of gonadotrophins and occurs prior to sexual maturity.

Interestingly, follicles of several different sizes are seen in the prepubescent ovary of women (Peters et al., 1975) and farm species (cattle, sheep and goats) (Rawlings et al., 2003). The discovery of anti-mullerian hormone (AMH) mRNA in granulosa cells of primary and pre-antral cow (Vigier et al., 1984) and rat (Baarends et al., 1995) follicles directed researchers towards its potential role in the human ovary. Weenen et al. (2004) later demonstrated that the functional role of AMH in stimulating follicular recruitment in the human ovary followed a similar pattern to that of other mammals (mice, cows, rats). More recently, the interactions between estrogen, AMH and FSH in the human ovary have been described (Dewailly et al., 2016) and the role of AMH as a biomarker for menopause has been proposed (Barad et al., 2016; Long et al., 2018). However, our understanding of factors regulating AMH expression and the initial recruitment of follicles from the ovarian reserve in the healthy or pathological mammalian ovary is limited (Moolhuijsen & Visser, 2020) and research in this area could benefit from appropriate, small animal models of ovarian function.

Overall, like later stage follicular growth, the endocrine control of early follicular growth in humans is broadly similar to other mammalian species. However, as mentioned earlier in this review, the oestrus cycle is largely influenced by environmental factors such as photoperiod and food intake; effects that are far less pronounced in the primate menstrual cycle.

Environmental influences of reproductive function

The functional ovary relies on several complex mechanisms regulating follicular activation and growth and the coordination of these mechanisms is generally well conserved in mammals. Crucially, however, the reproductive cycle of oestrus species (e.g cattle, sheep

and rodents) can be influenced by external or environmental factors including nutrition, photoperiod and copulation (Bellofiore, Cousins, et al., 2018).

Richards et al. (1989) described how a decreased food intake of 24% can induce nutritional anoestrus in cows. Diet restricted cows had reduced luteal activity as measured by the concentration and pulse frequency of LH. Additionally, increased nutrient intake after restrictions resulted in resumption of oestrus cycles and normal pregnancy rates. A follow up study by Rhodes et al. (1995) reported similar findings in heifers with a reduced food intake by 17% and a similar response to decreased food intake has been reported in several different breeds of ewe (Forcada & Abecia, 2006). Although underweight women are known to experience amenorrhea (Frisch & McArthur, 1974; Köpp et al., 1997), weight loss in women is often a more complex issue influenced by a variety of factors such as exercise and mental or physical stress, rather than an absolute reduction in food intake (Meczekalski et al., 2014; Stokić et al., 2005).

In species where daylight or photoperiod can regulate reproduction, melatonin secretion and the pineal gland are particularly important (Downey, 1980; Lincoln, 1992). Melatonin is secreted in low-light periods from the mammalian pineal gland and is known to affect the neuroendocrine (Reiter, 1991) and reproductive axes (Shi et al., 2013). As daylight increases during the breeding season, there is less downregulation of the pituitary gland by melatonin and, therefore, greater gonadotrophin secretion and a more functional ovary (Lincoln, 1992). To this end, more antral follicles are present in ewe ovaries during periods of increased light (Cahill, 1981). This also means a greater number of follicles can be ovulated naturally, or in response to hormone treatment than during the non-breeding season. Perhaps not surprisingly, the bovine reproductive cycle is also mediated by

photoperiod (De Rensis & Scaramuzzi, 2003) and mating is generally restricted to a specified breeding season (Downey, 1980; Zeron et al., 2001). The effect of photoperiod on pregnancy outcomes in humans have also been investigated. Liu et al. (2019) conducted a retrospective cohort study evaluating the effect of seasonality on birth outcomes following fertility treatment and reported no difference in pregnancy or birth rates across any season; providing further evidence for the lack of photoperiod influences of the human menstrual cycle.

In rodents and rabbits, the situation is more complicated. Although these species are also influenced by factors such as nutrition and photoperiod (Bronson & Perrigo, 1987; Kamwanja & Hauser, 1983; Lintern-Moore & Everitt, 1978), laboratory colonies are housed under controlled conditions with set parameters such as temperature, day-night cycles and *ad libitum* access to food and water (Haughton et al., 2016; Smith & Corrow, 2005). The ability to easily manipulate environmental conditions regulating the rodent or rabbit reproductive cycle is thus a very clear advantage in their experimental use. However, rabbits (and some rodents) are induced ovulators. Ovulation in these species only occurs following copulation where an excitatory threshold during coitus stimulates the release of LH at sufficient amounts to induce ovulation (Downey, 1980). This endocrine response to copulation is not present in humans (or any other menstruating primate species). Instead, ovulation occurs spontaneously following a pre-ovulatory surge of LH (Bellofiore, Cousins, et al., 2018).Thus, although rodents and rabbits have been useful in describing the endocrine control of folliculogenesis, coordination of ovulation in spontaneous ovulators is a process not appropriately modelled in induced-ovulating species.

Menstruating primates make excellent biological and physiological models of human reproduction (reviewed by (Bellofiore, Cousins, et al., 2018)). However, because of their evolutionary proximity to humans, they are also one of the most impractical models. Complex enrichment, food and housing requirements and large amounts of reagents/drugs required for experimentation make longitudinal studies financially and ethically taxing. As a result, their use in reproductive research is limited (Schatten & Constantinescu, 2017), and research into human reproduction would benefit from a small, menstruating animal model.

So far in this review, the similarities in several aspects of folliculogenesis and ovarian function between mammalian species have been discussed. Clearly, research in farm and laboratory species has been critical in increasing our understanding of natural human reproduction. However, model species have also played critical roles in the development of human ARTs, and many fundamental techniques owe their development to work conducted in model species. Thus, the following sections will discuss the impact of ARTs research in farm and laboratory animals on the development of human ovarian stimulation and *in-vitro* fertilisation.

1.3.4 Controlled ovarian stimulation (COS)

Collecting healthy, mature oocytes is a critical step for *in-vitro* research, animal colony management and treatment of infertility. However, in most cases, a low natural ovulation rate is inefficient and has led to the development of COS or superovulation. Growing demand in the cattle and sheep industries and simultaneous exploration of rodent genetics has led to major scientific and technical breakthroughs in our understanding and manipulation of ovarian function (Armstrong, 1993; Schatten & Constantinescu, 2017).

The last half-century has seen tremendous advances in mammalian superovulation and increased accessibility of ARTs for infertility treatment (Beall & DeCherney, 2012). Considering this, the incidence of COS-related complications such as ovarian hyperstimulation syndrome (OHSS) have increased significantly (Aboulghar, 2003) over the same period. Not only does COS require greater regulation, but also greater research in suitable animal models to mitigate the potentially lethal outcomes of COS.

Although we have learnt a great deal about COS from farm and laboratory species, substantial differences in reproductive physiology between humans and farm or laboratory species can limit translation to human infertility. This section will therefore review the impact of reproductive research in farm and laboratory species on COS development in humans. Specifically, the effect of exogenous gonadotrophins on ovarian function, development of agonist and antagonist cycles, and novel treatments for OHSS.

Exogenous stimulation of ovarian function

Edwards (1965) argued that whilst early studies of hormone stimulation on the mammalian ovary were 'highly satisfactory', complementary research in humans and other large primates was unsuitable due to complex experimental requirements, and the poorly understood side effects of COS in menstruating species. Therefore, our current understanding of ovarian stimulation largely stems from research in cattle, sheep, rodents and rabbits.

Soon after the isolation of pregnant mare serum gonadotrophin (PMSG) in 1930 (Cole & Hart, 1930), PMSG was shown to artificially increase the number of developing follicles and induction of estrus in the ewe; all during a period of natural anestrus (Cole & Miller,

1933). The physiological role of PMSG on the ovary was later demonstrated by Levin (1944) to strongly mirror mammalian FSH, and provided a valid explanation behind the increased follicular growth seen in the rat and ewe ovary (Cole & Hart, 1930; Cole & Miller, 1933). Moreover, considering the shared ovarian sensitivity to pituitary gonadotrophins between mammalian species (reviewed earlier), it is no surprise that PMSG was later demonstrated to induce substantial follicular growth in cows (Hammond & Bhattacharya, 1944), does (Pincus, 1940) and women (Rydberg & Pedersen-Bjergaard, 1943). Although PMSG can stimulate follicular growth in several mammalian species, PMSG was replaced by urinary (uFSH) or recombinant FSH(rFSH) in humans (Lunenfeld, 1960) to avoid the potential production of anti-PMSG antibodies (van Wely et al., 2011).

Interestingly, pituitary gonadotrophins within a particular species show some structural homology (Mullen et al., 2013). Considering this, although Levin (1944) demonstrated the similar physiological role of PMSG to FSH, PMSG can also produce minor LH-like activity. Similarly, uFSH (also called human menopausal gonadotrophins; hMG) has both FSH and LH activity (Lunenfeld, 2004). With this in mind, the sole use of PMSG or hMG has been shown to induce ovulation in ewes (Cole & Miller, 1933), and primates (including women; reviewed by (Bettendorf, 1990)) respectively. However, ovulation rates following hMG treatment were much lower compared to treatment with human chorionic gonadotrophin (hCG) (Bavister et al., 1984; Pincus, 1940; Zarrow et al., 1958), suggesting a conserved sensitivity to, and requirement for, more potent LH analogues for ovulation induction.

At the same time, there was increasing evidence for timed hormone administration to harness endogenous gonadotrophins and steroids for optimal COS success. For example, research in mice (Fowler & Edwards, 1957) and heifers (Lindsell et al., 1986) clearly

demonstrate a significantly increased number of ovulated oocytes when stimulation occurred during the follicular phase of the oestrous cycle. To this end, Kruip et al. (1984) argued that the intraovarian control mechanisms and endocrinology cannot be sufficiently controlled purely with exogenous gonadotrophins and it seems more appropriate to complement, rather than resist, natural ovarian function across the reproductive cycle. This situation sounds ideal. However, by significantly elevating the already high steroid and gonadotrophin levels, the ovary may become overstimulated and women may present with potentially lethal OHSS (Aboulghar, 2003). Moreover, as circulating hormone levels vary heavily between women, there is no one-size-fits-all method for ovarian stimulation (Fiedler & Ezcurra, 2012). Once again, we turn to reproductive research in farm and laboratory species where gonadotrophin releasing hormone (GnRH) analogues have been used effectively to control endogenous hormone levels prior to COS.

Regulation of ovarian function by GnRH analogues

The reproductive physiology of cattle and sheep is arguably the best understood of any mammal and intense pressures for increased reproductive efficiencies has led to rapid adoption and advances in ARTs. Extensive research was carried out in the mid-late 20th century investigating various hypothalamic, pituitary and gonadal hormones to regulate the oestrous cycle, and their implications in humans were indicated early in their development (Schally & Kastin, 1971). Increased folliculogenesis and ovulation rates, while extremely beneficial in the treatment of infertility, were also coupled with increased incidence of OHSS and multifetal pregnancies. This led to the subsequent development of GnRH analogues for effective superovulation whilst managing the potentially lethal side-effects of COS.

GnRH analogues

The effectiveness of oestrous cycle downregulation in the mid 20th century was limited, but promising (reviewed by (Britt et al., 1981; Dhindsa, 1963). For some time, combined treatment with progestogens and prostaglandins (PGs) appeared to be the most effective method for oestrus cycle synchronisation and ovulation in several species (Kumar et al., 2014). However, whilst PGs are required for oocyte maturation in mammals, their influence on ovulation is species-specific. Inhibition of PG synthesis in does (Espey et al., 1986) and cows (De Silva & Reeves, 1985) blocked ovulatory events, but not in primates (Pall et al., 2001), highlighting the inherent differences in the endocrinology of menstrual and oestrus species. Attention then turned to GnRH analogues which were more active and remained active for longer than endogenous GnRH in several mammalian species (reviewed by (Schally et al., 1980)).

While PGs showed a species-specific influence on ovulation, the action of GnRH agonists is more broadly conserved in mammals (Fraser et al., 1987; Koos & Lemaire, 1985; McNeilly & Fraser, 1987; Scott et al., 1993). Nillius et al. (1980) demonstrated a dose- and time-dependent effect of GnRH agonists on ovarian function, which sparked their use in short- and long-downregulation cycles, but also as triggers for follicle maturation and ovulation (Catt & Lingham, 2016). However, it was quickly discovered that the inhibitory effects of GnRH agonists extended into the luteal phase, leading to a defective corpus luteum and a non-receptive uterus (Smitz et al., 1988). This also occurs during agonist-based COS in farm species, but because females generally act as oocyte or embryo donors an active luteal phase is not required. Moreover, CLs inhibit further follicular growth and are generally inactivated and regress following ovulation induction to bring females rapidly back into estrus (Kumar et al., 2014). Considering this, and the differences

between the endocrinology of oestrus and menstrual species (reviewed earlier), luteal phase problems following agonist-based COS are not appropriately modelled in these species.

Until the late 1980s, a combination of HMG, clomiphene citrate (agonist) and hCG was the most common COS protocol in ART clinics (Rizk & Smitz, 1992). By inducing a flare of pituitary gonadotrophin release, this simultaneously lengthened the follicular phase, allowing for more oocytes to be matured, whilst also inhibiting ovulation. However, this treatment regime also led to an increased frequency of OHSS (8-14% moderate and severe cases) (Herman et al., 1990). OHSS is a potentially lethal iatrogenic side-effect of excessive ovarian stimulation, strongly associated with agonist-based COS (Navot et al., 1991). Therefore, a protocol for effective cycle downregulation without increasing the risk of OHSS or affecting luteal phase function was required.

Concurrently, research in heifers (Madill et al., 1994), ewes (Campbell et al., 1990) and macaques (Balmaceda et al., 1981) demonstrated rapid inhibition of the pituitary without a 'flare' release of gonadotrophins using GnRH antagonists. Considering this, the use of GnRH analogues in women has been the subject of several reviews (Al-Inany & Aboulghar, 2002; Filicori et al., 1996; Wang et al., 2017), with most authors concluding that, compared to agonists, antagonist cycles substantially reduced treatment time and OHSS risk without influencing pregnancy or live birth rates in normal responders.

However, the situation is more complicated in poor responders or women with gynaecological disorders. For example, women with polycystic ovarian syndrome (PCOS) or endometriosis are at higher risk of developing severe OHSS following ovarian

stimulation (Fedele et al., 1992; Wallach et al., 1996) and research into novel methods for menstrual cycle regulation, such as using kisspeptin analogues for at-risk women, are therefore vital to prevent or mitigate the risk of developing OHSS.

Kisspeptin analogues for ovarian hyperstimulation syndrome (OHSS)

Mild symptoms of OHSS are present in almost every stimulation cycle, whereas the moderate and severe forms (ovarian cysts, thromboembolism etc) are less common, but are potentially fatal (WHO, 1973). Interestingly, ovarian cysts are common in cows, and they present with similar symptoms of OHSS as women (BorŞ & BorŞ, 2020). However, despite over three decades of reports on ovarian cysts, the aetiology and pathogenesis of ovarian cysts and OHSS are still unclear due to their elusive pathophysiology. The most accepted hypothesis for cyst formation in cows is considered to be altered feedback of the HPG axis leading to abnormal ovarian vascular permeability (VP) (reviewed by (BorŞ & BorŞ, 2020)). While increased VP is a perquisite for CL formation, excessive estradiol and LH stimulation following ovarian stimulation induces abnormal luteinization and dysregulation of vascular growth factors (Yan et al., 1993).

Kisspeptins were only recently discovered to play an important role in GnRH signalling and initiation of puberty in several mammalian species (reviewed by (Caraty et al., 2007)). Kisspeptins were later demonstrated to regulate ovarian VP (Cho et al., 2009), and Zhai et al. (2017) reported decreased kisspeptin-10 (K-10) mRNA and receptors in luteinized granulosa cells in rat models of OHSS and in high-risk women. Kisspeptin gene expression is also similar in the human, marmoset and rat ovary (Gaytan et al., 2009), and a recent study reported promising results with kisspeptin-54 (K-54) as an oocyte maturation/ovulatory trigger in women at high risk of OHSS (Abbara et al., 2015). A follow

up study comparing the genes involved in ovarian steroidogenesis and LH/FSH receptors revealed significant differences to traditional triggers (hCG and agonists), but no increased risk of OHSS (Owens et al., 2018).

Owing to fundamental research in model species, it appears that treatment with kisspeptin analogues can safely induce final oocyte maturation/ovulation whilst mitigating the potentially fatal effects of excessive VP. While cows and sheep have been particularly useful in identifying the functional role of GnRH and kisspeptin analogues on ovarian function, their large size and cost limits their application in biomedical research. Similarly, rodent models of OHSS are generally immature (Kitajima et al., 2004; Ujioka et al., 1997), therefore eliminating key, natural endocrine interactions vital to understand the true pathologies of OHSS. Development of a small, menstruating animal model that exhibits comparable ovarian function in response to hormone treatment as women will significantly advance our ability to treat and diagnose complications of COS including OHSS, which remains poorly understood.

Collectively, we have learnt a great deal about COS, manipulation of the HPG axis and have defined novel treatments for OHSS from research in farm and laboratory species. However, another hurdle to overcome in human infertility is fertilisation of the mature oocyte. While human IVF is a broadly successful technique, some patients present with total and/or recurrent fertilisation failure. Like other aspects of human reproduction, researchers have turned to animal models to explain the aetiology of this, and propose novel treatments for, male- and female-factor fertilisation failure.

1.3.4 IVF and fertilisation failure

Following the first report of human IVF by Rock and Menkin (1944), the growth of ARTs over the following half century saw rapid technical advances and the application of IVF across a variety of taxa (Bavister et al., 1984; Brackett et al., 1982; Chang, 1959; Clulow et al., 1999; Whittingham, 1968). However, progress in human IVF was hindered by ethical and legal restrictions that limited access to human tissues. In contrast, studies in domestic and farm species rapidly advanced and showed that many of the underlying mechanisms and processes involved in fertilisation are conserved in mammals, and much of our understanding of fertilisation in humans stems from research in farm and laboratory species. However, due to the significant differences in reproductive physiology between humans and other mammalian species, there is currently no perfect model for human IVF or fertilisation failure (Vetharaniam et al., 2010).

With this in mind, fertilisation rates in humans rarely exceed 70% (Bhattacharya et al., 2001; De Munck et al., 2020), and some patients, although rare, still present with total fertilisation failure (TFF) (Suo et al., 2018). While deciphering the aetiology of TFF remains technically challenging, IVF in farm and laboratory species have provided substantial evidence for male- and female-factors affecting fertilisation success including DNA fragmentation, inadequate sperm capacitation, aneuploidy or poor oocyte activation. Therefore, this section will review the impact of IVF research in farm and laboratory species on our understanding and treatment of male- and female-factor fertilisation failure.

Male factor fertilisation failure

In the early 1950s, two independent researchers simultaneously described the necessary physiological changes referred to as ‘capacitation’ for rabbit spermatozoa to fertilise

oocytes *in-vivo* (Austin, 1951; Chang, 1951). Considering this, Yanagimachi and Chang (1963) achieved the first successful *in-vitro* sperm capacitation and fertilsation in hamsters using a chemical-defined medium. Although early attempts to capacitate human spermatozoa *in-vitro* were largely unsuccessful (Edwards et al., 1969; Edwards et al., 1966), the knowledge gained from these studies were critical for achieving the first human live-birth following IVF in 1978 (Steptoe & Edwards, 1978). Over the next two decades, a considerable amount of research, in rodents and rabbits in particular, revealed the molecular and structural changes during capacitation and the culture components necessary for supporting human *in-vitro* sperm capacitation (reviewed by (Puga Molina et al., 2018)).

In the early 1970s, Hanada and Chang (1972) demonstrated the penetration of zona-free hamster eggs by large numbers of mice and rat spermatozoa. Yanagimachi et al. (1976) later took advantage of this phenomenon by suggesting zona-free hamster egg binding as a novel test for evaluating human sperm function. In the following decade, several authors demonstrated a positive correlation between hamster egg binding and fertilisation rate in human IVF (Margalioth et al., 1989; Margalioth et al., 1986; Wolf et al., 1983). Considering this, in a bid to further improve our understanding of human sperm capacitation without using human tissues, the laboratory mouse became the *de facto* model for human sperm capacitation, acrosome reaction and male-factor fertilisation failure (De Jonge, 2017). However, male-factor fertilisation failure is distinct between humans and mice despite the wide-spread adoption of a mouse model.

Neuber and Powers (2000) argued that human fertilisation failure, generally results from the oocyte's cytoplasm failing to support pronuclear development. Contrastingly, the

absence of sperm penetration is more often the cause of fertilsation failure in mice. The authors suggested greater sperm selection occurs at the level of the ZP in mice, but within the oocyte cytoplasm in humans, and caution should be used when translating from mouse failed-fertilisation models to clinical settings. However, not *all* human fertilisation failure results from lack of oocyte cytoplasmic support. Poor sperm motility, zona binding or low sperm concentration are common features of male-factor fertilisation failure. Repeated fertilisation failure and patients with severe male-factor infertility prompted the development of novel techniques including zona drilling, subzonal insemination (SUZI) and intracytoplasmic sperm injection (ICSI) as alternative methods of fertilisation (Palermo et al., 2017).

Gordon and Talansky (1986) were the first to describe zona-drilling in mice as a method for bypassing the ZP, potentially enhancing fertilisation rates in males with poor semen parameters. The same authors subsequently applied this technique to human infertility, reporting a significantly increased fertilisation rate in couples experiencing male-factor fertilisation failure (Gordon et al., 1988). However, the polyspermy rate for zona-drilled oocytes was very high (50%). Thus, zona-drilling had great potential for treating male-factor fertilisation failure, but required further optimisation to reduce the rate of polyspermy. Three years later, Palermo and Van Steirteghem (1991) improved on this technique by directly injecting a single spermatozoon into the perivitelline space (SUZI); effectively negating the need for motile, acrosome-reacted sperm all together. Ironically, it was during SUZI, when the oolemma was accidentally punctured and a spermatozoon was delivered directly into the ooplasm, that intracytoplasmic sperm injection (ICSI) was subsequently established (Palermo et al., 1992).

However, compared to the primate, cow or hamster oocyte, mouse oocytes have a poor 'wound healing' capacity following mechanical manipulation and survival rarely exceeds 50% (Kimura & Yanagimachi, 1995; Yoshida & Perry, 2007). While hamster oocytes are more robust than murine oocytes, hamsters are considered a pest species in some countries (including Australia) and are prohibited from use in research. Similarly, ICSI in cows is very rarely performed due to the technical complexity, and additional requirement of oocyte activation following manipulation (Emuta & Horiuchi, 2001). Although ICSI is successful in non-human primates (Simerly et al., 2010; Sun et al., 2008; Wolf et al., 2004), the combined cost of maintaining captive primate colonies and the complexity of ICSI limits their use in biomedical research. Clearly, current animal models of ICSI and fertilisation failure present with several technical and biological limitations that limit their application as models for clinical use.

Of course, this is only one side of the story. While ICSI is an effective treatment for male-factor infertility, ICSI does not confer any benefit in cycles with female factor infertility (Bhattacharya et al., 2001; De Munck et al., 2020; Supramaniam et al., 2020). While semen is relatively easy to collect and study, collection of mature oocytes suitable for *in-vitro* research is far more difficult, and much of our understanding of female-factor infertility relies on comparative research in farm and laboratory species.

Female factor fertilisation failure

In women, underlying fertility issues often involve ovarian or ovulatory disorders, which can largely be overcome by appropriate COS regimes (Yeste et al., 2016). However, since factors influencing female fertility are very broad in nature, treatments can be limited (Yeste et al., 2016). Moreover, the study of oocyte function in women is restricted by

availability of viable tissues and ethical concerns. Therefore, various animal models have been used to studyspecific areas of female infertility, with advanced maternal age (AMA) undoubtedly the most significant factor influencing fertilisation success.

Reproductive aging was understood to affect fertility relatively early in the history of human ARTs (reviewed by (Talbert, 1977)). However, the physiological mechanisms influencing fertility were not well understood until the late 20th century. Pivotal research by Maudlin and Fraser (1978) demonstrated a significant effect of AMA on murine IVF success, specifically proposing increased aneuploidy in older oocytes as the primary cause of fertilisation failure. Three years later, Parkening et al. (1980) reported significantly reduced concentrations of pituitary gonadotrophins (FSH, LH) in aged (16-20 months old) C57BL/6 mice compared to younger (2 months old) mice of the same strain. A similar effect is seen in peri-menopausal women compared to younger women, and the reduced responsiveness of the aged ovary to pituitary gonadotrophins may also explain the reduced steroid concentrations in older women and mice (Pal et al., 1991; Pellicer et al., 1995).

While mice have been very useful in this area of research, the rapid aging of rodents is also a disadvantage to their use in reproductive modelling as the chronic effects of age on oocyte competency are comparatively short-lived compared to those of aged mare of cow models (Carnevale, 2008; Malhi et al., 2005). However, the expense of cow and mare models when compared to rodents far outweighs the benefit of more longitudinal aging studies, and mice are still the animal model of choice for reproductive aging (Schatten & Constantinescu, 2017). While a clear connection was emerging between aneuploidy and

IVF failure, failure of euploid oocytes to fertilise following IVF or ICSI was considered to be a result of poor oocyte activation (Rawe et al., 2000).

During mammalian fertilisation, spermatozoon-oocyte fusion triggers intracellular oscillations of calcium ions within the oocyte, activating several cellular processes and resumption of the cell cycle (Wakai et al., 2011). This process was initially identified in mice and hamsters before it's description in human fertilisation a few years later (reviewed by (Homa, 1994)). However, despite our increased knowledge about calcium ion signal transduction during fertilisation, the functional relationship between frequency, number and amplitude of calcium oscillations in human oocyte activation and fertilisation failure remains unclear (Meerschaut et al., 2014). In the early 2000s, assisted oocyte activation (AOA) in mice (Ducibella et al., 2002) and does (Ozil & Huneau, 2001) with precisely controlled calcium transients demonstrated the ability of exogenous cations to activate oocytes *in-vitro*. This also gave rise to AOA as a possible treatment option for couples with repeated oocyte activation or fertilisation failure, and Heindryckx et al. (2005) later reported normal fertilisation and pregnancy following AOA in patients with female-fertilisation failure. However, being a novel treatment method, there is currently no standardisation of ion concentrations or exposure times, and the effects of AOA on subsequent *in-vitro* growth or postnatal outcomes in subfertile humans are unknown (Meerschaut et al., 2014). In an attempt to optimise and standardise AOA methods, a recent study comparing several AOA protocols in phospholipase C ζ (PLCζ) null mice (sperm oocyte activation factor) reported comparable fertilisation and embryonic development irrespective of the oscillatory activity of several divalent cations (Ferrer-Buitrago et al., 2020). While this study did not compare pre- or postnatal data, two earlier papers (Bernhardt et al., 2018; Ozil et al., 2017) reported impaired implantation rates and

reduced pup weights following AOA in mice. While promising, future research in appropriate animal models is clearly needed before routine use of AOA in treating fertilisation failure.

