New insights into the Reproductive Physiology and Management of the Female Koala (*Phascolarctos cinereus*): Factors affecting the control of the oestrous cycle

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B.App.Sc. (Hons)

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School of Agriculture and Food Science
ABSTRACT

The objective of this thesis was to investigate the factors controlling the reproductive cycle of the female koala and develop an oestrous synchronization technique to facilitate the further refinement and efficacy of artificial insemination (AI) in the koala. A component of this development was the establishment of less invasive strategies to accurately monitor the reproductive status of the female koala, without the need for physical restraint or serial venipuncture. Chapter 2 of this thesis evaluated the efficacy of faecal oestrogen analysis, combined with the detection of oestrous behaviour as a control, to monitor ovarian cycles. Whilst faecal oestrogens did not correlate well with plasma oestradiol-17β to allow for an accurate estimate of cycle length or indicate or predict the precise timing of oestrus, the individual mean faecal oestrogen concentrations did, however, show a strong relationship to the individual mean plasma oestradiol-17β concentrations for each koala; in addition total faecal oestrogens were significantly higher in cycling females (P = 0.007).

Chapters 3 and 4 of this thesis examined factors controlling reproductive cyclicity in the female koala. The pattern of prolactin (Prl) secretion and its relationship to oestrous behaviour and pouch young (PY) development throughout lactation in the koala was investigated in Chapter 3. Oestrous behaviour was suppressed throughout the majority of lactation despite basal levels of Prl during early lactation. Koalas returned to oestrus some 102 days before PY had reached independence. The koala anterior pituitary also remained responsive in terms of luteinizing hormone (LH) secretion to injections of mammalian gonadotrophin-releasing hormone (mGnRH) during periods of high and low Prl secretion. Chapter 4 investigated the seasonality of oestrous behaviour (oestrous cycle activity) in a captive koala population in Southeast Queensland (SEQ). Although some individual koalas in the population showed signs of oestrous behaviour throughout the year, an obvious seasonality was apparent with significantly less females displaying oestrous
behaviour in late autumn and winter (May - August), than September to April (P < 0.0001). While average monthly photoperiod (P < 0.0001) and average monthly temperature (P < 0.0001) were associated with oestrous behaviour, rainfall was not (P = 0.097).

Chapters 5 and 6 report studies which investigated techniques, designed to allow for the manipulation and planning of timed insemination of female koalas. The impact of the gonadotrophin-releasing hormone (GnRH) antagonist, azaline B on LH and ovarian steroid hormone secretion in response to stimulation with mGnRH and its potential application in an oestrous synchronization protocol in cycling koalas was examined in chapter 5. In experiment 1, single sub-cutaneous (SC) injections of azaline B successfully blocked the ability of the anterior pituitary (AP) to respond to exogenous mGnRH in a dose dependant manner: 0 mg (n = 4) did not suppress LH response, 1 mg (n = 6) suppressed LH response for 24h (P<0.05), 3.3 mg (n = 8) suppressed LH response for 3h (P<0.05) and 10 mg (n = 4) suppressed LH response for 7 d (P<0.05). In experiment 2, daily 1 mg SC injections of azaline B over a 10 d period during seasonal anoestrus (June – July) (n = 6), suppressed (P<0.01) the LH response to mGnRH and the LH response did not recover four days after cessation of treatment. Experiment 3 was designed to test the efficacy of a daily 1 mg SC dose of azaline B over 10 days to suppress plasma LH and oestradiol-17β concentrations and ultimately synchronize timed return to oestrus during the breeding season. Whilst treatment with azaline B did not suppress basal LH or oestradiol-17β, oestrus was delayed in all treated females by 24.2 ± 5.0 days, but this period was highly variable (range 9 - 39d). Overall, this study demonstrated the GnRH antagonist azaline B in koalas is able to inhibit the LH response to exogenous mGnRH and successfully delay the return to oestrus. However, whilst azaline B appears to clearly disrupt folliculogenesis, it was not able to effectively synchronise return to oestrus in the koala.
The ability of the synthetic progestogen implant levenorgestrel (LNG) to control ovarian activity for the purposes of oestrous synchronisation in the koala was investigated in chapter 6. Following implantation, LNG treated koalas immediately ceased displaying oestrous behaviour, showed reduced oestradiol-17β secretion to basal levels and no evidence of urogenital cytology consistent with a koala in oestrus. In contrast, plasma oestradiol-17β levels in control koalas showed evidence of cyclic activity associated with periods of behavioural oestrus and a coincident increase in the proportion of cornified epithelial cells in the urogenital smear at day 33 to 35 after saline injection. LNG treated koalas showed a subsequent oestrus 13, 14, 17 and 30 days after LNG implant removal and continued to produce PY in subsequent breeding seasons. This suggests that LNG implants can inhibit oestrous behaviour and elevated secretion of oestradiol-17β, most likely through preventing sufficient development of a pre-ovulatory follicle. While removal of the implant resulted in the synchronous return of oestrus in 3 of the 4 treated koalas, further studies on a larger population are required to validate this finding.

Whilst a reliable oestrous synchronization protocol was not achieved, this thesis has provided significant insight into the mechanisms controlling reproductive activity in the koala which can now form the basis for future research. These include: 1) Preliminary evidence for the importance of the suckling stimulus, not prolactin secretion, in the suppression of koala oestrous behaviour during early lactation, 2) Evidence for a clear seasonality in captive female koala reproductive activity, 3) The ability to temporarily disrupt the koala follicular cycle using repeated doses of azaline B, 4) Preliminary evidence for the use of synthetic progestogen as a mechanism for oestrous synchronization in the koala.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly authored works that I have included in my thesis.

I have also clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis.

The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature


Publications included in this thesis


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<tbody>
<tr>
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The use of the GnRH antagonist Azaline B to control the oestrous cycle in the koala (Phascolarctos cinereus), Reproduction, Fertility and Development, doi.org/10.1071/RD14349

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Contributions by others to the thesis

Dr Stephen Johnston (SAFS) - Conception and design of experiments, critical revision of manuscript and thesis.

Dr Stephen Anderson - Critical revision of manuscript and thesis, statistical analysis, development and use of Prl and LH assays, assistance and supervision of hormonal analysis.

Mr Allan Lisle - Statistical analysis

Drs Michael Pyne and Vere Nicholson - Veterinary care and sample collection

Statement of parts of the thesis submitted to qualify for the award of another degree

None
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Oestrous synchronization, oestrous cycle, seasonality, GnRH antagonist, azaline B, synthetic progestogen, faecal metabolites, prolactin, *Phascolarctos cinereus*
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Fields of Research (FoR) Classification

FoR code: 0608, Zoology, 100%
List of Tables

Chapter 1

Table 1.1: Characteristics used to clarify the patterns of reproduction as proposed by Tyndale-Biscoe and Renfree (1987).  

Chapter 2

Table 2.1: Correlation coefficients between plasma oestradiol-17β and faecal total oestrogens with a 0, 2, 4, 6, 8, 10 and 12 day lag.  

Chapter 3

Table 3.1: Basal LH, LH response to mGnRH and Prl concentrations during mid and late lactation.
## List of Figures

### Chapter 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>The control of reproduction in female marsupials</td>
<td>1 - 15</td>
</tr>
<tr>
<td>1.2</td>
<td>Mean (± SEM) plasma concentrations of FSH and LH in brush tail possums that ovulated following removal of pouch young.</td>
<td>1 – 19</td>
</tr>
<tr>
<td>1.3</td>
<td>Mean daily plasma concentrations of LH, FSH, Prl and P4 in reproductively cycling female brushtail possums in relation to the day of ovulation.</td>
<td>1 - 20</td>
</tr>
<tr>
<td>1.4</td>
<td>Plasma progesterone concentration profiles of 9 marsupial species during pregnancy.</td>
<td>1 - 26</td>
</tr>
<tr>
<td>1.5</td>
<td>Mean (± SEM) concentration of (a) oestradiol-17β and (b) progestogen up to 49 days after mating in six pregnant and six non-pregnant koalas.</td>
<td>1 - 27</td>
</tr>
<tr>
<td>1.6</td>
<td>Mind map of thesis concepts.</td>
<td>1 - 35</td>
</tr>
</tbody>
</table>

### Chapter 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Individual koala profiles of faecal total oestrogens, plasma oestradiol-17β and oestrous behaviour in non-cycling and cycling koalas.</td>
<td>2 - 8</td>
</tr>
<tr>
<td>2.2</td>
<td>Association between mean total faecal oestrogens (log10) (±SEM) and mean plasma oestradiol-17β (log10) (±SEM) in cycling (closed circles) and non-cycling (open circles) koalas.</td>
<td>2 - 9</td>
</tr>
</tbody>
</table>

### Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Mean (± SEM) Prl concentrations throughout the lactation period of the koala.</td>
<td>3 - 7</td>
</tr>
</tbody>
</table>
Figure 3.2: Prl concentrations, developmental stages of pouch young (PY) and the occurrence of oestrous behaviour throughout the lactation period.

Chapter 4

Figure 4.1: Proportion of koalas in oestrus per calendar month in 2009, 2010, 2011, 2012, fitted with an average trend line.

Figure 4.2: Monthly percentage of koalas that showed oestrous behaviour (columns) and mean monthly temperature (°C) (± SD) in 2009, 2010, 2011 and 2012.

Figure 4.3: Percentage of koalas that showed oestrous behaviour (2009, 2010, 2011, 2012) and photoperiod (h).

Figure 4.4: Monthly percentage of koalas that showed oestrous behaviour and total rainfall (mm) in 2009, 2010, 2011 and 2012.

Figure 4.5: Monthly percentage of koalas that showed oestrous behaviour and mean monthly weight (g) (± SEM) in 2012.

Figure 4.6: Monthly percentage of births from successful matings (%) (2003-2012).

Figure 4.7: Number of male and female PY born per calendar month from 2003-2012.

Chapter 5

Figure 5.1: LH response to mGnRH challenge in koalas given different doses of azaline B.

Figure 5.2: (A) LH response to mGnRH challenge (T15 – T0) and (B) Plasma LH concentrations prior to mGnRH challenge (T0) in koalas before, during and post azaline B treatment.

Figure 5.3: Basal plasma oestradiol-17β and LH concentrations during (0-10d)
and after (10-60d) 10 daily 1 mg injections of azaline B. Azaline treatment was during the interoestrus phase of the female koala, commencing 3 days after cessation of oestrus.

**Chapter 6**

Figure 6.1: Individual animal profiles of plasma oestradiol-17β and luteinizing hormone concentrations, oestrous behavioural observations and positive oestrous urogenital smears in control treated and LNG implanted females.
List of abbreviations used in the thesis

ABT – Assisted breeding technology
AP - Anterior pituitary
AI - Artificial insemination
CL - Corpus luteum
CWS - Currumbin Wildlife Sanctuary
DW - Dreamworld
EE – Electro-ejaculation
FSH - Follicular-stimulating hormone
GnRH - Gonadotrophin releasing hormone
GnRH-R - Gonadotrophin releasing hormone receptors
GRB – Gene resource bank
hCG - Human chorionic gonadotropin
LNG - Levonorgestrel
LH - Luteinizing hormone
mGnRH - Mammalian gonadotrophin releasing hormone
NSW - New South Wales
PCR – Polymerase chain reaction
PM – (faecal) progestagens metabolites
PY - Pouch young
Prl – Prolactin
SD – Standard deviation
SEM – Standard error of the mean
SEQ – Southeast Queensland
SC – Sub-cutaneous
TABLE OF CONTENTS

PRELIMINARY PAGES
ABSTRACT.......................................................................................................................... I
DECLARATION BY AUTHOR ...............................................................................................V
PUBLICATIONS DURING CANDIDATURE .......................................................................... VI
PUBLICATIONS INCLUDED IN THESIS ............................................................................ VII
CONTRIBUTIONS BY OTHERS .......................................................................................XI
ACKNOWLEDGEMENTS .................................................................................................. XII
LIST OF TABLES ............................................................................................................... XIV
LIST OF FIGURES ........................................................................................................... XV
LIST OF ABBREVIATIONS ................................................................................................ XVIII

CHAPTER 1: INTRODUCTION............................................................................................. 1-1
CONSERVATION OF THE KOALA ..................................................................................... 1-1
FEMALE MARSUPIAL REPRODUCTION: A REVIEW .................................................. 1-7
THESIS CONCEPTS ......................................................................................................... 1-34
REFERENCES .................................................................................................................. 1-44

CHAPTER 2: MONITORING REPRODUCTIVE ACTIVITY IN THE KOALA USING
FAECAL OESTROGEN SECRETION AND BEHAVIOURAL OESTRUS .................... 2-1
ABSTRACT ....................................................................................................................... 2-1
INTRODUCTION .............................................................................................................. 2-2
METHODS ...................................................................................................................... 2-3
RESULTS .......................................................................................................................... 2-7
DISCUSSION .................................................................................................................... 2-10
REFERENCES .................................................................................................................. 2-13

CHAPTER 3: PLASMA PROLACTIN CONCENTRATIONS DURING LACTATION,
POUCH YOUNG DEVELOPMENT AND THE RETURN TO BEHAVIOURAL OESTRUS
IN CAPTIVE KOALAS (PHASCOLARCTOS CINEREUS) ................................................. 3-1
ABSTRACT ....................................................................................................................... 3-1
INTRODUCTION .............................................................................................................. 3-2
METHODS ...................................................................................................................... 3-3
RESULTS .......................................................................................................................... 3-6
DISCUSSION .................................................................................................................... 3-9

xix
CHAPTER 1: INTRODUCTION

CONSERVATION OF THE KOALA

Current status of the Koala

The koala is an Australian cultural icon and a major contributor to the tourist economy. In 1996, koala-based industries generated approximately $1.1 billion worth of revenue; this is equivalent to 9000 jobs (Hundloe and Hamilton, 1997). Consequently, the conservation and management of this species in SEQ and Northern New South Wales (NSW) is of major concern to both State and Local Governments. The Queensland koala population, once in the millions, has declined substantially over the past century with a population estimate of approximately 300000 in 1990 and a further decline of 43% over 10 years to approximately 170000 in 2010 (Threatened Species Scientific Committee, 2012).

The koala coast, identified as one of the most significant remaining koala habitats in Australia, covers 375 sq km of land located within the SEQ bioregion and stretches from the border of northern NSW to Gladstone and west to Toowoomba. The increasing urban expansion in SEQ, (currently the largest in Australia) poses the largest threat to the existing koala habitat and conservation. In 2004, the koalas in the SEQ Bioregion were classified as ‘vulnerable’ to extinction after scientific data indicated a significant decline in their population and a high level of threat from changing land uses (Environmental Protection Agency, 2006). The results of the 2008 Koala Coast koala survey showed a 51% decline in less than 3 yrs and a 64 percent decline in 10 yrs from more than 6200 in 1999 to an estimate of 2279 (Department of Environment and Resource Management, 2009). The most recent koala population survey (2010) estimated a further decline of 13% to around 2000 (Department of Environment and Resource Management, 2012).
Current Conservation Plan

In a bid to reduce the increasing threat to koala populations and to ensure the conservation of koala populations in Queensland, the Nature Conservation (Koala) Conservation Plan 2006 and Management Program 2006–2016 was developed. This plan addresses the threats of urban expansion along with a focus on koala management including public education, captive koala populations, research and rehabilitation. Whilst significant effort has been invested into the preservation and restoration of koala habitat in Queensland, little attention has been directed in these programs towards the active management of genetic viability.

The koalas in the Koala Coast have been reported to have a significantly lower level of heterozygosity and reduced allelic variation compared to other koala populations in SEQ (Lee et al., 2010). In an earlier study, Thompson (2006) provided evidence of habitat fragmentation due to urban development presenting as a barrier to gene flow and further isolation of local populations within fragmented urban landscapes. Given the current ‘vulnerable’ status and rapid decline of the koala coast population, genetic isolation could prove detrimental to population sustainability.

The establishment and collaborative management of genetically viable captive koala populations in association with zoological institutions or specific breeding facilities is an important conservation tool providing a valuable reservoir of genetic diversity, that can potentially be used to produce animals for re-introduction (Johnston et al., 2013a). Other Australian State governments such as Western and South Australia have already recognized the importance of captive populations in the management of genetics and the propagation of new individuals for release in recovery programs in a range of marsupials. For example, in 2005 a captive breeding program to establish a disease-free ‘insurance population’ for the Tasmanian devil (Sarcophilus harrisii), was developed across 17 zoos and parks across Australia (Zoo Aquarium Association, 2013) and Perth Zoo has
been instrumental in the conservation management of the numbat (*Myrmecobius fasciatus*), dibbler (*Parantechinus apicalis*) and the western swamp tortoise (*Pseudemydura umbrina*) (Perth Zoo, 2013).

There are currently seven *ex situ* koala populations in SEQ; Lone Pine Koala Sanctuary (n = 96), Dreamworld (n = 63) Currumbin Wildlife Sanctuary (n = 54), Australia Zoo (n = 60), Paradise Country (n = 26), Alma Park Zoo (n = 11) and David Fleay Wildlife Park (n = 3) (Australasian Regional Association of Zoological Parks and Aquaria, 2014). Managed as a single population through captive breeding and zoo exchange, there are a total of 313 koalas; this represents a significant population that could prove vital to the future conservation of the species. The allelic diversity of both the Dreamworld and Currumbin Wildlife Sanctuary populations has also been found to be similar to that of wild populations in SEQ, evidence that the heterozygosity of these populations appears to have been managed appropriately (Lee et al., 2009).

Reproductive Technology and Koala Conservation

Assisted breeding technology (ABT) is becoming a valuable tool for captive koala breeding and genetic management (Johnson and Holt, 2014). Although it is clear that it is still not a panacea for the loss of individual animals as a result of human based activity, reproductive technology has the potential to play an important role in supporting genetic viability in captive populations in anticipation for re-introduction to the wild. The current reproductive management technique for captive koalas is reliant upon oestrous detection (Johnston et al., 2000b) and natural mating. In the larger wildlife parks in SEQ, such as Lone Pine Sanctuary, Dreamworld and Currumbin Wildlife Sanctuary, koala breeding is vigilantly managed through the strict adherence to a Zoo and Aquarium Association studbook (Mucci pers. Comm.) to ensure maximum heterozygosity is maintained in the population, whereas genetic management of smaller captive populations is reliant
upon the occasional transfer of animals between wildlife parks; this is especially true for the captive populations held in overseas zoological institutions, which is currently achieved through the shipment of whole animals. With the development of AI technology, this task could be more efficiently conducted through the shipment and storage of semen.

Cryopreserved semen samples shipped both nationally and internationally negate the welfare issues and added cost of quarantine associated with the transfer of whole animals. Preserved semen can be screened by polymerase chain reaction (PCR) for infections such as Chlamydia (Bodetti et al., 2003) prior to insemination. Gene resource banks (GRB) also permit the indefinite storage of genetic material including spermatozoa, oocytes and embryos and combined with AI potentially permit precise reproductive and genetic management and facilitate production of all genetically viable animals in terms of both space and time.

The development of electro-ejaculation (EE) has advanced the capabilities of this technology even further, allowing for the collection of semen from wild individuals and injured animals that would not normally be able to mate (Johnston and Holt, 2014). The practical application of the procedure has recently been demonstrated by Allen et al. (2010) with the successful collection of repeated samples from both captive (monthly) and wild koalas (every 6 weeks) from the same individuals. In association with AI, semen collected via EE can be used to infuse genetic material from wild-born individuals into genetically stagnant ex situ populations or alternatively indefinitely cryopreserved in a GRB for future generations.

Despite significant advances in koala sperm cryopreservation (Johnston et al., 2006, Zee et al., 2008, Zee et al., 2007, Johnston et al., 2012, Johnston et al., 2013c), the quality of cryopreserved koala semen after thawing is still inadequate for successful AI. Although this precludes genome resource banking for the moment, chilled semen storage offers significant
logistical flexibility for the application of AI in the koala, particularly as it relates to the national and global movement of genetic material.

Allen et al. (2008b) has previously demonstrated the successful use of extended and extended-chilled koala semen collected via electro ejaculation. The study resulted in the birth of 18 joeys with a conception rate of 44%; similar to that determined previously by Johnston et al. (2003) and a favourable comparison with estimates of conception rates following natural mating in established zoological institutions that specialize in koalas (e.g., Lone Pine Koala Sanctuary) of between 43% and 57% (O'Callaghan, 1996). PY were produced from semen, which was extended and chilled for 72hrs (Allen et al., 2008b), which is plenty of time to allow for international air transportation to relevant overseas zoological institutions, thereby opening the way for the national and international exchange of koala genetic material. The same study also demonstrated the successful use of genetic exchange between institutions with the production of a PY in one wildlife park using a chilled semen sample collected in another sanctuary approximately 43 km away.

PY have also been produced from successful copulations up to Day 8 of oestrus, providing a significant amount of time flexibility for the collection and transportation of semen (Allen et al., 2008b). In a recent study Johnston et al. (2013c) examined the effect of chilled (5°C) semen storage for 16 days on sperm motility, membrane integrity, the percentage of sperm with relaxed chromatin and sperm fragmentation; that being 50%, 57%, 25% and 15% respectively. Although further AI studies are required, these results suggest that koala spermatozoa are potentially fertile after chilled storage for 16 days, increasing the degree of flexibility for the collection and transportation of semen even further.

Apart from its important use in ex situ conservation, koala AI also has the potential to have an important impact on the management of wild koala populations in SEQ. The combined use of
chilled spermatozoa with AI will allow the transfer of genetic material between land-locked koala populations for which there is limited opportunity for natural genetic exchange. Johnston et al. (2013a) recently introduced the idea of a living genome resource bank to assist with genetic exchange and population management. Utilizing AI and natural captive breeding, captive animals could become the repositories of important genetics of wild animals and could be used to facilitate gene flow. This proposal involves the development of a koala breeding centre where wild koalas could be held for a short period of time to allow successful genetic exchange with either captive animals or wild animals from other fragmented populations. Koalas with PY would be released back into their respective fragment ensuring greater genetic diversity and gene flow into both fragments and the propagation of additional captive offspring of high genetic worth. These captive populations, if genetically managed appropriately, could also be used for restocking rehabilitated or reclaimed empty habitat (Johnston et al., 2013a). The success of such a project, however, relies heavily on a thorough understanding of the genetic relationships within these populations and the adaptations to their specific environments (Thompson, 2006).

Oestrous Cycle Control

In order to further refine AI in the koala and minimise the holding time of wild koalas within captivity, a methodology that will enable the control of the timing of oestrus in the koala needs to be developed. The ability to synchronise oestrus in the koala, as is the situation in domestic species, would greatly facilitate the wide spread use of AI in the zoo industry and have implications for genetic exchange. If adequate control of the koala oestrous cycle could be attained, wild male koalas could be brought into captivity at a time that coincided with oestrus in captive females, so that mating could occur promptly and wild male koalas to be released almost immediately. Further, semen from both wild and captive animals housed in other zoological institutions could be collected, chilled and transported in unison with female oestrus, allowing multiple females to be
inseminated; this would be a far more cost effective approach to the transportation of whole animals especially in regards to the associated housing costs. Such an approach is vital until sperm cryopreservation is advanced in the koala. Consequently, this thesis will investigate the factors controlling the reproductive cycle of the koala and techniques which will allow for the manipulation and planing of insemination of females to facilitate the further refinement and efficacy of artificial insemination in the koala.

FEMALE MARSUPIAL REPRODUCTION: A REVIEW

Our understanding of the reproductive biology of marsupials is limited by the fact that a large proportion of the published literature is based on the tammar wallaby (*Macropus eugenii*). It would be extremely naïve to think that all marsupial reproductive physiology is the same as that found in the tammar wallaby, just as it would be naïve to consider that all eutherian mammals have similar reproductive strategies and physiologies. It is, therefore, important to summarize the range of reproductive physiology so far described in female marsupials and then ultimately compare what is known in the koala.

Patterns of Marsupial Reproduction

Tyndale-Biscoe and Renfree (1987) have classified marsupial female reproduction into 4 distinct categories defined in terms of the number of oocytes they ovulate per oestrous cycle (mono or poly ovular), frequency of the oestrous cycle (mono or poly oestrous), ratio of the gestation period to oestrous cycle length, timing of parturition with respect to the stage of the oestrous cycle, placental type and the presence or absence of embryonic diapause (Table 1.1). These definitions were extended by the inclusion of induced ovulation data in the koala (Johnston and Holt, 2001). A recent review by McAllan (2011) summarised these definitions into 3 broad categories, however
some of the unique sub categories were overlooked. This review will focus on the more descriptive grouping originally described by Tyndale-Biscoe and Renfree (1987) with reference to recent research on marsupial reproductive biology.

**Group 1**

The luteal phase of group 1 marsupials is comparable in length to their gestation period, which typically comprises less than 60% of the oestrous cycle. Birth typically correlates with the end of the luteal phase and subsequent follicular growth is suppressed by lactation (Tyndale-Biscoe and Renfree, 1987). Follicular growth recommences with the loss or removal of pouch young during the breeding season (Hinds, 1990). Marsupials displaying this mode of reproduction can either be mono or polyovular, mono or polyoestrous and posses a range of placental types (1, 2 and 3). This mode of reproduction is considered to be the most primitive and most diverse and is representative of the Dasyuridae, Didelphidae, Petauridae, Vombatidae and Phalangeridae. It also includes the Phascolarctidae (koala), which is the only marsupial described to date in which induced or reflex ovulation has been confirmed. It is likely that this classification of the mode of female reproduction should be further sub-divided to include the unusual reproductive physiology of the koala and monoestrous (semelparous) carnivorous marsupials such as the *Antechinus* and *Planigale* spp (Johnston and Holt, 2001).

