The use of urine as a tool to analyse the reproductive function of captive female southern hairy-nosed wombats (*Lasiorhinus latifrons*)

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Abstract

Southern hairy-nosed wombats (SHNW - *Lasiorhinus latifrons*) do not breed well in captivity. To overcome this, a better understanding of their reproductive physiology is essential. While faecal hormone analysis has provided useful information regarding progesterone excretion in the SHNW, biologically relevant changes in oestrogens have proven difficult to detect, and protein hormones such as luteinizing hormone (LH) are not readily excreted in faeces. The analysis of urinary hormone metabolites is a valuable tool for assessing reproductive function in other wildlife species and has been linked to changes in reproductive behaviour, which can then be used as an effective non-invasive method of detecting oestrus and oestrous cycle characterisation for captive breeding programs.

The overall aims of this research program were to: (1) determine if female captive SHNW could be conditioned for the reliable and frequent urine sample collection for this study, (2) develop and validate techniques to detect biologically relevant changes in urinary LH, oestrogens and progesterone metabolite concentrations in captive female SHNWs, (3) characterise the oestrous cycle of female SHNWs, (4) determine if changes in reproductive hormones could be mapped to changes in behaviours and urine sample characteristics, and (5) determine if male SHNWs could differentiate between oestrus and non-oestrus female urine. The overall objective of this research program was to increase our understanding of the female SHNW reproductive physiology and identify reliable, or improve current, methods of detecting oestrus to enhance captive breeding success of this species.

Captive female SHNWs (*n* = 11) were classically conditioned to provide daily urine samples during the 2013 and 2014 breeding seasons. Initially, the animal was conditioned to a tactile stimulus (light tapping of the percloacal region) and the presence of the collection tray under the rump during normal urination behaviour. Following three months of conditioning, collection success was 82%; however, some collection protocols were modified for timid or aggressive females, such as the use of a false floor, and aspiration of urine off a clean enclosure floor, to minimise stress associated with daily sample collection.

Fresh urine samples were analysed for physical characteristics, including volume, specific gravity, pH, leukocytes and qualitative index of the number of urogenital epithelial cells.
Samples were then stored at -20 °C for subsequent analysis of urinary creatinine, LH, oestradiol-17β (E2), estrone-3-glucuronide (E1C) and progesterone metabolites (P4M). Urinary reproductive hormone analysis techniques were validated using two exogenous hormone trials: (1) an injection of a high dose (4 and 10 µg) of exogenous gonadotrophin releasing hormone (GnRH) to induce an LH surge that could be detected in urine; and (2) two sequential and increasing injections of equine chorionic gonadotrophin (150 and 300 IU) to stimulate the secretion of urinary E2, E1C and P4M..

While serum and urinary LH increased significantly after the GnRH challenge, the collection of single daily urine samples was not sufficient for the detection of a naturally occurring pre-ovulatory LH surge. Similarly, biologically relevant changes in E1C concentrations were only detected after exogenous hormone injections and could not be used to accurately identify the different stages of naturally occurring oestrous cycles. While the analysis of urinary E2 resulted in higher overall oestrogen concentrations compared to E1C, E2 profiles were similar to E1C patterns, in that the different stages of the oestrous cycle were not reliably distinguishable. Further attempts to extract oestrogen metabolites from urine samples by enzyme hydrolysis or diethyl ether extraction also failed to improve the detection of distinct increases in oestrogen associated with oestrus. In contrast, the analysis for urinary P4M and changes in P4M concentrations could be used to identify the approximate onset and duration of both hormonally-induced and natural luteal phases.

The mean oestrous cycle length (the beginning of one luteal phase to the beginning of the next) was 35.1 ± 2.4 days, with a mean luteal phase of 20.8 ± 1.3 days. There was a high level of between and within animal variation in oestrous cycle dynamics as cycles ranged from 23 to 47 days (luteal phases ranged from 12 to 33 days) with some females exhibiting both long and short oestrous cycles within a single breeding season.

Changes in the physical characteristics of each urine sample were compared to changes in urinary P4M concentrations. When urinary P4M was ≤ baseline values, urine volume decreased, whereas urine concentration (as evaluated by creatinine and specific gravity) and urogenital epithelial cells increased significantly. To determine if changes in urinary P4M could be mapped to changes in behaviour, the frequency and duration of 26 general, defensive and reproductive
behaviours were analysed for seven females for eight days surrounding the onset of the luteal phase; three days when urinary P4M was ≤ baseline values (D-3, D-2, D-1), three days when urinary P4M was > baseline values [D0 (classified as the first day where urinary P4M was above baseline for 3 consecutive days), D+1, D+2)] and two days which served as behaviour control days (D-14 and D+14) when reproductive behaviours were least likely to occur. Behavioural analysis showed that the duration of urination decreased significantly when P4M was ≤ baseline values; however, there was no difference in the frequency or duration of feeding. Five of the seven females analysed exhibited pacing behaviour which increased significantly when P4M was ≤ baseline values. Rump biting (defensive behaviour) by the female also increased when urinary P4M was ≤ baseline values. Male-initiated urogenital sniffing was low when urinary P4M was ≤ baseline values, and the lack of difference in olfactory behaviour of males exposed to oestrus and non-oestrus urine samples confirmed that males did not preferentially investigate oestrus samples and therefore were unlikely to be able identify receptive females by evaluating urine alone.

The results from this research demonstrate that the collection and analysis of urine can be a valuable non-invasive tool for the reproductive assessment of captive female SHNW. The analysis of urinary P4M was effective for estimating the reproductive cycle dynamics of captive females; however, more research is required to refine techniques for the accurate assessment of biologically relevant changes in urinary LH and oestrogens. The collection of fresh urine samples from individual females and the analysis of urinary characteristics enabled the identification of markers which could be linked to changes in urination behaviour and locomotion, potentially useful non-invasive tools for monitoring the reproduction of this species and identify receptive females to optimise future breeding attempts.
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 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, financial support 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 higher degree by research 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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Conference abstracts


Publications included in this thesis


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Research Involving Humans or Animal Subjects

This research was conducted using a captive research population at the Australian Animals Care and Education wombat research facility. All research was approved by the University of Queensland Animal Ethics Committee.

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ANZSRC code: 030104, Immunological and Bioasssay Methods 30%
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Fields of Research (FoR) classification

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Dedications

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Glossary of Abbreviations

ABTS 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium
ACTH Adrenocorticotropic hormone
AMS Alyce Maree Swinbourne (Primary researcher)
AI Artificial insemination
ARTs Assisted reproductive technologies
ANZSRC Australian and New Zealand Standard Research Classifications
AACE Australian Animals Care and Education
BL Baseline
BFRW Black-footed rock wallaby
CW Common wombat
CL Corpus luteum
CL425 Progesterone antibody
Cr Creatinine
D0 Days, 0 days from X
DE Diethyl ether
DVR Digital video recorder
EHB Enzyme hydrolysis buffer
EIA Enzyme-immunoassay
eCG Equine chorionic hormone
E1C Estrone-3-glucuronide
F Female
FoR Field of Research
FSH Follicle stimulating hormone
FP Follicular phase
GAMG Goat anti-mouse gamma globulin
GARG Goat anti-rat gamma globulin
GnRH Gonadotrophin releasing hormone
GnRHa Gonadotrophin releasing hormone agonist
HRP Horseradish peroxidase
h Hour
HCG Human chorionic hormone
ID Identification
Inc Incorporated
IR Infrared
IUCN International Union for Conservation of Nature
IU International units
kg Kilograms
kms Kilometres
LP Luteal phase
LH Luteinizing hormone
518-B7 Luteinizing hormone antibody
µg Micrograms
1. Literature review

1.1. Lasiorhinus conservation

The family Vombatidae contains the largest burrowing mammals in Australia. There are two genera; the common or bare-nosed wombat (CW) (*Vombatus ursinus*), and two hairy-nosed species (*Lasiorhinus* spp.), the southern hairy-nosed wombat (SHNW) (*L. latifrons*) and the critically endangered northern hairy-nosed wombat (NHNW) (*L. krefftii*) (Taggart and Temple-Smith, 2008; Department of Environment and Heritage Protection, 2015; Woinarski and Burbidge, 2016). Given the critically low number of NHNW and the lack of a captive population, intensive manipulative research is currently not an option without the risk of potentially compromising their welfare. While the CW has previously been used as a model for NHNW research (Paris *et al.*, 2002; MacCallum and Johnston, 2005), the SHNW has similar morphological characteristics (Triggs, 2009), and like the NHNW, it is a distinct seasonal breeder (Murray, 1998). Additionally, the genetic divergence value between the two *Lasiorhinus* spp is 3.5% (Taylor *et al.*, 1994), such a low divergence value has been found among species that can hybridise (Cronin *et al.*, 1988; Lehman *et al.*, 1991). This means that compared to the CW, the SHNW makes a more appropriate model, or living reference, for NHNW research and reproduction. As such, the main focus of this review will be on the two *Lasiorhinus* spp with brief mentions to the CW.

1.1.1. Northern hairy-nosed wombat (*L. krefftii*)

Although local farmers reported wombats in the area from the 1880s (Crossman, 1988), confirmation of the NHNW in central Queensland did not occur until 1937 (Morning Bulletin, 1937). While it is suggested that the Queensland population was on the decline prior to European settlement, further contraction since then has been attributed to grazing competition from livestock, especially during drought periods (Crossman, 1988), and the introduction of rabbits (*Oryctolagus cuniculus*) during the 1940s (Stodart and Parer, 1988). As a result, the population declined to as little as 30 reported individuals during the latter half of the 20th century (Figure 1-1.) (Department of Environment and Heritage Protection, 2015).
The NHNW is listed as critically endangered on the IUCN Red List (Taggart et al., 2016a). Currently, there are no known free-ranging NHNW left in the wild, and the remaining individuals are restricted to two isolated populations in central and southern Queensland (Figure 1-2) both of which are contained within protected / fenced off areas. Today, the main population, consisting of approximately 200 individuals (Department of Environment and Heritage Protection, 2015), is located at Epping Forest National Park (NP) in central Queensland. Their range within the protected area is approximately 5 km², a sixth of the size of the park, and consists of open woodland with grassy understory.

As the single population is vulnerable to environmental catastrophes such as drought, flood and wild fires, a recovery plan has been established with the aims of (1) maximising the reproductive output of the remaining individuals, and (2) allowing new populations to become established (Horsup, 2004). The first part of the recovery plan involves in situ conservation, which included the protection and restoration of their natural habitat, as well as the establishment of a second translocated population. At Epping Forest NP, conservation methods such as the exclusion of cattle and wild dogs using predator-proof fencing, the manipulation of pasture through controlled wild fires, and methods to prevent the outbreak of diseases and parasites, have been employed (Horsup, 2011). Further conservation methods include the provision of
supplementary food and water, the restriction of human access within the protected areas, and new research into remote monitoring, such as the use of infrared cameras, and non-invasive DNA analysis to help monitor the population (Horsup, 2011; Department of Environment and Heritage Protection, 2015).

![Map of Epping Forest National Park and St George, Australia](image1.jpg)

Figure 1-2 Two *in situ* protected populations of northern hairy-nosed wombats.

Action was taken by the NHNW recovery team to establish a second protected area near St George, approximately 40 kms west of where NHNW were first reported by European settlers in 1861 (Swinbourne *et al.*, 2016b). Richard Underwood Nature Refuge (RUNR), an area of 130 hectares consisting of eucalypt woodland on old river levees (Department of Environment and Heritage Protection, 2015) was selected as suitable habitat for NHNW, and between 2009 and 2010, 15 NHNWs, six males and nine females, were translocated to RUNR. In 2010, an orphaned hand-raised female was introduced to the nature refuge, and in 2011 two births were recorded; however, only one was reported to have survived (A. Horsup, 2016, *pers comm*).

Recently, in June 2016, another male was moved to the refuge to increase breeding potential and genetic diversity. Since the initial translocations began in 2009, 19 NHNW have been
translocated from Epping Forest NP to RUNR. Currently, ten individuals remain, of which two are males (Department of Environment and Heritage Protection, 2015; A. Horsup, 2016, pers comm).

The knowledge gained from these two in situ populations, and conservation efforts employed at both areas, will pave the way for the second part of the recovery plan, ex situ conservation by means of the development of a viable captive population (Horsup, 2004). To date, an ex situ insurance population has yet to be established for NHNW. The drought and the declining population trend initially led to an attempt to establish a captive population of NHNWs at Taronga Western Plains Zoo in 1996. The first NHNW transferred into captivity did not habituate to the artificial environment and/or the captive diet, and as a result died of a twisted bowel (Horsup, 2011). Subsequently, the captive program was halted due to concerns regarding the stress and survivability of NHNWs in captivity (Horsup, 1999).

1.1.2. Southern hairy-nosed wombat (L. latifrons)

In contrast to the NHNW, the SHNW is abundant in semi-arid areas of grasslands, open plains, savannah and open woodlands across South Australia and south-eastern parts of Western Australia (Figure 1-3.). The main populations are located in the Murraylands, Eyre Peninsula, Gawler Ranges, along the Nullarbor Plains, and isolated colonies on the Yorke Peninsula (Swinbourne et al., 2016b). There is also a colony of approximately 300 animals on Wedge Island descended from six animals, which were translocated in the 1970s (Ostendorf et al., 2012).

Similar to the NHNW, the influences of European settlement are usually blamed for a decline in the range of the SHNW (St John et al., 1989). The effects of livestock grazing (Horsup, 2004), culling (Tartowski and Stelmann, 1998), and rabbits (Cooke, 1998) have undoubtedly contributed to their decline and current distribution. However, for the SHNW, the dog-proof fence has protected the majority of the population, and the clearance of Mallee and tea-tree scrub for farmland may have potentially increased their distribution by removing unfavoured habitat (Stott, 1998).
Information about whether the population of SHNW is declining or expanding appears to be based more on belief rather than on objective scientific analysis. Population studies have shown fluctuations in population estimates (Table 1-1.) with one study reporting a four-fold increase over 20 years between 1980 and 2002 (Biggs et al., 2002). While small populations within the Murraylands’ population have declined (Swinbourne et al., 2016b), largely in part due to drought, outbreaks of sarcoptic mange, and emaciated body condition attributed to the overgrowth of introduced weed species causing starvation and malnutrition (Woolford et al., 2014), the distribution of the entire Murraylands’ population has actually been increasing (Swinbourne et al., 2016b).

Figure 1-3. Distribution of southern hairy-nosed wombats. Adapted from Swinbourne et al., 2016.

As a result, contradictory actions to both control and protect SHNWs are being undertaken simultaneously by different sectors of the community. On one hand SHNWs are considered an agricultural pest and are subjected to illegal and legal culling practices to control numbers (St John et al., 1989; Ostendorf et al., 2009). On the other hand, it is believed that they are
vulnerable and need to be protected (Woinarski and Burbidge, 2016). Further research into the presence, or absence, of SHNWs in the wild needs to be undertaken if the species is to be appropriately managed.

1.2. Hairy-nosed wombat captive breeding

The development of a successful captive breeding program will play an important role in future NHNW conservation. However, before removing individuals from the wild, there is a need to firstly have a thorough understanding of their reproductive biology, behaviour and endocrinology. Access to NHNW is limited, and intensive manipulative research is not an option. Further, due to their fossorial and nocturnal behaviour, obtaining reliable information regarding their reproduction in the wild is limited. More research into their reproductive biology is required if we are ever going to develop an *ex situ* insurance population. In order to obtain this knowledge, we need to study their closely related cousin, the SHNW, to serve as a reproductive model.

SHNWs are long lived in the wild, up to 15 years (Triggs, 2009), and produce one pouch young every two years (Finlayson *et al.*, 2007). In captivity, whilst they live just as long, if not longer, they do not breed reliably (Hogan *et al.*, 2013). On average one pouch young is born into captivity each year, few of which survive to adulthood. (Hogan *et al.*, 2013). The mean annual recruitment rate for captive SHNW since 2003 is 2.5, with a mean annual loss of 7.3, resulting from death, export or release (Hogan *et al.*, 2013). Further, second generation breeding has yet to occur in Australia.
Table 1-1. Population estimates of southern hairy-nosed wombats since the 1980s.

<table>
<thead>
<tr>
<th>Year</th>
<th>Estimate</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td><strong>Nullarbor Plain (outside dog fence)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>109,051</td>
<td>(St John, 1998)</td>
</tr>
<tr>
<td><strong>Nullarbor Plain (inside dog fence)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1980</td>
<td>162,854</td>
<td>(Tiver, 1981)</td>
</tr>
<tr>
<td>1995</td>
<td>457,000</td>
<td>(Biggs et al., 2002)</td>
</tr>
<tr>
<td>2002</td>
<td>476,100</td>
<td>(Biggs et al., 2002)</td>
</tr>
<tr>
<td><strong>Murraylands - Entire Area</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1992</td>
<td>33,871</td>
<td>(MacGregor and Wells, 1998)</td>
</tr>
<tr>
<td>2012</td>
<td>148,448</td>
<td>(Ostendorf et al., 2016)</td>
</tr>
<tr>
<td><strong>Eyre Peninsula</strong></td>
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<td></td>
</tr>
<tr>
<td>1989</td>
<td>9,502</td>
<td>(St John, 1998)</td>
</tr>
<tr>
<td><strong>Gawler Ranges - Lake Harris</strong></td>
<td></td>
<td></td>
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<tr>
<td>1988</td>
<td>1,633</td>
<td>(St John, 1998)</td>
</tr>
<tr>
<td><strong>Gawler Ranges - Lake Acraman</strong></td>
<td></td>
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<tr>
<td>1985</td>
<td>12,740</td>
<td>(St John, 1998)</td>
</tr>
<tr>
<td><strong>Yorke Peninsula</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>696</td>
<td>Taggart, et al., unpublished data</td>
</tr>
</tbody>
</table>

As a reliable SHNW captive breeding program has yet to be established, it stands to reason that prior to removing NHNW from the wild to establish a captive breeding population, a reliable breeding program first needs to be established for the SHNW. In order to achieve this, it is vital to understand the factors that contribute to successful reproduction in captivity, and what aspects of the captive environment are hindering current SHNW reproductive efforts.

While captive environments offer a platform from which to intensively monitor animal behaviour, general health and reproduction (Hosey, 1997), they can also be radically different from those in the wild. Captive bred animals can undergo significant changes to their wild born counterparts. For example, behaviours can be vastly different, where tameness, a trait which
would be maladaptive in the wild, may be inadvertently selected for in captivity (Lynch and O'Hely, 2001). Animals in captivity can learn to adapt to their artificial environment to the point that carnivores can survive without hunting, predator avoidance is unnecessary, their daily diet is provided, and diseases and parasites are controlled (Frankham and Loebel, 1992; Moseby and Donnell, 2003). Lastly, captivity can result in changes in reproductive fitness (Frankham and Loebel, 1992) and gene expression (MacBean et al., 1971). Controlled environments, continual human interaction, imposed mate selection, nutrition and resource availability can also increase selective pressure on a species in captivity, negatively impacting captive breeding efforts (Lynch and O'Hely, 2001).

The low captive breeding rate of SHNWs suggests there are aspects of the captive environment which fail to mimic conditions found in the wild. These include limited space availability and the lack of environmental enrichment leading to boredom-induced stereotypic behaviours (Hogan and Tribe, 2007; Hogan et al., 2009; Hogan et al., 2011c; Descovich et al., 2012c). Inappropriate captive diets can lead to obesity or nutritional deficiencies (Gaughwin, 1982; Treby, 2005). Wild caught adults may be unable to adapt to their new captive environment (Lynch and O'Hely, 2001), which can lead to dire consequences as demonstrated by the previous attempt to house a NHNW (Horsup, 2011). Therefore, each aspect of the captive environment needs to be appropriately managed specifically for SHNW if a reliable captive breeding programs is to be established.

1.2.1. Enclosure design and space availability

The home range of wild SHNWs is approximately two to four hectares, and depending on the season, individuals will spend up to 9 hours per night above ground, travelling on average 221 metres (Finlayson et al., 2005; Finlayson et al., 2010). The minimum housing requirement for captive wombats is currently between 45 and 50 m² for two individuals (New South Wales Department of Primary Industries, 2006, p19), less than 1% of its range in the wild. As a result, many wombats in captivity demonstrate a wide range of stereotypic behaviours causing damage to either themselves, each other or to their enclosure. Due to their nocturnal behaviour, stereotypies may be primarily unseen due to the majority of their activity occurring outside of
zoo hours, and abnormal behaviours may not be recognised by caretakers or zoo staff (Descovich et al., 2012c).

Enclosure design and spatial availability can have a profound effect on the behaviour, welfare and subsequent reproduction of individuals in captivity (Remience et al., 2008; Brummer et al., 2010; Descovich et al., 2012c). The design of an enclosure is largely dependent on the purpose of the captive population (Gaughwin, 1982). Small enclosures are favourable for exhibition as it prevents the animal from removing itself from public view. However, small enclosures restrict resting and feeding locations, as well as foraging opportunities. These limitations can lead to increased stereotypic behaviour such as pacing, climbing, digging along fence lines to escape, and aggressive behaviour towards other captive individuals or to their carers (Eriksson et al., 2010).

Housing wombats in larger enclosures, up to 400 m², has been shown to reduce aggressive and stereotypic behaviours (Booth, 1999; Descovich et al., 2012c). Increasing space availability for captive SHNW from 75.5 m² to 224 m² has also been shown to increase positive behaviours such as grazing and enclosure exploration, while at the same time reduce fence line digging, pacing, excessive grooming and alert or vigilant behaviours. Additionally, larger enclosures improves group harmony as aggressive behaviour towards other captive individuals is decreased (Descovich et al., 2012c).

As well as the size of an enclosure, inappropriate designs of the captive environment can negatively impact reproductive behaviour and successful breeding (Hogan, 2004). For example, in small enclosures wombats may not be able to perform their elaborate courtship and mating rituals, so that natural mating behaviour is prevented (Hogan et al., 2011a). Environmental structures, such as narrow tunnels and/or burrows, may allow the female to escape the male’s grasp, ultimately hindering the male’s effort to successfully pursue, catch and copulate with the female (Hogan, 2004), all of which could be important pre-copulatory behaviours.
1.2.2. Nutrition

Understanding the nutritional requirements of animals in captivity can be difficult as captive facilities may not be able to duplicate their natural diet. For many marsupials, the basal metabolic rate is low compared to eutherian mammals (Hume, 2005). Wombats in particular have the lowest energy, protein and nitrogen requirements of any herbivorous marsupials, and require less food per kilogram of body weight (Wells, 1973; Barboza and Hume, 1992). When placed on captive diets, SHNWs are prone to obesity and can develop poor dental health as well as digestive disorders (Hume, 2005). For individuals within a captive breeding program it would be essential that they are supplied with a diet that is more representative of their wild diets in order maximise the animal’s reproductive potential.

Depending on rainfall, grazing competition and seasonal availability, wild SHNWs diet consists of a variety of plants including burr medic (Medicago polymorpha), stipa grass (Stipa nitida), chenopods (Chenopodiaceae), Ward’s weed (Carrichtera annua), emu bush (Eremophila), stork bills (Erodium cicutarium), daisies (Asteraceae) and thread iris (Moraea setifolia) (Taggart et al., 2016b). Enclosures which contain native vegetation would provide these grazing animals with a more natural component of their diet rather than restricting them to high concentrate captive diets. Additionally, as many animals in the wild spend much of their time seeking and processing food, captive animals should also be provided with similar opportunities (Hosey et al., 2013). Therefore, rather than simply supplying the captive animals with food, providing access to native vegetation within their enclosure will encourage natural foraging and exploratory behaviour, which in turn can reduce the onset of obesity and health disorders, and reduce boredom-induced stereotypic behaviours.

SHNW breeding occurs during winter / spring which coincides with changes in pasture growth and feed availability (Gaughwin et al., 1998). Significant changes in body weight between breeding and non-breeding seasons have been reported in SHNW (Taggart et al., 2005b), suggesting that animals in the wild undergo seasonal changes in depletion and repletion of body condition; a continual cyclic change imposed by the animal’s natural environment. These changes in feed availability may be an important environmental cue required to trigger the release of reproductive hormones (McEvoy and Robinson, 2003). In captivity, continual feeding regimes and high feeding levels provided throughout the year would not allow for natural
catabolism, nor would it allow for the seasonal release of specific nutrients. Therefore, limited access to natural or native vegetation, as well as an imposed and inappropriate diet provided throughout the year could potentially be detrimental to a wombat’s health in regard to obesity and nutritional disorders, and also may be preventing specific environmental cues required for timed reproduction.

1.2.3. Social structure

Another important factor for successful captive breeding is understanding social behaviours of SHNW in the wild which should to be mimicked in captive facilities. Behavioural observations of individuals in the wild suggest a tolerant relationship with conspecifics (Gaughwin, 1982). They are not strictly solitary in the wild as they often share warrens with a large number of individuals with up to 10 within a single warren system (Finlayson et al., 2005). However, burrow sharing may not be for just social interactions as it may also be a function of thermoregulation in colder conditions, reducing energy costs associated with digging and maintaining burrows, as well as increasing protection against predators (Finlayson et al., 2005; Walker et al., 2007).

Wombats in captivity can be housed individually, in mixed or same sex pairs, or housed in small groups; generally, one male with two females (Treby, 2005; Hogan et al., 2011a; Descovich et al., 2012c). The social structure for captive SHNW is unpredictable and either sex can be dominant. Orders of dominance and social hierarchy are quickly established and can be maintained with little displacement and mild aggressive behaviour (Gaughwin, 1982). However, this imposed social structure can negatively impact reproduction, especially if the female is dominant over the male, which may occur if she is larger and/or older than the male. For reproduction to be successful, a male overpowers the female, catching her and rolling her onto her side (Hogan et al., 2011a). If a male is subordinate, he is less likely to overpower the female and complete a successful copulation.

If a mating is successful, group housing can be detrimental to the survival of the pouch young. Gaughwin (1982) reported that while gestation was not affected by group housing, half of the pouch young (three of six) were lost during lactation from females housed with a single male.
As females do not cohabitate with males after copulation, the imposed social housing in captivity may be contributing to the poor success rate of captive breeding (Gaughwin, 1982; Hogan et al., 2013). Therefore, it makes sense that to maximise the reproductive output and increase pouch young survival, the female needs to be isolated from the male after mating or parturition.

1.2.4. Stress and welfare

Captive environments are markedly different to those in the wild, in that they are less complex, less varied and less spacious. Artificial environments can negatively impact the general health and welfare of an animal, resulting in aggression and stereotypic behaviours, and therefore, negatively impact reproductive performance (Mason, 2010). Inadequate enclosure design, poor diet, imposed social structure and group housing could possibly lead to chronic stress for some SHNW. Individuals that are continually harassed, chased or bitten can result in high levels of stress and poor body condition (Gaughwin, 1982).

Stress occurs when the environmental demand taxes or exceeds the adaptive capacity of an organism, resulting in sustained negative psychological, behavioural and biological changes (Cohen et al., 1997; Siegel and Gross, 2007). Stress stimulates a series of complex adaptive responses, and while not every stressor is considered ‘bad’, if the stress is experienced over a prolonged period, it can disturb the normal function of many systems within the body. For example, chronic stress can manifest as a loss of appetite and atrophy of tissue, maladaptive behaviour such as stereotypies, immunosuppression and / or suppressed hormone secretion, resulting in the inhibition of reproduction (Tilbrook et al., 2002; Siegel and Gross, 2007; McPhee and Carlstead, 2010; Chand and Lovejoy, 2011).

Previous studies have investigated the physiological and behavioural response to stress on SHNWs in captivity, specifically, human interaction and space availability (Hogan et al., 2011c; Descovich et al., 2012c). Hogan et al, (2011c) reported that while the reactivity to human interaction decreased, faecal cortisol metabolite secretion remained high in response to human contact. This indicates that while the animals appeared to be comfortable interacting with humans, it is more of a learned helplessness behaviour. Descovich et al. (2012c) found that negative stereotypic behaviours, indicative of underlining anxiety in captive individuals, were
increased in group housed SHNW in small enclosures. To improve breeding of any animal in captivity, the stress and welfare of the animal needs to be continually monitored and appropriately managed.

1.2.5. Health and disease

One of the benefits of captivity is the control and prevention of diseases. While captive facilities cannot ensure that animals under their care are completely free from disease or pain, these facilities can ensure that animals are effectively monitored and clinical and pathological problems are treated promptly and appropriately (Hosey et al., 2013). In the wild, wombats are susceptible to a range of infectious and non-infectious diseases (Vogelnest and Woods, 2008). The former can be managed through appropriate quarantine, hygiene and husbandry procedures, and the latter can be prevented in captivity by providing the animals with the appropriate diet, regular health checks, monitoring normal passage of urine and faeces, and reporting any changes in normal behaviour.

1.2.6. Implications

There are a range of factors that can negatively impact reproductive performance in captivity, including inadequate enclosure design, imposed social structure leading to social conflict and competition for resources, and inappropriate diets and feeding schedules which can lead to obesity and nutritional disorders. However, these factors can be effectively managed through the provision of appropriate nutrition, enclosure design and environmental enrichment, adequate social housing, and positive human interactions. Through the appropriate management of their captive environment, we can aim to minimise stress, and animals are more likely to exhibit natural behaviours. This will enable a better understanding of the natural physiological state of the species.

To further reduce stress caused by the captive environment, non-invasive techniques such as urine or faecal hormone analysis and remote behavioural monitoring using infrared cameras, may be integrated into a captive breeding program. Such techniques would provide insight into
the behaviour and biological function of this species with minimal human interaction or interference.

1.3. Assessing wildlife reproduction

Over the past three decades there has been a number of literature reviews on non-invasive hormone monitoring in a variety of captive and free-ranging wildlife species (Schwarzenberger et al., 1996; Schwarzenberger et al., 1997; Whitten et al., 1998; Brown, 2000; Möstl and Palme, 2002; Monfort, 2003; Schwarzenberger, 2007). While each of these reviews focusses on a particular aspect of hormone monitoring, a specific species or genera, type of biological sample, stress, welfare, reproduction or wildlife conservation, each review emphasises a number of important factors regarding the accuracy of hormones analysis techniques. These factors include understanding the difference between hormones and their metabolites, the hormone metabolic and excretion pathways, as well as sample collection and appropriate storage protocols. Further, these reviews also focus on the selection of the most appropriate analysis technique, the need for proper validation, both biological and biochemical validation of the hormone, confounding factors such as sex, age, diet, environment, and how individual or species-specific differences can influence the interpretation of the results.

All of these factors will contribute to a better understanding of wombat reproduction, not only through the development of proper hormone analysis techniques, but through the interpretation of the data. A greater understanding of wombat reproduction will lead to the appropriate reproductive management of individuals in captivity, and the subsequent development of a reliable captive breeding program, after which, ex situ conservation of the NHNW can begin. However, in order to achieve that, first each aspect of hormone analysis needs to be properly investigated, and where necessary, techniques may need to be developed and validated specifically for hairy-nosed wombats.

1.3.1 Hormones and their metabolic pathways

Hormones are involved in a large number of bodily functions, including reproduction, development and the expression of behaviours (Whitten et al., 1998). Plasma and serum provide
the most immediate endocrine information as hormones secreted from endocrine organs / glands such as gonads, adrenal and pituitary, are all secreted directly into the bloodstream (Whitten (Whitten et al., 1998; Schwarzenberger and Brown, 2013). While the analysis of hormones in non-invasive samples often reflects profiles found in the blood, the metabolic and excretion pathway may affect the amount and / or the structure of the hormone of interest (Pukazhenthi and Wildt, 2003). Therefore, in order to determine the best method of analysis, it is important to first understand the metabolic pathway of the hormone.

1.3.2 Reproductive protein hormones

Pituitary hormones are glycoproteins and are heavily metabolised by bacteria in the gastrointestinal tract. As a result, these hormones may be difficult to detect in biologically relevant concentrations (Whitten et al., 1998). The reproductive protein hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), are signal molecules which are released from the anterior pituitary into bloodstream and are transported to their target site, the gonads, and bind to specific target tissues. The hormone then triggers an effect, altering the cell activity or protein synthesis (Goymann, 2012). Once the hormone has reached its target site, the hormone is rendered inactive, it is progressively broken down by peptidase enzymes in the blood, removing one amino acid at a time. Larger proteins may be partially degraded while still in the blood, and consequently, present in the circulation as complete peptides (Norris and Carr, 2013).

As protein hormones are unstable and degrade rapidly, liver metabolism, excretion into the bile and gut transit time can prevent quantifiable amounts of proteins being excreted in some biological samples (Rozell and Okrainetz, 2009; Norris and Carr, 2013). For example, urinary LH is difficult to detect in the Asian elephant (Elephas maximus), African elephant (loxodonta africana) (Brown et al., 2010), and the Przewalski’s horse (Equus ferus przewalskii) (W. Collins, 2013, pers comm). However, urinary LH can be analysed in biologically relevant quantities in the giant panda (Ailuropoda melanoleuca) (Durrant et al., 2006), human and non-human primates (Li et al., 2002; Aramaki et al., 2010), Indian rhinoceros (Rhinoceros unicornis) (Stoops et al., 2004), five species callitrichid monkeys (Ziegler et al., 1993), and a small number of aquatic mammals such as the killer whale (Orcinus orca) (Robeck et al., 2004), bottlenose dolphin (Tursiops truncates) (Robeck et al., 2005), Pacific white sided dolphin (Lagenorhynchus
obliquidens) (Robeck et al., 2009), belugas (Delphinapterus leucas) (Steinman et al., 2012) and Amazonian manatee (Trichechus inunguis) (Amaral et al., 2014). As there are a wide variety of species in which urinary LH can be accurately analysed, with proper validation, there is potential that LH may be detectable in non-invasive samples collected from a SHNW.

1.3.3 Reproductive steroid hormones

Reproductive steroid hormones are not very soluble in aqueous solutions, and metabolism often requires alteration of the hormones after they have been cleared from circulation and modified by the gut flora (Goymann, 2012). Steroid metabolism involves the reduction or removal of side chains of attached groups and the joining of other molecules such as gluconate or sulphate (conjugation), transforming them into soluble molecules and rendering the steroid inactive (Norris and Carr, 2013). Once conjugation occurs, the steroid may be filtered from the blood by the kidneys and excreted in urine. Alternatively, steroids may be metabolised via oxidative pathways by the liver and broken down into metabolites, transported through the bile and excreted via the intestinal tract as portions of the former steroid (Norris and Carr, 2013). As steroid hormones are more stable than protein hormones, in some species the hormone, as well as its metabolite or conjugate, are excreted in non-invasive samples (Ziegler et al., 1996). This needs to be taken into consideration when selecting an analysis method for the species and hormone in question.

1.3.4 Immunoassays for hormone analysis

Wildlife conservation relies on accurate information regarding all aspects of animal reproduction. Understanding the basics of endocrine function is important as hormones play a key role in nearly every aspect of reproduction (Pukazhenthi and Wildt, 2003; Schwarzenberger and Brown, 2013). Measuring hormones enables researchers to evaluate the physiological response of an animal and their interaction/relationship with behaviour, ecology and conservation (Wielebnowski and Watters, 2007).

A common, convenient and economic method for hormone analysis is the use of competitive immunoassays, which measure the immunoreactive properties of a hormone (or
metabolite) within a biological sample (Wielebnowski and Watters, 2007; Hodges et al., 2010; Goymann, 2012). The principle of the immunoassay is the competition of a labelled antigen and an unlabelled hormone (within the sample) binding to an antibody. Hormone concentration is determined by the comparison of the labelled and unlabelled hormone within the solution against a previously calibrated standard curve of known hormone concentration (Hodges et al., 2010; Kleiman et al., 2010; Norris and Carr, 2013). The two most commonly used immunoassays available are radio- (RIA) and enzyme-immunoassays (EIA). RIAs use a $^{125}$Iodine tracer and are based on competitive binding between the hormone in the sample and the $^{125}$Iodine-labelled hormone to a specific antibody. EIA use antigens which are linked with an enzyme, such as horseradish peroxidase (HRP), or a biotin labelled hormone, which binds to HRP-labelled streptavidin, to provide competition to the binding of the antibody (Price and Newman, 1991; Norris and Carr, 2013).

While RIAs have been the standard assay for hormone analysis for many years, EIAs are becoming more commonly used in wildlife and conservation studies (Monfort, 2003; Schwarzenberger, 2007; Schwarzenberger and Brown, 2013). EIAs negate the need for a licence to use radio-isotopic tracers and the removal of radioactive material, rather relying on colour change for hormone quantification. As such, EIAs are less costly, reagents are easy to prepare, and have a highly stable and long shelf-life. In addition, EIAs can be easily developed for use in zoos and in the field, where sophisticated laboratory facility may not be available (Wielebnowski and Watters, 2007; Hodges et al., 2010).

There are two types of EIAs, single and double antibody. The single antibody immunoassay relies on labelled (HPR enzyme conjugate) and unlabelled (sample hormone) antigens competing for sites on the hormone-specific antibody. The addition of a substrate reacts with the bound enzyme conjugate and changes the colour, deeper colours representing high levels of binding affinity with the enzyme conjugate. For double antibody, the methodology is the same with the exception of a secondary antibody (anti-first antibody) used to coat the plate prior the addition of the primary antibody. The use of monoclonal antibodies can lose some or all of their binding affinity when absorbed directly onto plastic, i.e. coating a 96-well microtiter plate. The use of two antibodies in an assay helps to retain some of the binding affinity of the antibody when absorbed, increasing sensitivity and creating a more robust assay (Wild and Kusnezow, 2013).
The benefit of using a double, as opposed to a single antibody assay, is that less primary antibody is required as it binds to the secondary antibody rather than the plate. However, there is an additional overnight incubation period required to coat the plate prior to coating the plate with the primary antibody (Price and Newman, 1991; Wild and Kusnezow, 2013).

1.3.5 Biological and biochemical validation

Non-invasive steroid analysis has now become a well-established tool for monitoring reproductive and adrenal function in a variety of mammalian species (Monfort, 2003; Schwarzenberger, 2007; Kersey et al., 2010). However, in order to determine accurate hormonal activity in an animal, each assay needs to undergo validation to ensure that (1) the immunoassay is accurately detecting the hormone (or its metabolite) of interest, and (2) that the hormone concentration is being measured in biologically relevant quantities. This involves pairing known physiological events such as behavioural oestrus, ovulation or mating, with reproductive hormone profiles (Monfort, 2003; Hodges et al., 2010).

