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Reproductive biology of captive southern hairy-nosed wombats (*Lasiorhinus latifrons*). Part 1: oestrous cycle characterisation

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Abstract. Southern hairy-nosed wombats (SHNWs: *Lasiorhinus latifrons*) do not breed well in captivity. To better understand their reproduction, daily urine samples were collected from nine captive females and analysed for volume (mL), specific gravity and a qualitative index of the number of epithelial cells, then stored at −20°C until samples could be analysed for progesterone metabolites (P4M). The mean oestrous cycle length 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). 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 urinary characteristics. During the late follicular phase, urine volume decreased ($P = 0.002$) while urine specific gravity ($P = 0.001$) and concentration of epithelial cells ($P = 0.004$) both increased. The level of variability in oestrous cycle length suggests that some captive females may exhibit abnormal cycles; however, the changes in the urinary characteristics associated with the different stages of the oestrous cycle appear to offer a possible non-invasive means of monitoring the reproductive status of captive SHNWs.

Additional keywords: endocrinology, marsupial, progesterone, urine.

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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.* 2017*a*). However, their burrowing and nocturnal behaviour makes them a problematic species to study, both in the wild and in captivity. As a result, there is only limited information regarding their reproductive behaviour and physiology, so that breeding SHNWs in captivity has been challenging (Hogan *et al.* 2013), with only three births reported in Australian zoos (Australian Species Management Program, Zoological and Aquarium Association) since 2015 (V. Nicolson, pers. comm.). A better understanding of SHNW reproductive physiology would prove beneficial for the development and facilitation of future captive breeding programs not only for the captive SHNWs, but also as a model to better understand the reproductive physiology of other marsupial species, including the critically endangered northern hairy-nosed wombat (*Lasiorhinus krefftii*; Department of Environment and Heritage Protection 2015).

In order to improve current captive breeding programs and potentially develop assisted reproductive technologies, such as AI and cross-fostering (see Hogan *et al.* 2013), further data are needed regarding reproductive endocrinology of the oestrous cycle that would allow for the accurate assessment of the reproductive status of females in captivity. The collection and analysis of longitudinal blood samples has been investigated in the female SHNW (Finlayson *et al.* 2006); however, due to the large size of the SHNW (Jackson 2003*a*) and its non-domesticated nature, daily blood sampling is typically too invasive. Repeated capture, restraint and sedation of the animal has the potential to be costly and stressful for both the animal and the handler; for example, increased stimulation of the hypothalamic–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).

Non-invasive collection methods have proven to be an effective tool for monitoring reproduction in a variety of

wildlife species (Monfort 2003; Schwarzenberger 2007; Schwarzenberger and Brown 2013; Kersey and Dehnhard 2014), including marsupial species (Hamilton *et al.* 2000; Matson *et al.* 2008; Ditcham *et al.* 2009; Takahashi *et al.* 2009; Hogan *et al.* 2010*a*, 2010*b*, 2010*c*, 2011, 2012; Pollock *et al.* 2010; Lambert *et al.* 2011; Descovich *et al.* 2012*a*, 2012*b*; Keeley *et al.* 2012; Narayan *et al.* 2012, 2013; Du *et al.* 2017; Swinbourne *et al.* 2017*b*). In the SHNW, the non-invasive collection and analysis of faecal samples has provided helpful (Paris *et al.* 2002; Hogan *et al.* 2010*a*, 2010*b*, 2010*c*) albeit limited information regarding female reproduction based on faecal progesterone metabolites that appear to be stable and readily measured. However, other reproductive hormones, such as oestrogen, are heavily degraded and are difficult to measure in faeces (Hogan *et al.* 2010*c*).