**Group 2**

Group 2 marsupials have a similar reproductive pattern to that described for group 1, except that pregnancy is substantially shorter than the luteal phase occupying between 59 and 67% of the oestrous cycle length (Tyndale-Biscoe and Renfree, 1987). The luteal phase is extended into the lactation period so that parturition occurs during the mid-luteal phase. This mode of reproduction appears to be unique to the bandicoot (Peramelidae) and possibly, by phylogenetic association, the greater bilby (*Macrotis lagotis*) (Ballantyne et al., 2009).
Group 3

The reproductive biology of group 3 is typical of the Macropodidae and Potoridae. These species normally have a luteal phase that extends into the next oestrous cycle so that gestation occupies more than 90% of the cycle (Hinds, 1990). Follicular growth resumes in the last third of pregnancy such that post-partum oestrus and ovulation occurs. Parturition occurs at the termination of the luteal phase and further development of the recently formed CL and associated embryo in utero is arrested by the suckling stimulus associated with lactation (Tyndale-Biscoe and Renfree, 1987). In the absence of increased progesterone, the new embryo, which develops to a unilaminar blastocyst, enters embryonic diapause (Hinds, 1990). Removal of the suckling pouch young induces reactivation of the CL and the diapausing blastocyst (Hinds, 1990). In four macropod species, the parma wallaby (*Macropus pama*), the whip tail wallaby (*M. parryi*), the eastern grey kangaroo (*M. giganteus*) and the western grey kangaroo (*M. fulignosus*), the long gestation period is associated within an extended luteal phase so that gestation extends over 80% of the oestrous cycle. Post-partum ovulation rarely occurs in these species, but when it does, the CL is inhibited and embryonic diapause occurs. As in the non-macropodiod species, the suckling stimulus appears to suppress oestrus so that ovulation may occur towards the end of the lactation period, with the resulting corpus luteum (CL) becoming quiescent and the embryo entering diapause (Tyndale-Biscoe and Renfree, 1987).

Group 4

Group 4 marsupials are primarily small possums that also display embryonic diapause all be it in a slightly different guise. The cycle is characterized by a very pro-longed luteal phase and gestation in which the embryo grows relatively quickly to an unilaminar blastocyst stage before growth slows (Shaw, 2006). This period of slow growth can last for a period of up to several months. The endocrine control of this diapause is unknown but it may serve to synchronise births with peaks in food availability (Shaw, 2006). Unlike the kangaroo and wallabies, reactivation does
not appear to be controlled by lactation. The placental type, gestation ratio of oestrus and stage of the oestrous cycle at which parturition occurs in these marsupials is unknown, although all species are polyovular (Tyndale-Biscoe and Renfree, 1987). This pattern is seen in three families; the Tarsipidae - honey possum (*Tarsipes rostratus*); the Burramyidae, including the pygmy-possums *Cercartetus concinnus*, *C. nanus* and *C. lepidus* but not *Burramys parvus* and members of the Acrobatidae - the feathertail gliders *Acrobates pygmaeus* and *Distocechurus pennatus*.

Table 1.1. Characteristics used to clarify the patterns of reproduction as proposed by Tyndale-Biscoe and Renfree (1987).

<table>
<thead>
<tr>
<th>Reproductive Characteristic</th>
<th>Reproductive Pattern</th>
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<tr>
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<tr>
<td>Mono/Polyovular</td>
<td>P &amp; M</td>
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<tr>
<td>Ratio of the gestation period to oestrous cycle length</td>
<td>&lt;0.6*</td>
</tr>
<tr>
<td>Timing of parturition with respect to the stage of the oestrous cycle</td>
<td>Termination of luteal phase</td>
</tr>
<tr>
<td>Placental type</td>
<td>1**</td>
</tr>
<tr>
<td>Embryonic diapause</td>
<td>No</td>
</tr>
<tr>
<td>Examples</td>
<td>Dasyuridae, Didelphidae, Petauridae, Vombatidae, Phalangeridae, Phascolarctidae</td>
</tr>
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Table legend: P- Polyovular; M- Monovular; Placental types based on the condition of the allantois relative to the chorion , (Hughes, 1984) and according to the intimacy of the contact between the conceptus and maternal tissue after the break down of the shell membrane (Hughes, 1974).* Phascolarctidae has a gestation ratio of 0.7; ** *Dasyurus viverrinus* (Dasyuridae) is the only marsupial so far described with a type 2 placenta, Phascolarctidae is the only family described with a type 3 placenta.
Other unique reproductive patterns in female marsupials

There is now considerable evidence of male induced oestrus in some female marsupials. Studies conducted on the grey short-tailed opossum (Monodelphis domestica) (Fadem, 1985; Hinds et al., 1992) brush-tailed bettong (Bettongia penicillata) (Hinds and Smith, 1992; Smith, 1992; 1994) and bilby (Ballantyne et al., 2009) have shown that oestrus can be induced by the presence of the male, although it is uncertain whether ovulation follows spontaneously or also requires the physical act of coitus as the case for “true” reflex ovulators such as the cat or rabbit (Felis catus; Banks, 1986; Ramirez and Lin Soufi, 1994) that posses a copuloreceptive-reflex arc (Ramirez and Lin Soufi, 1994). Studies of the woolly opossum (Caluromys philander) conducted by Perret and M’Barek (1991), showed that despite evidence of enhanced and synchronized oestrus in response to male introduction or exposure to male pheromones, ovulation in this species occurred spontaneously. Hinds and Smith (1992) showed that female brush-tailed bettong had irregular oestrous cycle patterns when isolated from males. In contrast, females housed with males had a similar more regular reproductive pattern to that of most macropodids, in that the females which gave birth, showed a post-partum oestrus, were then subsequently mated, ovulated and entered a period of embryonic diapause (Smith, 1994). Similarly, peak progesterone concentrations indicative of ovulation and the onset of the luteal phase, were only observed in female bilbies upon male introduction (Ballantyne et al., 2009).

Johnston et al. (2000a) have provided convincing evidence that ovulation in the koala is induced by the physical act of mating or involves some form of ovulating factor in koala semen (Johnston et al., 2004). Non-mated cycles (33d) were found to be significantly shorter in duration than mated non-pregnant cycles (53d) and considered to be anovulatory with no luteal phase. Johnston et al. (2000b) initially suggested that the luteal phase of the oestrous cycle (including ovulation) may involve the triggering of a copulo-ceptive reflex similar to that described in the domestic cat (Banks, 1986) and rabbit (Ramirez and Lin Soufi, 1994). In a later study, however,
Johnston et al. (2004) noted an important role for semen in the successful induction of ovulation and the subsequent luteal phase, leading these authors to suggest that koala semen may contain a biochemical factor that promotes the induction of the luteal phase, similar to the way that semen induces ovulation in the Camelidae (Musa et al., 1993, Bravo et al., 1990). Despite being well documented in eutherian species, reflex or semen induced ovulation is yet to be found in any other marsupial, potentially placing the koala in a reproductive category of its own. The koala is typically monovular with a type 3 placenta, has a gestation period occupying 70% of its oestrous cycle and with parturition typically occurring before termination of the luteal phase.

The generalised Marsupial Hypothalmic-Pituitary-Gonadal

The principal endocrine control of the mammal reproductive system is exerted by the hypothalamus through the regulation of gonadotrophin secretion from the AP gland, which, in turn, governs hormone and gamete production by the gonads. Neurons in the medial basal hypothalamus synthesize GnRH, a neurohormone that directly regulates the synthesis and secretion of gonadotrophic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the AP (Rance, 2009). The AP does not receive direct neural input from the hypothalamus but axons from the cell bodies of the GnRH neurons project to fenestrated capillaries in the median eminence (Rance, 2009) to provide a dense plexus of neurosecretory terminals (Clarke and Tilbrook, 2009). GnRH is secreted from the axon terminals into the hypophyseal portal vessels, a capillary system that carries blood to the pituitary gland (Rance, 2009). Secretion into the hypophysial portal system reflects the phasic activity of the neurons and converts the neural transmission to an endocrine signal (Clarke and Tilbrook, 2009). The timing of the oestrous cycle is dependent upon the episodic pattern of GnRH, and therefore, gonadotrophin hormone secretion. Both the pulse rate and the amount of hormone released into the blood stream are important factors in stimulating follicular growth and oestradiol secretion by the ovary (Tyndale-Biscoe, 2005). In addition, multiple neural
inputs to hypothalamic GnRH neurons convey information on the status of various physiological inputs to optimize the timing of reproduction (Rance, 2009).

Mammalian GnRH has been purified from the hypothalamus of several marsupial species including the tammar wallaby, short-nosed bandicoot (*Isoodon macrourus*) eastern quoll (*Dasyurus viverrinus*) (King et al., 1989) the brushtail possum (*Trichosurus vulpecula*) (King et al., 1994) and its function can be inferred from an experiment conducted in the tammar wallaby in which an injection of GnRH induced a pulse of LH within one hour (Tyndale-Biscoe, 2005). The presence of GnRH1 and 2, FSHβ and LHβ have also recently been confirmed in the koala (Busby et al., 2014). GnRH1 and 2 cDNAs were identified within the koala brain, specifically the hypothalamus and midbrain, GnRH1 and 2, FSHβ and LHβ receptors were identified in the pituitary, and FSHβ and LHβ receptors have also been located within the koala testes (Busby et al., 2014).

In female mammals, LH and FSH target the ovaries via the systemic circulation to regulate folliculogenesis, ovulation, and the secretion of steroid and glycoprotein hormones. In turn, ovarian steroids, oestrogen and progesterone, exert positive and negative feedback actions on both the hypothalamus and the pituitary (Rance, 2009). The ovary also secretes glycoprotein hormones (such as inhibin and activin) that modulate FSH secretion at the level of the AP. The initially high plasma concentrations of FSH stimulate the growth and recruitment of immature ovarian follicles in the ovary (Shaw, 2006). As the follicles mature, they produce increasing amounts of oestradiol (Tyndale-Biscoe, 2005). At low concentrations, oestrogen has a negative feedback on FSH and LH by reducing GnRH pulse frequency, but at high concentrations it has a positive feedback effect increasing pulse frequency and inducing a surge of LH (Horn et al., 1985).

A rise in oestradiol concentration also has an effect on reproductive or behavioural oestrus as well as stimulating cell division and cellular secretions in the reproductive tract in preparation for
mating, sperm transportation and fertilization (Shaw, 2006). FSH progressively declines with rising oestradiol secretion from the maturing follicle as a result of the increasing potency of the negative feedback control by oestradiol (Tyndale-Biscoe, 2005). The oestradiol induced LH surge results in rupture of the mature dominant Graafian follicle and ovulation of the oocyte. The remaining theca and granulosa cells undergo reorganisation and transformation to become the corpus luteum (CL). The ovulated oocyte is then swept into the oviduct, where fertilization takes place if sperm is present (Shaw, 2006).

Increasing progesterone concentration as a result of the newly formed CL, causes a ‘luteal phase’ regardless of whether fertilization takes place or not (Tyndale-Biscoe and Renfree, 1987). In eutherian species, progesterone secreted from the CL slows the rate of pulsatile GnRH secretion with an increase in pulse amplitude (Clarke and Tilbrook, 2009). While this allows for some follicular development, it is not sufficient for the production of high levels of oestradiol. A similar mechanism may occur in the marsupial as follicles do not proceed beyond approximately 2.5 mm in diameter in the brushtail possum when a CL is present, whereas, at ovulation, the follicle reaches a diameter of approximately 5 mm (Eckery et al., 2002b). Similarly, Johnston (1999) has shown that oestrus, displayed in association with high oestradiol levels, is suppressed when a CL is present in both pregnant and non-pregnant koalas. For the majority of marsupial species, the bandicoot being the exception, the CL secretes progesterone throughout pregnancy until it regresses just prior to birth. Progesterone stimulates cell growth in the reproductive tract, particularly in the glands of the endometrium. After pregnancy, further follicular development and oestrous cycle activity is suppressed by the suckling young (Tyndale-Biscoe and Renfree, 1987). In the non-pregnant cycle, the CL regresses, progesterone feedback is lost in response to declining progesterone concentrations and the restraint on GnRH cells is lifted (Clarke and Tilbrook, 2009). An increase in frequency of pulsatile secretion of GnRH and LH and a reduction in amplitude stimulates follicular growth and a
new cycle begins (Clarke and Tilbrook, 2009). Figure 1.1 summarizes the control of the reproductive cycle in female marsupials.

**Oestrous cycles and pregnancy**

![Diagram of the control of reproduction in female marsupials]

Figure 1.1: The control of reproduction in female marsupials. Regulated by external and internal stimuli the hypothalamus modulates the production of GnRH, a hormone that controls FSH and LH by the pituitary. During the follicular phase, FSH stimulates maturation of follicles, which produce oestradiol. Oestradiol acts on the reproductive tract, preparing it for oestrus, as well as regulating its own production through a negative feedback loop at the hypothalamo-pituitary axis. High levels of oestradiol produced by mature follicles cause a surge of LH secretion that causes mature follicles to ovulate, and transform the remnant tissue to a CL that makes progesterone, a hormone essential to the establishment of pregnancy. [Modified from Shaw (2006)].
The Marsupial Follicular Phase

Female reproduction involves cyclic changes in ovarian activity initiating a series of interrelated neuro-physiological and behavioural processes. The central feature of the mammalian oestrous cycle is the periodic maturation of the oocytes that will be released at ovulation and luteinization of the follicles after ovulation to form the corpora lutea (Bronson, 1989). These two processes form the two distinct respective phases of the oestrous cycle in non-primate mammals, the follicular phase and the luteal phase.

Folliculogenesis

The follicular phase constitutes the period of follicular growth and maturation, oestrus and the first few days after ovulation (Tyndale-Biscoe and Renfree, 1987). The marsupial follicle consists of an oocyte, surrounding granulosa cells and outer layers of steroid producing theca cells. In eutherian species, follicular development involves two phases, basal follicular growth and terminal follicular growth. During basal follicular growth, follicles grow slowly and the follicular growth rate is tightly related to proliferation of granulosa cells (McGee and Hsueh, 2000a, Markstrom et al., 2002). Growth is primarily under the control of growth factors of paracrine origin. Terminal follicular growth is rapid and occurs by enlargement of the antrum, a fluid filled lumen (McGee and Hsueh, 2000a). These Graafian follicles are the major source of the cyclic secretion of ovarian oestrogens (McGee and Hsueh, 2000a).

The marsupial oocyte and follicular growth conform to a similar biphasic pattern with the major difference to eutherian mammals being that the diameter of the follicle, oocyte and oocyte nucleus are two to three times larger at maturation (Rodger et al., 2009). As in eutherian species, the final size of the pre-ovulatory follicle varies between species and appears to be correlated with adult body size (Tyndale-Biscoe and Renfree, 1987). Terminal follicular development in eutherian
mammals is strictly dependent on LH and FSH (Sjaastad et al., 2003). FSH plays determinant roles in enhancing granulosa cell differentiation, expansion of the fluid-filled antrum and secretion of oestrogen by the follicle cells before ovulation (Markstrom et al., 2002). LH stimulates steroidogenesis in theca cells and sustains terminal maturation of granulosa cells in the pre-ovulatory follicle (Monniaux et al., 1997). This is most likely also true for marsupials, as mRNA coding for LH and FSH receptors are found in the granulosa cells of the ovary in the brushtail possum (Eckery et al., 2002a). Further, similar to that observed in eutharian mammals, injections of FSH (McLeod et al., 1999) or PMSG followed by injections of synthetic GnRH (Rodger and Mate, 1988) or ovine LH (Glazier and Molinia, 1998) have been shown to stimulate the growth of multiple follicles in the brushtail possum suggesting FSH plays a key role in the regulating follicular growth.

Endocrinology of the marsupial follicular phase, oestrus and ovulation

While ovarian function and subsequent hormonal changes at post-partum oestrus and ovulation have been examined in various species of marsupial, endocrinological data on the regulation of oestrus and ovulation has only been determined for the brushtail possum (Curlewis et al., 1985) and the tammar wallaby (reviewed by Tyndale-Biscoe and Renfree, 1987). As the tammar wallaby belongs to a different reproductive group (group 3) to that of the brushtail possum and koala (group 1), the endocrinology of the brushtail possum follicular phase will be the focus here. In the brushtail possum, plasma oestradiol-17β concentrations remain undetectable throughout the cycle, except for a single rise during the early follicular phase (Curlewis et al., 1985, Crawford et al., 2011). Gonadotrophin stimulation of follicular growth initiates the cycle, leading to the secretion of oestradiol by the Graafian follicle (Harder et al., 1985). Initially, high plasma concentrations of FSH gradually decline with the progressive development of one or more large follicles, the subsequent emergence of a single pre-ovulatory follicle, and enlargement of the
reproductive tract in association with rising oestradiol concentrations (Crawford et al., 1999). In eutherian mammals, the growth of a dominant follicle is associated with increased serum concentrations of oestradiol and inhibin, which both act on the hypothalamus and pituitary to inhibit secretion of FSH (Findlay et al., 1990). It is likely that a similar mechanism occurs in the marsupial, as increased oestradiol concentrations have been measured during proestrus in the brushtail possum (Crawford et al., 1999) and in the vein draining the ovary containing the Graafian follicle in the tammar wallaby (Harder et al., 1984). Immediately before ovulation, FSH concentrations in the brushtail possum are undetectable (Crawford et al., 1999). Preovulatory surges of both FSH (6 - 9 h in duration) and LH (7 - 9h duration) induced by the positive feedback effects of oestrogen on the pituitary, occur 1 - 2 days before ovulation (Crawford et al., 1999). Peak concentrations of LH occur 3 - 6 h after initial elevation, the magnitude and duration of which are comparable to that of the tammar wallaby (Sutherland et al., 1980). Approximately 1h after the LH surge, there is a marked increase in FSH (Crawford et al., 1999). In some eutherian mammals, a peak in FSH has been reported after ovulation, evidently in response to the abrupt removal of the negative feedback effect of oestradiol and inhibin (Baird et al., 1981, Webb and England, 1982). The peak in FSH is thought to initiate growth of a new follicular wave. The elevated concentrations of FSH in the brushtail possum may serve a similar purpose. LH concentrations remain basal throughout the remaining oestrous cycle until the time of the pre-ovulatory surge in the subsequent cycle (Crawford et al., 1999). Figure 1.2 shows the mean FSH and LH concentrations in the brushtail possum through an ovulated oestrous cycle.

There is no discrete profile of Prl during the follicular phase of the brushtail possum, however, Crawford et al. (2006) did detect a distinct preovulatory biphasic surge of Prl tightly regulated prior to and immediately after the pre-ovulatory surges in three out of four possums which showed a typical pre-ovulatory LH surge as shown in figure 1.3. A Prl surge (3-5h in duration) was observed 2 - 6 h before the pre-ovulatory LH surge and a 2nd Prl surge (5 - 9 h in duration) occurred
coincidently with the delayed pre-ovulatory FSH surge (Crawford et al., 2006). A similar pattern in Prl secretion has also been reported in the Quoll (Hinds and Merchant, 1986).

Figure 1.2: Mean (±sem) plasma concentrations of FSH (●) and LH (○) in brushtail possums that ovulated following removal of pouch young in (a) Experiment 1 and (b) Experiment 2. In experiment 1, blood samples were collected every 1-2 days for 20 days and the time of ovulation was determined by laparoscopy. In experiment 2, blood samples were taken daily from -5 until 20 days after removal of pouch young and the day of ovulation was assigned retrospectively on the basis of vaginal cytology and hormone profiles (From Crawford et al., 1999).
Figure 1.3: Mean (± SEM) daily plasma concentrations of LH (○), FSH (□), Prl (●) and P₄ (●) in reproductively cycling female brushtail possums in relation to the day of ovulation. (From Crawford et al., 2006).

While the patterns of gonadotrophin secretion observed in the brushtail possum in relation to the oestrous cycle and to the time of ovulation basically conform to those reported for other eutherian species (Freeman, 1994), the koala is an induced ovulator (Johnston et al., 2000b, Johnston et al., 2000a, Johnston et al., 2004) and the occurrence of the LH surge is dependant on successful coitus and perhaps, some ovulating factor in the semen. Johnston et al. (2004) reported a LH surge 24 - 32h post-coitus, followed by an increase in progesterone. The precise timing of ovulation after coitus has not been determined and Prl levels have not yet been measured. If mating does not occur and ovulation is prevented, the koala will remain in behavioural oestrus for up to 10 days.
days on average, after which time the dominant follicle will presumably regress and become atretic (Johnston et al., 2000b). If the koala does not ovulate, there is no CL and subsequently, no progesterone secretion. Presumably, a new wave of folliculogenesis results in the development of new dominant follicle and another oestrus approximately 30 days later. The shorter, non-mated cycle consists of an inter-oestrous period interval ranging between 25d and 48d (mean 32.9 ± 1.2 d) coinciding with elevated oestradiol-17β concentrations (Johnston, 1999) but no attempt has been made to measure FSH in the koala.

Expression of oestrus

High oestradiol-17β concentrations have been shown to be associated with oestrous behaviour and changes in the urogenital epithelium in the brushtail possum (Curlewis et al., 1985), western quoll (Dasyurus geoffroii; Stead-Richardson et al., 2001), the tammar wallaby (Shaw and Renfree, 1984; Harder et al., 1985) and the koala (Johnston et al., 2000b). In the tammar wallaby, marked changes in the cells of the urogenital sinus, associated with changes in the pouch, occurred 1 day after behavioural oestrus and persisted for several days. In the western quoll, cornified epithelial cells were present on the day of behavioural oestrus and were associated with peak oestradiol concentrations (Stead-Richardson et al., 2001). It is presently not clear, as to which factors, hormonal or pheromonal, influence the attractiveness or receptivity of females to males in the oestrous period (Hinds et al., 1996).

For many eutherian species it is the withdrawal of progesterone which is necessary to induce behavioural oestrus (e.g. Sheep and Cattle) (Hinds et al., 1996). In the brushtail possum, progesterone withdrawal appears insignificant as the decline in progesterone occurs several days before oestrus in a non-pregnant cycle and may be separated by several months of lactation (Pilton and Sharman, 1962). Similarly in the female koala, an induced ovulator, progesterone
concentrations remain basal throughout a non-mated cycle and the female re-enters oestrus some 30 days later (Johnston et al., 2000b).

The captive female koala has distinctive behaviours indicative of oestrus; these include increased activity, twitching or jerking behaviour (in a ‘hiccupping manner’) and a form of standing oestrus (Johnston et al., 1999), which can be used to help determine the reproductive status of the female. These behavioural cues have been linked to elevated oestradiol-17ß in plasma and an increased presence of epithelial cells in smears of the koala urogenital mucosa (Johnston et al., 2000b). The decline in peripheral plasma oestradiol-17ß concentrations before the cessation of oestrous behaviour suggests that while elevated concentrations of oestradiol-17ß may be important for initiating oestrus, other factors are involved in its continuance (Johnston et al., 2000b). Increased levels of oestradiol-17ß without progesterone withdrawal do not appear to play a role in the initiation of oestrous behaviour in the southern hairy nosed wombat, as injections of oestradiol benzoate did not induce reproductive behaviour despite a spike of oestradiol-17ß metabolites in the faeces 3-4 days later (Hogan et al., 2010).

The Marsupial Luteal Phase

**Formation of the CL**

In marsupials, the CL is formed from the granulosa and theca cells of the ovulated follicle (Gemmell, 1995). Under the influence of the pre-ovulatory surge of LH from the anterior pituitary, the mature follicle ruptures and releases an oocyte (McCracken et al., 1999). The wall of the follicle collapses, capillaries and connective tissue invade the developing CL and granulosa cells and the inner layer of the theca cells fill the central cavity and undergo luteinization to form luteal tissue. This progression occurs in the koala, eastern quoll, long-nosed bandicoot, southern brown bandicoot, northern brown bandicoot and red-necked wallaby (Tyndale-Biscoe and Renfree, 1987).
The lutein cells synthesise and secrete progesterone (Shaw, 2006). In most marsupial species, the formation and subsequent production of progesterone plays a central role in the regulation of the oestrous cycle and is essential for the establishment of pregnancy (Gemmell, 1995). If fertilization does not take place, the CL regresses and a new cycle begins.

In many eutherian mammals the life of the CL is typically extended during pregnancy. The ability of the CL to persist and secrete progesterone, together with the more developed chorioallantoic placenta has allowed for the prolonged foetal intrauterine development and birth of a relatively mature neonate (Gemmell, 1995). In some eutherian mammals, such as the goat, the CL is required to produce progesterone throughout pregnancy, while in others, the placenta literally "hijacks" the production of progesterone from the CL. In most marsupial species, except for the bandicoot, the luteal lifespan is not pro-longed during the short pregnancy and luteolysis typically corresponds with birth of highly altricial young; these young then continue the greater part of their development in the pouch. The marsupial pattern of luteal growth and decline can be divided into 3 broad categories, as defined by the 3 of the 4 types of reproduction referred in section 1.2.1. Type 1 reproduction involves a short gestation, short luteal phase and is found in the majority of marsupials; the luteal phase occupies no more than 60% of the cycle and the CL regresses just prior to parturition. Type 2 reproduction is characterized by a short gestation and prolonged luteal phase, which is extended into lactation; this type of reproductive pattern is restricted to the bandicoot and possibly the bilby. Type 3 reproduction has a long gestation period which extends into the follicular phase of the next oestrous cycle and is characterised in the majority of macropodid species.

Endocrinology of the luteal phase and pregnancy

Typically, progesterone concentration profiles of pregnancy and the luteal phase of non-pregnant oestrous cycle in marsupials are not significantly different in their length or magnitude (Tyndale-Biscoe and Renfree, 1987). In the marsupials that have been studied thus far, progesterone
is elevated prior to birth, and peripheral plasma concentrations decrease in association with parturition. In the brushtail possum, progesterone concentrations increase slowly after ovulation until day 7, where after there is a sharp rise reaching a peak at day 13 (Curlewis et al., 1985). There are only minor differences in progesterone concentration between the pregnant and non-pregnant cycle.