For controlled experiments, a synthetic drug can be administered which helps to clarify hormone excretion pathways and time delay between stimulation of the endocrine gland and the appearance of hormonal metabolites in the excreta (Wielebnowski and Watters, 2007). To test adrenal function, an adrenocorticotropic hormone (ACTH) injection will result in a rise in plasma cortisol secretion. For reproduction, a gonadotrophin releasing hormone (GnRH) agonist is used to illicit changes in pituitary hormone secretion in a similar fashion (Monfort, 2003). Non-invasive samples can contain a large number of hormone metabolites, and the metabolite of interest may be present in low concentrations. This can interfere with the accuracy and reliability of the immunoassay (Whitten et al., 1998). To overcome this, either the use of group-specific antibodies which cross-react with many metabolites related to the hormone (Brown et al., 2010), or hormone-specific antibodies which cross-react with other components, can be used in the immunoassay (Paris et al., 2002). However, there is also the inherent problem that the measurement of a structurally related metabolite, which may have different physiological properties, can be misleading and resulting in the analysis of incorrect hormone concentrations (Hodges et al., 2010).
As immunoassays are highly sensitive, each needs to be biochemically validated prior to analysing samples (Hodges et al., 2010). Biochemical validation of immunoassays tests for (1) accuracy; the ability to detect the correct amount of hormone in the sample, (2) specificity (cross-reactivity); the hormone-specific antibody used, and the potential influences of interfering substances within the sample, (3) sensitivity; the minimum amount of hormone that can be detected, and (4) precision; within and between immunoassay repeatability, and parallelism of the dose-response relationship of the known and the unknown hormone concentration (Hodges et al., 2010). Ongoing validation is also conducted through monitoring the inter- and intra-assay variability (< below 15%), which will ensure consistency between assays, and allow for the accurate interpretation of results (Whitten et al., 1998; Hodges et al., 2010; Sheehan, 2013).

1.3.6 Sample collection and storage

Plasma provides the most immediate endocrine information as hormones secreted from endocrine organs such as gonads, adrenal and pituitary, along with peptides and growth factors, are all secreted directly into the bloodstream (Whitten et al., 1998; Schwarzenberger and Brown, 2013). As a result, studies which have used serial blood collection to analyse reproductive hormones have gained substantial information on a wide range reproductive functions, including gonadal function on the basis of sex, age and seasonality, the timing of spermatogenesis and ovulation in some species, the type of ovulation (spontaneous or induced), pregnancy diagnosis, methods to overcome infertility, and the development of assisted reproductive technologies (ARTs) (Pukazhenthi and Wildt, 2003).

Fortunately, some captive animals can be trained or conditioned for regular blood collections {non-human primates (Reinhardt, 1997), bottlenose dolphins (*Tursiops truncates*) (O'Brien and Robeck, 2012), African elephant (Brown et al., 2010), giant pandas (W. Boardman 2013, pers. comm) and South American tapir (*Tapirus terrestris*) (Gillet and Torres, 2008)}. However, for most wildlife species in captivity, sequential blood sampling is not an effective sample collection method. Blood sampling often requires capture, restraint and sedation, a method which is not practical as it is costly, stressful and potentially dangerous to both the animal and its handler (Hodges et al., 2010). Over a prolonged period, blood collections can be counter-productive as chronic stress can interfere with certain hormone secretions making it difficult to discern normal hormone profiles (Monfort, 2003).
Since the 1980s, hormones have been successfully analysed from a variety of biological samples which can be collected using non-invasive methods, such as urine, faeces, saliva, milk, hair (Hodges et al., 2010). Non-invasive hormones have been analysed to investigate not only natural hormone regulation and secretion, but also the interaction between hormones and animal welfare, ecology, behaviour, physiology and conservation biology (Ganswindt et al., 2012). While these samples can be comparatively easier to collect compared to blood (Whitten et al., 1998), interpretation of hormone concentration requires accurate knowledge regarding the physiology, hormone secretory patterns, and the metabolic and excretion pathways of the hormone of interest (Goymann, 2012). While there are commonalities between species concerning reproductive endocrinology, there are also species-specific differences, such as those seen among the rhinoceros species (Heistermann et al., 1998; Schwarzenberger et al., 1998; Brown et al., 2001), and not all analysis techniques are suitable for all non-invasive samples (Palme et al., 1996; Schwarzenberger et al., 1996; 1997; Graham et al., 2002; Palme, 2005).

The major benefit of non-invasive hormone monitoring is that samples can be collected without the need to physically touch or restrain the animal. For animals in captivity, animals can be conditioned for collection, or waste can be easily recovered off the enclosure floor during normal animal husbandry routines (Hodges et al., 2010). While a little harder for free-ranging animals, it is possible to collect fresh scats opportunistically, and small volumes of urine (< 1 mL) can be scavenged off vegetation (Knott, 1997; Robbins and Czekala, 1997). As there is little to no stress to the animal during sample collection, further benefits of non-invasive hormone analysis include the evaluation of reproductive hormones over long periods, such as over several breeding seasons, providing longitudinal hormonal data. This method can provide valuable information regarding long term reproductive assessment of an individual animal or a population (Monfort, 2003).

Once the sample has been collected, the hormone of interest may become unstable and undergo degradation. After excretion, environmental factors such as temperature and humidity, as well as bacterial enzymes can influence the immunoreactivity of the hormone (Touma and Palme, 2005). This can be overcome by placing the sample on ice temporarily or freezing it until hormone analysis can be conducted. However, this may be difficult for field collections or
impractical in remote locations, and preservatives may be required to reduce hormone degradation (Whitten et al., 1998).

Depending on the sample type, a range of preservatives or preservation methods have been used with some success (Livesey et al., 1983; Wasser et al., 1988; Kesner et al., 1995; Whitten et al., 1998; Foley et al., 2001; Tecot, 2001). The addition of ethanol to freshly collected faecal samples can extend time to sample processing (Wasser et al., 1988), which would be beneficial for field collection where immediate freezing is not an option. However, it is important to note that the addition of alcohol will initiate steroid extraction. In order to prevent sample loss or sample bias, standardisation methods of both the sample and alcohol should be employed at the time of collection to ensure consistency in sample collection and storage methods (Palme, 2005). Further faecal preservation methods include the use of drying methods either with silica, oven, solar radiation or ‘fire’ (Wasser et al., 1988; Whitten et al., 1998; Foley et al., 2001; Tecot, 2001).

For urine samples, adding glycerol to fresh samples can extend freezing time without significant loss of hormone immunoreactivity (Kesner et al., 1995). However, this is not the case for every species as some preservation protocols can result in immunoreactivity changes in hormone concentration (Hunt and Wasser, 2003). Storage time, temperature and freeze-thaw protocols can also impact the stability of the hormone (Livesey et al., 1980). Therefore, prior to conducting sample collection in the field, the collection, preservation and storage method needs to be properly validated for the sample type and the species of interest (Monfort, 2003).

When developing studies for reproductive hormone analysis, the choice in the sample to analyse is dependent on a range of factors including the species in question, the metabolic and excretion pathways and the analysis techniques available. Further considerations include the practicalities of the research, such as determining the type of information that you want to obtain will help to determine how many samples are required, the collection environment, free-ranging or captive, the reliability to obtain fresh, uncontaminated samples from the animal, and if a sample preservation method is required.
1.4 The application of non-invasive methods to assess southern hairy-nosed wombat reproduction

Information regarding SHNW reproductive endocrinology is limited. In both male and female SHNW, reproductive steroid hormones have been analysed in serum and faeces using both RIAs and EIAs (Gaughwin, 1981; Hamilton et al., 2000; Paris et al., 2002; Druery et al., 2003; Taggart et al., 2005b; Finlayson et al., 2006; Hogan et al., 2010a; Hogan et al., 2010b; Descovich et al., 2012a).

Samples have been collected from both wild and captive individuals over a number of breeding and non-breeding seasons. However, studies conducted on captive individuals have also enabled sequential sample collection from known individuals, allowing for longitudinal assessment of reproductive endocrinology. This level of observation has not been possible on wild individuals and should be taken into consideration when making comparisons.

1.4.1 Wombat reproductive steroid hormones

Reproductive steroid analysis has demonstrated that captive female SHNWs are polyoestrus, exhibiting at least 2 to 3 cycles per breeding season (Paris et al., 2002; Finlayson et al., 2006; Hogan et al., 2010b). In captivity, the oestrous cycle appears to be highly variable ranging between 27 to 41 days (Table 1-2), consisting of a short follicular phase (9 to 15 days) and a longer luteal phase (15 to 31 days) (Finlayson et al., 2006). Plasma oestradiol (E2), has shown to peak approximately five days prior to a sustained increase in P4 (Finlayson et al., 2006). However, this is based on interpolated data, as serial blood collection was too invasive (Figure 1-4). Non-invasive faecal hormonal analysis (Figure 1-5) has shown that there is a single progesterone (P4) peak during the luteal phase which occurs between 3 and 9 days after mating (Hogan et al., 2010b). The onset of a luteal phase has also been reported in non-mated females (Paris et al., 2002), indicating that ovulation in this species is likely to be spontaneous ovulation rather than induced by coitus.

A range of progesterone metabolites has been quantified in two wombat species, CW (Paris et al., 2002; West et al., 2004) and SHNW (Gaughwin, 1981; Finlayson et al., 2006;
Hogan et al., 2010b; Descovich et al., 2012a). In captive CW, plasma P4 ranged from <2.9 to 120 nmol/L (West et al., 2004), which is greater than the basal concentration of 3.6 nmol/L and peak concentrations of 139 nmol/L (Finlayson et al., 2006). In wild female SHNW, plasma P4 could only be measured in females with a corpus luteum, as such only a mean basal value of 4.52 nmol/L was reported (Gaughwin, 1981). As serial blood sampling in wombats can be quite invasive, Paris et al. (2002) validated three specific progesterone metabolites (P4M) for non-invasive faecal samples in captive wombats (5β-20α-OH-pregnane, 20-oxo-pregnane and 5α-20α-OH-pregnane). They determined that female wombats predominantly excrete 5β-reduced pregnanes, and reported P4 concentrations between 0 and >500 ng/g faeces. Hogan et al. (2010c) used a broad spectrum P4M to measure faecal progesterone, reporting basal concentrations of <20 to 135 ng/g faeces during peak luteal phase. Despite the differences in concentration, it appears that the progesterone metabolites which are excreted in biologically relevant quantities are quite effective in determining the reproductive status of a captive individual, in that changes in hormone concentrations can be matched to changes in physiological or behavioural events.

Plasma E2 has only been effectively measured in female SHNW using commercial RIA kits (DSL-4800) (Finlayson et al., 2006). This study reported cyclic changes in E2, ranging between 44 to 320 pmol/L. While an EIA has been used to detect E2 in non-invasive faecal samples (Hogan et al., 2010b), there was a high variability between females (10.6 to 283.2 ng/g faeces), and no significant variation or distinct cyclic changes in E2 concentrations throughout the reproductive cycle. The primary limitation was the inability to detect significant changes over time, indicative of the onset and duration of a follicular phase. This suggests that either E2 is heavily metabolised during the three-day gut transit (Hogan et al., 2011b) or the analysis techniques used thus far have not been suitable to measure biologically relevant concentrations of E2 in non-invasive samples. If the former is the case, it may be possible that a different non-invasive sample such as urine, may be more suitable of detecting change in wombat E2 concentrations. If it is the latter, then the use of an alternative E2 antibody may be required for analysis. Either way, both avenues warrant further investigation to refine the oestrogen profiles for wombats.
Table 1-2. Summary of studies that have investigated female southern hairy-nosed wombat oestrous cycle length (mean days and range), luteal phase (LP) and follicular phase (FP) based on plasma or faecal hormone metabolite analysis.

<table>
<thead>
<tr>
<th>Mean cycle length (days)</th>
<th>Mean LP (days)</th>
<th>Mean FP (days)</th>
<th>Measured by</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>41.1</td>
<td>27.6</td>
<td>13.3</td>
<td>Faecal P4 metabolites</td>
<td>(Paris et al., 2002)</td>
</tr>
<tr>
<td>35.6 (35-38)</td>
<td>23.3 (16-26)</td>
<td>11.3 (10-12)</td>
<td>Plasma P4</td>
<td>(Finlayson et al., 2006)</td>
</tr>
<tr>
<td>31.8 (27-38)</td>
<td>20.9 (15-31)</td>
<td>11.6 (9-15)</td>
<td>Faecal P4 metabolites</td>
<td>(Hogan et al., 2010b)</td>
</tr>
</tbody>
</table>

Source: (Hogan et al., 2013).

In male SHNW, significant seasonal differences have been recorded in androgen secretion (Hamilton et al., 2000), which almost doubles during the breeding season (July to September). For wild individuals, an increase in faecal testosterone (T) also correlated with increased growth of the androgen dependent prostate and bulbourethral glands (Hamilton et al., 2000). In captive individuals, there was significant seasonal variation in plasma T; however, faecal T remained relatively constant throughout the year (Hogan et al., 2010a). The mean (± standard error of mean) faecal T concentration of wild individuals was $17.9 \pm 2.15$ ng/g during the breeding, and $9.89 \pm 1.47$ ng/g during the non-breeding season. These concentrations are far greater than the mean faecal T concentration over the entire year for captive individuals ($1.8 \pm 0.26$ ng/g).

Reproductive accessory gland size, as measured by ultrasound (Hogan et al., 2010a), also remained constant in captive individuals compared to wild individuals (Hamilton et al., 2000). Despite the differences in analysis techniques between the two studies, RIA (Hamilton et al., 2000) versus EIA (Hogan et al., 2010a), these observations clearly indicate that seasonality is less prominent in captive male SHNW, suggesting that the captive environment may be negatively affecting androgen production and potentially hindering captive breeding efforts.
Figure 1-4. Linear interpolation of mean plasma oestradiol and progesterone concentrations from three female southern hairy-nosed wombats over an oestrous cycle. Source: (Finlayson et al., 2006).

Figure 1-5. Mean faecal hormone concentration of six female southern hairy-nosed wombats. Source: (Hogan et al., 2013).
One of the limitations to both serum and faecal hormone analysis is that venepuncture is often impractical and the three-day gut transit time prevents some reproductive hormones from being measured in biologically relevant quantities (Hogan et al., 2010b). The collection and analysis of non-invasive urine samples may be the key in refining the E2 profiles. CW urine samples have been collected and analysed for reproductive steroid hormones using alternative assay methods including fluorometric analysis and gas chromatography (Peters and Rose, 1979). While biological validation was conducted for urinary hormone analysis, the collection methodology used would now be considered highly stressful to the animals, wombats were kept in metabolic cages, and would likely interfere with normal secretory patterns of hormones under investigation. Since this study, much less invasive means of sample collection are now possible in a variety of species, including the detection of protein and steroid hormones in urine recovered through conditioning individual animals or direct collection off the enclosure floor (Hodges et al., 2010).

1.4.2 **Wombat reproductive protein hormones**

To date, only one study has examined gonadotrophins, specifically LH, in female SHNWs (Gaughwin, 1981). During 1976 and 1978, serum samples were collected from 31 individuals, and while serum LH was detected, the results only provided limited information as there was no proper biological validation to correlate changes in LH to specific reproductive events. Further research needs to be conducted whereby immunoassays are validated to known physiological events during each stage of the SHNW reproductive cycle to detect changes in LH concentrations, such as the pre-ovulatory LH. By understanding the secretion of LH, we can manage the reproduction of captive individuals appropriately.

1.4.3 **Biological validation of luteinizing hormone in marsupials**

While a GnRH challenge to measure gonadotrophins has yet to occur in SHNW, GnRH challenges have been conducted on other marsupials, providing a reference for marsupial LH secretion for future GnRH studies in wombats. In female tammar wallabies (*Macropus eugenii*), 10 µg GnRH agonist intravenous injection (Calbiochem, 10 µg/mL sterile 0-9% NaCl) resulted in a rise in plasma LH within 15 minutes, peaking at 25 minutes, and was cleared within two
hours after administration (Evans et al., 1980). More recently, lower doses of synthetic GnRH have been used in marsupial reproduction studies (2 to 4 µg) resulting in similar LH profiles. For example, in koalas (Phascolarctos cinereus), 4 µg GnRH (Buserelin) resulted in an LH rise within 30 minutes, peaking between three and four hours after administration (Allen et al., 2008a). In female western grey kangaroos (M. giganteus), administration of 2 µg GnRH (Fertagyl) resulted in LH peaks 15 minutes after injection, $T_0 = 0.544$ ng/mL and $T_{15} = 7.72$ ng/mL (Wilson et al., 2013). The differences in LH profiles between marsupials is likely to be attributed to the differences in synthetic GnRH used and / or the individual variation in metabolic pathways between species. These differences in concentration and excretions times demonstrates the need to conduct species-specific validation for each assay, even between marsupials.

Other biological validation methods have been employed, using gonadectomised and / or hypophysectomised animals (Hearn, 1974; Hearn, 1975; Catling and Sutherland, 1980; Evans et al., 1980; Sutherland et al., 1980; Tyndale-Biscoe and Hearn, 1981; Fletcher, 1989; Williamson et al., 1990; Rudd et al., 1999). The removal of the gonads and / or the pituitary gland modifies hormone secretion. The ablation of the pituitary gland prevents the secretion of gonadotrophins and results in limited detection in biological samples. Gonadoectomy results in increased gonadotrophin secretion into peripheral circulation, as these molecules are not being taken up by their target tissue, there is a subsequent lack of negative feedback on the hypothalamus and the anterior pituitary (Catling and Sutherland, 1980). No such study had been conducted on wombats.

1.4.4 Analysis of luteinizing hormone in marsupials

While there has been only one study investigating plasma LH in wombats (Gaughwin, 1981), research has been conducted investigating plasma LH in other marsupials (Table 1-3), mainly using tammar wallabies as the model. After rigorous validation, a double antibody RIA developed by Sutherland et al. (1980) effectively measured plasma LH in tammar wallabies. The basal concentration of plasma LH was < 0.20 ng/mL and increased at oestrus, after ovariectomy and after a GnRH challenge (7.0, 10.0 and 11.0 ng/mL, respectively). The biological validation of plasma LH and physiological events helped to determine that tammar wallabies undergo a post-partum oestrus (12 to 14 hours after birth). The pre-ovulatory LH surge occurs at or just
after mating, with ovulation approximately 24 hours later. Due to the rigorous validation of the double antibody RIA using antiovine LH antibody (GDN#15), the methodology from Sutherland et al. (1980) became the standard reference in a number of subsequent marsupial reproduction studies (Catling and Sutherland, 1980; Evans et al., 1980; Harder et al., 1985; Horn et al., 1985; Fletcher and Renfree, 1988; Fletcher, 1989; Fletcher et al., 1990; Williamson et al., 1990; Hinds et al., 1992).

The use of eutherian LH antibodies and reference standards to monitor LH concentration in tammar wallabies has not always resulted in the detection of baseline LH concentrations (Hearn, 1974; Hearn, 1975; Harder et al., 1985; Fletcher et al., 1990). As such, McFarlane et al. (1997) and Moore et al. (1996) suggested that the limitations of previous LH studies were that concentrations could only be expressed as reference standards to eutherian mammals and the actual quantities of LH could only be estimated. As a result, Moore et al. (1996) developed a homologous RIA using common brushtail possum (Trichosurus vulpecular) LH as the antigen and the reference standard. McFarlane et al. (1997) also developed a heterologous RIA using a monoclonal antibody raised against bovine LH (518B7), and tested it against purified tammar wallaby LH (ME-14B). Using specific marsupial LH as the antigen, both these studies were able to detect baseline and peak values of plasma LH, and both assays were biologically validated using either oestradiol benzoate and GnRH (McFarlane et al., 1997), and / or gonadoectomised animals (Moore et al., 1996). While the sensitivity of these assays decreased, and binding affinity was higher when using marsupial LH, concentrations in both studies did not differ significantly from previous results (Catling and Sutherland, 1980; Sutherland et al., 1980). This suggests that undetectable basal concentrations were undetectable due to other factors such as the use of different antibodies and antigens, the assay technique, individual animal variation or inappropriate sample storage, which may also impact on LH detection in the assay.

Plasma LH in female tammar wallabies ranged from undetectable limits \(\{< 2.0 \text{ ng/mL to peaks between 7.0 ng/mL to } > 50 \text{ ng/mL (Evans et al., 1980; Sutherland et al., 1980; Harder et al., 1985)}\}\). Using the same RIA techniques, plasma LH concentrations in female kowaris (Dasyuroides byrnie) ranged from 0.2 to 12.9 ng/mL (Fletcher, 1989). Basal LH concentration reported in female koalas range from undetectable values to 1.5 ng/mL, with peak concentrations between 6.6, 14 and 18 ng/mL (Allen et al., 2008a; Allen et al., 2008b). In all these studies, the
same LH antibody was used in the RIA; however, in all the koala studies a different ovine reference standard was used compared to the tammar wallaby and kowaris studies. The differences in LH concentrations between species needs to be taken into consideration when developing an assay with appropriate sensitivity limits, as this can affect the validity of the assay used. Samples may need to be diluted or concentrated to enable accurate and quantifiable LH profiles.

While the majority of studies investigating plasma LH in marsupials have been conducted using RIA, EIAs are now becoming common place when measuring and monitoring wildlife endocrinology, and two studies have measured marsupial plasma LH using EIA (Matson et al., 2009; Wilson et al., 2013). While it was developed and validated as an LH RIA to measure plasma LH in African elephants (Graham et al., 2002), Matson et al., (2009) used the same assay components, bovine LH β-subunit (518B7) monoclonal antibody and bovine LH reference standard (NIH-bLH-B10), to develop an LH EIA to analyse plasma LH in western grey kangaroos (M. fuliginosus ocydromus) and black-flanked rock wallabies (Petrogale lateralis lateralis). This was followed up by the analysis of plasma LH in eastern grey kangaroos (M. giganteus) (Wilson et al., 2013). The monoclonal antibody raised against bovine LH is versatile and has shown to have good cross-reactivity amongst a variety of mammalian species (Hodges et al., 2010), including tammar wallabies (McFarlane et al., 1997; Rudd et al., 1999).

Previous studies have shown that the bovine LH (518B7) EIA has the potential to measure urinary LH in a variety of species (Table 1-4) but has yet to be tested on a marsupial. While this assay was specifically developed and validated for African elephant serum, it failed to detect LH in elephant urine (Brown et al., 2010). The inability to detect urinary LH was attributed to the differences in LH metabolism and/or that LH was excreted in undetectable levels. To determine the validity of the assay for SHNW urine, a GnRH challenge will need to be conducted, with paired blood and urine samples collected over a known time period. This would not only validate the assay and but also determine the clearance rate and excretion lag time of LH in female SHNWSs, providing valuable information regarding the LH surge and ovulation, which to date, has yet to be identified.
Table 1-3. Plasma luteinizing hormone immunoassays in marsupials

<table>
<thead>
<tr>
<th>Marsupial Species</th>
<th>Sex</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>LH Label</th>
<th>Reference Standard</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tammar wallaby</td>
<td>6♀</td>
<td>Antisera to Human LH or HCG</td>
<td>Turkey anti-rabbit γ-G</td>
<td>125I-labelled human LH</td>
<td>Human LH</td>
<td>(Hearn, 1974)</td>
</tr>
<tr>
<td>(Macropus eugenii)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tammar wallaby</td>
<td>30♀</td>
<td>Antiovine LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
<td>125I-labelled ovine LH (G3-222B or LER1374A)</td>
<td>NIH-LH-S19</td>
<td>(Sutherland et al., 1980)</td>
</tr>
<tr>
<td>(M. eugenii)</td>
<td></td>
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<tr>
<td>Tammar wallaby</td>
<td>30♀</td>
<td>Antiovine LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
<td>125I-labelled ovine LH</td>
<td>NIH-LH-S19</td>
<td>(Evans et al., 1980)</td>
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<tr>
<td>(M. eugenii)</td>
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<tr>
<td>Tammar wallaby</td>
<td>13♂</td>
<td>Antiovine LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
<td>125I-labelled ovine LH</td>
<td>NIH-LH-S19</td>
<td>(Catling and Sutherland, 1980)</td>
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<tr>
<td>(M. eugenii)</td>
<td></td>
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</tr>
<tr>
<td>Southern hairy-nosed wombat</td>
<td>31♀</td>
<td>Rat LH</td>
<td>Sheep anti-rabbit γ-G</td>
<td>125I-labelled rat LH</td>
<td>NIH-LH-S19</td>
<td>(Gaughwin, 1981)</td>
</tr>
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<td>(Lasiorhinus latifrons)</td>
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</tr>
<tr>
<td>Tammar wallaby</td>
<td>8♀</td>
<td>Antiovine LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
<td>125I-labelled ovine LH</td>
<td>NIH-LH-S19</td>
<td>(Tyndale-Biscoe et al., 1983)</td>
</tr>
<tr>
<td>(M. eugenii)</td>
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</tr>
<tr>
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<td>Sheep anti-rabbit γ-G</td>
<td>NIH-LH-S19</td>
<td>(Harder et al., 1985)</td>
<td></td>
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<td>(M. eugenii)</td>
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<tr>
<td>Tammar wallaby</td>
<td>16♀</td>
<td>Antiovine LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
<td>NIH-LH-S19</td>
<td>(Horn et al., 1985)</td>
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<tr>
<td>(M. eugenii)</td>
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<td>14♀</td>
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<td>Sheep anti-rabbit γ-G</td>
<td>NIH-oLH-S23</td>
<td>(Lewis et al., 1986)</td>
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<td>(M. eugenii)</td>
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<td>Antiovine LH GDN#15</td>
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<td>NIH-oLH-S23</td>
<td>(Fletcher and Renfree, 1988)</td>
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<tr>
<td>(M. eugenii)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Fletcher, 1989)</td>
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</tr>
<tr>
<td>Kowari (Dasyuroidea byrnie)</td>
<td>9♀ 4♂</td>
<td>Antiovine LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
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<td>Species</td>
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<td>Antiviral LH</td>
<td>Species Source</td>
<td>Radioactivity</td>
<td>Antiviral LH Source and Year</td>
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<tr>
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<td></td>
<td>Antiviral LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
<td>NIADDK Rat LH-I-6</td>
<td>NIH-oLH-S23 (Fletcher et al., 1990)</td>
</tr>
<tr>
<td>Tammar wallaby (M. eugenii)</td>
<td>20♂</td>
<td></td>
<td>Antiviral LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
<td>NIADDK Rat LH-I-6</td>
<td>NIH-oLH-S23 (Williamson et al., 1990)</td>
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<tr>
<td>Bennett’s wallaby (M. rufigriseus)</td>
<td>~70♂</td>
<td></td>
<td>Antiviral LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
<td>125I-labelled ovine LH</td>
<td>NIH-oLH-S18 (Curlewis, 1991)</td>
</tr>
<tr>
<td>Eastern Quoll (Dasyurus viverrinus)</td>
<td>6♂</td>
<td></td>
<td>Antiviral LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
<td>NIH-LH-S19</td>
<td>(Bryant, 1992)</td>
</tr>
<tr>
<td>Tammar wallaby (M. eugenii)</td>
<td>8♀</td>
<td></td>
<td>Antiviral LH GDN#15</td>
<td>Sheep anti-rabbit γ-G</td>
<td>125I-labelled ovine LH</td>
<td>NIH-LH-S19 (Hinds et al., 1992)</td>
</tr>
<tr>
<td>Tammar wallaby (M. eugenii)</td>
<td>30♀</td>
<td></td>
<td>Antirat LH NIDDK-antiratLH-S-I0</td>
<td>Goat anti-rabbit γ-G</td>
<td>NIH-oLH-S23</td>
<td>(Renfree et al., 1994)</td>
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<tr>
<td>Brushtail possum (Trichosurus vulpecular)</td>
<td>4♂</td>
<td>4♀</td>
<td>Ovine LH</td>
<td>125I-labelled possum LH</td>
<td>Possum LH</td>
<td>(Moore et al., 1996)</td>
</tr>
<tr>
<td>Tammar wallaby (M. eugenii)</td>
<td>4♂</td>
<td>4♀</td>
<td>Monoclonal bovine LH (518B7)</td>
<td>Sheep anti-mouse γ-G</td>
<td>125I-labelled hCG</td>
<td>Tammar wallaby LH (ME-14B)</td>
</tr>
<tr>
<td>Brushtail possum (T. vulpecular)</td>
<td>39♀</td>
<td></td>
<td>Antiviral LH</td>
<td>Sheep anti-rabbit γ-G</td>
<td>125I-labelled ovine LH</td>
<td>Possum LH</td>
</tr>
<tr>
<td>Tammar wallaby (M. eugenii)</td>
<td>10♂</td>
<td>9♀</td>
<td>Monoclonal 518B7</td>
<td>Sheep anti-mouse γ-G</td>
<td>125I-labelled hCG</td>
<td>ME-14B</td>
</tr>
<tr>
<td>Tammar wallaby (M. eugenii)</td>
<td>24♂</td>
<td></td>
<td>Antiviral LH Wa-R oLH</td>
<td></td>
<td></td>
<td>Porcine LH</td>
</tr>
<tr>
<td>Koala (Phascolartos cinereus)</td>
<td>9♀</td>
<td></td>
<td>Antiviral LH GDN#15</td>
<td></td>
<td>125I-labelled ovine LH</td>
<td>NIADDK oLH-S18 (Johnston et al., 2004)</td>
</tr>
<tr>
<td>Species</td>
<td>Gender</td>
<td>Antiovine LH</td>
<td>Possum LH</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------</td>
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<td>------------------------------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tammar wallaby (<em>M. eugenii</em>)</td>
<td>16♀</td>
<td>Antiovine LH Wa-R oLH</td>
<td>Possum LH</td>
<td>(Herbert et al., 2005)</td>
<td></td>
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<tr>
<td>Brushtail possum (<em>T. vulpecular</em>)</td>
<td>20♀</td>
<td>Antiovine LH</td>
<td>Sheep ant-rabbit γ-G</td>
<td>(Crawford et al., 2006)</td>
<td></td>
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<tr>
<td>Tammar wallaby (<em>M. eugenii</em>)</td>
<td>20♂</td>
<td>Antiovine LH Wa-R oLH</td>
<td>Possum LH</td>
<td>(Herbert et al., 2007)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koala (<em>P. Cinereus</em>)</td>
<td>16♀</td>
<td>Antiovine LH GDN#15</td>
<td>NIH-bLH-B10</td>
<td>(Matson et al., 2009)</td>
<td></td>
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<tr>
<td>Koala (<em>P. Cinereus</em>)</td>
<td></td>
<td>Antiovine LH GDN#15</td>
<td>NIH-bLH-B10</td>
<td>(Matson et al., 2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tammar wallaby (<em>M. eugenii</em>)</td>
<td>16♀</td>
<td>Antiovine LH Wa-R oLH</td>
<td>Possum LH</td>
<td>(Herbert et al., 2013)</td>
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</table>

**Plasma Luteinizing Hormone Enzymeimmunoassay in Marsupials**

<table>
<thead>
<tr>
<th>Species</th>
<th>Gender</th>
<th>Monoclonal 518B7</th>
<th>Goat anti-mouse γ-G</th>
<th>Biotinylated Ovine LH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western grey kangaroo (<em>M. fuliginosus ocydromus</em>)</td>
<td>8♀</td>
<td>Monoclonal 518B7</td>
<td>Goat anti-mouse γ-G</td>
<td>Biotinylated Ovine LH</td>
<td>NIH-bLH-B10</td>
</tr>
<tr>
<td>Black-flanked rock wallaby (<em>Petrogale lateralis lateralis</em>)</td>
<td>19♀</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eastern grey kangaroo (<em>M. giganteus</em>)</td>
<td>109♀</td>
<td>Monoclonal 518B7</td>
<td>Goat anti-mouse γ-G</td>
<td>Biotinylated Ovine LH</td>
<td>NIH-bLH-B10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Gender</th>
<th>Monoclonal 518B7</th>
<th>Goat anti-mouse γ-G</th>
<th>Biotinylated Ovine LH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western grey kangaroo (<em>M. fuliginosus ocydromus</em>)</td>
<td>8♀</td>
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<td></td>
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<tr>
<td>Black-flanked rock wallaby (<em>Petrogale lateralis lateralis</em>)</td>
<td>19♀</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Eastern grey kangaroo (<em>M. giganteus</em>)</td>
<td>109♀</td>
<td>Monoclonal 518B7</td>
<td>Goat anti-mouse γ-G</td>
<td>Biotinylated Ovine LH</td>
<td>NIH-bLH-B10</td>
</tr>
<tr>
<td>Species</td>
<td>Sex</td>
<td>Primary Antibody</td>
<td>Secondary antibody</td>
<td>LH Label</td>
<td>Reference Standard</td>
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<td>---------------------------</td>
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</tr>
<tr>
<td>Killer whale (<em>Orcinus orca</em>)</td>
<td>6 ♀</td>
<td>AB # 15</td>
<td></td>
<td>NIADDK ovine LH</td>
<td>NIADDK ovine LH</td>
</tr>
<tr>
<td>Five species of Callitrichid Monkeys</td>
<td>16 ♀</td>
<td>Monoclonal 518B7</td>
<td>Goat anti-mouse IgG</td>
<td>hCG CR-125</td>
<td>HSP5-14-77</td>
</tr>
<tr>
<td>Killer whale (<em>Orcinus orca</em>)</td>
<td>3 ♀</td>
<td>Monoclonal 518B7</td>
<td>Goat anti-mouse IgG</td>
<td>125I-Labelled ovine LH</td>
<td></td>
</tr>
<tr>
<td>Bottlenose dolphin (<em>Tursiops truncates</em>)</td>
<td>10 ♀</td>
<td>Monoclonal 518B7</td>
<td></td>
<td>Biotinylated ovine LH</td>
<td>NIH- bovine</td>
</tr>
<tr>
<td>White-sided dolphin (<em>lagentorhynchus obliquidens</em>)</td>
<td>12 ♀</td>
<td>Monoclonal 518B7</td>
<td></td>
<td>Biotinylated ovine LH</td>
<td>NIH- bovine</td>
</tr>
<tr>
<td>Asian elephant (<em>Elephas maximus</em>)</td>
<td>6 ♀</td>
<td>Monoclonal 518B7</td>
<td>Sheep anti-mouse IgG (#M8645)</td>
<td>125I-Labelled ovine LH</td>
<td>NIH- oLH-I-4</td>
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<tr>
<td>African elephant (<em>Loxodonta africana</em>)</td>
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<td></td>
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<tr>
<td>Beluga whale (<em>Delphinapterus leucas</em>)</td>
<td>4 ♀</td>
<td>Monoclonal 518B7</td>
<td>Goat anti-mouse IgG</td>
<td>Biotinylated ovine LH</td>
<td>NIH-bovine</td>
</tr>
<tr>
<td>Amazonian manatee (<em>Trichechus inunguis</em>)</td>
<td>3 ♀</td>
<td>Monoclonal 518B7</td>
<td>Goat anti-mouse IgG</td>
<td>NIH- oLH-26</td>
<td>NIH-bovine</td>
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</tbody>
</table>
1.4.5 Assisted reproductive technologies

Assisted reproductive technologies (ARTs) have been successfully developed and are widely used in production and laboratory animals (Mapletoft and Hasler, 2005; Byers et al., 2006; Maes et al., 2011) as well as in humans (Hovatta et al., 1997; Wright et al., 1999). In some marsupial species, ARTs have been trialled in both common and threatened species with some success (Rodger et al., 2009). Artificial insemination (AI) technology has produced viable offspring in the koala, and this technique has now even been incorporated into state government koala policy (Queensland Government - Environment Protection Agency, 2006). AI has also been used to produce offspring in the tammar wallaby (Paris et al., 2005). Cross-fostering has accelerated breeding of the threatened brush-tailed rock-wallaby (*Petogale penicillata*) (Taggart et al., 2005a), while other ARTs such as hormone-induced superovulation have only been used as a basic research tool in the development of biological control and management of the common brushtail possum in New Zealand where it has become a feral pest species (Molinia et al., 2001).

In wombats, ARTs have been attempted in both males and females with varying results. In males, semen collection and cryopreservation has been conducted with some promising results with high collection and motility rates in both CW and SHWN reported (Table 1-5). However, in females, ARTs have been less successful (Table 1-6), which may be attributed to the limited knowledge regarding the female reproductive cycle. Previous ART studies (McDonald et al., 2006; Druery et al., 2007; West et al., 2007) were conducted on female wombats without the necessary fundamental knowledge of the oestrous cycle, which is vital for the success of ART. Superovulation techniques using a series of porcine LH and FSH or a single PMSG injection have been shown to increase ovarian activity (McDonald et al., 2006; Druery et al., 2007; West et al., 2007); however, daily hormone injections are invasive and until more information is gained regarding the basic reproductive cycle, especially oestrus and ovulation, the success of ARTs in female wombats will continue to be limited.
Table 1-5. Seminal characteristics of common wombats (CW) and southern hairy-nosed wombats (SHNW) at initial assessment and after cryopreservation.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ejaculate volume</th>
<th>Total sperm number (10⁶/mL)</th>
<th>% motile sperm</th>
<th>Sperm motility rating (0 – 5)</th>
<th>% live sperm</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHNW (wild)</td>
<td>≤ 7.5</td>
<td>9.0 ± 6.2</td>
<td>90-9 PF</td>
<td>4-5 PF</td>
<td>PF</td>
<td>Taggart et al., 1998 (ejaculate)</td>
</tr>
<tr>
<td>CW (wild)</td>
<td>≤ 7.5</td>
<td>7.6 ± 1.6</td>
<td>90-95 PF</td>
<td>5 PF</td>
<td>5 PF</td>
<td>(Bicknell et al., 2001) (epidydimal)</td>
</tr>
<tr>
<td>CW (wild)</td>
<td></td>
<td></td>
<td>70-90 PT</td>
<td>5 PT</td>
<td></td>
<td>(MacCallum et al., 2001) (ejaculate)</td>
</tr>
<tr>
<td>CW (wild)</td>
<td></td>
<td>45 PT</td>
<td>12-40 PT</td>
<td>3-4</td>
<td></td>
<td>(MacCallum, 2005) (fast and slow freezing)</td>
</tr>
<tr>
<td>CW (wild)</td>
<td></td>
<td>60-62 PF</td>
<td>30-70 PT</td>
<td>30-90</td>
<td></td>
<td>(MacCallum and Johnston, 2005) (ejaculate)</td>
</tr>
<tr>
<td>CW (captive)</td>
<td>3.9-7.8</td>
<td>3.8-78.5</td>
<td>83.4 ± 7.3 PF</td>
<td>3.6 PF</td>
<td>94.3 ± 2.6 PF</td>
<td>(Taggart et al., 2005b) (ejaculate)</td>
</tr>
<tr>
<td>CW (wild)</td>
<td>14-75</td>
<td>63.6 ± 3.2 PF</td>
<td>1.5-3 PT</td>
<td>30-60</td>
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</tr>
<tr>
<td>CW (fast)</td>
<td>10-35 PT</td>
<td>3.6 PF</td>
<td>3-4</td>
<td>70-90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CW (slow)</td>
<td>30-70 PT</td>
<td>1-4 PF</td>
<td>1-2 PT</td>
<td>58-72 PT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHNW (wild)</td>
<td>&lt; 1.0 (Jan)</td>
<td>2.7 ± 4.4 (Jan)</td>
<td>58.3 ±30.5 (Jan)</td>
<td>4.5 ± 0.5 (Sep)</td>
<td></td>
<td>(Taggart et al., 2005b) (ejaculate)</td>
</tr>
<tr>
<td></td>
<td>3-9 (Sep)</td>
<td>34 ±31.5 (Sep)</td>
<td>59.8 ±10.8 (Sep)</td>
<td>2.5 ± 1.3 (Nov)</td>
<td>% Intact plasma membrane (0-2 h)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;1.0 (Nov)</td>
<td>4.0 ± 4.7 (Nov)</td>
<td>64.8 ±6.9 (Nov)</td>
<td>% Intact heads (0-2 h)</td>
<td>55-91 (PT)</td>
<td>(Sparrow, 2009)</td>
</tr>
<tr>
<td>CW (wild)</td>
<td></td>
<td></td>
<td>8-84 (PT)</td>
<td>34-98 (PT)</td>
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</tr>
<tr>
<td>SHNW (swan reach)</td>
<td>7.2 ±0.5</td>
<td>15.8 ±4.2</td>
<td></td>
<td></td>
<td></td>
<td>(Johnston et al., 2006) (ejaculate)</td>
</tr>
<tr>
<td>SHNW (Urania)</td>
<td>3.6 ± 0.8</td>
<td>9.7 ± 4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHNW (Kulpura)</td>
<td>7.2 ±0.8</td>
<td>9.0 ±4.6</td>
<td></td>
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</table>

PF: pre-freeze; PT: Post thaw. Adapted from Hogan et al., 2013
Table 1-6. Ovarian superovulation studies in common wombats (CW) and southern hairy-nosed wombats (SHNW).