Collectively, *in-vitro* and *in-vivo* research in model species has revealed several underlying mechanisms of male- and female-factor fertilisation failure. However, the primary goal of ART treatment is to achieve a healthy, live birth and the transfer of a healthy embryo to a receptive uterus is a crucial step to establishing a viable pregnancy. However, pregnancy rates rarely exceed 30% following ART treatments (Coughlan et al., 2014; Fitzgerald et al., 2017) and, compared to other mammals, the exact mechanisms that regulate embryo implantation in humans remain elusive.

1.3.5 Embryo implantation

Embryo implantation is an intricately controlled process necessary for placental development and establishment of pregnancy and consists of three distinct phases: apposition, adhesion and invasion (Figure 3; (Paulson & Comizzoli, 2021)). However, due to ethical restrictions surrounding implantation research in humans, the physiological mechanisms of each of these phases are more readily studied in farm and laboratory species.

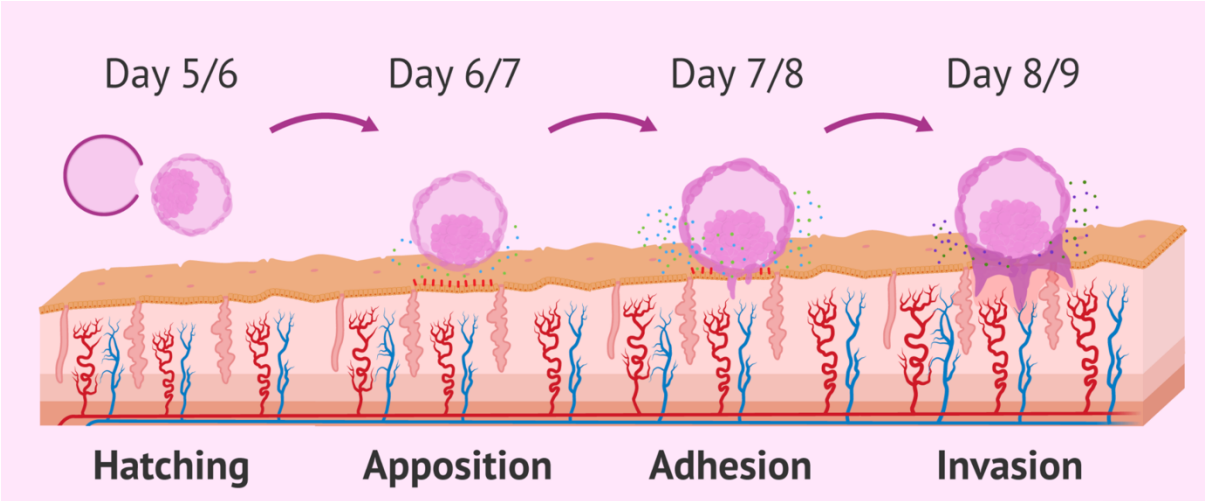

Figure 3: Schematic representation of the phases of human embryo transit and implantation. *Hatching: The blastocyst hatches from its zona pellucida to allow for trophectoderm cell adhesion. Apposition: The embryo sits directly adjacent to the endometrium and a dialogue is initiated between embryo and endometrium. The blastocyst then adheres to the uterine epithelium and inevitably invades the luminal epithelium and underlying stroma. Image sourced from (Muñoz et al., 2019)*

This section will review the impact on our knowledge of these three phases in human implantation from research using farm and laboratory animal models.

Embryo apposition

An intricate dialogue between blastocyst and uterine luminal epithelium (LE) is essential for initiation of embryo implantation. Apposition of the blastocyst to the LE is the first step of mammalian embryo implantation and lasts only a few hours in higher-order primates, making apposition difficult to study in depth (Enders, 2000). However, the prolonged apposition of ewes, cows and sows without invasion of the LE make them excellent candidate models to study the regulation and mechanisms of human embryo apposition (Lee & DeMayo, 2004; Schatten & Constantinescu, 2017).

Considering this, early research in ewes, sows and rats identified a complex array of uterine secretions critical for blastocyst survival and early stages of implantation (Bazer,

1975; Dickmann & Dey, 1974; Robertson & King, 1974). In the late 90s, ewe gene-knockout models were particularly useful to identify key genes expressed by the LE during embryo implantation (Spencer et al., 1999). Evidence from human and non-human primate species subsequently confirmed these findings and the unequivocal role of uterine secretions for production and maintenance of pregnancy in mammals (Bazer et al., 1979; Carson et al., 2000). However, ewes, sows and cows form an epitheliochorial placenta with minimal trophoblast invasion, limiting their use as models for later stages of embryo implantation and placentation.

While murid rodents form a similar placenta to humans, the rapidity of embryo implantation in mice (within 6 hours of blastocyst apposition) (Lee & DeMayo, 2004) limits their use in early implantation studies. However, the true strength of rodent models is in our immense understanding of rodent genetics and availability of gene manipulation technologies. For example, studies in mice described the importance of interleukin 11 and its receptor (IL-11, IL-11R) during embryo implantation and their expression was shown to be essential for decidual development (Bilinski et al., 1998; Robb et al., 1998). A similar pattern of IL-11 and IL-11R expression was later demonstrated in women and non-human primates during the window of implantation (Cork et al., 2001; Dimitriadis et al., 2003; Dimitriadis et al., 2006). Moreover, the requirement for ovarian steroids during early phases of embryo implantation appears to be conserved between oestrus and menstrual species (reviewed by (Carson et al., 2000; Lee & DeMayo, 2004)) and aberrant gene expression regulated by ovarian steroids is associated with implantation failure in mice and women (Koler et al., 2009; Lubahn et al., 1993). Despite the similar regulation of embryo apposition, significant differences are observed during embryo adhesion and invasion between domestic and primate species.

Embryo adhesion and invasion

Following apposition, the embryo trophectoderm adheres to the uterine LE in preparation for embryo invasion and placental development. Considering the ethical restrictions of collecting human implantation sites, there are only a handful of reports describing early-stage human implantation sites (Enders, 1989; Hertig, 1975; Knoth & Larsen, 1972) and neither embryo adhesion nor invasion have ever been seen in humans.Therefore, our understanding of embryo adhesion and invasion relies heavily on tissues collected from model species which has severely limited translation of this knowledge to the clinic.

In true menstrual species, decidualisation of endometrial stromal cells occurs spontaneously following ovulation (Bellofiore, Cousins, et al., 2018), whereas this process is triggered by embryo adhesion to the LE in oestrus species (Nimbkar-Joshi et al., 2015). Consequently, embryo adhesion is prolonged in these species to allow for decidualisation to take place (Lee & DeMayo, 2004), and adhesion is arguably more easily studied in these species (Carson et al., 2000). Considering this, research in farm and laboratory animals have revealed several physical and molecular requirements for primate embryo adhesion and invasion. For example, the spatial-temporal expression of several epidermal growth factors (EGF) localised to the apical border of LE cells during mouse embryo adhesion (Paria et al., 1994) was also confirmed during macaque implantation (Yue et al., 2000). Additionally, research in ewes (Gharib-Hamrouche et al., 1993), and later in humans (Smith et al., 2002), revealed high expression of EGF receptors (EGF-R) on trophoblasts during/just prior to embryo adhesion. Treatment with EGFs was later demonstrated to rescue trophoblast apoptosis by reactive oxygen species (ROS) (Moll et al., 2007), and the authors suggested a role for EGF treatment in mitigating implantation

failure in women with pre-eclampsia or intrauterine growth restriction. Despite the conserved expression pattern of growth factor between species, little is known about the direct effects of specific growth factors on human implantation and research in this area is encouraged to improve implantation rates following ART treatments.

In addition to growth factors, research in cows and rodents have revealed the critical role of adhesion molecules such as integrins and cadherins for successful embryo adhesion (reviewed by (D'Occhio et al., 2020)). For example, Liu et al. (2008) provided evidence for the role of adhesion molecules in facilitating mouse embryo adhesion and Sathanawongs et al. (2012) demonstrated a higher expression of cadherins in good quality bovine blastocysts compared to poor quality blastocysts. Both studies have provided insight into the mechanisms of implantation and pregnancy loss in women. With this in mind, the absence or aberrant expression of adhesion molecules during the window of implantation is associated with infertility in women (Lessey et al., 1992; Ordi et al., 2003). Collectively, these studies suggest a shared reliance on adhesion molecules and growth factors for embryo implantation between menstrual and oestrus species.

While embryo adhesion appears to be similar between mammals, depth of trophoblast invasion can differ dramatically between species. Menstruating primates present with aggressive, interstitial embryo implantation (Nimbkar-Joshi et al., 2009), whereas rodents show eccentric implantation with moderate trophoblast invasion (Figure 4). Moreover, adhesion and invasion of the uterine LE occurs within 6 hours in rodents, which limits the utility of this order as models for understanding the underlying physical mechanisms involved in early stages of human implantation (Lee & DeMayo, 2004). Cows and ewes, on the other hand, show centric embryo implantation with no invasion of the LE at all and

the fetus grows large within the uterine lumen (Figure 4). While non-menstruating primates such as common marmosets have been used as *in-vitro* models of primate embryo adhesion and protein secretion (Franek et al., 1999), marmosets also present with centric implantation (Enders & Lopata, 1999) and do not reflect the events of higher-order primates. Together, these studies highlight the significant species variations in mammalian embryo invasion and the need for a biologically relevant, menstruating model of human embryo implantation.

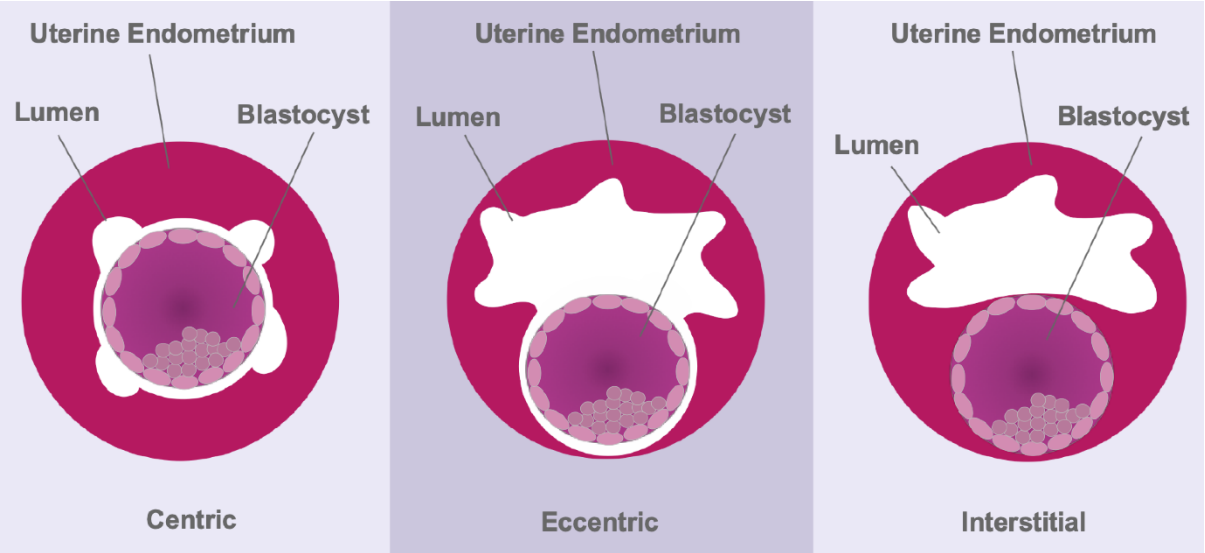

Figure 4: Schematic representing the three modes of implantation. *During centric implantation, the embryo is superficially attached to the uterine epithelium and trophoblasts do not invade the endometrium. Eccentric implantation involves the formation of a tear-drop shaped cavity within the endometrium with a moderate degree of trophoblast invasion. During interstitial implantation, the blastocyst completely embeds itself within the endometrial stroma with trophoblasts invading to the basal endometrium.*

1.3.6 Implantation in menstruating species

While four species of bat are known to exhibit menstrual-like events with aggressive trophoblast invasion, menstruation in these species is either confined to a single event per year, or decidual characteristics of endometrial stromal cells could not be confirmed (reviewed by (Bellofiore, Cousins, et al., 2018)). Similarly, menstruation in the elephant shrew (*Elephantulus myurus*) occurs seasonally, and the decidual response also differs

from menstruation in higher-order primates. Therefore, the most suitable models of human reproduction (including embryo implantation) are species with cyclical, human-like menstruation such as old-world monkeys, the great apes (Emera et al., 2012), and, more recently, the Egyptian spiny mouse (Bellofiore et al., 2017).

Throughout the 1980-90s, Professor Allen Enders collected and analysed reproductive tissues from several old-world monkeys detailing the events during embryo adhesion, invasion and early placental development (Enders, 1989, 1991; Enders et al., 1983; Enders & King, 1991). Whilst neither adhesion nor invasion of human embryos has been seen, Enders (1989) hypothesised that human trophoblasts will follow a similar pattern of endometrial invasion to other higher-order primates. To confirm this, Enders and King (1991) described syncytialtrophoblasts forming adjacent to the inner cell mass (ICM), penetrating between uterine epithelial cells and spreading along the residual LE basal lamina during macaque and baboon implantation. The earliest examples of human implantation sites are at the trophoblastic plate stage, several days after endometrial adhesion and invasion (Enders, 2000). However, this stage is broadly similar to the post-implantation sites described by Enders and King (1991) prior to maternal artery remodelling and placental development.

While old-world monkeys clearly present with aggressive trophoblast invasion, great-ape embryos invade even deeper within the endometrial stroma. Recently, trophoblast invasion and subsequent spiral artery remodelling in chimpanzees (Pijnenborg et al., 2011a) and gorillas (Pijnenborg et al., 2011b) were simultaneously described, both strongly resembling the processes in later stages of human implantation. Considering our phylogenetic relatedness, it is perhaps no surprise that deep trophoblast invasion

extending to the inner myometrium are features shared between all great apes, and implantation is most suitably modelled in these species.

As already mentioned, scientists are heavily restricted by human tissue use to definitively determine the timing and mode of human embryo implantation *in-vivo*. While great apes act as the most biologically appropriate models, they are perhaps the least practical. Thus, *in-vitro* and *in-vivo* studies in farm and laboratory species have been pivotal in increasing our understanding of human embryo implantation. While each species has advantages, it would be beneficial for future implantation research to be able to use a single animal species rather than examining specific aspects of implantation in several different species.

1.3.7 The spiny mouse (*Acomys cahirinus*)

Egyptian or common spiny mice (*Acomys cahirinus*), are a desert-adapted murid species native to arid regions of northern Africa and the Middle East (Osborn & Helmy, 1980). *A. cahirinus* were first used in biomedical research as models of renal function and type-2 diabetes (Gonet et al., 1966), but were more recently used as models of wound-healing (Seifert et al., 2012), perinatal development and birth asphyxia (Dickinson, 2006; Ireland et al., 2011; O'Connell et al., 2013). Spiny mice present with several human-like reproductive characteristics rare in rodent species, including precocial young, likely stemming from a relatively long gestation (~39 days; (Brunjes, 1990), human-like endocrinology and placentation (King & Hastings, 1977; Lamers et al., 1986; O'Connell et al., 2013; Quinn et al., 2013) and, most recently, a human-like menstrual cycle (Bellofiore et al., 2017).

Bellofiore, Cousins, et al. (2018) state that menstrual species have evolved a number of several key reproductive characteristics: cyclical ovulation, spontaneous decidualisation of

endometrial stromal cells, aggressive trophoblast invasion, spiral artery formation/remodelling and extensive remodelling prior to formation of a haemochorial placenta. To date, all but depth of trophoblast invasion and spiral artery remodelling have been confirmed in this species, and *A. cahirinus* is now an established laboratory animal. However, while the menstrual cycle and later stages of pregnancy have been investigated in this species, the events leading up to pregnancy including ovulation, embryogenesis and embryo implantation have not yet been characterized. Additionally, a paucity of information ARTs is available in spiny mice and there is therefore a need to develop specific laboratory-based techniques to make this model more relevant for biomedical research.

Therefore, the aims of this thesis are to characterise the events leading to early pregnancy in the spiny mouse including mating behaviour, ovulation, embryogenesis and embryo implantation, whilst developing robust ARTs to make this species a more accessible animal model of human reproduction.

Chapter 2: Good things come to those who mate: Analysis of the mating behaviour in the menstruating rodent, Acomys cahirinus

Submitted manuscript currently under review (Animal Behaviour)

Good Things Come to Those Who Mate: Analysis of the Mating Behaviour in the Menstruating Rodent, *Acomys cahirinus*

2.1 Abstract

The Egyptian spiny mouse is the only known rodent to exhibit true, human-like menstruation and postpartum ovulation, and is an important new model for reproductive studies. Spiny mice do not produce a visible copulatory plug, and calculation of gestational age is therefore restricted by the need to use mated postpartum dams. The current inefficient method of monitoring until parturition to provide a subsequent estimate of gestational age increases study duration and costs. This study aimed to address this issue by comparing the mating behaviour of spiny mice across the menstrual cycle and proposes a more accurate method for staging and pairing animals that provides reliable estimates of gestational age. Female spiny mice were paired at the follicular or luteal phases of the menstrual cycle to determine any effect on the pairing-birth interval (Experiment 1; n =10 pairs). In Experiment 2, mating behaviour was recorded overnight to collect data on mounting, intromission and ejaculation (Experiment 2; n = 5 pairs per stage) in spiny mice paired at menses and at early and late follicular and luteal phases of the menstrual cycle. We report a broad mating window of ~ 3 days during the follicular phase and early luteal phase of spiny mice. When pairing occurred during the late luteal phase or menses no mating activity was observed during the recording period. Males displayed a discrete 'foot twitch' behaviour during intromission and a brief copulatory lock during ejaculation. Litters were delivered after 40-43 days if pairing occurred during the mating window, compared with 46-48 days for spiny mice paired in the late luteal phase. This study clearly demonstrates an effect of menstrual cycle stage on spiny mouse mating

behaviour and provides a reliable and more effective protocol for estimating gestational age without the need for postpartum dams.

Keywords

Acomys cahirinus

Copulation

Ejaculation

Intromission

Menstruation

Spiny mouse

2.2 Introduction

Copulatory behaviour is tightly linked to species reproductive anatomy and physiology, including timing of ovulation (Dewsbury, 1975). While most mammals adopt a seasonal breeding strategy, whereby environmental cues trigger ovulation, in several species including cats and some rodents, ovulation requires the stimulus of copulation. During mating and subsequent stimulation of mechanoreceptors in the female reproductive tract, an excitatory threshold triggers the release of LH in sufficient amounts to induce ovulation (Bakker & Baum, 2000; Downey, 1980). In contrast, coitus induction of ovulation has not been observed in menstruating mammals. In these species, ovulation occurs spontaneously after a surge of LH linked to hormonal changes during the menstrual cycle (Bellofiore, Cousins, et al., 2018). Bignami and Beach (1968) have therefore argued that copulatory behaviour in species with spontaneous ovulation is generally less elaborate than in induced ovulators because excessive vagino-cervical stimulation is not required to trigger ovulation.

The Egyptian spiny mouse, *Acomys cahirinus,* is a rodent native to North Africa. This species has several reproductive characteristics that are rare in, or unique to, rodents including the birth of precocial young (Dickinson & Walker, 2007), as the result of a relatively long gestation for a small rodent (~39 days; (Brunjes, 1990), and, more recently, a natural, human-like menstrual cycle (Bellofiore et al., 2017); the first confirmation of menstruation in any rodent species. Interestingly, unlike most rodents, *A. cahirinus* produces no visible copulatory/seminal plug (Haughton et al., 2016). This provides significant challenges when using mated females for gestational studies because ejaculation, and therefore early pregnancy, cannot be confirmed non-invasively. However, based on observations in our breeding colony, female spiny mice also experience a

postpartum ovulation (Gilbert, 1984), in which mating occurs within 48 hours of parturition. Using this knowledge, estimates of gestational age are currently calculated from the date of delivery of the previous term litter (Ireland et al., 2011; O'Connell et al., 2013). While this method provides a useful and relatively accurate estimate of gestational age, it is time-consuming, costly and inefficient. It requires the monitoring of breeder pairs until parturition (a minimum of 40 days from pairing virgin females and no guarantee that females will have mated immediately upon pairing) plus the subsequent timing of the pregnancy to the required stage of gestation.

Previous observations from our laboratory have identified variable pairing-to-birth intervals, that ranged from 40-46 days, when females were paired at unknown stages of the menstrual cycle. This suggested that stage of the menstrual cycle influences mating behaviour that results in variations in gestational outcomes in *A. cahirinus*. Although copulatory behaviour in this species has been described (Dewsbury & Hodges, 1987), no attempt was made to correlate the phase of the reproductive cycle with the timing of copulation and birth outcomes, and this study also preceded the discovery of menstruation in *A. cahirinus*. Moreover, their study was conducted over 40 years ago, and the data recording methods used were not clearly detailed, and no video or photographic evidence of mating behaviour was provided. With the development of, and easy access to, improved recording technologies, a more accurate and effective method for timing mating, insemination and pregnancy can now be achieved. In this study, we have investigated the effect of stage of the menstrual cycle on receptivity, copulation and birth outcomes in the spiny mouse and used these data to propose a better method for estimating gestational age in the spiny mouse.

2.3 Methodology

2.3.1 Ethics

All experimental procedures carried out in this study adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All animals were sourced from our breeding colony and approved for use by the Monash Medical Centre Animal Ethics Committee (MMCB 2019/13BC). Sexually mature spiny mice (3 – 9 months of age) were housed in sex-segregated groups up to nine per cage or as breeding pairs under a 12:12 hour light:dark cycle at 25-28^{o}C and humidity of 30-40%. Cages were lined with wood shavings and plastic tunnels, cardboard boxes or tissue paper provided as environmental enrichment. Food (rat and mouse cubes; Specialty Feeds, Glen Forest WA) and water was provided ad libitum, with weekly supplements of fresh carrots and celery.

2.3.2 Experimental design

Two experiments were conducted in this study. The first experiment was used to determine the effect of menstrual cycle stage on spiny mouse pairing-birth intervals. The second experiment was designed to record and characterise the mating behaviour of spiny mice at different stages of the menstrual cycle.

2.3.3 Experiment 1 (n=10 pairs)

Stage of the menstrual cycle was determined (Bellofiore et al., 2017) in sexually mature females prior to pairing during the follicular or luteal phases (n=5 pairs per phase) of the cycle. As *A. cahirinus* are naturally crepuscular (Wang et al., 2020), they were paired at 1700 hours each day and then left together until there were visible signs of pregnancy, and pups were palpable (~ 30 days; unpublished observations from our lab).

2.3.4 Experiment 2 (n=5 pairs/phase of the menstrual cycle)

The aim of this experiment was to compare behavioural cues for copulation with those outlined by Dewsbury and Hodges (1987), and in particular, mounting, intromission and ejaculation. Spiny mice were paired at the early follicular, late follicular, early luteal, late luteal and menses phases of the cycle. The mating activity of each pair was recorded during the night for at least 6 hours, commencing at 5 pm. Although a published sleep study in this species showed a sharp decrease in activity two hours after dark onset under natural lighting (Wang et al., 2020), a preliminary study of our colony showed animals remained active for 5-6 hours after dark onset. Males and females were separated and returned to their respective home cages the following morning and females were lavaged vaginally on the morning of separation to check for signs of semen or a copulatory plug. Each female was then monitored for 30 days after pairing, when the signs of pregnancy outlined in Experiment 1 were used to confirm that pregnancy had been achieved from the observed copulatory activity. Females that were visibly pregnant were removed from their home cages and housed in a single cage to litter down; those that were not pregnant were left undisturbed in their home cage.

2.3.5 Recording

Mating behaviour was recorded in a clean cage, with fresh bedding and enrichment (as required by ethics), using a 1080p wireless security camera system (cctv-wf-cla-4c-4b; UL-Tech, Australia)(Figure 1). At least 6 hours of footage was examined after pairing. Mating behaviour was measured as described by Dewsbury and Hodges (1987) with time to live birth (pairing-birth interval) and number of pups born as additional measures.

The following behaviours were included in the behavioural assessment: mount latency (ML; time from pairing to first mount), intromission latency (IL; time from pairing to first intromission), intromission frequency (IF; number of intromissions prior to ejaculation), ejaculation latency (EJL; time from first intromission to ejaculation), ejaculation interval (EJI; time from ejaculation to the next intromission) and ejaculation frequency (EJF; number of ejaculations observed).