Progesterone secretion has been measured in a group of female tammar wallabies through an oestrous cycle and part of the subsequent pregnancy (Hinds and Tyndale-Biscoe, 1982). Pregnancy in most macropods extends almost to the next ovulation, so that oestrus occurs a few hours after birth (Tyndale-Biscoe and Renfree, 1987). The endocrinology of the non-fertile tammar wallaby oestrous cycle begins with an initial decline of progesterone that precedes oestrus where the concentration of progesterone remains low for approximately 7 days. This is followed by a brief increase in progesterone concentration, usually for only one day, a fall to basal levels and then subsequent elevation between day 10 and 15. The progesterone levels then remain elevated until a decline associated with the next oestrus. The length of a non-fertile oestrous cycle is 30 to 31 days. The progesterone secretory profile during the first 10 days of pregnancy is indistinguishable from the first 10 days of the non-fertile oestrous cycle, with a transient pulse of progesterone occurring on day 6 or 7, preceding the development of the CL and the expansion of the developing blastocyst.

A similar peak has also been observed in the koala at day 2 of the pregnant cycle, and day 2 of the mated but non-parturient cycle (Johnston et al., 2000b). This transient peak has also been observed in the kowari, *Dasyuroides byrnei* (Fletcher, 1983), striped-faced dunnart, *Sminthopsis macroura* (Menkhorst et al., 2009) brushtailed bettong, *Bettongia penicillata* (Hinds and Smith, 1992) the quokka, *Setonix brachyurus* (Cake et al., 1980) and the dibbler, *Parantechinus apicalis* (Mills et al., 2011). In contrast to that of the tammar wallaby and the koala, the transient peak in the kowari and quokka was not observed during the oestrous cycle (Fletcher, 1983, Cake et al., 1980).
Further study is required to ascertain the functional significance of this transient peak, before a full understanding of the progesterone profile in these species can be obtained. No other reproductive hormones have been measured through a full cycle or pregnancy in a marsupial species.

Variations are apparent in the progesterone profiles of several marsupial species during pregnancy (Figure 1.4). The basic progesterone profile, containing a monophasic peak, is represented by the opossum (Gemmell, 1995). This pattern has been modified in the brushtail possum with an extension of the pre-luteal phase and the northern brown bandicoot in which the post-luteal phase has been extended into lactation (Gemmell, 1995). The kowari appears to be an intermediate between the polyprotodonts and the macropodids displaying a bi-phasic progesterone profile. The macropodids, quokka, tammar wallaby and the bennett's wallaby, all have embryonic diapause and also display a bi-phasic pattern for plasma progesterone concentrations, extending the pre-luteal phase (Gemmell, 1995). As with placental mammals, a decline in progesterone is not always observed immediately prior to parturition. Plasma progesterone declines in the opossum at least four days prior to birth (Harder and Flemming, 1981) at least 2 days prior to birth in the quokka (Cake et al., 1980) and at least 24 h prior to birth in the tammar (Tyndale-Biscoe et al., 1983) and bennett's wallaby (Walker and Gemmell, 1983).

Progesterone concentrations in the koala increase again on day 11 after the initial rise at day 2 and reach a peak on day 28, before progressively declining in association with birth (day 34-35) and only dropping to basal levels 7 days after birth (Johnston et al., 2000b). The koala has a bi-phasic progesterone profile following coitus, similar in form to that reported for the majority of macropods and the kowari (Johnston et al., 2000b, Gemmell, 1995). In other marsupials, the early surge in progesterone post-coitus is considered to be important for preparation of the uterus for pregnancy (Tyndale-Biscoe and Renfree, 1987). Johnston et al. (2000b) noted progestogen concentrations throughout pregnancy in the female koala were significantly higher than those of the
luteal phase of mated but non-parturient koalas. This study, however, was based on results from 6 pregnant and 6 mated but non-parturient koalas and so requires further validation. There is also evidence that oestrus was inhibited during the luteal phase and post-partum, presumably due to the suckling stimulus of the pouch young; however, follicular activity and oestradiol-17β production was not.

Figure 1.4: Plasma progesterone concentration profiles of 9 marsupial species during pregnancy. The stage of pregnancy at which the primitive streak blastocyst stage during each pregnancy was observed is indicated with an arrow. The top 5 species have a monophasic peak, *D. byrnei* is an intermediate species, and the bottom 3 species have a bi-phasic peak and exhibit embryonic diapause (From Gemmell, 1995).
Figure 1.5: Mean (± sem) concentration of (a) oestradiol and (b) progestogen up to 49 days after mating in six pregnant and six non-pregnant koalas. C: day of mating; B: day of parturition; □, behavioural oestrus; ●, oestradiol concentration (pg ml⁻¹) of pregnant females; ○, oestradiol concentration (pg ml⁻¹) of non-parturient females; ■, progestogen concentration (pg ml⁻¹) of pregnant females; □, progestogen concentration (pg ml⁻¹) of non-parturient females (From Johnston et al., 2000b).

While oestradiol concentrations in the koala remain relatively low during periods of peak progesterone secretion (Figure 1.5), only increasing in pregnant animals immediately before
parturition, there was no evidence of pre-partum or post-partum oestrus so that the functional significance of this peak remains unclear. The absence of a similar oestradiol peak in non-parturient animals suggests that it may be associated with the onset of parturition, or alternatively is simply the result of the continuance of follicular development and production of a dominant follicle throughout the luteal phase.

Factors Affecting The Control Of The Koala Oestrous Cycle

Proximate and ultimate factors

In mammals, the distribution of births throughout the year is commonly dependant upon its environment (Bronson, 1989). Environmental factors operate at two levels and are known as ultimate and the proximate factors. Ultimate factors are important for the long-term survival of the species, whilst proximate factors are more closely associated with the immediate onset and cessation of reproductive activity (Bronson, 1989). From an ultimate perspective, annual reproductive patterns reflect a complex interaction between dietary and climatic factors that favour offspring survival (Bronson, 1989). The timing of the breeding season in many mammals is often related to successful lactation and the weaning of young at time that ensures high availability of food and beneficial climatic conditions for offspring reaching independence (Tyndale-Biscoe, 1979, Bowyer, 1991). Nutrition and photoperiod are two major environmental factors with the greatest influence on reproduction. Foraging conditions influence energy balance and are the ultimate cause of seasonal reproduction in all mammals and the proximate cause in many (Bronson, 2009). The energetic costs of reproduction are very significant, so births must occur when the muscle and thermoregulatory costs of obtaining food are most favourable. Photoperiod is a proximate factor acting as predictive cue to allow some species to prepare in advance for seasonal changes in foraging conditions (Bradshaw and Holzapfel, 2007).
Koalas are seasonal breeders, restricting breeding to the warmer months of the year (McLean and Handasyde, 2006, Ellis et al., 2010). Preliminary evidence of an influence of photoperiod on the timing of the breeding season of the koala was originally suggested by Johnston (1994), who noted a peak in breeding in captive Queensland koalas held in San Diego Zoo (32°45’ N) (Thompson, 1987) 5 months after their usual breeding peak in Queensland (27°30’ S). Despite a large distribution spanning some 2,000 km across the eastern edge of the Australian continent (White and Kunst, 1990), no significant variation between breeding peaks in koala populations within Australia have been recorded. Seasonality studies on Victorian koalas have reported a breeding period from September through to May (Handasyde, 1986, Handasyde et al., 1990, Martin and Handasyde, 1990, Mitchell and Martin, 1990) with a peak occurring from November to March. Queensland koalas breed from August and April (White and Kunst, 1990, Blanshard, 1994, Johnston, 1994, O’Callaghan, 1996) with a peak in mating behaviour between October and November (Johnston, 1994), although births have been recorded throughout the year (Ellis et al., 2010). McLean and Handasyde (2006) have reported that some Victorian populations produce about 90% of young between December and March, although other Victorian populations they studied over the same 4 month period produced only about half this proportion, suggesting other environmental differences such as food abundance and climate may also be influence breeding.

Previous studies on reproductive seasonality in mammals have reported a variation in the degree of responsiveness to photoperiod within single populations; the range is continuous from totally responsive to totally unresponsive (Bronson, 2009). This strategy allows some individuals to adopt a photoperiod-determined survival mode during the winter absent of any reproductive activity while other members can reproduce opportunistically, if conditions are favourable (Kerbeshian et al., 1994). As koalas are polycestrus, repeated oestrus may occur beyond the normal breeding period in the absence of mating or in the event of pouch young loss (Handasyde et al., 1990); thus it seems likely the koala can reproduce opportunistically.
The timing of the breeding season in many mammals is often related to lactation and the weaning of young to ensure a high availability of food and beneficial climatic conditions for offspring reaching independence (Tyndale-Biscoe, 1979). Female koalas lactate for 12 months so that breeding in the spring and summer months ensure favourable conditions for the offspring approaching independence in the next breeding season. Lactation has been shown to be one of the most influential factors determining the mating period in the koala (Handasyde et al., 1990). Handasyde (1990) found evidence of lactational anoestrus in free-ranging Victorian koalas when she showed the presence of an almost weaned offspring or the disappearance of back young was associated with a rise in oestradiol-17β concentration and a subsequent brief rise in progesterone concentration; this was interpreted as evidence of oestrus and a luteal phase. The seasonal peak in progesterone concentration was then followed by a decrease to basal levels where it remained during the lactation period until the next breeding season or the loss of a pouch young.

*Effect of lactation on the marsupial oestrous cycle*

The sucking stimulus (Renfree, 1979) and the posterior pituitary (Hearn, 1973) inhibit follicular growth during lactation. Due to the degree of variation between the reproductive patterns and timing of birth within the cycle held by each marsupial reproductive group (1-4), the hormonal conditions during lactation are dependant upon the stage of the ovary at the time of its arrest. For the majority of species (group 1) birth occurs after lysis of the CL at the end of the luteal phase and the suckling stimulus of the PY suppresses the next follicular phase and the subsequent oestrus and ovulation (Tyndale-Biscoe, 2005). By contrast, in macropod species displaying type 3 pattern of reproduction, as well as the honey and pygmy possums, birth occurs when a Graafian follicle is present on the ovary and ovulation occurs soon after birth. In these species, the sucking stimulus arrests the newly formed CL and the new embryo until the end of lactation; a phenomenon known as embryonic diapause.
The mechanism responsible for lactational anoestrus in group 1 marsupials has not been directly investigated, however in women, suckling increases the sensitivity of the hypothalamus to the negative feedback effect of oestradiol, suppressing the GnRH/LH pulse generator, so that small amounts of oestradiol secreted from developing follicles would be sufficient to switch off further GnRH pulsatile secretion thus inhibiting further follicular growth (McNeilly, 1994). The mechanism through which oestradiol sensitivity is increased is not known. There is some evidence that Prl may be involved in the suckling-induced suppression of GnRH/LH release in women (McNeilly, 2001), however, in the cow, pig and during early lactation in the rat, suppression of Prl during lactation appears to have little or no effect on gonadotrophin secretion or ovarian activity (McNeilly, 1994). Thus, the role of Prl in lactational anoestrus appears to be species dependant.

Studies conducted by Handasyde et al. (1990) on the seasonal pattern of Prl secretion in lactating and non-lactating female koalas found no significant difference in Prl secretion between lactating and non-lactating females until the young began to exit the pouch, thus it is unlikely that Prl suppresses ovarian activity at this time. As reported in other marsupials, reduction in the frequency of suckling, when the young approached independence, was associated with the resumption of oestrous cycle activity as indicated by the subsequent presence of pouch young. The growth of pouch young has not been directly correlated with changes in Prl concentrations throughout lactation in the koala, nor has the occurrence of oestrous behaviour. This information would provide greater insight into the mechanisms controlling oestrus during lactation.

*Exogenous hormones - Progesterone*

In eutherian species, progesterone secreted by the CL inhibits GnRH secretion from the hypothalamus, which directly controls the release of FSH and LH. These gonadotrophin hormones subsequently drive folliculogenesis and ovulation in females. The administration and timed removal
of progesterone implants serves to mimic the endocrine function of the CL and ‘reset’ the follicular wave allowing for timed oestrus in treated animals. The current theory on progesterone secretion from the marsupial CL indicates that it has little ability to inhibit follicular activity, although the majority of work in this area is based on macropod species which conform to an entirely different reproductive pattern (group 3) to that of the koala (Tyndale-Biscoe and Renfree, 1987).

Crawford et al. (2011) conducted a series of experiments utilizing progesterone implants to synchronize follicular development and ovulation in the brushtail possum. Insertion of progestogen implants commonly used in eutherian species (0.75 mg progestagen norgestamet) failed to block ovulation. In a second experiment, higher dose progestogen implants (which produced a mean progestogen concentration of 11.7 ± 0.9 ng/ml when implanted in ovariectomized possums), effectively blocked ovulation in intact brushtail possums, however, the degree of synchrony in follicle development and ovulation was not sufficient for practical use in research (Crawford et al., 2011).

In a recent study, Hynes et al. (2010) indicated that female koalas receiving the synthetic progestin levonorgestrel (0.70mg progestagen) for contraceptive purposes, did not reproduce during implantation and came back into oestrus once the progesterone implant had been removed. While interesting, the contraceptive effect of this procedure may have little to do with suppressing follicular activity per se but be more about the prevention of the development of a pre-ovulatory follicle that is potentially capable of ovulation following sufficient coital stimulation: this phenomena requires further investigation and could provide some interesting insights into factors controlling ovarian activity in the koala.
Exogenous hormones - GnRH antagonists and agonists

As for eutherian species, the hypothalamic secretion of GnRH ultimately regulates the marsupial oestrous cycle. Continuous occupancy of the GnRH receptors with antagonists or by the continuous stimulation of the GnRH receptors by agonists (desensitization) inhibits endogenous GnRH binding and in doing so has the ability to suppress LH and FSH secretion (Al-Inany et al., 2006). GnRH agonists bind to GnRH receptors and mimic the cellular response induced by endogenous GnRH (Conn and Crowley, 1994). Initial treatment induces a large increase in LH and FSH (‘flare effect’), which can last for several days, followed by a return to basal levels (D’Occhio et al., 2000). Continuous stimulation with an agonist causes receptors to be internalized, resulting in down-regulation and desensitization of GnRH receptors on gonadotroph cells (Hazum and Conn, 1988). After the initial burst of gonadotropin production, levels of LH, and to a lesser extent levels of FSH, are suppressed and gonadal steroid production is diminished. In female mammals, follicular development is arrested whilst FSH secretion is suppressed; suppression of FSH also inhibits the oestrogen secretion induced positive feedback to the hypothalamus and prevents ovulation (see review by Fraser, 1993).

The use of GnRH agonists to control reproductive function in marsupials has been largely limited to studies of contraception with positive results (Herbert et al., 2007, Herbert et al., 2004, Herbert and Trigg, 2005, Herbert et al., 2006). In light of growing interest and increased success in the use of long acting GnRH agonists and steroid implants to suppress ovarian activity in marsupials [tammar wallaby, brushtail possum (Herbert et al., 2005, Nave et al., 2000)], these synthetic molecules have also recently been applied to the induction of ovulation for koala AI. While the GnRH agonist, buserelin, has been found to induce a normal luteal phase in female koalas (Allen et al., 2008a) similar to that described for human chorionic gonadotropin (hCG) (Johnston et al., 2000a), the procedure, is only applicable to females currently in oestrus with a dominant follicle.
GnRH antagonists bind to GnRH receptors on the gonadotrophic cell membrane but don’t induce stimulatory activity (Herbert and Trigg, 2005). Receptor occupancy by GnRH antagonists blocks binding of endogenous GnRH, causing an immediate suppression of gonadotropin release (Conn and Crowley, 1994, Schalley, 1999). A major benefit of GnRH antagonists is that they do not provoke the initial stimulatory “flare” effect of agonists and their effects are immediate. No studies have investigated the use of GnRH antagonists as a method for oestrous synchronization in marsupials, although, GnRH antagonists have successfully been employed to inhibit the LH surge and ovulation in gilts (Brussow et al., 2001), prevent ovulation but not follicular development in the domestic cat (Risso et al., 2010) and they have been used to help determine the endocrine control of follicular development in a range of species [e.g. mares and heifers] (Checura et al., 2009, Haughian et al., 2013).

In theory, it is possible that GnRH antagonists could be used to control the oestrous cycle in female koalas, by inhibiting follicular activity and gonadotrophin synthesis. For example, injections of the GnRH antagonist, acyline have been shown to suppress LH secretion in male koalas for up to 21 h after administration (Allen et al., 2008a). It would thus seem possible to control the oestrous cycle in female koala at the level of the anterior pituitary, by preventing gonadotrophin synthesis and follicular activity through the administration and withdrawal of GnRH antagonists.

THESIS CONCEPTS

Figure 1.6 is a mind map of the studies conducted under the broad title of this thesis “The control of the koala oestrous cycle”. Each study has been presented as a chapter in a sequence that reflects the logical flow and synthesis of the thesis, rather than the chronological order in which they were conducted.
Control of the Koala Oestrous Cycle

**FACTORS CONTROLLING THE FEMALE OESTROUS CYCLE**
- The effect of lactation on the koala oestrous cycle. *Chapter 3*
- The influence of season on female koala reproductive activity. *Chapter 4*

**MONITORING THE KOALA OESTROUS CYCLE**
- Non-invasive methods of monitoring the koala oestrous cycle. *Chapter 2*

**POTENTIAL PATHWAYS TO OESTROUS CYCLE MANIPULATION**
- Oestrous synchronization based on the GnRH antagonist Azaline B. *Chapter 5*
- The use of progesterone implants to control the timing of oestrus. *Chapter 6*

Figure 1.6: Mind map of thesis concepts.

Monitoring reproductive activity in the koala using faecal oestrogen secretion and behavioural oestrus.

While koala artificial insemination is now a well-established and successful technique (Johnston, 1999, Johnston et al., 2004, Allen et al., 2008b, Rodger et al., 2009) resulting in the production of a total 34 pouch young (Johnson and Holt, 2014) continued research is required to improve the efficiency of the technique. Part of this development is the need to establish less invasive strategies to accurately monitor the reproductive status or cycle of female koalas, without the need for physical restraint or serial venipuncture.
Successful reproductive monitoring requires the collection of repeated samples for hormone evaluation, often over a pro-longed period of time. Given that most studies of koala physiology are conducted on captive animals within zoos or wildlife parks, such vigorous sample schedules are often difficult to implement. While not as definitive as hormone analysis in the blood, faecal steroid analysis is a non-invasive technique that is becoming increasingly used for monitoring reproductive function in a range of marsupial species (Bradshaw et al., 2004, Hesterman et al., 2008a, Kusuda et al., 2009, Woodd et al., 2006, Hesterman et al., 2008b, Stead-Richardson et al., 2001, Stead-Richardson et al., 2010); this technique allows increased frequency of sample collection and a greater access to a larger number of koalas that might otherwise be excluded from such studies, due to their lack of tractability or requirement for exhibition.

Concentrations of steroid metabolites in the faeces are an indirect measure of their concentrations in the plasma (Schwarzenberger et al., 1996). Steroids are metabolized by the liver before excretion via urine or bile into the faeces. While progesterone is present in the faeces predominately as a series of \(5\alpha\)- or \(5\beta\)-reduced pregnanes [pregnanediones, mono- and di-hydroxylated pregnanes (Schwarzenberger et al., 1996)], oestrogens are the end products of steroid metabolism and, therefore, the actual chemical composition of these compounds in plasma and faeces are similar (Schwarzenberger et al., 1996). Radiometabolism studies have confirmed the presence of oestrogens in the form of oestradiol and / or oestrone in faecal samples, thus their concentrations can be relatively readily determined using specific assays or a total oestrogen assay (Schwarzenberger, 2007).

Due to the pulsatile secretory patterns of some plasma steroid hormones, such as oestrogen or testosterone, a blood sample is representative of the plasma steroid concentration for only a narrow time frame (Goymann et al., 1999). The intestinal or gut passage of faecal steroid metabolites causes a lag-time between the circulation of steroids in the plasma and their appearance
in the faeces, so that steroid metabolites in the faeces can represent a pooled fraction of plasma steroids, and may, therefore, potentially provide a more integrated measure of the hormonal status of the animal with less interference from daily rhythm and acute stress (Goymann et al., 1999, Schwarzenberger, 2007). The delay time between circulation of steroids in the plasma and the appearance of faecal steroid metabolites approximately correlates with the passage of digesta and varies considerably both among and between the species (Palme et al., 1996). The lag time of faecal steroids has been reported to be approximately 24 h in the honey possum (*Tarsipes rostratus*) (Oates et al., 2004), the spotted-tailed quoll (*Sarcophilus maculatus*) and the Tasmanian devil (*Sarophilus harrissii*) (Hesterman et al., 2008a).

In the koala, Kusuda et al. (2009), reported a significant positive correlation coefficient between faecal serum P4 and faecal progestagens (PM) between blood and faeces collected on the same day, the following day and 2 days later. Johnston et al. (2013b) recorded a significant increase in faecal corticosterone metabolite secretion 24 hours following adrenocorticotropic hormone administration, but no subsequent elevated levels over the following 9 day sample period. These differing results are surprising given the gut transit time in the koala digesta has been determined to be 99 h (4.2 days) for particles and 213 h (8.9 days) for solute respectively (Cork and Warner, 1983). Johnston et al. (2013b) proposed the early-elevated levels of corticosterone metabolites was most likely due to urine contamination of the faecal sample and that the failure to detect elevated corticosteroid metabolite concentrations in koala faeces could potentially be explained by the long gut transit in the koala resulting in an extended breakdown of the hormones into metabolites that do not cross-react with the chosen antisera. Given that marsupials possess a cloaca for common exit of faeces and urine, the possibility of urine contamination is high. The type and passage of metabolised steroid hormone in the urine is typically significantly different and faster (no lag effect in urine) than in faeces, such that this disparity may potentially cause difficulty with the interpretation of hormone levels determined in faecal if the sample is contaminated with urine.
Whilst excretory patterns of both hormones have been studied in a wide variety of eutherian mammals, the technique has only been applied to marsupial species in recent years. Faecal levels of oestrogens were first measured in the chuditch (*Dasyurus geoffroii*) by Stead-Richardson et al. (2001) and patterns of excretion of PM and oestrogens were used to monitor the oestrous cycle in the greater bilby (*Macrotis lagotis*) (Curnow et al., 2001). Faecal PM was also used to define the oestrous cycle and detect oestrous behaviour in both the common wombat (*Vombatus ursinus*) and the southern-hairy nosed wombat (*Lasiorhinus latifrons*) by Paris et al. (2002). Changes in faecal steroid excretion and urinary cytology have also been reported in the squirrel glider (*Petaurus norfolcensis*) (Woodd et al., 2006) and a series of papers have been published on the reproductive biology of the small nectarivorous honey possum *Tarsipes rostratus* using faecal steroids (Bradshaw et al., 2004, Oates et al., 2002, Oates et al., 2007). Characterisation (oestrogens, PM) of the oestrous cycles of two of the largest dasyurid species, the spotted-tailed quoll (*Sarcophilus maculatus*) and the Tasmanian devil, using both faecal and plasma samples have also recently been reported by Hesterman et al. (2008a,b).

Despite the past success in the use of faecal steroid monitoring in some marsupials, elevations in oestrogens have equally been undetectable in a variety of other marsupial species. Changes in faecal oestrogens were not helpful in the accurate prediction of oestrus or ovulation in the red-tailed phascogale (*Phascogale calura*; (Foster et al., 2008)), the gilbert’s potoroo (*Potorous gilbertii*; (Stead-Richardson et al., 2010)) or the numbat (*Myrmecobius fasciatus* (Hogan et al., 2012)). Elevated oestrogens also provided little instructive information on the timing of ovulation in the Julia Creek dunnart (*Sminthopsis douglasi*) (Pollock et al., 2010). Hogan et al. (2010) has also noted that whilst faecal PM successfully mapped oestrous cycle activity in the southern hairy-nosed wombat, it was not useful for the prediction of oestrus, and faecal oestrogens provided little instructive information on cyclic activity and was not associated with oestrus. Faecal oestrogens are yet to be measured in the female koala.
The inability to detect elevated oestrogens could be attributed to several factors. Firstly, in some species such as the red-tailed phascogale, elevations in oestrogens are brief and may only be detectable in a single faecal sample (Foster et al., 2008). Secondly, whilst radiometabolism studies have affirmed that oestrogens in the form of oestradiol and/or oestrone are present in faecal samples, there is a high species-specific differentiation in steroid metabolism, in even closely related species (Schwarzenberger, 2007). In addition, as measurements of the partitioning of oestrogen excretion between urine and faeces is unknown in the majority of marsupials, it is possible that the major proportion of the biologically active form of oestrogen may be primarily excreted in the urine of these species (Palme et al., 1996, Schwarzenberger et al., 1996). To date, radiometabolism studies in marsupial species have been limited to a single study conducted on the honey possum (Bradshaw et al., 2004); this study revealed 63% of recovered 14C-oestradiol-17b in the faeces, 37% in the urine and less than 20% of recovered oestrogens in the faeces was excreted unconjugated. Bradshaw et al. (2004) have suggested that the low proportion of oestrogens excreted in the unconjugated form is likely to be attributed to the comparatively rapid transit of digesta through the intestine in this nectarivorous species. Maximum levels of radioactivity appeared in the faeces 6h after injection and 95% of the total was eliminated between 12 and 20h post-injection (Bradshaw et al., 2004).

Oestrous behaviour in the female koala is commonly used as an effective tool for the detection of oestrus in most captive koala populations. The behaviours have previously been described in great detail by Smith (1980), Blanshard (1994) and reviewed by Johnston et al. (2000b). Captive female koalas held at Dreamworld (DW) and Currumbin Wildlife Sanctuary (CWS) have been ‘teased’ for oestrous activity daily since 2004. Signs of oestrous behaviour (i.e vocalization, pseudo-mounting homosexual behaviour etc) are easily identified and have been routinely recorded by keeper staff. This husbandry practise plays an essential role in the monitoring the reproductive status of individual female koalas and the timing of male introduction for
successful breeding. As oestrus has also been shown to correspond with an elevation in oestradiol-17β (Johnston et al., 2000b), it also serves as a valuable method of biological validation for faecal oestradiol assays. The utilization of faecal steroid analysis (total oestrogens) combined with oestrous behaviour monitoring as an alternative to plasma oestradiol analysis in female koalas for future application in koala reproductive physiology studies was investigated in chapter 2 of this thesis.