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Treatment</th>
<th>Follicle number</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW</td>
<td>11</td>
<td>Unstimulated common wombat</td>
<td>15.5/ wombat</td>
<td>(Cleary <em>et al.</em>., 2003)</td>
</tr>
<tr>
<td>SHNW</td>
<td>10</td>
<td>8 x 25 mg pFSH every 12 hr</td>
<td>10</td>
<td>(McDonald <em>et al.</em>., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 mg pLH 12 hr post final pFSH</td>
<td>(0-19)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8 x 6 mg pFSH every 12 hr</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 mg pLH post final pFSH</td>
<td>(0-35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14 x 16.5 mg pFSH every 12 hr</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 mg pLH post final pFSH</td>
<td>(0-18)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1 x 150 IU PMSG</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 mg pLH 72hr after PMSG</td>
<td>(0-23)</td>
<td></td>
</tr>
<tr>
<td>CW</td>
<td>11</td>
<td>8 x 6 mg pFSH every 12 hr</td>
<td>21.1 ± 3.0/ wombat</td>
<td>(West <em>et al.</em>., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 4 mg LH 12 hr post final pFSH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHNW</td>
<td>6</td>
<td>14 x 14.28 mg pFSH every 12 hr (total of 200 mg pFSH)</td>
<td>5.91 ±1.98</td>
<td>(Druery <em>et al.</em>., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 25 mg pLH 12 hr post final pFSH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8 x 25 mg pFSH every 12 hr (total 200 mg)</td>
<td>1.67 ± 0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 25 mg pLH 12 hr post final pFSH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1 x 150 IU PMSG</td>
<td>2.17 ± 1.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 25 mg pLH 72 hr later</td>
<td></td>
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</table>
1.5 Potential non-invasive methods for the detection of oestrous

Once the SHNW reproductive cycle has been properly characterised, correlations between reproductive hormones and other non-invasive methods, such as reproductive behaviour, changes in the reproductive tract and/or urinary cytology, or the use of olfactory cues, can be developed and implemented into captive SHNW breeding programs.

1.5.1 Reproductive behaviour

The identification and validation of specific reproductive behaviours, either from the male or female, can negate the need for regular biological sampling and hormone analysis. Reproductive behaviours have been recorded in a variety of marsupial species (Table 1-7), and have been used to determine accurate timing for pairing individuals for natural mating (Pollock et al., 2010) and artificial insemination (Allen et al., 2008b).

The type of behaviour exhibited by males and females can be species-specific. A female koala in oestrus exhibits a range of antagonistic, restless behaviours, such as pseudo-male mounting and bellowing (Johnston et al., 2000). Female Julia creek dunnarts (Sminthopsis douglasi) exhibit a period of increased activity, indicated by increased wheel running in captivity (Pollock et al., 2010). Female South American grey short-tailed opossums (Monodelphis domestica) exhibit a sequence of behaviours leading up to copulation all of which appear to be invitations to the male; however, copulation does not occur until the female is sexually receptive (Baggott et al., 1987). While specific reproductive behaviours have been observed, changes in normal behaviours have also been reported around times of oestrus or mating. For example, the female Tasmanian devil goes through periods of decreased food consumption which is used to identify the onset of oestrus in females, and subsequently, the appropriate time for the induction to a male (Keeley et al., 2012).
Table 1-7. Reproductive behaviours observed in marsupials.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Behaviour</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quokka (<em>Setonix brachyurus</em>)</td>
<td>♂</td>
<td>Standing erect, Swiping tail rapidly, Urogenital smelling, Urine smelling, Following female, Grasping tail, Chase/pursuit, Penis partially everted</td>
<td>(Sharman, 1955)</td>
</tr>
<tr>
<td>Red kangaroo (<em>M. rufus</em>)</td>
<td>♂</td>
<td>Urogenital smelling, Urine smelling, Following female, Grasping tail, Chase/pursuit, Penis partially everted</td>
<td>(Sharman and Calaby, 1964)</td>
</tr>
<tr>
<td>Tasmanian bandicoot (<em>Permales gunnii</em>) and</td>
<td>♂</td>
<td>Close following and sniffing hind quarters, Pawing females tail and sniffing, lips parted and curled in the corners, Mounting</td>
<td>(Heinsohn, 1966; Stodart, 1966)</td>
</tr>
<tr>
<td>Long-nosed bandicoot (<em>P. nasuta Geoffroy</em>)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red-necked wallaby (<em>M. rufogriseus banksianus</em>) and Bennett’s wallaby (<em>M. r. rufogriseus</em>)</td>
<td>♂</td>
<td>Approach female with penis partly everted, Postpartum mating</td>
<td>(Merchant and Calaby, 1981)</td>
</tr>
<tr>
<td><em>Sminthosis murina, S. Virginiens</em>, <em>Planigale maculatus</em></td>
<td>♂</td>
<td>Vocalisations to attract male</td>
<td>(Van Dyck, 1979; Taplin, 1980; Fox and Whitford, 1982)</td>
</tr>
<tr>
<td>South American grey short-tailed opossums (<em>Monodelphis domestica</em>)</td>
<td>♂</td>
<td>Naso-cloacal contact, Slow circling, Chasing, Hunching, Chattering, Mounting</td>
<td>(Baggott et al., 1987)</td>
</tr>
<tr>
<td>Matschie’s tree kangaroo (<em>Dendrolagus matschiei</em>)</td>
<td>♂</td>
<td>Allogrooming, Urogenital sniffing, Pouch investigation</td>
<td>(Hutchins et al., 1991)</td>
</tr>
<tr>
<td>Koala (<em>Phascolartos cinereus</em>)</td>
<td>♂</td>
<td>‘jerking’ or convulsive behaviour – hiccoughing, Pseudo-male mounting, Bellowing, Increased activity and restlessness</td>
<td>(Johnston et al., 2000)</td>
</tr>
<tr>
<td>Julia creek dunnart (<em>S. douglasi</em>)</td>
<td>♂</td>
<td>Increased activity – wheel running</td>
<td>(Pollock et al., 2010)</td>
</tr>
<tr>
<td>Tasmanian devils (<em>Sarcophilus harrisi</em>)</td>
<td>♂</td>
<td>Decreased appetite, Receptivity to male</td>
<td>(Keeley et al., 2012)</td>
</tr>
</tbody>
</table>

In order for behavioural observation to be used as a reliable non-invasive method of detecting oestrus in females, behaviours need to be matched to accurate hormone profiles. This requires validation trials where hormone profiles in biological samples can be correlated to...
behavioural changes over time. This type of research requires intensive longitudinal observation to gain accurate information about ‘normal’ behaviour and observable changes during the oestrous period. For SHNW, the use of remote monitoring and non-invasive faecal hormone analysis has enabled such a study to be conducted (Hogan et al., 2010b); however, the limitation of hormone analysis prevented confirmation between changes in hormone profiles to changes in behavioural activity surrounding the oestrous period (Hogan et al., 2010b).

1.5.2 Wombat reproductive behaviour

Due to their nocturnal behaviour, it can be difficult to regularly observe reproductive behaviours of both male and female SHNWs. However, behavioural observations have been made for both wild and in captive populations (Gaughwin, 1978; Wells, 1978; Gaughwin, 1979; Gaughwin, 1981; Gaughwin, 1982; Hogan et al., 2011a). In the wild, sexual behaviours have been reported to occur over a 2 to 3 day period initiated by the male following investigation of female urogenital region or urine (Gaughwin, 1979). One report found there was chase / pursuit behaviour, with the male following the female to her preferred burrow. This was followed by loud vocalisations and ‘scuffling’, suggesting that copulation most likely occurs in the burrow with the male pinning the female in the blind end of a tunnel, preventing her escape (Gaughwin, 1981). Similar behavioural observations have also been reported in captive SHNWs (Wells, 1971; Stenke, 2000), which was later confirmed after an intensive behavioural study conducted using 12 captive SHNW housed at Rockhampton Zoo (Hogan et al., 2011a). Using infrared cameras and lights installed above wombat sleeping chambers and yard enclosures, a full range of general and reproductive behaviours were identified and characterised in both male and female SHNWs (Table 1-8). It was reported that females in captivity exhibit a series of precopulatory behaviours including attracting actions towards or in the presence of the male - nudging, rump presentation and increased urination. However, the limitation of this study was the inability to accurately identify the onset and duration of oestrus by means of E2 faecal hormone metabolites (Hogan et al., 2010a). While these behaviours may indicate the onset of oestrus, there has yet to be a study which confirms these behaviours are associated with elevated E2.
Table 1-8. General and reproductive behaviours of 12 adult captive southern hairy-nosed wombats.

<table>
<thead>
<tr>
<th>General</th>
<th>Courtship</th>
<th>Mating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digging</td>
<td>Urine Smelling (M)</td>
<td>Rump Bite (M)</td>
</tr>
<tr>
<td>Exploring</td>
<td>Approach (M)</td>
<td>Grasp and Restrainment (M)</td>
</tr>
<tr>
<td>Feeding</td>
<td>Urogenital Smelling (M)</td>
<td>Kicking and Turning (M)</td>
</tr>
<tr>
<td>Foraging</td>
<td>Evading (F)</td>
<td>Catch and Release (M/F)</td>
</tr>
<tr>
<td>Grooming</td>
<td>Nudging (F)</td>
<td>Coitus (M/F)</td>
</tr>
<tr>
<td>Sitting-at-rest</td>
<td>Attraction Actions (F)</td>
<td>Break (M)</td>
</tr>
<tr>
<td>Sun Basking</td>
<td>Chase (M/F)</td>
<td></td>
</tr>
<tr>
<td>Sleeping</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stereotypic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from (Hogan et al., 2011a).

1.5.3 Changes in female reproductive tract

In female marsupials, the reproductive tract undergoes distinct morphological changes during each stage of the oestrous cycle (Tyndale-Biscoe and Renfree, 1987). During the follicular phase, uterine endometrial and epithelial cells increase, and the vaginal complex and urogenital sinus are enlarged by hyperplasia and hypotrophy. However, these changes are species-specific. For example, in the Virginian opossum (*Didelphis virginiana*) and tammar wallabies, the lateral vaginal canal enlarges (Renfree, 1983), which differs to the common brushtail possum where this occurs in the median vagina (Pilton and Sharman, 1962), and in the vaginal caecum for bandicoots (Lyne, 1976), banded hare-wallaby (*Lagostrophus fasciatus*), and the ‘boodie’ (*Bettongia lesueur*) (Tyndale-Biscoe, 1968). In the female koala, the clitoris becomes hyperaemic and the hairless pericloacal region becomes enlarged and oedematous (Brown, 1987). However, no consistent pattern in the changes in pouch condition or teat morphology, or in the appearance of these structures were not considered useful for the determination of each stage of the oestrous period (Johnston et al., 2000).

The mucosal lining of epithelium of the median vagina and vaginal caecum are highly secretory during oestrus. The lateral vaginae and the urogenital sinus are lined with squamous epithelium that become cornified and slough in clumps into the lumina. The assessment of the rate and concentration of the epithelial ‘sloughing’ can be used to characterise each stage of the oestrous cycle (Tyndale-Biscoe and Renfree, 1987). The presence and concentration of epithelial cells from vaginal swabs or voided urine has been used to determine accurate timing of pairing.
for natural mating for an number of small marsupials, including captive numbats (*Myrmecobius fasciatus*) (Power *et al.*, 2003), Julia creek dunnarts (Pollock *et al.*, 2010), bandicoots (Lyne, 1976), swamp antechinus (*Antechinus minimus maritimus*) (Wilson, 1986), and the brown antechinus (*Antechinus stuartii*) (Woolley, 1966).

In wombats, morphological changes in female reproductive biology such as urogenital cytology, pouch and genitalia appearance have previously been investigated as possible methods of detecting oestrus (Peters and Rose, 1979; West *et al.*, 2004; MacCallum, 2005; Finlayson *et al.*, 2006). In CW, daily vaginal smears showed a reduction in para-basal cells and leukocytes was recorded four to five days prior to oestrus, and the presence of cornified epithelia cells coincided with vaginal tumescence and increased activity around the period of oestrus (Peters and Rose, 1979). However, there are no consistent patterns in the changes in vaginal smears which could be correlated to changes in urinary reproductive steroid hormones and, therefore, could not be used to accurately predict the reproductive status in CW. Another study on CW broadly correlated the proportion of para-intermediate epithelial cells to faecal P4M (West *et al.*, 2004); however, MacCallum (2005) reported that urogenital cytology could not be used to accurately determine the length of the oestrous cycle in the CW. Nor could changes in anatomical structures, such as the appearance of the clitoris, pericoacal region or pouch morphology be reliably used to characterise cyclic activity or predict the onset of oestrus in the CW.

One study found that due to the level of individual variation between females, changes in epithelial cell concentration could not be correlated to changes in reproductive steroid hormones (Finlayson *et al.*, 2006). Similar to the CW, pouch morphology, such as depth, opening size, wall thickness, cleanliness and teat length was found to be a useful indicator of the reproductive status of the female, these characteristics could not be used to accurately identify individual phases of the oestrous cycle in female SHNW (Finlayson *et al.*, 2006).

While vaginal cytology has been effective in characterising distinguishable changes throughout the oestrous cycle in some marsupials, the variation in wombat vaginal cytology has been ineffective in determining cycle length or accurately identifying the timing of oestrous in both the CW and SHNW. The anatomy of the urogenital sinus makes it difficult to be consistent
with vaginal smears from the epithelium of the sinus wall, and the variation in sinus length between individual females makes it difficult for vaginal cytology to be used as an accurate indexing method of oestrous detection. The methods previously used to collect this information required capture and restraint (Peters and Rose, 1979) and in other cases sedation (West et al., 2004; MacCallum, 2005; Finlayson et al., 2006), methods which would not be practical for long term reproductive assessment. Alternatively, urinary cytology is relatively non-invasive and has previously been used to predict the onset of oestrus in some small female marsupials. The characterisation of urine collected from female SHNW throughout repeated oestrous cycles may provide more insight into vaginal cytology and the natural ‘sloughing’ of epithelial cells during each stage of the oestrous cycle.

1.5.4 Olfaction and pheromones

Odours are a protracted form of communication providing information about territory, reproduction, social status, predators and prey, and the general health of an animal (Dehnhard, 2011). Odours from bodily fluids such as urine, saliva, faeces or cutaneous gland secretions, particularly those of the mouth, chin, pouch and chest, contain a variety of volatile and non-volatile chemicals, pheromones, which animals are able to discriminate, even at very low concentrations (Delbridge et al., 2010). Compared to domestic and laboratory animals, only a small number of studies have investigated different aspects of olfaction and vomeronasal organ in the tammar wallaby (Schneider et al., 2008; Frankenberg et al., 2011; Schneider et al., 2012), and glandular secretions and scent marking in a variety of marsupial species (Fadem, 1987; Mallick et al., 1994; Stoddart et al., 1994; Day et al., 2000; Harder and Jackson, 2003; Zabaras et al., 2005; Tobey et al., 2009; McLean et al., 2012); however, the retrospective roles of these systems in marsupial reproduction is not clearly understood.

There are two types chemical signals related to reproduction, releaser and primer pheromones, which are vital for eliciting different neuroendocrinological and behavioural changes in the ‘target’ animal (Sankar and Archunan, 2004). Releaser pheromones can elicit short-latency behavioural changes such as territory marking, mating or aggressive attacks, along with the modification of physiological functions, such as penile erection and mounting. Primer pheromones induce a delayed neuroendocrinological response, influencing an individual’s
reproductive status, such as the onset of puberty or oestrus in an anoestrous female (Tirindelli et al., 2009; Dehnhard, 2011).

Glandular secretions and scent marking have been investigated in a small number of marsupials and have shown to provide individual and community-specific information. Dominant male sugar gliders (*Petaurus breviceps*), the individual with the highest plasma testosterone concentration, will continually mark themselves, their mates and other individuals within their colony using his gular and/or frontal scent glands (Mallick et al., 1994; Stoddart et al., 1994). Suprasternal scent glands secretions from the male South American grey short-tailed and Virginian opossums can induce oestrus in anoestrous females (Fadem, 1987; Harder and Jackson, 2003). The common brushtail possum is mainly solitary (Day et al., 2000); however, they use their chin, sternal and cloacal glands to mark surfaces as signature mixture to indicate dominance, home territory and dens, allowing for individual recognition, avoid conflicts and prevent mating of close kin (McLean et al., 2012). Male koalas communicate their identity, health status, sexual maturity and dominance through sternal gland secretions, secretions which are influenced by the age of the individual and the breeding season (Tobey et al., 2009). Significant seasonal variation in sternal gland secretions have been reported in the female tammar wallaby which coincide with the breeding season (Zabaras et al., 2005). While glandular secretions and scent marking have not been reported in wombats, this does not mean that other forms of scent marking, urine, faecal, rubbing / scratching, are used to provide information to, and about, conspecifics.

Olfaction in SHNW has previously been investigated determining the differential response to the presence of faeces from different species and conspecifics (Descovich et al., 2012b). This study reported that males were able to differentiate between species and sexes; however, the use of faecal samples collected outside the breeding season prevented the observation of behavioural changes from males to oestrus females. Odours and reproductive chemical signals are linked directly to the hypothalamus which influences mammalian behaviour through neuroendocrine function (Tirindelli et al., 2009). This warrants further investigation in male wombats to determine how odours and pheromones in urine and faeces from conspecifics at different stages of the oestrous cycle may affect male behaviour. Distinct behavioural changes which occur in
males, such as increased exploratory behaviour or exhibition of the ‘flehmen’ response, may possibly be used as a non-invasive method of oestrus detection in SHNW reproduction.

1.5.5 Flehmen response

Oestrus-related pheromones in urine and vaginal secretions can induce behavioural changes in males, such as the flehmen response, which has been observed in a number of animals, including Asian elephants (Thitaram et al., 2009), domestic cattle (Sankar and Archunan, 2004) and sheep (Bland and Jubilan, 1987), water buffalo (Karthikeyan et al., 2013) and donkeys (Canisso et al., 2010). The mechanism of flehmen enables air to be drawn through the nasal cavity into the lumen of the vomeronasal organ, inducing vasoconstriction and dilation of blood vessels lateral to the lumen, acting like a pump, and stimulating the autonomic nervous system (Sankar and Archunan, 2004). A male displaying flehmen after exposure to female urine could potentially be used as an indication of the female’s reproductive status. However, in eutherian mammals, such as the Asian elephant, flehmen has been reported as a response to any novel odour and may be associated with curiosity rather than directly associated with reproduction (Dehnhard, 2011).

The flehmen response has only been recorded in a few marsupial species, mainly macropods including the Antilopine wallaroo (M. antilopinus) (Croft, 1982), red kangaroos (M. rufus) (Coulson and Croft, 1981) and eastern grey (M. giganteus) and western greys kangaroos (M. fuliginosus) (Coulson, 1997), and the SHNW (Gaughwin, 1979). In SHNW, flehmen was observed followed by erratic sniffing of conspecific urine, occasionally expelling air or snorting through the nostrils and pawing at the ground. Between bouts of intense sniffing, it was reported that the male raised his head and retracted his upper lip, signifying a mechanism of the classical flehmen response in SHNW (Gaughwin, 1979). This description differs to macropods, whereby the upper lip retracts or curls up over the incisors. In SHNW, the upper lips are split resulting in lateral upward retraction and exposure of the large incisors (Figure 1-6).
In the male kangaroos, flehmen was observed after the male nosed a fresh stream of urine from the female which was then followed by copulation (Coulson, 1997). Similarly, in a male SHNW exposed to female excreta (Gaughwin, 1979), after the male had sniffed the female’s cloacal region, or when presented with a swab of the urogenital sinus of a female suspected to be in oestrus (Gaughwin, unpublished data). Consequently, the flehmen response in marsupials has often been associated with reproduction. However, as these observations in the SHNW were based on a single captive male, more information is required to determine if this behaviour is exhibited by all male SHNW or if it is in fact specifically related to reproduction.

Even though hundreds of chemical components of pheromones have been identified, deciphering the role of these olfactory messages requires extensive bioassays of compounds, both individual and as a mixture, and involves the expertise of chemists, physiologists and
behavioural scientists. To better understand the role of pheromones and flehmen in wombat reproduction as a non-invasive method of detecting oestrus, a controlled study needs to be undertaken whereby males are exposed to both urine and faeces from oestrus and non-oestrus females. Such a study would allow for the characterisation of flehmen in direct response to the female’s reproductive status.

1.6 Conclusion and further research

The survival of any species is strongly dependent on successful reproduction. Change in endocrinology is the driving force behind reproduction, and is therefore responsible for major reproductive activities, both behavioural and physiological. The accurate analysis of reproductive hormones can provide valuable information regarding the reproductive function of an individual animal, a species or a population (Ganswindt et al., 2012). Comprehensive understanding in reproductive function of a species is a key component to the development of self-sustaining in and ex situ populations.

The analysis of non-invasive faecal hormones has provided useful, yet limited, information regarding oestrus and ovulation in the female SHNW. As such, an alternative non-invasive biological sample such as urine requires further investigation as a potential tool to providing the important information regarding key reproductive events such as the onset of oestrus and the mechanism of ovulation. A comprehensive analysis of urinary reproductive hormones and urinary characteristics, such as cytology, pH and specific gravity, as well as reproductive behavioural observations, may provide key information about SHNW reproduction, knowledge which may be directly applied to a NHNW conservation and captive breeding.

However, as one cannot assume what works for one species can be directly applied to another, proper validation of all analysis techniques, including collection and storage protocol, and immunoassays for LH and E2, all need to be thoroughly conducted for SHNW urine. Once properly validated, the relationship between changes in urinary hormones and characteristics can potentially be linked to changes in female reproductive behaviour, which should be the subject of further investigation. Additionally, specific behavioural cues from the male during the oestrus period may also provide valuable information regarding wombat reproduction.
The following research chapters have been designed to validate the use of urine as a non-invasive tool for the monitoring reproduction in captive female SHNW. These chapters include the development of a non-invasive collection protocol for the biological validation and assessment of urinary reproductive hormones including LH, E2, and P4. Once validated, these analytical methods will be applied to identifying the relationship between changes in urinary reproductive hormones and reproductive behaviour, as well as the investigation of the flehmen response in males as a potential non-invasive method of detecting oestrus in the female SHNW.
2. Non-invasive urine collection in the female southern hairy-nosed wombat (Lasiorhinus latifrons) with the aid of classical conditioning

Chapter published as

Abstract

We propose that regular urine samples can be used to monitor and characterise the reproductive cycle of the wombat, but this approach has never before been attempted in a marsupial. We conducted a three stage conditioning process for non-invasive urine collection methods in captive wombats which included (1) initial habituation and observation of urination patterns; (2) classical association of a stimulus with urination; and (3) urine collection with the classically conditioned stimulus. Four of the five female wombats selected for this trial were successfully conditioned for urine collection. During stage 2, the animals responded to tactile stimulation 96 times from 208 attempts (46%). In stage 3, urine was successfully collected 399 times from 485 attempts (82%), with the majority of samples being collected in the morning (280/388). Hand-raised females that were previously conditioned for toileting purposes as pouch young responded to the stimulus faster than juvenile females with no prior conditioning. This study is the first to describe the successful collection of urine by classical conditioning in a marsupial.

2.1 Introduction

The development of a successful captive breeding program for the southern hairy-nosed wombat (SHNW – Lasiorhinus latifrons) has been difficult to achieve, as the relationship between reproductive endocrinology and oestrus behaviour is still poorly understood (Hogan et al., 2010a). In addition, the closely related northern hairy-nosed wombat (NHNW – Lasiorhinus kreffitii) is critically endangered with less than 200 individuals left in the wild. While a recovery plan has been developed to manage this species (Horsup and Parks, 2004), limited knowledge of reproduction in both Lasiorhinus sp. has hindered the establishment of an ex situ insurance population, In order to build a profile of the reproductive hormone
changes occurring in the female SHNW prior to and during oestrus, it is important to be able to collect regular biological samples. Current methods of assessing reproductive hormones from the peripheral circulation require capture, restraint and sedation for venepuncture, methods which can be highly stressful, costly and potentially dangerous to both humans and wombats, and can interfere and confound normal hormone secretion and reproduction (Monfort, 2003; Waiblinger et al., 2006).

Non-invasive faecal collection has previously been used to examine reproductive function in both wild and captive wombats; however, to date only androgen and progestogen metabolites have been successfully detected in faecal samples (Hamilton et al., 2000; Paris et al., 2002; Hogan et al., 2010b; 2010c). Thus far, it has been difficult to accurately detect oestrogen metabolites throughout the oestrous cycle, and the rapid degradation of protein hormones, such as luteinizing hormone (Norris and Carr, 2013), and the 3-day delay for digesta (Hogan et al., 2010c) are likely to prevent faecal analysis in the SHNW from being a useful means of either oestrus detection or measurement of reproductive protein hormones. Therefore, and alternative biological samples is required. Similar to faeces, urine is produced constantly and contains various classes of hormone and their metabolites, in particular both reproductive protein and steroid hormones. While Peters and Rose (1979) previously investigated urinary steroid hormones in common wombats (Vombatus ursinus) using metabolic cages, such an approach was likely to be stressful on the animal, resulting in increased cortisol secretion; a phenomenon which has been demonstrated in Wied’s black tufted-ear marmosets (Callithrix kuhl; Smith and French, 1997). A variety of alternative non-invasive collection methods have been employed in eutherian mammals using operant conditioning (Robeck et al., 2005), opportunistic collection methods (Creel et al., 1993), and direct aspiration of the ground (Collins et al., 2012), thereby removing the need for metabolic cages, capture restraint or sedation. In marsupial, one study has investigated the use of classical conditioning, in which a neutral stimulus is paired with a biologically important one to create a conditioned stimulus (Harris, 2010) to train quokkas (Setonix brachyurus) to fear foxes (McLean et al., 2000). The success of this study demonstrated that it is possible to condition a marsupial species, therefore, the objective of this study was to determine if female wombats could provide daily urine samples with the aid of a non-invasive conditioning process.
2.2 Methods and materials

2.2.1 Animals

All female SHNW were housed and managed at the Australian Animals Care and Education (AACE) research facility in Mount Larcom, Central Queensland (23.75°S, 151.00°E) in mixed or same sex groups of two or three individuals. The trial ran from July to October 2013 and was approved by the University of Queensland Animal Ethics Committee (SAFS/171/13AACE).

Of the 11 female SHNWs within the breeding population, only five were chosen for conditioning as they initially tolerated the presence of, and tactile stimulation from, humans. The other six females were too aggressive or extremely timid and were not included due to safety and welfare concerns for both the animals and the researcher. Three of the five females chosen were hand-raised as joeys (~200g) at the AACE facility (F1 - 2006; F2 - 2009; F3 - 2009) and as part of this care were conditioned to eliminate after bottle feeding. This involved the animal being placed in lateral recumbence, with the periculoacal region being lightly scratched until elimination occurred. The other two trial animals had been brought into the facility as juveniles (less than two years of age; F9: 2011 and F10: 2012) and were not exposed to any elimination conditioning.

2.2.2 Conditioning and collection

There were three stages to the procedure for conditioning of urine collection. Stage 1 consisted of a period of habituation and general observation and was conducted over seven days as part of the daily husbandry routine. Habituation involved the researcher being introduced to the wombats via the regular cleaning of the dens and yard enclosures, which was conducted daily between 0700 h and 0900 h, with feeding at 1600h. During this time observations were made of the animal’s natural elimination times and locations by means of direct or digital video surveillance (Figure 2-1A-C).
Stage 2 was a period of classical conditioning of the urination response, conducted over a period of 35 days. Conditioning was conducted twice daily at 0600 h (AM) and 1530 h (PM), just prior to husbandry and feeding. The animal was approached, and if asleep, was awoken, and a small collection tray [(120mm diameter frying pan (Tefal Australia)] placed under the urogenital region. A tactile stimulation of a light scratching in the general proximity of the percloacal region was applied until urination ensued and was continued throughout the elimination process. Conditioning was also attempted if the animal was already awake and there was no evidence of prior urination. The period from application of the tactile stimulus to the commencement of urination was recorded. A conditioning session would last until the animal urinated or for 25 minutes, after which time the test was deemed to have failed. Occurred first. In the latter case, the attempt was considered to have failed.

Stage 3 was the collection of urine. The objective of this stage was to collect a single urine sample per animal per day. The collection methodology utilized for each animal during stage 2 was applied in the morning and if this was unsuccessful, a second attempt was made in the afternoon.

2.3 Results and Discussion

Observations from stage 1 revealed that shortly after waking the wombats urinated in specific areas or preferred elimination areas (PEA) within their dens and yard enclosures, and both stage 2 and stage 3 of the trial were conducted in the PEA identified for each animal. For example, one wombat used the spare sleeping chamber in her den system as her PEA. For hygiene purposes, this den was closed at all other times except during urine collection.
During stage 2 one female became highly aggressive (F2) towards the researcher and was removed from the trial. The remaining four animals responded positively to this method of conditioning and urinated in response to the tactile stimulation 96 times out of 208 attempts (46%). The mean (± SEM) times to urination for all four animals were 256 ± 10.7 seconds (AM) and 315 ± 20.2 seconds (PM). Table 2-1 shows the individual variation between each of the females in the trial. The mean time to urination for the two hand-raised animals (F1 and F3) was shorter than that for the non-hand-raised animals (F9 and F10) (AM: 227 ± 158.4 versus 285 ± 66.7 seconds; PM: 142 ± 47.4 versus 487 ± 112.6 seconds).

Collection success increased during stage 3, with urine being successfully collected 399 times from 485 attempts (82%), with a similar success rate for both AM collections and PM collections (280/338 - 82% versus 119/147 - 81%). The mean (± SEM) time to urination were 167 ± 9.7 seconds in the morning and 187 ± 10.2 seconds in the afternoon. Unlike in stage 2, the mean time to urination varied between the individual females rather than between hand-raised and non-hand-raised groups. F3, who was hand-raised, had the shortest response time (95 ± 9.2 seconds) followed by F10 who was not hand-raised (108 ± 6.9 seconds). F10 had a higher collection success (98%) compared to the other three females (F1: 54%; F3:83%; F9: 82%) despite not receiving previous elimination conditioning.

Figure 2-3 shows the collection success and changes in the mean-time to urination for stages 2 and 3 of the trial. Collection success increased from week four and time to urination decreased from week five, with most collections occurring under 240 seconds. For the females used in this study, 35 days of conditioning appeared to be sufficient to associate the tactile stimulus with the elimination behaviour for both hand raised and non-hand-raised
females, resulting in the expression of a classical conditioning paradigm for this methodology.

Table 2-1. Individual variation of urination behaviour and collection success of four captive southern hairy-nosed wombats during stage 2 and 3 of conditioning. Time expressed as mean seconds (secs) ± standard error of mean (SEM).

<table>
<thead>
<tr>
<th>Wombat ID</th>
<th>Collection time</th>
<th>Time to urination (mean ± SEM)</th>
<th>Collection attempt</th>
<th>Urinated prior to collection (n)</th>
<th>Collection success (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 2: Conditioning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1a</td>
<td>AM</td>
<td>274 ± 136.8</td>
<td>4/9</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>0 ± 0.0</td>
<td>0/23</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>F3a</td>
<td>AM</td>
<td>180 ± 180.0</td>
<td>1/29</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>284 ± 94.8</td>
<td>9/21</td>
<td>2</td>
<td>43</td>
</tr>
<tr>
<td>F9</td>
<td>AM</td>
<td>413 ± 103.2</td>
<td>16/29</td>
<td>0</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>535 ± 133.7</td>
<td>16/24</td>
<td>0</td>
<td>67</td>
</tr>
<tr>
<td>F10</td>
<td>AM</td>
<td>157 ± 30.2</td>
<td>27/29</td>
<td>2</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>439 ± 91.5</td>
<td>23/24</td>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td>Stage 3: Collection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1a</td>
<td>AM</td>
<td>275 ± 37.4</td>
<td>40/60</td>
<td>2</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>83 ± 83.3</td>
<td>1/16</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>F3a</td>
<td>AM</td>
<td>80 ± 9.7</td>
<td>77/93</td>
<td>4</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>135 ± 20.3</td>
<td>29/34</td>
<td>1</td>
<td>85</td>
</tr>
<tr>
<td>F9</td>
<td>AM</td>
<td>223 ± 25.2</td>
<td>72/91</td>
<td>8</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>374 ± 38.9</td>
<td>55/63</td>
<td>2</td>
<td>87</td>
</tr>
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<td>F10</td>
<td>AM</td>
<td>91 ± 7.7</td>
<td>91/94</td>
<td>3</td>
<td>97</td>
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<tr>
<td></td>
<td>PM</td>
<td>156 ± 11.3</td>
<td>34/34</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

*aHand Raised females
Figure 2-3. Mean time to urination and collection success during stages 2 (weeks 1-5) and 3 (weeks 6-18) of conditioning of 4 captive female southern hairy-nosed wombats.

For keepers and investigators, classical conditioning can be a powerful tool for decreasing stress associated with husbandry procedures (Wilson, 2013). To our knowledge this is the first study to report a successful method of collecting non-invasive urine samples from a marsupial species using classical conditioning with the animal associating a tactile stimulus to the desired behaviour. Females without previous elimination conditioning successful responded to the tactile stimulus and provided daily urine samples on demand. However, those animals that were hand-raised and received previous conditioning responded faster to the tactile stimulus. Classical, as opposed to operant, conditioning was used as a preliminary investigation found that food was not an effective bridge as it was often left uneaten. As these captive SHNW were able to associate the tactile stimulus with the desired behaviour, this suggests that an alternative stimulus such as a non-tactile verbal cue or sound (whistle or clicker) may be equally effective in conditioning wombats for urine collection and should be the subject of further investigation.

It should be noted that this method of conditioning was only suitable for 4 of the 11 wombats in this captive colony, so that alternative urine collection methods will also need to
be employed. While not part of the current conditioning trial, we have also successfully employed the use of false floors with drain holes in the animal’s sleeping chamber to collect urine into an underlining tray. Nevertheless, care needs to be taken with this approach as samples in this manner may be prone to contamination with conspecific urine or micro-organisms.

2.4 Conclusion

To our knowledge this is the first study to describe successful collection of urine by classical conditioning in any marsupial species and was achieved for wombats entering captivity both as pouch young to be hand-raised or as juveniles. While not appropriate for all wombats in the colony, we nevertheless now have the ability to regularly (AM and PM) collect a clean mid-stream urine sample from four captive wombats. This will now provide us with a unique opportunity to investigate changes in biologically active hormones or their metabolites secreted in urine at key stages of the reproductive cycle.
2.5 References


[doi](https://doi.org/10.1016/j.anireprosci.2010.01.006)


3. Validation to techniques to measure reproductive hormones in the 
urine of female southern hairy-nosed wombats (*Lasiorhinus latifrons*)

*Chapter published as*
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wombats (*Lasiorhinus latifrons*). *General and Comparative Endocrinology, 252*, 130-141.
doi: 10.1016/j.ygcen.2017.08.003

**Abstract**

Southern hairy-nosed wombats (SHNW) do not breed well in captivity and the 
collection of serial data to accurately evaluate the female reproductive cycle has been limited. 
Three separate trials were conducted to biologically validate and evaluate enzyme-
immunoassays (EIA) for the detection and analysis of luteinizing hormone (LH), oestrogen 
and progesterone metabolites in urine samples collected non-invasively from captive female 
SHNW. Two separate biological hormone challenges were conducted for the biological 
validation of the enzyme-immunoassays; (1) a gonadotrophin releasing hormone challenge to 
experimentally induce an LH surge, and (2) administration of equine chorionic gonadotrophin 
to stimulate gonadal production of oestrogen and progesterone. Urine samples were analysed 
using double antibody EIAs for LH, oestradiol-17β (E2), estrone-3-glucuronide (E1C) and 
progesterone metabolites (P4M). Parallelism was confirmed for each assay and urine was 
standardised for creatinine content. The results from this study showed that the LH and E1C 
EIAs were suitable for the detection for the hormonally induced changes in urinary 
reproductive hormones; however, biologically relevant changes in concentrations of both 
these hormones could not be detected during natural reproductive cycles. Further attempts to 
extract oestrogen metabolites from the samples by enzyme hydrolysis (with a β-
Glucuronidase/Arylsulfatase based buffer) or diethyl ether extraction failed to improve the 
detection of distinct increases associated with oestrus. In contrast, the progesterone EIA was 
suitable for the detection of urinary progesterone concentrations related to both hormonally 
induced and natural oestrous cycle, with patterns proceeding changes in faecal metabolites 
concertation by 2 to 3 days. Therefore, urinary P4M analysis is suitable for the longitudinal 
reproductive assessment of captive female wombats. This is the first study to analyse urinary 
reproductive hormones in SHNW, and while the sampling frequency or the LH EIA was
unable to detect a natural LH surge, and oestrogen EIAs were not effective in measuring endogenous changes in urinary hormones during natural oestrous cycles, the information gained from this research is still valuable. These techniques have the potential to be used for endocrinology research in a variety of marsupial species, and is the first step into the refinement of urinary reproductive hormone analysis techniques for the SHNW.

3.1 Introduction

Southern hairy-nosed wombats (SHNW – *Lasiorhinus latifrons*) do not breed well in captivity (Hogan *et al*., 2013). The Australian captive population is currently not self-sustaining with an average of only one pouch young born per year (Skipper, 2013), many of which do not survive to weaning (V. Nicolson, 2013, personal communication, previous SHNW studbook keeper). In order to improve the captive breeding success of SHNW, there is an urgent need to have a thorough understanding of female reproductive physiology, but information regarding the SHNW female reproductive function is still limited. For example, characterisation of the full endocrinology of the oestrous cycle, especially in terms of the timing of ovulation, has yet to be elucidated (Hogan *et al*., 2013).