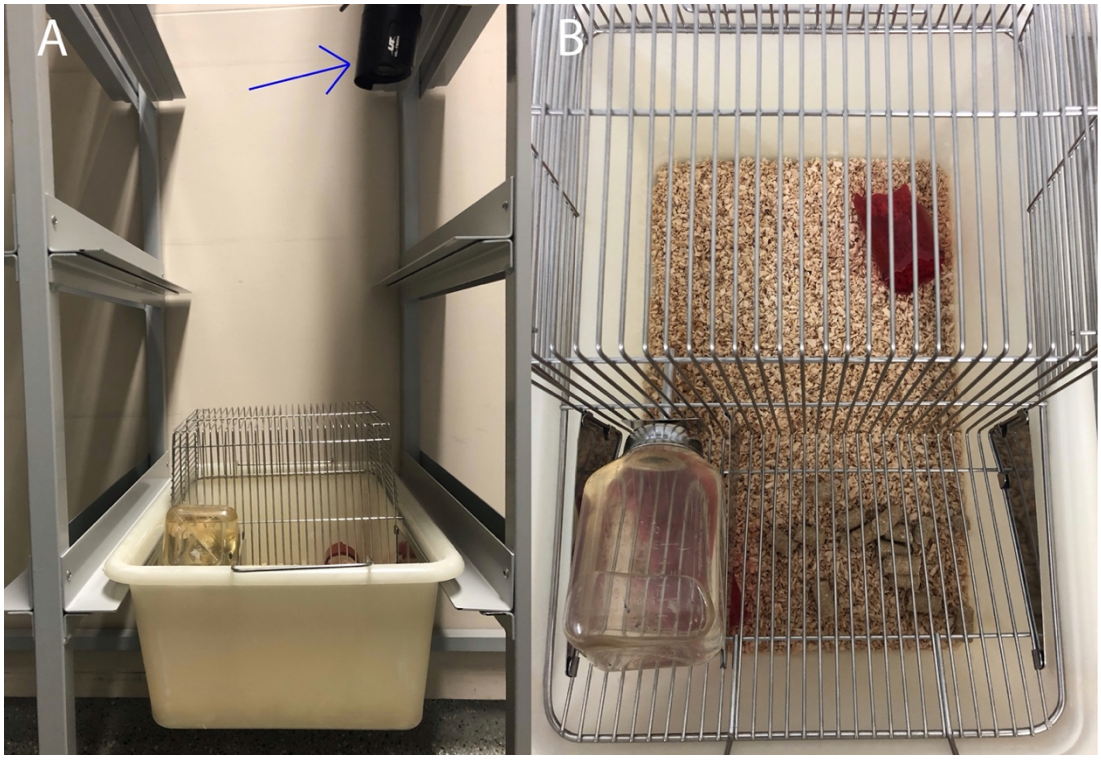

Figure 2 Recording setup used for recording spiny mouse mating behaviour. Cameras (arrow) are attached to the cage racks and placed two shelves above the breeder cage (A). Water is provided on top of the cage lid, whilst food and enrichment devices are provided within the cage (B).

2.3.6 Statistical analysis

All data from Experiment 2 were analysed using Prism 8 software (GraphPad) and samples were tested for normality using the Shapiro–Wilk test before further analysis. One-way analysis of variance (ANOVA) was used to compare data between cycle phase and the Grubb's test used to identify any outliers. All data reported at mean ± standard deviation (SD) and statistical significance set at $p<0.05$.

2.4 Results

2.4.1 Experiment 1

In Experiment 1, only 4/5 females paired in the follicular phase and 1/5 paired in the luteal phase gave birth within 45 days of pairing. The female paired in the luteal phase which gave birth before 45 days had vaginal cytology containing a mixture of cornified epithelial cells (CECs), leukocytes and nucleated epithelial cells (NECs) (Figure 2A) that was more typical of an early luteal phase vaginal smear in *A. cahirinus*. The remaining 4 females from the luteal phase pairings all showed vaginal cytology typical of the late luteal phase at pairing (Fig 2B).

Litters were born between 39 and 42 days after pairing (mean 41.7 ± 1.2 SD) by all females paired in the follicular and early luteal phases. In contrast, the remaining 4 females paired in the late luteal phase gave birth between 46-48 days after pairing (mean 46.6 ± 0.9 SD).

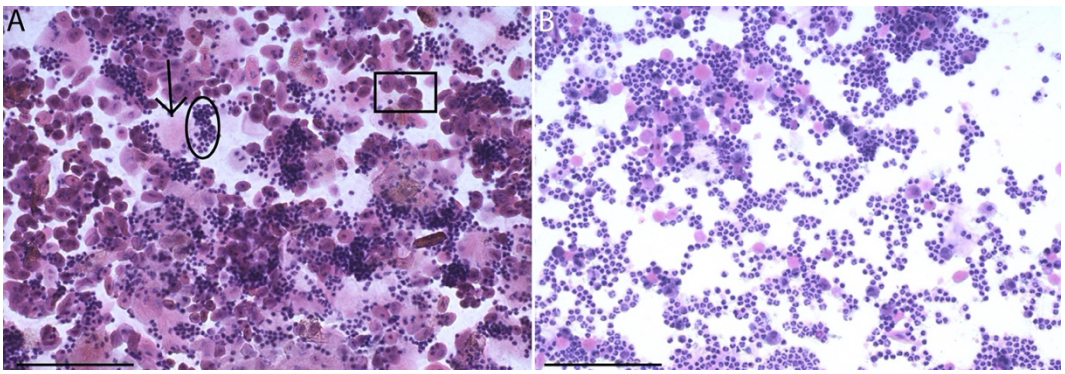

Figure 2 Comparative vaginal cytology from the early (A) and late (B) luteal phase. The early luteal phase cytology contained a mixture of cornified epithelial cells (CECs; arrow), leukocytes (circle) and nucleated epithelial cells (NECs; square). Late luteal phase cytology was dominated by leukocytes with a comparatively smaller number of CECs and no NECs. Scale bars = 100um

2.4.2 Experiment 2

Male spiny mice approached females from behind prior to mounting (Figure 3, Supplementary videos 1-2) and females either moved away, sometimes climbing the wire cage top to prevent unwanted advances or were receptive and allowed the male to mate with her.

Mounting was usually initiated rapidly after introduction and was defined as the male clasping the female's back with his forepaws. Mount latencies were generally under 10 minutes in all phases of the menstrual cycle (Figure 3A), with the exception of the late luteal phase where mounting was only observed in one of the five pairs and occurred 85 minutes after introduction. No mounting behaviour was seen when pairing occurred during menses.

Intromission was a discrete event in which the female stood still while the male climbed further along her back with no apparent biting of the female's back or nape. Males also display a clear, and previously unrecorded, foot twitch during the brief period of intromission (~1 second; Supplementary videos 1-2; figshare.com/s/0b83b3d7d9848f32076a and figshare.com/s/7125e0acfbf20de04820) followed by a ballistic dismount.

Figure 3 Mounting behaviour during the lights-on (A, B) and lights-off (C, D) periods in spiny mice. Male spiny mice approach females from behind and place front paws on the middle of the female's back. Females will either allow mating to occur or move to prevent unwanted mating.

Intromission was seen in all (5/5) late follicular phase pairings, but only in 3 each of the 5 early follicular and 5 luteal phase pairings (Figure 4B). The first intromission typically occurred approximately one hour after introduction (Figure 4B) followed by a series of between 6 and 18 intromissions (11.3 ± 3.4 SD; Figure 5A). All pairings in which intromission was seen also resulted in ejaculation, which was confirmed by the presence of spermatozoa in the vaginal lavage. There were no behavioural signs that intromission or, by its association, ejaculation had occurred in any of the late luteal or menses phase pairs. Intromissions were confirmed as non-ejaculatory as females smeared after each intromission event contained no spermatozoa.

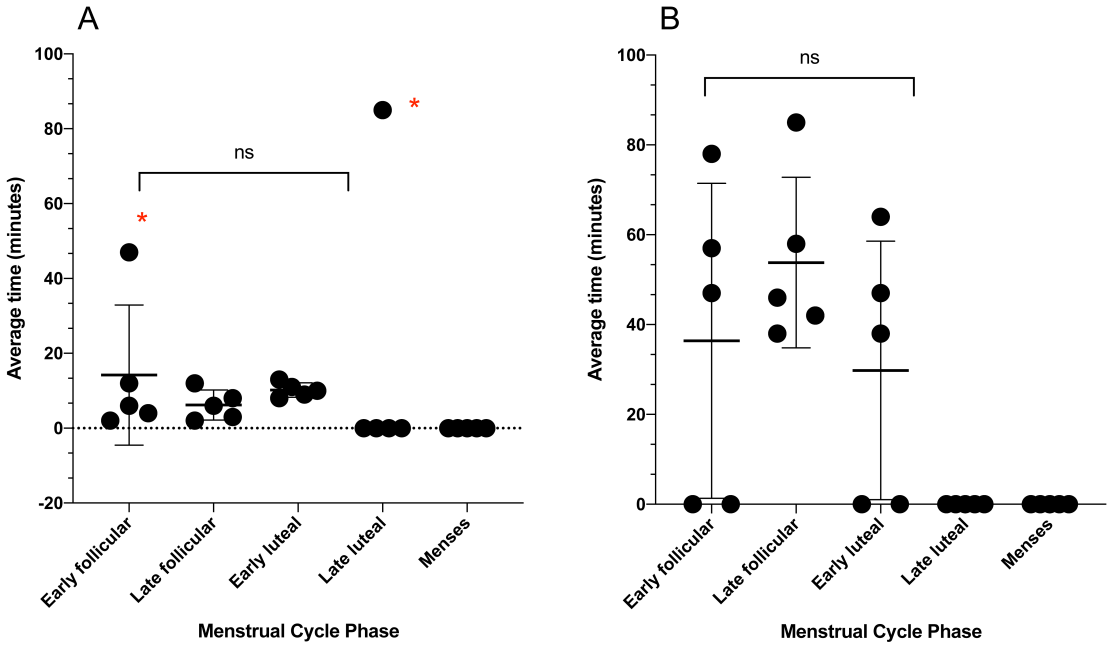

Figure 4 Mount latency (A; ML) and intromission latency (B; IL) in spiny mice paired at different stages of the menstrual cycle. ML and IL are similar between early and late follicular and early luteal phases, but significantly different to the late luteal and menses phases. Mounting was only seen in 1/5 pairs during the late luteal phase and no intromission was seen in these animals. No mounting or intromission behaviours were seen during menses. Intromission events were seen in 3/5 pairs during the early follicular and early luteal phases. All data are mean ± SD, significance set at $p<0.05$ and * reflect outliers within cycle stages. ANOVA for ML: $F_{4,20} = 0.6197$, $P = 0.6537$. ANOVA for IL: $F_{4,20} = 5.761$, $P = 0.003$.

Ejaculation was distinguished from intromission by the difficulty of the mating pair to separate (Supplementary videos 3-4; figshare.com/s/1f52244d4fb412bb90c8 and figshare.com/s/b72064faa2f9c3f5599d) following a discrete, but extremely brief, copulatory lock (~1 second). Copulatory locks were also confirmed to be ejaculatory events by the presence of spermatozoa in vaginal smears of females immediately following locking events (Supplementary figure 1; figshare.com/s/ab45a29a49aef428bcfe). In all 3 phases of the menstrual cycle where mating was successful, ejaculation occurred between 7 and 24 minutes (16.3 ± 5.3 SD) after the first intromission (Figure 5B) and always followed a series of non-ejaculatory intromissions.

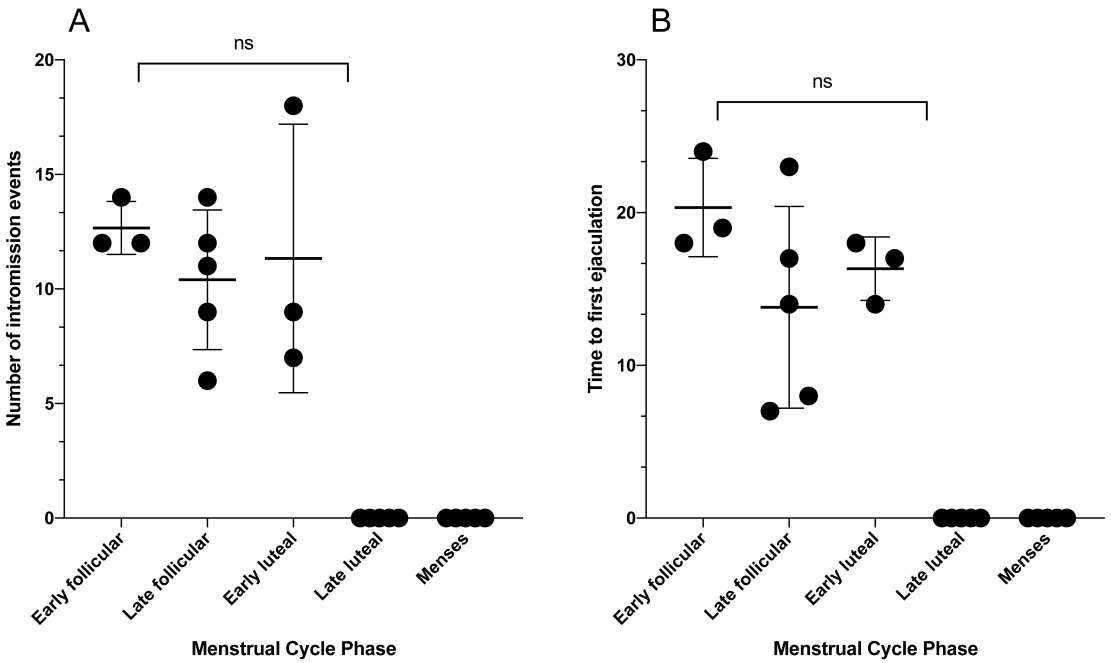

Figure 5 Intromission frequency (A; IF) and ejaculation latency (B; EJL) in spiny mice paired at different stages of the menstrual cycle. IF and EJL are similar between those cycle stages where intromission occurred. The late luteal phase and menses pairing were clearly different from the other three phases with no intromissions, and therefore no ejaculations, observed. Intromission occurred in a series between 6 and 18 events (A) and EJL occurred between 7 and 24 minutes after the first intromission (B). All data are mean ± SD and significance set at $p<0.05$. ANOVA for IF: $F_{4,16}= 24.89$, $P < 0.0001$. ANOVA for EJL: $F_{4,16}= 28.75$, $P < 0.0001$.

Most pairings resulted in a single ejaculation (8/11) during the observational period, however, based on observed locking events, three males ejaculated twice (2 in late follicular, 1 in early luteal) with a mean interval between ejaculations of 36 ± 11 minutes. No visible pelvic thrusting was evident during either intromission or ejaculation and genital grooming followed all intromission and ejaculation sequences. In all pairings for which ejaculation was observed, vaginal lavages from the following morning revealed no spermatozoa or visible seminal plugs. Also, litters in this experiment were born with a mean pairing to birth interval of 40.9 ± 0.8 days and an average litter size of 2.4 ± 0.1 pups (Figure 6A and B).

2.5 Discussion

This study has described an improved analysis of spiny mouse copulatory behaviour from that published by Dewsbury and Hodges (1987) and provided new data on the relationship between mating success and the phases of the spiny mouse menstrual cycle in our captive colony. In their original study, Dewsbury and Hodges argued that male copulatory patterns cannot be predicted from knowledge of the female estrous cycle. Our study challenges this assertion. We have demonstrated the clear presence of a broad 'mating window' in which mating can occur during the early follicular, late follicular and early luteal phase phases of the menstrual cycle, but not during the late luteal or menses phases. In mammals, gonadal steroids are known to affect sexual behaviour and mating receptivity (Jennings & de Lecea, 2020). This relationship is true in female rodents (Voss, 1979), and also women, where copulation is significantly more frequent during their ovulatory window (Wilcox et al., 2004; Wilcox et al., 1995) when testosterone and dihydrotestosterone (DHT) concentrations are the highest (Rothman et al., 2011). Although androgen concentrations during the menstrual cycle in *A. cahirinus* have not been reported, our observations that females may also be receptive to mating several days either side of ovulation suggest a possible relationship between copulation frequency and changing androgen levels.

Unexpectedly, pairing-birth intervals across each stage where litters were produced were similar. However, the time between the early follicular and early luteal phases of the menstrual cycle may differ by up to 2 days in the spiny mouse (Bellofiore et al., 2017). Regardless, it appears that individual pairs established during the early follicular to early luteal phases of the cycle produce litters after a similar period to those reported in postpartum female spiny mice (41 days ± 4.4 days (SD); unpublished data from our colony). Thus, our method of timing pairing to a particular phase of the menstrual cycle, as

outlined here, resulted in similar gestational outcomes to mated postpartum dams, while reducing study duration and costs.

Another distinction between our study and that of Dewsbury and Hodges (1987), is the use of vaginal lavage to confirm behavioural cues for, and the timing and location of, ejaculation within the female reproductive tract. No spermatozoa were present in vaginal lavages taken immediately after individual, non-locking, intromission events. Despite interruptions to mating activities to obtain vaginal smears, and the potential stress involved in this procedure, spiny mice pairs rapidly resumed sexual activity after each consecutive vaginal lavage. This is an interesting and important observation for future mating studies because, although spiny mice are known to be susceptible to stress (unpublished observations from our colony), it appears that the mating drive is strong enough to overcome any stress caused by the disturbance of removing females briefly for vaginal lavage. Spermatozoa were seen in vaginal smears of females immediately after a copulatory lock, suggesting intravaginal ejaculation. However, as post-coital reproductive tracts of females were not examined in this study, we cannot rule out the possibility of intrauterine or intracervical insemination, as occurs in some other species like pigs and the camelids (Hawk, 1983).

Our mating behavioural analysis reveals several similarities to Dewsbury and Hodges (1987). We observed no pelvic thrusting during either intromission or immediately prior to ejaculation, and a series of intromissions always precedes ejaculation. We also observed no obvious lordosis in female spiny mice during intromission or ejaculation; a feature that is typical of murid copulation (Hull et al., 2007). Instead, we observed a distinct male foot twitch behaviour, which was not reported by Dewsbury and Hodges (1987). Interestingly, a

similar behaviour, ‘thumping’, was reported in Mongolian gerbils in which either individual taps its hind feet against the cage floor immediately following coitus (Kuehn & Zucker, 1968). However, the cause of this behaviour is ambiguous as it presents in both sexes in other non-coital settings and has been considered a sign of stress (Clark & Galef Jr, 1977). In contrast, the male spiny mouse foot twitch that occurred only during coitus, we interpret as a behavioural response of males to pre-ejaculatory penile insertion during mounting on a female not presenting any clear lordosis. Multiple intromissions have been suggested as a necessary requirement in rodents, and rabbits, where extensive vaginal-cervical stimulation is required to trigger ovulation (Dewsbury, 1975, 1990). However, given that a menstrual cycle with spontaneous ovulation and an active luteal phase, rather than the usual rodent oestrous cycle, has been confirmed in the spiny mouse (Bellofiore et al., 2017), this relationship seems unlikely and multiple intromissions are a more likely prerequisite to stimulate ejaculation, rather than stimulating ovulation.

We also confirm the presence of a copulatory lock and no obvious copulatory plug in *A. cahirinus*. In an extensive review of 118 mammalian species, Dewsbury (1975) categorised mating behaviour into 16 categories; the most common patterns being # 9 (no lock, intravaginal thrusting, multiple intromissions and ejaculations) and #13 (no lock, no intravaginal thrusting, multiple intromissions and ejaculations). Interestingly, all species in these two categories were either primates or rodents, with the majority of rodents falling into category #13. From this, it appears that mating behaviour in *A. cahirinus* is broadly similar to that of other rodent species but with the addition of a copulatory lock. Seminal plugs are common in rats and guinea-pigs, but only observed in a few mouse species (Voss, 1979), and these non-plugging species generally have copulatory locks and reduced or underdeveloped accessory glands (reviewed by (Hartung & Dewsbury, 1978)).

Further, Voss (1979) argued that if there is a causal relationship between the presence of copulatory locks and the absence of plug formation, they 'must serve much the same function(s) as the plugs they presumably replaced'.

Male spiny mice have a normal complement of accessory glands typical of many murid rodents (Peitz et al., 1979), with a large well-developed seminal vesicle but a comparatively small prostate and coagulating glands. Hartung and Dewsbury (1978) have suggested that well-developed accessory glands are required for rodent seminal plug formation, but no copulatory plug has been observed in spiny mice. However, coagulation studies using spiny mouse accessory gland secretions (Peitz et al., 1979), especially mixing of extracts from the seminal vesicles and the coagulating glands, shows coagulum formation. Together, this suggests the possibility of a covert post-ejaculatory seminal plug in spiny mice, perhaps deep within the vagina against the cervix or within the cervical canal.

The functional role of copulatory plugs in rodents has been debated for centuries (Stockley et al., 2020) and several hypothesis have been suggested. These include prevention of insemination by rival males, assisting sperm transport, induction of pseudopregnancy and prevention of sperm leakage from the vagina (Voss, 1979). None of these hypothesis are likely to apply in *A. cahirinus* considering the extremely brief lock compared to true locking species (Dewsbury, 1972) and the demonstrated inability to induce pseudopregnancy in this species (Bellofiore et al., 2020). An alternative explanation is that, despite the brief ejaculatory lock, spiny mice deposit most or all of the ejaculate directly into the cervix or uterus like camelids and pigs (Hawk, 1983). Although spermatozoa were seen in vaginal lavages immediately following the brief locking events, these spermatozoa may be

flowback through the cervix or leakage from the penis during withdrawal from the vagina. Considering this, if ejaculation does occur within the cervix or the uterus, formation of a small, very temporary, seminal plug may assist in maintaining spermatozoa at the site of ejaculation. Future studies of female spiny mouse reproductive tracts following coitus may provide answers to these questions on the site of insemination and presence of post-coital seminal plug, and provide new information on sperm concentration, survival and transit through the female tract.

2.6 Conclusion

This study has extended and improved the analysis of spiny mouse mating behaviour reported by Dewsbury and Hodges (1987) and, importantly, it provides a comprehensive description of mating behaviour across the recently discovered menstrual cycle of this species. Here, we report predictable mating behaviour, pairing to birth intervals and a more reliable method for staging gestational age in female spiny mice paired at particular phases of the menstrual cycle. Ejaculation was seen in 11/15 pairs (73%) during the mating window, and an average of 2 pups were delivered from 10/11 (91%) pairs between 38 and 43 days later. Moreover, females paired during the late luteal or menses phases were not receptive to mating, and males displayed no mating behaviour during these phases. This study has confirmed its original aim of providing a method for more reliably estimating gestational and fetal age in pregnant spiny mice. This will improve gestational studies by reducing both the research time and financial costs of using postpartum animals.

Chapter 3: Postpartum ovulation and early pregnancy in the menstruating spiny mouse, Acomys cahirinus

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Postpartum ovulation and early pregnancy in the menstruating spiny mouse, *Acomys cahirinus*

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3.1 Abstract:

Egyptian spiny mice are the only known species to have human-like menstruation and a postpartum ovulation. Unfortunately, no endocrine or morphological evidence has been provided for a postpartum ovulation in spiny mice, and while later stages of pregnancy have been well studied, early events including embryo implantation and spiral artery remodelling have not been reported. This study compared the sex steroid endocrinology and reproductive tract morphology of dams at eight timepoints (n=40) postpartum to determine the timing of ovulation and the timing and invasiveness of embryo implantation in *A. cahirinus*. Reproductive tracts were fixed and stained for histology and immunohistochemistry, and plasma was prepared for enzyme-linked immunosorbent assay. Ovarian histology and estradiol-17B concentrations indicate ovulation within 48hrs of parturition and then immediate resumption of follicular growth. Uterine histology and immunohistochemistry revealed progressive epithelial repair, endometrial growth and spiral artery assembly and remodelling in dams postpartum. Blastocysts were seen in the uterine lumen at day 4-5 postpartum and embryos had implanted superficially with minimal stromal invasion by day 5-6. This study provides further evidence for the unique, humanesque reproductive biology of spiny mice and for a postpartum ovulation using endocrine and morphological changes observed during early pregnancy. Taken together, our data suggest that spiny mice may act as appropriate models of human pregnancy disorders such as implantation failure or pre-eclampsia.

3.2 Introduction

Several species of mammals experience a postpartum ovulation (PPO) during which they ovulate and copulate within 24 hours of parturition (Carrillo-Martínez et al., 2011). In rodents, the PPO is primarily controlled by endogenous steroid hormones (Blaustein, 2008) and maximises reproductive efficiency through a shorter generation interval between litters. In menstruating species, ovulation occurs spontaneously in response to a surge of estradiol and luteinizing hormone (LH) (Lorenzen et al., 2015). Following ovulation, formation of the corpus luteum (CL) and increased progesterone secretion induce terminal differentiation of endometrial stomal cells (spontaneous decidualisation; SD) in preparation for embryo implantation (Bellofiore, Cousins, et al., 2018). While these processes occur naturally during the primate menstrual cycle, ovulation, SD and embryo implantation have not been reported in any menstruating species postpartum; this is due to the suppression of ovarian function by lactational amenorrhea (Rolland et al., 1975).

Further, Gray et al. (1987) reported anovulation for 45 days on average postpartum in non-breastfeeding women, compared to an average 189 days in those who consistently breastfed. In contrast, the Egyptian spiny mouse (Acomys *cahirinus*), the only rodent to have a primate-like menstrual cycle (Bellofiore et al., 2017), also experience a PPO (Gilbert, 1984). Considering the highly consistent 40-day inter-birth interval (Dickinson & Walker, 2007), PPO has been assumed to occur 24 hours after litter delivery and the sequence has been used to estimate gestational and fetal age in recent studies (Hułas-Stasiak et al., 2017; Ireland et al., 2011; O'Connell et al., 2013) owing to the lack of a discernible vaginal plug after mating (Bellofiore et al., 2020). Additionally, because spiny mice mothers actively nurse their young from birth (observations from our colony), and

assuming spiny mice have similar endocrine control of ovulation as humans, dams would be expected to remain anovulatory whilst nursing.

Moreover, while murid rodents form the same type of placenta as humans (haemochorial), embryo implantation in mice is superficial with minimal trophoblast invasion compared to the interstitial, aggressive implantation in humans (Bellofiore, Cousins, et al., 2018). While spiny mice also form a haemochorial placenta (O'Connell et al., 2013), the mode of embryo implantation and the morphology and endocrinology of the postpartum reproductive tract have not been characterised. These basic observations are required to further assess the role of *A. cahirinus* as a suitable model for female reproductive function, and to understand the evolutionary biology of menstruation in this species. Therefore, this study aims to 1) identify the timing of PPO through evaluation of sex steroid endocrinology, 2) evaluate the gross morphology of the postpartum reproductive tract, and 3) to describe the timing and invasiveness of embryo implantation in *A. cahirinus*.

3.3 Methodology

3.3.1 Animals

All experimental procedures in this study adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and complies with the ARRIVE guidelines. All spiny mice were sourced from our Monash University breeding colony and approved for use by the Monash Medical Centre Animal Ethics Committee (MMCB 2019/13BC).