Control of reproductive activity during lactation

Similar to most mammals, reproductive activity in the female koala is thought to be suppressed during lactation with the recommencement of ovarian activity only occurring when lactation ceases due to the loss of PY or as young reach independence (Handasyde et al., 1990). Prl has previously been considered as one of the key factors causing suppression of ovarian activity in lactating mothers (McNeilly, 2001) and Handasyde et al. (1990) formerly suggested the prevention of cyclic activity during lactation in the female koala may be induced by the increase in Prl associated with milk let down. The role of Prl in the control of GnRH is reported to be species-dependant, with varying levels of importance both between species and the different stages of lactation (McNeilly, 1994). Studies on marsupial species have typically shown a low secretion of Prl during early lactation when follicular activity is suppressed (Curlewis et al., 1986, Hinds and Merchant, 1986, Muths and Hinds, 1996, Crawford et al., 2011).

Recent studies have revealed the importance of the intensity of the suckling stimulus acting via a neural pathway to inhibit oestrous cycle activity in the rat (Rattus spp) through the suppression of gonadotrophin release - namely, LH (Tsukamura and Maeda, 2001). Tsukamura and Maeda (2001) discovered pulsatile LH release was strongly inhibited during the first half of lactation in ovariectomized rats (Tsukamura and Maeda, 2001). Further, LH release returned 18 - 24 h after pup
removal and was suppressed within 4 - 7 h of reattachment (Tsukamura and Maeda, 2001), thus suggesting the neural stimulus of suckling itself is the primary signal to the hypothalamus in this species, rather than the hormonal milieu.

In women, McNeily (2001) discovered the suckling stimulus induced an increase in the sensitivity of the hypothalamus to the negative feedback effect of oestradiol on the GnRH/LH pulse generator; however, the precise mechanism responsible is unknown. In the tammar wallaby, denervation of the sucked teat and associated mammary gland resulted in the growth of the quiescent CL and diapaused embryo to a stage in development equivalent to that expected if PY had been removed (Renfree, 1979). Chapter 3 of this thesis investigated the control mechanisms responsible for lactational anoestrus in the koala by characterising serial Prl secretion throughout lactation and correlating this data with the development of PY, oestrous behaviour and the ability of the AP to respond to GnRH stimulation and release LH.

**Seasonality in the Female Koala**

While koala births have been recorded throughout the year at most locations in the wild (Ellis et al., 2010), the majority of observations suggest, that despite the geographical location of koala populations, (mainland Australia, southern Victoria, central Queensland or island populations off the Australian coast) about 60% of births in the Southern Hemisphere occur in summer and early autumn (December–March). To date, the evidence of seasonal reproduction has been primarily been based on an estimate of PY young age. Whilst this technique is useful when investigating the influence of season on successful mating, it only indirectly measures the influence of season on female koala oestrous cycle activity. Captive koala populations provide a unique opportunity to study the effect of season on oestrous expression without the confounding influences of lactational anoestrus, rainfall and food availability; in this way, it will be possible to focus with
more confidence, on the effect of temperature and photoperoid on reproductive cyclicity. Seasonal influences on the koala oestrous cycle are investigated in chapter 4.

**Oestrous Synchronization - GnRH Antagonists**

A prospective alternative method for oestrous control in the marsupial is the regulation of gonadotropin secretion at the level of the AP (Allen et al., 2008a). As in eutherian species, hypothalamic secretion of GnRH from the marsupial AP regulates LH and FSH secretion (Tyndale-Biscoe, 2005). Administration of GnRH antagonists has the ability to suppress LH and FSH secretion in eutherian mammals due to the competitive blockage of GnRH receptors (Herbert and Trigg, 2005) and preliminary studies in the male koala, appear to suggest a similar occurrence in marsupial species (Allen et al., 2008a). Hypothetically, administration of GnRH antagonists could be used to manipulate the female koala oestrous cycle through the temporary suppression of gonadotrophin secretion and follicular development. Rapid metabolic clearance of the antagonist should result in the availability of GnRH-R, the re-binding of endogenous GnRH to the receptor and the reactivation of FSH and LH secretion and consequent recommencement of oestrous cycle activity. This would allow the ovary to be potentially ‘reset’ in terms of its follicular wave, providing a means by which females could be synchronised to the same stage of the follicular phase for timed oestrus and insemination. Results from a pilot study conducted by Johnston (Unpublished) of four female koalas clearly showed that treatment with acycline (a potent GnRH antagonist) (Samant et al., 2005); prevented an increase in LH plasma concentrations following the injection of mGnRH. This finding supports the rationale and underlying hypothesis of the experiments conducted in chapter 5.
Obtaining adequate control of the reproductive cycle in order to manipulate the timing or synchronization of oestrus and ovulation is a fundamental aspect of a successful AI program. Despite the recent increase in assisted reproductive techniques in marsupials (Rodger et al., 2009), few studies have investigated the development of oestrous synchronization protocols in these species (Johnston and Holt, 2001). Studies on the use of synthetic progestogens for oestrous synchronization in the brushtail possum have revealed that while synthetic progestin implants prevented ovulation, the timed removal of implants did not synchronise oestrus (Crawford et al., 2011). Hynes et al. (2010) have used the synthetic progestogen levonorgestrel (LNG) for the successful contraception of the koala, preventing the birth of pouch young but also proving that the females returned to reproductive activity once the implants had been removed. The contraceptive effect of this procedure may have little to do with suppressing follicular activity per se but be more about the prevention of the development of a pre-ovulatory follicle that is potentially capable of ovulation following sufficient coital stimulation (Johnston et al., 2004). While the ability of luteal or exogenous progesterone to inhibit follicular growth in the koala has not been specifically investigated, Johnston (1999) has shown that oestrus is at least suppressed during the luteal phase in both pregnant and non-pregnant koalas. The ability of LNG implants to suppress oestrous cycle activity in the koala and ultimately synchronize oestrus after its removal is the focus of chapter 6.

Summary

The primary objective of this thesis was to improve and refine the current AI procedure in koalas by gaining a greater understanding of the factors controlling the oestrous cycle and timing of oestrus in the female koala in order to develop a protocol to reliably synchronise oestrus in this species. The development of such a protocol will facilitate the use of AI for the purposes of genetic
management in both wild and captive populations. The primary aims of the thesis were to (1) investigate the use of faecal steroid metabolite analysis to monitor the female koala oestrous cycle via total faecal oestrogens, (2) characterise prolactin secretion throughout lactation and investigate the relationship between prolactin, oestrous cycle activity and the developmental stages of pouch young, (3) investigate the strength of seasonality in captive Queensland koalas, (4) develop a koala oestrous synchronisation program based on the use of the GnRH analogues and (5) determine if it was possible to control ovarian activity (oestrous) in the koala using the synthetic progestogen levonorgestrel. All these aims can be broadly grouped under the general area of “The control of the koala oestrous cycle”.

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CHAPTER 2: MONITORING REPRODUCTIVE ACTIVITY IN THE KOALA USING FAECAL OESTROGEN SECRETION AND BEHAVIOURAL OESTRUS

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Presented as submitted for publication in Zoo Biology

ABSTRACT

This study evaluated the practical application of faecal oestrogen analysis and the detection of oestrous behaviour to monitor ovarian cycles in future studies aimed at improving artificial breeding techniques in the koala. Faecal and plasma samples were collected from a total of 8 koalas (4 cycling and 4 non-cycling) every 2-4 days over a 6 week period or until females returned to oestrus. Total faecal oestrogens and plasma oestradiol-17β concentrations were evaluated to determine if faecal oestrogens gave an accurate index of plasma oestradiol-17β levels. Although spikes in total faecal oestrogens were detected in association with oestrous behaviour and elevated plasma oestradiol-17β, there were also elevated concentrations detected that did not correlate with plasma levels, despite allowing for a digestive tract processing lag affect. A possible explanation for this incongruence could be the combination of urine contamination and a long and differential digestion rate which ranges from 99 – 213 h depending on digesta particle size (99h - 213h); this can lead to an inconsistent pooling of oestrogen levels in faecal samples. Whilst faecal oestrogens did not correlate with plasma oestradiol-17β to allow for an accurate estimate of cycle length or indicate the precise timing of oestrus, the individual mean faecal oestrogen concentrations did,
however, show a strong relationship to the individual mean plasma oestradiol-17β concentrations for each koala and total faecal oestrogens were significantly higher in cycling females.

**INTRODUCTION**

The most critical factors for effective captive animal management and the development of assisted breeding techniques (ABT) is the detailed assessment and knowledge of reproductive physiology (Schwarzenberger et al., 1996). Recent success and continued research into the development of an ABT program in the koala (Johnston, 1999, Johnston et al., 2004, Allen et al., 2008, Rodger et al., 2009) have highlighted the importance of developing strategies to accurately monitor reproductive status of the female koala. Reproductive monitoring requires the collection of repeated samples for hormonal evaluation over a pro-longed period; typically at least 1 oestrous cycle or 2 periods of behavioural oestrus. While previous studies have relied upon repeated blood sampling (Johnston et al., 2000a;b), frequent venipuncture is not desirable, particularly for zoo exhibit based animals, so that a greater emphasis should be placed on establishing reliable non-invasive techniques to monitor the reproductive status in the koala.

Faecal steroid analysis is a valuable method for monitoring the female ovarian cycle (Schwarzenberger et al., 1997) and is increasingly being used for monitoring reproductive function in marsupial species (Bradshaw et al., 2004, Hesterman et al., 2008a, Kusuda et al., 2009, Woodd et al., 2006, Hesterman et al., 2008b, Stead-Richardson et al., 2001, Stead-Richardson et al., 2010). The technique is exceptionally useful in cases of frequent and long-term sampling as it reduces the risk of injury and stress that may result from animal capture, immobilisation and venipuncture. Although, faecal oestrogen is yet to be measured in the female koala, a recent study successfully monitored the female oestrous cycle and ovarian activity using a combination of faecal progesterone metabolites (PM) and assessment of behavioural oestrus (Kusuda et al., 2009); this
was the first established technique for non-invasive reproductive endocrine monitoring in koalas using faeces. While this was an important advance, ovulation in the koala is induced following coitus so that progesterone metabolites will have limited value in documenting repeated oestrous cycle activity in animals that do no participate in mating.

Oestrous behaviour in the female koala is commonly used as an effective tool for the timing of natural or artificial insemination in most captive SEQ koala populations. These behaviours have been described in great detail by Smith (1980), Blanshard (1994) and reviewed by (Johnston et al., 2000b). Captive female koalas held at Dreamworld (DW, Coomera, Queensland) and Currumbin Wildlife Sanctuary (CWS; Currumbin Beach, Queensland) have been ‘teased’ for oestrus activity daily since 2004; signs of oestrous behaviour (i.e vocalization, homosexual, “jerking” behaviours) are easily identified and routinely recorded. This husbandry practise plays an essential role in the monitoring of the reproductive status of individual female koalas and the timing of male introduction for successful breeding. As oestrus has been shown to correspond with elevated oestradiol-17ß secretion (Johnston et al., 2000b), such profiles also provide valuable information on the reproductive physiology and status of the species.

It is the aim of this study to evaluate the efficacy of faecal total oestrogen analysis combined with the occurrence of oestrous behaviour to monitor ovarian cycles in the female koala and to assess the practical application of such techniques in future studies aimed at improving artificial reproduction techniques in the koala.

METHODS

Animals

This study was carried out on eight sexually mature captive koalas held at Currumbin Wildlife Sanctuary (153°29’12”E, 28°07’64”S) and Dreamworld (153°26’24”E, 28°04’12”S) on
the Gold Coast, southeast QLD. All animals were fertile as indicated by previous birthing records and remained clinically healthy throughout the experimental period. The study was approved by the University of Queensland Animal Ethics Committee (SAS/450/12/DREAMWORLD) and was conducted over a 12-week period within the breeding season commencing in January 2012.

Study design

The oestrous cycle of four sexually mature female koalas was closely monitored via assessments of endocrinology (plasma and faecal samples for oestrogens) and signs of oestrous behaviour commencing on day 2 of a non-mated anovulatory cycle until day 2 of the following oestrous period. In order to compare total faecal oestrogen levels in cycling and non-cycling female koalas, an additional four non-cycling koalas (Implanted with synthetic progestin levonorgestrel 70mg silicone implants and confirmed to have basal plasma oestradiol-17β; see chapter 6) were also analysed for total faecal oestrogens for a period of 45 days. Plasma and faecal samples were collected in unison every 2 - 4 days throughout the study period. Female koalas were teased for signs of oestrous behaviour daily (Blanshard, 1994).

Detection of behavioural oestrus

Oestrous detection in this study used the method previously described by Blanshard (1994) and Johnston et al. (2000a). A “teaser’ male was introduced into the female enclosure and allowed to scent mark objects with his sternal gland, bellow and urinate. Females that were sexually receptive, immediately displayed behaviours characteristic of oestrus, which included bellowing vocalizations, ear flapping, “jerking”, homosexual mounting and strong interest in the presence of the male. The male was also presented individually to each female in the enclosure to ensure all females had equitable exposure but no direct physical contact was permitted. Females were identified as in oestrus if they displayed the majority of the oestrous behaviours listed above. Oestrous detection was conducted between 8 am and 9 am daily at DW and CWS and continued
throughout the study period. The ‘teaser’ males are regularly rotated to avoid habituation of both males and females to the oestrous detection procedure.

**Plasma collection**

Venipuncture was conducted on conscious koalas as previously described by Blanshard (1994). Blood samples (2 mL) were collected sequentially from the cephalic vein using a 12 mm 25 gauge needle attached to a 0.5 mm x 39.5 mm winged infusion set (Terumo, Tokyo) and a 3 mL syringe. The sample was divided equally into 1 mL heparinized tubes (Becton Dickinson, NJ) and centrifuged at 1600g for 3 min. Plasma samples were stored in a –20 °C freezer. As koalas held at DW and CWS are handled regularly by staff, the collection of blood samples resulted in little or no distress (minor struggling) to the animal.

**Faecal collection and sample preparation**

Fresh faecal samples (7-12 g) were collected during blood collection every 2 - 4 days, placed into a plastic bag and frozen at -20 °C until analysis. Faecal extraction was modified from that previously described in Schwarzenberger *et al.* (2000); briefly, wet faecal samples were accurately weighed between 0.48 g - 0.51 g and vortexed with 1 mL distilled water and 4.5 mL methanol for 1 hr. After centrifugation 1 mL of methanol supernatant was transferred into a separate vial, mixed with 0.25 mL of a 5% NaHCO₃ solution and 5.0 mL of diethyl ether then vortexed for 30 sec. The ether phase (2 mL) was transferred into a separate vial, evaporated to dryness and the residue re-dissolved in 1.5 mL assay buffer. Samples were capped and stored in 4 °C overnight.

**Oestrogen faecal hormone analysis**

Faecal extracts were assayed for oestrogen metabolites using ImmuChem™ *Double Antibody* total oestrogens RIA kits (MP Biomedicals, LLC Diagnostics Division Orangeburg, NY).
Total oestrogens were expressed in ng/g of wet faeces after correcting for dilution losses during the extraction process. Serial dilutions of faecal extracts produced a displacement curve parallel to the standard curve. A 67-percent recovery of faecal oestrogen metabolites was calculated by adding a spike of 2ng/ml^-1 oestradiol-17β (Sigma Aldrich, Australia) to wet 0.5g faecal samples (n = 3). Spiked and non-spiked faecal samples were run through the extraction process and assayed in duplicate to calculate the percentage recovery of faecal oestrogen metabolites (corrected for extraction losses). The sensitivity of the assay was 1.25 pg / mL and the intra-assay and inter-assay coefficient of variation was 11.7% and 17.2%, respectively. All samples were assayed in duplicate. The oestrogen antibody cross-reacted with oestradiol-17β (100%), oestrone (100%), oestriol (9%), oestradiol-17α (7%), Equilin (2.5%) and <1% in all other steroids tested (MP Biomedicals Europe, Belgium).

**Plasma oestradiol-17β analysis**

The concentration of oestradiol-17β in the peripheral blood of koalas was assayed directly from plasma using commercially available ultra-sensitive estradiol RIA kits (Immunotech, Prague – Czech Republic). The assay sensitivity was 1.0 pg/ml, and the intra-assay and inter-assay coefficient of variations were 7.5% and 7.6% respectively. All samples were assayed in duplicate. The reported oestradiol antibody cross-reactivity was oestrone (2.4%), D-Equilenin (3.4%), 17-β-oestradiol-3-glucuronide (2.6%) and <1% for all other steroids tested compared to 17β oestradiol at 100% (Immunotech, Prague, Czech Republic).

**Statistical analysis**

Correlation coefficients between faecal total oestrogens and plasma oestradiol-17β concentrations were calculated using a partial Pearson correlation test; data were log (x) transformed before analysis, data was grouped into 2 day bins and lags for 0, 2, 4, 6, 8,10 and 12 days were calculated. Analysis was carried out using the SAS General Linear Model procedure.
allowing for differences between individuals. Faecal and plasma oestrogens in cycling and non-cycling koalas were analysed using a repeated measures ANOVA fitted with status (cycling or non-cycling), individual within status and day (sample days were pooled into 2 day pairs). The SAS MIXED procedure was used for analysis.

RESULTS

Correlation coefficients between plasma and faecal oestrogens were calculated with a zero, two, four, six, eight, ten and twelve day lag. A significant positive correlation was calculated at lag zero (P < 0.001) but not for two – twelve day lags (Table 2.1). Significant positive correlations were also calculated for lag zero in cycling females (P < 0.05) and non-cycling females (P < 0.001) when analysed separately.

**Table 2.1:** Correlation coefficients between plasma oestradiol-17β and faecal total oestrogens with a 0, 2, 4, 6, 8, 10 and 12 day lag.

<table>
<thead>
<tr>
<th>Lag (day)</th>
<th>Number of paired plasma and faecal samples</th>
<th>Partial Pearson</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>110</td>
<td>0.33</td>
<td>0.0006</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>-0.11</td>
<td>0.4614</td>
</tr>
<tr>
<td>4</td>
<td>71</td>
<td>-0.09</td>
<td>0.4548</td>
</tr>
<tr>
<td>6</td>
<td>61</td>
<td>-0.10</td>
<td>0.4287</td>
</tr>
<tr>
<td>8</td>
<td>60</td>
<td>0.01</td>
<td>0.9665</td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td>-0.15</td>
<td>0.2685</td>
</tr>
<tr>
<td>12</td>
<td>41</td>
<td>-0.17</td>
<td>0.3197</td>
</tr>
</tbody>
</table>
Figure 2.1: Individual koala profiles of faecal total oestrogens (closed circles), plasma oestradiol-17β (open circles) and oestrous behaviour (black bars) in non-cycling (left side) and cycling (right side) koalas.

The relationship between plasma and faecal oestrogen concentrations and oestrous behaviour in individual koalas with a zero lag is shown in figure 2.1. Oestrous behaviour coincided with increases in plasma oestradiol-17β and total faecal oestrogens, however increases in total faecal oestrogens also occurred that did not correlate with oestrous behaviour or increases in plasma.
oestradiol-17β. A significant group effect was found between cycling and non-cycling faecal oestrogen concentrations (P = 0.007) with significantly higher total faecal oestrogen concentrations in cycling koalas than non-cycling koalas. A similar difference was not significant between cycling and non-cycling plasma oestradiol-17β concentrations (P = 0.611). Mean plasma oestradiol-17β (log10) and faecal oestrogen (log10) concentrations for each koala are compared in figure 2.2. With the exception of koala K6, increases in mean plasma oestradiol-17β were associated with increases in mean faecal oestrogen concentrations within each koala.

![Graph](image_url)

**Figure 2.2:** Association between mean total faecal oestrogens (log10) (±SEM) and mean plasma oestradiol-17β (log10) (±SEM) in cycling (closed circles) and non-cycling (open circles) koalas with a line of best fit.
DISCUSSION

The primary objective of this study was to assess the ability of faecal steroid analysis combined with the occurrence oestrous behaviour to monitor oestrous cycle activity in the female koala. Levels of total faecal oestrogens ranged from 0.7 – 3.9 ng/g of wet faeces; however, as no measurements have been made on the partitioning of oestradiol-17β excretion between urine and faeces in the koala, the concentrations of total faecal oestrogens may only represent a small fraction of the total oestradiol-17β excreted; for example, studies conducted in the honey possum (Tarsipes rostratus) recovered 63% of 14C-oestradiol-17β in the faeces and 37% in the urine (Bradshaw et al., 2004).

Faecal steroids mimic the pattern of circulating hormone levels in plasma, however due to their passage through the gut, excretion is delayed (Schwarzenberger et al., 1996). In the current study, correlation coefficients between plasma oestradiol-17β and faecal total oestrogens were calculated with a 0, 2, 4, 6, 8, 10 and 12 day lag because the lag time of these metabolites (number of days for circulating oestradiol-17β to be excreted in faeces) in the koala are unknown. Interestingly, a significant positive correlation was only calculated for lag zero in cycling, non-cycling and combined data sets. Kusuda et al. (2009) also reported a significant positive correlation coefficient between koala plasma P4 and faecal progestagens (PM) at lag zero. A similar finding was also reported by Johnston et al. (2013) when they recorded a significant increase in faecal corticosterone metabolite secretion 24 hours following adrenocorticotropic hormone administration, but no subsequent elevated levels over the following 9 day sample period. Given the koala rate of passage of digesta in the koala has been reported to be approximately 99 h (4.2 d) for particles and 213 h (8.9 d) for solute respectively (Cork and Warner, 1983), these results are surprising. Johnston et al. (2013) proposed the early-elevated levels are most likely to be due to urine contamination of
the faecal sample so that this may account for the positive correlation between plasma oestradiol-17β and faecal total oestrogen in this study 24h after ACTH injection. Given that marsupials possess a cloaca for common exit of faeces and urine, the possibility of urine contamination is high.

The type and passage of metabolised steroid hormone in the urine is typically faster (no lag effect) than in faeces, such that this disparity may potentially cause difficulty with the interpretation of hormone levels determined in faeces, particularly if the sample is contaminated with urine (Schwarzenberger et al., 1996). Whilst elevated total faecal oestrogens were detected in some koalas in association with oestrous behaviour and elevated plasma oestradiol-17β, high oestradiol-17β concentrations were also detected that did not correlate with plasma levels despite adjusting for a digestive lag affect. It is probable that the differential digestion rate in relation to digesta particle size (99h - 213h) (Cork and Warner, 1983) in the koala most likely results in an inconsistent pooling of oestrogen metabolites levels in faecal samples. In the koala, fine particles are retained in the caecum and proximal colon facilitating the rapid passage of large fibrous particles (Moore and Foley, 2000). Highly fibrous faecal samples thus represent a pooling of plasma concentrations over a shorter period of time than non-fibrous samples containing a higher percentage of fine particles; this also suggests that the water balance of koalas may influence faecal oestrogen measurements. Given the variability in faecal fibrous content both within and between sample animals during this study and the high risk of urine contamination, it is not surprising that the relationship between elevated plasma oestradiol-17β and faecal total oestrogens reported in this study was not linear.

Whilst the pattern in secretion of faecal total oestrogens did not accurately correlate with the pattern in plasma oestradiol-17β, a relationship between increasing faecal oestrogen concentrations and increasing plasma oestradiol-17β concentrations was observed. Koalas with relatively high plasma oestradiol-17β also showed a higher concentration of faecal total oestrogens; in fact, faecal samples collected from cycling koalas had a significantly greater concentration of total faecal
oestrogens than samples collected from non-cycling koalas. Cycling females also appeared to show a greater level of variation in oestrogen concentrations when compared to non-cycling females, which showed a consistently low concentration of total oestrogen secretion. Long-term faecal steroid analysis thus could still provide an indication of cycling females and may have an application in long-term contraceptive studies.

A notable observation from this study is the variation in plasma oestradiol-17β concentrations between individual koalas. Disparity in steroid levels and cycle length has previously been reported by Johnston et al. (2000a) and is most likely a result of individual variation. Successful reproductive monitoring in the koala therefore requires the sequential collection of hormone samples in conjunction with oestrous behaviour monitoring over a time period equivalent of at least one complete oestrous cycle to ensure peak oestradiol concentrations are recorded.

The purpose of this study was to assess the ability of faecal steroid analysis in association with oestrous behaviour detection to accurately monitor the oestrous cycle and its application in future research studies in the area of assisted reproduction. Whilst oestrous behaviour detection remains a successful technique for the identification of oestrus and increased plasma oestradiol-17β concentrations, faecal oestrogens did not provide an accurate indication of elevated plasma oestradiol-17β levels in this study to allow for the precise monitoring of the oestrous cycle. Despite this, individual mean faecal oestrogens were concurrent to mean plasma oestradiol-17β concentrations and variations in plasma oestradiol-17β secretion indicative of cyclic activity were associated with variations in faecal oestrogen secretion. Although future studies involving the use of radioactive oestradiol and HPLC/mass spectrophotometry would assist in characterizing the metabolic pathway of oestradiol excretion in the koala, preliminary results from this study indicate that there is not a strong correlation between the pattern of faecal and plasma oestradiol-17β
secretion so that faecal oestrogens as an indirect measure of plasma oestradiol-17β will therefore need to be regarded with caution.

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CHAPTER 3: PLASMA PROLACTIN CONCENTRATIONS DURING LACTATION, POUCH YOUNG DEVELOPMENT AND THE RETURN TO BEHAVIOURAL OESTRUS IN CAPTIVE KOALAS (PHASCOLARCTOS CINEREUS).