While venipuncture may provide immediate information regarding hormones in systemic circulation, it is an impractical methodology for most captive wildlife species, as it typically requires repeated capture, restraint and sedation, which can result in chronic stress and interfere with normal hormone secretion (Waiblinger *et al*., 2006). Alternatively, non-invasive faecal hormone analysis has proven to be useful for the monitoring of progesterone metabolites in female SHNW (Paris *et al*., 2002; Hogan *et al*., 2010a), the measurement of elevated oestrogens indicative of the follicular phase has been problematic (Hogan *et al*., 2010a). Additionally, depending on the species, protein hormones such as follicle stimulating hormone and luteinizing hormone (LH) are not typically detectable in biologically relevant quantities in faeces (Pukazhenthi and Wildt, 2003).

Hormones and/or their metabolites can be secreted in urine within hours of their secretion in blood (Monfort *et al*., 1991; Munro *et al*., 1991; Cano and Aliaga, 1995). Urinary hormone immunoassays are a non-invasive analysis procedure which can be employed for the detection of timing of ovulation (LH), demonstrated in a limited range of mammals, such as
the Indian rhinoceros (*Rhinoceros unicornis*) (Stoops et al., 2004), five species of callitrichid monkeys (*Saguinus Oedipus, Leontopithecus rosalia, L. chrysomelas, Callithrix jacchus, Cebuella pygmaea* (Ziegler et al., 1993), killer whale (*Orcinus orca*) (Robeck et al., 2004), and the Pacific white sided dolphin (*Lagenorhynchus obliquidens*) (Robeck et al., 2009). Therefore, the analysis of urine may provide a more comprehensive evaluation of reproductive hormones in the female SHNW.

Four studies have investigated urinary reproductive steroid hormone detection methods in three marsupial species, urinary progesterone metabolites (P4M) in female numbats (*Myrmecobius fasciatus*) (Matson et al., 2008; Ditcham et al., 2009), urinary oestrogen and progesterone metabolites in two aged Tasmanian devils (*Sarcophilus harrisii*) (Crichton et al., 2003), and a single female koala (*Phascolarctos cinereus*) (Takahashi et al., 2009). Unfortunately, in all these studies, a biological validation associated with a reproductive event was not conducted, and while hormone metabolite concentrations were detected, it is unknown if they were secreted in biologically relevant concentrations. It is important to note that due to species-specific differences in hormone composition and excretion routes (Hodges et al., 2010), each immunoassay needs to be properly validated for each species and biological sample in question.

To date, no study has reported the use of immunoassays for the measurement of urinary reproductive protein hormones in marsupials. Given that it is possible to collect urine from captive female SHNW using either classical conditioning methods and/or direct collection off the floor of their enclosure (Swinbourne et al., 2015), the detection of LH, oestrogen and progesterone metabolites in urine could be highly beneficial for SHNW reproductive assessment. The use of either a synthetic or natural sequence mammalian gonadotrophin releasing hormone (GnRH) to trigger an LH surge, has been successfully used to validate immunoassays for the detection of serum LH in a range of marsupials (Sutherland et al., 1980; Tyndale-Biscoe et al., 1983; Fletcher, 1989; Bryant, 1992; Moore et al., 1996; Rudd et al., 1999; Johnston et al., 2004; Allen et al., 2008a; Matson et al., 2009; Wilson et al., 2013; Ballantyne et al., 2016a; Ballantyne et al., 2016b).
To test the efficacy of urine as a suitable biological sample for the measurement of reproductive hormones (LH, oestrogen and progesterone metabolites) in female SHNWS, a series of biological and immunoassay validation trials were conducted using two exogenous hormones. In the first trial, a supermaximal dose of GnRH agonist (GnRHa) was used to challenge the anterior pituitary to secrete a surge of LH that could then be analysed in both blood and urine. The second trial involved two sequential but increasing doses of equine chorionic gonadotrophin (eCG) in order to stimulate gonadal steroid secretion. Further assay validation included urinary steroid extraction, the analysis of longitudinal sample to evaluate the urinary hormone profiles of a pregnant female and a mated non-pregnant female, as well as a comparison of hormone profiles between matched urine and faecal samples.

3.2 Methods and materials

3.2.1 Animals and animal management

All wombats were housed and managed at Australian Animals Care and Education wombat research and breeding facility in Mount Larcom, Central Queensland (23.75° S, 151.00° E). This study was conducted during two wombat breeding seasons (August to January 2013/14 and 2014/15). Each season is considered as a separate data set as wombats were examined over two distinct breeding seasons. Individual animal, age, weight, housing and allocation of wombats to the different experimental treatments is reported in Table 3-1. Each hormone trial was approved by the University of Queensland Animal Ethics Committee (GnRHa challenge: SAFS/171/13AACE; eCG trial: SAFS/271/14).

Each enclosure consisted of an indoor temperature controlled (air-conditioned) area (5.8 m²) with individual sleeping chambers (3 x 0.6 m² each). The inside enclosure had an adjoining large outdoor yard (between 90 and 100 m²) consisting of soil substrate, partial grass vegetation, and a dirt mound for enrichment. All animals were fed a daily mixed ration of 120 g rolled oats (COLES Smart Buy, Australia), 120 g Gumnut® pellets (MITAVITE, Australia), 35 g oaten chaff (Rich River Chaff and Grain, Australia), 200 g sliced sweet potato. Half cob of corn was also provided three times a week when available and water was available ad libitum.
Indoor infrared dome cameras (SUMO® - Model: CAM35IRHR) and weatherproof outdoor infrared cameras (SUMO® - Model: CAM78IRHR) were installed in all wombat enclosures. Footage was recorded on to a digital video recorder (DVR - KOBI® 16 channel) surveillance system and reviewed daily using XQ Pro Series DVR surveillance software to confirm the occurrence and timing of attempted mating and birthing behaviours.

3.2.2 Daily non-invasive sample collection

Unless otherwise stated, fresh urine samples were collected daily from female SHNW using the methodology previously described by Swinbourne et al. (2015). Urine samples were kept on ice until they could be divided into 2 mL aliquots (original and duplicate samples) and stored at -20 °C. Where possible, uncontaminated fresh faecal pellets were also collected daily directly off the enclosure floor, sealed in a labelled snaplock bag, immediately placed on ice, then stored at -20 °C until hormone analysis could be conducted.

3.2.3 Experiment 1: GnRHa challenge to experimentally induce an LH surge

The first GnRHa challenge was conducted in October 2013. All animals (n = 4; Table 3-1) were restricted to their sleeping chamber and were sedated by an intramuscular hand injection of Zoletil (10 mg/kg) using a 21-gauge needle. Following recumbency, the animal was transported to the adjacent veterinary surgery and maintained on mask using 2 – 5% gaseous isoflurane at a flow rate of 0.3/minute oxygen in a closed-circuit system. A single baseline blood sample (1 mL) was collected from the cephalic vein using a 23-gauge wing-infuser set and a 3 mL syringe and immediately transferred to a 3 mL lithium / heparin blood tube and stored on ice. Following the initial blood collection, 4 µg GnRHa (Buserelin, Intervet, Australia) was administered intravenously and serial blood samples were collected from the cephalic vein on alternative limbs at 5, 10, 20, 30, 60 and 120 minutes post GnRHa injection. After the final blood sample was collected (120 minutes), all samples were centrifuged for 10 minutes at 1800 rpm and the serum stored at -20 °C until hormone analysis.

Urine samples for this challenge were collected at approximately 48, 24, 6 and 3 hours prior to injecting GnRH. Urine samples were collected from anaesthetised wombats at 20, 30,
60, 120 and 180 minutes post GnRHa injection by palpation of the abdomen, applying firm pressure to the bladder until urine was released from the urethra into the urogenital sinus. After the final urine sample was collected, the animal was placed back into their locked sleeping chamber and recovery was monitored using both direct visual observation and indoor infrared video cameras. Once the animal had completely recovered from the anaesthesia, urine was subsequently collected at approximately 8 hour intervals for four days following the challenge.

The GnRHa challenge trial was repeated in January 2015 (n = 4; Table 3-1). The sampling schedule was the same as that described for the first GnRHa challenge; however, for the second trial two baseline blood samples (1 mL each) were collected 15 and 5 minutes prior to the GnRHa injection, and a higher dose of GnRHa (10 µg) was used. To ensure an adequate dose was administered to induce a detectable LH surge, two females (F7 and F11) were injected with an additional 5 µg GnRHa 30 minutes following the first injection.

3.2.4 Experiment 2: Equine chorionic gonadotrophin (eCG) stimulation to experimentally induce changes to the urinary secretion of oestrogen and progesterone metabolites

Equine chorionic gonadotrophin (eCG) stimulation was conducted during the 2014 wombat breeding season (October to December 2014). Each female was administered two sequential and increasing doses of eCG (Folligon, Intervet, Australia), 150 IU was administered on day 1 and another 300 IU was injected 53 days later. For each injection, females (n = 4; Table 3-1) were confined to their sleeping chamber and received an intramuscular injection in their hind quarter / rump. Each female was monitored for approximately one hour after the injection, then released. Fresh, urine samples were collected daily using the non-invasive methods previously described by Swinbourne et al. (2014). All urine samples were stored at -20 °C until hormone analysis could be conducted. No sedation or anaesthetic was used in this stimulation trial.
3.2.5 Enzyme-immunoassay validation for urinary reproductive hormones

Parallelism for urinary LH was confirmed using pooled animal samples (information provided in section 3.2.4.2) whereas parallelisms for urinary steroid hormones were confirmed for individual females (parallelism graphs are not shown). Information regarding sample dilution range has been provided under each hormone assay sub-section.

3.2.5.1 Urinary creatinine

Urine samples were standardised for water content using a creatinine (Cr) assay (Cayman Chemicals, Michigan, USA). Briefly, 100 µL diluted samples (dilution range: 1:500 to 1:2000), standards (0.0005 – 0.03 mg/mL) and high and low controls were dispensed in duplicate into a 96-well microtiter plate (Corning Inc., USA). Fifty µL of 0.75M NaOH was added to each well immediately followed by 50 µL 0.04M picric acid. The plate was incubated at room temperature for 10 minutes. The optical density for all assays was measured at 490 nm with a reference filter of 650 nm on a Biotek (Elx808) plate reader with Gen5 software (Millennium Science, Australia). All urinary hormone concentrations were expressed as ng/mg Cr.

3.2.5.2 Urinary luteinizing hormone (LH)

Serum and urinary LH was quantified using a monoclonal anti-bovine LH antibody (518-B7: provided by Janet Roser from the UC Davis, USA) EIA previously validated for serum LH in eastern grey kangaroos (Macropus giganteus) (Wilson et al., 2013). Briefly, 50 µL of undiluted serum samples and diluted urine samples (1:2 in assay buffer), standards (0.156 – 20 ng/mL) and high and low controls were dispensed in duplicate into goat anti-mouse gamma globulin (GAMG) pre-coated 96-well microtiter plate (Corning Inc., USA). LH antibody (final LH antibody dilution in assay buffer was 1:400 000) was added into each well (except blank or zero well) and the EIA was incubated in the dark overnight at room temperature (approximately 24 °C). Biotinylated labelled conjugate (final dilution 1:300 000) was added to each well and incubated for 3.5 hours at room temperature before utilising streptavidin and a 3,3’,5,5’-tetramethylbenzidine (TBM, Sigma Aldrich, Australia, catalogue # T2885) colour reaction steps to evaluate hormone concentrations.
Table 3-1. Age, weight, housing, validation method and trial date for female southern hairy-nosed wombats used for validation of urinary luteinizing hormone, estrone-3-glucuronide, oestradiol 17β, and progesterone metabolite enzyme-immunoassays.

<table>
<thead>
<tr>
<th>Wombat ID</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Housing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GnRH trail #1: October 2013</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td>6*</td>
<td>31.1</td>
<td>Female only pair</td>
</tr>
<tr>
<td>F6</td>
<td>6*</td>
<td>28.3</td>
<td>Female only pair</td>
</tr>
<tr>
<td>F9</td>
<td>4</td>
<td>20.6</td>
<td>Female only pair</td>
</tr>
<tr>
<td>F10</td>
<td>3</td>
<td>23.5</td>
<td>Female only pair</td>
</tr>
<tr>
<td><strong>eCG trial: October to December 2014</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F6</td>
<td>7</td>
<td>30.8</td>
<td>Female only pair</td>
</tr>
<tr>
<td>F7</td>
<td>10*</td>
<td>21.5</td>
<td>Female only pair</td>
</tr>
<tr>
<td>F8</td>
<td>10*</td>
<td>22.7</td>
<td>Female only pair</td>
</tr>
<tr>
<td>F11</td>
<td>11*</td>
<td>30.8</td>
<td>Female only</td>
</tr>
<tr>
<td><strong>Urinary steroid extraction. Samples collected from August to December 2014</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>9</td>
<td>24.6</td>
<td>Breeding pair</td>
</tr>
<tr>
<td>F2</td>
<td>5</td>
<td>22.0</td>
<td>Breeding pair</td>
</tr>
<tr>
<td>F9</td>
<td>5</td>
<td>22.6</td>
<td>Breeding pair</td>
</tr>
<tr>
<td>F10</td>
<td>4</td>
<td>23.5</td>
<td>Breeding pair</td>
</tr>
<tr>
<td><strong>Biological validation: pregnant cycle in 2013 and mated, non-pregnant cycles in 2014</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>4</td>
<td>24.8</td>
<td>Breeding pair</td>
</tr>
<tr>
<td>F10</td>
<td>4</td>
<td>23.5</td>
<td>Breeding pair</td>
</tr>
</tbody>
</table>

*Age is estimated as the female was rescued and brought into captivity as an adult. All other females were hand raised as pouch young or brought into captivity as a juvenile.

GnRH#1 and GnRH#2: wombats used for gonadotrophin releasing hormone agonist
eCG: wombats injected with equine chorionic gonadotrophin for gonadal steroid stimulation.
Steroid extraction: wombats used for additional oestrogen extraction studies.

3.2.5.3 Urinary esterone-3-glucuronide (E1C)

Urine samples were analysed for E1C (antibody R522-2 provided by Coralie Munro, UC Davis, USA) using a modified EIA previously validated for bottlenose dolphins (*Tursiops truncates*) (Robeck et al., 2005). Briefly, antibody (1:150 000 in phosphate assay buffer) was dispensed into a goat anti-rabbit gamma globulin (GARG) pre-coated 96-well microtiter plate (Corning Inc., USA) and incubated in the dark overnight at room temperature. Following incubation, 50 μL of diluted samples (dilutions range between 1:5 and 1:16), standards (0.078 – 5.0 ng/mL), high and low controls and E1C horseradish peroxidase enzyme conjugate (HRP label; 1: :200 000) were dispensed in duplicate into the plate and incubated for 3 hours. An
2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt colour reaction (ABTS - Sigma Aldrich, Australia, catalogue# A1888) was used to evaluate hormone concentrations.

### 3.2.5.4 Urinary oestradiol-17β (E2)

Urine samples were analysed for E2 (antibody R4972 provided by Coralie Munro, UC Davis, USA) using a modified EIA previously evaluated for SHNW faecal samples (Hogan et al., 2010a). Briefly, E2 antibody (1:185 000) was dispensed into a GARG-coated 96-well microtiter plate and incubated overnight in the dark at room temperature. Following incubation, 50 µL of diluted samples (1:4), standards (0.049 – 25.0 ng/mL), high and low controls and E2 HRP label (1:320 000) were dispensed in duplicate into the plate and incubated for 3 hours. An ABTS colour reaction was used to evaluate hormone concentrations.

### 3.2.5.5 Urinary and faecal progesterone metabolites (P4M)

Prior to analysis, daily faecal samples were extracted following the methodology described in Hogan et al. (2010a). Both urinary and extracted faecal samples were analysed using a modified P4M EIA (antibody CL425 provided by Coralie Munro, UC Davis, USA) previously validated for SHNW faecal samples (Hogan et al., 2010a). Briefly, antibody (1:80 000) was dispensed into a pre-coated GAMG-coated 96-well microtiter plate (Corning Inc., USA) and incubated in the dark overnight at room temperature. Following incubation, 50 µL diluted samples (dilutions ranged between 1:32 and 1:128), standards (0.0156 – 4.0 ng/mL), high and low controls and P4M HRP label (1:400 000) were dispensed in duplicate into the plate and incubated for 3 hours. An ABTS colour reaction was used to evaluate hormone concentrations.

### 3.2.5.6 Enzyme-immunoassay validation

To determine if compounds within the urine sample were interfering with accurate detection of hormone concentrations, a hormone recovery test was conducted for LH, E1C
and P4M. Individual animal urine pools were spiked with known volumes of concentrated LH, E1C or P4M standard and analysed to evaluate for accuracy of hormone detection. For LH, three separate animal urine pools were each divided into four dilutions (neat, 1:2, 1:3 and 1:8). Dilutions were further divided into four aliquots, three aliquots were spiked with an LH concentration of either 2.5, 1.25 or 0.625 ng/mL, and one aliquot was left untreated. For E1C, animal urine pools were diluted neat, 1:4, 1:8 and 1:16, and for progesterone, animal urine pools were diluted neat, 1:8, 1:32 and 1:64. E1C and progesterone aliquots were spiked at either 1.25, 0.625 or 0.313 ng/mL of hormone standard, and one aliquot from each dilution was left untreated. Spiked hormone concentrations were chosen based on values found in the middle of each standard curve, and dilutions were chosen based on individual animal parallelisms for each assay. Samples were analysed in quadruplicate.

As each EIA had been previously validated for a variety of species, information regarding cross-reactivity of the antibodies can be found elsewhere; LH (518-B7) (Matteri et al., 1987), E1C (R522-2) (Monfort et al., 1990), E2 (R4972) and progesterone (CL425) (Hogan et al., 2010a). The sensitivity for each EIA, based on 85 – 90% specific maximum binding was: LH: 0.156 ng/mL; E1C: 0.078 ng/mL; E2: 0.098 ng/mL; P4M: 0.313 ng/mL and Cr 0.001 ng/mL. The intra and inter-assay coefficients of variation based on the analysis of the high and low controls was: LH: 7% and 13%; E1C: 2% and 12%; E2: 3 and 13%; P4M: 4% and 13%, and Cr; both 2%. For E1C, P4M and Cr, parallelism was confirmed for individual animal urine pools for each assay, which also enable the identification of accurate sample dilutions for each animal. Parallelism for the LH assay was confirmed using grouped animal serum and grouped animal urine pools.

3.2.6 Urinary oestrogen sample preparation and steroid extraction

To determine the optimal urine handling and analysis process for the detection of oestrogen metabolites, different extraction or processing methods were trialled for four females (Table 3-1) for one complete oestrous cycle each. Urine samples were divided into four treatment groups; (1) analysis of E1C from untreated samples, (2) the analysis of oestradiol 17β (E2) from untreated samples, (3) steroid extraction with an enzyme hydrolysis buffer, then analysis of E2 (E2+EHB), and (4) double extraction using an enzyme hydrolysis buffer, followed by liquid extraction with diethyl ether, then analysed for E2 (E2+EHB/DE).
3.2.6.1 Enzyme hydrolysis

The steroid extraction methods used in this study have been described previously in Heisterman et al., (1993). Briefly, the urine sample was thawed, inverted to mix, then centrifuged at 2500 rpm at 4 °C for 10 minutes. Two hundred microliters of urine were incubated for 20 hours in a water bath at 37 °C with 200 µL enzyme hydrolysis buffer containing 0.15 M sodium acetate, pH 4.6, 1:200 000 L-ascorbic acid, and 1:100 β-Glucuronidase/Arylsulfatase (Roche Diagnostics GnnH, Germany, catalogue# 10 127 060 001). After incubation, the solution was inverted to mix and 200 µL was removed into a separate 5 mL plastic tube for further extraction in diethyl ether. The remaining sample was diluted in assay buffer (1:4), and analysed for E2.

3.2.6.2 Liquid extraction

A modified liquid extraction method was developed using information previously described in Heger and Neubert (1983) and the methodology recommended by Arbor Assays® (USA. http://www.arborassays.com/resources/#protocols). Briefly, 1 mL diethyl ether (Merck Pty. Ltd., Australia, catalogue# 1.00921.2500) was added to a 5 mL plastic test tube containing the 200 µL enzyme hydrolysed urine solution (1:5 v/v solution:solvent ration). The tube was vigorously vortexed for 2 minutes, then placed on a rotating shaker at 200 rpm for 10 minutes. After shaking, the tubes were left to stand for 5 minutes to allow the solvent layer to separate, then stored at -80 °C until the bottom layer was completely frozen.

After freezing, the solvent layer was decanted into a clean 5 mL plastic test tube, sealed and placed into the freezer. The remaining frozen solution was thawed at room temperature, and 1 mL diethyl ether was added to the original tube. The liquid extraction method was repeated with the second solvent layer added to the first, making a total 2 mL diethyl ether extracted solution. The 5 mL tube was placed into a heating block and the solution was dried under air at 37°C until the solution had completely evaporated. The desiccated sample was reconstituted in assay buffer (1:2 dilution) and shaken for 5 minutes before being analysed for E2.
3.2.7 Reproductive hormone characterisation

Baseline values for all urinary hormone profiles were calculated using the iterative process described in Moreira et al. (2001). All values > 2 SD from the mean were temporarily removed from the data set and the mean recalculated. This process was repeated until no more values > 2 SD could be removed. The remaining mean was then adopted as the baseline hormone concentration. Values ≥ 2 SD were considered as a significant increase in hormone concentration and peak hormone concentrations were classified as the maximal hormone concentration before returning to baseline values. To characterise reproductive cycles, a sustained increase in P4M concentration above baseline for ≥ 3 consecutive days was defined as the onset of the luteal phase which concluded when P4M concentration returned to baseline values. The period between the end of one luteal phase and the beginning of the next was classified as either the follicular phase or inter-oestrous period (Hogan et al., 2010a). To confirm biological validation, the hormone profiles from challenged females (n = 4) were compared to two natural reproductive events; (1) an oestrous cycle with a successful mating and parturition, and (2) a three consecutive non-pregnant oestrous cycle. Faecal samples from the same females were analysed and compared to urinary P4M profiles.

3.2.8 Data analysis

Parallelism of serially diluted (neat to 1:16) serum and urine samples against the LH standard curve was measured using simple linear regression (Brown et al., 2010). LH serum and urine data was logarithm transformed and a correlation between serum and urine LH profiles was estimated using regression analysis (Paris et al., 2002). An analysis of variance was conducted in SPSS® (PASW Statistics 18. IBM® Corporation) to determine differences in baseline hormone concentrations between natural cycling females and hormone challenged females. Urinary oestrogens were analysed using a linear mixed model on logarithm transformed data to determine the effect of treatment on hormone concentration. Samples were nested within individual animals, and the model used a Bonferroni adjustment. Significance levels were set at P < 0.05 and mean hormone concentrations were expressed as mean ± standard error of mean (SEM). Pearson’s correlation test was used to determine the relationship between urinary and faecal P4M hormone profiles. First, the samples collected on the same calendar day (unadjusted) were analysed, then to compensate for the excretion
lag time, the same data set was adjusted so that the P4M peaks in urine and faeces were aligned (adjusted) and the data set was reanalysed (Shideler et al., 1993). Significance levels were set at P < 0.05, and mean hormone concentrations were expressed as mean ± pooled SEM.

3.3 Results

The pooled serum samples showed a stronger parallelism to the bovine LH standard ($R^2 = 0.99$, $P = 0.001$) compared to the pooled urine samples ($R^2 = 0.69$, $P = 0.01$. Figure 3-1A). The mean recovery of spiked urine pools for LH, P4M and E1C was 112%, 102% and 101% (Figure 3-1B), revealed little matrix interference during the analysis of urine samples.

3.3.1 Experiment 1: Measurement of luteinizing hormone

Analysis of the individual undiluted serum samples from the first GnRH challenge (4 µg) failed to show a significant rise (> 2 SD baseline) in LH in any of the four females (Figure 3-2). The mean (± SEM) pre-GnRH serum LH was 0.87 ± 0.15 ng/mL and the mean peak serum LH concentration was 1.08 ± 0.15 ng/mL ten minutes after the GnRHa injection. As no significant LH elevation was found in the serum sample analysis, individual urine samples from the first GnRH challenge were not analysed.

For the second GnRH challenge (10 µg dose), serum samples were analysed as neat and urine samples were analysed at a 1:2 dilution. The mean (± SEM) pre-GnRH serum LH was $1.7 ± 0.60$ ng/mL ($F6: 3.2; F7: 0.8; F8: 0.7; F11 2.2$ ng/mL) which increased to between 1.8 and 4.2 ng/mL approximately ten minutes following GnRHa injection (Figure 3-3A). The mean (± SEM) pre-GnRH urinary LH was $0.07 ± 0.02$ ng/mL Cr ($F6: 0.05; F7: 0.08; F8: 0.12; F11 0.06$ ng/mg Cr) and peaked between 30 and 60 minutes following the GnRHa injection (peak range $0.12 – 0.23$ ng/mg Cr. Figure 3-3B). It was not possible to collect urine from F11 between T0 and T60, nor was it possible to collect urine from anesthetised F7 (T-5 to T120) by means of palpation of the bladder.
Figure 3-1. A) Serial dilution (neat to 1:8) of pooled serum (circles) and pooled urine (triangles) samples compared against bovine luteinizing hormone (LH) standard curve (squares). B) Hormone recovery assays of individual animal urine pools spiked with 2.5, 1.25 or 0.625 mg/mL LH standard, 2.5, 1.25 or 0.625 mg/mL progesterone standard (P4), or 1.25, 0.8, or 0.5 ng/mL estrone-3-glucoronide (E1C) standard.
Figure 3-2. GnRHa challenge 1 (2013). Mean (± SEM) serum luteinizing hormone concentration (ng/mL) of serial blood collections from four captive female southern hairy-nosed wombats injected with 4 µg GnRHa (Buserelin: Intervet). Baseline LH: 0.87 ± 0.15 ng/mL and peak LH: 1.08 ± 0.15 ng/mL.
3.3.2 Experiment 2: Measurement of urinary reproductive steroid hormones

Baseline (± SEM) hormone concentration for urinary E1C for F6, F7, F8 and F11 were 0.50 ± 0.02, 0.39 ± 0.02, 0.61 ± 0.03 and 0.33 ± 0.03 ng/mg Cr, respectively. Baseline hormone concentration for urinary P4M for F6, F7, F8 and F11 were 2.87 ± 0.17, 2.22 ± 0.09, 4.32 ± 0.16, and 1.34 ± 0.27 ng/mg Cr, respectively. The urinary steroid hormone analysis showed within and between animal variation (Figure 3-4A-B) in hormone concentrations or
patterns of hormone response. Females responded to one or both eCG injections which resulted in a significant increase in E1C followed by a subsequent rise in P4M between ten and 15 days following the injection. F6 appeared to be in the peak of a luteal phase when administered 150 IU eCG which resulted in peak E1C approximately seven days post-injection, followed by a rise in P4M from day 17 post-injection. F6 did not respond to the second, higher eCG injection as both urinary hormones remained around baseline values for the remainder of the 2014 breeding season. F7 appeared to be in anoestrum prior to the hormone stimulation as changes in urinary hormones only occurred after both eCG injections. The 150 IU eCG injection resulted in a sustained increased in both urinary E1C and P4M for F7; however, the second higher eCG injection (300 IU) resulted in a stronger response, as peak hormone concentrations more than doubled in value compared to the first injection. F8 was at the end of the luteal phase when stimulated with 150 IU eCG resulting in a slow rise in E1C, which was then followed by a sustained increase in P4M. Once again, F8 appeared to be at the end of the luteal phase when administered the second higher eCG, while there was no significant rise in E1C, P4M peaked eight days following the injection. F11 was in an interoestrous period when injected with the 150 IU. There was no significant rise in E1C after the 150 IU injection; however, there was a sustained P4M increase 14 days post-injection. Based on urinary P4M, the second eCG injection for F11 was administered during the mid-luteal phase. Two days following the injection, E1C significantly increased for seven days and P4M continued to rise, peaking 13 days following the 300 IU injection.
Figure 3-4. Results of hormone analysis from daily urine samples collected from four captive female southern hairy-nosed wombats injected with equine chorionic gonadotrophin (eCG) during the 2014 wombat breeding season. Vertical lines indicate day of eCG injection: D₀ = 150 IU; D₅₃ = 300 IU. A) Urinary oestrogen metabolites (E1C). B) Urinary progesterone metabolite (P4M).

3.3.3 Urinary oestrogens: samples preparation and steroid extraction

After extraction, there was a significant treatment effect on absolute urinary oestrogen metabolite concentrations (F₃, 591.7 = 265.3; P < 0.001). The mean hormone concentrations for E1C, E2, E₂+EHB and E₂+EHB/DE were 0.42 ± 0.02, 0.77 ± 0.02, 0.88 ± 0.02 and 0.35 ± 0.02 ng/mg Cr, respectively. Urine samples extracted using only the enzyme hydrolysis buffer (E₂+EHB) had consistently higher hormone concentrations compared to the other three treatments, with the double extraction method using diethyl ether (E₂+EHB/DE) having the
lowest mean concentration of urinary oestrogens. However, despite the significant extraction treatment effects on oestrogen metabolite concentrations, when compared to changes in urinary P4M concentrations, there was no significant difference found in the pattern or magnitude of changes between each treatment group that could be associated with the follicular phase, oestrus or ovulation in this species (Figure 3-5).

Figure 3-5. The use of different oestrogen analysis techniques to characterise the female southern hairy-nosed wombat (SHNW) oestrous cycle. Urine samples were analysed for oestrogen metabolites (E1C), oestradiol-17β (E2), and E2 after two different steroid extraction methods; enzyme hydrolysis buffer (E2+EHB) and enzyme hydrolysis buffer + diethyl ether (E2+EHB/DE). There was a significant extraction treatment effect on overall hormone concentration (P < 0.001); however, neither the unextracted or extracted E2 or E1C concentrations could be used to identify the follicular phase, oestrus or the onset of ovulation in the female SHNW.
3.3.4 The application of the urinary hormone EIAs during reproductive activity of two female wombats

The urinary hormone profiles from a pregnant female (F3) and a naturally cycling female (F10) were used here to demonstrate and compare urinary hormone profiles between natural cycling and hormone challenged females (Figure 3-6 and Figure 3-7). There were no differences between baseline urinary LH and E1C concentrations between the two groups (P > 0.05); however, mean baseline P4M of the challenged females was significantly greater than those of natural cycling females (2.69 ± 0.28 versus 1.55 ± 0.19 ng/mg Cr, respectively. P = 0.038).

Daily urinary LH concentrations for the pregnant female ranged from undetectable (below the lower limits of the LH assay sensitivity) to 0.38 ng/mg Cr (Figure 3-6). While there was a significant single day spike (> 2 SD above baseline) in urinary LH two days following the last mating bout, the fluctuation of urinary LH surrounding mating was at a similar magnitude and concentration, so that a distinct pre-ovulatory LH surge could not be detected. E1C was significantly above baseline values for the first ten days of urine collection (during the luteal phase), returning to baseline values and remained relatively constant during the oestrus, mating period and early gestation (collection days 11 to 42; Figure 3-6). E1C then increased significantly above baseline two days prior to partition and remained elevated throughout early lactation. Urinary P4M was above baseline values for the first 17 days of urine collection, dropping below baseline from day 18 to 27 (Figure 3-6). Mating bouts were observed from day 23 to day 25 of urine collection. A sustained increase in urinary P4M started two days following mating indicated the onset of a luteal phase. Urinary P4M peaked on day 46 (7.026 ng/mg Cr), then returned to baseline levels from day 54 of urine collection. Based on the recorded behavioural video, parturition was observed on day 46 of urine collection, suggesting an approximate 20-day gestation from the last mating bout.

Female SHNW, F10, was mated multiple times during the 2014 wombat breeding season, and urinary P4M profiles show three distinct luteal phases. Therefore, it was assumed that this female was cycling normally (Figure 3-7). After bouts of mating and the detection of a retrograde seminal plug on the enclosure floor, the female was removed from the male and monitored for birth; however, no pouch young was ever discovered. Urinary LH showed
similar patterns to F3, whereby LH was significantly increased sporadically above baseline levels before and after mating; however, a distinct pre-ovulatory LH surge was not detected in daily urine samples from F10 (Figure 3-7). Similarly, there were no distinct differences in E1C profiles during the first two oestrous cycles as E1C fluctuated around baseline values from day 12 and 29, both prior to and after the onset of the luteal phase (day 21 of urine collection). While there was a distinct increase in E1C prior to the onset of the third luteal phase, E1C was also significantly increased throughout the duration of the third luteal phase (Figure 3-7). Similar to F3, there were no consistent discernible changes in urinary E1C during the first two oestrous cycle for F10. While E1C increased significantly prior to the onset of the final luteal phase, it was significantly above baseline for 19 days (collection day 80 to 98), including six days into the luteal phase. As a result, the inconsistencies in the E1C concentrations could not provide valuable information regarding the follicular phase, oestrus or ovulation for this female. Peak urinary P4M concentrations progressively increased for each subsequent luteal phase (Figure 3-7). For example, during the first luteal phase, peak urinary P4M was 7.17 ng/mg Cr, 9.20 ng/mg Cr during the second luteal phase, and 10.16 ng/mg Cr during the third luteal phase.

There were individual differences between urine and faecal P4M concentrations and secretory patterns observed for F3 and F10 (Figure 3-8). F3 had a lower peak P4M concentrations compared to F10 (peak urinary P4M: 5.31 versus 8.54 ng/mg Cr, respectively, peak faecal P4M: 159.18 versus 348.14 ng/g). With respect to P4M secretory patterns, the correlation coefficient between the unadjusted urinary and faecal P4M profiles for F3 was 0.52 (P > 0.001), which increased to a correlation coefficient of 0.80 (P > 0.001) when adjusted for the three-day faecal lag time. For F10, the correlation coefficient between the unadjusted urinary and faecal P4M was lower compared to F3; 0.40 (P > 0.001), and increased to 0.64 (P > 0.001) when adjusted for the faecal time lag. While both females were mated, only F3 produced a pouch young during the research period.
Figure 3-6. Urinary reproductive hormone profile of a female southern hairy-nosed wombat (F3) prior to and including a successful mating and gestation. The grey box indicates a three-day mating period. Baseline urinary LH was 0.21 ± 0.02 ng/mg Cr and increased to 0.376 ng/mg Cr two days following the last bout of mating. Baseline E1C and P4M were 0.38 ± 0.03 and 1.72 ± 0.14 ng/mg Cr, respectively.

Figure 3-7. Urinary reproductive hormone profile from a cycling female southern hairy-nosed wombat (F10) during the 2014 breeding season. The grey box indicates a four-day mating period. Baseline urinary LH was 0.09 ± 0.02 ng/mg Cr and peaked at 0.76 ng/mg Cr on day 30 of urine collection, during the mid-luteal phase of the first oestrus cycle. Baseline urinary E1C and P4M were 0.34 ± 0.06 and 2.26 ± 0.23 ng/mg Cr, respectively.
Figure 3-8. Unadjusted urinary progesterone metabolite (P4M) profiles from matched urine samples and uncontaminated faecal samples collected using non-invasive methods from two female southern hairy-nosed wombats at different stages of the reproductive cycle. The grey box indicates period of mating. A) P4M profile of a successful mating and gestation of female F3. B) P4M profile of a cycling female during the 2014 wombat breeding season of female F10.

3.4 Discussion

This is the first study to evaluate and validate EIAs for the detection and analysis of urinary reproductive hormones in female SHNWs, as well as the first to report a biological challenge to detect luteinizing hormone excreted in marsupial urine. Despite positive detection of changes in urinary hormone patterns after exogenous hormone injections, a lack of detection of expected hormone of LH and oestrogen patterns in natural oestrous cycles suggest that further research is warranted to refine detection techniques or find more suitable hormone metabolite specific EIA systems.
Of the four published studies that have investigated reproductive steroid hormones in marsupial urine, two were validation studies associated with numbat urine, but reproductive hormone profiles were not assessed in detail (Ditcham et al., 2009; Matson et al., 2009). Urinary oestrogens were analysed in a single female koala (Takahashi et al., 2009), and changes in both urinary oestrogen and progesterone were detected in samples collected from two aged Tasmanian devils (Crichton et al., 2003). For all these studies, while validation of the assay was conducted through parallelism of the standard curve, limited biological validation was performed to determine if the immunoassay was appropriate for the hormone of interest or the species in question. For example, in the koala, changes in urinary reproductive hormones could not be correlated to changes in reproductive behaviour (Takahashi et al., 2009). This may be due to the female not being provided with access to a male or similarly to the results of this study, the EIA used to measure urinary oestrogen may not have been appropriate for the matrix analysed – marsupial urine.

In the current study, each of the EIAs were thoroughly validated through experimental biological validation, assay parallelism tests, hormone recovery tests, and the mapping of known reproductive events (e.g. mating and parturition) to changes in hormone concentrations, and the comparison between a previously validated method, faecal analysis to urinary hormone concentrations. The results from this study show that the P4M antibody, CL425 (Coralie Munro, UC Davis, USA), was suitable for the longitudinal reproductive assessment in captive female SHNWs utilising either urinary or faecal sampling, which also confirmed a 2 to 3-day lag between the excretion of progesterone metabolites in urine and faeces. While LH and E1C antibodies were suitable for detecting respective urinary hormones changes when the females were experimentally challenged, the assays were not able to detect natural changes in the wombat reproductive cycle associated with an LH surge or oestrus, thereby currently limiting their potential use to conduct reproductive assessment on this species in urine.

The 518-B7 antibody (Jan Roser, UC Davis, USA) was chosen for the LH urinary assay as it cross-reacts with circulating and urinary LH in a diverse range of mammalian species (Matteri et al., 1987), including the successful detection of serum LH surge in the Sumatran
rhinoceros (*Diceros sumatrensis*) (Roth *et al.*, 2001), Asian (*Elephas maximus*) and African elephants (*Loxodonta Africana*) (Brown *et al.*, 2004; Brown *et al.*, 2010; Czekala *et al.*, 2003; Graham *et al.*, 2002) as well as the detection of urinary LH surges in species such as the bottlenose dolphin (Robeck *et al.*, 2005), and beluga (*Delphinapterus leucas*) (Steinman *et al.*, 2012). It has also been used to detect natural and artificially induced LH activity in detected serum from marsupials, such as the koala (Ballantyne *et al.*, 2016a; Ballantyne *et al.*, 2016b), tammar wallaby (*M. eugenii*) (Sutherland *et al.*, 1980; Rudd *et al.*, 1999), western grey kangaroo (*M. fuliginosus ocydromus*), black-flanked rock wallaby (*Petrogale lateralis lateralis*) (Matson *et al.*, 2009), and the eastern grey kangaroo (Wilson *et al.*, 2013) but its potential to detect LH in marsupial urine had yet to be tested.

In order to validate the LH assay for SHNW urine, an initial GnRH challenge was conducted in 2013 using only 4 µg Buserelin (GnRHa). The same dose has been used successfully to determine the steroidogenic capacity (testosterone) of the SHNW testis (Hogan *et al.*, 2010b), as well as during a successful biological challenge in female koalas (Allen *et al.*, 2008a). The 4 µg GnRHa resulted in a sustained increased in serum LH from 60 minutes, and peak serum LH concentrations at 240 minutes post-injection in the koala (Allen *et al.*, 2008). Based on these two studies, we initially anticipated a detectable rise in both serum and urinary LH; however, more than double that dose (10 µg GnRHa) was required to adequately stimulate the SHNW anterior pituitary and elicit a significant rise in LH sufficient for detection in serum.