3.3.2 Tissue collection

Female spiny mice (n=40, >80 days and multiparous [≥ 1 litter]) were assessed at eight time points postpartum (pp): Day 0-6 and Day 10pp. Day 10pp animals were included to confirm embryo implantation and to rule out the possibility of embryonic diapause or delayed implantation. Females were anaesthetised with isoflurane prior to cardiac puncture and vaginal lavage (Bellofiore et al., 2017). Whole blood (0.5-1mL) was collected in heparinised tubes, centrifuged (3000 RPM at 4^{o}C for 10 minutes) and plasma aspirated and stored at -80^{o}C for later analysis. The reproductive tract with both ovaries attached was dissected out and trimmed of fat and uterine mesentery. Each ovary was then separated from its respective uterine horn and fixed in 10% buffered formalin (NBF). Uterine horns were separated by cutting through the cervix and uterine fundus and each horn fixed separately in NBF. Tissues were processed to paraffin wax, embedded and sectioned at 5um thick onto super-frost plus microscope slides (ThermoFisher, Australia). Slides were dried overnight and baked at 60^{o}C for 15 minutes prior to histology or immunohistochemistry.

3.3.3 Hormone analysis

Spiny mouse plasmas were analysed for oestradiol-17b (E2) (ES180S-100, Calbiotech, USA), progesterone (P4) (55-PROMS-E01, ALPCO, USA). Each sample was assayed in duplicate and the validity of each assay was tested and confirmed with spike-recovery and linearity of dilution procedures following the manufacturer's instructions. In-house ELISA data was also analysed externally by radioimmunoassay (RIA; P4) and liquid chromatography mass-spectrometry (LC-MS; E2) by Monash Pathology (Melbourne, Australia).

3.3.4 Ovarian and Uterine morphology

Ovary and uterine sections were stained with haematoxylin and eosin as previously reported (Bellofiore et al., 2017). Ovaries were cut at 5um thickness and every 8th section collected. Large antral follicles (>400um; AF) and CLs were counted at 10X magnification using an EVOS M7000 imaging system and AFs and CLs classified as previously described in this species (Hułas-Stasiak et al., 2017). Endometrial thickness was measured at 20X magnification from three randomly selected fields of view (FOV) taken per animal at each time point using ImageJ software (National Institutes of Health) and mean values ± standard deviation (SD) calculated and compared across each time point. Endometrial thickness was defined as the distance from the myometrium to the uterine epithelium as described by Bellofiore et al. (2017). As the uterine epithelium was not apparent in day 10pp sections, endometrial thickness was not recorded at this time point.

3.3.5 Immunohistochemistry

FOX01 immunostaining was used to assess luminal epithelial receptivity (Vasquez et al., 2018). Tissues were dewaxed, and antigen retrieval conducted in citrate buffer pH 6.0)

using a harsh retrieval method (boiled in the microwave for 9 minutes, simmered for 7 minutes and allowed to sit for 40minutes). Slides were washed in Tris-buffered saline (TBS) with Tween-20 (0.05%) (TBS-T, 2X 5 minutes), followed by one wash in TBS. Endogenous peroxides were blocked in 3% hydrogen peroxide solution for 10 minutes at room temperature, prior to washing as described. Serum block (X0909, Dako, USA) was then applied for one hour at room temperature prior to primary antibody incubation (C29H4, Cell signalling, USA; 1:100) overnight at 4^{o}C. Negative control sections were incubated with host species IgG in place of primary antibody at the same concentration. Slides were washed thrice in TBS before incubation with secondary biotinylated antibody (BA-1000, Vectorlabs, USA; 1:250) for 30 minutes. Slides were then washed and incubated in avidin-biotin complex (Vectastain Elite ABC kit, Vector Labs) for 45 minutes at room temperature. Slides were washed once more and 3,3'-Diaminobenzidine (DAB) applied to each section in 30 second increments until stain developed. Slides were then rinsed in dH_2O and counterstained in 10% Harris hematoxylin for 3-5minutes. Slides were dehydrated and coverslipped as above.

3.3.6 Immunofluorescence

To assess changes to vasculature and epithelium integrity postpartum, uterine tissue sections were subjected to double immunofluorescent staining for alpha smooth-muscle-actin (aSMA; #M0851, Dako) and cytokeratin (#15367, Santa-cruz, USA), as previously described (Bellofiore, Rana, et al., 2018) with modifications. Slides were baked at 60^{o}C for 20 minutes and dewaxed through 3X changes of xylene, graded ethanols and rehydrated in dH_2O for 5minutes. Antigen retrieval was performed in citrate buffer (2.94g Tris-sodium dihydrate in dH_2O, pH 6.0) using a mild retrieval method (boiled for 5 minutes in the microwave and allowed to sit for 20 minutes). Slides were washed in PBS-T (0.05%) (2X 5

minutes), followed by one wash in PBS. Glycine solution (1.877g of glycine in 250mL PBS) was then applied for 30minutes at 4°C in the dark. Slides were washed thrice (as above) and incubated in 3X changes of sodium borohydride solution (1g/100mL dH_2O) for 8 minutes and washed again. Serum block (Dako) was applied for 1 hour at room temperature in the dark prior to primary antibody incubation. Mouse anti-aSMA (#M0851, Dako; 1:200) and rabbit anti-cytokeratin (#SC-15367, Santa-cruz; 1:200) were applied to each section and incubated at 4°C overnight. Negative controls were incubated with serum block in place of primary antibodies. Slides were thrice washed in PBS prior to secondary antibody incubation. Goat anti-mouse (#A11029, Invitrogen, Australia; 488nm) and donkey anti-rabbit (#R37115, Invitrogen; 594nm) were applied at 1:500 to all sections in the dark for 30 minutes at room temperature. Slides were washed thrice in PBS and mounted with anti-fade with DAPI (#P36931; Invitrogen) prior to analysis.

3.3.7 Image acquisition

Immunofluorescent images were captured using an Olympus BX43 upright fluorescent microscope and all other images captured using an EVOS M7000 imaging system. To analyse DAB stained tissues, three randomly selected FOVs were taken at 20X magnification and were blindly analysed by covering slide labels and randomly assorting slides prior to analysis using ImageJ software. For immunofluorescent tissues, three FOVs were taken of each fluorescent channel separately and blindly analysed as above. Area coverage (%) was used as a measure of immunopositive structures in the postpartum reproductive tract and each FOV was calculated to give a mean value per sample and compared against other time points. As the stratum functionalis was not apparent in day 0-2pp uterine sections, vasculature was not assessed at these time points. Similarly, as the

uterine epithelium was not apparent in day 10pp sections, epithelial integrity (cytokeratin) and receptivity (FOX01) was not assessed at this timepoint.

3.3.8 Statistical analysis

All statistical analyses were conducted using Prism 8.0 (Graphpad Prism Software Inc, USA). Data was normally distributed and compared by a one-way ANOVA between time points and data expressed as mean ± SD. The Shapiro-Wilk test was used to verify normal distribution and statistical significance was set at $p<0.05$ for all datasets.

3.4 Results

3.4.1 Comparative ovarian morphology and endocrinology between time points

Antral follicles are present in all postpartum time points except for day 2pp (Figure 1). The mean number of AFs was not significantly different between days 0, 4-10pp or between days 1-4pp ($p>0.05$; Figure 1E). The number of CLs present in the postpartum ovary was not significantly different between any time points ($p>0.05$; Figure 1D). Circulating E2 and P4 levels were detected in spiny mouse plasma across all time points (Figure 1G-H, Appendix Figures 1-2). E2 levels were significantly elevated on days 0-1 and 5-10pp compared to all remaining time points ($p<0.05$; Figure 1G) whereas P4 concentrations were similar across all time points postpartum ($p>0.05$; Figure 1H).

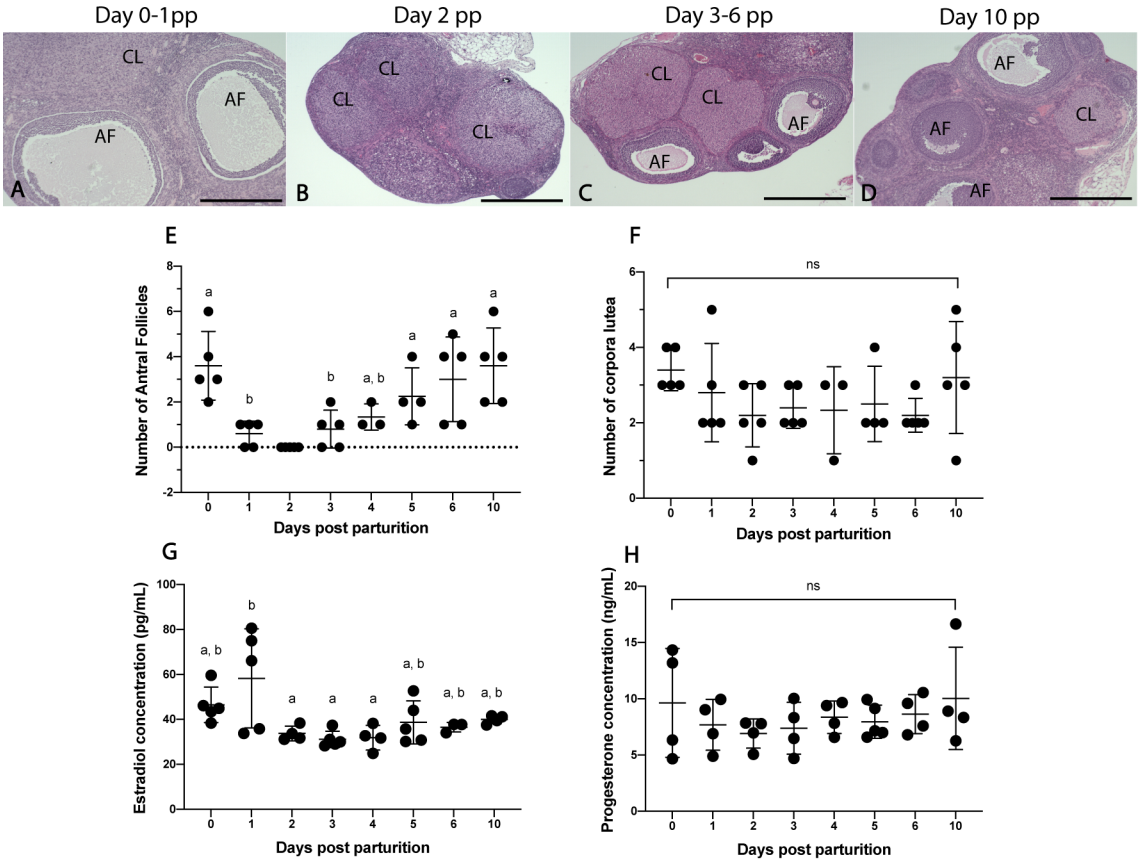

***Figure 3* Comparative ovarian morphology and endocrinology between time points**. Large antral follicles are present at all time points except for day 2pp and corpora lutea are present at all time points (A-D). Mean antral follicle counts on days 1, 4-10pp were similar and significantly different to days 1-3pp (E). Mean number of corpora lutea was not significantly different (ns) across all time points (F). E2 levels on days 0-1 and 5-10pp were similar but were higher than all remaining timepoints (G). P4 levels were similar across all timepoints (H). Graphs depict mean ± SD and data with different letters (a or b) differ significantly ($p<0.05$; ANOVA). Scale bars for A-D indicate 500um. Antral follicle (AF), corpus luteum (CL).

3.4.2 Vaginal cytology and uterine morphology between time points

Vaginal lavages from animals on days 0-1pp were almost entirely comprised of erythrocytes, with some samples also containing shed uterine tissue (Figure 2A-B). By day 2pp, erythrocytes have decreased in number and smear cytology consists of mainly leukocytes. Cytology from the remaining timepoints (days 3-6pp, 10pp) consisted predominantly of leukocytes and small numbers of cornified epithelial cells (CECs) and nucleated epithelial cells (NECs) (Figure 2C-D). These cytological features were indicative of the secretory/luteal phase in spiny mice. No spermatozoa were identified in the vaginal lavages at any timepoint.

From histological evaluations, changes in the thickness of the uterine epithelium and endometrium were observed during early pregnancy in the spiny mouse uterus postpartum. The stratum functionalis was shed at parturition and the uterine endometrium remained as a very thin layer (<300um) until day 3pp (Figure 2E-G). On days 0-1pp, the luminal epithelium was mostly low cuboidal, but in some areas, it was absent often with remnants found sloughed into the lumen (Figure 2E). By day 2pp, an increase in endometrial thickness was observed with less sloughed tissue in the uterine lumen (Figure 2F). By day 3pp the epithelium consisted of tall, columnar cells, each with a noticeable vacuole (Figure 2G). Moreover, the endometrium was fully grown by day 3pp and endometrial thickness was similar to days 4-6 (Figure 2G, I). The uterine lumen was closed by day 10pp and the luminal epithelium had regressed (Figure 2H-I).

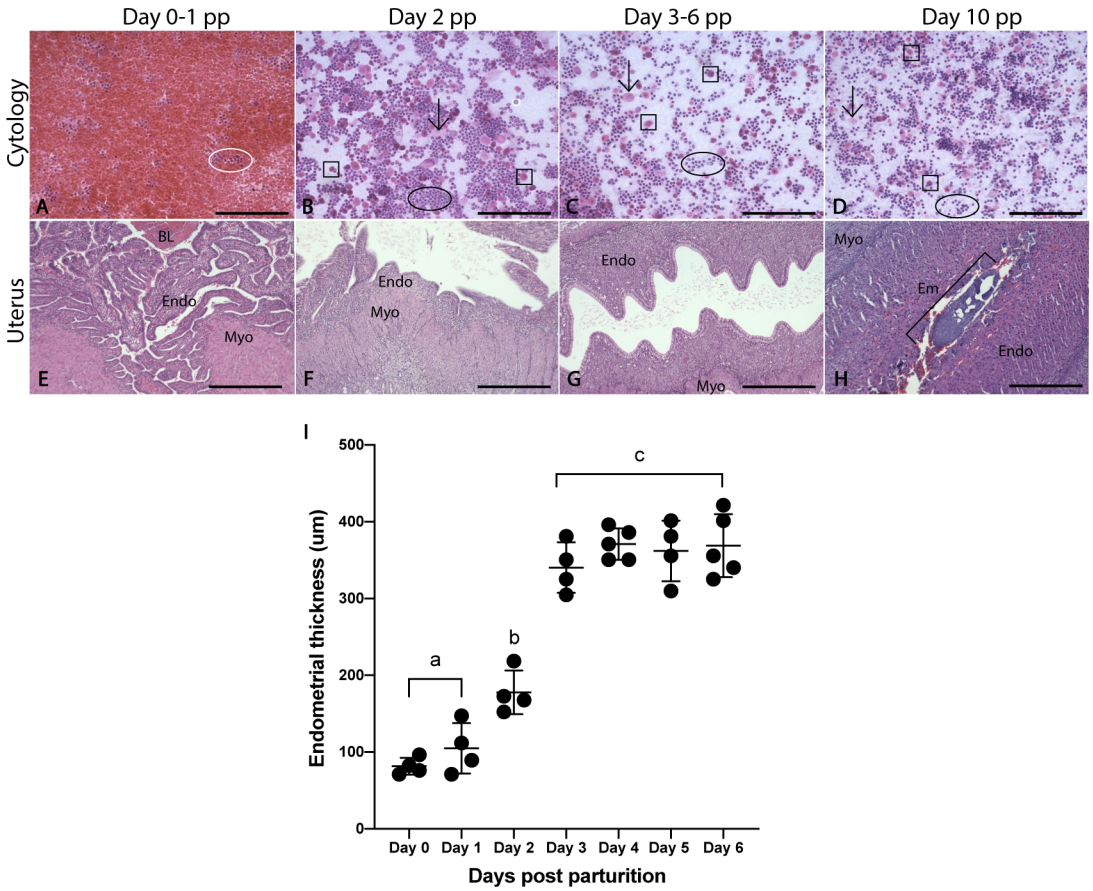

***Figure 4* Comparative vaginal cytology and morphological changes to the postpartum uterus between time points.** Vaginal cytology (A-D) across all remaining timepoints is dominated by leukocytes (circles) with a smaller number of CECs (arrows) and NECs (squares). Endometria on days 0-1pp are thin (<200um) and are not-significantly different but are significantly different to the remaining timepoints (E, I). On day 2pp, the endometrium begins thickening rapidly and reaches maximum thickness by day 3pp (F-I). Endometrial thickness on days 3-6 are similar (I). Day 10 data are excluded from analysis due to closure of the uterine lumen and regression of the luminal epithelium. Graphs depict mean ± SD and data with different letters differ significantly ($p<0.05$; ANOVA). Myometrium (Myo), endometrium (Endo), embryo (Em). Scale bars are 100um for cytology (A-D) and 500um for uterine sections (E-H).

3.4.3 Timing and invasiveness of embryo implantation

Preimplantation embryos are seen in the uterine lumen by day 4pp (Figure 3A, B) and early, superficial embryo implantation sites are evident in day 5-6pp (Figure 3C, D). By day 10pp, implantation has advanced in the endometrium (Figure 3E) as depicted by luminal closure though with minimal stromal invasion.

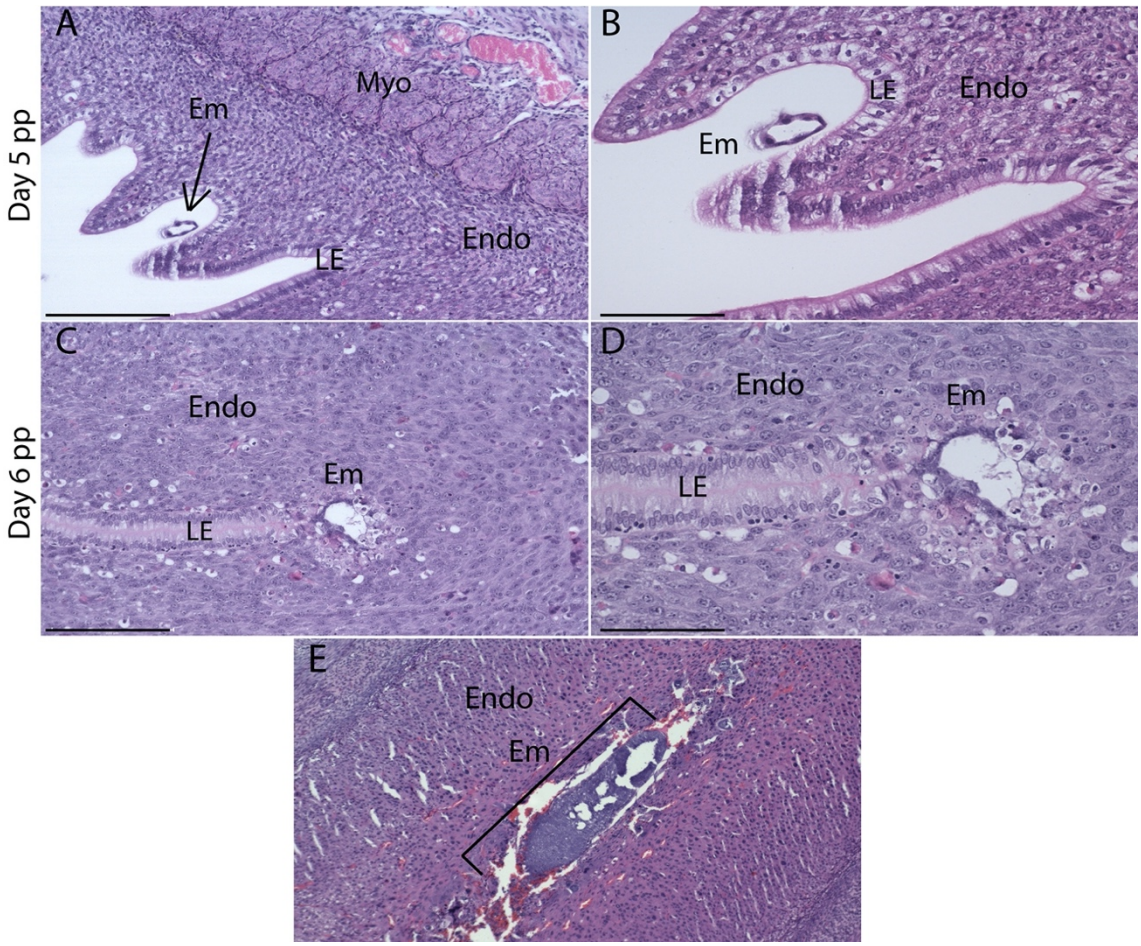

Figure 3 Histological sections of preimplantation embryos and implantation sites in postpartum uteri. Blastocyst (arrow) in the day 5pp uterine lumen apposing sections of luminal epithelium (A, B). Embryo implantation sites were seen on day 5pp (C, D). A larger, more advanced implantation site is visible in day 10pp uteri with no remaining epithelium. Scale bars for A, C and E are 200um, and scale bars for B and D are 100um. Luminal epithelium (LE), embryo (Em). Endometrium (Endo), myometrium (Myo), luminal epithelium (LE), embryo (Em).

3.4.4 Spiral arteriole remodelling and epithelial integrity

During days 0-2pp, large arteries were present in the basal endometrium and were immunopositive for aSMA (Figure 4A-B, D, J); no cytokeratin expression was detected (Figure 4C, D, I) Vasculature in the basal endometrium from day 3-6pp tissues were smaller than at previous time points and were immunopositive for aSMA (Figure 4 E-F, H, J). Spiral artery assemblies in the superficial endometrium were immunopositive for aSMA, and the luminal epithelium was positive for cytokeratin (Figure 4E-I). No endometrial tissues or structures were positive for aSMA on day 10pp and cytokeratin was undetectable (Figure 4I-J).

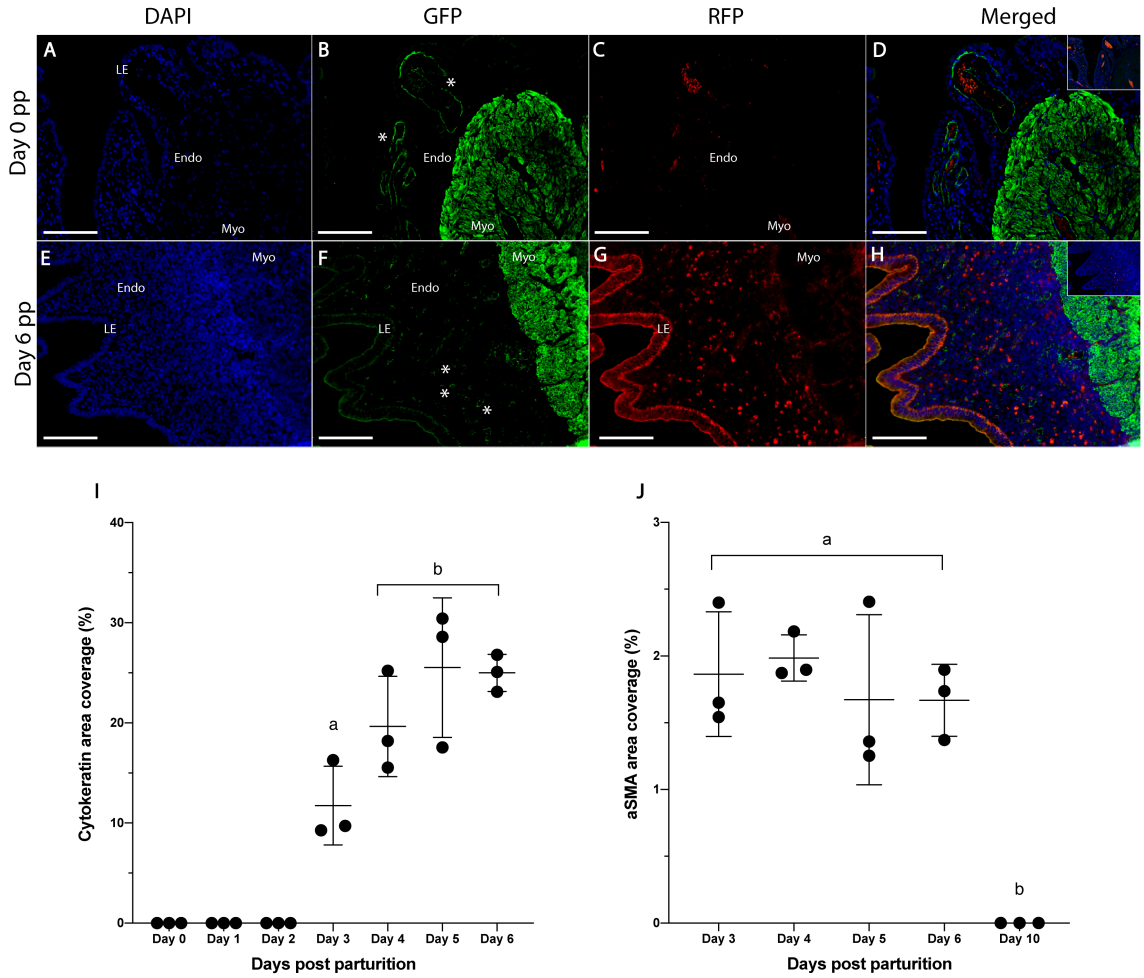

Figure 4 Comparative changes in cytokeratin and aSMA expression in the spiny mouse postpartum endometrium. Blue = DAPI, Green =aSMA, Red = Cytokeratin. A cluster of aSMA positive arteries in the endometrium (* in B) and strong aSMA positive staining in the myometrium of day 0pp sections. Arteries in the superficial endometrium of day 6pp sections (* in F) are aSMA positive. Spiral arteries were first seen on day 3pp (J) and the area coverage of aSMA positive tissue was not significantly different across days 3-6pp but was significantly higher than day 10pp (J). The day 0-2pp luminal epithelium is negative for cytokeratin (C), however cytokeratin is detected in the luminal epithelium by day 3pp (I). Cytokeratin reached maximal expression by day 4pp (I). Day 0-2pp endometria were omitted from aSMA analysis as there was no clear stratum functionalis. As the uterine lumen was closed by day 10pp, this timepoint was removed from cytokeratin analysis. Inset squares (D, H) are negative controls of the same tissue. Data depict mean ± SD and data with different letters differ significantly ($p<0.05$; ANOVA). Scale bars for A-H are 100um. Endometrium (Endo), myometrium (Myo), luminal epithelium

3.4.5 Epithelial receptivity to implantation

Minimal staining of FOX01 was detected during the follicular and menses phases; it remained restricted to the nuclei of myometrial and luminal epithelial cells (LECs; Figure 5A, C). FOX01 expression was nuclear and cytoplasmic of LECs during the luteal phase and was significantly increased compared to the follicular and menses phases (Figure 5B, J; $p<0.05$). On days 0-1pp, LECs were negative for FOX01, however stromal cell and myometrial cell nuclei were faintly positive (Figure 5D). Day 2pp LECs showed minor

nuclear staining (Figure 5E). FOX01 expression was elevated on day 3pp with expression in the nuclei and cytoplasm of LECs (Figure 5F) and reached maximal expression by day 4pp (Figure 5G, J). Day 10pp uterine tissue (Figure H) and negative control tissues (Figure 5I) were negative for FOX01.