Presented as submitted for publication in Reproduction, Fertility and Development.

ABSTRACT

Plasma Prl concentrations in captive koalas during lactation were determined by serial blood sampling. Prl levels were low (1.3 ± 0.1ng mL⁻¹, n = 5) during early lactation until PY began to emerge from the pouch (about day 130) before significantly (P < 0.05) increasing between days 161 - 175 (5.3 ± 1.0 ng mL⁻¹). A significant (P < 0.001) peak in Prl (7.7 ± 0.6 ng mL⁻¹) coincided with maturing young between days 189-231. All females failed to display any signs of oestrous behaviour until day 268.8 ± 8.5 (n = 4), some 102 ± 18.5 days before PY were weaned after achieving target weights of 2.5 – 2.7 kg. Throughout lactation, plasma LH concentrations were relatively high (range 4.9 - 8.7 ng mL⁻¹) and LH responses to exogenous GnRH were observed in all koalas at all times during lactation. In conclusion, oestrus is suppressed during the majority of koala lactation but this phenomenon appears to be unrelated to PRL secretion.
INTRODUCTION

Handasyde et al. (1990) have indicated that timing of reproductive activity in the female koala is closely related to the weaning of back young associated with marked changes in the concentration of Prl. In marsupial species, the sucking neonate induces a natural suppression of ovarian activity so that deliberate or accidental removal of pouch young allows the female to return to oestrus (Tyndale-Biscoe and Renfree, 1987). In fact, pouch young removal is a well-established experimental protocol for oestrous cycle control in marsupials (Paris et al., 2005), but unfortunately, it also has obvious ethical issues for endangered and vulnerable species, as it requires the sacrifice or transfer of the neonate (Johnston and Holt, 2001).

While the precise mechanisms responsible for lactational anoestrus in the koala are unknown, Prl does play a key role in ovarian function and lactation across a broad range of mammalian species (Freeman et al., 2000). Surprisingly there is only limited evidence that prolactin may be involved in the suckling-induced suppression of GnRH/LH secretion. McNeilly (2001) discovered that the suckling stimulus in women induced an increase in the sensitivity of the hypothalamus to oestradiol negative feedback on the GnRH pulse generator, but the precise mechanism (sucking stimulus versus Prl per se) is still unknown. In other species, including cows and pigs, and at least during early lactation in the rat, suppression of Prl during lactation appears to have little or no effect on gonadotrophin secretion or ovarian activity (McNeilly, 1994). Thus, the role of Prl in lactational anoestru is likely to be species dependant.

Studies conducted by Handasyde et al. (1990) on the seasonal pattern of Prl concentrations in wild female koalas showed that a marked increase in Prl levels occurs in lactating females when the young begin to exit the pouch. Further, a rapid fall in Prl concentrations and the recommencement of breeding activity was also associated with the reduction in the sucking
frequency as koala young approached independence. Such changes in Prl in lactating koalas are similar to that previously reported for many other marsupials [Tammar wallaby *Macropus eugenii* (Stewart, 1984); Bennetts wallaby *Macropus rufogriseus* (Curlewis et al., 1986) eastern quoll *Dasyurus viverrinus* (Hinds and Merchant, 1986) and red kangaroo *Macropus rufus* (Muths and Hinds, 1996)].

To date, a detailed serial longitudinal study of Prl concentrations throughout lactation in koalas has not been conducted, nor has there been any direct correlation with oestrous behaviour. A better understanding of this relationship could be important for determining the reproductive status of koalas with young, or as a potential mechanism for manipulative control of oestrus in captive breeding programs. The aim of this study was to characterize Prl concentrations throughout the lactation period of captive koalas and to determine the relationship of Prl with key events in PY development and also the recommencement of oestrus. Furthermore, the LH responsiveness of anterior pituitary gonadotrophs to a GnRH challenge was also investigated to determine if the gonadotrophin / pituitary axis is suppressed during lactation.

**METHODS**

*Animals*

This study was carried out on 7 captive sexually mature koalas held at Dreamworld (153°26′24″E, 28°04′12″S) on the Gold Coast in southeastern QLD. As determined by breeding history records, all females were fertile and remained clinically healthy throughout the experimental period. Of the 7 koalas studied, 5 successfully reared PY to weaning. The study was approved by the University of Queensland Animal Ethics Committee (SAS/339/10/DREAMWORLD/CWS) and was conducted over a 12 month period beginning in early January 2010.
Blood collection

Venipuncture was conducted on conscious koalas as previously described by Blanshard (1994). Blood samples (2 mL) were collected sequentially from the cephalic vein using a surflo 25g x 3/4" winged infusion set (Terumo, Tokyo) and a 3 mL syringe. The sample was centrifuged at 1600 g for 3 min. Plasma samples were stored in a –20 °C freezer. As koalas at DW are handled regularly by keeping staff, the collection of blood samples resulted in little (minor struggling) or no discomfort to the animal.

Detection of behavioural oestrus

Oestrous detection in this study used the method previously described by Blanshard (1994) and Johnston et al. (2000b). A “teaser’ male was introduced into the female enclosure and allowed to scent mark objects with his sternal gland, bellow and urinate. Females that were sexually receptive immediately displayed oestrous behaviours, which included bellowing vocalizations, ear flapping, “jerking”, homosexual mounting, increased agitated behaviour and strong interest in and approach to the male. The male was also presented individually to each female to ensure all females had equitable exposure, but no direct physical contact was permitted. Females were identified as in oestrus, if they displayed the majority of the oestrous behaviours listed above. Oestrous detection was conducted between 8 am and 9 am daily at DW and continued throughout the study period; ‘teaser’ males were regularly rotated to avoid habituation of both males and females to the oestrous detection procedure.

Study design

Seven female koalas were mated on days 2 - 4 of their behavioural oestrus. A blood sample was taken 1 week after parturition and fortnightly throughout lactation to assess changes in plasma Prl. For a short period during the study, an injection of natural sequence mGnRH (30 µg) (Peptide Biology Laboratory, The Salk Institute, La Jolla, CA) and a blood sample taken at T₀ and T₁₅ mins
after administration, was conducted once a month to test the responsiveness of the pituitary to GnRH during lactation. A total of 21 challenges were conducted in 6 koalas from day 110 until the return to oestrus (~Day 280). An increase in LH concentrations at T_{15} was indicative of the functional competence and responsiveness of the koala anterior pituitary and has been reported here as Δ LH (T_{15} – T_0). Throughout all of lactation and weaning, females were ‘teased’ for signs of oestrus and the developmental stages of PY recorded (Blanshard 1994).

Hormone analysis

Koala samples were assayed for Prl using a double-antibody RIA, as described previously for the Tammar wallaby (Hinds and Tyndale-Biscoe, 1982) and the eastern quoll (Hinds and Merchant, 1986). The assay used antiserum 33/1-8 raised in guinea pigs against human Prl together with ovine Prl (NIH-P-S12) as the standard and tracer. All samples were assayed in duplicate. Intra-assay and inter-assay coefficients of variation were 0.4% and 1.1% respectively for a quality control sample of 3.5 ng mL^{-1}. The assay sensitivity was 0.2 ng mL^{-1} with a measurable range up to 20.0 ng/ml. Koala plasma samples were also assayed for LH concentration using a heterologous RIA as described by Curlewis (1991), but with a mouse monoclonal antiserum (518B7; Monoclonal Antibodies, Inc, Mountain View, CA, USA) and ovine LH as standard and iodinated radioligand. This antibody is well-characterized to cross react with a diverse range of species (Matteri et al, 1987), including marsupials (McFarlane et al, 1997). Koala plasma diluted in parallel with ovine standards in this assay (data not shown). All samples were assayed in duplicate. The intra-assay coefficient of variation was 9.1% for a quality control sample of 4.0 ng mL^{-1}. The lowest detection limit of the assay was 0.4 ng mL^{-1}.

Statistical analysis

Plasma Prl concentrations were analysed over time using a repeat measures one-way ANOVA, with data being log (x+1) transformed before analysis. Significant differences between
time points were determined by post-hoc Dunnett’s multiple comparison tests. Statistical analysis was carried out using GraphPad Prism software version 6. Results are presented as mean ± SEM.

RESULTS

Changes in prolactin secretion during lactation

The mean (± SEM) and individual Prl concentrations throughout lactation and changes with PY development and the expression of behavioural oestrus are shown in figures 3.1 and 3.2, respectively. Two koalas (K6 and K7) lost their PY early during the study and for these animals PRL either did not increase (K6) or exhibited a transient increase (K7). For the other koalas (n = 5), Prl concentrations changed significantly over time, (P < 0.0001), with initial low concentrations (1.3 ± 0.1 ng mL$^{-1}$, n = 7) during early lactation (days 7-130), increasing between days 161 and 175 (5.3 ± 1.0 ng mL$^{-1}$) around the time that PY began to mature and emerge from the pouch. Peak Prl concentrations (7.7 ± 0.6 ng mL$^{-1}$) at days 189 to 231, coincided with maturing young eating leaf and often seen either on the mother’s back or sitting on own.

Expression of oestrus during lactation

All females failed to display any sign of behavioural oestrus until about day 268.8 ± 8.5 (n = 4), some 102 ± 18.5 days before PY were weaned after achieving a target weaning weight of 2.5 – 2.7 kg (Figures 3.1 and 3.2). For the two koalas that lost PY early during the study (K6 and K7 in Figure 3.2), koala K7 displayed oestrous behaviour 32 days later, whilst K6 failed to return to oestrus. Koala K3 died of lymphosarcoma 30 days after the collection of the final blood sample (day 273) and the PY, too young to rear, was euthanized.
Figure 3.1: Mean (± SEM) Prl concentrations throughout the lactation period of the koala; * P < 0.05, *** P < 0.001, ** P < 0.01 significantly different from value at day 7. Developmental stages of PY are reported along with the timing of the first oestrus (n = 5, except for day 217, 231 and 245 where n = 2). OP - PY seen out of the pouch, P – PY eating pap, BR - PY seen on mothers back, L - PY eating leaf, E – Mother’s first display of oestrous behaviour.

**LH Response to GnRH challenge during lactation**

Basal LH, changes in LH (T15 – T0) concentrations in response to injections of mGnRH, and PRL concentrations during mid and late lactation are shown in Table 3.1. Challenges were restricted to a 3 month period so individual LH data is not available throughout the entire lactation period. All koalas (n = 6, excluding K6) showed a LH response to mGnRH injection during lactation, regardless of whether PRL concentrations were low (days 112 to 154) or high (days 161 to 254). LH concentrations before injection of mGnRH (T0) were relatively high (range 4.9 - 8.7 ng mL⁻¹) throughout the study period.
Figure 3.2: Prl concentrations, developmental stages of pouch young (PY) and the occurrence of oestrous behaviour throughout the lactation period. HO - PY head out of pouch, OP - PY seen out of the pouch, P - PY eating pap, L - PY eating leaf, BR - PY seen on mothers back, E – Mother’s first display of oestrous behaviour, W – PY weaned.
Table 3.1: Basal LH, LH response to mGnRH and Prl concentrations during mid and late lactation. The LH response was calculated from blood samples taken at T₀ and T₁₅ (T₁₅ – T₀) after an injection of mGnRH. Key PY events: OP; PY seen out of the pouch, L; PY eating leaf, BR; PY on mothers back, E; first oestrus displayed by mother.

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<th>Day 112 to 154</th>
<th>Day 161 to 245</th>
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<tr>
<td><strong>Basal LH (ng mL⁻¹)</strong></td>
<td>6.8 ± 0.4 (n = 6)</td>
<td>6.5 ± 0.3 (n = 14)</td>
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<tr>
<td><strong>LH response to mGnRH (ng mL⁻¹)</strong></td>
<td>2.0 ± 0.5 (n = 6)</td>
<td>1.9 ± 0.3 (n = 14)</td>
</tr>
<tr>
<td><strong>Prl (ng mL⁻¹)</strong></td>
<td>2.1 ± 0.4 (n=15)</td>
<td>6.9±0.5 (n=29)</td>
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<tr>
<td><strong>Key PY events</strong></td>
<td>PY not seen out of pouch</td>
<td>OP, P, L, BR, E</td>
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**DISCUSSION**

The distinct pattern of Prl concentration during lactation in the captive koala reported in this current study was similar to that previously reported by Handasyde et al. (1990) for wild koalas and that previously observed in other marsupial species (Curlewis et al., 1986, Hinds and Merchant, 1986, Muths and Hinds, 1996, Crawford et al., 2011). The developmental stages of PY recorded in this study are also similar to those previously reported by Thompson (1987) and Blanshardt (1994). During early lactation (days 7 - 130), PRL concentrations remain low but then began to increase in association with maturing PY and the first emergence from the pouch (days 130 - 170). Peak PRL concentrations coincided with permanent pouch exit of the young onto the back of the adult at days 200 – 230, after which time PRL then decreased.

The low Prl concentrations in koalas during early lactation are most likely due to an insufficient suckling stimulus from immature young, despite the fact that the PY is permanently
attached to the teat. A similar pattern has been reported in the brushtail possum, where plasma Prl concentrations remain basal until the growth of PY fur, increased periods of young emergence from the pouch, as well as changes in suckling intensity (Crawford et al., 2006). Peak Prl secretion in the brushtail possum was also associated with permanent pouch exit of the young and decreased concentrations coincided with the observed regression of the mammary glands and weaning.

The female koala, similar to most mammals, experiences lactational anoestrus with the recommencement of reproductive activity only occurring when suckling stimulus markedly declines or ceases due to the loss of PY or as young reach independence (Handasyde et al., 1990). Females in the current study failed to display oestrous behaviour throughout the majority of lactation period, despite being ‘teased’ with a male on a daily basis. All 4 females, which successfully reared young to weaning, displayed oestrous behaviour approximately 102 days (range 60 – 145) before PY were fully weaned and PRL concentrations had begun to decrease. These results suggest that a reduction in suckling intimacy or the frequency of suckling associated with permanent pouch exit may be the potential trigger for recommencement of the reproductive cycle. A single female (K7) also displayed oestrous behaviour 32 days after the early loss of her PY. The delay in the recommencement of reproductive activity for K7 was similar in duration to the koala non-ovulatory cycle of 33.2 ± 1.2 days (Johnston et al. 2000)

Handasyde (1990) previously suggested in wild koalas that the prevention of cyclic activity during lactation is possibly due to increased PRL, but data presented in this current study indicates that this is unlikely, especially in early lactation where PRL concentrations remained low. Whilst Prl has long been considered as one of the key factors causing suppression of ovarian activity in lactating women (McNeilly, 2001), in other, species, including pigs, cows and at least in early lactation in the rat, suppression of Prl during lactation appears to have little or no effect on gonadotrophin secretion or ovarian activity (McNeilly, 1994). Results from the current study on
captive koalas are in accord with previous studies in other marsupial species, showing low concentrations of Prl during early lactation when follicular activity is suppressed (Curlewis et al., 1986, Hinds and Merchant, 1986, Muths and Hinds, 1996, Crawford et al., 2011). Together, these results suggest a separate mechanism inhibits cyclic activity at this time, rather than PRL per se. In some species, the intensity of the suckling stimulus also plays an important role in inhibiting oestrous activity (McNeilly 1994; Renfree 1979; Tsukamura et al. 1988). For example, in the Tammar wallaby, denervation of the sucked teat and associated mammary gland resulted in the growth of the CL and embryo to a stage in development equivalent to that expected if PY had been removed (Renfree 1979). Whether the suckling stimulus from immature koala PY is important in suppressing oestrus activity remains to be determined but circumstantial evidence suggests that this is likely. We found that expression of the first oestrus following parturition in the koala was typically associated with permanent exit of joey from the pouch, and presumably a corresponding reduction in suckling intensity and the ability to eat and process leaf.

The absence of oestrous activity during lactation has been reported to be largely due to the suppression of gonadotrophin release, particularly LH (McNeilly, 2001). Pulsatile LH release is strongly inhibited during the first half of lactation in ovariectomized rats and returns some 18 – 24 h after pup removal and can be suppressed again within 4 – 7 h of reattachment (Tsukamura and Maeda, 2001). Similarly in the brushtail possum, circulating LH concentrations are low throughout the lactation period (Crawford et al., 2011), albeit FSH concentrations are moderate (Crawford et al., 2006). Whilst the pulsatile secretory pattern of LH has not been accurately measured in female koalas, basal LH (T₀) concentrations in the current study (4.9 - 8.7 ng mL⁻¹, n = 21) were within the range of LH concentrations previously collected from four cycling koalas throughout a normal oestrous cycle (2.7 - 11.5 ng mL⁻¹ n= 53) (Ballantyne et al. 2015), implying that the suckling stimulus does not appear to suppress the ability of gonadotrophs to respond to GnRH. Further, injections of mGnRH in lactating female koalas consistently induced LH responses indicating that
the suckling stimulus does not act to suppress the ability of gonadotrophs to respond to GnRH. The development of a FSH assay would provide valuable information on FSH concentrations throughout lactation and the return to oestrus. Busby et al. (2014) have recently described the amino acid sequence of koala FSH and the FSH receptor so that development of homologous FSH assay for koala is possible.

During lactation in the rat, the oestrogen-positive feedback mechanism controlling GnRH induced LH surge remains intact despite vigorous suckling stimulus from pups (Tsukamura and Maeda, 2001). In the rat and Tammar at least, it seems possible that the suckling stimulus acts upon the hypothalamus, most likely through a specific neural pathway not through hormonal factors such as Prl to suppress pulsatile GnRH/LH secretion and thereby inhibit oestrous cycle activity (Renfree, 1979, Tsukamura and Maeda, 2001). Whether a similar mechanism exists in the koala is uncertain given the continued secretion of LH during the current study. Further studies could focus on serial blood collection using an indwelling catheter and the temporary removal or transfer of PY to determine the ability of the suckling stimulus to disrupt the mode of GnRH/LH secretion and ultimately suppress oestrous cycle activity in the koala; however, PY removal studies in this species would be difficult to undertake due to ethical considerations.

In conclusion, oestrus is suppressed during the majority of koala lactation from parturition to approximately 270 days post-partum but this phenomenon appears to be unrelated to PRL secretion. The suckling stimulus (intimacy/frequency) is the most probable mechanism responsible for the suppression of ovarian activity, but whether this is through the suppression or disruption of GnRH/LH pulse activity needs further investigation. While pouch young removal has previously been utilized as methods of oestrous synchronization in marsupial species (Paris et al., 2005), such techniques need to be implemented in conjunction with functional cross fostering programs.
REFERENCES


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CHAPTER 4: SEASONAL OESTROUS CYCLE ACTIVITY OF CAPTIVE FEMALE KOALAS IN SOUTHEAST QUEENSLAND

K. Ballantyne, A. Lisle, A. Mucci and S.D. Johnston

Presented as submitted for publication in Australian Mammalogy

ABSTRACT

This study investigated the seasonality of behavioural oestrous in a captive koala population in southeast Queensland. A total of 33 sexually mature koalas were examined over a 4 year period (2009 - 2012) to determine the influence of temperature, photoperiod and rainfall on the incidence of expression of behavioural oestrous without the confounding effect of lactation or limiting food resource availability. Although signs of behavioural oestrus were detected throughout the year, an obvious seasonality was apparent, with significantly less females displaying oestrus in late autumn and winter (May - August), than September to April (P < 0.0001). While average monthly photoperiod (P < 0.0001) and average monthly temperature (P < 0.0001) were significantly related with oestrous behaviour, rainfall was not (P > 0.05). A better understanding of the seasonality of reproductive function in the female koala will facilitate the use of reproductive management assisted breeding technology to enable improved genetic management in captive populations.

INTRODUCTION

In mammals, the distribution of births throughout the year is often dependent upon the environment (Bronson, 1989); environmental factors operate at two levels, known as ultimate and...
proximate factors. Ultimate factors are important for the long-term survival of the species, and produce the evolutionary pressure to result in seasonal breeding (Goodman, 1999), whilst proximate factors are associated with the environmental cues that a mammal uses to time reproductive activity (Goodman, 1999). From an ultimate perspective, annual reproductive patterns reflect a complex interaction between dietary and climatic factors that can impact upon energy balance and ultimately the survival of offspring (Bronson, 2009). In many seasonally breeding mammals, the period of copulation is related to timing late lactation and the weaning of young to coincide with higher food availability and beneficial climatic conditions in order to maximise the survival of both the dam and the offspring (Tyndale-Biscoe, 1979, Bowyer, 1991, Goodman, 1999, Bronson, 2009).

Female koalas lactate for approximately 12 months (McLean and Handasyde, 2006) so that breeding in the spring or summer months typically ensures favourable conditions for the offspring approaching independence in the next breeding season. Lactation is one of the most influential factors determining the mating period in post-parturient koalas (Handasyde et al., 1990); presumably, the sucking stimulus prevents subsequent oestrous cycle activity during the lactation period. Handasyde et al. (1990) found evidence of lactational anoestrus in free-ranging Victorian koalas when she showed that the presence of an almost weaned offspring or the disappearance of back young was associated with a rise in oestradiol concentration and a subsequent brief rise in progesterone concentration.

Ovulation in the koala is induced by coital stimulus and the possible presence of ovulating factors in the semen (Johnston et al., 2004). If copulation does not occur, a CL does not develop and consequently, there is no progesterone secretion, (Johnston et al., 2000a), so that the hormonal changes previously reported by Handasyde et al. (1990) were clearly evidence of an oestrus period, successful ovulation (mating) and a luteal phase (Johnston et al., 2000a). The seasonal peak in
progesterone concentration reported by Handasyde et al. (1990) was then followed by a decrease to basal levels, where it remained during the lactation period until the next breeding season or the loss of a pouch young.

Although koala births have been recorded to occur throughout the year at most wild locations in their range (Ellis et al., 2010), about 60% occur from the start of summer to early autumn (December–March) (Handasyde, 1986, Handasyde et al., 1990, Martin and Handasyde, 1990, Mitchell and Martin, 1990). McLean and Handasyde (2006) have also reported that 90% of koala births in two Victorian populations occurred between December and March. However, the same study reported only approximately 45% of births during the same 4 months in two other Victorian populations, suggesting other local environmental factors, such as koala population density, food abundance, soil type and climate may be influencing the timing of breeding.

To date, the evidence of seasonal reproduction in the koala has primarily been based on an estimate of pouch young age, an indirect measure of seasonal influence on female koala oestrous cycle activity. Behavioural oestrus has been shown to correspond with elevated oestradiol-17β secretion (Johnston et al., 2000b) and is commonly used as an effective tool for the timing of natural or artificial insemination in most captive SEQ koala populations (Johnston, 1999, Johnston et al., 2004, Johnston et al., 2000a, Johnston et al., 2000b, Allen et al., 2008). Captive koala populations provide a unique opportunity to study the effect of season on oestrous expression, without the confounding influence of lactational anoestrus and food resource availability, and in this way it will be possible to elucidate, with more confidence, the effect of temperature, photoperiod and rainfall on reproductive oestrous cycle activity.
METHODS

Animals and detection of behavioural oestrus

This study was carried out on captive koalas held at Dreamworld (DW) located on the Gold Coast in south-eastern QLD (153.31°E, 27.86°S). Female koalas used in this study were housed in single sex enclosures (4 m x 5 m x 3 m) containing between 3 - 5 individuals. Male and female enclosures where separated by 2 m with no physical contact between enclosures. A total of 33 sexually mature (between 2-9 years old) female koalas were examined over a 4-year period (2009-2012); the number of animals from which data was collected each month over the 4 year period is documented in Table 1. All females chosen were fertile and reproductively viable, as indicated by previous birthing and oestrous behaviour records, and remained clinically healthy throughout the full observation period.

Oestrous detection in this study used the method previously described by Blanshard (1994) and Johnston et al. (2000a). A ‘teaser’ male was introduced into the female enclosure and allowed to scent mark objects with his sternal gland, bellow and urinate. Females that were sexually receptive, immediately displayed oestrous behaviours that included bellowing, vocalizations, ear flapping, jerking, homosexual mounting, a strong interest in the male. The male was also presented individually to each female in the enclosure to ensure all females had equitable exposure but no direct physical contact was permitted. Females were identified as being in oestrus, if they displayed the majority of the oestrous behaviours listed above. Oestrous detection was conducted between 8am and 9am daily. The ‘teaser’ males were also rotated on a daily basis to avoid habituation of selected males to the oestrous detection procedure.
Data collection and analysis

Captive female koalas held at DW have been ‘teased’ regularly (every 1-3 days) for oestrous cycle activity since 2004 but daily data collection has only been documented since 2009 with respect to the incidence of oestrous behaviour and its relationship to the occurrence of copulation, births, lactation and environmental factors such as rainfall, photoperiod and temperature. Non-mated female koalas possess an anovulatory oestrous cycle of 32.9 ± 1.1 days and remain in oestrus for 10.3 ± 0.9 days, essentially entering a period of oestrus once a month, whereas mated non-fertile females ovulate and enter a luteal phase with a cycle length of 49.5 ± 1.0 days (Johnston et al., 2000b). Due to the extended length of the ovulatory cycle, only female koalas that did not copulate or which experienced an anovulatory cycle were included in this analysis, which allowed for the calculation of a monthly percentage of teased koalas displaying oestrous. The proportion of females excluded from the study due to mating or lactation was 22.5 ± 8.9 % (mean ± SD) during the warmer months (Sept – Apr) and 23.9 ± 2.2 % during cooler months (May – August). Behavioural oestrous periods that straddled the end and start of two months were recorded as occurring in the month for which the onset of oestrus was first observed. Data on the incidence of matings and births at DW have been recorded since 2003 and were pooled (2003 – 2012) to obtain a monthly birth rate as a percentage of matings. The number of matings that resulted in the birth of a PY was calculated as a percentage from the total matings for that month; the sex of PY and month of birth was also recorded. Koalas held at DW are weighed only 2 - 3 times a month. Weight data collected from non-lactating females (n = 12) during 2012 was collated to obtain an average monthly weight for females in the study throughout 2012. Monthly mean rainfall and temperature data for the study site were sourced from the Australian Bureau of Meteorology, Gold Coast Seaway Station, 153.43° E, 27.94° S (Australian Bureau of Meteorology). Photoperiod was calculated as the time between sunrise and sunset and was sourced from Geoscience Australia.
Statistical methods

The dataset consisted of 48 observation time intervals taken over a 4 yr period (Table 1). Each observation interval calculated the number of females entering oestrus and the number of females observed in that month. Independent variables considered were; individual year (2009 – 2012), month (1 – 12), non-breeding season (May-August), average temperature recorded in each month (varied between years), total rainfall recorded in each month (varied between years) and the average photoperiod for each month (constant between years). A range of different models were examined using logistic regression, predicting the probability of a female entering oestrus as a function of the various temporal and climatic variables. Daily Minimum and Maximum temperatures were not appropriate for monthly resolution of the oestrous data. Tests were adjusted to take account for overdispersion by estimating the scale parameter from the residual mean deviance. Analysis was carried out using the SAS GENMOD procedure. Monthly live weights were analysed using a repeated measures ANOVA with a first order autoregressive error structure. The model included terms for calendar month and the occurrence of oestrus, with animals considered as subjects. The SAS MIXED procedure was used for analysis. Analysis between month of birth and sex of PY were carried using a chi-squared test. For all statistical analyses the sample size was presented and the null hypothesis rejected if $P < 0.05$.