Further, the LH antibody was able to measure acute artificial changes in female wombat urinary LH, but not able to detect biologically meaningful or relevant concentrations, such as the pre-ovulatory LH surge, during natural oestrous cycles or prior to pregnancy with the current sample collection frequency. The longitudinal LH profile from F3 (Figure 3-6) showed fluctuations in LH prior to mating, followed by a significant increase two days after the final mating bout. While this may appear to be a pre-ovulatory LH surge, the magnitude of the increased urinary LH was similar to the preceding fluctuations observed following the increase in urinary P4M, prohibiting the detection of levels indicative of a pre-ovulatory LH surge as seen in the other mammals, such as the bottlenose dolphin (Robeck *et al.*, 2005). The urinary LH of F10 was similar to F3, in that urinary LH increased significantly prior to a sustained increase in P4M. However, urinary LH also increased to a similar concentration.
approximately 10 days later during the luteal phase. The subsequent LH peak from F10 (Figure 3-7) may have occurred during the mating period; however, the concentration was lower than two previous peaks, the last LH peak that was observed in this female also occurred during the luteal phase making its biological significance ambiguous. It is possible that these peaks observed during the luteal phase are associated with follicular waves, as previously reviewed in domestic water buffalo (*Bubalus bubalis*) (Perera, 2011), but the lack of amplitude difference between the SHNW LH peaks suggests it is more likely “noise” or unresolved variation in the data. Perhaps, more research needs to be conducted into alternative LH analysis, such as the analysis of the LH beta core-fragments which can also be used to identify LH surges in urine, when the real LH surge has been obscured by cross-reactivity (O'Connor *et al.*, 1998).

A limitation for detecting LH during natural cycles in female SHNW may be that the urine collection protocol employed for the longitudinal assessment of LH may not have been appropriate to allow satisfactory differentiation of the LH surge. In the female bottlenose and white sided pacific dolphin, urine sample collection frequency increased from daily to up to five time a day when females were in the periovulatory period. This enabled the timely identification of the LH surge, which in conjunction with ultrasonography, was used to develop an artificial insemination protocol in these species (Robeck *et al.*, 2005; Robeck *et al.*, 2009) In the tammar wallaby, the total duration of the LH surge is about 12 hours; which included a rapid increase to peak levels over the first four hours followed by a slower decline back to baseline (Sutherland *et al.*, 1980). The pre-ovulatory LH surge in the nocturnal brushtail possum (*Trichosurus vulpecula*) last between seven and nine hours and occurs early in the morning, between 2 and 6am (Crawford *et al.*, 1999). If the SNHW is similar to the tammar wallaby and the brushtail possum, it is likely that the ovulatory LH surge could easily be missed if samples were collected only once per day or if the pooling of hormones within the urine masked the LH surge if urination was infrequent.

Additionally, during the second GnRH challenge, urinary LH more than doubled 30 minutes after GnRHa was administered, returning to around baseline levels between one to two hours post-injection. This suggests that the clearance rate of LH may be rapid in female SHNW and was only detectable during the challenge where urine collections were 15 to 60 minutes apart. A study conducted on male rats showed that labelled ovine LH had a half-life
of 5 minutes in circulation, and degraded labelled LH was present in the tissue of the liver, testicle, kidneys and excreted in the urine 60 to 90 minutes after injection (Ascoli et al., 1975). The circulatory half-life of human LH occurs between 30 and 60 minutes in middle-aged women, and the LH found in the urine 24 hours later was mostly degraded (Kohler and Parmele, 1967).

Consequently, the limited sampling frequency, in conjunction with the possibility of hormone degradation between urine collection times or as a result of freezer storage, may have prevented the accurate evaluation and detection of biologically relevant changes in urinary LH in female SHNW. We therefore recommend further research into the rate of LH clearance and possible degradation of LH in wombat urine. Increased sampling times during both day and night may improve the detection of the LH surge in female SHNW, as well as the use of an LH tracer, which would enable the accurate identification of the LH metabolic pathway and rate of decay within this species. While, the addition of a preservative to the urine samples has been shown to decrease post-collection degradation of the hormone during freezing and processing (Kesner et al., 1995), caution is required as some preservatives may also alter the immunoreactivity of the hormone (Hunt and Wasser, 2003). Furthermore, the use of ultrasound to observe follicle growth and ovulation, a non-invasive methodology employed for monitoring reproduction in marine mammals (Robeck et al., 2005), would also provide valuable information regarding timing of the LH surge and ovulation in female SHNW, but there are of course logistical difficulties in applying this technology without anaesthesia to behaviourally unconditioned wombats.

To validate EIAs for SHNW urinary reproductive steroid hormones, biological validation using eCG injection was used to increase gonadal stimulation in each of the trial animals. A single dose of 150 IU eCG had previously been used to increase ovarian activity in the SHNW (McDonald et al., 2006; Druery et al., 2007). Both studies reported a high level of individual animal variability in response to the hormone treatment, as some females did not respond at all to the eCG while others produced over 20 ovarian follicles ≥1 mm. Hence, a single injection of eCG appeared to be suitable starting point for gonadal stimulation in at least some female wombats. Therefore, in the current study, we anticipated an increase in urinary oestrogen metabolites and subsequent rise in P4M after administration. The initial eCG dose was then followed up with a higher eCG dose to ensure reproductive steroid
hormones could be detected in urine samples. Our preliminary results suggest that the response to the eCG was dependent on which stage of the reproductive cycle the female was at when the hormone was administered. Additionally, urinary hormone concentrations were greater in the eCG females (Figure 3-4) than the concentrations reported during the two natural oestrous cycles (Figure 3-6 and Figure 3-7), which is likely to be associated with the artificial stimulation of the gonad as noted in oestrus-induced bitches (England and Allen, 1991).

The E1C antibody, R522-2, (Coralie Munro, UC Davis, USA) has been shown to be effective in the longitudinal reproductive assessment of urinary oestrogens in range of eutherian species, including the Yunnan snub-nosed monkey (Rhinopithecus beiti) (He et al., 2001), killer whales (Robeck et al., 2004) and bottlenose dolphins (Robeck et al., 2005). In the current study, E1C concentration yielded biologically measurable and meaningful concentrations from hormonally challenged females, but the same could not be said for naturally cycling females. In SHNW, the E1C profiles during natural cycles were similar to those reported in saddle-back tamarins (Saguinus fuscicollis), whereby E1C concentrations fluctuated but remained relatively constant during both the follicular and luteal phases (Heistermann and Hodges, 1995) limiting its use to detect changes associated with oestrus. Therefore, to refine the detection and characterisation of oestrogen secretion in SHNW urine we conducted further steroid extraction methods and explored the use of another E2 antibody, R4972 (Coralie Munro, UC Davis, USA).

A single extraction method with an enzyme hydrolysis buffer resulted in higher E2 concentrations (0.108 – 3.350 ng/mg Cr) compared to the double extraction with diethyl ether (0.023 – 1.309 ng/mg Cr), untreated E1C (0.139 – 0.957) and untreated E2 (0.034 – 1.998 ng/mg Cr) methods. However, while extraction with just the enzyme hydrolysis buffer appeared to be effective for E2 extraction, significant changes in urinary oestrogens over time were still not detectable in biologically relevant patterns during natural reproductive cycles. It may, therefore, be the case that oestrogens are heavily metabolised and or are degraded during metabolism (Hogan et al., 2010a), and were only measurable from increased artificial stimulation. Once again, the use of a hormone tracer would enable the accurate mapping of the oestrogen metabolic and excretion pathway, which would enable the development of the most appropriate oestrogen metabolite antibody and analysis technique for SHNW urine.
The progesterone antibody, CL425, chosen for this study cross reacts with a number of progestogens and has previously been used for the detection of P4M in marsupial urine (Ditcham et al., 2009), and faeces (North and Harder, 2008; Ballantyne et al., 2009; Pollock et al., 2010; Descovich et al., 2012a; Hogan et al., 2012; Keeley et al., 2012). The CL425 antibody has also been validated for SHNW faeces and was effective for the reproductive assessment of female wombats in captivity (Hogan et al., 2010a). The validation of the CL425 assay for SHNW urine was supported by the hormone profiles observed from the eCG treated females, the natural cycling females and the matched urine and faecal P4M profiles. The secretory patterns of the urinary P4M between the naturally cycling and hormone challenged females were consistent with progesterone profiles previously described in female SHNW (Finlayson (Paris et al., 2002; Finlayson et al., 2006; Hogan et al., 2010a), showing distinct and regular progesterone cycles throughout the wombat breeding season. Additionally, while there were individual differences regarding excretion lag time in urinary versus faecal P4M between F3 and F10, changes in urinary P4M were reflected in faecal P4M profiles two to three days later.

Despite the difficulties in detecting relevant changes in urinary LH and E1C, the collection and analysis of urine for hormone analysis still provided a more immediate assessment for urinary P4M compared to the analysis of faeces. While the collection of faecal pellets from captive SHNW can be conducted without direct contact with individual animals, unless animals are housed individually, the collection of daily urine samples negates the need for a faecal marker, such as non-toxic glitter (Hogan et al., 2011). Urine samples are also collected fresh and can be stored almost immediately, reducing hormone degradation. Further, for hormone analysis, urine samples can be used neat or diluted in assay buffer without prior steroid extraction necessary for faecal hormone analysis.

3.5 Conclusion

The results of this study represent a major step forward for the development of non-invasive monitoring of reproductive protein and steroid hormones in SHNW and marsupials in general. Under experimental conditions (GnRH and eCG challenges) we have demonstrated the successful validation of LH, oestrogen and progestogen metabolites detection in wombat urine. Although the LH and oestrogen antibodies used for EIA assay in
this study were not effective at detecting changes associated with ovulation and oestrus, respectively, some of this apparent lack of capability in LH measurement is no doubt likely to be associated with the frequency of the sampling schedule and the short biological half-life of the hormone.

While the P4M urinary assay appeared to provide biologically relevant information consistent with our current understanding of the wombat oestrous cycle and pregnancy, it is clear that more refined analysis is required for the detection of oestrogens in this species. The ability to detect changes in LH, as well as oestrogen secretion in SHNW urine will not only be valuable for the characterisation of the SHNW oestrous cycle, but also for determining oestrus and the timing of ovulation in females for techniques such as timed artificial insemination. Until then, the analysis of urinary P4M provides more immediate information compared to faecal P4M analysis and can be used effectively to assess the reproductive function of captive females.
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4. The reproductive biology of captive southern hairy-nosed wombat
   (Lasiorhinus latifrons): Oestrous cycle characterisation

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Abstract

The southern hairy-nosed wombat (SHNW: Lasiorhinus latifrons) does not breed well in captivity. To better understand the reproductive physiology of this species, nine captive females were conditioned for daily urine samples collected using non-invasive methods during the wombat breeding seasons of 2013 and 2014. Fresh urine samples were assessed for volume (mL), pH, specific gravity, leukocyte concentration and a qualitative index of the number of epithelial cells, and then stored at -20 ºC until further analysis. Frozen-thawed urine samples, standardised for creatinine (Cr), were analysed for progesterone metabolites (P4M) using a previously validated enzyme-immunoassay. Commencement of the luteal phase was defined as the first day of a sustained increase in urinary P4M above the baseline concentration determined using an iterative process. Baseline urinary hormone P4M varied between females and ranged from 0.85 to 4.04 ng/mg Cr. Peak urinary P4M (range 6.99 – 16.32 ng/mg Cr) varied between cycles within females and between females. The mean oestrous cycle length, defined as the end of one luteal phase to the end of the next, was 35.1 ± 2.4 days; however, individual cycle length ranged from 23 to 47 days. The mean luteal phase length was 20.8 ± 1.3 days (range: 12 to 33 days). Females exhibited two to three oestrous cycles during each sampling period, and females housed in breeding pairs had higher overall mean urinary P4M concentrations compared to non-breeding females (2.98 ng/mg Cr versus 1.99 ng/mg Cr, respectively. P = 0.001). Urinary P4M was divided into four oestrous cycle stages; (1) early follicular phase, (2) late follicular phase, (3) early luteal phase, (4) late luteal phase, and analysed against urine volume, pH, urine concentration as evaluated using both specific gravity and creatinine concentration, leukocyte and urogenital epithelial cell concentration to identify possible physiological changes associated with changes in urinary hormones. During the late follicular phase, urine volume decreased (P = 0.002), while urine concentration [specific gravity (P = 0.001) and creatinine concentration (P = 0.001)] and the
index of urinary epithelial cells (P = 0.004) all increased. The level of variability in SHNW oestrous cycles length and hormone concentration observed in this study suggests that some captive females may exhibit abnormal or infertile cycles. Nevertheless, urinary P4M was a useful tool for determining the cyclic activity of captive SHNW, and the physiological changes in the measured urinary characteristics associated with the stages of the oestrous cycle offer an alternative, potentially useful means for the non-invasive monitoring of the reproductive status.

4.1 Introduction

The southern hairy nosed wombat (SHNW - *Lasiorhinus latifrons*) is a large fossorial species that is found in fragmented populations across semi-arid southern Australia (Swinbourne *et al.*, 2016b). Their cryptic, burrowing and nocturnal behaviour makes them a relatively difficult species to study, both in the wild and in captivity and as a result, there is limited information regarding the reproductive behaviour and physiology of this species, and breeding them in captivity has been challenging (Hogan *et al.*, 2013). A better understanding of their reproductive physiology would prove beneficial for the development and facilitation of future captive breeding of the closely related, and critically endangered, northern hairy-nosed wombat (*L. krefftii*), of which only an estimated 230 individuals remain (Department of Environment and Heritage Protection, 2015).

To improve captive breeding and develop assisted reproductive technologies, such as artificial insemination and cross-fostering, more data are needed regarding the reproductive endocrinology that would allow for the accurate assessment of reproductive status of the female hairy-nosed wombat (Hogan *et al.*, 2013). The large size of the SHNW (Jackson, 2003a) and its non-domesticated nature are factors that do not allow for daily blood sampling which is typically too invasive as it requires capture, restraint and sedation. Repeated anaesthesia also has the potential to be costly and stressful for both the animal and the handler, and increased pressure on the hypothalamus-pituitary-adrenal axis can interfere with normal reproductive hormone secretion, hindering or masking the detection of reproductive hormones in blood samples (Waiblinger *et al.*, 2006; Hodges *et al.*, 2010).
The collection and analysis of reproductive hormones in biological samples collected using non-invasive methods is an effective tool for characterising the reproductive biology and assessing the reproductive activity of a number of captive wildlife species (Schwarzenberger, 2007; Schwarzenberger and Brown, 2013; Kersey and Dehnhard, 2014), including captive female SHNW (Paris et al., 2002; Hogan et al., 2010; Swinbourne et al., 2017). While urinary and faecal progesterone metabolites have provided insight into the reproductive biology of captive female SHNW (Paris et al., 2002; Hogan et al., 2010), examination of the oestrous cycle in these studies were limited by the analysis of females over a single breeding season, and by the small number of females in each study [\( n = 2 \) (Paris et al., 2002) and \( n = 8 \) (Hogan et al., 2010)]. Consequently, more research is required to gain a better understanding of SHNW reproductive cycle.

It is also possible that analysis of wombat urine is not only useful for the assessment of reproductive status (Swinbourne et al., 2017a), but physiochemical changes in its composition and cytology may have interpretive value. For example, changes in urine volume have been linked to changes in the oestrous cycle of female house mice (\textit{Mus domesticus}) (Drickamer, 1995), and the presence and concentration of urogenital epithelial cells and leukocytes, collected in urine samples or urogenital swabs, has been used as an effective method of detecting oestrus in a range of marsupials, including the Julia Creek dunnart (\textit{Sminthopsis douglasi}) (Pollock et al., 2010), squirrel glider (\textit{Petaurus norfolcensis}) (Woodd et al., 2006), Gilbert’s potoroo (\textit{Potorous gilbertii}) (Stead-Richardson et al., 2010) the greater bilby (\textit{Macrotis logotis}) (Ballantyne et al., 2009) and numbat (\textit{Myrmecobius fasciatus}) (Power et al., 2009).

There have also been a number of studies that have investigated the use of urogenital cytology to estimate the length of the oestrous cycle in both the common wombat (\textit{Vombatus ursinus}) (Peters and Rose, 1979; West et al., 2004) and the SHNW (Finlayson et al., 2006). While in the common wombat, epithelial cells were high when faecal progesterone was low (West et al., 2004), in the SHNW, the anatomy of the urogenital sinus and the variation in sinus length between individual females makes it particularly challenging to be consistent with the sampling of vaginal smears from the epithelium of the sinus wall, making urinary cytology from vaginal swabs an unreliable indexing method of oestrous cycle characterisation (Finlayson et al., 2006).
The aim of this study was to collect daily urine samples using non-invasive methods from captive female SHNW to evaluate oestrous cycle dynamics within individual females, and to determine if changes in urine characteristics (volume, specific gravity, pH, leukocytes and urogenital epithelial cell concentration) are potentially associated with changes in urinary progesterone metabolites (P4M) as a non-invasive method to improve our understanding of the oestrous cycle and reproductive biology of this species.

4.2 Materials and Methods

4.2.1 Animals and animal management

This study was conducted during two wombat breeding seasons (July 2013 to January 2014; August 2014 to December 2014) and was approved by the University of Queensland Animal Ethics Committee (SAFS/171/13AACE). All wombats (Table 4-1) were housed and managed at the Australian Animals Care and Education (AACE) wombat research facility in Mount Larcom, Central Queensland (23.75oS. 151.00oE). Animals were housed in a temperature-controlled building consisting of eight large indoor enclosures, each containing a common area (5.8 m²) with three individual sleeping chambers (0.6 m² each). Each animal had access to a large outdoor enclosure consisting of soil substrate, partial grass vegetation, logs, tree branches and a dirt mound for enrichment. Animals were fed a daily mixed ration of 120 g rolled oats (Coles, Smart Buy, Australia), 120 g gumnut pellets (Mitavite, Australia), 35 g oaten chaff (Rich River Chaff and Grain, Australia), 200 g sliced sweet potato (locally farmed produce), and water was available ad libitum.

Indoor infrared dome cameras (SUMO® - Model: CAM35IRHR) and weatherproof outdoor infrared cameras (SUMO® - Model: CAM78IRHR) were installed in all wombat enclosures. Footage was recorded and stored on to a digital video recorder (DVR - KOBI® 16 channel) surveillance system, and reviewed using XQ Pro Series DVR Surveillance software, which allowed for real-time and retrospective observation of wombat behaviours for further reproductive behaviour analysis (Chapter 5) and to confirm the occurrence and timing of attempted mating and birthing behaviours.
Table 4-1. Body weights and housing configuration of nine captive female southern hairy-nosed wombats during two consecutive wombat breeding seasons. Each breeding season was considered a separate data set as animals were examined over two distinct breeding seasons: \( n = 12 \) individual animal data sets.

<table>
<thead>
<tr>
<th>Season</th>
<th>Wombat ID</th>
<th>Animal age</th>
<th>Mean weight (kg)</th>
<th>Housing</th>
<th>Den mate</th>
<th>Mean weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>F3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>25.1</td>
<td>Breeding pair</td>
<td>M6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>25.2</td>
<td>Non-breeding pair</td>
<td>M4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.4</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>29.5</td>
<td>Female pair</td>
<td>F6</td>
<td>29.6</td>
</tr>
<tr>
<td></td>
<td>F6</td>
<td>5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>29.6</td>
<td>Female pair</td>
<td>F5</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>F9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>20.5</td>
<td>Female pair</td>
<td>F10&lt;sup&gt;^&lt;/sup&gt;</td>
<td>21.5</td>
</tr>
<tr>
<td></td>
<td>F10&lt;sup&gt;^&lt;/sup&gt;</td>
<td>3</td>
<td>21.5</td>
<td>Female pair</td>
<td>F9</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>F11&lt;sup&gt;*&lt;/sup&gt;</td>
<td>10&lt;sup&gt;*&lt;/sup&gt;</td>
<td>26.4</td>
<td>Female only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>F1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9</td>
<td>25.0</td>
<td>Breeding pair</td>
<td>M1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>36.4</td>
</tr>
<tr>
<td></td>
<td>F2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5</td>
<td>22.0</td>
<td>Breeding pair</td>
<td>M2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.7</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>28.6</td>
<td>Breeding pair</td>
<td>M6&lt;sup&gt;^&lt;/sup&gt;</td>
<td>38.6</td>
</tr>
<tr>
<td></td>
<td>F9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5</td>
<td>22.7</td>
<td>Breeding pair</td>
<td>M1</td>
<td>36.4</td>
</tr>
<tr>
<td></td>
<td>F10&lt;sup&gt;^&lt;/sup&gt;</td>
<td>4</td>
<td>23.6</td>
<td>Breeding pair</td>
<td>M1</td>
<td>36.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Female was successfully mated in August 2013.
<sup>b</sup>Vasectomised male
<sup>c</sup>Hip injury as a joey limited reproductive behaviours as an adult
<sup>*</sup>Age is estimated as the animal was rescued and brought into captivity as an adult.
<sup>^</sup>Animal was hand raised as pouch young or brought into captivity as a juvenile.

4.2.2 Non-invasive urine sample collection

Daily urine samples were collected from all females (Table 4-1) using non-invasive methods previously described by Swinbourne et al. (2015). Briefly, female SHNW were conditioned to provide daily urine samples on demand, either directly into a small collection tray placed under the rump of the animal or aspirated off the clean den floor. Urine was transferred into a 70 mL polypropylene specimen container (SARSTEDT, Germany) and then maintained on ice throughout processing.
4.2.3 Urine characteristics and cytology

Each urine sample was initially measured for volume (mL), pH (Fisher Scientific, Fisherbrand, catalogue # FB33011), leukocyte concentration using a Combur-Test9 urine test strip (four-point scale: 0 – 3) (Roche Diagnostics Ltd, Switzerland), and specific gravity using a handheld refractometer (Bellingham + Stanley) (range 1.000 – 1.080), then divided into 2 x 2 mL aliquots and stored at -20 °C for reproductive hormone analysis using an enzyme-immunoassay (EIA). From the daily urine sample collected, 79/1161 (6.8%) were not analysed for urogenital epithelial cells due to low volume. Depending on the remaining volume of the collected urine, approximately 11 mL was transferred into a 15 mL conical Falcon tube (Fisher Scientific, Fisherbrand, catalogue # 14-959-53A) and centrifuged for 10 minutes at 2000 rpm. After centrifugation, the supernatant was decanted until 1 mL of urine and the pellet remained in the tube. The pellet was resuspended in the 1 mL urine supernatant and a drop of this solution transferred onto a microscope slide and covered with a cover slip. Using a light microscope (Olympus CH2 microscope, Olympus, Japan) at 40X magnification, the relative number of epithelial cells present in each sample was estimated using a four-point scale: 0: none / clean urine; 1+: low level; 2+: moderate number of cells; 3+: high number of cells (Jackson, 2003b). The presence or absence of spermatozoa in the urine was also assessed to determine if a successful mating had occurred.

4.2.4 Urinary progesterone metabolite analysis

The analysis of urinary P4M has previously been described for SHNW (Swinbourne et al., 2017a). The P4M enzyme-immunoassay sensitivity, based on 85 – 90% specific maximum binding, was 0.313 ng/mL and the intra and inter-assay coefficients of variation based on the analysis of the high and low controls were 4 and 13%, respectively. Urine samples were standardised for water content using a creatinine (Cr) assay (Cayman Chemicals, Michigan, USA). The intra- and inter-assay coefficient of variation for the creatinine assay were both 2%. Hormone concentrations are expressed in ng/mg Cr.

4.2.5 Reproductive cycle characterisation

Baseline hormone concentrations were calculated for each female using the iterative process described in Moreira et al. (2001). All values > 2 SD from the mean were temporarily
removed from the data set and the mean recalculated. This process was repeated until no more values > 2 SD could be removed. The remaining mean was then adopted as the baseline hormone concentration. Values ≥ 2 SD were considered as a significant increase in hormone concentration and peak hormone concentrations were classified as the maximal hormone concentration during a sustained increase in urinary P4M before returning to baseline values. A sustained increase in P4M concentration above baseline for ≥ 3 consecutive days was defined as the onset of the luteal phase, and the first day of the sustained rise was classified as day 0 (D₀). The luteal phase concluded when urinary P4M returned to baseline values. The period between the end of one luteal phase and the end of the next was classified as an oestrous cycle (Hogan et al., 2010).

4.2.6 Statistical analysis

All statistical analysis was conducted using PASW Statistics GradPack 18 (formally SPSS® IBM®). The oestrous cycle of the SHNW was previously calculated as approximately 36 days, consisting of a 12-day follicular phase and a 24-day luteal phase (data extrapolated from Paris et al., 2002; Finlayson et al., 2006; Hogan et al., 2010) and formed the basis of determining what constituted early versus late follicular and luteal phase stages for evaluation against urine parameters. Each follicular and luteal phase was divided into two separate stages, for a total of four different oestrous cycle stages (Figure 4-1); (1) the early follicular phase occurred from day -12 to day -7, (2) the late follicular phase consisted of day -6 to day -1, (3) the early luteal phase was the first seven days following a sustained increase in urinary P4M above baseline (day 0 to day 6), and (4) the late luteal phase was the last seven days prior to urinary P4M returning to baseline levels (to account for variable luteal phase lengths). For cycles that had less than a 12-day follicular phase period, the six days prior the onset of the early luteal phase was counted as the late follicular phase and the remaining days prior were considered part of the early follicular phase.

All data were log₁₀ transformed and tested for normal distribution by the Shapiro-Wilk test (Villasenor Alva and Estrada, 2009). An analysis of variance (ANOVA) was conducted to determine the relationship between the four oestrous cycle stage periods and individual urinary characteristics, including urine volume, pH, urine concentration as evaluated using both specific gravity and creatinine concentration. A paired T-test was
performed to determine the relationship between changes in leukocyte and urogenital epithelial cell concentration during each of the four oestrous cycle stages (Pollock et al., 2010). A Pearson’s correlation test was conducted to determine the relationship between the two urine standardisation methods; specific gravity and urinary creatinine concentration, during each stage of the oestrous cycle, and tested to determine the relationship between urinary progesterone values standardised for creatinine versus urinary progesterone values standardised for specific gravity. Lastly, females were divided into two groups; (1) breeding pair or (2) female pairs only or single housed female, to determine whether the presence of the male had any effect on urinary progesterone concentration and luteal phase length.

4.3 Results

Of the nine females that were evaluated during both the 2013 and 2014 sampling period, data were obtained for 19 luteal phases but only 16 complete oestrous cycles (Figure 4-2). Unfortunately, data obtained for F5 and F6 only provided partial information on oestrous cycle activity due to an extended period required to successfully condition them for urine collection and because they had previously had been used in a GnRH validation trial conducted in October 2013 (Swinbourne et al., 2017a). Consequently, data collected from these two females prior to the GnRH challenge trial was included in the urine characteristics analysis only. F9 and F10 also were involved in the same GnRH validation trial in 2013; however, as they responded more rapidly to conditioning for urine collection, data on natural reproductive activity prior to the trial could be included in both the oestrous cycle and urinary characteristics analysis.

There was a high degree of variability in the urinary P4M concentration and the oestrous cycle length between and within individual females (Table 4-2). Urinary P4M profiles indicated that these captive females were polyoestrous, cycling between two or three times within each sampling period (Figure 4-2). The mean oestrous cycle length was $35.1 \pm 2.4$ days (range between 23 to 47 days) and the mean luteal phase length was $20.8 \pm 1.3$ days and ranged between 12 and 33 days. Baseline urinary P4M ranged from 0.84 to 4.04 ng/mg Cr (Table 4-2), and peak urinary P4M concentrations ranged from 6.99 to 16.32 ng/mg Cr and varied between cycles within females. F1, F2, F4 (2014), and F11 exhibited higher peak urinary P4M at the beginning of the breeding season (September/October: Figure 4-2B), and
F10 (2014) had higher peak urinary P4M concentrations later in the breeding season (November/December: Figure 4-2J). Females housed in breeding pairs had a higher overall mean urinary P4M concentration (mean 2.99 ± 0.09 ng/mg Cr) compared to female only pairs or single housed females (mean 1.92 ± 0.13 ng/mg Cr. F_{1,1032} = 40.819; P = 0.001). While the individual luteal phases varied between females, there was no difference in the mean luteal phase length between females housed in breeding pairs compared to female only pairs or single housed females (19.4 ± 2.3 versus 22.8 ± 2.2 days. F_{1,15} = 1.155; P = 0.299).

During 2013, one female (F3; Figure 4-2A) had a successful mating (over three consecutive nights), birthing behaviours were observed (using the indoor infrared cameras) 21 days from the last bout of mating and a pouch check 60 days following mating confirmed the presence of a pouch young. During the same breeding season (2013), F4 was housed with a vasectomised male (M6), and while no mating behaviour was ever observed between the two animals, hormone analysis showed that F4 had three complete oestrous cycles (Figure 4-2F). The oestrous cycle lengths for F4 increased over the course of the 2013 sampling period (23 days, 25 days, then a 30-day oestrous cycle). F9 and F10 were housed together in a female only pair in 2013 and both females had low baseline urinary P4M levels compared to the other females (0.85 and 1.01 ng/mg Cr). F9 was in a luteal phase at the beginning of the 2013 sampling period which lasted 30 days before returning to baseline values briefly for 6 days, then significantly increasing again for 33 days. Urinary P4M returned to baseline levels again for 21 days indicating an extended period of baseline P4M prior to the next luteal phase (Figure 4-2D). For F10, urinary P4M remained below baseline values until day 68 of urine collection when urinary P4M significantly increased for 17 days, returning to baseline values for four days, then remained significantly elevated for the remainder of the 2013 sampling period (Figure 4-2E). Urinary P4M for F11 (Figure 4-2B) demonstrated two contrasting oestrous cycles in 2013, one lasting 22 days from the end of the luteal phase to the end of the subsequent luteal phase (consisting of a 17-day luteal phase). This was followed by a longer oestrous cycle (38 days) which consisted of a 14-day luteal phase.

In the 2014 sampling period, nine individual oestrous cycles were evaluated, and mating behaviour was observed in multiple pairings, but no pouch young were produced. F4 exhibited a very different urinary P4M profile in 2014 compared to 2013. In 2014, luteal phases for F4 were difficult to accurately identify due to elevated urinary P4M during the first
half of the sampling period (Figure 4-2H), with the only notable difference being that she was housed with a larger male (M5) who demonstrated persistent mating behaviour (chasing, rump bites, grasp/restraint, occurring between 10 and 33 days apart over 5 to up to 9 consecutive days). Two complete oestrous cycles were measured for F9 (2014), and while the variability in luteal phase length between the 2 seasons was greater in 2014 (20 and 33 days) compared to 2013 (26 and 27 days), the mean luteal phase length was similar (2013: 25.5 days versus 2014: 26.5 days). There was no increase in urinary P4M following the end of the second luteal phase suggesting that F9 entered an anoestrous state for the remainder of the 2014 sampling period (Figure 4-2I). F10 had three distinct oestrous cycles throughout the 2014 sampling period, with the last oestrous cycle having an extended period prior to the commencement of the following luteal phase (Figure 4-2J).

Urine concentration, evaluated as both specific gravity ($F_{3,1097} = 8.532; P = 0.001$) and urinary creatinine concentrations ($F_{3,1097} = 17.735; P = 0.001$), was highest during the late follicular phase compared to the other three stages of the oestrous cycle (Table 4-3). There was also a significant correlation between the two urine standardisation methods ($P = 0.001$; Figure 4-3); however, when analysing individual hormone profiles, the correlation between urinary progesterone values standardised for creatinine and urinary progesterone standardised for specific gravity varied between females, demonstrating both weak and strong relationships (Table 4-4). Both standardisation methods produced similar urinary progesterone profiles as there was no observable difference in the pattern of the urinary progesterone profiles (Figure 4-4).
Figure 4-1. Schematic of the estimated southern hairy-nosed wombat (SHNW) 36-day oestrous cycle divided into the follicular phase and the luteal phase (based on data extrapolated from Paris et al., 2002; Finlayson et al., 2006; Hogan et al., 2010). Each phase is further divided into two stages; the early follicular phase from day -12 to day -7; the late follicular phase from day -6 to day -1; early luteal phase from day 0 to day 6; the late luteal phase the last seven days prior to urinary P4M returning to baseline levels (eg. day 17-23 if the luteal phase = 23 days).

Table 4-2. Reproductive cycle dynamics of ten captive female southern hairy-nosed wombat data sets during the 2013 and 2014 wombat breeding seasons. The luteal phase was classified as the beginning of a sustained increase in urinary P4M above baseline values to when urinary P4M returned to baseline values. An oestrous cycle length was the period between the end of one luteal phase to the end of the subsequent luteal phase.

<table>
<thead>
<tr>
<th>Season</th>
<th>Animal</th>
<th>Urinary P4M (ng/mg Cr)</th>
<th>Luteal phase length (days)</th>
<th>Oestrous cycle length (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>Range</td>
<td>n / sampling period</td>
</tr>
<tr>
<td>2013</td>
<td>F3*</td>
<td>1.72</td>
<td>0.14 – 7.03</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>1.37</td>
<td>0.11 – 5.00</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>F9</td>
<td>0.85</td>
<td>0.03 – 5.89</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>F10</td>
<td>1.01</td>
<td>0.17 – 7.62</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F11</td>
<td>1.56</td>
<td>0.34 – 12.32</td>
<td>2</td>
</tr>
<tr>
<td>2014</td>
<td>F1</td>
<td>1.63</td>
<td>0.82 – 12.88</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>1.94</td>
<td>0.73 – 8.17</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>4.04</td>
<td>0.87 – 12.96</td>
<td>3</td>
</tr>
</tbody>
</table>

*F3 was successfully mated during the 2013 breeding season and data on previous oestrous cycles were unavailable.
Figure 4-2. The urinary progesterone metabolite (P4M) profiles of captive female southern hairy-nosed wombats during the 2013 and/or 2014 breeding season. The grey boxes indicate periods when urinary P4M was above individual baseline values. Baseline (BL) urinary progesterone values for each female: A) F3 (2013): successful mating (broken line box) and gestation; B) F11 (2013); C) F4 (2013); D) F9 (2013); E) F10 (2013); F) F1 (2014); G) F2 (2014); H) F4 (2014); I) F9 (2014); J) F10 (2014).
Table 4-3. Urinary characteristics; volume (Vol), specific gravity (SG), pH, and urinary creatinine concentration (Cr), of daily urine samples collected and analysed for captive female southern hairy-nosed wombats during the early and late follicular phase (FP) and early and late luteal phase (LP) based on urinary progesterone metabolites concentration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Early FP</th>
<th></th>
<th>Late FP</th>
<th></th>
<th>Early LP</th>
<th></th>
<th>Late LP</th>
<th></th>
<th>P-Value (df 3,1027)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Range</td>
<td>Mean ± SEM</td>
<td>Range</td>
<td>Mean ± SEM</td>
<td>Range</td>
<td>Mean ± SEM</td>
<td>Range</td>
<td>F</td>
</tr>
<tr>
<td>Vol (mL)</td>
<td>26 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1-142</td>
<td>25 ± 1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1-100</td>
<td>31 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1-122</td>
<td>30 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2-156</td>
<td>4.071</td>
</tr>
<tr>
<td>SG</td>
<td>1.036 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00-1.07</td>
<td>1.039 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00-1.07</td>
<td>1.033 ± 0.001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.00-1.08</td>
<td>1.033 ± 0.001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.00-1.08</td>
<td>6.758</td>
</tr>
<tr>
<td>pH</td>
<td>6.5 ± 0.07</td>
<td>4.0-9.0</td>
<td>6.4 ± 0.08</td>
<td>5.0-9.0</td>
<td>6.3 ± 0.08</td>
<td>4.0-9.0</td>
<td>6.4 ± 0.04</td>
<td>4.0-9.0</td>
<td>1.990</td>
</tr>
<tr>
<td>Cr (ng/mg)</td>
<td>5.6 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35-14.4</td>
<td>6.07 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3-18.1</td>
<td>4.8 ± 0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2-15.2</td>
<td>4.3 ± 0.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3-14.72</td>
<td>18.412</td>
</tr>
</tbody>
</table>

Bold data points indicate significant hormonal / urine characteristic relationship
<sup>abc</sup> superscripts determine the significant differences between each stage of the oestrous cycle.
Figure 4-3. The correlation between two urine standardisation methods; specific gravity and creatinine concentration ($R^2 = 0.71$; correlation coefficient 0.840; $P = 0.001$).

Table 4-4. Individual animal correlation between urinary progesterone standardised for creatinine versus urinary progesterone standardised for specific gravity.

<table>
<thead>
<tr>
<th>Season</th>
<th>Animal ID</th>
<th>$n$ samples</th>
<th>$R^2$ Value</th>
<th>Correlation coefficient</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>F3</td>
<td>76</td>
<td>0.20</td>
<td>0.452</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>103</td>
<td>0.48</td>
<td>0.694</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>38</td>
<td>0.74</td>
<td>0.861</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>F6</td>
<td>55</td>
<td>0.16</td>
<td>0.395</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>F9</td>
<td>95</td>
<td>0.54</td>
<td>0.734</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>F10</td>
<td>93</td>
<td>0.21</td>
<td>0.460</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>F11</td>
<td>104</td>
<td>0.09</td>
<td>0.304</td>
<td>0.002</td>
</tr>
<tr>
<td>2014</td>
<td>F1</td>
<td>111</td>
<td>0.52</td>
<td>0.718</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>120</td>
<td>0.44</td>
<td>0.666</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>124</td>
<td>0.52</td>
<td>0.719</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>F9</td>
<td>117</td>
<td>0.54</td>
<td>0.736</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>F10</td>
<td>125</td>
<td>0.43</td>
<td>0.655</td>
<td>0.001</td>
</tr>
</tbody>
</table>
While urine volume was significantly lower ($P = 0.007$) during the late follicular phase compared to the early follicular phase or during both luteal phase stages (Table 4-3), there were no significant changes in urinary pH between each of the oestrous cycle stages ($P = 0.114$; Table 4-3). Regarding urinary cytology, urogenital epithelial cells increased significantly during the late follicular phase ($P = 0.001$; Figure 4-5) and decreased during the luteal phase; however, there was no significant relationship between changes in urinary leukocyte concentration during each stage of the oestrous cycle ($P = 0.334$; Figure 4-5). No spermatozoa were found in any of the urine samples collected following bouts of mating, including the female which had a successful mating and gave birth in 2013.
Figure 4-5. Changes in urogenital epithelial cells and leukocytes across the different stages of the captive female southern hairy-nosed wombat oestrous cycle; early and late follicular phase (FP), early and late luteal phase (LP). abc superscripts determine the significant differences (P < 0.05) between each stage of the oestrous cycle.

4.4 Discussion

This study examined whether changes in the physiochemical characteristics and cell types released in wombat urine could be used as an index of reproductive activity in captive female SHNW. Using changes in urinary P4M concentration to define the characteristics of the reproductive cycle (oestrous cycle, luteal phase length, and changes in P4M levels across cycles), the results of this study revealed not only significant variation in the length and frequency of the oestrous cycle between individuals and sampling periods, but also significant changes in the volume of urine recovered, urinary specific gravity and creatinine concentration, as well as the concentration of urogenital epithelial cells throughout the oestrous cycle. These urinary characteristics have the potential to be used as non-invasive markers for monitoring the reproductive status in this species in captivity.