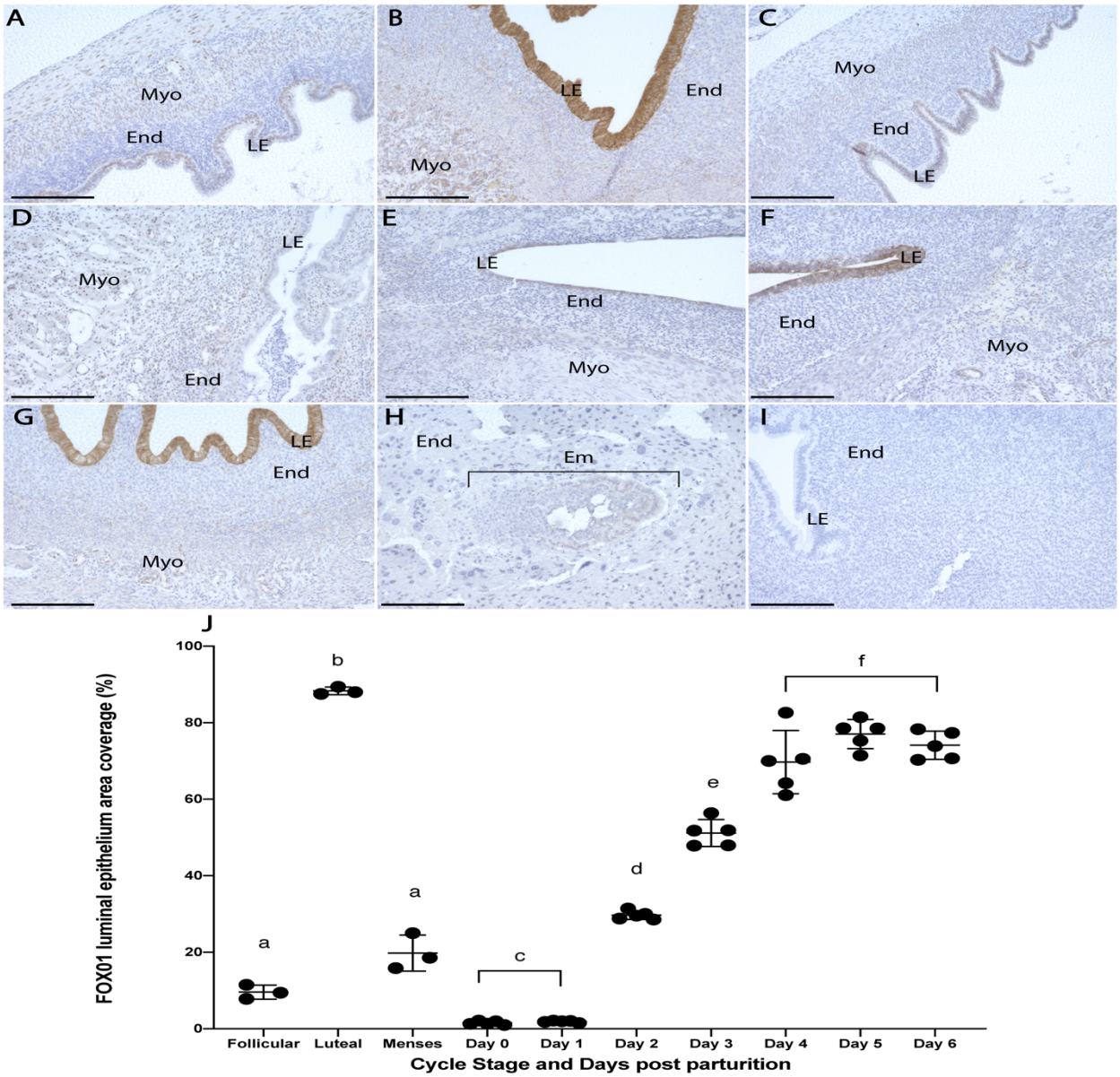

Figure 5 Comparative FOX01 expression during the menstrual cycle and 7 days postpartum. Follicular phase tissue (A) exhibited minor nuclear staining in luminal epithelial and myometrial cells. (B) Luteal phase luminal epithelial cells showed strong expression of FOX01 and menses phase tissue (C) were similar to the follicular phase. On days 0-1pp (D), luminal epithelial cells were negative for FOX01, but stromal and myometrial cell nuclei were faintly positive. By day 2pp (E), the luminal epithelium showed minor nuclear expression of FOX01. FOX01 expression was primarily cytoplasmic on day 3pp (F) and showed minor nuclear and major cytoplasmic staining in the luminal epithelium on day 4pp (G). Day 10pp tissues (H) were negative for FOX01 and negative control tissue is provided in (I). FOX01 expression was similar between follicular and menses phases but was significantly increased during the luteal phase (J). A significant increase of FOX01 staining was seen in the luminal epithelium on day 2pp compared to days 0-1pp. Similarly, day 3pp expression was significantly higher than on day 2pp and by day 4 FOX01 expression had peaked (J). Data depict mean ± SD and data with different letters differ significantly ($p<0.05$; ANOVA). Scale bars indicate 200um. Endometrium (End), myometrium (Myo), luminal epithelium (LE), embryo (Em).

3.5 Discussion

This study is the first to provide evidence for postpartum ovulation in a menstruating species. Our study conflicts with longstanding dogma of lactational amenorrhea in menstruating species (Rolland et al., 1975) and questions the regulation of the hypothalamic-pituitary-gonadal axis in *A. cahirinus.*

The absence of large preovulatory follicles on day 2pp suggests a postpartum ovulation within 48 hours of parturition for female spiny mice in our colony. Folliculogenesis resumes following PPO and large antral follicles (>400um) are seen at all remaining time points. These AFs do not reach the preovulatory size seen in day 0-1pp ovaries suggesting that they are either destined for atresia or maintained through pregnancy acting as endocrine glands until parturition. A similar pattern of follicular growth and maintenance has been reported in rodents (Peters et al., 1975; Schwartz & Talley, 1968) and women (Dekel et al., 1977) where follicles of all sizes, except preovulatory, are seen throughout pregnancy. We cannot confirm from this study if folliculogenesis continues throughout gestation in postpartum spiny mice, but future studies of >day 10pp tissues may reveal the presence and role of antral follicles in later stages of pregnancy.

The number of CL present in the *A. cahirinus* postpartum ovary was similar across all time points. In women, CL regresses between weeks 3-7 of gestation when the placenta assumes the role of the CL and takes over P4 secretion (Hennebold, 2018). Contrastingly, the rodent placenta does not produce enough P4 to maintain pregnancy, therefore P4 secretion is required from corpora lutea and the placenta (Takiguchi et al., 2004) and there are often several CL from recent ovulations and the previous pregnancy present at

parturition. Although both spiny mice and women form a haemochorial placenta (Bellofiore, Cousins, et al., 2018) at around the same point in gestation (O'Connell et al., 2013; Yaron et al., 2002), our results suggest a similar endocrine dependence on corpora lutea during pregnancy as in other rodent species. However, placentation in *A. cahirinus* occurs around gestational day 10-15 (O'Connell et al., 2013) and tissues >day 10 were not collected in this study. Moreover, as large corpora lutea were seen on day 0pp, we cannot rule out the possibility of a pre-partum ovulation as near-term tissues were not collected in this study. Tissue collected near-term and during the period of placental development may reveal the role of the placenta and corpora lutea on progesterone secretion in *A. cahirinus*.

In mammals, a preovulatory E2 surge induces a reciprocal LH surge from the anterior pituitary, which then causes rupture of the dominant follicle, ovulation and formation of the CL (Bellofiore, Cousins, et al., 2018). To our knowledge, a PPO has not been reported in any species with a human-like menstrual cycle. The reason for this is likely to be caused by the sharp decrease in estrogen levels at birth after removal of the placenta (Hendrick et al., 1998), and the onset of lactational amenorrhea (Rolland et al., 1975). However, our hormonal data suggest that an extended period of E2 stimulation up to 48hrs after parturition may exceed the ovulatory threshold in *A. cahirinus*. Interestingly, the E2 concentrations measured here are similar to late proestrus rats and postpartum mice (McCormack & Greenwald, 1974; Nilsson et al., 2015) suggesting this relationship may be conserved in rodents despite the pronounced differences in the reproductive cycle. However, we were unable to measure circulating LH (or FSH) levels in this study using commercially available mouse/rat or human assays. Although the amino acid sequence of peptide hormones is similar across mammalian species (Mullen et al., 2013), failure of current human and rodent assays to measure FSH and LH in the spiny mouse will now

await further investigations to design species-specific antibodies for these immunoassays. Combined with our data, circulating LH levels will provide key evidence for confirming postpartum ovulation in spiny mouse dams.

Circulating progesterone levels were similar across all time points and were similar to concentrations reported during the luteal phase in women (Soules et al., 1989) and rats (Nilsson et al., 2015). However, our data differ from concentrations reported in the mouse during the luteal phase and early pregnancy (McCormack & Greenwald, 1974; Murr et al., 1974). Steroid hormones during pregnancy are known to vary significantly between menstruating species (Chambers & Hearn, 1979) and between murine strains (McCormack & Greenwald, 1974; Murr et al., 1974). Considering this, circulating P4 levels during the luteal phase of cycling spiny mice are considerably elevated (5-15 fold) compared to postpartum females (Bellofiore et al., 2017). Prolactin is known to influence fertility in nursing mammals by inhibiting the release of GnRH, decreasing LH pulsatile frequency and therefore reducing ovarian steroid production (Grattan et al., 2007; Henderson et al., 2008; Rolland et al., 1975). As spiny mice actively nurse their young, this may explain the considerable reduction in P4 concentrations in postpartum animals compared to cycling animals.

Another explanation for the decreased P4 concentration may be changes to progesterone receptor (P4R) expression. The effects of P4 in the reproductive tract are tightly mediated by its interaction with the P4R (Patel et al., 2015) and altered expression of the P4R has been shown to impede embryo implantation in mice (Franco et al., 2012; Wetendorf et al., 2017) and women (Mazur et al., 2015); generally through an impaired decidual reaction (Wetendorf & DeMayo, 2012). Although markers of decidualisation were not measured

here, implantation sites and cellular morphology in endometria on days 5, 6 and 10pp suggest adequate decidualisation and therefore normal P4 or P4R levels. Analysis of uterine P4R expression and concentrations of hypothalamic or pituitary hormones (including prolactin) have not been reported in this species and remain a future direction for spiny mouse research.

During the first 3 days postpartum, the endometrium is thin, consisting essentially of the stratum basalis. In menstruating species, decidualisation of the endometrium occurs solely in the stratum functionalis, which is later shed during menses or parturition (Bellofiore, Cousins, et al., 2018; Bellofiore et al., 2017). Moreover, endometrial thickness during days 0-2pp is similar to the endometrial thickness during the early follicular phase in this species and by day 3pp, repair and growth of the endometrium has begun. The endometrium at this stage is structurally similar to the luteal phase in *A. cahirinus* (Bellofiore et al., 2017), suggesting a similar timeframe for postpartum endometrial repair and growth as during the menstrual cycle. Thus, our data show that the postpartum reproductive tract has similar structural and physiological characteristics to follicular and luteal phase reproductive tracts of spiny mice during the menstrual cycle.

Rodents undergo eccentric embryo implantation by gestational day 5 with the blastocyst forming an implantation ‘chamber’ in the superficial layer of the endometrium without fully invading the underlying stroma (Carson et al., 2000; Vasquez et al., 2018). Conversely, human embryos aggressively invade the uterine luminal epithelium, and are completely embedded within the endometrial stroma (interstitial) (Dey et al., 2004). Our observations show preimplantation embryos in the uterine lumen 4-5 days postpartum and early implantation sites in the superficial endometrium on days 5-6pp. Larger, more advanced

implantation sites were also observed in day 10pp uteri, however minimal endometrial invasion was seen. Considering this, our data suggests that spiny mice embryos implant at a similar time postpartum and with a similar level of invasiveness to other rodents.

Epithelial receptivity and spiral artery assembly begins 72 hours after parturition

An advantage of the spiny mouse as a model of female reproduction is cyclical assembly of spiral arteries during the luteal phase; a feature characteristic of menstruating species (Bellofiore, Rana, et al., 2018). Our data indicate a spatiotemporal expression pattern of endometrial aSMA during early pregnancy. Presence of aSMA positive vasculature in the endometrium by day 3pp provides evidence for simultaneous endometrial growth/repair and spiral artery assembly; similar to the events during the menstrual cycle of this species (Bellofiore, Rana, et al., 2018). However, immunopositive spiral arteries were not detected in the decidua on day 10pp. In menstruating primates, invading trophoblasts break down the smooth muscle layers of spiral arteries resulting in dilated, low-resistance vessels for perfusion of the growing placenta (Kim et al., 1999; Petroff & Hunt, 2005). Further, impaired remodelling of endometrial vasculature has been observed in women with preeclampsia and can significantly affect pregnancy outcomes (PrabhuDas et al., 2015). It appears spiny mice experience a similar breakdown of endometrial vascular smooth muscle during early placental development (around day 10), which further validates the use of this species to investigate pregnancy/angiogenic related disorders such as pre-eclampsia. Considering this and the moderate trophoblast invasion reported here, further investigations are needed to define more clearly spiral artery function and remodelling during placental development in spiny mice.

Cytokeratins have been used as traditional markers for epithelial cell structural integrity (Wonodirekso et al., 1993). Our data demonstrate absent cytokeratin staining in the uterine luminal epithelial cells on days 0-2pp, which is similar to the mid-late menses phase in women (Centola et al., 1984) and cycling spiny mice (Bellofiore, Rana, et al., 2018) where the luminal epithelium has been destabilised and shed during menstruation. Similarly, a significant increase ($p<0.05$) in immunopositive luminal epithelial cells from day 3pp reflects the re-epithelisation and repair process occurring during the late menses phase. Structural integrity of luminal epithelial cells was apparent until day 10pp when the luminal epithelium was largely regressed following embryo implantation; a similar process is seen during rodent embryo implantation (Dey et al., 2004) suggesting a conserved process post-implantation in spiny mice.

While this study is the first to describe the morphology and endocrinology in postpartum spiny mice and highlights its potential as a novel laboratory model for early human pregnancy, we acknowledge several limitations to future research in this species including the lack of commercially produced suite of spiny mouse-specific antibodies. Moreover, although the spiny mouse transcriptome has been drafted (Mamrot et al., 2017), a fully sequenced genome is not yet available to conduct protein sequence homology which has limited our ability to conduct a more comprehensive analysis of decidualisation and hormonal control of PPO and early pregnancy.

However, we have provided convincing, endocrine and morphological evidence for a PPO in spiny mice and described postpartum changes in angiogenesis, epithelial integrity and receptivity, and in the timing and invasiveness of embryo implantation. This study further highlights the uniqueness of *A. cahirinus* reproductive biology and its importance as a

novel, small animal model of human reproduction and we encourage future research in this species to reveal more about their humanesque reproduction.

3.6 Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Chapter 4: Technical report:

Successful sperm cryopreservation in Egyptian spiny mice Acomys cahirinus

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Technical report

Successful sperm cryopreservation in Egyptian spiny mice *Acomys cahirinus*

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Abstract. The menstruating Egyptian spiny mouse has recently been proposed as a new animal model for reproductive health research. Unfortunately, little is known about reproduction in males. This study compared several characteristics of sperm function before and after cryopreservation. Epididymal spermatozoa were cryopreserved in different concentrations of raffinose and skim milk and tested for motility and membrane integrity (Experiment 1). Further evaluations of motility, plasma membrane and acrosome integrity, mitochondrial membrane potential and DNA integrity were conducted with the addition of L-glutamine to the extender (Experiment 2). The results show that, following cryopreservation, motility and membrane integrity were reduced, but were better maintained in the presence of L-glutamine ($P < 0.05$). Moreover, although all sperm parameters were significantly reduced following cryopreservation ($P < 0.05$), most cryopreserved spermatozoa retained acrosome, membrane and DNA integrity while also maintaining motility and mitochondrial membrane potential. This study provides a new step towards the development of assisted reproductive techniques and archiving the important genetics of the world's only known menstruating rodent.

Keywords: *Acomys cahirinus*, acrosome reaction, cryopreservation, DNA integrity, Egyptian spiny mice, fluorescence microscopy, membrane integrity, mitochondrial potential, sperm, spermatozoa.

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Introduction

Sperm cryopreservation combined with IVF is a commonly used assisted reproductive technique (ART) in laboratory species for colony maintenance, the preservation of strain genetics and reproductive studies.

Until recently, the most commonly used murine sperm freezing extender was a combination of 18% w/v raffinose and 3% w/v skim milk, as defined by Okuyama *et al.* (1990). However, Takeo and Nakagata (2010) reported improved post-thawing sperm parameters in C57BL/6 mice by supplementing Okuyama's extender with 100 mM L-glutamine. Mammalian spermatozoa are particularly susceptible to oxidative stress (Aitken *et al.* 2014) and supplementation with antioxidants such as L-glutamine is known to mitigate oxidative damage following cryopreservation (Gharagozloo and Aitken 2011).

The Egyptian spiny mouse *Acomys cahirinus* is a desert-adapted rodent species that exhibits several human-like reproductive characteristics that are rare traits in laboratory rodents, including precocial young (Brunjes 1990), long gestation (Dickinson and Walker 2007) and a menstrual cycle (Bellofiore *et al.* 2017), the latter being present in <2% of all mammalian species (Emera *et al.* 2012). Because *A. cahirinus* thrives in laboratory breeding colonies (Haughton *et al.* 2016), it has great potential as an animal model to study human reproductive function and dysfunction. The aims of the present study were to examine the functional parameters of cauda epididymal spermatozoa and to develop a robust sperm cryopreservation technique for archiving spiny mouse spermatozoa samples for genetic management and future assisted reproductive studies in the species.

Materials and methods

Experimental design

Two *in vitro* experiments were designed to: (1) evaluate the efficacy of six different extender concentrations in preserving the motility and membrane integrity of spiny mouse spermatozoa following cryopreservation (Experiment 1); and (2) assess the effect of L-glutamine supplementation on sperm motility and membrane integrity while optimising tests for the analysis of acrosome integrity, mitochondrial membrane potential (MMP) and DNA integrity (Experiment 2).

Animals

All experiments were approved by the Monash University/Monash Medical Centre Animal Ethics Committee (MMCA/2017/14). The animals used in this study ($n = 30$) were sourced from the Monash research colony and housed under controlled conditions described by Dickinson and Walker (2007).

Table 1. Extender solutions containing different concentrations of raffinose and skim milk

Treatment group	Cryopreservation solutions (% w/v in Milli-Q water)		Abbreviation
1	18% raffinose	3% skim milk	18R3SM
2	15% raffinose	3% skim milk	15R3SM
3	15% raffinose	5% skim milk	15R5SM
4	10% raffinose	5% skim milk	10R5SM
5	15% raffinose	10% skim milk	15R10SM
6	10% raffinose	10% skim milk	10R10SM

Experiment 1 sperm analysis

Epididymal spermatozoa were cryopreserved in six different extenders containing different concentrations of raffinose and skim milk (Table 1). Sperm extenders were prepared as described by Takeo and Nakagata (2010).

Sperm collection and swim-up procedure

Mice were killed by inhalation of an overdose of isoflurane in a sealed chamber containing 0.2 mL isoflurane per animal before excision of the cauda epididymidis. Each cauda epididymidis was incised several times and placed into 1.5-mL warmed tubes (Thermo Fisher) containing either 500 µL extender before cryopreservation or 500 µL M2 handling media (Sigma-Aldrich). Spermatozoa were allowed to swim up in the medium for 30 min at 37°C to isolate the motile population before analysis.

Sperm freezing and thawing

After selecting spermatozoa using the swim-up procedure, 10-µL aliquots of each sperm suspension (30×10^6 spermatozoa mL^{-1}) were loaded into 0.25-mL cryopreservation straws and sealed with polyvinyl alcohol (PVA). The straws were then held in liquid nitrogen vapour (−150°C) for 10 min before plunging and storage in liquid nitrogen for at least on week prior to thawing. Samples were thawed in air for 5 s and then in a water bath at 37°C for 10 min. Each sample was expelled into a 50-µL drop of warmed M2 handling medium at 37°C and incubated for at least 2 h under oil (Sigma) before analysis (Fig. S1, available as Supplementary Material to this paper).

Experiment 1

A 10-µL aliquot of sperm suspension was added to a prewarmed Makler chamber (Origio) and percentage motility was assessed by scoring the number of motile and immotile spermatozoa in randomly selected microscope fields. At least 200 spermatozoa were counted, and two replicates prepared per sample. A qualitative assessment of sperm motility was also performed (+++, fast progressive motility; ++, slow progressive motility; +, non-progressive motility).

Sperm plasma membrane integrity was assessed using a combination of propidium iodide (PI; final concentration 7.0 µg mL^{-1}; Sigma) and H33342 (final concentration 0.21 µg mL^{-1}; Thermo Fisher) staining. After staining, 10 µL sperm mixture was placed on a microscope slide, coverslipped and examined using an Invitrogen EVOS M7000 imaging system (Fig. S1). Fluorescence was detected using 4′,6′-diamidino-2-phenylindole (DAPI; 500 nm) and red fluorescent protein (RFP; 590 nm) emission filters (Table S1).

Experiment 2

The best extender composition determined from Experiment 1 was chosen for Experiment 2 and supplemented with 100 mM L-glutamine. In addition to motility and membrane integrity, spermatozoa frozen in this extender were evaluated for acrosome integrity, MMP and DNA integrity. At least 200 spermatozoa were counted, and two replicates prepared per sample.

Double- and single-stranded DNA breaks were assessed using terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP–digoxigenin nick end-labelling (TUNEL; Roche Diagnostics) with minor modifications to the manufacturer's protocol. Briefly, a 10-µL aliquot of spermatozoa was dried at 37°C on a microscope slide for 30 min, sprayed with methanol fixative (Leica) and left for 1 h at room temperature. Slides were then washed twice with phosphate-buffered saline (PBS) and permeabilised with 0.1% v/v Triton-X in PBS for 10 min on ice. The slides were then washed twice in PBS and incubated with 50 µL TUNEL reaction mix (TdT enzyme + labelling solution) in a humidified atmosphere in the dark for 1 h at 37°C. Spermatozoa were counterstained with H33342 (final concentration 0.22 µg mL^{-1}; Sigma) for 5 min. Slides were washed in PBS, coverslipped and fluorescence was detected using DAPI (500 nm) and fluorescein isothiocyanate (FITC; 535 nm) emission filters on an Invitrogen EVOS M7000 imaging system (Table S1; Fig. S2).

Plasma membrane integrity, acrosome integrity and MMP were assessed simultaneously using a 'quad' stain of H33342, PI, FITC-conjugated peanut agglutinin (PNA-FITC) and 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1). For staining, H33342 (final concentration 0.21 µg mL^{-1}), PI (final concentration 7.0 µg mL^{-1}), PNA (final concentration 1.7 µg mL^{-1}; Sigma) and JC-1 (final concentration 0.2 µg mL^{-1}; Thermo Fisher) were added to a 10 µL sample from each sperm suspension. The mixture was then incubated in the dark for 15 min at 37°C and fluorescence analysed using an Invitrogen EVOS M7000 imaging system (Fig. 1; Fig. S1; Table S1).

Statistical analysis

All data were analysed using Prism 8 software (GraphPad) and samples were tested for normality using the Shapiro–Wilk test and for homogeneity using the Brown–Forsythe test before further analysis. For Experiment 1, one-way repeated-measure

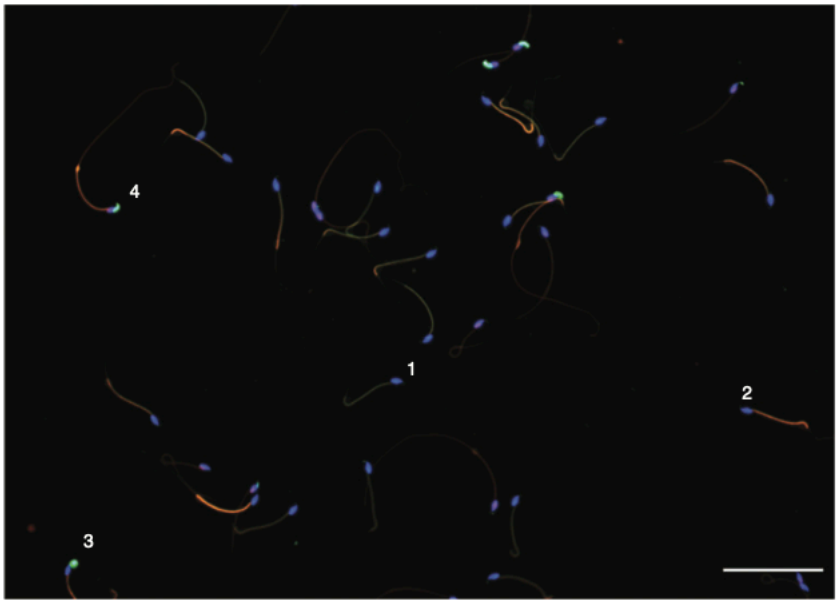

Fig. 1. Fluorescent micrograph showing sperm staining patterns. 1, living spermatozoa with intact acrosomes and low mitochondrial membrane potential (MMP); 2, living spermatozoon with intact acrosome and high MMP; 3, living spermatozoon with a damaged acrosome and high MMP; 4, damaged spermatozoon with a damaged acrosome with high MMP. Scale bar = 50 µm.

analysis of variance (ANOVA) was used to compare data between cryopreservation groups and to determine any inter-subject variability, whereas paired *t*-tests were used to determine any intrasubject variability. For Experiment 2, all sperm parameters were analysed by independent-sample *t*-tests. Statistical significance was set at two-tailed $P < 0.05$ for all experiments.