RESULTS

The seasonality of oestrus expression

The monthly percentage of koalas in an anovulatory cycle that entered oestrus in 2009, 2010, 2011 and 2012 are shown in figure 4.1. Although some individual koalas in the population showed signs of oestrous behaviour throughout the year, an obvious seasonality was apparent, with significantly less females displaying oestrous behaviour in late autumn and winter (May - August), than September to April ($F_{1,43} = 106.32, P < 0.0001$). A significant difference in the proportion of
koalas displaying oestrous was also observed between the year of observation ($F_{1,43} = 24.83$, $P < 0.0001$), with the highest levels observed in 2009 (average 80.2%) and lowest in 2012 (average 35.7%). The inclusion of a second climatic variable into the analysis provided no significant improvement in the fit of the model.

The monthly percentage of koalas in an anovulatory cycle that entered oestrus is displayed with the mean monthly temperature ($\pm$ SD), photoperiod and total rainfall throughout 2009, 2010, 2011 and 2012 in figures 4.2, 4.3 and 4.4, respectively. Allowing for overall differences between years and individual months, behavioural oestrus was significantly related to average monthly photoperiod ($F_{1,43} = 68.85$, $P < 0.0001$) and average monthly temperature ($F_{1,43} = 60.54$, $P < 0.0001$). Time of year, as either breeding or non-breeding (2 divisions) had a statistically stronger relationship with oestrous behaviour ($F_{1,43} = 106.32$, $P < 0.0001$) than temperature and photoperiod. Rainfall did not appear to be associated with expression of oestrous behaviour ($F_{1,43} = 2.88$, $P = 0.097$) and interactions between variables were not significant. Models incorporating a lag variable, while significant, did not provide as good an explanation as the unlagged variable. That is Year + Temperature was a better predictor of the likelihood of oestrus than Year + lag (Temperature).

Koala weights are recorded 2-3 times a month at DW and have been collated into a monthly average and presented in figure 4.5 to show the relationship between body mass (kg) and oestrous expression of female koalas during 2012. There was no significant correlation between live-weight and calendar month ($F_{11,109} = 1.61$, $P = 0.105$) or oestrus ($F_{1,10} = 2.45$, $P = 0.149$).
Table 4.1: Number of koalas in which behavioural oestrus was recorded each month over the four years of the study

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Figure 4.1: Proportion of koalas in oestrus per calendar month in 2009 (O), 2010 (■), 2011 ( ), 2012 (•), fitted with an average trend line (n values mirror those reported in figure two).
Figure 4.2: Monthly percentage of koalas that showed oestrous behaviour (columns) and mean monthly temperature (°C) (± SD) (line) in 2009(A), 2010(B), 2011(C) and 2012(D). Number of koalas observed per month is represented at the bottom of each column.
Figure 4.3: Percentage of koalas that showed oestrous behaviour (columns) (2009 - black, 2010 – white, 2011 – dark grey, 2012 – light grey) and photoperiod (h) (line).

Seasonal changes in birth rate and sex of PY

Data on the occurrence of matings and births at DW has been recorded since 2003 and collated to obtain a monthly birth rate as a percentage of successful matings. Figure 4.6 shows the mean birth rate throughout the year from 2003 until 2012 from a total of 103 matings. Being a captive population, the incidence of mating is under human control and is typically restricted to the warmer months of the year, September to March. Nevertheless, these results suggest that while successful breeding is possible throughout the entire breeding season, the percentage that results in the birth of PY decreased towards the end of the season in April. Figure 4.7 shows the number of male and female PY born per calendar month from 2003 -2012. No sexual bias (P = 0.38) in the timing of male or female births in the breeding season was observed.
Figure 4.4: Monthly percentage of koalas that showed oestrous behaviour (columns) and total monthly rainfall (mm) (black line) in 2009(A), 2010(B), 2011(C) and 2012(D). Number of koalas observed per month is represented at the bottom of each column.
Figure 4.5: Monthly percentage of koalas that showed oestrous behaviour (columns) and mean monthly weight (g) (±SEM) (line) (n=12) in 2012.

Figure 4.6: Monthly percentage of births from matings (%) (2003-2012). Number of matings observed per month is represented at the bottom of each column.
DISCUSSION

This is the first study to investigate the influence of season (time of year) on expression of behavioural oestrus in a captive koala population without the confounding influences of lactational anoestrus, food availability or shelter. Our results are in agreement with previous studies from both wild and captive populations in southern Australia and Queensland that record a strong seasonal influence upon koala births (Ellis et al., 2010, White and Kunst, 1990, Blanshard, 1994, Johnston, 1994, O'Callaghan, 1996, McLean and Handasyde, 2006). Seasonal variation has also been reported in wild and captive male koalas, which displayed an increase in testicular volume and steroidogenic capacity of the testis over spring and summer (Allen et al. 2010). Seasonality studies on Victorian koalas have reported a breeding period from September through to May (Handasyde, 1986, Handasyde et al., 1990, Martin and Handasyde, 1990, Mitchell and Martin, 1990) with a peak occurring from December to March (McLean and Handasyde, 2006). Ellis et al. (2010) previously reported that Queensland koalas have a peak in births between December and March. In the current study, an increased prevalence of oestrous expression was recorded in early spring through to early autumn (September – March) with more than 65% of observed koalas displaying behavioural

Figure 4.7: Number of male (black columns) and female (grey columns) PY born per calendar month from 2003-2012.
oestrus during this time; a peak in the expression of oestrus (approximately 75%) was recorded from November – January and again in March. Whilst the general pattern of seasonal oestrous expression remained similar from 2009 to 2012, the proportion of females displaying this behaviour declined significantly in 2012. Despite the number of koalas available for the study remaining essentially consistent over the 4 years, the lower level of oestrous expression was not accounted statistically by changes in temperature, rainfall or photoperiod.

Although the proximate factors responsible for the sharp increase in koalas displaying oestrus at the end of spring are unknown, the months of peak oestrous expression in the current study was strongly associated with the months of increasing or long day length. Despite year to year variation, the commencement of the breeding season (> 60% of koalas expressing oestrous) typically occurred approximately 1 to 2 months after the winter solstice in a period of increasing day length. Similarly, a distinct nadir in oestrous behaviour (< 30%) was observed in late autumn (May) and winter (June - July) coinciding with the approach of winter solstice and a period of decreasing or short day length. Preliminary evidence of an influence of photoperiod on the timing of the breeding season of the koala was originally suggested by Johnston (1994), who alluded to a peak (March to May) in breeding in captive Queensland koalas held in San Diego Zoo (32°45’ N) (Thompson, 1987), approximately 6 months after their usual breeding peak in Queensland (27°30’ S).

A distinct nadir in oestrous expression was also associated with a decrease in temperature to 17 - 19 °C in May - August, compared to the higher temperatures of 23 - 26 °C recorded during late spring to early autumn (November - March) when oestrous behaviour was at its peak. Furthermore, in the current study, the percentage of births from matings was also observed to decline at the end of the breeding season (April) in association with cooler temperatures (22°C). The warmer weather conditions may allow koalas to reallocate energy usually reserved for thermoregulation to reproductive activities such as lactation and searching for mates (Withers,
Koala young reach independence at approximately 12 months of age (Handasyde et al., 1990); hence, restricting breeding to the warmer months may ensure that independence is reached when temperatures are favourable for survival. Given the variability in the climatic and environmental conditions within the koala habitat range, minor adjustments in the breeding season of individual populations are likely to occur. Whilst koalas so far studied appear to restrict breeding to the warmer months of the year, it is likely that populations located in hotter or more humid regions may potentially limit breeding during these times to avoid heat stress. It is unclear from our observations as to whether temperature is simply a co-correlate of photoperiod or whether it is functioning as ultimate factor in the evolution of seasonal reproduction in the koala.

A previous study on wild Queensland koala populations reported a pattern between annual birth patterns in koala populations in semiarid regions and rainfall patterns (Ellis et al., 2010). The most restricted timing of births occurred at the site with the most variable rainfall occurring over a shorter a period, whilst at the third study site (south-east Queensland) a more even distribution of annual rainfall was associated with a less defined peak in births. Due to the captive nature of the koalas in the current study, food availability and shelter were presumed not to be limiting factors, thus as expected, rainfall had little impact on seasonality under these conditions. Similarly, given the constant food source, it is not surprising that female koala weight did not change significantly during the year.

Mclean and Handasyde (2006) previously suggested a difference in the seasonality of births between male and female juveniles in some koala populations in southern Australia, with 50% of male births before the end of November and 50% of female births towards the end of December. They proposed a possible explanation for this as the ‘Early Bird’ hypothesis, whereby species that show male biased sexual dimorphism produce male young early in the season to allow these individuals more time to grow before they reproduce the following year. In the current study, there
was no evidence of sexual bias in the timing of births throughout the breeding season. This is in agreement with previous studies on wild Queensland koala populations, where sex difference in the timing of births was also not detected (Ellis et al. 2010).

Goodman (1999) has noted that seasonally breeding mammals can be divided into two broad categories, those that use the duration of light (photoperiod) and those that use food availability. Those using photoperiod are usually larger mammals, with a relatively long lifespan and that employ the “K-type” of a “Planner” reproductive strategy of producing only a few offspring at a time and a major investment of energy in postnatal development. “R-type” strategists or opportunists, produce a large number of offspring at a time and almost no energy after weaning. While the results of the current study appear to support the premise that the koala is an increasing day length “K-type” seasonal breeder, it is probably best classified as a facultative seasonal breeding species, at least in captivity. It is possible that captivity has resulted in a degree of domestication in this species such that regular and predictable provision of high quality food resources have led to sufficient energy to allow occasional out of season births. Future studies should investigate the neuroendocrine mechanisms of seasonal breeding in the koala by examining secretion of melatonin and leptin.

In conclusion, our results on captive Queensland koalas are in agreement with previous studies on wild Queensland and Victorian populations, suggesting that despite habitat type (captive or wild), lactational anoestrus, rainfall or differential temperatures and photoperiods across latitudes in Australia, female koalas have a strong tendency to restrict reproductive behaviour to the warmer months of the year and that these ecological factors may pose minor alterations to the timing of the koala breeding season. As females retained their seasonal breeding pattern despite the provision of food and water ad lib, proximate factors, other than food availability, are likely to be influencing the timing of reproductive behaviour. This study has clearly demonstrated an underlying seasonal
influence on breeding pattern strongly associated with photoperiod and mean monthly temperature. However, the relative importance of photoperiod and temperature as proximate factors requires further investigation, as does the synergistic effect of these and other environmental factors.

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Withers PC (1992) 'Comparative Animal Physiology'. (Saunders College Pub, Fort Worth, TX.)
CHAPTER 5: THE USE OF GNRH ANTAGONIST AZALINE B TO CONTROL THE OESTROUS CYCLE IN THE KOALA (PHASCOLARCTOS CINEREUS)


Presented as submitted for publication in Reproduction, Fertility and Development.

ABSTRACT

This study examined the effectiveness of the gonadotrophin-releasing hormone (GnRH) antagonist, azaline B to suppress plasma LH and oestradiol-17β concentrations in koalas and its potential application for oestrous synchronization. In experiment 1, single sub-cutaneous (SC) injections of azaline B successfully blocked the LH response to exogenous mGnRH in a dose dependant manner; while controls injected with vehicle (n = 4) did not suppress LH response, 1 mg (n = 6) suppressed LH response for 24h (P<0.05), 3.3 mg (n = 8) suppressed LH response for 1-3 days and 10 mg (n = 4) suppressed LH response for 7 d (P<0.05). In experiment 2, daily 1 mg SC injections of azaline B over a 10 d period during seasonal anoestrus (June – July) (n = 6), also suppressed (P<0.01) the LH response to mGnRH, and the LH response had not recovered four days after cessation of treatment. Experiment 3 was designed to test the efficacy of a daily 1 mg SC dose of azaline B over 10 days to suppress plasma LH and oestradiol-17β concentrations and ultimately synchronize timed return to oestrus during the breeding season. Whilst treatment with azaline B did not suppress basal LH or oestradiol-17β, oestrus was delayed in all treated females by 24.2 days, but with high variability (range 9 - 39d). Overall, this study demonstrates the GnRH antagonist azaline B in koalas is able to inhibit the LH response to exogenous mGnRH and successfully delay
the return to oestrus. However, whilst azaline B appears to clearly disrupt folliculogenesis, it has not been able to effectively synchronise return to oestrus in the koala.

INTRODUCTION

Hypothalamic release of Gonadotrophin-Releasing Hormone (GnRH) controls marsupial reproduction through the regulation of luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion from the anterior pituitary gland (Tyndale-Biscoe, 2005). Exogenous GnRH analogs acting on GnRH receptors (GnRH-R) are either able to competitively block the receptor (GnRH antagonists) or desensitize the receptor (GnRH agonists) resulting in suppression of LH and FSH secretion in eutherian mammals (Herbert and Trigg, 2005a). Preliminary investigations in the male koala, appear to suggest this also to be the case in marsupials (Allen et al., 2008). However, most previous studies manipulating GnRH to control reproductive function in marsupials have focussed on the potential contraceptive properties of the GnRH agonist deslorelin (Herbert et al., 2007, Herbert et al., 2004a, Herbert and Trigg, 2005b, Herbert et al., 2006, Herbert et al., 2004b). Although GnRH agonists generally mimic the gonadotrophin response induced by endogenous GnRH (Conn and Crowley, 1994), their continuous occupancy of the GnRH-R causes receptor internalization, resulting in down-regulation and desensitization of GnRH receptors to GnRH (Hazum and Conn, 1988). Whilst this mechanism of action has been successfully shown to inhibit ovarian activity in marsupial species (Herbert et al., 2004a, Herbert et al., 2006, Herbert et al., 2005), the duration of agonist action is variable and reports have indicated that some individuals undergo several unsuccessful cycles before they are capable of breeding successfully after deslorelin treatment (Herbert et al., 2005). Such observations suggest that desorelin is likely to be unsuitable for the purpose of oestrous synchronization of marsupials.
Alternatively, GnRH antagonists bind to GnRH-R on gonadotrophs without either stimulatory activity or down-regulation of receptor expression (Herbert and Trigg, 2005b). Therefore, such treatments could be potentially used to control the oestrous cycle, temporarily decreasing gonadotrophin release. Metabolic clearance of the antagonist should result in availability of GnRH-R to again bind endogenous GnRH, resumption of gonadotrophin secretion and consequently ovarian activity. This would allow the ovarian folliculogenesis to be ‘reset’, providing a means by which females could be synchronised for timed artificial insemination.

Azaline B [((Ac-D-Nal\(^1\), D-Cpa\(^2\), D-Pal\(^3\), Aph\(^5\) (atz), D-Aph\(^6\) (atz), Ilys\(^8\), D-Ala\(^10\))-GnRH] is a potent and selective GnRH receptor antagonist with little or no anaphylactoid activity in animal models (Campen et al., 1995). Because of its limited solubility in aqueous buffers, its clinical development was abandoned (Samant et al., 2005), however, previous studies have indicated its potential in the control of reproductive endocrine activity in many animal species (Shangold et al., 1995, Campen et al., 1995). This antagonist has been shown to suppress several reproductive processes in rats including ovulation, and inhibiting gonadotrophin release in response to the GnRH agonist, histrelin, from rat pituitary cells in vitro (Campen et al., 1995). Daily injections of azaline B have also been shown to suppress oestradiol-17\(\beta\) concentrations in cynomogus monkeys, with subsequent ovulation occurring within 15-20 days after discontinuation of azaline B administration (Shangold et al., 1995). Altogether, these studies suggest azaline B might be a potential oestrous synchronization agent.

Previous studies (Zee et al., 2007, Zee et al., 2008, Johnston et al., 2012, Johnston et al., 2013) have failed to develop an effective method for the cryopreservation of koala spermatozoa to assist with genetic exchange, so AI protocols incorporating oestrous synchronization with the use of chilled semen are required in the short-term. The primary objective of this study was to investigate the potential application of the synthetic GnRH antagonist, azaline B, to synchronize oestrus in the
koala. Experiments examined whether azaline B could be used to temporarily suppress gonadotrophin release and prevent ovarian cyclicity. Experiment 1 investigated the duration of action of a single dose of the GnRH antagonist azaline B to competitively block the GnRH-R in the female koala. Experiment 2 aimed to ascertain the effect of repeated daily doses of azaline B to block the GnRH-R. In both experiments, blockade of GnRH-R was determined by measuring the LH response to a GnRH challenge. Experiment 3 was conducted in the breeding season and designed to test the efficacy of a daily dose of azaline B given during the interoestrus period to reset ovarian folliculogenesis and synchronise oestrus in female koalas once antagonist administration had been withdrawn.

METHODS

Animals

This study was conducted on captive koalas held at Currumbin Wildlife Sanctuary (153°29’12”E, 28°07’64”S) and Dreamworld (153°26’24”E, 28°04’12”S) on the Gold Coast, in south-eastern QLD. All females chosen were fertile (as indicated by previous birthing records) and remained clinically healthy throughout the experimental period. The study was approved by the University of Queensland Animal Ethics Committee (SAS/348/09/DREAMWORLD/ CURRUMBIN) and experiments were conducted between September 2009 and November 2010.

Blood collection and drug injections (azaline B and mGnRH)

Venipuncture was conducted on conscious koalas as previously described by Blanshard (1994). Blood samples (2 mL) were collected sequentially from the cephalic vein using a 12 mm, 25 gauge needle attached to a 0.5 mm diameter, 39.5 mm long winged infusion set (Terumo, Tokyo) and a 3 mL syringe. The sample was divided equally into 1 mL heparinized tubes (Becton Dickinson, NJ) and centrifuged at 1600 g for 3 min. Plasma samples were stored in a −20 °C
freezer. Injections of azaline B (Peptide Biology Laboratory, The Salk Institute, La Jolla, CA) dissolved in 5 ml of 5% mannitol (Sigma, Australia), and natural sequence mGnRH (30 µg) (Peptide Biology Laboratory, The Salk Institute, La Jolla, CA) were administered subcutaneously between the shoulder blades of conscious koalas using a 21-gauge needle. Due to the regular handling of koalas at DW and CWS by staff, the collection of blood samples and administration of azaline B resulted in little or no distress (only minor struggling) to the animal so that no sedation or anaesthesia was required.

Detection of behavioural oestrus

Oestrous detection in this study used the method previously described by Johnston et al. (2000). A “teaser” male was introduced into the female enclosure and allowed to scent mark objects with his sternal gland, bellow and urinate. The male was also presented individually to each female in the enclosure to ensure all females had equitable access but no direct physical contact was permitted between the teaser and female koalas. Females that were sexually receptive, immediately displayed oestrous behaviours; which included bellowing vocalizations, ear flapping, “jerking”, homosexual mounting and a strong interest in the male. Females were identified as in oestrus if they displayed the majority of the oestrous behaviours, as previous studies have established a clear relationship between oestrous behaviour and elevated levels of oestradiol-17β (Johnston et al., 2000b). Oestrous detection was conducted between 8 am and 9 am daily and continued throughout the study period. The ‘teaser’ males were regularly rotated to avoid habituation of both males and females to the oestrous detection procedure.

Experimental design.

Experiment 1: Effect of a single dose of Azaline B dose on the LH response to GnRH

This experiment was designed to investigate the duration of action of a single dose of the
GnRH antagonist azaline B to competitively block the GnRH-R on gonadotrophs in koalas. Twenty-two female koalas in seasonal anoestrus (July – August) were used for the trial. The responsiveness of gonadotrophs in each koala was first established by challenge with a subcutaneous (SC) injection 30 µg mammalian GnRH. Blood samples were taken prior to (T₀) and 15 mins (T₁₅) after administration. An increase in LH secretion at T₁₅ (LH response) was indicative of gonadotrophic response. Approximately 3h later, each koala was administered with either 0 mg (control) (n = 4), 1 mg (n = 6), 3.3 mg (n = 8) or 10 mg ( n = 4 ) of azaline B subcutaneously in a 5 mL 5% mannitol solution. Approximately 3h after azaline B treatment, a second mGnRH challenge was conducted. Further mGnRH challenges were conducted on days 1, 2, 3, 5, 7, 9, 12, 15 and 19 after azaline B treatment. Failure to induce an LH response following each mGnRH challenge was interpreted as evidence of antagonist efficacy on gonadotrophs.

Experiment 2: Effect of continuous, low dose (1mg) azaline B treatment on the LH response to GnRH and subsequent gonadotroph recovery after azaline B

Six female koalas were subcutaneously injected with a 1 mg dose of azaline B daily for 10 days during a period of seasonal anoestrus (June – July) and recovery monitored over 4 days. Again the responsiveness of gonadotrophs was determined by mGnRH challenge on Day 0 prior to azaline B treatment and further challenges conducted every second day prior to azaline B treatment. Daily doses of azaline B ceased on day 9, and gonadotrophin responsiveness was assessed by mGnRH challenges on days 10 to 13. Again, failure to induce an LH response (T₁₅ – T₀) was interpreted as evidence of antagonist efficacy on gonadotrophs.

Experiment 3: The effect of azaline B on the oestrous synchronisation of koalas

Experiment 3 aimed to determine the effect of low dose azaline B administered during the inter-oestrous period of the koala oestrous cycle and its ability to synchronise follicular development following withdrawal. During the breeding season (January – March) six sexually
mature females were administered daily 1 mg dose of azaline B for 10 days commencing 3 days after the cessation of oestrus (inter-oestrous period of an anovulatory cycle). At this time, the dominant follicle of the last oestrus was presumably atretic, and moreover, there would be no functional CL, as mating induced ovulation had not occurred. During the 10d administration period and until return to oestrus, each female was bled every 2d to determine plasma oestradiol-17β (E2) and LH concentrations.

Hormone analysis

Koala plasma samples were assayed for LH concentration using a heterologous RIA as described by Curlewis (1991), but with a mouse monoclonal antiserum (518B7; Monoclonal Antibodies, Inc, Mountain View, CA, USA) and ovine LH as standard and iodinated radioligand. This antibody is well-characterized to cross react with a diverse range of species (Matteri et al, 1987), including marsupials (McFarlane et al, 1997). Koala plasma diluted in parallel with ovine standards in this assay (data not shown). The assay detection limit was 0.4 ng.ml⁻¹, and the intra-assay and inter-assay coefficient of variations were 11.7% and 14.9% respectively. All samples were assayed in duplicate. The plasma concentration of oestradiol-17β (E₂) was determined directly from plasma using commercially available ultra-sensitive oestradiol RIA kit (Immunotech, Prague – Czech Republic). The assay sensitivity was 1.0 pg.ml⁻¹, and the intra-assay and inter-assay coefficient of variations were 8.8% and 12.7% respectively. All samples were assayed in duplicate. The reported oestradiol antibody cross-reactivity was oestrone (2.4%), D-Equilenin (3.4%), 17-β-oestradiol-3-glucuronide (2.6%) and <1% for all other steroids tested compared to 17β oestradiol at 100% (Immunotech, Prague, Czech Republic).

Statistical analysis

A one-way ANOVA was used to examine the treatment effect (LH responses and basal LH concentrations) across time within an azaline B dose. Following a significant main treatment effect,
each time point was compared to pre-treatment (T-3) using a post-hoc Dunnett’s multiple comparison test. Values are reported as Mean ± SEM. P<0.05 was used for statistical significance.

**RESULTS**

*Experiment 1*

Injections of a single dose of azaline B successfully blocked the ability of gonadotrophs to respond to mGnRH in a dose dependant manner (Figure 5.1). All koalas showed a LH response to mGnRH (2.4 ± 0.4 ng.mL$^{-1}$) 3h before treatment. Control (0 mg) females continued to show robust LH responses to mGnRH (2.4 ± 0.3 ng.mL$^{-1}$, range 0.9 - 6.6 ng.mL$^{-1}$) throughout the study period. A single dose of 1mg of azaline B significantly (P<0.05) suppressed the LH responses for a 24hr period immediately following azaline B treatment (Figure 5.1). In contrast, a single 10 mg dose significantly (P<0.05) suppressed the LH responses for up to 7 days and returned to pre-treatment levels on day 9. A single azaline B dose of 3.3 mg produced variable results of LH suppression between animals, [3hr (n = 3), 24hr (n = 1), 48hr (n = 1) and 96hr (n = 3)], with significant (P<0.05) LH suppression only noted at 3hr post-azaline.