The longitudinal urinary P4M profiles analysed in this study were similar to the serum and faecal progesterone profiles previously reported for captive female SHNWs (Paris et al., 2002; Finlayson et al., 2006; Hogan et al., 2010). In the current study, the urinary P4M profiles confirmed that female captive SHNWs are polyoestrous, and on the central coast of
Queensland (Mt Larcom), may have at least two to three oestrous cycles per breeding season. As there was evidence of reproductive activity beginning prior to the sampling period (T. Janssen personal observation) and continuing past the end of the sampling period (F10 produced pouch young in February 2015), it is likely that some females in this captive population in central Queensland have an extended breeding season beyond that of the wild SHNW in southern Australia which occurs between July to December (Gaughwin et al., 1998).

While the mean oestrous cycle length was similar to previous reports, the variability in the oestrous cycle length of the females in the current study (range between 23 to 47 days) was greater than the range of those previously reported \( [n = 2: 41.1 \text{ days (Paris et al., 2002); } n = 8: 35 \text{ to } 38 \text{ days (Finlayson et al., 2006); } n = 8: 27 \text{ to } 38 \text{ days (Hogan et al., 2010)}] \). Similarly, the variability of luteal phase length between females within this study (12 to 33 days) was slightly larger than the variability previously reported for captive females SHNW [27.6 days (Paris et al., 2002); 16 to 26 days (Finlayson et al., 2006); 15 to 31 days (Hogan et al., 2010)]. Interestingly, Hogan et al., (2010) measured a luteal phase during the first months of their sampling period (March to April 2006), which again was, outside the normal breeding season for wild SHNWs. Unfortunately, as longitudinal reproductive hormone analysis has not been conducted on wild female SHNWs, we cannot accurately determine what might be considered normal oestrous cycle variability within this species and therefore are unsure if our results are reflective of normal SHNW reproductive biology or are altered as an artefact of captivity.

While urinary P4M analysis was a useful tool for the assessment of general reproductive activity of captive SHNWs, the analysis of urinary P4M alone does not provide specific enough information to fully understand the irregular cyclic activity displayed by some female captive SHNWs in this study. The variability in luteal phase length reported in captive females may be a consequence of only measuring P4M (Paris et al., 2002; Hogan et al., 2010) without the accompanying analysis of meaningful changes in oestrogen and luteinizing hormone, which so far, have been difficult to accurately measure in samples collected using non-invasive methods (Hogan et al., 2010; Swinbourne et al., 2017). In the current study, the luteal phase length could only be estimated, as the precise timing of ovulation to the formation of a functional corpora luteum (CL) is currently unknown in
The time from ovulation to an established luteal phase is species-specific in marsupials, and unlike eutherian mammals, the CL does not form rapidly following ovulation (Bradshaw and Bradshaw, 2011). Depending on the species and the gestation period, the timing from ovulation to CL development and a detectable increase in circulating and excreted progesterone can be as little as 1 to 4 days in the striped-faced dunnart (*Sminthopsis macroura*) (Menkhorst et al., 2009), or between 6 and 8 days in the brown antechinus (*Antechinus stuartii*) (Hinds and Selwood, 1990). For this study, the length of the luteal phase was estimated based on the sustained increase in urinary P4M above baseline values which may have resulted in over- or underestimation in the luteal phase length for each female.

The iterative process to determine baseline values may have also contributed to the luteal phase variability between females. This process, in terms of what values to remove, varies depending on the biological sample analysed and the species of interest. For example, when evaluating faecal P4M in marsupials, it is common to remove values greater than the mean ± 1.5 standard deviation (SD) (Keeley et al., 2012) or the mean ± 1.75 SD (Oates et al., 2004; Hogan et al., 2010; Hogan et al., 2012; Mills et al., 2012). While Moreira et al. (2001) initially used the same methodology, they reported more biologically relevant profiles when baseline values were calculated using mean ± 2 SD. This was also the case for the profiles reported in the current study. For example, when baseline values greater than the mean ± 1.75 SD were removed during the process, urinary P4M for F11 and F4 remained above baseline for 47 and 85 days, respectively. Similar to what was used by Moreira et al. (2001), the use of mean ± 2 SD resulted in more biologically relevant hormone profiles compared to mean ± 1.5 SD which has previously been used for marsupials. When the precise timing of ovulation is established, we will be able to determine which iterative process is appropriate for each female or indeed if the iterative process needs to be evaluated and adjusted for each female.

While there is a data interpretation element to analysing urinary P4M profiles, the variability in luteal phase length may also be attributed to abnormal ovarian function or even stress. Abnormal ovarian function, such as luteinized unruptured follicles, a persistent CL on the ovary or the formation of an ovarian cyst, can result in prolonged, erratic and infertile anovulatory cycles which have been reported in a variety of eutherian mammals [some deer (*Cervinae*) species (Adam et al. 1985; Curlewis et al. 1988), domestic cattle (Sheldon et al. 2006), breeding mares (King et al. 2010), African black (*Diceros bicornis*), white
(Ceratotherium simum) (Brown et al. 2001) and Indian rhinoceros (Rhinoceros unicornis) (Stoops et al. 2004)]. Another possibility worth investigating is the effects of stress and increased adrenal response, which have shown to disrupt or reduce reproductive fitness (Bonier et al. 2009). Persistent mating behaviour exhibited from the males towards the female irrespective of her receptivity may be a contributing factor to the prolonged periods of progesterone secretion. More research needs to be conducted to determine if the long luteal phases reported in some females in the current study are a result of abnormal ovarian function or a response to the intrinsic factors within the captive environment. This will help to identify either ideal females for captive breeding or appropriate husbandry and management strategies for captive individuals.

The variability in the intraovarian function and regulation of the marsupial CL (Tyndale-Biscoe and Renfree, 1987; Gemmell and Sernia, 1995) also needs to be taken into consideration. Unlike most eutherian mammals, where CL function is regulated by prostaglandin F2α induced luteolysis, CL regulation in marsupial appears to be programmed by intrinsic factors. Once formed, the CL, influenced by luteinizing hormone (Stewart and Tyndale-Biscoe, 1982), functions autonomously (Hearn, 1973), and is therefore more susceptible to variation. There is also evidence in the Tammar wallaby (Macropus eugenii) to suggest that the foetus or placenta may exert a luteolytic effect on the CL (Tyndale-Biscoe and Renfree, 1987). Unfortunately, the analysis of urinary P4M alone does not provide specific enough information to fully understand the irregular cyclic activity displayed by some captive female SHNWs in this study, so that further research is required to understand luteolysis of the SHNW CL, especially in the non-pregnant female.

In the wild, SHNW are winter/spring breeders (Gaughwin et al., 1998), which coincides with increased rainfall and pasture growth (Taggart et al., 2005b). However, other environmental factors such as day length or photoperiod may also play a role in SHNW reproduction. For some of the females in the current study; F2, F4, F9, F10, and F11, the increase in days between luteal phases coincided with increased day length during the sampling period. The influence of photoperiod on reproduction has been investigated in the brushtail (Trichosurus vulpecula) (Gemmell and Sernia, 1995) and honey possums (Tarsipes rostratus) (Oates et al., 2007), and some Antechinus species (Dickman, 1985; McAllan and Dickman, 1986; McAllan and Geiser, 2006); these studies have revealed that an increase in
day length had a negative impact on reproduction, resulting in increased oestrous cycle length or reduced fertility. While data on wild female SHNWs is extremely limited, a long term study on wild male SHNW reproduction showed significant reductions in ejaculate volume, spermatozoa number and motility, and the size of sex accessory glands occur from November to January (Taggart et al., 2005b), this time period coincides with increased photoperiod in South Australia (Geoscience Australia, 2017). It is currently unknown what influence photoperiod may have on SHNW reproduction, and further research could include investigate the use of artificial light schedules to identify the role that photoperiod might have on SHNW reproduction and as a means of altering the captive environment to optimise reproduction.

If captive breeding managers are willing to invest the necessary time for conditioning individual females for daily urine collection, as per the methodology described in Swinbourne et al. (2015), the evaluation of urinary characteristics such as urine volume, specific gravity and urinary cytology appear to be a useful and relatively easy non-invasive tool to identify females in different stages of the oestrous cycle. Firstly, when urinary P4M was below baseline values, collected urine volume decreased, which coincided with decrease toileting behaviour observed during the same sampling period (Swinbourne et al., submitted manuscript). As urine can easily be collected and transferred into a measuring cylinder, this may be a useful reproductive management tool for potential method of detecting oestrus. Secondly, the concentration (both urinary specific gravity and urinary creatinine values) was significantly higher when urinary P4M was below baseline values, which may be due to a decrease in urine volume and urination frequency.

The correlation between the two urine standardisation methods to accurately measure urine concentration (urinary specific gravity versus urinary creatinine concentration; P = 0.001) has previously been reported in other species (Haddow et al., 1994; Carrieri et al., 2000; Heavner et al., 2006; Cone et al., 2009). While there was a strong correlation between the two urine standardisation methods, the analysis of individual female profiles demonstrated a strong correlation between the two methods (F9 (2014): R² = 0.583) while for other females it was weak (F11: R² = 0.09). When urinary hormone profiles were reanalysed for progesterone standardised for specific gravity, the profile themselves did not appear to vary significantly (Figure 4-4). However, evaluation of the data to determine specific gravity P4M baseline values (data not shown) showed that the luteal phases did not directly match
those analysed for creatinine, demonstrating slightly shorter and/or slightly longer luteal phase lengths. Unfortunately, as the correlation between the two methods appeared variable and weak for some females, we cannot be certain which standardisation method (specific gravity or creatinine) is the most appropriate for evaluating urinary hormone concentration. More urinary hormone analysis may help to determine the appropriate standardisation method; however, until then the use of specific gravity may be more beneficial for assessing urinary concentration in the field and be used to determine the appropriate dilution for hormone analysis.

While previous analysis of urogenital epithelial cells swabbed directly from the urogenital sinus has proven inconclusive for assessing reproductive status of captive wombats (Peters and Rose, 1979; Finlayson et al., 2006), the results from the current study showed that the number of naturally sloughed urogenital epithelial cells increased during the late follicular phase. Therefore, we propose that for the accurate assessment of the reproductive status of captive female SHNW, it should be possible for captive breeding managers (zookeepers or research/captive breeding facilities) to use a combination of the urinary characteristics (changes in urine volume, urine specific gravity and an index of urogenital epithelial cells) as simple non-invasive markers, that are relatively inexpensive and require only basic equipment (measuring cylinder, handheld refractometer and a 40X light microscope) to facilitate the identification of female SHNW reproductive status (cyclicity) without the need for hormone analysis, which in contrast requires specialised equipment, training and laboratory facilities.

Due the level of individual variation of these urinary characteristic, if they are to be used as effective non-invasive markers for reproductive status in captive SHNW, it may be prudent to first evaluate each urine parameter characteristic against individual urinary hormone profiles to allow for the identification of individual variability, determine the range for each female, and identify periods of significant change in the marker compared against changes in the urinary hormone. Once these parameters have been established for each female, then they can be used as non-invasive markers to monitor the reproductive status of captive female SHNW.
4.5 Conclusion

Urinary P4M assays were used in this study to provide clear evidence of cyclicity in the SHNW. In addition, the identification of three non-invasive markers (urinary volume, specific gravity and urogenital epithelial cell numbers) which changed in response to changes in urinary P4M concentrations suggests that urinary parameters are also useful for the non-invasive assessment of reproductive status in captive SHNW. It is still unclear as to why mated oestrous cycles of many SHNWs in this study did not result in pouch young and why there was so much variability in the length of the oestrous cycle. Further studies are required to better map the urinary hormone profiles of the follicular phase, to confirm the timing of ovulation, and better understand the effects that stress may have on female reproduction.
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5. The reproductive biology of the captive female southern hairy-nosed wombat (*Lasiorhinus latifrons*): Oestrous behaviour

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Abstract

A lack of understanding of what characterises oestrous behaviour in the southern hairy-nosed wombat (SHNW - *Lasiorhinus latifrons*) and its relationship to circulating reproductive hormones has contributed to a relatively low level of captive breeding success for this species. Over two wombat breeding seasons (August to December 2013; 2014), the use of two non-invasive tools were investigated; (1) the collection of daily urine samples from seven female wombats, and (2) the use of infrared cameras for 24-hour remote behavioural monitoring. The aim of this research was to determine if female behaviour could be used to identify reproductive status. Frozen-thawed urine samples were analysed for progesterone metabolites (P4M) using an enzyme-immunoassay to confirm the relationship between behaviour and stage of the oestrous cycle. Urinary P4M was divided into two concentrations; (1) ≤ baseline values and (2) > baseline values, and evaluated against urine volume (mL) and the duration (seconds) and frequency of 23 general, defensive and reproductive behaviours recorded on day D-14, D-3, D-1, D0, D+1, D+2 and D+14, with D0 denoted as the estimated beginning of the luteal phase based on changes in urinary P4M values. When urinary P4M was ≤ baseline, the duration of urination (P = 0.03) and volume (P = 0.04) both decreased, whereas the duration (P = 0.01) and frequency (P = 0.02) of pacing and the duration (P = 0.04) and frequency (P = 0.04) of rump bites by the female towards the male increased. The frequency of grasping / restraint (P = 0.04) and guarding of the female by the male (P = 0.01) both increased when urinary P4M ≤ baseline values. These results suggest that there were detectable behavioural changes which can be mapped to the stages of the SHNW oestrous cycle, and therefore may be used as behavioural indicators by captive wombat managers to identify the reproductive status of females.
5.1 Introduction

The northern hairy-nosed wombat (NHNW - *Lasiorhinus kreftii*) is a critically endangered Australian marsupial located in two protected populations in central Queensland (Department of Environment and Heritage Protection, 2015), Epping Forest National Park, which has over 200 individuals, and a small translocated population at Richard Underwood Nature Refuge in St George, which currently has 11 individuals remaining from 20 introductions (A. Horsup, 2016, *pers comm*). A recovery plan was established for this species (Horsup, 2004) and initially facilitated through *in situ* methods, which include predator-proof fencing, vegetation management and supplementary feeds where necessary. While an *ex situ* captive breeding population was also recommended, the low number of individuals originally estimated and the limited information regarding the wombat’s breeding behaviour and physiology, delayed the establishment of a captive colony. However, a recent increase in the Epping Forest National Park population (approximately 230) now means that the establishment of an *ex situ* insurance population can once again be considered.

In preparation for the ultimate implementation of a captive insurance colony of the NHNW, there has been a significant investment to improve the captive husbandry and breeding success of the closely related southern hairy-nosed wombat (SHNW; *Lasiorhinus latifrons*) (Hogan *et al.*, 2009; Hogan *et al.*, 2010a; Hogan *et al.*, 2010b; Hogan *et al.*, 2010c; Hogan *et al.*, 2011a; Hogan *et al.*, 2011b; Descovich *et al.*, 2012b; Descovich *et al.*, 2012c; Swinbourne *et al.*, 2015; Swinbourne *et al.*, 2017b). However, one of the major challenges associated with breeding SHNW in captivity is the inability to accurately identify the reproductive status of captive females (Hogan *et al.*, 2010a; Hogan *et al.*, 2010b). Non-invasive tools, such as remote behavioural monitoring and faecal hormone analysis, have been proven to be useful for characterising the reproductive behaviour and physiology in many wildlife species (Monfort, 2003; Schwarzenberger, 2007; Schwarzenberger and Brown, 2013). In the SHNW (Paris *et al.*, 2002; Hogan *et al.*, 2009; Hogan *et al.*, 2010a; Hogan *et al.*, 2010b), they have been used in the identification and characterisation of behaviours, especially reproductive, exhibited by captive individuals of this nocturnal species (Hogan *et al.*, 2010a). Although the analysis of faecal progesterone metabolites (P4M) accurately reflects the longitudinal progesterone profiles of captive females (Hogan *et al.*, 2010b), changes in reproductive behaviours have not shown correlations with changes in faecal progesterone metabolites (Hogan *et al.*, 2010b), which may be a factor of females undergoing
a silent oestrous or becoming indifferent or habituated to the male, or the low libido from the captive male to instigate reproductive behaviours in receptive females (Hogan et al., 2010a).

Rather than just focusing on observing reproductive behaviours, which are infrequent, cryptic and therefore hard to detect, changes in general behaviours could be used to identify the reproductive status of SHNW, as they have in other captive marsupials. For example, the female Tasmanian devil (Sarcophilus harrisii) undergoes periods of decreased appetite during oestrus (Keeley et al., 2012), and the female Julia creek dunnart (Sminthopsis douglasi) displays periods of increased activity when in oestrus as detected by increased wheel running behaviour (Pollock et al., 2010). As a result, changes in these or similar behaviours may be used as valuable tools for identifying the optimum timing for mate introductions for captive breeding programs. Additionally, physiological changes, such as urination behaviour, have also been linked to changes in reproduction in the house mouse (Mus domesticus) (Drickamer, 1995), and may, therefore, also be a useful tool to identify reproductive changes in female wombats as well. Consequently, the current study aimed to identify changes in SHNW general and reproductive behaviours which could be mapped against changes in urinary progesterone profiles in an attempt to elucidate non-invasive measures that could be utilised in the reproductive management of this species.

5.2 Methods and materials

5.2.1 Animals and animal management

The study was conducted over two wombat breeding seasons (August to December 2013; 2014) and was approved by the University of Queensland Animal Ethics Committee (SAFS/171/13AACE). Wombats were housed and managed at the Australian Animals Care and Education wombat research facility in Mount Larcom, Central Queensland (23.75oS, 151.00°E). Table 5-1. documents the wombat housing arrangements (pairings or single) of nine females over the two breeding seasons. Each enclosure consisted of an indoor air-conditioned area (5.8 m²) with three individual sleeping chambers (0.6 m² each) and an adjoining large outdoor yard enclosure (4 x 90 m² and 4 x 100 m²), consisting of soil substrate, partial grass vegetation, logs, tree branches and a dirt mound for enrichment. The daily diet consisted of a mixed ration of 120 g rolled oats (Coles, Smart Buy, Australia), 120
g gumnut pellets (Mitavite, Australia), 35 g oaten chaff (Rich River Chaff and Grain, Australia), 200 g sliced sweet potato (locally farmed produce). Animals had ad libitum access to water.

Table 5-1. Body weights and housing configuration of nine captive female southern hairy-nosed wombats during two consecutive wombat breeding seasons (August to December 2013; 2014)

<table>
<thead>
<tr>
<th>Season</th>
<th>Wombat ID</th>
<th>Animal age</th>
<th>Mean weight (kg)</th>
<th>Housing</th>
<th>Den mate</th>
<th>Mean weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>F3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>25.1</td>
<td>Breeding pair</td>
<td>M6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>25.2</td>
<td>Non-breeding pair</td>
<td>M4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.4</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>29.5</td>
<td>Female pair</td>
<td>F6</td>
<td>29.6</td>
</tr>
<tr>
<td></td>
<td>F6&lt;sup&gt;£&lt;/sup&gt;</td>
<td>5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>29.6</td>
<td>Female pair</td>
<td>F5</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>F9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>20.5</td>
<td>Breeding pair</td>
<td>M3&lt;sup&gt;£&lt;/sup&gt;</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td>F10&lt;sup&gt;£&lt;/sup&gt;</td>
<td>3</td>
<td>21.5</td>
<td>Female pair</td>
<td>F9</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>F11</td>
<td>10&lt;sup&gt;*&lt;/sup&gt;</td>
<td>26.4</td>
<td>Female only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>F1&lt;sup&gt;£&lt;/sup&gt;</td>
<td>9</td>
<td>25.0</td>
<td>Breeding pair</td>
<td>M1&lt;sup&gt;£&lt;/sup&gt;</td>
<td>36.4</td>
</tr>
<tr>
<td></td>
<td>F2&lt;sup&gt;£&lt;/sup&gt;</td>
<td>5</td>
<td>22.0</td>
<td>Breeding pair</td>
<td>M2&lt;sup&gt;£&lt;/sup&gt;</td>
<td>30.7</td>
</tr>
<tr>
<td></td>
<td>F9&lt;sup&gt;£&lt;/sup&gt;</td>
<td>5</td>
<td>22.7</td>
<td>Breeding pair</td>
<td>M1</td>
<td>36.4</td>
</tr>
<tr>
<td></td>
<td>F10&lt;sup&gt;£&lt;/sup&gt;</td>
<td>4</td>
<td>23.6</td>
<td>Breeding pair</td>
<td>F9</td>
<td>22.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Female was successfully mated in August 2013.
<sup>b</sup>Vasectomised male
<sup>c</sup>Hip injury as a joey limited reproductive behaviours as an adult
<sup>*</sup>Age is estimated as the animal was rescued and brought into captivity as an adult.
<sup>£</sup>Animal was hand raised as pouch young or brought into captivity as a juvenile.

### 5.2.2 Non-invasive urine sample collection

Daily urine samples were collected from females using non-invasive methods previously described by Swinbourne et al. (2015). Female SHNW were conditioned to provide daily urine samples on demand, and samples were collected either directly into a small collection tray placed under the rump of the animal or aspirated off the clean den floor. Urine was transferred into a polypropylene specimen container (either 70 or 150 mL, SARSTEDT, Germany) and maintained on ice. The volume of urine (mL) and concentration as determine by specific gravity measured using a handheld refractometer (range 1.000 –

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1.080. Bellingham + Stanley) were recorded and the sample divided into 2 mL aliquots and stored at -20 °C for subsequent urinary hormone analysis.

5.2.3 Reproductive cycle characterisation

Urine samples, standardised for creatinine (Cr) were analysed for progesterone metabolites (P4M) using an enzyme-immunoassay (EIA) previously described and validated for SHNW urine (Swinbourne et al., 2017b). An iterative process, described in Swinbourne et al. (submitted manuscript), was used to determine the baseline P4M values for individual captive female SHNWs. A sustained increase in P4M concentration above baseline for ≥ 3 consecutive days was defined as the onset of the luteal phase, and the first day of the sustained rise was classified as day 0 (D0). An oestrous cycle was defined as the end of one luteal phase to the end of the next (Hogan et al., 2010b).

5.2.4 Behavioural monitoring

Remote behavioural observations were conducted using a digital video surveillance system. One infrared (IR) indoor camera (SUMO® - Model: CAM35IRHR) and one IR weatherproof bullet outdoor camera (SUMO® - Model: CAM78IRHR) were installed in each of the eight wombat enclosures (total 16 cameras). Cameras were connected to a digital video recorder (DVR - KOBI® 16 channel) surveillance system, and footage was reviewed using XQ Pro Series DVR surveillance software which allowed 24-hour continuous behavioural observations. Animal identification was achieved based on individual coat markings / colour, and animal size (e.g. females were generally paired with a larger male). Footage from all IR cameras was transferred onto an external hard drive (5 x 4TB Seagate Expansion Desktop) until retrospective behavioural analysis could be conducted.

5.2.5 Retrospective behavioural analysis

An ethogram (Table 5-2) was developed based on the general and reproductive behaviours previously described in Hogan et al. (2010a) and Hogan et al. (2010b). As SHNW are typically only sexually receptive for approximately 13 hours (Hogan et al., 2010a), both
general and reproductive behaviours were analysed for six days surrounding $D_0$ of the luteal phase and two additional days which were used as “behavioural control days” where it was anticipated that reproductive behaviours were least likely to occur. These days were separated into two P4M levels; (1) $\leq$ baseline values which included $D_{-3}$, $D_{-2}$, $D_{-1}$, and (2) $>$ baseline values which included $D_0$, $D_{+1}$, $D_{+2}$, and two behavioural “control days”; $D_{-14}$ and $D_{+14}$, where reproductive behaviours were least likely to occur. Using CowLog 2.0 software (Hänninen and Pastell, 2009), the duration (seconds) and frequency (number) of 23 mutually exclusive general ($n = 10$), defensive ($n = 3$) and reproductive ($n = 10$) behaviours were analysed for the first 5 of every 30-minute interval throughout each day using continuous focal sampling methods observed by the same investigator (AMS) for each female.

5.2.6 Statistical analysis

All statistical analysis was conducted using IBM® PASW Statistics GradPack 18 (formally SPSS® Statistics). Residual plots of raw data did not demonstrate normal distribution and were therefore log$_{10}$ transformed and residual plots were retested for normal distribution by the Shapiro-Wilk test (Villasenor Alva and Estrada, 2009). An analysis of variance (ANOVA) was conducted to determine the relationship between the two urinary P4M concentrations and the volume of urine and specific gravity, and the duration and frequency of individual behaviours, examining the differences between individual animals and the population. An additional ANOVA was conducted to identify changes in behaviours against females which underwent short or long cycles, with short cycle lasting $< 35$ days and long cycles lasting $> 35$ days. For all ANOVAs, a Post-Hoc analysis was conducted which included a Least Significant Difference and a Bonferroni correction to identify individual animal variation between the expression of behaviours. Lastly, a principal component analysis was conducted to determine behavioural clustering. Data for clustered behaviours was collated and reanalysed using the same ANOVA parameters for individual behavioural analysis. Residuals for clustered behaviours were tested for normal distribution. For all statistical analysis, significance levels were set at $P \leq 0.05$. 
Table 5-2. Ethogram of wombat general and reproductive behaviours

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General Behaviours (female only)</strong></td>
<td></td>
</tr>
<tr>
<td>Digging</td>
<td>Repeated pawing at the ground or the den walls.</td>
</tr>
<tr>
<td>Exploring/Foraging</td>
<td>Investigation of, or searching for, both edible and inedible objects within indoor / outdoor enclosures</td>
</tr>
<tr>
<td>Feeding</td>
<td>Consumption of food and water provided by keepers</td>
</tr>
<tr>
<td>Grooming</td>
<td>Scratch body part with hind limbs or rubbing against a stationary object</td>
</tr>
<tr>
<td>Lying awake</td>
<td>Sternal recumbency, eyes open, ears erect, doing nothing else</td>
</tr>
<tr>
<td>Sitting</td>
<td>Sitting on hind quarters and front paws, doing nothing else</td>
</tr>
<tr>
<td>Sleeping</td>
<td>Lying with neck recumbent on the ground, curled into a ball or on their back, ears relaxed, eyes closed.</td>
</tr>
<tr>
<td>Pacing</td>
<td>Traversing the same path three of more times consecutively</td>
</tr>
<tr>
<td>Urination</td>
<td>Stopping to eliminate waste, leaving visible pools of urine or on the ground. (Difficult to determine/observe just defecation behaviour using IR cameras).</td>
</tr>
<tr>
<td><strong>Defensive Behaviours (female only)</strong></td>
<td></td>
</tr>
<tr>
<td>Defensive</td>
<td>Female uses rump to deter the den mate’s approach. Usually blocking the entrance of the sleeping chamber or indoor den access.</td>
</tr>
<tr>
<td>Kicking</td>
<td>Female uses her back legs to repeatedly kick others in the enclosure if they approach.</td>
</tr>
<tr>
<td>Evading</td>
<td>Female avoids the den mate’s approach by walking or running away.</td>
</tr>
<tr>
<td><strong>Reproductive Behaviours (male and female)</strong></td>
<td></td>
</tr>
<tr>
<td>Urogenital Sniffing</td>
<td>Male approaches female and sniffs the urogenital sinus, associated with female’s acceptance.</td>
</tr>
<tr>
<td>Urine Smelling</td>
<td>Approaches freshly voided urine and places snout in urine sample</td>
</tr>
<tr>
<td>Approach</td>
<td>Either male or female approaches the other to investigate, e.g. sniff, nudge, bite.</td>
</tr>
<tr>
<td>Chase</td>
<td>Male approaches female and follows her at a close distance (&lt; 1 m). The female may initiate chase by approaching the male first.</td>
</tr>
<tr>
<td>Rump bite</td>
<td>Approaches animal and delivers a significant bite to the rump. A male may use this behaviour to initiate chase with the female, or the female may deliver a bite as defensive behaviour to deter the male.</td>
</tr>
</tbody>
</table>
Grasp/Restraint  Male jumps onto back of female, grasps her hips with his forelimbs and rolls the female onto her side.

Turning  The male turns the female on her side, kicks out her hind limbs and pulls her rump towards his urogenital region.

Coitus  Male directs his penis into the female’s urogenital region, followed by rhythmic pelvic thrusting.

Break  After a bout of coitus, the female is released from the male and both rest in isolation.

Cohabitation  During the courtship/mating period both animals share a single sleeping chamber.

Modified from (Hogan et al., 2010a; Hogan et al., 2010b)

5.3 Results

While urine samples were collected from nine females during both sampling periods, behavioural analysis was only conducted for seven females (eight individual data sets). While urinary P4M was evaluated for all females (Swinbourne et al., submitted manuscript), for behavioural analysis data sets for F11 as she was not under continual video surveillance in 2013, F6 was blind and did not demonstrate normal behaviours, and the hormone profiles for F10 (2013) and F4 (2014) did not demonstrate normal reproductive activity. For behavioural analysis, seventeen distinct oestrous cycles could be paired with daily behavioural footage, which composed of six non-mated oestrous cycles and one mated pregnant cycle in 2013, and nine mated cycles and one non-mated cycle during 2014 (Table 5-3).

An analysis of toileting behaviour showed that when urinary P4M was ≤ baseline the duration was lower compared to when urinary P4M was > baseline (Table 5-4); however, there was no difference in the frequency of toileting behaviour over the duration the analysis period (Table 5-5). Urinary volume was also lower when P4M was ≤ baseline (22.8 ± 2.8 mL versus 30.4 ± 2.4 mL. $F_{1,100} = 4.434, P = 0.04$), and urine concentration, evaluated using specific gravity and creatinine, was high when P4M was ≤ baseline compared to when urinary P4M was > baseline [Specific gravity: 1.036 ± 0.001 versus 1.035 ± 0.001 ($F_{1,1174} = 12.545 \; P = 0.001$). Creatinine: 6.28 ± 0.18 versus 5.43 ± 0.15 ng/mg. ($F_{1,1174} = 44.111, P = 0.001$)].

The duration (Table 5-4) and frequency (Table 5-5) of pacing behaviour increased by up to four-fold when urinary P4M was ≤ baseline values. However, there was considerable
individual animal variability in this behaviour ($F_{7.110} = 5.629$, $P < 0.01$), as pacing was only observed in six of the eight data sets analysed, for example, F4 and F5 were not observed pacing during the each of the study periods. When comparing behaviours between females with short cycles versus long cycles, the duration of evading was significantly longer for short cycling females compared to females with long cycles ($0.8 \pm 0.2$ versus $0.7 \pm 0.1$ seconds. $F_{(1,117)} = 4.292$; $P = 0.04$). The frequency of sitting decreased significantly in females who underwent short cycles compared to females who had long cycles ($0.9 \pm 0.03$ versus $1.1 \pm 0.03$ times. $F_{(1,117)} = 5.424$; $P = 0.02$). When urinary P4M was $\leq$ baseline values, females who has long cycles had significantly increased duration of pacing ($1.3 \pm 0.23$ versus $0.29 \pm 0.17$. $F_{(1,72)} = 7.108$; $P = 0.01$) compared to when urinary P4M was $> baseline$. There were no significant behavioural relationships found for short cycling females when urinary P4M was $\leq$ or $> baseline$ values ($P > 0.05$)

Table 5-3. Reproductive cycle dynamics of eight captive female southern hairy-nosed wombat data sets during the 2013 and 2014 wombat breeding seasons. The luteal phase was classified as the beginning of a sustained increase in urinary P4M above baseline values to when urinary P4M returned to baseline values. An oestrous cycle was the end of one luteal phase to the end of the subsequent luteal phase.

<table>
<thead>
<tr>
<th>Season</th>
<th>Animal</th>
<th>$n = cycles$ analysed</th>
<th>Luteal phase (days)</th>
<th>Oestrous cycle (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>2013</td>
<td>F3</td>
<td>1</td>
<td>22-day gestation period. No luteal phase identified</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>3</td>
<td>13.6</td>
<td>12 – 15</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>1</td>
<td>24.0</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>F9</td>
<td>2</td>
<td>26.5</td>
<td>26 – 27</td>
</tr>
<tr>
<td>2014</td>
<td>F1</td>
<td>3</td>
<td>23.3</td>
<td>18 – 30</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>2</td>
<td>23.5</td>
<td>23 – 24</td>
</tr>
<tr>
<td></td>
<td>F9</td>
<td>2</td>
<td>27</td>
<td>20 – 33</td>
</tr>
<tr>
<td></td>
<td>F10</td>
<td>3</td>
<td>22.3</td>
<td>22 – 23</td>
</tr>
</tbody>
</table>

*F3 was successfully mated during the 2013 breeding season and information on previous oestrous cycles was unavailable.

The frequency and duration of rump bites exhibited by the female towards the male when in breeding pairs or female den mate when housed in female only pairs, was higher when urinary P4M was $\leq$ baseline (Table 5-4 and Table 5-5). However, similar to the pacing behaviour, there was a high level of individual animal variation regarding both the duration ($F_{7.109} = 2.900$, $P = 0.01$) and frequency ($F_{7.109} = 2.608$, $P = 0.02$) of rump biting, as only four females exhibited this behaviour when housed as breeding pairs. Rump biting was observed
in F1, F2, F3 and F10 a total of 6, 3, 42 and 23 times /5 mins /30 mins, respectively, when urinary P4M was ≤ baseline compared to 3, 4, 0 and 0 times /5 mins /30 mins, respectively, when urinary P4M was > baseline. The duration of female rump biting also increased when urinary P4M was ≤ baseline (mean 65.1 ± 31.6 versus 1.4 ± 0.9 seconds/5 mins /30 mins). Similarly, the mean frequency of rump bites exhibited by males towards females was higher when urinary P4M was ≤ baseline (8.5 ± 2.7 times versus 2.3 ± 1.1 times/5 mins /30 mins $F_{1,104} = 6.901$, $P = 0.01$).

The duration of courtship and mating behaviours exhibited by the male (grasping and restraining the female and guarding behaviour) was increased when urinary P4M was ≤ baseline values (Table 5-4). In addition, the mean frequency of males grasping and restraining the female was six times higher when urinary P4M was ≤ baseline compared to > baseline (Table 5-5). In contrast, the frequency of urogenital sniffing by the male was low when urinary P4M was ≤ baseline (Table 5-5).

Coitus was observed in only four females; F2, F3, F9 and F10, on seven different days during the behavioural analysis period. In 2013, following the mating of F3 and M6, a retrograde seminal plug was found on D+2 when urinary P4M was > baseline. From this mating, a pouch young was born 21 days following the first mating bout as confirmed via video surveillance (pouch check confirmation 60 days following mating), and the young was weaned at the end of the 2014 breeding season. Following the mating of F10 and M1 in 2014, a seminal plug was found on D+1, when urinary P4M was > baseline; however, this mating failed to result in a pouch young.

The analysis of behaviours again cycle length showed that the duration of evading was significantly longer ($F_{1,117} = 4.292; P = 0.041$) for short cycling females (0.8 ± 0.2) compared to females with long cycles (0.7 ± 0.1), and the frequency of sitting decreased significantly ($F_{1,117} = 5.424; P = 0.022$) in females with underwent short cycles (0.9 ± 0.03) compared to females who had long cycles (1.1 ± 0.03). The principal component analysis conducted for behavioural clustering showed three distinct behavioural clusters; (1) reproductive, (2) defensive, and (3) general behaviours. However, there was no relationship found for either the duration or frequency of these clustered behaviours to changes in urinary P4M ($P > 0.05$. Table 5-6).
Table 5-4. The mean logarithmic duration (log<sub>10</sub> secs / 5 mins / 30mins) of behaviours recorded from eight female southern hairy-nosed wombat data sets when urinary progesterone metabolites (P4M) were ≤ or > individual baseline values.

<table>
<thead>
<tr>
<th>Behaviours</th>
<th>≤ baseline urinary P4M</th>
<th>&gt; baseline urinary P4M</th>
<th>P value (F, 1, 117)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>±SEM</td>
</tr>
<tr>
<td>Digging</td>
<td>2.6</td>
<td>0.0   - 4.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Exploring</td>
<td>3.9</td>
<td>1.9   - 4.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Feeding</td>
<td>2.9</td>
<td>0.0   - 3.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Grooming</td>
<td>2.1</td>
<td>0.0   - 4.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Lying Awake</td>
<td>3.7</td>
<td>0.0   - 4.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Sitting</td>
<td>3.5</td>
<td>1.9   - 4.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Sleeping</td>
<td>4.7</td>
<td>4.3   - 4.8</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Pacing</strong></td>
<td><strong>1.1</strong></td>
<td><strong>0.0</strong>   - <strong>4.0</strong></td>
<td><strong>0.2</strong></td>
</tr>
<tr>
<td>Sniffing</td>
<td>1.1</td>
<td>0.0   - 3.5</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Toilet</strong></td>
<td><strong>0.6</strong></td>
<td><strong>0.0</strong>   - <strong>3.2</strong></td>
<td><strong>0.1</strong></td>
</tr>
<tr>
<td>Defensive</td>
<td>2</td>
<td>0.0   - 4.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Approach</td>
<td>0.5</td>
<td>0.0   - 3.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Evading</td>
<td>0.9</td>
<td>0.0   - 3.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Nudging</td>
<td>0.1</td>
<td>0.0   - 2.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Chase</td>
<td>0</td>
<td>0.0   - 1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Urogenital Sniffing</td>
<td>0</td>
<td>0.0   - 0.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Urine Smelling</td>
<td>0.1</td>
<td>0.0   - 1.8</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Rump Bite</strong></td>
<td><strong>0.4</strong></td>
<td><strong>0.0</strong>   - <strong>3.2</strong></td>
<td><strong>0.1</strong></td>
</tr>
<tr>
<td>Grasp/Restraint</td>
<td>0.5</td>
<td>0.0   - 3.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Turning</td>
<td>0.1</td>
<td>0.0   - 2.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Coitus</td>
<td>0.3</td>
<td>0.0   - 3.2</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Break</strong></td>
<td><strong>0.2</strong></td>
<td><strong>0.0</strong>   - <strong>3.6</strong></td>
<td><strong>0.1</strong></td>
</tr>
<tr>
<td>Cohabitation</td>
<td>0.1</td>
<td>0.0   - 3.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Guarding</td>
<td>0.0</td>
<td>0.0   - 1.3</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Bold data points indicate significant behaviour-urinary P4M interaction.
Table 5-5. The mean logarithmic frequency (log\(_{10}\) \(n / 5\) mins / 30 mins) of behaviours recorded from eight female southern hairy-nosed wombat data sets when urinary progesterone metabolites (P4M) were \(\leq\) or \(>\) individual baseline values.