Alternatively, the non-invasive collection and analysis of urine samples has been successfully used to assess reproduction in a variety of eutherian species, such as the Indian rhinoceros (*Rhinoceros unicornis*; Stoops *et al.* 2004), callitrichid monkeys (*Saguinus oedipus*, *Leontopithecus rosalia*, *Leontopithecus chrysomelas*, *Callithrix jacchus*, *Cebuella pygmaea*; Ziegler *et al.* 1993), killer whale (*Orcinus orca*; Robeck *et al.* 2004), the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*; Robeck *et al.* 2009) and the Amazonian manatee (*Trichechus inunguis*; Amaral *et al.* 2014). Although research regarding urinary hormone analysis in marsupials is comparatively limited (Matson *et al.* 2008; Takahashi *et al.* 2009; Pollock *et al.* 2010; Du *et al.* 2017, 2018; Swinbourne *et al.* 2017*b*), the collection and analysis of urine samples for assessing and monitoring reproduction in captive marsupials may prove to be beneficial for their non-invasive reproductive management.

In addition to urinary hormone analysis, physiochemical changes in urine sample composition and cytology may also provide potential novel indicators reflecting the reproductive status in females. Changes in urine volume have been linked to changes in oestrous cycle phases in the female house mouse (*Mus domesticus*; Drickamer 1995), and the presence and concentration of urogenital epithelial cells and leucocytes, collected through non-invasive urine samples or urogenital swabs, has been used as an effective method for the detection of oestrus in a variety of marsupials, including the Julia Creek dunnart (*Sminthopsis douglasi*; Pollock *et al.* 2010), squirrel glider (*Petaurus norfolcensis*; Woodd *et al.* 2006), Gilbert's potoroo (*Potorous gilbertii*; Stead-Richardson *et al.* 2010), the greater bilby (*Macrotis logotis*; Ballantyne *et al.* 2009) and the numbat (*Myrmecobius fasciatus*; Power *et al.* 2009).

Further, there have been several studies that have investigated the use of urogenital cytology to estimate the length of the oestrous cycle in both the common wombat (*Vombatus ursinus*; Peters and Rose 1979; West *et al.* 2004) and the SHNW (Finlayson *et al.* 2006). Although the proportion of epithelial cells was high when faecal progesterone was low in the common wombat (West *et al.* 2004), the anatomy of the urogenital sinus and the variation in sinus length between individual SHNW females makes it particularly challenging to be consistent with the sampling of vaginal smears from the epithelium of the sinus wall, making urinary cytology swabbed from the vaginal or urogenital mucosa an unreliable index of oestrous cycle characterisation (Finlayson *et al.* 2006).

As a non-invasive methodology has been successfully used for the daily collection of urine samples from both male and female SHNWs (Swinbourne *et al.* 2015, 2017*b*; Du *et al.* 2017, 2018), the aim of the present study was to determine whether the analysis of urine samples and urinary hormones could be used as an effective tool for the longitudinal assessment and monitoring of reproduction in female SHNWs. The relationship between specific reproductive and general behaviours and urine steroid metabolites is covered in a parallel analysis of the same SHNW data in this Journal (see Swinbourne *et al.* 2018).

Materials and methods

Animals and animal management

The present study was conducted during two wombat breeding seasons (July 2013–December 2014; August–December 2014) and was approved by the University of Queensland Animal Ethics Committee (SAFS/171/13AACE). All wombats (nine females, six males; Table 1) were housed and managed at the Australian Animals Care and Education (AACE) wombat research facility in Mount Larcom, central Queensland (23°50′09.8″S 150°58′39.9″E). Animals were housed in a temperature-controlled building consisting of eight large indoor enclosures, each containing a common area (5.8 m^2) with three individual sleeping chambers (0.6 m^2 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), 120 g gumnut pellets (Mitavite), 35 g oaten chaff (Rich River Chaff and Grain) and 200 g sliced sweet potato (locally farmed produce), with water available *ad libitum*.