LH concentrations prior to mGnRH challenge (T$_0$) were not affected by azaline B treatment in animals administered with a single dose of either 1.0 mg or 3.3 mg of azaline B (data not shown). However a significant (P<0.05) suppression of basal (T$_0$) LH concentrations was noted on Days 7 and 9 (0.7 ± 0.3 and 0.5 ± 0.2 ng.mL$^{-1}$ respectively) after the 10 mg dose of azaline B, compared to LH concentrations in the same animals prior to azaline B treatment (2.0 ± 0.4 ng.mL$^{-1}$).
Figure 5.1: LH response to mGnRH challenge in koalas given different doses of azaline B (0mg n = 4; 1mg n = 6; 3mg n = 8; 10mg n = 4). LH responses are expressed as the change in LH concentrations from 15 minutes after mGnRH challenge compared to prior to challenge ($T_{15} - T_0$).

* $P<0.05$, ** $P<0.01$ significantly different from value at time 0 (pre-treatment). Values are mean ± SEM.
Figure 5.2: (A) LH response to mGnRH challenge ($T_{15} - T_0$) in koalas before azaline B treatment (day 0; white shaded area), during daily 1mg treatment with azaline B (days 1-9; light grey shaded area), and post azaline B treatment (days 10-13; dark grey shaded area). * azaline B injection, * $P<0.05$ significantly different from value at day 1 (pre-treatment). (B) Plasma LH concentrations prior to mGnRH challenge ($T_0$) in koalas before azaline B treatment (day 0; white shaded area), during daily 1mg treatment with azaline B (days 1-9; light grey shaded area), and post azaline B treatment (days 10-13; dark grey shaded area). ↑ azaline B injection, * $P<0.05$ significantly different from value at day 1 (pre-treatment). Values are mean ± SEM.
Figure 5.3: Basal plasma oestradiol (●) and LH (○) concentrations during (0-10d) and after (10-60d) 10 daily 1 mg injections of azaline B. Azaline treatment was during the interoestrus phase of the female koala, commencing 3 days after cessation of oestrus. The approximate expected return to oestrus is indicated by grey shaded area. Actual return to oestrus for each animal is indicated by an arrow.
**Experiment 2**

Daily 1mg doses of azaline B during seasonal anoestrus, significantly ($P<0.01$) suppressed the LH response to exogenous mGnRH ($T_{15}-T_0$) over a 10d treatment period and for another 4 days after azaline B ceased (Figure 5.2A). However, following cessation of azaline B treatment, a significant ($P<0.05$) increase in LH concentration prior to mGnRH challenge ($T_0$) was observed (Figure 2B), despite no change in LH response to the mGnRH challenge.

**Experiment 3**

Daily 1mg doses of azaline B over a 10 day period delayed oestrus in all 6 treated koalas (Figure 5.3). The delay in oestrus, based on a mean oestrous cycle length of 33 days (Johnston et al., 2000b), was $24.2 \pm 5.0$ days (range 9 to 39 days). Plasma LH and oestradiol-17β concentrations were variable between animals, with no obvious suppression being noted during azaline B administration (Figure 5.3).

**DISCUSSION**

The successful ability of GnRH antagonists to reversibly block GnRH receptors in humans has lead to its inclusion in several assisted reproductive treatment schedules (Coccia et al., 2004). In women, competitive blockage of GnRH-R by antagonist administration leads to significant falls in basal plasma LH, and to a lesser extent in FSH secretion (Coccia et al., 2004) but the same effect was not observed in the koala. The results of the current study demonstrates that the GnRH antagonist azaline B does not suppress basal LH or oestradiol-17β concentrations in the koala, however, it does appear to suppress the ability of anterior pituitary to respond to an exogenous GnRH challenge; prolonged treatment (10d) also successfully disrupts folliculogenesis, delaying the return to oestrus.
A limitation of this study was the difficulty of measuring pulsatile LH concentrations in the koala. It was not possible, ethically or practically (zoo animals on display), to catheterise koalas to allow more continuous blood sampling. This difficulty was in a large part overcome with the use of mGnRH challenges to test the availability of GnRH receptors on the pituitary during and after antagonist treatment. Therefore, we examined LH responsiveness to mGnRH using just two blood samples, rather than pulsatile LH release.

Our results indicated that basal LH secretion in general continued unabated after treatment with either a single dose of azaline B (1 – 10 mg dose; 0.2 – 2 mg/kg) or prolonged daily (1mg for 10d) treatment. The only evidence of LH suppression was noted on days 7 and 9 after a single 10 mg dose of azaline B, nevertheless LH concentrations were not suppressed prior to or after these days. The general lack suppression of basal LH by azaline B in koalas is surprising given injections of 0.2 mg/kg of the antagonist in ovariectomized rats has been shown to suppress LH concentrations for up to 3 days, and doses of 2 mg/kg suppress LH for 15 days with a gradual recovery by day 30-35 (Shangold et al. 1995).

Interestingly, in experiment 2 where koalas were administered with daily 1mg doses of azaline B and challenged with injections of mGnRH every two days, LH concentrations measured before a mGnRH injection (T₀) increased in concentration so that LH concentrations post azaline B treatment where significantly higher then those prior to treatment. This appeared to be an isolated phenomenon, as a similar trend was not observed in experiment 1 or 3 despite a similar treatment schedule.

The primary aim of experiments 1 and 2 was to ascertain the dose of azaline B required to block the GnRH-R for a period of time. As these experiments were not focussed on the ovarian response of azaline B, experiments were conducted in July to August outside of the recognised
koala breeding season (September to April). Results indicated that a 1 mg dose of azaline B blocked
the anterior pituitary’s ability to respond to a GnRH challenge for 24 h, whilst a dose of 10 mg
appeared to have suppressed LH response for up to 7d with a slow recovery back to normal function
between days 9-19. Given the relatively rapid recovery (24 hrs) of the LH response following 1mg
administration, the prolonged suppression in the 10mg was surprising. Down-regulation or
desensitization of GnRH-R due to the repeated exposure to mGnRH, is unlikely given control
females continued to show a sustained LH response to mGnRH throughout the study period.
Azaline B has been documented to form a slow releasing depot when injected subcutaneously in
rats in 5% aqueous mannitol solutions at concentrations from 5 to 20 mg mL⁻¹ (Gray et al., 1994).
However, the formation of such a depot seems unlikely in the current study, as doses of azaline B
(0.2 – 2 mg mL⁻¹) were below the gel forming range noted in rats.

When koalas where treated with a daily 1 mg dose over 10 days in experiment 2, LH
response to mGnRH had not recovered 4 days after azaline B treatment had ceased; in fact, this
suppressive effect occurred for a period of at least 13 d, similar to that observed with a single 10 mg
injection (Experiment 1). This result suggests, that a single low (1 mg) dose does not cause down-
regulation of GnRH-R, however multiple low doses appear to impose a similar response to that of a
single high (10 mg) dose. While it is documented that GnRH antagonists can competitively block
GnRH-R without the desensitization or down-regulation of GnRH-R observed with GnRH agonists
(Loumaye et al., 1984), there are nevertheless numerous reports concerning decreases in pituitary
GnRH-R mRNA during pro-longed GnRH antagonist treatment (Weiss et al., 2006). Treatment
with high doses of the GnRH antagonist cetrorelix have also produced considerable down-
documented that decreased GnRH-R mRNA expression during pro-longed GnRH antagonist
treatment was similar to that caused by a GnRH agonist. Similarly, Halmos et al. (1996), reported a
time-dependant decrease in both occupied and unoccupied GnRH receptors in cetrorelix treated
rats. The continued occupancy of receptors with GnRH antagonist has been reported to down-regulate GnRH-R mRNA expression indirectly by inhibiting the up-regulation of the receptor expression induced by endogeneous GnRH in the rat (Kovács and Schally, 2001, Kovács et al., 2001, Horvath et al., 2002). Given the LH response in the current study recovered 24 h after a single injection, but failed to recover for at least 3 d after prolonged treatment (10 days), it is possible that a similar mechanism also occurs in the koala. Additional research is required to investigate the exact pathway or combination of mechanisms responsible for the sustained suppression of pituitary response.

Since daily treatment with a 1 mg dose of azaline B suppressed the LH response in koalas during the anoestrous season (Experiment 2), the same protocol was applied during the breeding season (Experiment 3) to determine the ability of azaline B to prevent ovarian cyclicity via suppression of LH after azaline B withdrawal, to potentially synchronize the return to oestrus. It was expected that the time taken for the female koala to return to oestrus post-azaline B would be approximately equal to the period between the decline in progesterone and the return to oestrus in an ovulatory cycle, some 15 days (Johnston et al., 2000a). Surprisingly, treatment with azaline B also appeared to have had little inhibitory affect on the concentrations of oestradiol-17β. Given basal LH secretion was not suppressed, it is likely that basal oestradiol-17β secretion would also continue, however, unexpectedly oestrus was delayed substantially in 5/6 treated females. Further, previous studies on cynomolgus monkeys reported daily injections of azaline B at doses of 15, 25 and 40 µg/kg suppressed oestradiol-17β concentrations for the duration of the treatment, recovering within 1 to 5 days and ovulation occurred 15 - 20 days after discontinuing azaline B (Shangold et al., 1995). In contrast, the results of our study indicated a high level of variability in the recovery from azaline B with respect to the return to oestrus (19, 30, 33, 33, 40 and 49 days after treatment). Due to the extended length of the return to oestrus and difficulty of collecting repeated blood samples due to animal ethics committee restrictions on the number of samples that could be
collected, blood collection was discontinued before oestrus behaviour was expressed; consequently a peak in oestradiol-17β coincident with oestrus was not recorded in this study.

Although plasma FSH concentrations and ovarian follicular development was not directly measured during this study, the delay in the return to oestrus clearly indicates some disruption in follicular development associated with a delayed growth of the dominate follicle associated with insufficient production of oestradiol to induce oestrous behaviour. Follicular oestradiol production is dependant upon both FSH stimulation of aromatase in granulosa cells and LH stimulation of androstenedione production by the theca (McGee and Hsueh, 2000). Our results suggest that azaline B treatment in the koala may be able to suppress gonadotrophin support somewhat and inhibit growth of the dominate follicle, but still allow sufficient basal FSH and LH secretion to allow some follicular development and low levels of oestradiol production.

Haughian et al. (2013) reported similar findings in heifers where by treatment with the GnRH antagonist acycline, effectively blocked GnRH-stimulated LH and FSH secretion during the preovulatory gonadotrophin surge, but did not decrease periovulatory circulating FSH and had no effect on early follicle development. Whilst we are unable to measure koala FSH due to the lack of an appropriate assay, it would seem unlikely that basal FSH levels would be suppressed by azaline B, as basal FSH release in marsupials is continuous (Crawford et al., 2009), probably via a constitutive pathway independent of GnRH secretion, like other mammals. In GnRH antagonist treated heifers, circulating LH is reduced and the growth of a single dominant follicle is prevented (Haughian et al., 2013), suggesting LH secretion plays a critical role in the selection of a dominate follicle. Further, at the time of dominate follicle selection in cattle, there is an increase in LH concentration (Ginther et al., 2001), increased LH receptor expression in granulosa cells (Lucy, 2007, Beg et al., 2001, Xu et al., 1995, Luo et al., 2011, Sartori et al., 2001) and increased LH responsiveness (Sartori et al., 2001). Final maturation of preovulatory follicles has also been shown
to be LH, not FSH dependent in pigs (Brussow et al., 2001). It is likely that LH plays a similar role in marsupial folliculogenesis, as LH receptors are also present in granulosa cells of follicles in the brushtail possum (Eckery et al., 2002) and induction of ovulation has been achieved by single injections of ovine LH in superovulation protocols in the brushtail possum (Glazier and Molinia, 1998). While results from the current study indicate that basal LH concentrations were not suppressed during azaline B treatment, GnRH stimulated LH release was inhibited indicating that perhaps the GnRH dependant episodic or pulse release of LH has been disrupted impairing the selection of a dominate follicle. LH analysis of serial blood samples collected from antagonist and non-antagonist treated koalas during the follicular phase would obviously provide more valuable data regarding the influence of GnRH antagonists on episodic LH release but this was not possible in the current study.

Based on an anovulatory oestrous cycle in the koala being about 33 days (Johnston et al., 2000b), we estimated that oestrus expression in experiment 3 was delayed. Although 3 of the 6 koalas entered oestrus within 3 days of each other, suggesting a degree of synchrony, the variability in the remaining 3 females suggests the technique currently remains unreliable as a successful oestrous synchronization tool. It is likely that the degree of variability is attributed to the continued development of early follicles combined with inherent variability in the length of the koala oestrous cycle 25-45 days; (Johnston et al., 2000b). The length of recovery is also surprising and possibly indicates down-regulation of pituitary GnRH-R. If GnRH receptors are not down-regulated during azaline B treatment, then perhaps the addition of an appropriate single dose of GnRH agonist would help promote synchronous folliculogenesis and improve the timing of oestrus as it has been used in domestic species (Twagiramungu et al., 1995).

The current study has demonstrated the ability to block GnRH receptors with the GnRH antagonist azaline B, inhibiting a LH response to injections of exogenous mGnRH and delaying the
return to oestrus in the koala after prolonged (10d) treatment. Whilst these results suggest that it may be possible to disrupt folliculogenesis in the koala, we have not been able to obtain adequate control over the recommencement of follicular growth. It seems likely that azaline B disrupts follicular development in the koala through the disruption of episodic LH secretion imposed by the competitive blockade of GnRH-Rs. Further studies on follicular development, FSH concentrations and LH pulsatility during azaline B treatment are required to determine the exact mechanism through which this GnRH antagonist disrupts the oestrous cycle in the koala. This study is the first to investigate the effects of a GnRH antagonist as a possible method of oestrous synchronization in a marsupial and has provided further insight into the regulation of the female marsupial reproduction.

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CHAPTER 6: THE USE OF SYNTHETIC PROGESTERONE, LEVONORGESTREL (LNG) TO CONTROL THE OESTROUS CYCLE IN THE KOALA


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ABSTRACT

This study investigated the efficacy of a synthetic progestogen LNG to control koala ovarian activity for the purposes of oestrous synchronisation. Captive koalas were administered either saline control or a 70 mg LNG implant on day 2 of oestrus and urogenital cytology, oestrous behaviour, plasma oestradiol-17β and LH concentrations were monitored over a 6 week period. After LNG implant removal females were monitored to determine if the return to oestrus was synchronized. LNG treated koalas immediately ceased displaying oestrous behaviour, showed no evidence of “cornified” epithelial cells in smears of urogenital cytology, and exhibited reduced plasma oestradiol-17β concentrations throughout the implantation period. In contrast, oestradiol-17β levels in control koalas showed evidence of continued cyclic activity associated with behavioural oestrus and increased cornified epithelial cells in the urogenital smears on days 33 to 35 after saline injection. After implant removal, LNG treated koalas exhibited oestrus at 13, 14, 17 and 30 days post-implantation. Plasma LH concentrations varied throughout the study period with no significant time (P = 0.49) or treatment (P = 0.13) effect. Overall results from this study suggest LNG implants in koalas can inhibit oestrous behaviour and reduce circulating oestradiol-17β levels, most likely by
preventng development of the pre-ovulatory follicle. However, there was no evidence of LH suppression by the LNG implants. Removal of LNG implants resulted in the synchronous return to oestrus in 3 of the 4 treated koalas. Further studies on a larger population are required to validate these findings.

INTRODUCTION

Endocrine-based systems to synchronise ovarian function and the precise timing of ovulation are well-established procedures of assisted breeding for humans and domestic animals. In many eutherian mammals, it is possible to synchronize oestrus by the timed administration and removal of progestogen implants or by the removal of the inhibitory effects of progesterone secreted from the corpus luteum (CL) using exogenous analogues of PF2α in order to cause regression of the CL. While it is generally accepted that progesterone secretion from the marsupial CL has little inhibitory effect on follicular activity during pregnancy or a luteal phase (Shaw 2006), the majority of this work has been based on the tammar wallaby Macropus eugenii, which exhibits a “type 3” reproductive strategy (Tyndale-Biscoe and Renfree, 1987); this reproductive strategy is characterised by a gestation period that extends into the follicular phase of the next oestrous cycle and an associated post-partum oestrus.

The koala, in contrast, has a modified type 1 pattern of reproduction (Johnston and Holt, 2001) with its gestation period (≈35d) substantially less than the length of a normal oestrous cycle (≈50d) (Johnston et al., 2000b). Further, ovulation in the koala is induced following coitus, requiring the stimulus of intromission and seminal plasma to induce the LH surge and therefore ovulation (Johnston et al., 2004). If copulation does not occur, ovulation is prevented and the koala will typically remain in behavioural oestrus for up to 10 days, after which time the dominate follicle presumably regresses and become atretic (Johnston et al., 2000b). In the absence of ovulation, a CL
does not develop and consequently, there is no progesterone secretion. Most likely, a new wave of folliculogenesis results in the development of a new dominant follicle and another oestrus approximately 33 days later (Johnston et al., 2000b).

Studies on the use of synthetic progestogens for contraception in the tammar wallaby (Hynes et al., 2007) have revealed that although the progestogen levonoregestrel (LNG) was capable of preventing a LH surge, it was unable to suppress follicular activity, and consequently this compound is unlikely to be suitable as an oestrous synchronization agent in the tammar. Further, a recent study by Crawford et al. (2011) concluded that whilst progestogen implants were capable of blocking ovulation in the brushtail possum, *Trichosurus vulpecula*, the degree of ‘synchronous’ antral follicle development and ovulation after implant withdrawal was not sufficient for practical use with oestrous synchronisation (Crawford et al., 2011).

Interestingly, whilst Middleton et al. (2003) and Hynes et al. (2010) demonstrated successful use of LNG as a contraceptive in wild female koalas, Hynes et al. (2010) reported no difference in the prevalence of cornified epithelial cells in urogenital smears between control and treated females. This observation provided indirect evidence that follicular development was possibly not suppressed by LNG treatment, as is the case in women (Croxatto, 2002). In women, LNG implants do not suppress oestradiol secretion or follicular development, however, the release of gonadotrophins are profoundly altered resulting in abnormalities in follicular growth and ovulation (Croxatto, 2002). It is, therefore, possible that LNG may deter mating in the koala, possibly via the suppression of behavioural oestrus, leading to an insufficient release of LH and hence prevent ovulation.

LNG has been shown to successfully prevent pregnancy via the suppression of behavioural oestrus in the domestic cat, another species in which ovulation is induced via a copulo-receptive
reflex (Baldwin et al., 1994). Alternatively, ovulation in LNG treated koalas may be prevented, even if mating is achieved through inhibition of the LH surge, as is the case in many other spontaneous ovulating species including the tammar wallaby (Baldwin et al., 1994, Bettinger et al., 1997, Savage et al., 2002, Hynes et al., 2007). The ability of luteal or exogenous progestogen to suppress gonadotrophin secretion and subsequently inhibit follicular growth in the koala has not been specifically investigated, however, Johnston (1999) has shown that behavioural oestrous is at least suppressed during the luteal phase in both pregnant and non-pregnant koalas.

The principal aim of this study was to determine whether exogenous progestogen (LNG) was capable of inhibiting oestrus activity in the koala and if this could potentially be used to manipulate ovarian activity for the purposes of oestrous synchronisation. The present study focused on the physiological and behavioural effects on individual koalas following administration of 70 mg LNG silicone implants over a 6 week period.

METHODS

Animals

This study was carried out on eight sexually mature captive koalas held at Currumbin Wildlife Sanctuary (153°29’12”E, 28°07’64”S) and Dreamworld (153°26’24”E, 28°04’12”S) on the Gold Coast in southeastern QLD; all females were fertile as indicated by birthing records and remained clinically healthy throughout the experimental period. The study was approved by the University of Queensland Animal Ethics Committee (SAS/450/12/DREAMWORLD) and was conducted over a 12-week period within the breeding season commencing in January 2012.
Blood collection

Venipuncture was conducted on conscious koalas as previously described by Blanshard (1994). Blood samples (2mL) were collected sequentially from the cephalic vein using a 12 mm 25 gauge needle attached to a 0.5 mm x 39.5 mm winged infusion set (Terumo, Tokyo) and a 3 mL syringe. The sample was divided equally into 1 mL heparinized tubes (Becton Dickinson, NJ) and centrifuged at 1600 g for 3 min. Plasma samples were stored in a –20 °C freezer. As koalas held at DW and CWS are handled regularly by staff the collection of blood samples resulted in little or no distress (minor struggling) to the animal.

Detection of behavioural oestrus

Oestrous detection in this study used the method previously described by Blanshard (1994) and Johnston et al. (2000a). A “teaser’ male was introduced into the female enclosure and allowed to scent mark objects with his sternal gland, bellow and urinate. Females that were sexually receptive immediately displayed oestrous behaviours; which included bellowing vocalizations, agitated behaviour, jerking, homosexual mounting, and strong interest in the presence of the male. The male was also presented individually to each female in the enclosure to ensure all females had equitable exposure, but no direct physical contact was permitted. Females were identified as in oestrus if they displayed a majority of the oestrous behaviours listed above. Oestrous detection was conducted between 8am and 9am daily at DW and CWS and continued throughout the study period. The ‘teaser’ males were rotated to avoid habituation of both males and females to the oestrous detection procedure.

Urogenital cytology method

As ovulation in the koala is induced by coitus (Handasyde et al., 1990, Johnston, 1994), care was taken when obtaining cells from the urogenital sinus to not accidently induce ovulation. Cells from the luminal surface of the urogenital sinus were therefore collected from conscious koalas
using a technique previously described by Johnston (1999) which caused little discomfort or stimulation to the urogenital tract. A glass eye dropper with a rounded tip containing 1 mL sterile physiological saline was gently inserted approximately 40 mm into the opening of the urogenital sinus. Cells were lavaged from the wall of the urogenital lumen by repeated infusion and aspiration of sterile saline. Aspirates were then smeared on to a microscope slide, air dried, fixed in methanol and stained with Diff Quick stain (Lab Aids, Australia). Smears were analysed and interpreted based on the methodology described by Hynes et al. (2010). Briefly, slides were examined under a microscope at 400X magnification for the presence and prevalence of cornified versus non-cornified epithelial cells, and leucocytes. The whole slide was scanned and the density and proportion of epithelial cells in the smear were given a score from 1 - 5. A score of 4 was given if >75% of the field of view was covered in cells with at least 50 % of these being cornified. A score of 5 was given if between 90% and 100% of the field of view was covered in cornified epithelial cells. These were usually seen as a sheet of cells. A score of 4 or 5 was recorded as an oestrous smear.

Study design

Four females were implanted with synthetic progestogen levonorgestrel (70mg) silicone implants (40mm x 2.5mm o.d) on day 2 of behavioural oestrus. Control females (n = 4) were given a saline injection (1mL) on day 2 of oestrus, as empty implants were not available for administration. The oestrous cycle was monitored by daily signs of oestrous behaviour, urogenital cytology every 2 – 3 days and blood samples for hormone analysis every 2 - 3 days. The progestogen implants were removed after a 6 wk period and females were closely monitored for signs of oestrous behaviour to establish whether the timing of oestrus return could be reliably predicted or was synchronous.
Implant insertion and removal

All treated females received a single implant containing 70 mg LNG in a silastic tube (40mm x 2.5mm o.d; Norplant; Leiras). As these implants are not manufactured currently they were provided as a gift from Prof Marilyn Renfree, Department of Zoology, The University of Melbourne, Australia. This implant releases 30 mg LNG per day for a period of 12 months (Sivin, 1988). Implants were inserted subcutaneously between the shoulder blades guided by an implant needle (60 mm x 5 mm o.d; SX10, part # 509873; Fort Dodge Laboratories INC, Fort Dodge, IOWA, USA) inserted through a 3 - 5mm skin incision. A single treatment female received the implant under local anaesthetic (1 mL lignocaine hydrochloride 20 mg mL\(^{-1}\), injected subcutaneously between the shoulder blades), but due to difficulty in implant placement, the remaining 3 treated females were implanted whilst under general gaseous isoflurane anaesthesia (McGowan et al., 1995). Before and after implant insertion, the site was cleaned with citridine (Mavlab, Australia) in ethanol, and the wound closed with a single mattress suture. The site of implantation was checked visually and by gentle palpation at the time of sample collection throughout the study. Implants were removed under general anaesthesia through a small incision (10 mm) transverse to the implant using forceps; the site was cleaned with citridine in ethanol, and the wound closed with a single mattress suture.

Hormone analyses

The concentration of oestradiol-17\(\beta\) in the peripheral blood of koalas was assayed directly from plasma using commercially available ultra-sensitive estradiol RIA kits (Immunotech, Prague, Czech Republic). The assay sensitivity was 1.0 pg mL\(^{-1}\), and the intra-assay and inter-assay coefficient of variations were 7.5% and 7.6% respectively. All samples were assayed in duplicate. The reported oestradiol antibody cross-reactivity was oestrone (2.4%), D-Equilenin (3.4%), 17-\(\beta\)-oestradiol-3-glucuronide (2.6%) and <1% for all other steroids tested compared to 17\(\beta\) oestradiol at 100% (Immunotech, Prague, Czech Republic). Plasma samples were also assayed for LH using a
heterologous RIA as described by Curlewis (1991), but with a mouse monoclonal antiserum LH518B7 and ovine LH as standard and iodinated radioligand. Previous studies with this assay (Johnston et al., 2004) have demonstrated that koala pituitary extract and koala plasma diluted in parallel with ovine standards for this assay. The intra-assay coefficient of variation was 9.1%. The lowest detection limit of the assay was 0.4 ng mL\(^{-1}\). All samples were assayed in duplicate.

**Statistical analysis**

A repeated measure mixed model was used to examine the effect of treatment and time on plasma LH and oestradiol-17\(\beta\) concentrations. The homogeneity of variance within subjects was examined using a Levene test (Minitab Release 16). Given time was not a significant factor and variance within subjects was not equal, a weighted general linear model with subject means was used to examine hormone levels between control and treated animals. Oestradiol-17\(\beta\) data was log transformed before analysis. Measurements are reported as mean ± SEM.