<table>
<thead>
<tr>
<th>Behaviours</th>
<th>(\leq) baseline urinary P4M</th>
<th>&gt; baseline urinary P4M</th>
<th>P value ((1, 117))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>±SEM</td>
</tr>
<tr>
<td>Digging</td>
<td>0.7</td>
<td>0.0 -</td>
<td>1.5</td>
</tr>
<tr>
<td>Exploring</td>
<td>1.3</td>
<td>0.6 -</td>
<td>1.9</td>
</tr>
<tr>
<td>Feeding</td>
<td>0.6</td>
<td>0.0 -</td>
<td>1.3</td>
</tr>
<tr>
<td>Grooming</td>
<td>0.8</td>
<td>0.0 -</td>
<td>1.3</td>
</tr>
<tr>
<td>Lying Awake</td>
<td>0.9</td>
<td>0.0 -</td>
<td>1.3</td>
</tr>
<tr>
<td>Sitting</td>
<td>1.1</td>
<td>0.3 -</td>
<td>1.6</td>
</tr>
<tr>
<td>Sleeping</td>
<td>0.9</td>
<td>0.6 -</td>
<td>1.2</td>
</tr>
<tr>
<td>Pacing</td>
<td>0.2</td>
<td>0.0 -</td>
<td>1.3</td>
</tr>
<tr>
<td>Sniffing</td>
<td>0.3</td>
<td>0.0 -</td>
<td>1.0</td>
</tr>
<tr>
<td>Toilet</td>
<td>0.2</td>
<td>0.0 -</td>
<td>0.8</td>
</tr>
<tr>
<td>Defensive</td>
<td>0.6</td>
<td>0.0 -</td>
<td>1.7</td>
</tr>
<tr>
<td>Approach</td>
<td>0.2</td>
<td>0.0 -</td>
<td>1.0</td>
</tr>
<tr>
<td>Evading</td>
<td>0.3</td>
<td>0.0 -</td>
<td>1.6</td>
</tr>
<tr>
<td>Nudging</td>
<td>0.0</td>
<td>0.0 -</td>
<td>0.8</td>
</tr>
<tr>
<td>Chase</td>
<td>0.0</td>
<td>0.0 -</td>
<td>0.8</td>
</tr>
<tr>
<td>Urogenital Sniffing</td>
<td>0.0</td>
<td>0.0 -</td>
<td>0.8</td>
</tr>
<tr>
<td>Urine Smelling</td>
<td>0.0</td>
<td>0.0 -</td>
<td>0.8</td>
</tr>
<tr>
<td>Rump Bite</td>
<td>0.2</td>
<td>0.0 -</td>
<td>1.2</td>
</tr>
<tr>
<td>Grasp/Restraint</td>
<td>0.5</td>
<td>0.0 -</td>
<td>0.9</td>
</tr>
<tr>
<td>Turning</td>
<td>0.1</td>
<td>0.0 -</td>
<td>0.7</td>
</tr>
<tr>
<td>Coitus</td>
<td>0.5</td>
<td>0.0 -</td>
<td>0.7</td>
</tr>
<tr>
<td>Break</td>
<td>0.1</td>
<td>0.0 -</td>
<td>0.9</td>
</tr>
<tr>
<td>Cohabitation</td>
<td>0.0</td>
<td>0.0 -</td>
<td>0.8</td>
</tr>
<tr>
<td>Guarding</td>
<td>0.0</td>
<td>0.0 -</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Bold data points indicate significant behaviour-urinary P4M interaction.
Table 5-6. The mean duration (Dur: log_{10} secs / 5 mins / 30 mins) and frequency (Freq: log_{10} n / 5 mins / 30 mins) of clustered behaviours expressed by captive southern hairy-nosed wombats when urinary progesterone metabolites (P4M) were ≤ or > individual baseline values.

<table>
<thead>
<tr>
<th>Behaviours</th>
<th>Analysis</th>
<th>≤ Baseline P4M Mean ± SEM</th>
<th>Range</th>
<th>&gt; Baseline P4M Mean ± SEM</th>
<th>Range</th>
<th>P Value (1,127)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1: Reproductive behaviours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chasing</td>
<td>Dur</td>
<td>0.3 ± 0.1</td>
<td>0 - 3.7</td>
<td>0.4 ± 0.1</td>
<td>0 - 4.3</td>
<td>3.308 0.07</td>
</tr>
<tr>
<td>Urine Smelling</td>
<td>Freq</td>
<td>0.2 ± 0.1</td>
<td>0 - 3.7</td>
<td>0.1 ± 0.1</td>
<td>0 - 1.9</td>
<td>1.803 0.18</td>
</tr>
<tr>
<td>Urogenital sniffing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nudging</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chin Resting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohabitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 2: Defensive behaviours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defensive</td>
<td>Dur</td>
<td>3.2 ± 0.3</td>
<td>0 - 8.8</td>
<td>2.4 ± 0.2</td>
<td>0 - 9.3</td>
<td>0.009 0.92</td>
</tr>
<tr>
<td>Evading</td>
<td>Freq</td>
<td>0.2 ± 0.1</td>
<td>0 - 2.3</td>
<td>0.2 ± 0.1</td>
<td>0 - 1.1</td>
<td>0.063 0.80</td>
</tr>
<tr>
<td>Kicking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 3: General behaviours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exploring /</td>
<td>Dur</td>
<td>12.2 ± 0.2</td>
<td>7.9 - 15.5</td>
<td>12.3 ± 0.2</td>
<td>7.5 - 15.5</td>
<td>0.002 0.97</td>
</tr>
<tr>
<td>Foraging</td>
<td>Freq</td>
<td>3.8 ± 0.1</td>
<td>1.9 - 5.9</td>
<td>4.0 ± 0.1</td>
<td>2.0 - 5.7</td>
<td>0.661 0.42</td>
</tr>
<tr>
<td>Sitting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digging</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grooming</td>
<td></td>
<td></td>
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</tbody>
</table>

5.4 Discussion

This study represents the first attempt to examine the relationship between general and reproductive behaviours and urinary P4M levels in the captive female SHNW. The results from this study showed that there was a high level of individual animal variation between the reproductive cycle dynamics based on urinary progesterone (Table 5-3) and the general and reproductive behaviours exhibited by each of the captive females (Table 5-4, Table 5-5, Table 5-6). Nevertheless, there were specific behavioural changes in pacing, aggression and toileting behaviour that have the potential to be used as markers to identify the reproductive status of female SHNW in captivity.

Both the duration of toileting and the urinary volume (mL) decreased during periods when urinary P4M was ≤ baseline values. As there was no difference observed in the frequency of urination nor in the frequency or duration of feeding, this evidence suggests that female SHNW may be retaining water prior to ovulation, resulting in more concentrated urine
and lower urine volume. Water retention has previously been reported to occur in both Sprague-Dawley® and Holtzman® female rats, where the accumulation of intraluminal uterine fluid coincides with the decline in progesterone levels (Armstrong, 1968; Nequin et al., 1979). In Merino ewes (Ovis aries), oviductal fluid volume was three times higher around the time of oestrus compared to volumes measure mid-cycle (Sutton et al., 1984). In human females, basal plasma osmolality was found to be decreased during the luteal phase (Stachenfeld et al., 1999), and females administered with exogenous 17β-oestradiol for 14 days had lower urine output compared to control females injected with saline (Stachenfeld et al., 1998). The phenomenon of decreased urine output and duration observed in the female SHNW may therefore be an important non-invasive behavioural / physiological link to reproductive hormones, such that monitoring individual animal changes in urination behaviour in breeding females could potentially be used to identify the reproductive status.

The significant relationship between changes in pacing behaviour and urinary P4M may also be a useful marker to identify the different stages of the oestrous cycle. Both the duration (P = 0.05) and frequency (P = 0.01) of pacing were significantly increased during periods of low urinary P4M. As pacing was more obvious in four of the seven females analysed, it is an important consideration for captive animal managers that animal care staff are familiar with the individuality of their breeding animals as the individual differences exhibited by the females in this study, may be overlooked. For example, F1, F2 and F3 demonstrated the most significant changes in pacing behaviour associated with changes in reproductive hormones, however, F9 and F10 were not pacing as frequently as the other females and F10 paced when urinary P4M was above and below baseline values. Regarding the pacing behaviour of the other two females, F4 was never seen pacing; however, it may be possible that she exhibited this behaviour along the fence line behind the dirt mound hidden from the camera. F5 was a rescued animal, and injuries sustained prior to this study resulted in her demonstrating slower and / or and limited mobility. For this reason, she was not used as a breeding female, but it may have also contributed to her not displaying pacing behaviours as seen in the other four captive females.

Increased activity levels and/or locomotion during oestrus have been observed in domesticated water buffalo (Bubalus bubalis) and dairy cows (Bos taurus) (Williams et al., 1986; Schofield et al., 1991; Arney et al., 1994; Maatje et al., 1997), in laboratory animals such as the golden hamster (Mesocricetus auratus) (Richards, 1966), and in a range of captive
wildlife species, including female giant pandas (*Ailuropoda melanoleuca*) (Owen *et al.*, 2016), and a female Malaysian sun bear (*Helarctos malayanus*) (Rog *et al.*, 2015), the stripe-faced dunnart (*S. macroura*) (Francis and Coleman, 1990), and the Julia Creek dunnart (Pollock *et al.*, 2010). Captive female common wombats (*Vombatus ursinus*) were reported to exhibit increased pacing behaviour at oestrus (Peters and Rose, 1979); however, in a previous study on the SHNW, no such relationship was found (Hogan *et al.*, 2010b). The lack of a significant observable increase in activity in female SHNW may be due to high level of between animal variation as seen in other variables in the current study, differences in the captive environment, or it could be due to the fact that the device used for monitoring activity levels in female SHNW in the Hogan *et al.* (2010b) study was attached by a collar to the neck of the female and were not pedometers per se. Hence, it is possible that the collar device may not have been sensitive enough to resolve pacing behaviour from other more general activities.

Wild female SHNW disperse more (visit more burrows) throughout the population compared to males (Walker *et al.*, 2008); however, the link between locomotion and oestrous cycle in wild female SHNW has yet to be investigated. While such a task would be difficult logistically, it is important to investigate whether this increased exploratory behaviour in wild females was correlated with changes in reproductive hormones or the onset of oestrus, in an attempt to locate or increase the female’s probability of encountering a male partner during her peak receptive period. More research is required to further investigate locomotion and pedometry in captive female SHNW to better understand its role in reproduction, and to confirm if the pacing behaviour observed in captive females is linked to reproduction, i.e. a female searching for a mate, or simply a reflection of stereotypic behaviours commonly exhibited by captive individuals (Clubb and Vickery, 2006; Hogan and Tribe, 2007). If the latter was true, then this behaviour would be less variable during the oestrous cycle, increased study animals might be able to confirm this.

In addition to pacing behaviour, rump bites exhibited by the female also increased significantly when urinary P4M was $\leq$ individual baseline values; however, females exhibited rump bites more frequently when housed in mating pairs compared to female only pairs. This change in behaviour suggests that when urinary P4M was low, females were more vigilant and aggressive towards their male den mates. This increased aggressive behaviour expressed by the female when urinary P4M was low could contribute to the decrease frequency in
urogenital sniffing bouts by the male during this period, as the female may not have permitted the male to investigate.

It is possible that the increase in aggressive behaviour observed in the female was a result of the increased number of mating attempts (grasp/restraint behaviours) initiated by the male when urinary P4M was low. While mating behaviours were observed throughout the female’s oestrous cycle, the frequency of mating attempts initiated by the male increased during what would be considered the optimum time for successful mating. However, as no other courtship and mating behaviours were correlated with changes in urinary P4M, this suggests that some females were not receptive to their male den mate, and that other factors, such as female mate selection, may prevent mating attempts (grasp/restraint behaviour) becoming successful mating bouts.

While the majority of mating behaviours observed in this study occurred during the normal breeding season in the wild (July to December, Gaughwin et al., 1998), males in this captive population have also been observed attempting to mate females during March (T. Janssen, personal observations), which is outside the normal SHNW breeding season, with one pouch young produced in February 2015. In addition, males in the current study were observed to mate females during the luteal phase (Appendix 9.4). It may be possible that in captivity, males mate females opportunistically rather than relying on the female to be receptive, which would explain why males appear to be unable to differentiate between oestrous and non-oestrous female urine (Swinbourne et al., 2017a). Forced cohabitation in captivity impacts the female’s ability to choose her mate, which may be a contributing factor to the poor captive breeding rate. For example, F2 was housed with a larger male, M5, and his prior injury prevented him from expressing normal mating behaviours, including chasing. While he did attempt to grasp, restrain and turn F2, she did not appear be receptive to him despite being at the optimum time for mating. F2 was aggressive towards M5 and displayed grasp/restraint behaviour towards him. Further, F4 was housed with M4 in 2013, and mating behaviours were never observed between the two animals, despite F4 having three oestrous cycles during that season. Female mate-selection would explain why some females in this study were not receptive to their male den mate at the optimum time for mating. It is possible that for some captive individuals housed together can become incompatible due to being indifferent to each other or overly aggressive (Zhang et al., 2004), and the increased burrow visits wild females (Walker et al., 2008) might suggest that females are seeking an unfamiliar
male to mate, which also explains why captive female exhibit increased pacing observed prior to the onset of the luteal phase.

For those females that were receptive to the male, it is difficult to accurately determine why the coital bouts observed just prior to the onset of the luteal phase did not result in a pouch young, including the mating bouts which resulted in a seminal plug in 2014. While the social structure of this species is not clearly understood, the number of burrow visits (Walker et al., 2008) suggests that conspecifics do no cohabitate for long, if at all. This is in direct contrast to the current captive management strategy where individuals are forced to cohabitate throughout the year, a housing strategy which may be a contributing factor to the low captive breeding rate. For example, the persistent reproductive behaviour exhibited by males in captivity, whether it be the male asserting dominance over the female as a conspecific or a stereotypic expression of boredom, has the potential to result in periods of chronic stress in the female which can negatively affect the normal secretion of reproductive hormones (Waiblinger et al., 2006), or potentially cause the loss of pouch young shortly after birth (Gaughwin, 1982; Lambert et al., 2011). As chronic stress can affect normal reproductive hormone secretion, (Waiblinger et al., 2006), the variability between and within animals regarding the length of oestrous cycles and luteal phases recorded in the females used in this study (Table 5-3) may be an indication of this stress, for example, being mated outside the female’s receptive period. Consequently, it may be prudent that future wombat reproduction research investigates different housing strategies such as single housing and paring during breeding seasons, to determine the effects of stress in females during the mating seasons and identify any negative impacts that unwanted mating attempts may have on female reproduction and the successful production of viable offspring.

5.5 Conclusion

When urinary P4M was low, female SHNW excreted less urine and exhibited more vigilant, agitated and aggressive behaviours, such as pacing and rump biting, with respect to their den mates. We conclude, therefore, that a thorough monitoring and elevation of these behaviours on an individual animal basis may be useful management tool for identifying reproductive status of this species in captivity. For example, the analysis of pacing behaviour in conjunction with changes in urination duration, and urine characteristics (volume and
concentration) of individual animals may be more profitable rather than relying on clearly defined behaviours that are contiguous across the population.

The current inability to use urinary oestrogens and LH to identify oestrous cycle state (Swinbourne et al., 2017b), or identify strongly expressed specific reproductive behaviours or events of mating is not seen, means that the detection of oestrus in this species remains a significant challenge. This phenomenon is further complicated by the fact that male captive SHNW reproductive behaviour (e.g. attempted mating) was observed in the luteal phase and outside the normal mating season, and is, therefore, not always a reliable predictor of oestrus in the female. We propose that further research is required into understanding the social structure and reproductive strategy of this species in the wild, so that we can better evaluate the potential impact of social structure in the reproductive management of SHNW in captivity.
5.6 References


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wombats (*Lasiorhinus latifrons*). General and Comparative Endocrinology, 
doi:10.1016/j.ygcn.2017.08.003


6. Olfactory behavioural responses in captive male southern hairy-nosed wombats (*Lasiorhinus latifrons*) exposed to oestrous and non-oestrous urine samples

Chapter published as

Abstract

Whilst flehmen behaviour has been observed in some marsupials, including the southern hairy-nosed wombat (*Lasiorhinus latifrons* - SHNW), it is unknown if it, or other male specific behaviours are exhibited differentially in response to the detection of oestrus females, which if proven, could assist in the detection of oestrus in breeding programmes for captive SHNW. Six captive male SHNW were exposed to urine samples collected from a single female that was confirmed to be in oestrus and mid-pregnancy. Urine samples were presented to the males simultaneously and subsequent behaviours recorded for five minutes. There were no significant differences in the number of times flehmen behaviour was displayed in response to oestrous and non-oestrous urine samples (P = 0.577), the number of times the male sniffed each sample (P = 0.595), or the time spent investigating each urine sample (P = 0.414). Our results suggest that neither male flehmen behaviour nor any specific male related pre-copulatory behaviour was associated with the olfaction of urine from a female in different stages of the oestrous cycles and is thus unlikely to be a reliable management tool for the detection of oestrus.

6.1 Introduction

The southern hairy-nosed wombat (SHNW – *Lasiorhinus latifrons*) is a large herbivorous burrowing species, and its nocturnal behaviour makes it difficult to study in the wild (Finlayson *et al.*, 2003; Taggart and Temple-Smith, 2008). Individuals kept in captivity for research or display can suffer from poor welfare, manifested as obesity, aggression, stereotypic behaviours, and restricted reproductive output (Jackson, 2003; Hogan *et al.*, 2013). While reproductive behaviours in both male and female SHNW have been
characterised (Hogan et al., 2011a), female specific behaviours have not been directly correlated with the onset and duration of oestrus (Hogan et al., 2010b). A study of the male’s behaviour towards a female when her reproductive state is known may provide insight into the relevance of behaviours associated with reproduction.

Pheromones present in biological fluids play a vital role in communication between and within species and oestrus-related pheromones in female urine and vaginal secretions can induce both behavioural and physiological changes in males (Baggott et al., 1987; Gomez-Diaz and Benton, 2013). One such behaviour, the flehmen response, commonly displayed after physical contact with conspecific excreta, is believed to facilitate transfer of pheromones into the olfactory organs (Tirindelli et al., 2009). Ethologically, it is a grimace in which an animal raises its head, retracts the upper lip, opens its mouth and draws air over the lumen of the vomeronasal organ which subsequently stimulates the autonomic nervous system (Sankar and Archunan, 2004). The flehmen response has been observed in many mammalian species and has been reported to occur more frequently and vigorously by males in the presence of an oestrous female (Estes, 1972). It is often displayed in conjunction with reproductive behaviours, including mounting and penile erection in cattle (*Bos indicus*) (Reinhardt, 1983), buffalo (*Bubalus bubalis*) (Rajanarayanan and Archunan, 2004), and goats (*Capra hircus*) (Sankarganesh et al., 2014).

The flehmen response has also been reported in a few marsupials, including the red kangaroo (*Macropus rufus*) (Coulson and Croft, 1981), antilopine wallaroo (*M. antilopinus*) (Croft, 1982), Doria’s tree kangaroo (*Dendrolagus dorianus*) (Gansloßer 1979, cited in Coulson & Croft 1981), tammar wallaby (*M. eugenii*) (Renfree et al., 1989; Schneider et al., 2008) eastern grey (*M. giganteus*) and western grey kangaroo (*M. fuliginosus*) (Coulson, 1997), and the SHNW (Gaughwin, 1979; Hogan et al., 2011a). Coulson (1997) has reported that male kangaroos would display flehmen after presenting their nose to a fresh stream of urine from the female which would occur prior to copulation, consequently, it was thought that flehmen was associated with reproduction in macropods. Flehmen has also been reported in a male SHNW exposed to female excreta, after the male had sniffed the female’s cloacal region, and when presented with a swab of the urogenital sinus of a female suspected to be in oestrus (Gaughwin unpublished data). However, as this was based on the observations of a single male in captivity and oestrus was not confirmed in the female, it is unknown whether this was representative of the species as a whole.
If it is determined that this behaviour or its frequency in the male SHNW is increased in response to oestrous cues from the female, it may have the potential to be used as a non-invasive method of detecting oestrus. However, it has not been experimentally tested to determine if the SNHW flehmen response is related to a specific reproductive state or is exhibited in response to other novel odours, such as scents from unfamiliar females. Therefore, the aim of this study was to examine the flehmen response and subsequent behaviours in male SHNW to determine their relationship to oestrous versus non-oestrous related odours in female urine and whether these behaviours might be used as a tool for reproductive management.

6.2 Methods and materials

6.2.1 Animals and animal management

Six healthy, sexually mature captive male SHNW, aged 4 to 8 years old, were kept / housed at the Australian Animals Care and Education wombat research and breeding facility in Mount Larcom, Central Queensland (23.75° S, 151.00° E). One male (M4: 27.8 kg) was housed individually, four males were housed in breeding pairs (M1: 33.3 kg, M2: 26.3 kg, M3: 23.9 kg, M6: 37.5 kg) and one male was housed as a breeding trio with two females (M5: 27.3 kg). Each enclosure consisted of an indoor temperature-controlled (air-conditioned) area (5.8 m²) with individual sleeping chambers (3 x 0.6 m² each). Each inside enclosure had an adjoining large outdoor yard enclosure 90 to 100 m², which contained a soil substrate, partial grass vegetation, and a dirt mound for enrichment.

All animals were fed a daily mixed ration of 120 g rolled oats (COLES® Smart Buy, Australia), 120 g Gumnut® pellets (MITAVITE, Australia), 35 g oaten chaff (Rich River Chaff and Grain, Australia), and 200 g sliced sweet potato. A half cob of corn was included three times a week when available and water was available ad libitum. The trial ran from September to October 2014 and was approved by the University of Queensland Animal Ethics Committee (SAFS/269/14).
6.2.2 Urine sample collection

Urine samples used in this trial were collected from a single healthy (25.3kgs) sexually mature female (four years of age) at two stages of reproductive activity, using a non-invasive methodology previously described in Chapter 3. Briefly, the female was conditioned to urinate in response to a light, tactile stimulation of the peri-cloacal region. The sample was collected into a small sterile collection tray placed posteriorly beneath the animal’s urogenital sinus, then immediately transferred into a 70 mL polypropylene specimen container (SARSTEDT, Germany) and stored on ice. Eight individual aliquots (5 x 2 mL and 3 x 5 mL) were removed from the original sample and then all urine samples were stored frozen (-20 °C) until urinary reproductive hormones could be measured using enzyme-immunoassay (EIA).

The urine samples representing oestrus (O) consisted of daily samples collected three days prior to and on the day mating (August 2013). After observations of mating and the presence of an expelled seminal plug three days following mating, the female was isolated. Urine samples collected 12 to 15 days after mating (September 2013), were used to represent a period in which the female was no longer in oestrus (Non-Oestrus: NO = Luteal phase). Visual confirmation of a pouch young six weeks following mating confirmed that the urine samples used for testing were representative of the female’s oestrus and non-oestrus (mid-gestation) period (Hogan et al., 2013). The urine samples selected for the O and NO urine were thawed approximately 12 months post-collection, pooled into their respective urine sample types, divided into 12 aliquots each, and then stored at -20 °C until required for the flehmen trial (within 3 weeks post first thaw).

6.2.3 Enzyme-immunoassay of urinary reproductive hormones

Daily urine samples were analysed for urinary progesterone metabolites (P4M) using a methodology previously validated for faecal P4M in female SHNW, and information regarding cross-reactivity of antibodies can be found in Hogan et al. (2010). Validation was confirmed through parallelism to a known standard concentration, and a recovery evaluation. All urine samples were standardised using a creatinine (Cr) assay (Cayman Chemicals, Michigan, USA), and hormone concentration is expressed as ng/mg Cr.
P4M antibody, CL425 (supplied by Coralie Munroe, UC Davis, USA), was dispensed into a goat anti-mouse gamma globulin pre-coated 96-well microtiter plate and incubated overnight at room temperature. Following incubation, 50 µL of diluted samples, standards (0.0156 to 4.0 ng/mL), high and low controls and P4M horseradish peroxidase enzyme conjugate were dispensed in duplicate into the plate and incubated at room temperature for three hours. A 2,2-Azino-bis(3-ethylbenzothiaoline-6-sulfonic acid) (ABTS: Sigma Aldrich, Australia) colour reaction was used to evaluate hormone concentrations and optical density was measured at 450 nm with a reference filter of 650 nm on a Biotek (Elx808) plate reader with Gen5 software. The P4M EIA sensitivity, based on 85 – 90% specific maximum binding, was 0.313 ng/mL. The intra-assay coefficient of variation was 4.4%, and inter-assay coefficient of variation based on the analysis of the high and low controls was 13%.

6.2.4 Presentation and flehmen testing

Two identical 1.8 L metal bowls (glued to thick conveyer belt rubber to prevent the wombats from knocking the bowl over) served as urine presentation vehicles. To prevent cross-contamination of odours, each bowl was clearly labelled as either O or NO, and after each trial, the bowls were cleaned and sterilised. Each male was individually tested between 1000 and 1300 h twice a day (1 to 2 hours apart) over 12 days, giving a total of 12 trials per male and 72 individual trials.

Initially, the bowls were presented to the males to determine if they were an appropriate presentation vehicle and to habituate the males to the presence of the bowls within their enclosure (three to five minutes per day for two weeks). Once habituated, prior to commencing each trial, the male was confined to his sleeping chamber and the den floor was mopped with disinfectant and allowed to dry to prevent odour transmission between trials. The O and NO urine was poured into its designated metal bowl, then the O sample was randomly assigned to either the far left or far right end of the enclosure, with the NO sample placed on the opposite side (bowls were approximately 110 cm apart and 150 cm from the door of the male’s sleeping chamber). The male was released from his sleeping chamber and behaviours were recorded for five minutes, after which, the male was removed from the testing area by either ushering him back into his sleeping chamber or releasing him into the outdoor yard enclosure.
An ethogram (Table 6-1) was developed, based on previous olfactory behavioural observations conducted on marsupials, both in the wild and captivity (Gaughwin 1979; Coulson and Croft 1981). Behavioural observations were recorded and analysed by the same person. Recorded behaviours included the frequency (number) and duration (seconds) of sniffing the sample in either bowl, exhibition of flehmen as defined previously, a raised head without flehmen, as well as behaviours directed at the urine sample (biting, pawing, moving, nosing the bowl) and other behaviours such as exploring the testing area, sitting or lying down, and failure to leave sleeping chamber.

Table 6-1. The description of behaviours observed by each of the six male southern hairy-nosed wombat when exposed to oestrus and non-oestrus urine samples for five minutes.

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sniffing</td>
<td>Inhalation of air through the nose in a repeated, shallow fashion.</td>
</tr>
<tr>
<td>Flehmen</td>
<td>After bouts of sniffing, the male raises its head, retracts the upper lip and inhales multiple times.</td>
</tr>
<tr>
<td>Raised head</td>
<td>After bouts of sniffing the male raises his head and inhales multiple times. Appearance of smelling the air.</td>
</tr>
<tr>
<td>Biting</td>
<td>Bites at either the bowl or the rubber.</td>
</tr>
<tr>
<td>Pawing</td>
<td>Uses paw to dig at the bowl or the rubber.</td>
</tr>
<tr>
<td>Moving</td>
<td>Uses paw to move the bowl around the testing area</td>
</tr>
<tr>
<td>Nosing</td>
<td>Uses nose to move the bowl around the testing area</td>
</tr>
<tr>
<td>Exploring the den</td>
<td>After bouts of sniffing, the animal moves away from both bowls and investigates the testing area. This includes sniffing or digging at the walls and floors and the other sleeping chambers.</td>
</tr>
<tr>
<td>Lying down</td>
<td>After bouts of sniffing, the animal moves away from both bowls and lies down either in the testing area or returns to their sleeping chamber.</td>
</tr>
<tr>
<td>Fail</td>
<td>Did not leave sleeping chamber to investigate the urine samples.</td>
</tr>
</tbody>
</table>

Each trial was recorded using three methods; (1) direct observations by the researcher (AMS) standing behind a barrier approximately 1.5 metres from the testing area, (2) a SONY® Handycam mounted on a tripod (VELBON® - Model: CX440) placed in the spare sleeping chamber, and (3) an indoor infrared (IR) dome camera (SUMO® - Model:
CAM35IRHR) installed on the roof of the den. All IR cameras were remotely connected to a digital video recorder (DVR - KOBI® 16 channel) surveillance system, and footage was reviewed using XQ Pro Series DVR surveillance software.

6.2.5 Data analysis

Recorded footage was analysed using the CowLog 2.0 software and statistical analysis was conducted using the PASW Statistics GradPack 18 (SPSS® IBM®). To determine differences in the frequency and duration of behaviours directed at the O or NO urine samples, an analysis of variance was conducted for individual males. To account for repeated measures combined data was also analysed with males nested within each trial. Residuals were checked for normal distribution, and for data that was not normally distributed, a non-parametric Moods median test was employed. Significance levels were set at $P < 0.05$, and data was expressed as mean ± standard error of mean (SEM).

6.3 Results

Analysis of urinary progesterone metabolites (Figure 6-1) indicates that the urine samples used for this study were collected at two different endocrinological phases of the female reproductive cycle. The mating which occurred during this reproductive cycle resulted in the production of a pouch young, further validating the reproductive distinctiveness of the urine samples used to define oestrus and non-oestrus periods of the cycle.

Of the 72 trials, 10 failed to produce a response. Of the remaining 62 trials, there was no difference found in the duration or frequency of behaviours that were analysed (Table 6-2), including the mean time spent investigating each urine sample, mean number of visits to each, mean duration of flehmen, or the mean number of times flehmen was exhibited. No differences were found between the mean duration or mean frequency of biting, pawing, moving or nosing behaviours directed towards either urine sample.
Figure 6-1. Urinary hormone analysis from a female SHNW over an oestrous cycle which resulted the birth of a pouch young. The grey boxes indicate the two different stages of the cycle; an oestrus (mating period) and mid-gestation.

Table 6-2. The mean duration (seconds) and frequency (number) of observed behaviours from six captive male southern hairy-nosed wombats exposed to oestrus (O) and non-oestrus (NO) urine samples for five minutes.

<table>
<thead>
<tr>
<th>Duration (secs)</th>
<th>Behaviour</th>
<th>O</th>
<th>NO</th>
<th>± SEM</th>
<th>P Value(df = 1,142)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sniffing</td>
<td>43.4</td>
<td>38.1</td>
<td>5.52</td>
<td>F=1.024 P=0.414</td>
</tr>
<tr>
<td></td>
<td>Flehmen</td>
<td>2.0</td>
<td>2.3</td>
<td>0.77</td>
<td>F=0.644 P=0.666</td>
</tr>
<tr>
<td></td>
<td>Raised Head</td>
<td>5.4</td>
<td>4.5</td>
<td>0.96</td>
<td>F=0.458 P=0.838</td>
</tr>
<tr>
<td></td>
<td>Moving</td>
<td>1.3</td>
<td>0.7</td>
<td>0.34</td>
<td>F=0.991 P=0.416</td>
</tr>
<tr>
<td></td>
<td>Biting</td>
<td>3.7</td>
<td>2.4</td>
<td>1.54</td>
<td>F=1.323 P=0.266</td>
</tr>
<tr>
<td></td>
<td>Pawing</td>
<td>2.7</td>
<td>2.8</td>
<td>0.89</td>
<td>F=0.414 P=0.798</td>
</tr>
<tr>
<td></td>
<td>Nosing</td>
<td>1.5</td>
<td>1.7</td>
<td>0.52</td>
<td>F=1.741 P=0.146</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frequency (n)</th>
<th>Behaviour</th>
<th>O</th>
<th>NO</th>
<th>± SEM</th>
<th>P Value(df = 1,142)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sniffing</td>
<td>13.4</td>
<td>12.7</td>
<td>3.14</td>
<td>F=0.770 P=0.595</td>
</tr>
<tr>
<td></td>
<td>Flehmen</td>
<td>0.5</td>
<td>0.6</td>
<td>0.19</td>
<td>F=0.765 P=0.577</td>
</tr>
<tr>
<td></td>
<td>Raised Head</td>
<td>1.2</td>
<td>1.0</td>
<td>0.19</td>
<td>F=0.141 P=0.990</td>
</tr>
<tr>
<td></td>
<td>Moving</td>
<td>0.3</td>
<td>0.3</td>
<td>0.84</td>
<td>F=1.149 P=0.338</td>
</tr>
<tr>
<td></td>
<td>Biting</td>
<td>0.4</td>
<td>0.3</td>
<td>0.15</td>
<td>F=1.277 P=0.284</td>
</tr>
<tr>
<td></td>
<td>Pawing</td>
<td>0.7</td>
<td>0.7</td>
<td>0.19</td>
<td>F=0.571 P=0.684</td>
</tr>
<tr>
<td></td>
<td>Nosing</td>
<td>0.4</td>
<td>0.5</td>
<td>0.13</td>
<td>F=1.631 P=0.172</td>
</tr>
</tbody>
</table>

When the data were examined at the individual animal level (Appendix Table 9-2) there was also no differences in the mean duration or mean frequency for the behaviours that were observed. Out of the six males, four males exhibited the flehmen response as previously described, and the other two raised their head after bouts of sniffing without retraction of the
upper lip. The mean exploration frequency was 1.0 ± 0.11 (mean duration 30.0 ± 6.54 s) and males sat or lay down an average of 0.3 ± 0.11 times (mean duration 13.1 ± 5.20 s) during testing periods.

6.4 Discussion

The objective of this study was to determine if olfactory related behaviours in the male SHNW could be used as a non-invasive method of detecting oestrous in female SHNW, but the data suggest that there were no population or individual differences between the number of times or the length of time spent investigating the O urine sample compared to the NO urine sample. Consequently, olfactory responses in the male do not appear to be useful as an accurate index of the reproductive status in the female SHNW. Our results suggest that for captive male SHNW, the flehmen response also appears to be specific to certain individuals, as only four out of the six males displayed flehmen during the testing period. The lack of differentiation between oestrous and non-oestrous urine olfactory related behaviours was surprising given that SHNWs can differentiate between conspecific odours (Descovich et al., 2012b).

There is no reliable non-invasive method of accurately determining when a female SHNW was in oestrus (Finlayson et al., 2006; Hogan et al., 2010b). Therefore, in order to provide an oestrous urine sample for this study, we collected urine from a single female that had experienced a successful mating and gestation. Urine collected over a three-day mating period was pooled and used as the O sample, and urine collected during three days of mid-gestation was pooled and used as the NO sample. As no other successful mating occurred during the trial period, we were restricted to the urine samples of just a single female. As the volatility (scent) of urine can differ between individuals (Schaefer et al., 2001), this is a potential limitation of the current study, as there was no possible way to determine if the sample used was of sufficient volatility to produce an olfactory response in all the test subjects.

Furthermore, the female used in the trial had been housed with M6 for over two years prior to commencing urine collection, and upon visual confirmation of a pouch young, the female was isolated from direct contact with all males. Consequently, this test subject (M6)
would most likely have been exposed to this particular O and NO urine prior to conducting the experiment and may have been habituated to her pheromones. While the other five males were in close proximity to the female (within 2 to 15 metres), there was no direct contact with either the female or with the O and NO urine samples, and as such, both urine samples would have been a novel stimulus to these males.

Another contributing factor to the lack of differentiation of urine sample type may be that while the urine samples were collected fresh and stored frozen within an appropriate time frame of three hours, the repeated freeze-thaw procedure for the flehmen test may have altered the odoriferous and pheromonal parameters of the urine. One study reported that vocalisations from male mice differed significantly when exposed to fresh female urine samples when compared to urine samples that were frozen for 24 to 28 hours at -20 °C (Hoffmann et al., 2009). In humans, the biochemical properties of urine change significantly when samples are frozen (-20°C) and then thawed six and 24 months later (Manley et al., 1992). Unfortunately, for this study freezing the daily urine samples was unavoidable as the only accurate method for determining a female in oestrus required retrospective analysis once mating and a pouch young presence had been confirmed.

It is also possible that in the case of captive male SHNWs that flehmen behaviour is displayed more in response to novel stimuli (the scent of an unfamiliar female) rather than flehmen being directly associated with reproduction. While flehmen is often closely associated with reproduction, this is not always the case (Marinier et al., 1988; Stahlbaum and Houpt, 1989; Swaisgood et al., 2000; Allen et al., 2015). Flehmen behaviour has been observed in stallions during field and controlled studies, where males exhibit flehmen regardless of the female’s reproductive status (Marinier et al., 1988). In both male and female foals flehmen was display in response to biological fluids collected from related and non-related females (Weeks et al., 2002). In Asian elephants (Elephas maximus), both sexes exhibit flehmen in response to conspecific odours. For example, a bull will exhibit flehmen in response to the inspection of the genitals and urine of the cow during the female’s oestrus period (Rasmussen et al., 1982; Thitaram et al., 2009), and female’s exhibit flehmen as a way of determining musth in bulls (Perrin and Rasmussen, 1994). Furthermore, flehmen in elephants has also been reported as a response to a novel odour which was associated with curiosity rather than directly related to reproduction (Dehnhard, 2011).
The use of olfactory enrichment also has conservation implications for captive breeding, rehabilitation and/or release programs. The use of predatory odours can be an effective conditioning method for predator aversion upon release (Apfelbach et al., 2005). In marsupials, exposure to dingo urine can elicit fear-based responses, and has been shown to deter foraging behaviour in some macropod species (Parsons and Blumstein, 2010a; Parsons and Blumstein, 2010b). Similarly, in SHNWs both in captivity (Descovich et al., 2012b) and in the wild (Sparrow et al., 2016), individuals appear more vigilant and will avoid excreta from dingos. However, it should be remembered that there is also a potential negative impact of introducing novel scents from conspecifics, prey or predators into captive environments. Olfactory enrichment may increase stress and agitation in some captive housed animals, resulting in more aberrant behaviours, and ultimately doing more harm than good (Wells, 2009). However, the results from this study show that captive males investigated both urine samples equally, and as such, it may be beneficial to investigate the use of novel odours as a form of positive enrichment for captive SHNW.

6.5 Conclusion

The results of this study suggest that in captive male SHNW olfactory behaviour in response to conspecific urine was not directly associated with the reproductive state of the female. As a result, the identification of such behaviours would not make an effective tool for monitoring female reproduction in captivity. Nevertheless, this study did produce some interesting findings regarding captive male SHNW behaviour. For example, the flehmen response, which has been observed in a number of mammalian species, was not exhibited by every male in this study, nor was it displayed exclusively after exposure to urine sample collected during the oestrus period. While there were some limitations identified in this study, it was evident that males did investigate both urine samples equally irrespective of female reproductive status. Further research needs to be conducted into the identification of non-invasive method of detecting oestrus for female SHNW which will enable real-time identification of oestrus and improve reproductive management of this species in captivity.
6.6 References


7. General discussion

The research reported in this thesis focused on methodologies which could be used to improve our understanding of the reproductive function of the southern hairy-nosed wombat (SHNW – *Lasiorhinus latifrons*). While currently it is not necessary to intensively breed SHNW in captivity, they are a valuable research proxy for understanding the reproductive function of the critically endangered northern hairy-nosed wombat (NHNW – *L. kreffti*), of which an estimated 230 individuals remain (Department of Environment and Heritage Protection, 2015). Through an understanding of the reproductive function of the SHNW we can develop reproductive technologies, such as artificial insemination and cross fostering techniques, for the conservation of the NHNW.