Digital video camera monitoring

Indoor infrared dome cameras (Model CAM35IRHR; SUMO) and weatherproof outdoor infrared cameras (Model CAM78IRHR; SUMO) were installed in all wombat enclosures to allow for real-time and retrospective observation of wombat behaviours for reproductive behaviour analysis (Swinbourne *et al.* 2018) and to confirm the occurrence and timing of attempted mating and birthing behaviours. Footage was recorded and stored on a digital video recorder (DVR; KOBI 16 channel) surveillance system and reviewed using XQ Pro Series DVR surveillance software.

Non-invasive urine collection

Daily urine samples were collected non-invasively from all females (Table 1) using the methodology described previously 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 concrete den floor. Urine was transferred into a 70-mL polypropylene specimen container (SARSTEDT) and then maintained on ice throughout processing.

Table 1. Bodyweight and housing configuration of nine captive female southern hairy-nosed wombats during two consecutive wombat breeding seasons

Each breading season was considered a separate dataset because animals were examined over two distinct breeding seasons ($n = 12$ individual animal datasets)

Season	Wombat ID	Animal age (years)	Mean weight (kg)	Housing	Den mate	Mean weight (kg)
2013	F3[AE]	4	25.1	Breeding pair	M6[E]	35.8
	F4	6[D]	25.2	Non-breeding pair	M4[BE]	27.4
	F5	6[D]	29.5	Female pair	F6	29.6
	F6	5[D]	29.6	Female pair	F5	29.5
	F9[E]	4	20.5	Female pair	F10[E]	21.5
				Breeding pair	M3[E]	23.3
	F10[E]	3	21.5	Female pair	F9	20.5
	F11	10[D]	26.4	Female only		
2014	F1[E]	9	25.0	Breeding pair	M1[D]	36.4
				Breeding pair	M2[E]	30.7
	F2[E]	5	22.0	Breeding pair	M2[E]	30.7
				Non-breeding pair	M5[CE]	27.6
	F4	7[D]	28.6	Breeding pair	M6[E]	38.6
	F9[E]	5	22.7	Breeding pair	M1	36.4
				Female pair	F10[E]	23.6
	F10[E]	4	23.6	Breeding pair	M1	36.4
				Female pair	F9	22.7

[A]Female was successfully mated in August 2013.
[B]Vasectomised male.
[C]Hip injury as a joey limited reproductive behaviours as an adult.
[D]Age is estimated because the animal was rescued and brought into captivity as an adult.
[E]Animal was hand-raised as pouch young or brought into captivity as a juvenile.

Urine characteristics and cytology

Each urine sample was initially measured for volume (mL), pH (Catalogue no. FB33011; Fisher Scientific), leucocyte concentration (using a Combur-Test[9] urine test strip (four-point scale from 0 to 3); Roche Diagnostics) and specific gravity (using a hand-held refractometer (Bellingham + Stanley); range 1.000–1.080), then divided into two 2-mL aliquots and stored at −20°C until analysis of reproductive hormones using enzyme immunoassay (EIA). In all, 79 of 1161 urine samples (6.8%) were not analysed for urogenital epithelial cells because of low urine volume. Depending on the remaining volume of the collected urine, approximately 11 mL was transferred into a 15-mL conical Falcon tube (Catalogue no. 14-959-53A; Fisher Scientific) and centrifuged at 720*g* for 10 min at room temperature. 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 was transferred onto a microscope slide and covered with a coverslip. Using a light microscope (CH2 microscope; Olympus) at ×40 magnification, the relative number of epithelial cells present in each sample was estimated using a four-point scale: 0, none or clean urine; 1, low level; 2, moderate number of cells; 3, high number of cells (Jackson 2003*b*). The presence or absence of spermatozoa in the urine was also assessed to determine whether a successful mating had occurred. Male infertility was not suspected because a subsequent study confirmed the males used in the present study were producing spermatozoa (Du *et al.* 2018).