Results

Experiment 1

No post-thaw motility was observed in spiny mouse spermatozoa cryopreserved in solutions containing 10% skim milk (15R10SM, 10R10SM); this concentration of skim milk was not used in further experimentation. Moreover, post-thaw sperm motility and membrane integrity were low for all treatments when assessed immediately after thawing. However, when samples were allowed to swim-out in M2 for 2 h after thawing, membrane integrity and total motility were significantly improved ($P < 0.0001$) and a qualitative assessment showed an increase in progressive motility (Table S2).

Motility and membrane integrity in the remaining four extenders were significantly reduced compared with fresh controls (Fig. 2*a*; $P < 0.05$). Moreover, motility and membrane integrity in solutions 18R3SM, 15R3SM and 15R5SM were similar ($P > 0.05$), but significantly higher than that in 10R5SM ($P < 0.05$). Inter- and intrasubject variability in all treatments was not significant ($P > 0.05$). Because post-thaw motility was comparable in Treatments 1–3 (18R3SM, 15R3SM and 15R5SM), Treatment 1 (18R3SM) was selected for use in Experiment 2.

Experiment 2

Membrane integrity and total motility were significantly increased in spermatozoa cryopreserved in 18R3SM + L-glutamine compared with 18R3SM alone (Fig. 2*b*; $P < 0.01$). Moreover, mean (±s.d.) motility (67.0 ± 3.7 vs 75 ± 2.3), membrane integrity (74.2 ± 4.3 vs 79.4 ± 1.8), MMP (70.5 ± 4.2 vs 87.5 ± 2.2), acrosome integrity (78.6 ± 1.8 vs 88.2 ± 2.2) and DNA integrity (80.8 ± 2.5 vs 89.2 ± 1.5) remained high after thawing (Fig. 2*c*). (Values here in parentheses represent spermatozoa %, i.e. frozen-thawed % and fresh % respectively.) Qualitative assessment also showed an improvement in progressive motility in spermatozoa cryopreserved in 18R3SM + L-glutamine compared with 18R3SM alone (+++ vs ++). However, all parameters from frozen–thawed spermatozoa were significantly reduced compared with fresh spermatozoa ($P < 0.05$).

Discussion

Cryopreservation has an essential role in the long-term storage of cells and tissues for fertility preservation and for the archiving of important genetic material. Sperm cryopreservation has been routinely performed in many mouse strains (Mochida *et al.* 2014; Taft 2017), as well as in the closely related Mongolian (Sato *et al.* 2000) and Indian (Koshimoto *et al.* 2009) gerbils. However, sperm cryopreservation has not been previously reported for any species in the large murid subfamily Deomyinae.

Optimal conditions for sperm cryopreservation can differ significantly between species due to inherent variability in membrane permeability and osmotic tolerances (Shaw and

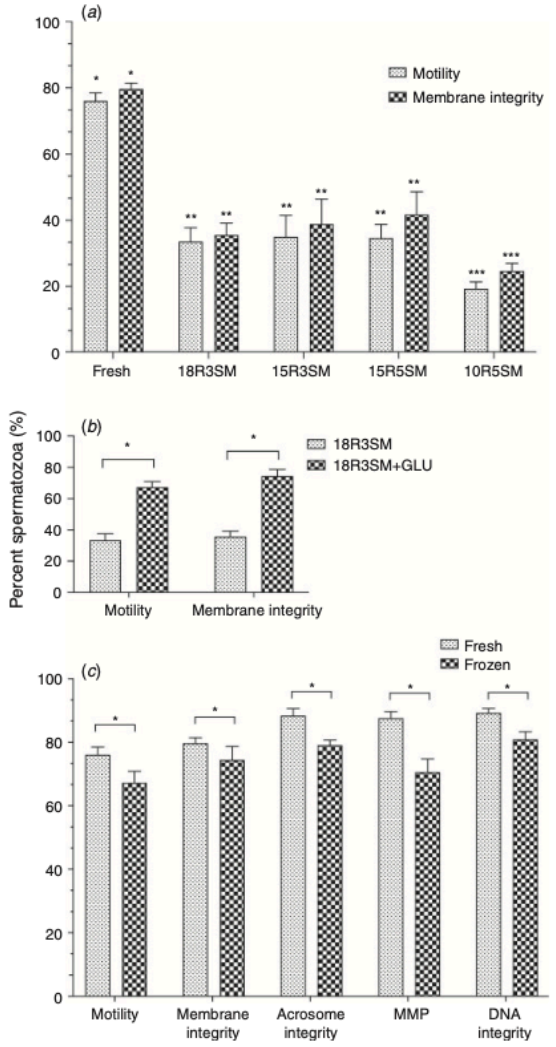

Fig. 2. (*a*) Post-thaw sperm motility and membrane integrity in four extender solutions, (*b*) post-thaw sperm motility and membrane integrity with the addition of L-glutamine to the extender solution and (*c*) various sperm function parameters between fresh spermatozoa and spermatozoa cryopreserved using an extender solution containing 18% raffinose, 3% skim milk (18R3SM) and 100 mM L-glutamine (GLU). Data are the mean ± s.d. $*P < 0.05$. 15R3SM, 15% raffinose and 3% skim milk; 15R5SM, 15% raffinose and 5% skim milk; 10R5SM, 10% raffinose and 5% skim milk; 15R10SM, 15% raffinose and 10% skim milk; 10R10SM, 10% raffinose and 10% skim milk; MMP, mitochondrial membrane potential.

Jones 2003). Despite this, murine spermatozoa are generally well protected against cryodamage using a combination of raffinose and skim milk (Sztein *et al.* 2001), and the results from Experiment 1 in this study demonstrate similar cryotolerance of *A. cahirinus* spermatozoa to those of other mice (Takeo and Nakagata 2010) and gerbils (Sato *et al.* 2000; Koshimoto *et al.* 2009). Moreover, Experiment 2 revealed that, as in mice (Takeo and Nakagata 2010), spiny mouse spermatozoa are successfully cryopreserved in the presence of L-glutamine (Figs 1, 2). L-Glutamine is a known antioxidant and is able to mitigate excessive oxidative damage from reactive oxygen species (Aitken *et al.* 2014). Therefore, the observation of improved functional parameters when spermatozoa are cryopreserved with L-glutamine may be due to the increased resistance of spiny mouse spermatozoa to oxidative stress. Future studies of other parameters, including freezing distances, cryoprotectants and cryopreservation tools, are encouraged to further optimise sperm cryopreservation in this species.

In summary, we have developed a successful protocol for sperm cryopreservation in the Egyptian spiny mouse *A. cahirinus*. An extender consisting of raffinose, skim milk and L-glutamine significantly improved all post-thaw sperm parameters over controls and other extenders used in this study. A reliable and successful cryopreservation protocol and associated assays provide new possibilities for future reproductive research, including assisted reproduction, in spiny mice and a successful method for preserving and archiving the genetics of the world's only known menstruating rodent.

Conflicts of interest

The authors declare no competing interests.

Acknowledgements

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Supplementary Material

Successful sperm cryopreservation in Egyptian spiny mice *Acomys cahirinus*

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Fig. S1. Flowchart of sperm analysis procedure for both Experiment 1 and 2 adapted from Takeo and Nakagata (2010).

Fig. S2. Micrograph displaying sperm DNA fragmentation.

Table S1. Staining patterns of fluorescently labelled sperm.

Table 2. Comparative post-thaw motility and membrane integrity of spiny mouse spermatozoa when assessed immediately after thawing and following a two-hour incubation in warmed handling media.

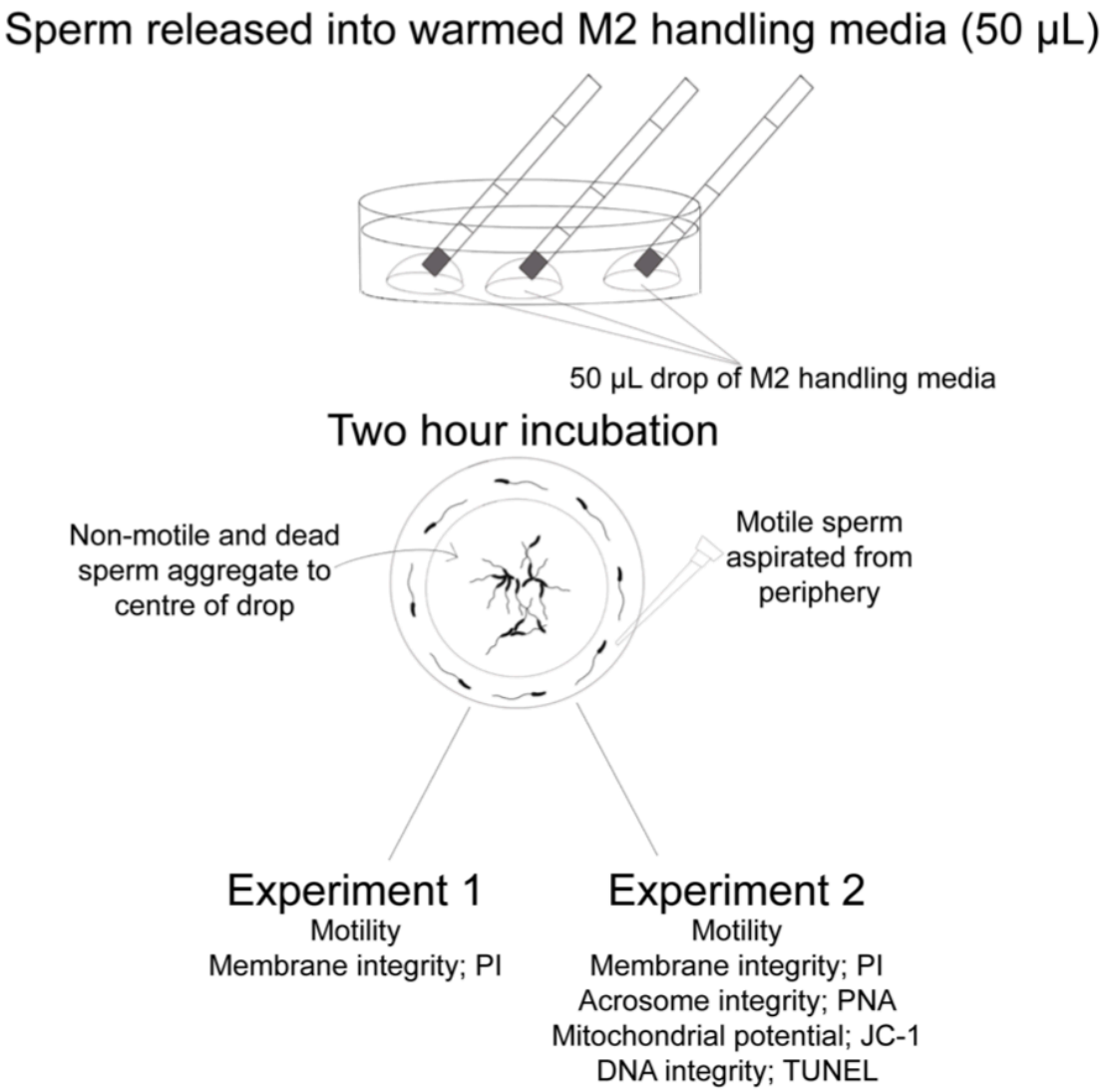

Fig. S1. Flowchart of sperm analysis procedure for both Experiment 1 and 2 adapted from Takeo and Nakagata (2010).

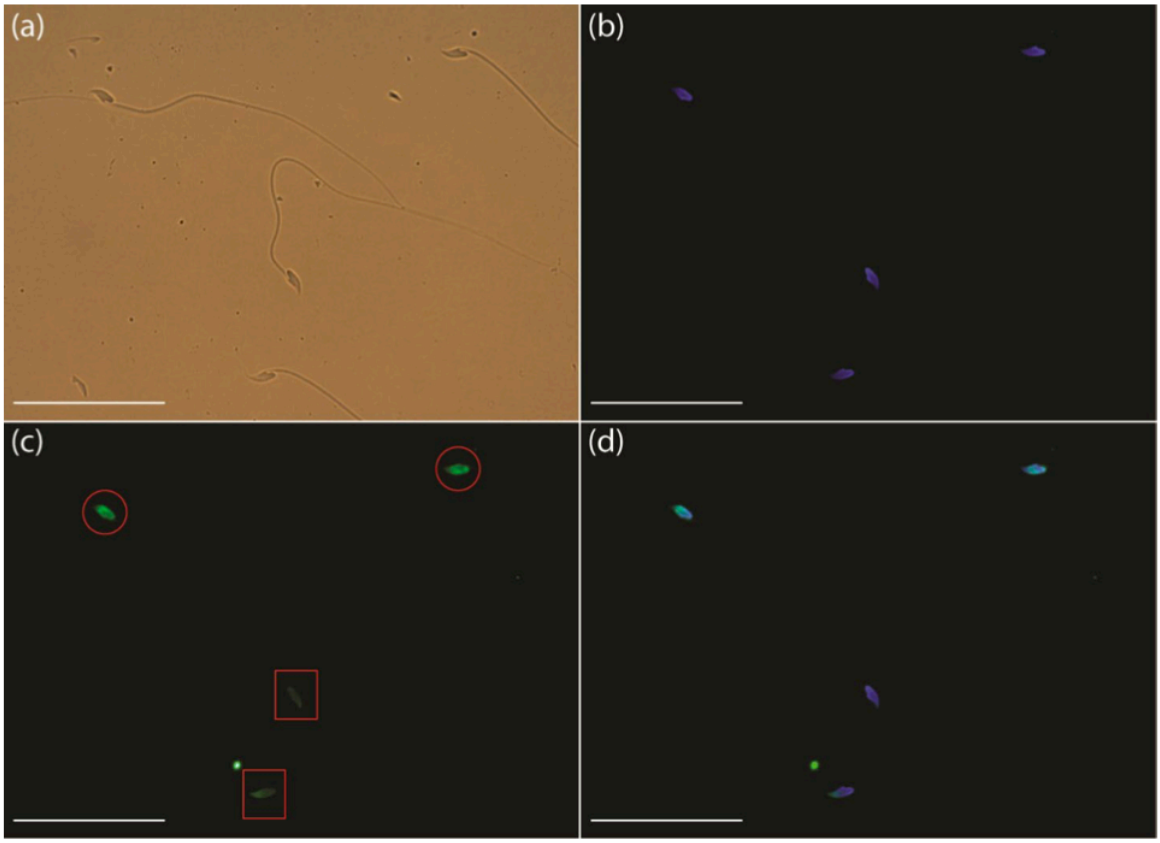

Fig. S2. Micrograph displaying sperm DNA fragmentation. (*a*) Bright field image of *A. cahirinus* spermatozoa; (*b*) H33342 positive sperm; (*c*) TUNEL positive (circles) and negative (squares) spermatozoa; (*d*) Merged image showing TUNEL and H33342 positive (circles) and negative (rectangles) spermatozoa. Scale bars indicate 50 μm.

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Table S1. Staining patterns of fluorescently labelled sperm

+, fluorescent positive; -, fluorescent negative

Staining pattern			Sperm classification		
PI + (purple) - (blue)	PNA + (green) - (absent)	JC-1 + (orange/yellow) - (green)	Membrane integrity	Acrosome integrity	Mitochondrial potential
-	-	+	Intact	Intact	High
		-			Low
-	+	+	Intact	Damaged	High
		-			Low
+	-	+	Damaged	Intact	High
		-			Low
+	+	+	Damaged	Damaged	High
					Low

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Table 2. Comparative post-thaw motility and membrane integrity of spiny mouse spermatozoa when assessed immediately after thawing and following a two-hour incubation in warmed handling media

Data are mean of six animals and two slides per animal. Motility quality is described as + non-progressive, ++ slow-progressive and +++ rapid-progressive motility

	Immediate assessment				Two-hour incubation			
Cryopreservation solution	18R3S M	15R3S M	15R5S M	10R5S M	18R3S M	15R3S M	15R5S M	10R5S M
Average motility (%)	14	17	17	19	34	35	35	19
Motility quality	++	+	+	+	++	++	++	++
Average membrane integrity (%)	24	22	24	27	36	39	42	25

Chapter 5: A human-based assisted reproduction protocol for the menstruating spiny mouse, Acomys cahirinus

5.1 Introduction

The laboratory mouse is undoubtedly the most widely used animal model for biomedical research. Small size, short gestation, ease of colony management and widely available assisted reproductive techniques (ARTs) make the laboratory mouse a very efficient, accessible model species. Access to robust ARTs not only streamlines colony management procedures, but also allows for more efficient *in-vitro* and *in-vivo* research to better understand aspects of human health such as embryogenesis, embryo implantation and pregnancy. While the spiny mouse menstrual cycle and later stages of gestation have been characterized, protocols for fundamental ARTs such as gamete cryopreservation, IVF and embryo transfers are yet to be developed. Given the growing interest in spiny mice, and their potential role as a small animal model of human reproductive function, there is a need to develop specific laboratory-based ARTs to make this model more relevant to biomedical research. Thus, this chapter aimed to successfully 1) collect and fertilise mature oocytes with fresh and frozen-thawed spermatozoa, 2) cryopreserve and thaw viable two-cell embryos, and 3) transfer fresh and frozen-thawed two-cell embryos to achieve live births.

This chapter is presented in two sections:

1) My attempts to repeat a published superovulation protocol in *A. cahirinus.*

2) Novel protocols for superovulation, IVF/IVC, embryo cryopreservation and embryo transfer in *A. cahirinus* accepted for publication.

5.2 Attempts to repeat a published superovulation protocol in *A. cahirinus*

To successfully collect viable, mature spiny mouse oocytes, I attempted to repeat a published protocol for superovulation in this species. The methods outlined by Pasco et al. (2012) involved 2 injections of pregnant mare serum gonadotrophin (PMSG, 10IU each, 9hrs apart) and a single injection of human chorionic gonadotrophin (hCG, 20IU, 60hrs later) to induce follicular growth and ovulation respectively. Stimulated animals were mated at the time of hCG injection with stud males and 1-2 cell embryos were collected 33hrs later. Of the 17 superovulation protocols tested, this was the most effective protocol and between 6-26 embryos (mean 14) were collected following hormone administration. Considering this, I attempted to repeat this protocol to collect mature oocytes for IVF, and embryos for cryopreservation or embryo transfer.

Of the 26 animals tested, I was only successful in collecting viable oocytes from 1 animal. In this situation, only 4 oocytes were collected (Figure 1, Table 1). Considering the average litter size of spiny mice in our colony of 2-3 pups (unpublished observations), this led us to believe this animal was not stimulated by the exogenous hormones, because this was more likely a natural ovulation rate. Regardless, these oocytes were fertilised using fresh spiny mouse spermatozoa following a protocol for murine IVF in our lab. While oocytes appeared to be fertilised and have undergone early *in-vitro* cleavage, (Figure 1), cleavage was unexpectedly fast when compared to the *in-vitro* timeline for murine embryo cleavage. Spiny mouse oocytes had divided within 3hrs of insemination and reached the 4-cell stage in under 30 hours.

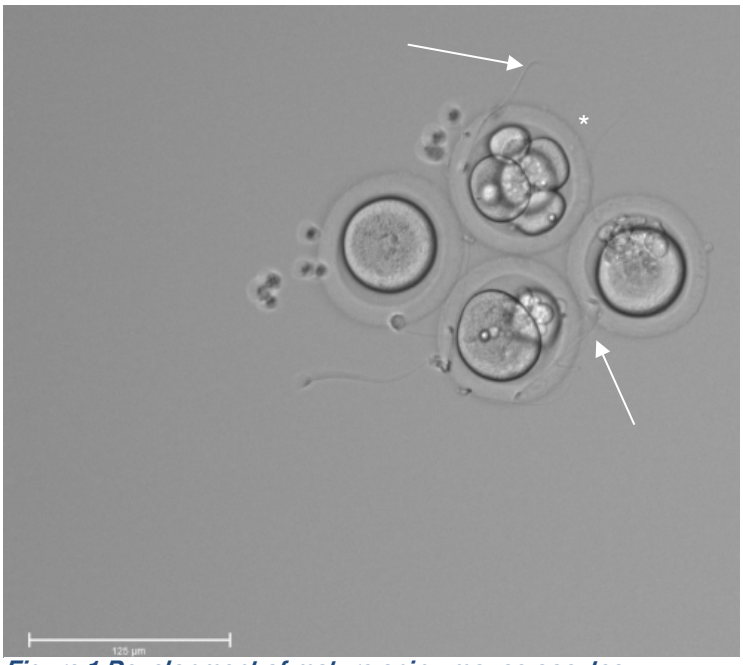

Figure 1 Development of mature spiny mouse oocytes inseminated with fresh spermatozoa. *Thirty hours post-insemination, spermatozoa (arrows) are seen bound to the zona pellucida of spiny mouse oocytes and one oocyte developed to the four-cell stage (*). The remaining oocytes were unfertilised and begun degenerating.*

While unexpected, as our knowledge of spiny mouse IVF and embryogenesis is extremely limited, I believed this may be a natural phenomenon. However, pronuclei were not seen during time-lapse observations, which suggested these oocytes were more likely parthenogenically activated than fertilised.

I also collected oocytes of unknown maturity following this protocol – to be referred to as metaphase II + bulge (MII+B) from here on. Interestingly, MII+B's were found in both the ovary and in the oviduct of several females, which questioned the maturity of these oocytes. To further investigate this, two MII+B's were stained with HOECHST (Figure 2) to check if the suspected polar bodies were true (contained DNA) and whether or not the 'bulge' sectioned also contained DNA.

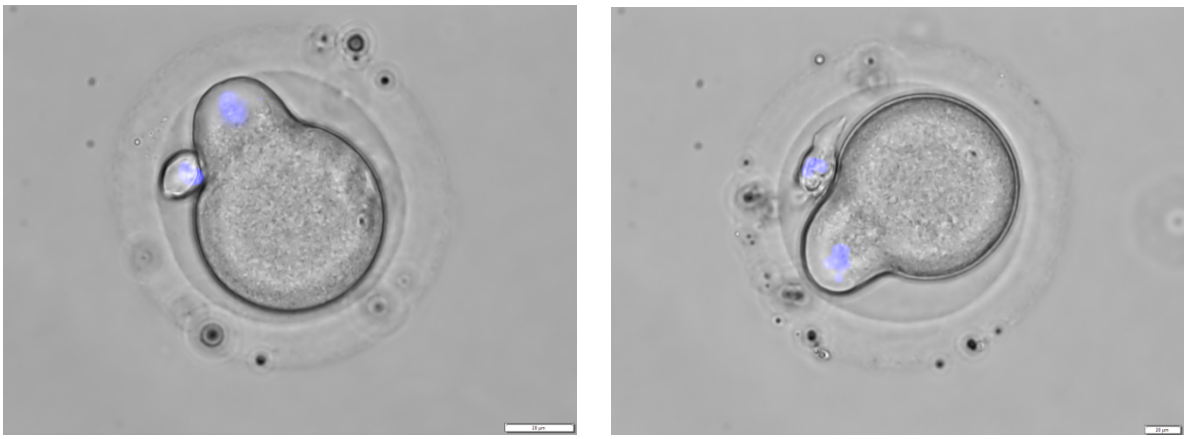

Figure 2: HOECHST staining in spiny mouse MII+B oocytes. *Positive HOECHST staining (blue) was local to both 'bulge' and potential polar bodies in two spiny mouse MII+B oocytes.*

These oocytes were later inseminated with fresh spiny mouse spermatozoa, but cleavage was not seen, and these oocytes degenerated within 24 hours of insemination.

Considering the unexpectedly poor outcomes when following a successful and published stimulation regime in this species, I attempted to collect mature oocytes from virgin animals during the late follicular or early luteal phase of the cycle; the suspected period of ovulation in this species. Spiny mice were lavaged vaginally, and menstrual cycles staged as reported in this species by Bellofiore et al. (2017). A summary of my collection results is provided in Table 2.

***Table 1 Raw data of eggs collected and collection times post exogenous hormone injections**. GV: germinal vesicle, MI: metaphase 1, MII+B: metaphase 2 and bulge*

Animal age	PMSG injection issues?	hCG injection issues?	Collection time post-hCG	No. mature eggs collected	No. immature eggs collected
80 days	Fine	Fine	33hrs	4 degenerated	4 degenerated
80 days	Fine	Slight leak	33hrs	0	0
80 days	Fine	Fine	33hrs	2 degenerated	2 degenerated
80 days	Fine	Fine	33hrs	0	0
75 days	Fine	Fine	33hrs	0	3 GV
75 days	Fine	Fine	33hrs	0	2 MI
75 days	Fine	Fine	33hrs	0	0
75 days	Fine	Fine	33hrs	0	2 GV
75 days	Leak on 2nd PMSG, reinjected no issue	Fine	24hrs	0	2 MII+B
75 days	Fine	Fine	24hrs	0	0
75 days	Fine	Fine	24hrs	0	9 pre-GV
75 days	Fine	Fine	24hrs	0	0

75 days	Fine	Fine	24hrs	0	2 GV
80 days	Leak on 2nd PMSG, reinjected no issue	Fine	27hrs	0	2 GV
80 days	Fine	Fine	27hrs	0	14 MI, 6 GV
80 days	Fine	Fine	27hrs	0	0
80 days	Fine	Slight leak	27hrs	0	0
80 days	Fine	Fine	27hrs	0	5 MI
130 days	Fine	Fine	27hrs	0	2 MI
130 days	Fine	Fine	27hrs	0	0
130 days	Fine	Fine	27hrs	0	7 MI
130 days	Fine	Fine	27hrs	0	2 MI
130 days	Fine	Fine	27hrs	4	0
130 days	Fine	Fine	27hrs	0	2 GV, 2 MI
130 days	Fine	Fine	27hrs	0	MII+B
130 days	Fine	Fine	27hrs	0	0

Table 2 Raw data of eggs collected from unstimulated females. *No mature oocytes were collected from unstimulated animals during the late follicular or early luteal phases of the spiny mouse menstrual cycle.*

Age	Cycle stage 24hr prior to collection	No. mature eggs collected	No. immature eggs collected
180 days	Late follicular	0	3 GV
181 days	Early luteal	0	2 GV
182 days	Early luteal	0	1 MI
183 days	Late follicular	0	2 MII+B
184 days	Late follicular	0	2 GV
365 days	Late Follicular	0	3 GV
365 days	Early luteal	0	2 GV

Considering this failure to repeat the published protocol and collect mature oocytes, I then attempted to confirm the proposed ovulation and *in-vivo* developmental timepoints proposed by Pasco et al. (2012). Three two-cell embryos were collected from 3 unstimulated, staged females 48hrs after pairing (Table 3). Whilst 2 cell embryos were collected, our collection time did not agree with the published paper, as embryos are seen up to 15hours earlier than I report.