While the information on the hairy-nosed wombat reproductive endocrinology is limited (Hogan *et al.*, 2013), the results reported throughout this thesis provide insight into how different non-invasive methods, such as remote behavioural monitoring and the analysis of urine samples, may be applied to monitor female SHNW reproduction in captivity. The chapters in this thesis highlight the benefits and challenges associated with (1) characterising the reproductive cycle of the female SHNW in captivity, and (2) identifying specific physiological and behavioural changes in females which could be used to accurately identify the reproductive status of females in captivity.

**The development of a reliable non-invasive sample collection method for the evaluation of reproductive function (Chapter 2)**

Based on a toileting method for rearing pouch young (T. Janssen, *pers comm*), the use of a tactile stimulus in conjunction with classical conditioning enabled the longitudinal collection of daily urine samples from captive females during two consecutive wombat breeding seasons. While this thesis reported the collection of urine from females, a modified version of this methodology has been successfully applied for the collection and analysis of urinary reproductive and stress hormones in captive male SHNW (Du *et al.*, 2017).

An ideal urine collection method is one where each sample could be collected directly from the animal into a sterile collection vehicle, such as a small tray slid under the rump of the animal, so that each sample remains free from contamination. Successful urine collection
relied on understanding the animal’s normal sleeping routine which allowed for enough urine to pool in the bladder for the analysis of both the physical and chemical properties (volume, pH, specific gravity, and urinary cytology) and reproductive hormones.

While the use of tactile stimulus was the preferred collection method applied throughout this research project, some samples were collected via aspiration off the clean enclosure floor or through the use of a false floor. The use of the tactile stimulus collection method meant that urine samples were collected, for the most part, on demand, and samples could be collected from group housed individuals without the use of a non-toxic sample marker often required for faecal collection (Hogan et al., 2011b). A disadvantage of this collection method was the time initially invested into training animals, dedicating more than two hours both in the morning and the afternoon to training. For some captive breeding facilities this type of time investment may not be a viable option.

Aspiration off the clean enclosure floor would be a practical sample collection method especially in a time-poor breeding facility where fresh urine can be collected during normal husbandry practices with minimal direct contact with individual animals. Similar to the tactile method, an understanding of the animal’s normal urination behaviour and preferred latrine was essential in order to collect samples as fresh as possible. However, studies need to be conducted to determine if collection off the floor changes some of the physical characteristics of the urine sample.

The last collection method, the use of a false floor, was applied to two timid females who took longer to condition for daily urine sample collection. For one female (F5), this collection method was first applied to conditioning her to the presence of humans during urination, then over time gradually transitioned from the use of the false floor to collection into a sterilised tray. The second female (F6) was blind and kept to the indoor enclosure, with her preferred latrine being her sleeping chamber. For this female, the false floor was effective not only for daily urine collection but for managing her waste and the general cleanliness of her enclosure. While this method was effectively applied for the collection of daily samples from these two females, it would be an impractical method for an entire captive breeding population as the false floor was heavy, it needed to be thick enough to support the weight of a wombat and large enough to cover the sleeping chamber floor, and needed to be removed,
cleaned and sterilised daily (on occasion multiple times a day). For an entire captive population this method would be quite time consuming on top of normal animal husbandry routines, which again, would not be a practical collection method in a time-poor breeding facility.

The effectiveness of all urine collection methods relied on the temperament of the individual animal, with collection methodologies modified for aggressive or timid individuals. When comparing the collection modifications with the evaluated hormone profiles of the females in this study, it may be worth investigating a link between the response to conditioning and normal hormone secretion. For example, the females where collection methods had to be modified were not ideal for breeding. F5 and F6 were rescued animal and their injuries prevented them from being paired with a male for breeding. F4 was aggressive and the majority of samples collected were aspirated off the clean enclosure floor. In 2013, F4 did appear to cycle normally; however, in 2014 her urinary hormone profile did not represent normal reproductive cycles. In contrast, the other females that responded well to the tactile conditioning, especially F3 and F10, went on to produce pouch young. One area of further research may include temperament assessment of individual SHNWs in captivity as a means of identifying animals suitable for breeding compared animals more suitable for exhibition or display purposes only.

Another factor that was not investigated during this project was the effect of stress during conditioning for urine collection which required direct human-animal interaction or minimal human interaction i.e. waking the animal. Hogan et al. (2011c) reported learned helplessness behaviour in captive SHNWs, where individuals displayed less vigilant behaviours in captivity while faecal cortisol levels remained relatively high. Their research was conducted on wild-caught adults who acclimatised to the captive environment, whereas some of the females used in the current research were hand-raised as pouch young (~200 g). While the use of classical conditioning and collection directly from the animal may appear minimally invasive, it would be interesting to determine what effect, if any, direct human-animal interaction had during urine sample collection, both for the hand-raised young and rescued adults. Such research would allow captive SHNW managers to identify, modify and optimise collection procedures to minimise stress and allow for normal reproductive hormone secretion.
The validation and assessment of reproductive protein and steroid hormones in urine collected from captive female SHNW (Chapter 3)

The analysis of reproductive hormones in sample collected non-invasively is not a simple one-test-fits-all approach. An essential aspect of wildlife endocrinology is the validation of each biological sample for the species and hormone (or metabolite) of interest. Chapter 1.3 highlights the importance of validation to identify and overcome species-specific differences in hormone secretion, metabolic and excretion pathways to allow for the accurate interpretation of hormonal data. The results reported in Chapter 3 demonstrate the importance of using both biological challenges and in-house assay validations to accurately analyse hormone profiles. While the hormone profiles from females challenged with exogenous hormones demonstrated biologically relevant profiles, only progesterone metabolites could be accurately measured in naturally cycling (unchallenged) females.

This is not the first study to report a biological challenge conducted on captive SHNWs where significant hormonal changes could only be detected in plasma or the hormone could only be measured during the intensive sampling period. Hogan et al. (2010a) reported a significant increase in both plasma and faecal testosterone (faecal metabolites) in male SHNW following a 4µg GnRH agonist injection. However, natural seasonal changes in testosterone could only be measured in plasma but not in faeces. Similarly, Du et al. (2017) reported significant changes in plasma and urinary testosterone metabolites following a GnRH challenge. However, only marginal seasonal changes were measured in longitudinal urine samples (Du, 2017, Du et al., 2018). In contrast, significant seasonal changes in both plasma and faecal testosterone have been reported for wild male SHNWs (Hamilton et al., 2000). The fact that hormones can be detected following exogenous hormone challenges indicates that the analysis techniques applied were appropriate for that biological sample and hormone of interest. However, naturally occurring (not challenged with exogenous hormones) urinary hormone concentration measured in this study that did not appear to correspond or correlate with the expected degree of biological relevance tend to suggest there may be factors within the captive environment which may be affecting normal hormone metabolic processing and excretion pathways, making it difficult to accurately measure hormones in some captive SHNWs.
It may be possible that the differences between the diets of captive and wild SHNW may be a contributing factor to the difficulties in detecting certain hormones or their metabolites during natural reproductive cycles. An increase in food and water availability, diet composition and gut biota can alter the way hormones are metabolised, subsequently distorting the estimation of hormone metabolite concentration measured in non-invasive collected samples (Goymann, 2012). While more research needs to be conducted into refining the technique for the analysis of reproductive hormones in biological samples collected using non-invasive methods, the ability to detect and analyse reproductive hormones, such as oestrogen and testosterone, may rely on a captive diet which is more representative of the composition, quantity and seasonal variation found in the wild (composition of wild diets discussed in section 1.2.2). This warrants further investigation, as the effects of the captive diet may be limiting our ability to accurately measure hormones in samples collected using non-invasive methods during natural (unchallenged) periods.

The characterisation of the oestrous cycle of the captive female SHNW based on changes in urinary progesterone metabolites (Chapter 4)

While the results of this research highlight the potential use of urine as a tool to monitor reproduction in captive female SHNWs, there were limitations to the hormonal information gained from the analysis of urine from captive female SHNWs. Unfortunately, the timing of ovulation is still unknown, as too is information regarding the timing from ovulation to the formation of the corpus luteum and the subsequent detectable, sustained increase in progesterone secretion. While the use of progesterone metabolites has previously been used to describe the reproductive function in marsupials (Paris et al., 2002; Hogan et al., 2010b; Pollock et al., 2010), the urinary P4M profiles described in Chapter 4 can only be used as an estimate of the oestrous cycle dynamics in captive SHNWs as information on other essential reproductive hormones, such as luteinizing hormone and oestrogen, is still missing.

As described in Chapter 4, the level of variability observed between and within the females in this research population was not unusual for this species in captivity (Paris et al., 2002; Finlayson et al., 2006; Hogan et al., 2010b). However, with very limited reference to wild females (Gaughwin, 1981), the only longitudinal data regarding female reproductive endocrinology has been collected from captive individuals, which may in itself, be affected by the captive environment. For example, following the end of the second sampling period in
January 2015, F10 was successfully mated and gave birth to pouch young in February 2015. This occurred well outside the normal breeding season for SHNW in southern Australia (Gaughwin et al., 1998), but right in the middle of the NHNW breeding season in central Queensland [(November to April (Department of Environment and Heritage Protection, 2015)]. It may be possible that captive SHNW in central Queensland are acclimatising to the different environmental conditions between semi-arid condition across southern Australia and the sub-tropical conditions in central Queensland. Du et al. (2018) reported increased aggressive and reproductive behaviours from captive males between October and February which supports the acclimatisation hypothesis. However, the urinary P4M profiles of the other females within the captive breeding colony demonstrated that in some females peak urinary P4M appeared to decline with each subsequent oestrous cycle and oestrous cycle length increased towards the end of the SHNW breeding season. So why was F10 successfully mated at the end of January 2015? Unfortunately, this birth occurred outside the sampling period and information on the reproductive function leading up to that successful mating is unavailable. Nevertheless, the effects of the differences in the environmental conditions due to the location of the captive breeding facility should be investigated. It may be that SHNWs acclimatise to their captive environment, i.e. location, and captive breeding managers may need to either adjust their breeding program accordingly to accommodate for the differences between NHNW and the SHNW breeding seasons or prepare for extended breeding seasons for captive individuals.

Over the course of this research, only one pouch young was produced during a sampling period (another produced after the end of the second sampling period). Mating behaviour was observed on multiple occasions (Appendix 9.4) during both phases of the oestrous cycle; however, none of these other mating bouts resulted in pouch young, as measured by negative pouch checks. What was different about the mating of F3 and F10 which resulted in a successful production of a pouch young? One possibility is that these females were young, both being 4 years old when successfully mated. F1 was 9 years old during the sampling period and was mated multiple times during the 2014 breeding season (Appendix Figure 9-26); however, neither a seminal plug nor a pouch young was observed following mating bouts.

A captive born female SHNW at Brookfield Zoo in Chicago, USA, was 12 years old when she produced her fourth pouch young in 2012 (ZooBorns, 2012), with the sire being 20
years old. In November 2016, a wombat survey field trip conducted at Swan Reach in South Australia reported the recapture of a female with pouch young who was originally caught and tagged as an adult 15 years prior (D. Taggart, pers comm). This confirms that female SHNWs, both in captivity and in the wild, have the potential to produce young over many years. If it is not the age of the female that limits their reproductive potential in captivity, then there may be other factors such as the male’s inability to identify periods of oestrus, or male infertility and/or stress from group housing, which are hindering the success of mating bouts. Further analysis of other reproductive hormones (and/or their metabolites) may help to determine why females are not producing viable pouch young in captivity, even when females are mated at the appropriate time and a seminal plug is produced (F10, 2014).

Remote observations of reproductive behaviours in captive female SHNWs (Chapter 5 and Chapter 6)

The use of infrared cameras (IR) are a valuable tool for monitoring behaviour, development and ecology of many wildlife species (Fleming et al., 2014; Burton et al., 2015). IR cameras have become common practice in wombat research, both in the wild (Borchard and Wright, 2010; Thornett et al., 2017) and in captivity (Hogan and Tribe, 2007; Hogan et al., 2009; Hogan et al., 2011a; Hogan et al., 2011c; Descovich et al., 2012b; Descovich et al., 2012c). Hogan et al. (2009; 2011a) outlined the importance that IR cameras play in monitoring and analysing SHNW general and reproductive behaviours in captivity, and the use of IR cameras were fundamental to many aspects of the research reported in this thesis (Chapter 2; Chapter 5; Chapter 6). IR cameras were used to identify the optimum time of day to undertake conditioning for urine collection, as changes in general behaviours, such as sleep patterns and preferred latrines, could be observed daily. Rare reproductive behaviours, such as mating and a birth, were also monitored (and recorded) using IR cameras. Stereotypic behaviours, which would normally go unnoticed in some captive facilities, were also monitored daily and husbandry practices could be adjusted accordingly, such as swapping or adding logs/tree branches or adding extra dirt for digging as sources of enrichment within the captive environment.

The use of IR cameras was also fundamental to observing changes in general and reproductive behaviours which could be linked to changes in reproductive hormones. One of the most significant findings from the research described in Chapter 5 was the increase in
pacing behaviour when urinary P4M was low. Initially observed as a classic stereotypic
behaviour commonly displayed by captive animals (Clubb and Vickery, 2006), the
association with urinary hormone analysis puts pacing behaviour in context of reproduction.
A significant increase in pacing from 30 minutes when urinary P4M was above baseline
values to close to three hours per night when urinary P4M was below baseline values
provides insight into how female activity changes, possibly to seek mates, during the
optimum time for mating.

While activity levels in captive SHNWs (Hogan et al., 2009), and ranging behaviour
(Finlayson et al., 2005) and dispersal behaviour (Walker et al., 2008) of wild individuals have
been investigated, this is the first study to link locomotion to reproduction in female SHNWs.
The increased pacing behaviour exhibited by females in this study, and the female dispersal
behaviour in the wild (as evaluated by the increased number of burrows visited by females
compared to males) (Walker et al., 2008), suggests a female mate-choice component to their
reproductive strategy. Unfortunately, little is known about the natural social structure and
mating strategy of the SHNW.

In the wild, SHNWs are not strictly solitary, rather they live in a tolerant coexistence
with other individuals (Finlayson et al., 2005). They do not appear to be territorial as large
warrens can be used by up to 10 or more different individuals, not necessarily all at the same
time (Wells, 1989). Females tend to disperse, leaving their weaned pouch young in the
maternal burrow, whereas males tend to remain local and form small kinship groups (Walker
et al., 2008). This is in contrast to how individuals are housed in captivity. Despite the fact
there is little published evidence to support this, males are often seen as being the dominant
sex (Treby et al., 2005; T. Janssen pers comm). Males and females in captivity can coexist as
equals (pers obs), and larger or older females can be dominant and aggressive towards their
male den mate (Gaughwin, 1982; Chapter 5). Captive males are generally housed with one or
two females (Treby, 2005), and while individuals can live harmoniously for extended periods
within this configuration (Gaughwin, 1982), there is no evidence to show that this is the
optimum housing strategy for successful reproduction in captivity.

Captive breeding programs for some marsupial species, such as the sandhill dunnart
(Sminthopsis psammophila) (Lambert et al., 2011), dibbler (Parantechinus apicalis) (Mills et
al., 2012), koala (*Phascolarctos cinereus*) (Allen *et al*., 2008b) and Tasmanian devil (*Sarcophilus harrisii*) (Keeley *et al*., 2012), house animals individually or in small same sex groups and pair animals with the opposite sex for mating. This strategy enables managers to have complete control of reproduction within the captive breeding program, while also adhering to the natural socialisation behaviour of each species, e.g. the Tasmanian devil is considered solitary or has low levels of social interaction outside the breeding season (Hamede *et al*., 2009) and is therefore housed individually and paired for breeding (Keeley *et al*., 2012). The same approach should be investigated for captive SHNW within a breeding colony.

It is possible that the continual forced cohabitation of SHNW in captivity may be negatively impacting both male and female reproduction. While animals may be genetically compatible, pair incompatibility due to forced cohabitation has been identified as a contributing factor for low reproductive success in other captive species (Asa *et al*., 2011). Inappropriate housing for captive breeding also poses some potential welfare issues that need to be considered if the male is continually attempting to mate the female when she is not receptive. Indeed, if it is the female that seeks a mate when she is ready (as suggested by an observed increase in pacing), then the current housing strategy implemented into the SHNW captive breeding populations (1M:1F or 1M:2F) may not be optimum for successful breeding. While a more appropriate housing strategy may be isolation until a brief period of mate introductions, it is important to note that the timing of introductions for captive breeding relies heavily on the ability to monitor and reliably predict the female’s reproductive status. The results from this research describe the use of potential non-invasive tools to do just that; however, more research is required to overcome or understand the level of variability between and within females in a captive breeding population. Nonetheless, appropriate housing for captive breeding SHNW warrants further investigation, as a misunderstanding of this may be a strong contributing factor to the low captive breeding rate.

Further research should also be conducted to determine the effects of persistent mating bouts on male fertility. Hogan *et al.* (2011a) reported that females are only receptive for 13 hours; however, in the current study, F3 and F10 were receptive to their male den mate for longer than 13 hours, with the female being mated over three or four-day periods. Both studies reported the birth of pouch young, which further demonstrates the amount of variability in the reproductive function/behaviour of this species in captivity. If females are
receptive for a short period, then the persistent mating behaviour observed during this research and the lack of differentiation between oestrus and non-oestrous females reported in Chapter 6 suggest that captive males may be mating females opportunistically. An opportunistic mating strategy would explain why males mate females irrespective of their reproductive status. It makes sense that in the wild a male will attempt mate a female seeking a mate, however, in the wild an unreceptive female can escape, an option which is not provided to captive females. Unfortunately, for wild individuals there is currently no information on the duration of a mating bout, nor the frequency of mating bouts, or how many females a male will mate with during a single breeding season. It would be interesting to determine if the mating behaviour observed in captive males is normal or if the frequency of mating attempts negatively effects the male’s potential fertility.

What is perceived as normal reproductive behaviour and physiology in SHNW is only what has been reported in captive individuals, both wild-caught adults (Gaughwin, 1982; Hogan et al., 2011a), or hand-raised as pouch young (Chapter 5). The development of a captive breeding program, including housing, socialisation, dominance, and diet, is based on limited knowledge of wild individuals, most of which was conducted on the Murraylands population in South Australia (Gaughwin, 1981; Tiver, 1981; Gaughwin et al., 1998; Taggart et al., 1998; Hamilton et al., 2000; Finlayson et al., 2003; Finlayson et al., 2005; Taggart et al., 2005b; Walker et al., 2008; Finlayson et al., 2010; Ostendorf et al., 2012). The fact that SHNW do not breed well in captivity yet appear to be expanding their distribution in the wild (Swinbourne et al., 2016; Swinbourne et al., 2017; Swinbourne et al., submitted manuscript) suggests that there is something intrinsically wrong with current captive management approach for this species. If assisted reproductive technologies are to be developed, which in turn can be applied to a captive breeding program for the critically endangered NHNW, there are still some fundamental questions that need to be answered, especially regarding appropriate housing, social structure, diet and reproductive behaviour of the SHNW. Until then, we are extrapolating data collected from studies on captive individuals which is not representative of the conditions in the wild, and making assumptions as to what we think are the best conditions for captive breeding.
7.1 Summary of findings and conclusions

The development of appropriate management and housing conditions of SHNW in captivity is vitally important if they are to be used as a model for captive breeding of the critically endangered NHNW. The current captive population of SHNW is not self-sustaining and individuals have to be sourced from wild populations, either as hand-raised pouch young or rescued/injured adults which cannot be re-released (Hogan et al., 2013). Information on many aspects of SHNW physiology, behaviour and ecology is still limited, and a fundamental misunderstanding of these aspects may be detrimental to the success of captive breeding. The high level of within and between animal variation of the individual’s behaviour and reproductive physiology reported in this research clearly demonstrates the complexity of breeding this species in captivity.

There were a number of results gained from this research that can be implemented into captive breeding programs, not just for hairy-nosed wombats, but for other marsupial captive breeding programs.

The main findings of this research were:

1) The ability to condition females for daily urine sample collection, using non-invasive methods. By adapting the collection methodology for each female’s urination behaviour, samples could be collected regularly with minimal interference to normal animal behaviour.
2) The assay validation methods for the assessment of urinary reproductive hormones; luteinizing hormones, oestrogen (E1C, E2) were limited to challenged females; however, progesterone (P4M) could be accurately assessed during natural reproductive cycles.
3) There was a high level of variability within and between the reproductive cycle dynamics of the captive female SHNW. While this level of variability has previously been reported in other captive females, the limitation of only measuring urinary P4M in biologically relevant quantities means that predicting the reproductive status of captive female SHNW will continue to be challenging.
4) The identification of urinary characteristics; volume, concentration and urogenital epithelial cells, have the potential to be used as non-invasive markers for monitoring female reproduction in captivity.
5) Behavioural changes which were correlated to changes in urinary P4M profiles can also serve as non-invasive markers for monitoring captive female reproduction. However, some behaviours were not universally displayed by all females, i.e. pacing, indicating that more research needs to be conducted into understanding the reproductive hormone-behaviour interaction.

6) Due to the inconsistency and variability of observed male behaviours exhibited towards the female, males cannot be used as a reliable indicator of the reproductive status of female SHNWs in captivity.

7) Based on the level of individual animal variability, captive female SHNWs should be monitored individually rather than using the same changes in behavioural/physiochemical parameters for all females within the population.

The results from this research were promising regarding the validation methods for hormone analysis and the identification of some potential behavioural and urinary changes, and the knowledge gained will contribute to our current understanding of the reproductive physiology of the female SHNW. Despite this, we still have more to learn about the accurate timing of oestrus or ovulation in this species, and subsequently, more research needs to be undertaken into refining these analysis techniques in order to have a complete understanding of reproductive function of this species. The development of assisted reproductive technologies, such as artificial insemination and cross-fostering of pouch young, relies heavily on our understanding, accurate prediction, and the ability to manipulate the female oestrous cycle. To date, the information regarding the female SHNW’s oestrous cycle is still limited. If these technologies are to be successfully applied for conservation and management of both hairy-nosed wombat species, the SHNW should be breeding reliably in captivity and the captive population should be self-sustaining prior to removing any NHNW from an already vulnerable population for the purpose of captive breeding.
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### 9. Appendix

#### 9.1 Urinary progesterone profiles standardised for urinary specific gravity

Table 9-1. Reproductive cycle dynamics of seven captive female southern hairy-nosed wombat data sets based on urinary progesterone metabolites standardised for specific gravity. The luteal phase was classified as the beginning of a sustained increase in urinary P4M above baseline values to when urinary P4M returned to baseline values. An oestrous cycle was the end of one luteal phase to the end of the subsequent luteal phase.

<table>
<thead>
<tr>
<th>Season</th>
<th>Animal</th>
<th>Urinary P4M (ng/mL SG)</th>
<th>Luteal phase length (days)</th>
<th>Oestrous cycle length (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>Range</td>
<td>n / sampling period</td>
</tr>
<tr>
<td>2013</td>
<td>F3*</td>
<td>0.15</td>
<td>0.02 – 0.98</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>0.06</td>
<td>0.01 – 0.23</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>0.17</td>
<td>0.07 – 0.30</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>F6</td>
<td>0.12</td>
<td>0.06 – 0.57</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>F9</td>
<td>0.11</td>
<td>0.01 – 0.73</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>F10</td>
<td>0.33</td>
<td>0.02 – 11.32</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>F11</td>
<td>0.17</td>
<td>0.04 – 0.90</td>
<td>3</td>
</tr>
<tr>
<td>2014</td>
<td>F1</td>
<td>0.05</td>
<td>0.02 – 0.30</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>0.10</td>
<td>0.06 – 0.37</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>F4^</td>
<td>0.33</td>
<td>0.05 – 2.46</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F9</td>
<td>0.16</td>
<td>0.08 – 0.55</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>F10</td>
<td>0.41</td>
<td>0.03 – 1.28</td>
<td>3</td>
</tr>
</tbody>
</table>

*F3 was successfully mated during the 2013 breeding season and data on previous oestrous cycles were unavailable.

^F4: Urinary P4M was elevated above baseline values for 72 days of urine collection

n/a: Information not available
9.2 Urinary hormone standardisation methods

Figure 9-1. Urinary progesterone profile for F3 2013. Grey boxes indicate periods of elevated P4M above baseline values based on SG.

Figure 9-2. Urinary progesterone profile for F4 2013. Grey boxes indicate periods of elevated P4M above baseline values based on SG.
Figure 9-3. Urinary progesterone profile for F5 2013. Grey boxes indicate periods of elevated P4M above baseline values based on SG.

Figure 9-4. Urinary progesterone profile for F6 2013. Grey boxes indicate periods of elevated P4M above baseline values based on SG.
Figure 9-5. Urinary progesterone profile for F9 2013. Grey boxes indicate periods of elevated P4M above baseline values based on SG.

Figure 9-6. Urinary progesterone profile for F10 2013. Grey boxes indicate periods of elevated P4M above baseline values based on SG.
Figure 9-7. Urinary progesterone profile for F11 2013. Grey boxes indicate periods of elevated P4M above baseline values based on SG.

Figure 9-8. Urinary progesterone profile for F1 2014. Grey boxes indicate periods of elevated P4M above baseline values based on SG.
Figure 9-9. Urinary progesterone profile for F2 2014. Grey boxes indicate periods of elevated P4M above baseline values based on SG.

Figure 9-10. Urinary progesterone profile for F4 2014. Grey boxes indicate periods of elevated P4M above baseline values based on SG.
Figure 9-11. Urinary progesterone profile for F9 2014. Grey boxes indicate periods of elevated P4M above baseline values based on SG.

Figure 9-12. Urinary progesterone profile for F10 2014. Grey boxes indicate periods of elevated P4M above baseline values based on SG.
9.3 Urinary progesterone metabolite urogenital epithelial cells

Figure 9-13. Urinary progesterone and urogenital epithelial cell profiles for F3 2013. Grey triangles indicate estimated urogenital epithelial cell concentration.

Figure 9-14. Urinary progesterone and urogenital epithelial cell profiles for F4 2013. Grey triangles indicate estimated urogenital epithelial cell concentration.
Figure 9-15. Urinary progesterone and urogenital epithelial cell profiles for F5 2013. Grey triangles indicate estimated urogenital epithelial cell concentration.

Figure 9-16. Urinary progesterone and urogenital epithelial cell profiles for F6 2013. Grey triangles indicate estimated urogenital epithelial cell concentration.
Figure 9-17. Urinary progesterone and urogenital epithelial cell profiles for F9 2013. Grey triangles indicate estimated urogenital epithelial cell concentration.

Figure 9-18. Urinary progesterone and urogenital epithelial cell profiles for F10 2013. Grey triangles indicate estimated urogenital epithelial cell concentration.
Figure 9-19. Urinary progesterone and urogenital epithelial cell profiles for F11 2013. Grey triangles indicate estimated urogenital epithelial cell concentration.

Figure 9-20. Urinary progesterone and urogenital epithelial cell profiles for F1 2014. Grey triangles indicate estimated urogenital epithelial cell concentration.
Figure 9-21. Urinary progesterone and urogenital epithelial cell profiles for F2 2014. Grey triangles indicate estimated urogenital epithelial cell concentration.

Figure 9-22. Urinary progesterone and urogenital epithelial cell profiles for F4 2014. Grey triangles indicate estimated urogenital epithelial cell concentration.
Figure 9-23. Urinary progesterone and urogenital epithelial cell profiles for F9 2014. Grey triangles indicate estimated urogenital epithelial cell concentration.

Figure 9-24. Urinary progesterone and urogenital epithelial cell profiles for F10 2014. Grey triangles indicate estimated urogenital epithelial cell concentration.
9.4 Observed mating behaviour

Figure 9-25. Observed mating behaviour of F3: 2013. Grey diamonds indicate days of male-initiated mating attempts.

Figure 9-26. Observed mating behaviour of F1: 2014. Grey diamonds indicate days of male-initiated mating attempts.
Figure 9-27. Observed mating behaviour of F1: 2014. Grey diamonds indicate days of male-initiated mating attempts.

Figure 9-28. Observed mating behaviour of F4: 2014. Grey diamonds indicate days of male-initiated mating attempts.
Figure 9-29. Observed mating behaviour of F9: 2014. Grey diamonds indicate days of male-initiated mating attempts.

Figure 9-30. Observed mating behaviour of F10: 2014. Grey diamonds indicate days of male-initiated mating attempts.
### 9.5 Individual analysis of male behaviour

Table 9-2. The mean duration (seconds) and frequency (number) of observed behaviours from for each of the six captive male southern hairy-nosed wombats exposed to oestrous (O) and non-oestrous (NO) urine samples for five minutes.

| Animal: M1 | Duration (secs) |  |  |  |
|------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Behaviour  | O SEM P Value (df = 1,22) | NO SEM P Value (df = 1,22) | | |
| Sniffing   | 59.5 12.21 F=0.511 P=0.487 | 57.5 12.21 F=0.511 P=0.487 | | |
| Flehmen    | 5.5 3.61 F=0.885 P=0.522 | 9.3 3.61 F=0.885 P=0.522 | | |
| Raised Head| 30.3 1.27 F=0.178 P=0.680 | 2.1 1.27 F=0.178 P=0.680 | | |
| Moving     | 0.9 1.09 F=0.006 P=0.940 | 2.3 1.09 F=0.006 P=0.940 | | |
| Biting     | 2.7 2.45 F=0.464 P=0.507 | 2.6 2.45 F=0.464 P=0.507 | | |
| Pawing     | 1.4 1.54 F=0.055 P=0.818 | 4.3 1.54 F=0.055 P=0.818 | | |
| Nosing     | 1.1 1.40 F=0.166 P=0.689 | 3.3 1.40 F=0.166 P=0.689 | | |

| Animal: M2 | Duration (secs) |  |  |  |
|------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Behaviour  | O SEM P Value (df = 1,22) | NO SEM P Value (df = 1,22) | | |
| Sniffing   | 8.9 3.99 F=0.893 P=0.358 | 14.1 3.99 F=0.893 P=0.358 | | |
| Flehmen    | | | | |
| Raised Head| 0.9 0.76 F=1.620 P=0.220 | 1.8 0.76 F=1.620 P=0.220 | | |
| Moving     | 1.0 0.47 F=0.105 P=0.750 | 0.6 0.47 F=0.105 P=0.750 | | |
| Biting     | 17.2 8.17 F=3.570 P=0.076 | 7.9 8.17 F=3.570 P=0.076 | | |
| Pawing     | 6.1 3.38 F=0.985 P=0.335 | 5.3 3.38 F=0.985 P=0.335 | | |
| Nosing     | 1.7 1.45 F=3.237 P=0.090 | 2.6 1.45 F=3.237 P=0.090 | | |

| Animal: M3 | Duration (secs) |  |  |  |
|------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Behaviour  | O SEM P Value (df = 1,22) | NO SEM P Value (df = 1,22) | | |
| Sniffing   | 67.1 14.32 F=2.757 P=0.116 | 88.0 14.32 F=2.757 P=0.116 | | |
| Flehmen    | 3.4 1.27 F=1.248 P=0.280 | 2.0 1.27 F=1.248 P=0.280 | | |
| Raised Head| 6.7 2.06 F=1.583 P=0.226 | 7.2 2.06 F=1.583 P=0.226 | | |
| Moving     | 0.1 0.05 F=0.222 P=0.644 | 0.0 0.05 F=0.222 P=0.644 | | |
| Biting     | 0.1 0.05 F=0.128 P=0.725 | 0.1 0.05 F=0.128 P=0.725 | | |
| Pawing     | 1.7 0.66 F=0.041 P=0.843 | 0.2 0.66 F=0.041 P=0.843 | | |
| Nosing     | 0.9 0.52 F=2.773 P=0.115 | 0.5 0.52 F=2.773 P=0.115 | | |

| Animal: M4 | Duration (secs) |  |  |  |
|------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Behaviour  | O SEM P Value (df = 1,22) | NO SEM P Value (df = 1,22) | | |
| Sniffing   | 57.4 10.81 F=0.150 P=0.703 | 47.7 10.81 F=0.150 P=0.703 | | |
| Flehmen    | 0.5 0.49 F=1.065 P=0.317 | 0.4 0.49 F=1.065 P=0.317 | | |
| Raised Head| 10.7 2.71 F=0.900 P=0.357 | 7.3 2.71 F=0.900 P=0.357 | | |
| Moving     | 4.6 1.19 F=3.504 P=0.075 | 0.8 1.19 F=3.504 P=0.075 | | |
| Biting     | 1.0 1.46 F=0.006 P=0.941 | 2.9 1.46 F=0.006 P=0.941 | | |
| Pawing     | 6.3 3.26 F=2.677 P=0.121 | 6.9 3.26 F=2.677 P=0.121 | | |
| Nosing     | 5.0 2.12 F=1.942 P=0.182 | 3.5 2.12 F=1.942 P=0.182 | | |

<p>| Animal: M5 | Duration (secs) |  |  |  |
|------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Behaviour  | O SEM P Value (df = 1,22) | NO SEM P Value (df = 1,22) | | |
| Sniffing   | 11.6 5.64 F=1.993 P=0.175 | 6.8 5.64 F=1.993 P=0.175 | | |
| Flehmen    | 2.2 1.73 F=0.070 P=0.794 | 2.1 1.73 F=0.070 P=0.794 | | |</p>
<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Duration (secs)</th>
<th>Frequency (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O  NO ± SEM</td>
<td>F= P=</td>
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<tr>
<td>Raised Head</td>
<td>2.5 5.1 2.80</td>
<td>F=0.949 P=0.343</td>
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<tr>
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<tr>
<td>Biting</td>
<td>0.7 0.9 0.83</td>
<td>F=3.261 P=0.088</td>
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<tr>
<td>Pawing</td>
<td>0.3 0.2 0.25</td>
<td>F=1.457 P=0.243</td>
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<tr>
<td>Nosing</td>
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<td>Animal: M6</td>
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<tr>
<td>Sniffing</td>
<td>50.6 14.4 13.48</td>
<td>F=1.298 P=0.269</td>
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<tr>
<td>Flehmen</td>
<td></td>
<td></td>
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<tr>
<td>Raised Head</td>
<td>7.9 3.6 2.34</td>
<td>F=0.193 P=0.665</td>
</tr>
<tr>
<td>Moving</td>
<td>0.5 0.54 0.00</td>
<td>F=0.000 P=1.000</td>
</tr>
<tr>
<td>Biting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pawing</td>
<td>0.3 0.30 0.00</td>
<td>F=0.000 P=1.000</td>
</tr>
<tr>
<td>Nosing</td>
<td>0.7 0.71 0.00</td>
<td>F=0.000 P=1.000</td>
</tr>
</tbody>
</table>
9.6 Animal ethics certificates

ANIMAL ETHICS APPROVAL CERTIFICATE

15-Jul-2013

Activity Details:
Chief Investigator: Dr Steve Johnston, Agriculture and Food Sciences
Title: Wombat reproductive and assisted breeding biology (Wombat Artificial Insemination)
AEC Approval Number: SAFS/171/13/AACE
Previous AEC Number:
Approval Duration: 16-Jul-2013 to 16-Jul-2016
Funding Body: SAS
Group: Native and exotic wildlife and marine animals
Other Staff/Students: Clive Phillips, Tina Janssea, Vees Nicolson, Alyce Swinbourne

Location(s): Other Queensland Location

Summary

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<thead>
<tr>
<th>Subspecies</th>
<th>Strain</th>
<th>Class</th>
<th>Gender</th>
<th>Source</th>
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<th>Remaining</th>
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</thead>
<tbody>
<tr>
<td>Wombats</td>
<td>Southern Hairy-Nosed wombat</td>
<td>Adults</td>
<td>Mix</td>
<td>Institutional Breeding Colony</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Wombats</td>
<td>Adults</td>
<td>Female</td>
<td>Female</td>
<td>Institutional Breeding Colony</td>
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Permit(s):
Wildlife Demonstrator License WIDL04775411 22-Oct-2010 to 21-Oct-2013

Approval Details:

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<th>Description</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Wombats (Female, Adults, Institutional Breeding Colony) 9 Jul 2013 Initial approval</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Wombats (Southern Hairy-Nosed wombat, Mix, Adults, Institutional Breeding Colony) 9 Jul 2013 Initial approval</td>
<td>14</td>
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</tr>
</tbody>
</table>
Animal Ethics Approval Certificate

Please check all details below and inform the Animal Welfare Unit within 10 working days if anything is incorrect.

Activity Details:
Chief Investigator: Dr Steve Johnston
Title: Urinary hormone validation of induced ovulation in captive female southern hairy-nosed wombats
AEC Approval Number: SAFS/271/14
Previous AEC Number:
Approval Duration: 16-Sep-2014 to 16-Sep-2016
Funding Body: Internal Funds - UQ
Group: Native and exotic wildlife and marine animals
Other Staff/Students: Clive Phillips, Tina Janssen, Alyce Swinbourne
Location(s): Other Queensland Location

Summary
Subspecies | Strain | Class | Gender | Source | Approved | Remaining
--- | --- | --- | --- | --- | --- | ---
Wombats | Southern Hairy-Nosed wombat | Adults | Female | Pound/Refuge | 4 | 4

Permit:

Provision
Approval Details:
Description | Amount | Balance
--- | --- | ---
Wombats (Southern Hairy-Nosed wombat, Female, Adults, Pound/Refuge) 9 Sep 2014 Initial approval | 4 | 4

Please note the animal numbers supplied on this certificate are the total allocated for the approval duration

Please use this Approval Number:
1. When ordering animals from Animal Breeding Houses
2. For labelling of all animal cages or holding areas. In addition please include on the label, Chief Investigator's name and contact phone number.
3. When you need to communicate with this office about the project.

It is a condition of this approval that all project animal details be made available to Animal House OIC. (UAEC Ruling 14/12/2001)

The Chief Investigator takes responsibility for ensuring all legislative, regulatory and compliance objectives are satisfied for this project.

This certificate supersedes all preceding certificates for this project (i.e. those certificates dated before 15-Sep-2014)
Animal Ethics Approval Certificate

Please check all details below and inform the Animal Welfare Unit within 10 working days if anything is incorrect.

Activity Details:
Chief Investigator: Professor Clive Phillips
Title: The fleshmen response in male southern hairy-nosed wombats (Lasiorhinus latifrons)
AEC Approval Number: SAFS/269/14
Previous AEC Number: 
Approval Duration: 16-Sep-2014 to 16-Sep-2016
Funding Body: Internal Funds - UQ
Group: Native and exotic wildlife and marine animals
Other Staff/Students: Steve Johnston, Tina Janssen, Alyce Swinbourne
Location(s): Other Queensland Location

Summary
<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Strain</th>
<th>Class</th>
<th>Gender</th>
<th>Source</th>
<th>Approved</th>
<th>Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wombats</td>
<td>Southern Hairy-Nosed wombat</td>
<td>Adults</td>
<td>Male</td>
<td>Pound/Refuge</td>
<td>7</td>
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</tbody>
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Permit:

Provision:

Approval Details:

Description | Amount | Balance |
-------------|--------|---------|
Wombats (Southern Hairy-Nosed wombat, Male, Adults, Pound/Refuge) 12 Sep 2014 Initial Approval | 7 | 7 |

Please note the animal numbers supplied on this certificate are the total allocated for the approval duration.

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