Urinary progesterone metabolite analysis

The analysis of urinary progesterone metabolites (P4M) has previously been described for SHNW (Swinbourne *et al.* 2017*b*). Based on 85–90% specific maximum binding, the specificity of the P4M EIA was 0.313 ng mL^{-1}, with intra- and interassay CVs based on analysis of high and low controls of 4% and 13% respectively. Urine samples were standardised for water content using a creatinine (Cr) assay (Cayman Chemicals). The intra- and interassay CVs for the creatinine assay were both 2%. Hormone concentrations are expressed in units of ng mg^{-1} Cr.

Reproductive cycle characterisation

Baseline hormone concentrations were calculated for each female using the iterative process described in Steinman *et al.* (2012). All values >2 s.d. from the mean were temporarily removed from the dataset and the mean recalculated. This process was repeated until no more values >2 s.d. could be removed. The remaining mean was then adopted as the baseline hormone concentration. Values >2 s.d. 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. The luteal phase concluded when urinary P4M returned to baseline values. The period between the

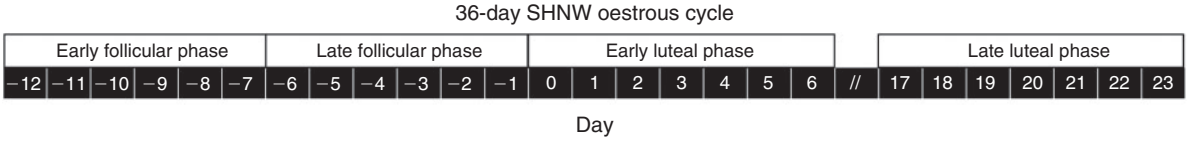

Fig. 1. Schematic of the estimated 36-day oestrous cycle in the southern hairy-nosed wombat (SHNW) divided into the follicular phase and luteal phase, based on data extrapolated from Paris *et al.* (2002), Finlayson *et al.* (2006) and Hogan *et al.* (2010*c*). Each phase is further divided into two stages: the early follicular phase from Day −12 to Day −7; the late follicular phase from Day −6 to Day −1; the early luteal phase from Day 0 to Day 6; and the late luteal phase the last 7 days before urinary progesterone metabolites (P4M) return to baseline levels (e.g. Days 17–23 if the luteal phase is 23 days).

end of one luteal phase and the end of the next was classified as an oestrous cycle (Hogan *et al.* 2010*c*).

Statistical analysis

All statistical analyses were performed using PASW Statistics GradPack 18 (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) and Hogan *et al.* (2010*c*)) 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 (Fig. 1): (1) the early follicular phase occurred from Day −12 to Day −7; (2) the late follicular phase was from Day −6 to Day −1; (3) the early luteal phase was the first 7 days following a sustained increased in urinary P4M above baseline (Day 0 to Day 6); and (4) the late luteal phase was the last 7 days before urinary P4M returned to baseline levels (to account for variable luteal phase lengths). For cycles that had a <12-day follicular phase period, the 6 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 $\log_{10}$ 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 (evaluated using both specific gravity and Cr concentration). Paired *t*-tests were performed to determine the relationship between changes in leucocyte and urogenital epithelial cell concentrations during each of the four oestrous cycle stages (Pollock *et al.* 2010). A Pearson correlation test was conducted to determine the relationship between the two urine standardisation methods; specific gravity and urinary Cr concentration were evaluated for each stage of the oestrous cycle and further tested to determine the relationship between urinary progesterone values standardised for Cr versus urinary progesterone values standardised for specific gravity. Finally, females were divided into two groups (i.e. breeding pair and female-only pairs or single-housed females) to determine whether the presence of the male had any effect on urinary progesterone concentration and/or luteal phase length.