**RESULTS**

Oestrous behaviour was observed in all koalas 1 day prior to either LNG or saline treatment and behavioural observations were confirmed by cornified epithelial cells in urogenital cytology. In LNG treated koalas, oestrous behaviour immediately ceased the day after LNG implantation and no further signs of oestrous behaviour or presence of cornified epithelial cells in urogenital cytology were observed throughout the 45 day implantation period. After implant removal, LNG treated koalas returned to behavioural oestrus 13, 14, 17 and 30 days after LNG implant removal. In contrast control animals continued to demonstrate oestrous behaviour following saline injection for 7.3 ± 2.3 days, and all animals exhibited oestrous behaviour in the next oestrous cycle on day 33 to 40 (35.7 ± 1.5) after treatment. Oestrous behavioural observations in control and LNG treated
animals coincided with the presence of cornified epithelial cells in smears of their urogenital cytology.

Figure 6.1: Individual animal profiles of plasma oestradiol (closed diamonds) and Luteinizing Hormone (open circles) concentrations, oestrous behavioural observations (black bars), and positive oestrous urogenital smears (grey bars) in control treated (left panels) and LNG implanted (right panels) females. Period of LNG implantation is indicated by grey vertical dashed lines.
Plasma oestradiol-17β concentrations in LNG treated koalas declined sharply after implantation and remained close to or below the detection limit of the assay (1 pg/mL) during the implantation period (Figure 6.1). In contrast, plasma oestradiol-17β concentrations in control females declined steadily and showed evidence of cyclic activity increasing after day 30 in association with the return to oestrus in the next cycle as indicated by both oestrous behaviour observations and urogenital cytology. Due to the high variability in plasma oestradiol-17β concentrations between koalas, data are presented as individual profiles (Figure 6.1). Overall mean oestradiol-17β concentrations in LNG treated females were lower and less variable throughout the implantation period [4.0 ± 0.6 pg mL⁻¹ (n = 4); individual means 1.6 ± 0.2; 1.0 ± 0.0; 1.7 ± 0.8; 10.0 ± 0.6 pg mL⁻¹] than control koalas [6.7 ± 0.9 pg ml⁻¹ (n = 4); individual means 3.1 ± 0.6; 2.7 ± 0.4; 19.4 ± 1.8; 1.5 ± 0.5 pg mL⁻¹]. For the majority (3 out of 4) of LNG treated animals, oestradiol-17β concentrations in most samples were close to the assay sensitivity (1.0 pg mL⁻¹). Plasma LH concentrations varied throughout the study period with no significant effect of time (P = 0.49) or treatment group (P = 0.13).

DISCUSSION

The results of this study suggest that treatment with the exogenous progestogen LNG suppresses oestrous cyclicity in the female koala, as evidenced by the absence of oestrous behaviour, low plasma oestradiol-17β concentrations and lack of cornified epithelial cells in urogenital cytology over the 45 d implantation period. In contrast, control koalas demonstrated normal cyclic activity as evident by increased oestradiol-17β concentrations leading to oestrous behaviour about 35 days later; within the range of a typical anovulatory oestrous cycle in the koala (Johnston et al., 2000b).
In eutherian species, follicular oestrogen production and maintenance of the Graafian follicle is dependant upon gonadotrophin (FSH and LH) secretion (Hsueh et al., 1984). Pituitary support has also been shown to be essential for the later stages of follicular growth including support of the Graafian follicle in the tammar wallaby (Hinds et al., 1996). In the current study, oestradiol-17β levels declined to basal levels soon after LNG administration and remained low in all animals throughout the implantation period. These observations suggest that LNG suppressed oestradiol secretion from the existing dominant Graafian follicle (at oestrus) and also subsequently prevented development of the dominant follicle of the next cycle. These results are consistent with progestogen studies in eutherian species, but somewhat contrary to what has been observed in the tammar wallaby (Hynes et al., 2007).

Plasma oestradiol-17β secretion in the koala has been reported to remain low during the luteal phase of the mated non-pregnant cycle (Johnston et al., 2000b), but follicular activity in the ovary during this period remains to be fully elucidated. While Johnston et al. (2000b) do report a peak in oestradiol-17β during the pregnant luteal phase of the koala, follicular development in both pregnant and non-pregnant luteal phases does not occur to the point of sufficient oestradiol production to induce behavioural oestrus or increased cornification of the lower lateral vaginal mucosa. Such observations, appear to be consistent with the present results reported in this study with administration of exogenous progestogen, and suggest the possible existence of some form of progestogen negative feedback on the hypothalamic-pituitary axis in the koala; particularly in the non-pregnant luteal phase.

Whilst progesterone implants blocked ovulation in the brushtail possum (Crawford et al., 2011), it was not determined whether follicular development and/or oestradiol secretion was also suppressed. Whilst here we have not determined if LNG treatment blocked ovulation in the koala, it certainly blocked oestrous behaviour in this induced ovulatory species. Interestingly, our results in
koalas and that of Crawford et al. (2011) in the possum, appear to be in contrast to those previously reported by Hynes et al. (2007) for the tammar wallaby, where by follicular activity continued unabated during progestogen treatment. This difference between species is not surprising given follicular growth in the tammar wallaby occurs during a period of elevated progesterone in the final third of pregnancy (Tyndale-Biscoe and Renfree, 1987). Consequently, it is likely that progestogen treatment prevents pregnancy in the tammar wallaby, through the suppression of the LH surge, thereby inhibiting ovulation (Hynes et al., 2007) but in the koala, LNG appears to inhibit mating success by the suppression of adequate secretion of oestradiol from the dominant follicle, preventing behavioural oestrus and consequently coitally or semen induced ovulation (Johnston et al., 2004).

The pattern of LH concentrations during the normal oestrous cycle and during LNG administration in the current study in general were not informative about potential progestogen negative feedback in the koala. Certainly, it should be noted that LNG does not suppress plasma LH concentrations. However, due to the pulsatile nature of LH, any interpretation of the data based on single blood samples needs to be made with some caution. It remains a possibility that FSH secretion from the anterior pituitary is more sensitive to progestogen feedback than LH, but this question can only be answered convincingly with the development of a species-specific FSH assay. Busby et al. (2014) have recently described the amino acid sequence of koala FSH and the FSH receptor so that development of homologous FSH assay for koala is possible.

During LNG implantation we failed to observe cornified epithelial cells in urogenital cytology of any of our captive animals. This result contrasts with that previously reported by Hynes et al. (2010), who found no difference in the prevalence of oestrous smears in either cycling and LNG treated animals in a wild koala population. Due to the field nature of the Hynes et al. (2010) study, cytology samples where only collected every 4-6 weeks; so it is possible that infrequent
sampling was not able to provide an accurate reflection of oestrous cyclicity. In the current study of captive koalas, oestrous cycle characterisation was based on daily testing for oestrus, together with serial blood sampling for oestradiol-17β and urogenital cytology every 2 to 4 days. The sudden cessation in oestrous behaviour in LNG treated females is similar to that previously reported by Smith (1980) in naturally mated koalas, where by oestrus ceased the day after a successful copulation; a finding later confirmed by (Johnston et al., 2000b). Johnston et al. (2000b) also noted the rapid cessation of oestrous behaviour was coincident with a decrease in oestradiol-17β concentration and an increase in progesterone concentration.

The mechanisms responsible for induction and / or maintenance of oestrous behaviour in the koala is not known. For many eutherian species it is the withdrawal of progesterone (Hinds et al., 1996) via a phenomenon known as progesterone priming (Scaramuzzi et al., 1972, Jabbour et al., 1992). However, the koala, as an induced ovulator has low progesterone concentrations throughout a non-mated cycle, but even so the female expresses oestrus some 30 days later (Johnston et al., 2000b). Apparently, high oestradiol-17β concentrations are not required for the continuation of oestrous behaviour in the koala, as plasma oestradiol-17β concentrations typically decline before the cessation of oestrus (Johnston et al., 2000b). In the absence of mating, females continue to display oestrous behaviour up to 10 days on average. A similar result was seen in our control animals in the current study. However after LNG implantation, we observed a sudden cessation of oestrous behaviour in all animals, which is similar to that seen after copulation in an ovulatory cycle (Johnston et al., 2000b). Together these results appear to suggest that progesterone in the female koala plays a pivotal role in the termination of oestrous behaviour.

In the current study, three of the four LNG treated koalas returned to oestrus 13, 14 and 17 d after implant removal. Although 3 of the four koalas returned within 4 d of each other, suggesting somewhat successful oestrous synchronization, the fourth female did not show oestrus until 30 days
after implant removal. Although 30 days is considerably longer, this koala previously had been recorded to exhibit an above average anovulatory cycle length upwards of 42 days, compared to both the usual 33 day cycle reported by Johnston et al. (2000b), and 35 day cycle lengths observed in the 4 control females in the current study. Furthermore, the LNG implant in this particular koala was removed towards the end of the breeding season (March), when oestrous activity has been shown to be more variable (Blanshard, 1994).

In the brushtail possum, ovulation of 18/40 animals was achieved between 8-14 days after the removal of progesterone implants inserted for 2 weeks (Crawford et al., 2011). Although it is difficult to draw comparisons between the results of the current study (small sample size) and those obtained by Crawford et al. (2011), a tighter level of synchrony appears to have been achieved in the koala. In the current study the time to return to oestrus after LNG treatment for most treated animals was similar to that in a naturally mated non-pregnant ovulatory cycle of approximately 16 days (Johnston et al., 2000b). In koalas displaying oestrous cycle activity, the time difference between the normal decline in progesterone and subsequent increase in oestradiol-17β levels coinciding with the return to oestrous behaviour is between 12-19 d, (n = 6) (Johnston, 1999). Given female koalas express oestrous behaviour for an average of 10 days, and have previously been successfully mated up to day 10 of oestrus and produced PY (Allen et al., 2008), a range of 4 days in females entering oestrus after LNG treatment would appear to be acceptable in terms of successful oestrous synchronization. In the current study, LNG implants appear to have successfully inhibited ovarian activity during peak oestradiol-17β secretion. While further validation on larger number of animals is required, it does appear that LNG implants should be capable of suppressing ovarian activity when inserted at any stage of the cycle, although the effect of LNG on luteolysis of the endogenous CL needs to be investigated. LNG implants will therefore provide a flexible and relatively non-labour intensive method of oestrous synchronization that is applicable in both captive and potentially wild koala populations. Implant removal can be performed during any stage of the
cycle (provided the administration period exceeds 30 days) allowing for multiple females to be synchronized.

In conclusion, the results from this study have provided insight into the mechanism by which LNG implants can prevent pregnancy in the female koala (Hynes et al., 2010) and by doing so, we have cautiously inferred a potential role of progesterone in the control of oestrous cyclicity in this species. Whilst follicular activity during the luteal phase or LNG treatment has not been assessed, it appears progesterone, mostly likely secreted from the koala CL, regulates cyclic activity via a similar mechanism to that previously observed in eutherian species. Further studies are required to fully investigate the potential negative feedback of progesterone in the koala oestrous cycle.

Results from the current study also provide promising evidence for the successful use of progesterone as a possible oestrous synchronization tool. A future study involving the timed administration and removal of LNG implants from koalas during different stages of their anovulatory cycle would allow the effectiveness of LNG to be fully assessed. Given the results of this study, it may also now be prudent to investigate the mechanism of luteolysis of the koala CL and the potential use of prostaglandin F2alpha as an additional protocol for controlling oestrous cycle activity (Hinds et al. 1990). The degree of control that progestogen exerted on the koala oestrous cycle was completely unexpected and is a timely reminder that that marsupial reproductive physiology is quite diverse, so that studies on individual species are important.
REFERENCES


CHAPTER 7: GENERAL CONCLUSIONS

The overarching objective of this thesis was to investigate the mechanisms responsible for the timing of reproductive activity and oestrus in female koalas in order to improve and refine the current artificial insemination protocol. The primary aims of the thesis were to (1) examine non-invasive methods for accurately monitoring the female koala oestrous cycle (Chapter 2), (2) investigate the underlying factors controlling the timing of reproductive activity in the koala such as lactation (Chapter 3) and season (Chapter 4), (3) the preliminary development of an oestrous synchronization protocol based on gaining control of anterior pituitary function (Chapter 5) and (4) to examine potential methods to manipulate the oestrous cycle using progesterone implants (Chapter 6).

NON-INVASIVE MONITORING OF THE KOALA OESTROUS CYCLE

The first research question addressed in this thesis was whether faecal steroid metabolites combined with oestrous behaviour monitoring could be used to accurately monitor the female oestrous cycle for research purposes. In an attempt to answer this question, 8 female koalas were monitored for oestrous behaviour and plasma and faecal samples were collected in unison over a 6 week period or until females returned to oestrus. Total faecal oestrogens were aligned with plasma oestradiol-17β concentrations to determine if faecal oestrogens gave an accurate indication of plasma oestradiol levels. Whilst individual mean faecal oestrogens were linked to mean plasma oestradiol-17β concentrations, a significant correlation between plasma oestradiol-17β and faecal oestrogens was not found despite adjusting for a time lag effect. The most likely explanation is a combination of faecal pellet urine contamination and a differential digestion rate in digesta particle size (99h - 213h) in the koala (Cork and Warner, 1983). Highly fibrous faecal samples pass through
the gut at a faster rate and thus represent a pooling of plasma oestradiol-17β over a shorter period. Similarly, the passage of metabolised steroid hormone in the urine is typically faster (no lag effect), thus resulting in an inconsistent pooling of oestradiol-17β levels subject to possible urine contamination and fibre content. Mean faecal oestrogens, did however, show a strong relationship to mean plasma oestradiol-17β concentrations providing an indication of cyclic activity and may, therefore, be of use in longitudinal contraception studies. For the purposes of this thesis, faecal oestrogen metabolite analysis did not provide an accurate indication of plasma oestradiol-17β secretion to allow for the identification of oestrus and was therefore abandoned as a technique for monitoring the koala oestrous cycle in the subsequent experiments of the thesis.

FACTORS CONTROLLING REPRODUCTIVE ACTIVITY IN THE FEMALE KOALA

Lactational anoestrus

The second research question of this thesis focussed on the underlying environmental factors controlling the timing of reproductive activity in the koala. A specific focus was the importance of lactational anoestrus and its association with Prl secretion. Chapter 2 examined the pattern of Prl secretion and its relationship to oestrous behaviour and PY development. Single injections of mGnRH were also administered during this time to test the capacity of the anterior pituitary to respond to mGnRH during lactation. Despite being ‘teased’ on a daily basis, oestrous behaviour was suppressed throughout the majority of lactation until Day 269, some 102 days before PY were weaned following achieving target weights of 2.5–2.7 kg. Basal levels of Prl during early lactation suggested a separate mechanism, most likely the suckling stimulus, was responsible for the suppression of oestrous activity during this time. Furthermore, the koala anterior pituitary remained responsive to injections of mGnRH during periods of high and low Prl secretion, indicating
gonadotrophs remain responsive throughout lactation. The suckling stimulus is the most probable mechanism responsible for the suppression of ovarian activity, whether this is through the inhibition, or disruption of GnRH/LH pulse activity, will require further investigation. These results demonstrated the strength of lactational anoestrus in the koala, excluding the possibility of early mating whilst PY remained in close contact with their mother. While pouch young removal and cross fostering have previously been utilized as methods of oestrous synchronization in marsupial species (Paris et al., 2005), such techniques are not applicable when dealing with an iconic species such as the koala. The results from this study raised the question as to what extent is the apparent seasonality in female koalas influenced by the timing of breeding and consequently lactation.

Seasonality

Chapter 4 investigated the extent of seasonal influence on the timing of reproductive behaviour in the female koala in a total of 33 sexually mature koalas housed at Dreamworld over a 4-year period (2009-2012). Non-lactating and unmated koalas were monitored daily for the expression of oestrous behaviour. Data on the occurrence of oestrous behaviour throughout each year was then correlated with temperature, rainfall and photoperiod patterns. Although some individual koalas in the population showed signs of oestrous behaviour throughout the year, an obvious seasonality was apparent with significantly less females displaying oestrous behaviour in late autumn and winter (May - August), than September to April (P < 0.0001). The distinct nadir in oestrus expression (< 30%) was consequently associated with a decrease in day length and temperature. Such a strong seasonal effect was somewhat surprising given that captive koalas are reported to be capable of breeding all year round (Blanshard, 1994) and limiting factors such as lactational anoestrus, food availability and shelter were controlled for in this study. The results of chapter 4 clearly showed a strong seasonal influence on female koala reproductive behaviour in
southeast Queensland. Hence, the timing in which further reproductive research or oestrous synchronization techniques are applied needs greater consideration in terms of breeding season. While the relative importance of these proximate factors to wild koala populations requires further investigation, it may be possible in captive animals, to manipulate these factors with respect to their influence over reproductive physiology and behaviour in order to improve reproductive success and productivity in a similar way to that achieved in domestic species (e.g. sheep and horse).

POTENTIAL METHODS TO MANIPULATE THE FEMALE KOALAS OESTROUS CYCLE AND IMPROVE THE TIMING OF AI

The final research question of this thesis was to develop techniques that would improve the timing of AI through the manipulation of the female koala oestrous cycle. This subject was originally addressed through the examination of the role of prolactin in preventing oestrous cycle activity during lactation in chapter 3. The results of chapter 3 indicated that prolactin secretion was not the primary mechanism inhibiting oestrous expression, at least in the first half of lactation. Due to the timing in the return to oestrus after PY weaning it seems highly likely that the suckling stimulus acts via a neural pathway to suppress oestrous behaviour throughout lactation. Subsequent studies in this thesis focused on the manipulation of hormonal pathways in order to gain control of the koala oestrous cycle.

GnRH antagonists

Given that previous research on the progesterone secretion from the marsupial CL had little inhibitory effects on follicular activity (Shaw, 2006) and studies on the synthetic progesterone LNG in the tammar wallaby did not suppress follicular activity, research in this thesis was first directed to
gaining control of the AP. Preliminary studies on the GnRH antagonist acycline in male koalas appeared to suppress a LH surge via the competitive blockage of GnRH receptors (Allen et al., 2008), thus suggesting that a similar mechanism could also be adopted in the female koala to manipulate FSH and LH secretion and ultimately follicular activity. Chapter 5 examined the potential application of the GnRH antagonist azaline B in an oestrous synchronization protocol for koalas. In experiment 1, single injections of azaline B successfully blocked AP function in a dose dependant manner; (0 mg; 0h, 1 mg; 24h, 3.3 mg; 1 - 4 d and 10 mg; 7 d). Results from the 1 mg dose gave the greatest consistency and its application to an oestrous synchronization protocol was determined to provide the greatest level of control and flexibility. Experiment 2 aimed to ascertain the ability of a daily dose of azaline B to continuously block endogenous GnRH binding (as measured by GnRH stimulation test) over a 10 day period. LH response was continually suppressed over this period and did not recover for a further four days after the cessation of treatment. Given the relatively rapid recovery of the LH response following 1 mg administration, the prolonged suppression after the 10 day period was surprising. This result suggests multiple low doses impose a similar response to that of a high dose (10 mg) and may cause down-regulation of GnRH receptors. Additional research is required to investigate the exact pathway or combination of mechanisms responsible for the sustained suppression of pituitary response. Experiment 3 was designed to test the efficacy of a daily dose of azaline B given during the interoestrus period to reset folliculogenesis once its administration had been withdrawn, and ultimately synchronize the return to oestrus in female koalas. While treatment with azaline B did not suppress basal LH or oestradiol-17β, oestrus was delayed in all treated females 24.2 days, but with high variability (range 9 - 39d). These results suggest that basal gonadotrophin secretion continues, despite azaline B treatment; however, secretion of a high enough oestradiol-17β concentration to induce oestrous behaviour was inhibited. Although FSH and follicular development was not directly measured during this study, the delay in the return to oestrus clearly indicates a disruption to follicular activity and a delay in the growth of a dominate follicle capable of secreting sufficient oestradiol to induce oestrous
behaviour. If GnRH receptors are not down-regulated during azaline B treatment, then perhaps the addition of a GnRH agonist would help promote synchronous folliculogenesis and improve the timing of oestrus as it has been used in domestic species (Twagiramungu et al., 1995). Based on previous GnRH antagonist studies in heifers (Haughian et al., 2013), it seems likely that azaline B disrupts folliculogenesis in the koala through the disruption of episodic LH secretion imposed by the suppression of GnRH secretion via competitive blockage of GnRH-R. The large variability in the time period taken to return to oestrus in the koala, suggested that while azaline B administration was able to disrupt folliculogenesis and expression of oestrus, it was unable to obtain sufficient influence over gonadotrophin secretion to control the timing of the development of the dominant follicle. It was concluded that further studies on follicular development, FSH and episodic LH secretion during azaline B treatment were required before further oestrous synchronization trials using this strategy are performed.

**Synthetic progestogens**

The final research experiment of this thesis aimed to clarify the current dogma on the inability of synthetic progestogens to inhibit follicular activity in marsupials. Previous contraception studies using LNG implants conducted by Hynes et al. (2010) in the koala had previously reported no difference between urogenital cytology slides between LNG treated and control wild koalas. This preliminary evidence suggested follicular activity continued unabated during LNG treatment and thus LNG implants would be unsuitable for the purposes of oestrous synchronization. Surprisingly, results from this thesis showed LNG treated koalas, immediately ceased displaying oestrous behaviour after implantation, had reduced oestradiol-17β concentrations and showed no evidence of urogenital cytology typical of an oestrus smear. In contrast, plasma oestradiol-17β levels in control koalas showed evidence of cyclic activity associated with periods of behavioural oestrus and coincident increases in cornified epithelial cells in the urogenital smear at
day 33 to 35 after saline injection. LNG treated koalas also showed a subsequent oestrus 13, 14, 17 and 30 days after LNG implant removal. Whilst follicular activity during the luteal phase or LNG treatment has not been assessed, results from this thesis suggest LNG implants inhibit oestrous behaviour and elevated secretion of oestradiol-17β possibly through preventing sufficient development of a pre-ovulatory follicle. It is possible that progesterone, mostly likely secreted from the koala CL, regulates cyclic activity via a similar mechanism to that previously observed in eutherian species. While further studies are required to fully investigate the potential negative feedback of progesterone in the koala oestrous cycle, results from the current study have nevertheless provided promising evidence for the potential use of progesterone as a possible oestrous synchronization tool. A future study involving the timed administration and removal of LNG implants from koalas during different stages of their anovulatory cycle would allow the effectiveness of LNG as an oestrous synchronization tool to be fully assessed. Given the results of this study, it may also now be prudent to also investigate the mechanism of luteolysis of the koala CL and the potential use of prostaglandin f2α as an additional protocol for controlling oestrous cycle activity.

SUMMARY OF NEW AND SIGNIFICANT FINDINGS

This thesis has produced important novel insights into female koala reproductive biology and the mechanisms controlling the timing of reproductive activity, including:

1. The utility of faecal steroid metabolite analysis to provide an indication of plasma oestradiol-17β concentrations and the presence of cyclic activity.

2. Preliminary evidence for the importance of the suckling stimulus, not prolactin secretion, in the suppression of koala oestrous behaviour during early lactation.
3. Continued LH surge capacity of the anterior pituitary surge centre during lactation.

4. Evidence for a clear seasonality in captive female koala reproductive activity that was closely linked to annual patterns in temperature and photoperiod.

5. The ability to block GnRH receptors through competitive binding with the GnRH antagonist azaline B and subsequent inhibition of a LH surge in response to injections of exogenous mGnRH.

6. Continued occupancy of GnRH receptors with repeated daily doses of azaline B.

7. The ability to temporarily disrupt the koala follicular cycle using repeated doses of azaline B.


9. The ability to temporarily suppress oestrous cycle activity with the use of synthetic progestogen implants (LNG).

10. Preliminary evidence for the use of synthetic progestogen as a mechanism for oestrous synchronization in the koala.

CONCLUSIONS

Assisted breeding technology is becoming an increasingly valuable tool for captive koala breeding and genetic management (Johnson and Holt, 2014) and has recently been recognized by the Queensland government in their koala planning and conservation documents. Although it is clear that it is still not a panacea for the loss of individual animals, as a result of human based activity, reproductive technology can play an important role in supporting genetic viability in
captive populations in anticipation for ultimate re-introduction into the wild. Advances in this technology are reliant upon a thorough comprehension of reproductive biology, an understanding that has been limited in marsupials by the fact that a large proportion of the published literature is based on the tammar wallaby. This thesis has provided convincing evidence that marsupial reproductive physiology, and in this the case, the koala, is indeed a diverse entity and should be considered on a species to species basis.

This thesis has built on the previous achievements of the most successful AI program in a marsupial to date (Johnston and Holt, 2014). Whilst a conclusive and reliable oestrous synchronization protocol was not achieved, this thesis has provided significant insight into the mechanisms controlling reproductive activity in the koala, which will form a solid basis for future research. Results of this thesis have demonstrated the ability of GnRH antagonists to temporarily disrupt follicular activity and the potential use of LNG implants to synchronize oestrus in the koala. Future research on follicular development, FSH and episodic LH secretion during azaline B treatment would aid in the identification of the exact mechanisms through which this antagonist disrupts the oestrous cycle, providing the necessary information to gain adequate control of the timing of oestrus in the koala. A further study on LNG implants in a larger sample size will likely confirm preliminary results from the current thesis and result in the successful development of an oestrous synchronization protocol. Until sperm cryopreservation is advanced in the koala, oestrous synchronization will prove vital to the genetic exchange and management between both captive and isolated wild populations and form a major component to the success and development of a ‘live’ genome resource bank (Johnston and Holt, 2014). The next step will be the application of an oestrous synchronization protocol to facilitate the insemination of several females with chilled spermatozoa collected and transported from either a wild or captive population.
REFERENCES