Table 3 Raw data of embryos collected from unstimulated, paired females. *One and two-cell embryos were collected 48hrs from 1 animal each paired in the late follicular and early luteal phases of the menstrual cycle.*

Age	Cycle stage at pairing	Days post pairing	Embryos collected?
365 days	Early luteal	2	1X two cell
365 days	Late follicular	2	2X two cell
180 days	Late follicular	2	0

As I was unable to collect mature, viable oocytes or embryos following the published superovulation protocol in this species, even after extensive modifications, I decided this protocol was unrepeatable and elected to define human-based protocols for future testing.

The following section includes an accepted registered-report manuscript outlining novel, human-based ARTs for Egyptian spiny mice.

5.3 Published research article (*Plos* One)

PLOS ONE

REGISTERED REPORT PROTOCOL

A human-based assisted reproduction protocol for the menstruating spiny mouse, *Acomys cahirinus*

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This is a Registered Report and may have an associated publication; please check the article page on the journal site for any related articles.

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Abstract

The Egyptian or Common spiny mouse (*A. cahirinus*) is the first rodent species to show human-like menstruation and spontaneous decidualisation. We consider from these, and its other, human-like characteristics that this species will be a more useful and appropriate small animal model for human reproductive studies. Based on this, there is a need to develop specific laboratory-based assisted reproduction protocols including superovulation, in-vitro fertilisation, embryo cryopreservation and transfer to expand and make this model more relevant. Because standard rodent superovulation has not been successful in the spiny mouse, we have selected to test a human protocol. Female spiny mice will receive a subcutaneous GnRH agonist implant and be allowed to recover. Menstrual cycle lengths will then be allowed to stabilize prior to ovarian stimulation. After recovery, females will be injected IP once a day for 4 days with a FSH analogue, to induce follicular growth, and on day 5 will be injected IP with a hCG analogue to trigger ovulation. Females will either be culled 36hrs after trigger to collect oocytes or immediately paired with a stud male and two cell embryos collected 48hrs later. Mature oocytes will be inseminated using fresh spiny mouse spermatozoa and all in-vitro grown and in-vivo collected two cell embryos will be cryopreserved using methods developed in a close spiny mouse relative, the Mongolian gerbil. For embryo transfer, vitrified embryos will be rapidly warmed and non-surgically transferred to surrogate mice. Surrogates will be monitored until pregnancy is apparent (roughly 30 days) and then left undisturbed until birth, 38–40 days after transfer. By successfully developing robust assisted reproduction protocols in *A. cahirinus* we will be able to use this rodent as a more effective model for human reproduction.

Introduction

The Egyptian or common spiny mouse (*A. cahirinus)* is a small, desert-dwelling rodent native to northern Africa and the Middle East. Spiny mice exhibit several unique, sometimes human-

Competing interests: The authors have declared that no competing interests exist.

like, characteristics among rodents, including a relatively long gestation (39 days; [1]), small litter sizes (1–4; observations from our colony) and the recent discovery of human-like menstruation [2]. This latter trait in particular, is expressed in <2% of all mammalian species and is primarily found in higher-order primates such as gorillas and orang-utans [3]. Although other non-primate species including the elephant shrew [4] and short-tailed fruit bat [5] exhibit menstrual-like events, *A. cahirinus* is, to our knowledge, the only species with human-like, cyclical menstruation. However, as the spiny mouse is a comparatively novel laboratory species, limited information is available regarding their reproductive physiology and endocrinology and how this compares to other menstrual species. Despite this, human-like, cyclical menstruation in spiny mice has established this species as an important new small animal model for studies into human reproductive and gynaecological disorders.

While other aspects of reproduction, such as the later stages of gestation, in *A. cahirinus* have been well documented [6–9], protocols for various assisted reproductive techniques (ARTs), including gamete and embryo cryopreservation, in-vitro fertilisation (IVF) and embryo transfers (ET) have yet to be developed in this species. Access to robust ARTs in *A. cahirinus* will allow for more efficient in-vitro and in-vivo research to understand better embryogenesis, implantation and pregnancy to evaluate further its role as a small animal model of human reproduction.

In women, superovulation involves two primary steps, 1) suppression of ovarian function by gonadotrophin-releasing hormone (GnRH) agonist or antagonists followed by, 2) direct stimulation using exogenous follicle stimulate hormone (FSH) [10, 11]; a feature absent from the original *Acomys* protocol [12]. Ovarian downregulation using Deslorelin, a potent GnRH agonist, has been successful in several mammalian species [13–17] and we hypothesise that Deslorelin will also be effective for producing large numbers of mature metaphase II oocytes in female spiny mice from our colony.

Successful ovulation and IVF of mature oocytes will then allow us to produce pre-implantation embryos that can either be frozen or transferred fresh to surrogates. In light of advances in human embryo vitrification, frozen-embryo transfers (FET) are now the preferred technique in human IVF [18]. Subsequent development of embryo vitrification in this *Acomys* species will streamline embryo transfer procedures, and provide the foundation for future creation of transgenic strains and for improved breeding efficiencies for captive spiny mouse colonies.

Our aim with this protocol is to develop a successful agonist-based superovulation technique for *A. cahirinus* that will provide large numbers of oocytes for IVF, and also embryos for culture, cryopreservation and transfer, and enable us to more effectively use the spiny mouse as a model for human reproduction.

Methodology

Animals

Monash University/Monash Medical Centre Animal Ethics Committee have approved the experimental use of this protocol (MMCB/2019/10). The animals for this study are sourced from our research colony of spiny mice maintained under a 12:12 hour light:dark cycle at 25–27˚C and humidity of 30–40% [19]. Sexually mature mice (>60 days old) are housed in groups up to nine per cage (female only) or as breeding pairs. Wood shavings are used to line cage floors and plastic tunnels, rings and tissue paper provide environmental enrichment. Food (rat and mouse cubes; Specialty Feeds, Glen Forest WA) and water is provided freely at all times, with weekly supplements of fresh carrots and celery.

Sample size and experimental design

Female spiny mice on average deliver litter sizes of 3 pups (our colony observations) but can range from 1–5 naturally. We have based our sample size calculations (80% power, alpha 0.05, sigma 2.5) on collecting 3 eggs per female from controls and 6 eggs from stimulated animals. Thus, the required number of females to show significant increases in egg/embryo numbers compared to controls is 9. This also includes an additional 3 animals to account for natural biological variation.

18 males are required for each of the 18 females which will be paired to collect embryos.

The success of the NSET is approximately 30% in laboratory mice [20]. To achieve 3 sets of live births, and show a significant effect of treatment, we will need a minimum of 12 surrogate spiny mice per group.

The experimental design consists of five experiments (Fig 1) conducted in three phases (Table 1):

1. to develop a novel agonist-based superovulation protocol for the reliable collection of spiny mouse oocytes.

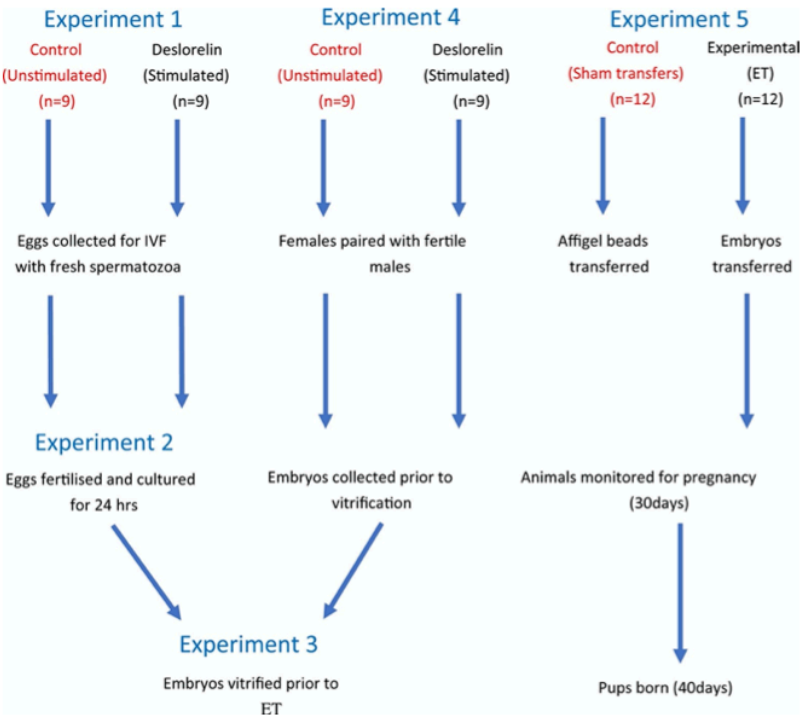

Fig 1. Overall experimental design. Experiment 1) Oocytes will be collected from stimulated (experimental) and unstimulated (control) animals prior to IVF. Experiment 2) Oocytes will be fertilised with fresh spiny mouse spermatozoa and cultured to the two cell stage prior to vitrification. Experiment 3) Embryos will be vitrified, stored and then thawed prior to ET. Experiment 4) Embryos will be collected from stimulated (experimental) and unstimulated (control) animals prior to vitrification. Experiment 5) Embryos will be thawed and transferred to surrogate female spiny mice. Dams will be monitored for pregnancy (~30 days) and for live births (~40days). A group of sham-transfer controls have affigel beads transferred in place of embryos.

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Table 1. Research timeline for ART development in *Acomys cahirinus*.

		Months								
		1	2	3	4	5	6	7	8	9
Phase 1	Transfer training									
	Implant surgery training									
	SHAM transfers									
Phase 2	Oocyte donor control collections									
	Surrogate transfers + litter down									
	Embryo donor control collections									
	Surrogate transfers + litter down									
Phase 3	Oocyte donor implant surgery									
	Embryo donor implant surgery									
	Oocyte collections									
	Surrogate staging + litter down									
	Embryo collections									

Experiments are conducted in three phases: Phase 1 consists of technique training and embryo transfer controls, phase 2 includes control oocyte and embryo collections, whilst phase 3 includes experimental oocyte and embryo collections.

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2. to define an IVF protocol for mature spiny mouse oocytes using fresh or cryopreserved epididymal spermatozoa.
3. collect viable two cell embryos from superovulated, mated spiny mice.
4. to successfully vitrify and warm *in vivo* flushed and in-vitro grown two cell embryos.
5. to compare the pregnancy success rates and birth outcomes of in-vitro grown and in-vivo collected embryos after non-surgical embryo transfer (NSET).

Tissue collections

Mature male mice are euthanised by isoflurane inhalation and the epididymides are removed through a midline abdominal incision by retracting each epididymis through its inguinal canal into the abdominal cavity. Each cauda epididymis is isolated, incised several times with fine iridectomy scissors and placed in warmed collection tubes containing 500uL of pre-equilibrated MT6 fertilisation medium (37˚C; 5% O_2, 6% CO_2, 79% N_2; modified Tyrode's solution [21]). Spermatozoa are allowed to swim out and begin capacitation in MT6 in a humidified incubator (37˚C, 5% O_2, 6% CO_2) for at least 30 minutes to provide a sperm sample with rapid forward motility for IVF (Experiment 3).

Oocytes are collected from females euthanised by isoflurane inhalation; their oviducts are removed immediately post-mortem and placed into 2mL of warmed (37˚C) HEPES-based handling medium (M2; Sigma, CAT #M7167). The oviducts are then placed into a 60mm culture dish (Sigma) on a heated stage at 37˚C for dissection. Each oviduct is gently opened using sharp micro-forceps to release cumulus oocyte complexes (COCs). The COCs are placed into a 50uL drop of pre-equilibrated MT6 and transferred into an incubator (37˚C, 5% O_2, 6% CO_2) prior to IVF (see Experiment 3). For embryo collections, female spiny mice are killed and dissected, as described for oocyte collections above. Embryos are released into a 60mm culture dish (Sigma, CAT #SIAL0166) on a heated stage containing warmed M2 handling medium (37˚C) and washed three times in fresh 50uL drops prior to vitrification (see Experiment 3).

Experiment 1: Unstimulated control oocyte collections

Spiny mouse menstrual cycles are monitored and characterised using vaginal cytology to identify 1) cycling individuals and 2) those in late follicular phase according to published criteria [2, 22] prior to hormone treatment. Females are then killed when they reach the late follicular stage, and oocytes collected for use in IVF, embryo vitrification and ET (Figs 1 and 2; Experiments 2–3, 5).

Experiment 1: Deslorelin stimulated oocyte collections

The vagina of females undergoing superovulation is lavaged as described previously, and the vaginal cytology used to identify menstruation (day 1–3 of the cycle). A subcutaneous Deslorelin implant (Virbac, Australia) is then surgically inserted into each menstruating female spiny mice as follows:

1. Each female receives 5mg/kg subcutaneous Carprofen analgesia immediately prior to surgery. Anaesthesia is induced with 5% isoflurane in room air via an automated anaesthetic machine (Univenter 400 anaesthetic unit; Zejtun, Malta). When adequately anaesthetised, the level of isoflurane is reduced to ~2.0–2.5% to maintain an appropriate level of

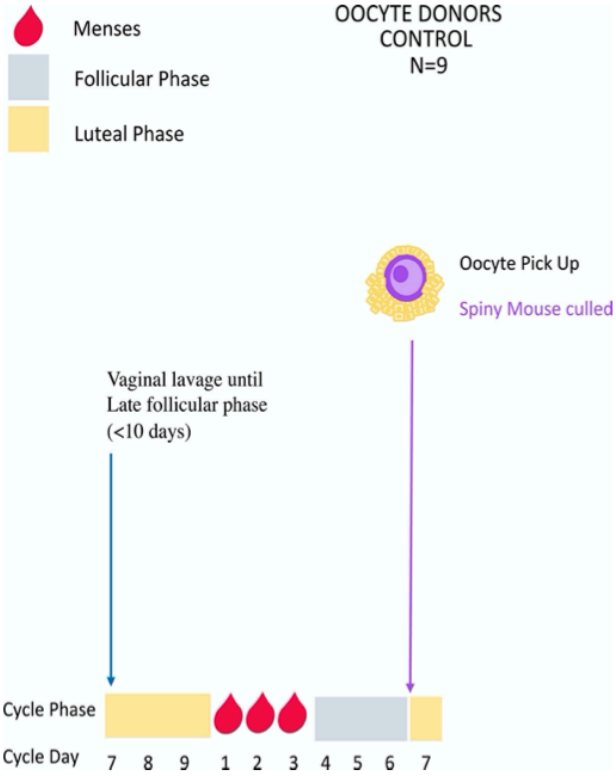

Fig 2. Control unstimulated oocyte collections. Mice are lavaged daily until the late follicular phase (~day 6 of the menstrual cycle) when they will be killed and oocytes collected for use in IVF (Experiment 2).

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anaesthesia during surgery. Each female is placed in a supine position on a warming pad (37˚C) and depth of anaesthesia monitored using foot and eye reflexes.

2. A 3mm interscapular incision is made in the skin and a 2cm x 2cm pocket formed with blunt dissection. The incision is flushed with 0.2mL topical analgesic (Bupivacaine) and interrupted the Deslorelin implant inserted into the pocket which is closed with 6/0 Ethilon sutures (Ethicon, Australia).
3. The nails on the front paws of the anaesthetised females will be trimmed to prevent scratching causing wound and skin tears and removing sutures.
4. Females are monitored every 2hrs for 8hrs after surgery, twice daily thereafter for 3 days and daily until 7 days after surgery.
5. Implanted females are allowed 27 days to recover before commencing gonadotrophin injections.

After recovery, females are injected (intraperitoneal; IP) once a day for 4 days with Gonal-F (Merck Serono, Australia), an FSH analogue, to induce follicular growth (Figs 3 and 4). On Day 5 of treatment, females are injected IP with Ovidrel (Merck Serono, Australia) to trigger ovulation. Females are killed 36hrs later by isoflurane inhalation, the ovaries and oviducts removed by fine dissection and ovulated oocytes released from the oviducts into fertilisation medium (MT6) for use in IVF and, subsequently, embryo vitrification and ET (Figs 1 and 2; Experiments 2–3, 5).

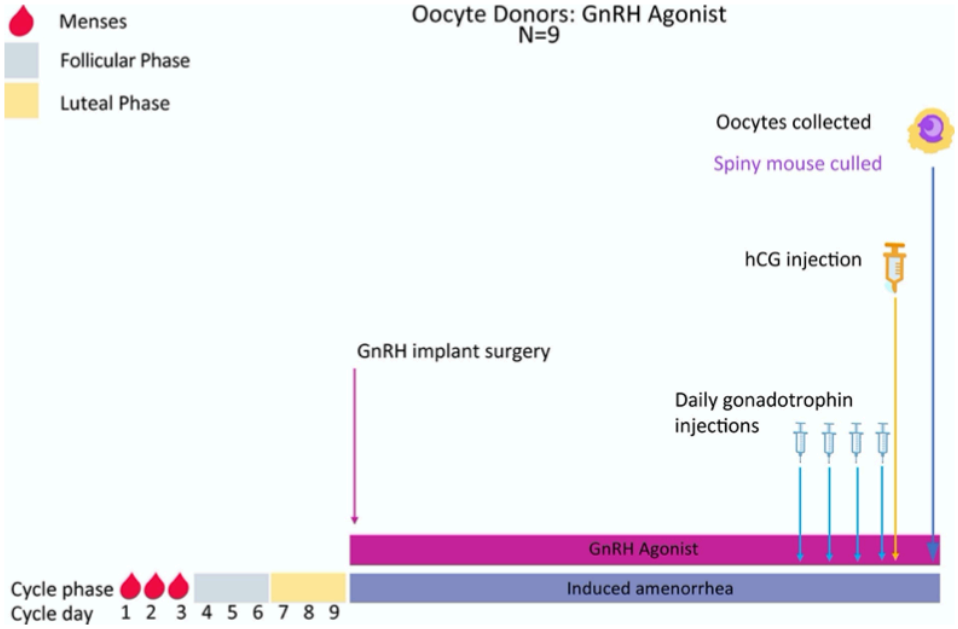

Fig 3. Superovulation protocol and oocyte collections in Deslorelin implanted mice. Mice are vaginally lavaged until the menstrual phase (~ day 1–3 of the cycle) and a Deslorelin implant then subcutaneously implanted into each mouse. All mice will be given 3 cycle lengths (27 days total) to recover from surgery and for the agonist implant to have taken effect. Mice will then be injected IP once daily for 4 days with Gonal-F followed by a single injection of Ovidrel on day 5. Females are culled 36 hours later and oocytes collected for use in IVF.

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Fig 4. Rotation of injection sites for hormone IP injections. Injection sites are rotated across the lower abdomen of spiny mice to prevent injury from repeated injection sites.

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Experiment 2: In-vitro fertilisation (IVF)

A 10uL aliquot of highly motile spermatozoa is aspirated, after a 30-minute swim-up at 37˚C from the top of the collection tube for motility assessment as previously reported [23]. A volume (roughly 2-4uL) containing at least 250,000 progressively motile spermatozoa is added to the drop of MT6 containing the COCs and returned to the incubator. After three hours incubation, the inseminated oocytes are washed 3 times in 50uL drops of M16 culture media (Sigma, CAT #M7292) and cultured in fresh media droplets for 24hrs in a humidified incubator (37˚C, 6% CO_2, 5% O_2). Twenty-four hours after insemination, fertilisation rates are estimated by the number of two cell embryos produced divided by the number of oocytes inseminated. Embryos are removed from M16 and washed 3 times in handling media (M2) prior to vitrification (Experiment 3).

Experiment 3: Embryo vitrification

In-vitro grown and in-vivo collected two cell embryos are vitrified and stored to better manage the timing of embryo transfers with IVF and embryo collections. Embryo vitrification will be

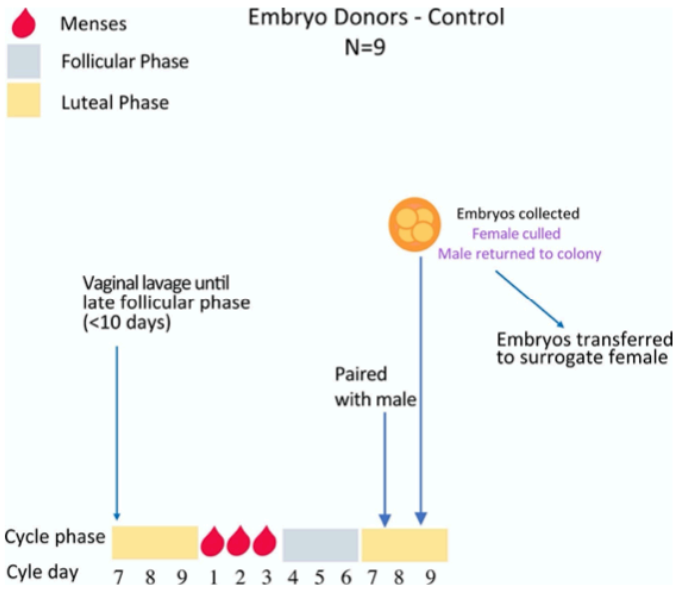

Fig 5. Embryo collections from unstimulated spiny mice. Females are lavaged daily until the late follicular phase (~day 6 of the cycle) when they will be paired with a fertile male and left undisturbed to mate overnight. Female mice are then killed 48hrs later and embryos collected for immediate vitrification (Experiment 3).

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performed according to the methods developed by Mochida, Wakayama, et al. [24] for Mongolian gerbil embryos with minor modifications. Up to 20 embryos are placed in 500uL of equilibration solution [ES; M2 containing 20% v/v ethylene glycol (EG), 30% Ficoll w/v and 0.5M sucrose) for two minutes at room temperature. Embryos are then transferred to 500uL of vitrification solution [ES; M2 containing 40% v/v ethylene glycol (EG), 30% Ficoll w/v and 0.5M sucrose) for thirty seconds at room temperature. The embryos are then aspirated inside 0.25mL cryopreservation straws (INV technologies, India) and a 1cm air gap aspirated immediately after the VS. The straws are then sealed with polyvinyl alcohol (PVA) and immersed directly in liquid nitrogen (LN2) for storage.

For warming, each straw is removed from LN2 and placed in a 22˚C waterbath for 10 seconds. The straws are then cut between the PVA seal and the VS, and embryos expelled into 800uL of M2 medium containing 0.5M sucrose. The embryos are then transferred to a fresh drop (50uL, 5embryos/drop) of M2 medium containing 0.5M sucrose for five minutes. After warming, the embryos are transferred to fresh 50uL drops of M2 medium prior to ET.

Experiment 4: Embryo collections from unstimulated control and Deslorelin-treated females

After vaginal lavage, when females reach the late follicular phase, they are paired in the morning (Day 6) with a sexually mature stud male with proven fertility and left undisturbed to mate. Females are killed 48hrs later and embryos collected for immediate vitrification (Figs 1 and 5).

As per oocyte collections above, the female spiny mice in this protocol receive Deslorelin slow-release implants. After recovery from implant surgery (3 cycle lengths; Fig 6), each female

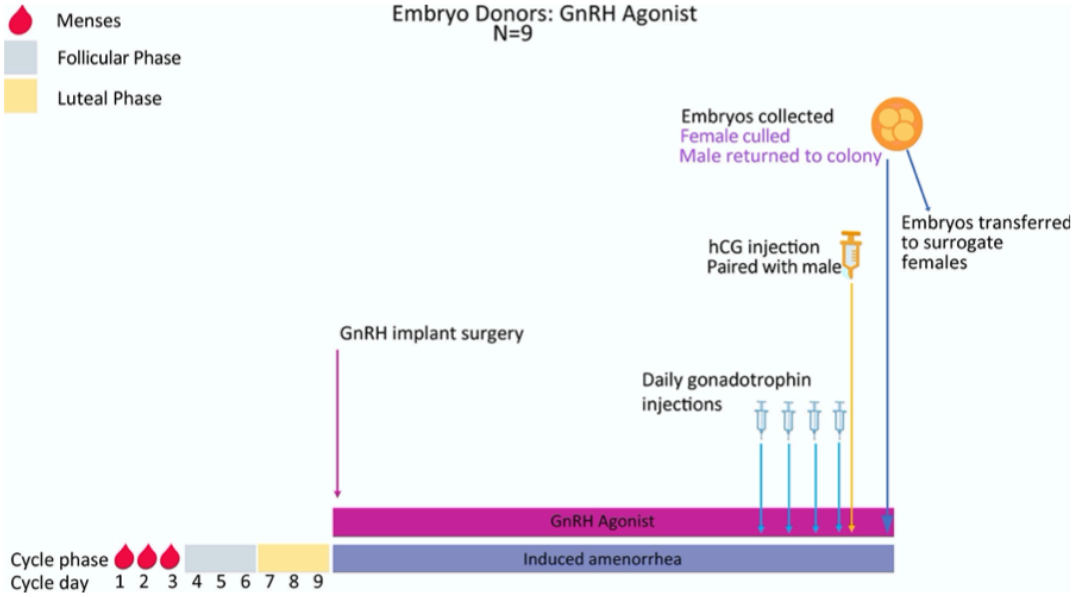

Fig 6. Superovulation protocol and embryo collections in Deslorelin implanted spiny mice. Females are vaginally lavaged until the menstrual phase (~ day 1–3 of the menstrual cycle) and a Deslorelin implant then subcutaneously implanted into each mouse. Females will be allowed 3 cycle lengths (27 days total) to recover and for the agonist implant to have taken effect. They will then be injected IP once daily for 4 days with Gonal-F followed by a single injection of Ovidrel on day 5. Females are then paired with a fertile male and left undisturbed to mate overnight. Forty-eight hours after the Ovidrel injection, the females are killed, embryos collected and immediately vitrified (Experiment 3).

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is placed on the same routine of 4 daily injections of gonadotrophins followed by an ovulation trigger. Immediately after the ovulation trigger, each female is paired with a male, and embryos are collected 48hrs later for vitrification (Experiment 3).

Experiment 5: Embryo transfer

Non-fragmented, morphologically normal two cell embryos are selected and transferred using a NSET device (Paratechs, USA) as previously reported [20] but with the following modifications. As spiny mice are unable to become pseudopregnant [25], surrogates are lavaged daily for up to 7 days to identify the mid-late luteal phase [2] at which females are most receptive to implantation and pregnancy. A maximum of 4 embryos, as opposed to the 20 transferred in a previous study [20], are transferred into surrogate spiny mice. Spiny mice have comparatively small litter sizes (average 2–3; [1, 19]) compared to laboratory mice (average 8–9; [26]), and thus, transfer of more than four embryos may negatively affect pregnancy or birth outcomes.

The NSET is attached to a 2.5uL pipette (Eppendorf) and set to 1.0uL. The plunger is depressed to half the first stop, embryos aspirated, and the pipette reset to 1.2uL to allow a bubble to form at the tip of the NSET. Spiny mice are held supine and the speculum inserted into the vagina to open the canal prior to embryo transfer (Fig 7A–7C). If needed, the smaller speculum is replaced by the larger speculum to better visualise the cervix. The NSET cannula is inserted into the vagina and passed through the cervix until the base of the cannula reaches the base of the speculum (Fig 7D). The pipette plunger is depressed to the first stop to expel the

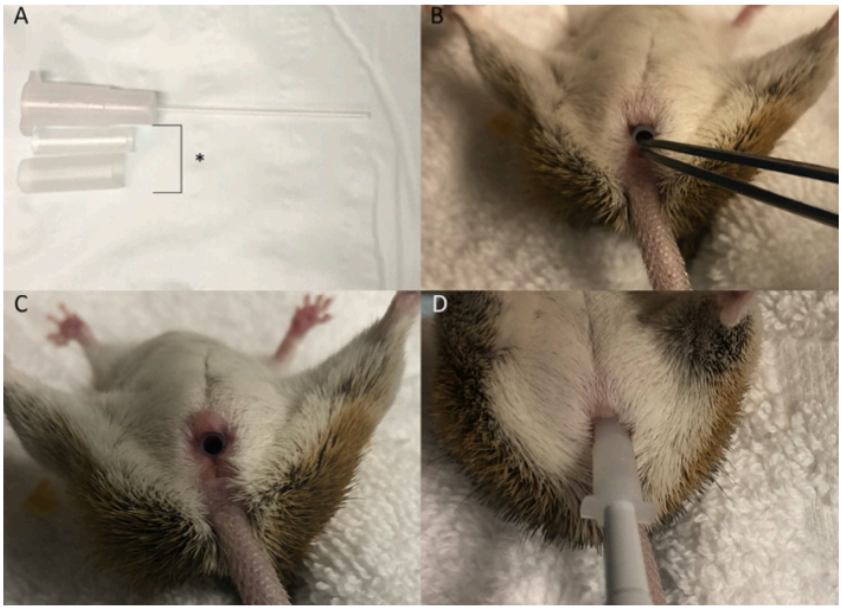

Fig 7. NSET transfer device and handling procedure. A) Both speculum (*) and the NSET cannula, B) Insertion of the smaller speculum, C) Speculum fully inserted in the vaginal canal, D) Insertion of NSET needle through the speculum and cervical opening.

https://doi.org/10.1371/journal.pone.0244411.g007

embryos, and the device slowly withdrawn as the plunger is depressed to the second stop to prevent embryos being aspirated back through the cervix.

The NSET tip is then checked under a dissecting microscope to confirm that all embryos have been transferred into the uterus. Each female is then returned to her respective cage and monitored twice daily for 3 days from the day of transfer and then daily until pregnancy is obvious (~30 days gestation; observations from our colony). The females are then left undisturbed until birth (roughly 38–40 days; Fig 8) or returned to colony if non-pregnant.

In order to observe any effects of the NSET device and transfer technique on spiny mouse behaviour and gestation, a control group of sham-treated animals will have non-toxic artificial blue beads (Affi-gel) transferred using the NSET in place of embryos.

Statistical analysis

1. All data will be tested for normality using the Shapiro-Wilk normality test prior to further analysis.
2. Number of oocytes and embryos collected from control and experimental animals will be compared statistically using a one-way ANOVA and the Tukey test used for post-hoc analysis; significance is set at $p<0.05$
3. IVF and developmental rates following unstimulated and stimulated oocyte collections will be compared using a one-way ANOVA and the Tukey test used for post-hoc analysis and significance set at $p<0.05$.

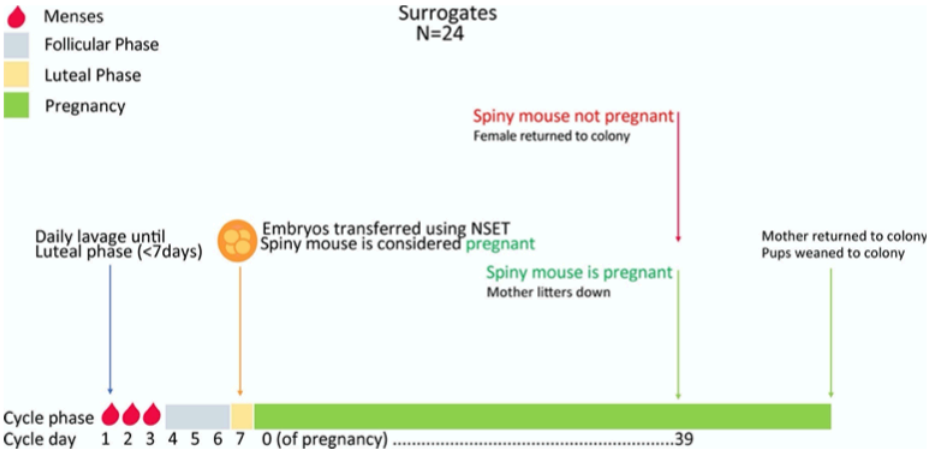

Fig 8. Embryo transfer procedure for surrogate spiny mice. Mice are vaginally lavaged until the luteal phase (~ day 7 of the menstrual cycle) and a maximum of 4 embryos will be transferred per female using the NSET device. Surrogate mice are presumed pregnant and will be monitored for external signs of pregnancy (~30 days). If mice are considered non-pregnant, they are removed from experimentation and returned to colony. If mice are pregnant, they are left to litter-down (~8–10 days later).

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4. Embryo survival rates post-warming will be compared between i*n-vitro* grown and in-vivo collected groups using an unpaired t test and significance set at $p<0.05$.
5. Live birth rates after embryo transfer will be compared between 1) control (unstimulated) and stimulated cycles; 2) In-vitro produced and in-vivo collected embryos. All live birth data will be analysed using a two-way ANOVA and the Tukey test used as post-hoc analysis with significance set at $p<0.05$

Discussion

Collection of progressively motile spermatozoa, and sufficient numbers of oocytes and embryos are critical steps for developing assisted reproductive techniques in any species. Currently, the most successful murine superovulation protocol involves single injections of equine chorionic gonadotrophin (eCG), to stimulate follicular growth, and human chorionic gonadotrophin (hCG) for ovulation induction [27]. However, the efficacy of this protocol is known to vary with age, genetic background and stage of oestrus cycle [28–31]. Although *A. cahirinus* is a rodent, no other rodent species, including other *Acomys* species, have been shown to menstruate. We consider that failure of our previous attempts to superovulate this species using the published A. *cahirinus* superovulation protocol [12] is due to its unusual reproductive strategy and hormonal profile [32]. Here, because of many similarities between spiny mouse and human reproduction, we have developed a novel superovulation and embryo transfer protocol for *A. cahirinus* based on a human agonist cycle.

Although GnRH agonists have been used in superovulation protocols for other menstruating species [33–36] such as baboons [35] and cynomolgus monkeys [33–36] they were injected, not implanted, with the GnRH agonists. In our experience, simultaneous hormone and agonist injections are likely to cause excessive, investigator-induced handling stress [37] and negatively affect superovulation outcomes. Given the human-like menstruation in *A. cahirinus*, its small size and high susceptibility to stress, an agonist-based superovulation

protocol using a GnRH agonist implant is considered a more appropriate and potentially successful approach to superovulation in this species.

Embryo vitrification is an essential murine ART for preserving strain genetics, colony management and streamlining ET procedures [38]. Although murine ETs are often performed using in-vitro grown blastocysts [20, 39], Momozawa, Matsuzawa, et al. [40] showed that healthy offspring can be produced either from vitrified blastocysts or two cell embryos. Interestingly, *A. cahirinus* experience an in-vitro developmental block at the four cell stage (unpublished; observations from our lab). Currently, the developmental capacity of blocked four cell embryos and the in-vitro requirements to progress past this stage are unknown. Thus, in-vitro grown, or in vivo collected, two cell embryos will be immediately vitrified prior to ET using a published vitrification method for two cell embryos from Mongolian gerbils; the closest relative of A. cahirinus [41–43].

Embryo transfers in mice, unlike in humans, are more commonly performed under general anaesthesia and usually embryos are surgically transferred directly into the oviduct [44–46]. Although NSET was first described nearly 70 years ago [47], poor live birth rates (8.5%) hindered its adoption compared to surgical embryo transfer (15–35%; [48]). Recently, however, NSET has become more successful with studies reporting comparable pregnancy and live birth rates to surgical methods [20, 39]. Thick, rigid tubing used by Beatty [47] were replaced with more flexible plastic tubing to more easily pass through the cervix and to prevent side-effects like uterine scratching and puncture that have been attributed to the low implantation and pregnancy rates in earlier reports [39]. Furthermore, considering the replacement, reduction and refinement requirements of ethics in animal research, refinements in NSET devices have reduced the number of animals needed and the stress/pain they may experience during the procedure [49, 50].

We chose embryo transfer using the NSET device to reduce stress, recovery time and potential complications with anaesthesia and post-operative care. However, a further complication in this species is that *A. cahirinus* do not produce a visible seminal plug after mating [1, 19] and pseudopregnancy cannot be induced by traditional methods [25, 39, 51, 52]. Females therefore need to be subjected to daily vaginal lavage for 2–7 days and staged with H&E as reported previously [2]. Similar to humans [11], spiny mouse surrogates must be in the mid-late luteal phase of their menstrual cycle prior to ET [2], to provide the optimal uterine environment for embryo implantation. Because of significant species-specific differences that exist in reproduction between the mouse and spiny mouse, even well-defined laboratory techniques for the laboratory mouse require modification before they can be applied to *A. cahirinus*.

Considering the unique reproductive biology of *A. cahirinus* and its potential as a model for contraceptive discovery and human reproductive disorders, the development of successful protocols for assisted reproduction in spiny mice will provide a better understanding of its embryogenesis, implantation and pregnancy whilst acting as a more relevant small animal model for women's reproductive health.

Limitations and future directions

Being a comparatively new laboratory species, limited information is available regarding spiny mouse reproductive biology and physiology and we acknowledge several potential limitations to the use of the protocols we have outlined.

Data on changes in LH and FSH across the menstrual cycle in this species is currently unavailable because of the lack of specific LH and FSH immunoassays and, despite the use of Deslorin to suppress the oestrous cycle of various mammals including some rodents (reviewed by [53]) we are unable to determine in advance if Deslorelin will induce amenorrhea in the

spiny mouse. Moreover, we are unable to collect standard, repeated blood-draws from ear or tail veins in this species due to the fragility of these tissues owing to the spiny mouse's unique ability for skin autotomy [54]. Longitudinal studies of changes to ovarian steroid production within individuals are therefore currently not possible.

Similarly, while we have an understanding of follicular growth in the cycling spiny mouse [2], follicle dynamics following hormone treatment are unknown. Considering this, ovarian histology may prove useful in optimising administered hormone concentrations and provide insights into the potential role of the spiny mouse as a model for ovarian hyperstimulation syndrome (OHSS).

Spiny mice remain fertile until at least 2 years of age in our colony but a decline in fertility has been observed between 3 and 4 years of age (unpublished). While the ovarian hormone profile (estrogens and progesterone) of older females is unknown, the impact of exogenous hormonal stimulation in these animals is likely to be reduced if they are to experience similar changes during reproductive aging as other mammals [55–57]. Future Investigations of reproductive aging in this species may not only suggest a role in studies of human reproductive aging, but also in improving ovarian stimulation for women of advanced maternal age.

To our knowledge, embryo cryopreservation has not been attempted in this species and we cannot confirm that the cryoprotectants described in this protocol will successfully preserve spiny mouse embryos. If not successful, future studies may need to compare different concentrations of Ficoll, different permeating cryoprotectants or cooling rates on spiny mouse embryo cryopreservation to improve the success of embryo cryopreservation in spiny mice.

If successful, our protocols will benefit future in-vitro and in-vivo reproductive research in this species by providing viable cells and tissues to more effectively use the spiny mouse as a model for human reproduction.

Author Contributions

Conceptualization: Jarrod McKenna, Nadia Bellofiore, Peter Temple-Smith.

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Chapter 6: General Discussion

6.1 Overall findings and significance

Egyptian spiny mice (*Acomys cahirinus*) were recently shown to exhibit a natural, human-like menstrual cycle (Bellofiore et al., 2017); an exceptionally rare trait among mammals (<2%) and unheard of in any other rodent species. This thesis provides a unique analysis of some fundamental aspects of spiny mouse reproductive physiology and behaviour, and paves the way for the use of the spiny mouse as an *in-vivo* and *in-vitro* model of human reproduction.

Our knowledge of laboratory mouse (*Mus musculus*) reproductive physiology dates back to the early 1930s, and researchers have access to an array of peer-reviewed or commercially available laboratory protocols. While spiny mice have been used as laboratory animals for over half a century (Gonet et al., 1966), spiny mouse reproductive physiology has been examined for just over a decade, and there are current only a few captive colonies in the world. Additionally, published research in its murid relatives does not necessarily transfer directly to spiny mice, and publications in this species often lack transparency, repeatability or require further optimisation. Together, the novelty of the spiny mouse in research and the limited access to captive populations makes this species challenging to work with, and explains why little has been published previously on its reproductive physiology.

In this thesis, I describe how the menstrual cycle affects spiny mouse mating behaviour, fertilisation and pregnancy outcomes, improved colony breeding efficiency by providing a protocol for reliable estimates of gestational age without using postpartum animals. I have also investigated the previously overlooked phenomena of a postpartum ovulation in a true-menstruating species. I have characterised the endocrinology and morphology of the

postpartum spiny mouse reproductive tract, and describe the timing and invasiveness of embryo implantation. I have also defined and optimised a robust protocol for sperm cryopreservation, which not only makes the spiny mouse model more accessible for future in-vitro reproductive research, but also archives the important genetics of our breeding colony. Lastly, I have designed several human-based ART protocols, which, when successfully tested and combined with my published sperm cryopreservation protocol, will establish the spiny mouse as a more relevant, accessible animal model of human reproductive function.

6.1.1 Mating behaviour

Prior to my study described in chapter 2, a single publication has described the mating behaviour of captive spiny mice (Dewsbury & Hodges, 1987). While the authors described mounting, intromission and ejaculatory behaviours, this study preceded the discovery of menstruation in *A. cahirinus* and no attempt was made to correlate the phase of the reproductive cycle with the timing of copulation and pregnancy or birth outcomes, which may prove to be of vital importance in a menstruating species

Considering spiny mice experience a postpartum ovulation (Strasser, 1968), estimates of gestational age are calculated from the date of delivery of the previous term litter (Ireland et al., 2011; O'Connell et al., 2013). While this method provides a useful and relatively accurate assessment of gestational age, it is time-consuming, costly and inefficient. Therefore, the rationale for exploring mating behaviour in spiny mice was to provide a simpler, cheaper alternative to using postpartum animals for gestational studies. My study confirms, and extends, the original report of spiny mouse mating behaviour by Dewsbury and Hodges (1987). I provide clear video evidence of spiny mouse mating behaviour,

outlining the relationship between mounting, intromission, ejaculation and pregnancy outcomes. I also provide evidence that copulatory locks are ejaculatory events by the presence of spermatozoa in vaginal smears of females immediately following locking events. Lastly, this study demonstrates a clear effect of the spiny mouse menstrual cycle on mating behaviour and pregnancy outcomes, and provides the information required to reliably estimate gestational age without the use of postpartum dams. As a result, breeding efficiency and maintenance of one of the few captive research colonies of spiny mice has been refined, and the results from this chapter has been drafted as a manuscript and submitted for publication in the Q1 journal Animal Behaviour.

6.1.2 Postpartum ovulation in a menstruating rodent

To our knowledge, spiny mice are the only known mammalian species with a human-like menstrual cycle and a postpartum ovulation (PPO) (Strasser, 1968). PPO was suggested by Stasser in 1968, but his report was lacking histological or endocrine evidence and at that time spiny mice were not known to menstruate. During this thesis, I evaluated postpartum spiny mouse sex steroid endocrinology, whilst characterising the gross morphological changes to the postpartum reproductive tract and describing the timing and invasiveness of embryo implantation.

While ovarian histology in the spiny mouse showed a similar pattern of folliculogenesis during pregnancy to women (Dekel et al., 1977), the endocrinology and persistence of corpora lutea throughout gestation is similar to other rodents (mice and rats; (Hilliard, 1973; Takiguchi et al., 2004)). Moreover, when comparing the postpartum reproductive tract to cycling spiny mice (Bellofiore, Rana, et al., 2018) , there were similar structural and

physiological characteristics including re-epithelialisation, increased endometrial growth and epithelial receptivity prior to embryo implantation.

In line with those of its murid relatives (Vasquez et al., 2018), spiny mouse embryos were implanted superficially by day 5 postpartum with minimal trophoblast invasion. However, postpartum animals also showed spiral artery formation and remodelling events similar to those occurring around human embryo implantation (Kim et al., 1999; Petroff & Hunt, 2005). Therefore, this study revealed similarities in early pregnancy to both rodent and humans. While this study highlights the potential of the spiny mouse as a model of early human pregnancy, there are several limitations to current and future research.

Relative to *M. musculus*, spiny mice are still a novel biomedical research species. Currently, no commercially produced, spiny mouse species-specific antibodies are available for immunohistochemical and protein-based methods. Because of this I was unable to measure circulating LH, FSH or prolactin using commercially produced ELISA kits, despite trialling antibodies which detect mouse/rat (LH, FSH) and human (LH, FSH and prolactin) proteins, which would have provided another important layer of knowledge about reproduction in this species. Although the amino acid sequence of these peptide hormones are generally conserved across mammalian species (Mullen et al., 2013), some of the spiny mice reproductive peptides and proteins apparently have a unique structures to other mammals, including other murid rodents, which showlittle cross-reactivity and/or do not share sufficient sequence homology with murids or humans to assay successfully using standard ELISA kits. Although the spiny mouse transcriptome has been drafted (Mamrot et al., 2017), a fully sequenced and assembled genome is not yet available to conduct protein sequence homology. Moreover, I was also unable to optimise

immunohistochemical methods for detection of traditional markers of decidualisation including interleukin 11 (IL-11) or insulin-like growth factor binding protein 1 (IGFBP-1). Thus, an in-depth analysis of spiny mouse endocrinology and decidualisation was limited.

Despite these challenges, I have provided new and robust evidence for a postpartum ovulation within 48hrs after parturition, and described angiogenesis, epithelial integrity and receptivity, and superficial embryo implantation 5-6 days after parturition in spiny mice. The experiments described in this chapter have been submitted as a manuscript for publication in the Q1 journal Scientific Reports.

6.1.3 Assisted reproductive techniques

M. musculus is undoubtedly the most widely used animal model in biomedical research, and a plethora of ARTs are available for in-vitro and in-vivo research. Prior to my published studies in chapters 4 and 5, there was a paucity of information regarding ARTs in spiny mice. Only a single (Pasco et al., 2012) proposing a superovulation protocol had been published, and no methods for gamete cryopreservation, IVF or embryo transfers were available. A clear need to develop specific laboratory-based assisted reproduction protocols was identified, not only to expand and make the spiny mouse model more relevant but to safeguard our unequivocal breeding colony for future research.

Development of a robust sperm cryopreservation protocol in spiny mice posed several challenges. Published slow-cooling and vitrification protocols for mouse spermatozoa were comparatively unsuccessful in spiny mice and commercially available protocols for DNA damage (TUNEL assay) were not immediately applicable to spiny mice and required extensive optimisation. However, during my candidature, spiny mice spermatozoa were

successfully cryopreserved, and a species-specific TUNEL protocol for DNA damage was optimised (chapter 4). These published protocols (Reproduction, Fertility & Development; Q1) provide new possibilities for future ARTs research whilst preserving and archiving the genetics of the world's only known menstruating rodent.

A significant change to the direction of my original thesis research plan was the discovery that a published successful superovulation protocol (Pasco et al., 2012) could not be repeated despite numerous attempts. I was only able to collect healthy, mature oocytes from 1/27 females, and embryo collection timings conflicted with the original report. Moreover, modifications to injection and collection timings were unsuccessful, and several collected oocytes were of either unknown maturity, or considered parthenogenetically activated due to extremely rapid cleavage rates. As this protocol did not include downregulation of the HPG axis, and therefore ovarian/pituitary function, we believe that this protocol was not appropriate and required further optimisation. Thus, I turned to other, true-menstruating species to define a novel, effective superovulation protocol in *A. cahirinus.* In chapter 5, I defined protocols for human-based, agonist-superovulation, IVF, embryo cryopreservation and transfer and these protocols were published as a registered-report protocol in *Plos* One (Q1). However, due to time restraints and lab/animal inaccessibility resulting from COVID-19 restrictions, these protocols have yet to be tested. If these human-based protocols successfully assist reproduction in the spiny mice they have the potential to provide a better understanding of embryogenesis, implantation and pregnancy and therefore a more relevant, accessible menstruating model for reproductive studies, especially for modelling some aspects women's reproductive health that are difficult or impossible using the current non-menstruating small animal models.

6.1.4 Future directions

Future directions for this work include collection and analysis of post-coital female reproductive tracts to determine the location of insemination and provide further insight into seminal plug formation, in-vivo fertilisation, sperm transit and survival in spiny mice. Characterisation of pre-partum reproductive tissues (uteri, cervix, vagina) and mating behaviour would be of interest to examine further the timing of ovulation and insemination in relation to parturition, and to compare pre-partum folliculogenesis to both cycling and postpartum animals. Additionally, optimisation and analysis of immunomarkers for decidualisation (IGFBP-1, IL-11 etc) will provide further insight into postpartum uterine receptivity prior to embryo implantation. Considering the data from chapter 4, future studies of other freezing parameters such as freezing distances, cooling velocities and use of different cryoprotectants (permeable and non-permeable) are encouraged to optimise further sperm cryopreservation in this species. Finally, testing of the protocols outlined in chapter 5 including superovulation, IVF and embryo cryopreservation in spiny mice are important future directions from the work described in this thesis, with the intention to make the spiny mouse a more accessible and relevant small animal model of human reproductive function.

6.1.5 Conclusion

Overall, Egyptian spiny mice display similar physiological characteristics to other menstrual species, whilst providing researchers with the ease and efficiency of a typical laboratory rodent colony. This thesis provides new data to confirm the unique, human-esque reproductive biology of Egyptian spiny mice. By applying the knowledge from my in-depth analysis of spiny mouse mating behaviour, I have increased the breeding efficiency

of our captive spiny mouse colony and provided new protocols that give more reliable estimates of fetal and gestational age than previous methods. Analysis of postpartum reproductive tissues has provided robust evidence for postpartum ovulation in the menstruating spiny mouse. Lastly, I have provided the first assisted reproductive techniques (ARTs) and protocols in this species allowing for further evaluation of the utility of spiny mice as a non-primate, small animal model of human reproduction.

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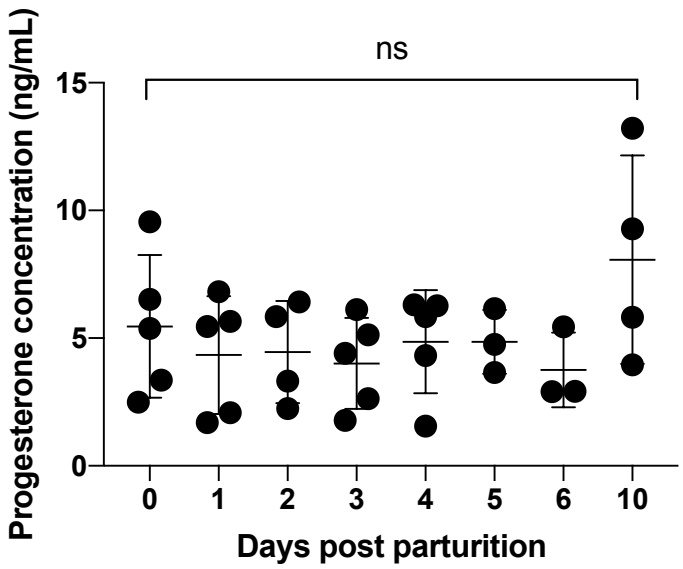
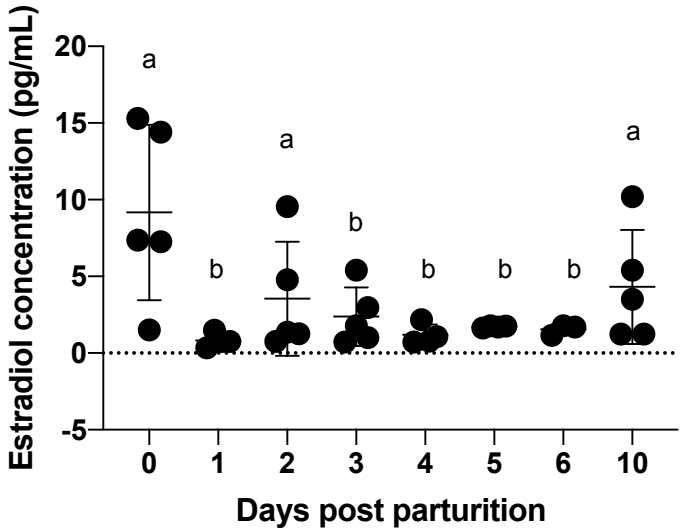
Appendix



Appendix figure 1 Progesterone concentration (ng/mL) determined by radioimmunoassay. Progesterone concentrations were non-significantly different at all timepoints postpartum and reflects the progesterone concentrations determined in-house through enzyme-linked immunosorbent assay (ELISA). Graphs depict mean ± SD and data with different letters differ significantly.



Appendix figure 5 Estradiol concentrations (pg/mL) determined by liquid chromatography-mass spectrometry (LC-MS). Estradiol (E2) concentrations were similar on days 0, 2 and 10pp, but were significantly reduced compared to the remaining timepoints postpartum. Postpartum E2 concentrations determined by LC-MS follow a similar pattern to E2 concentrations determined in-house through enzyme-linked immunosorbent assay (ELISA). Graphs depict mean ± SD and data with different letters differ significantly.