Results

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

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

During 2013, one female (F3; Fig. 2*a*) 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

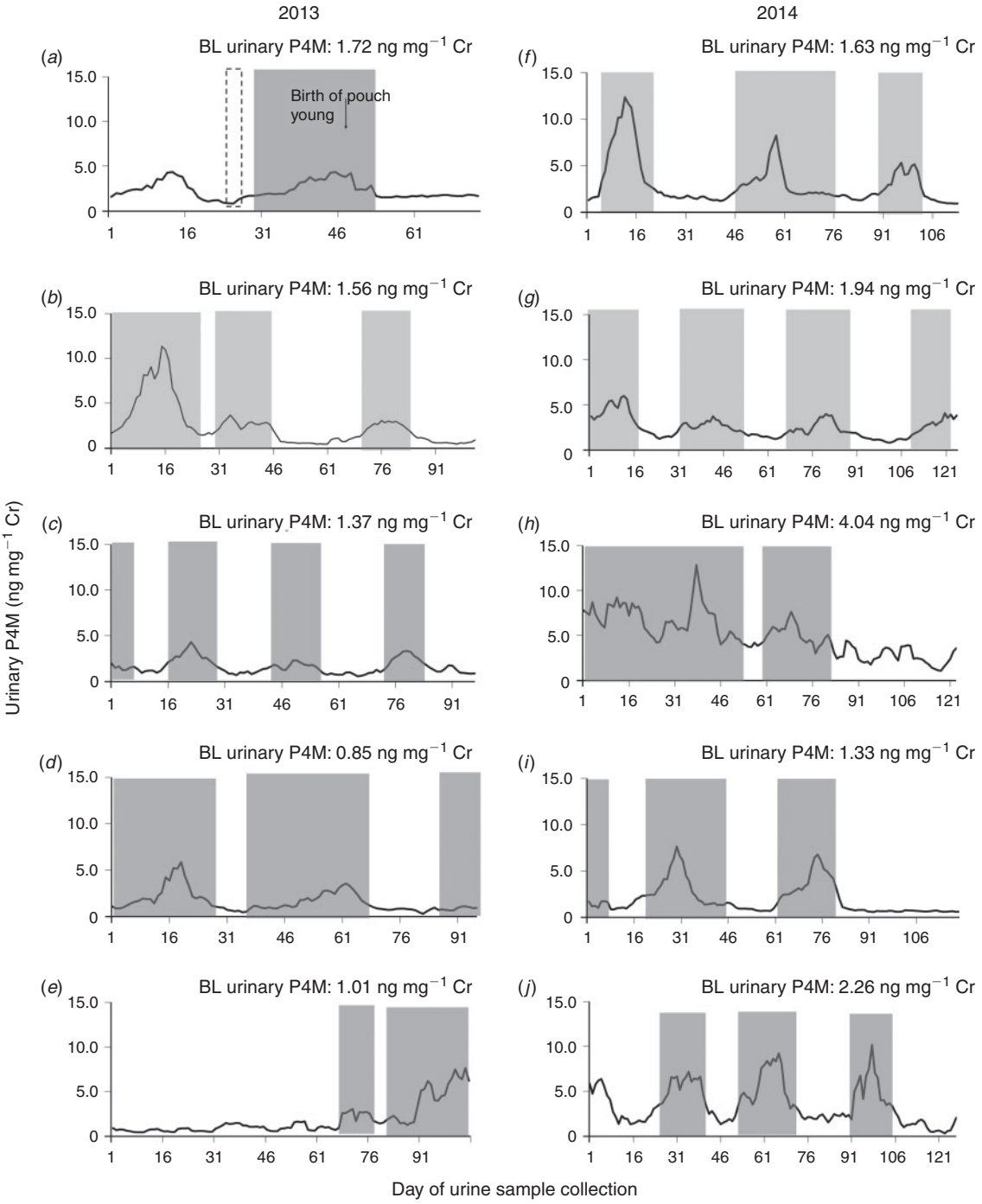

Fig. 2. Urinary progesterone metabolite (P4M) profiles of individual captive female southern hairy-nosed wombats during the 2013 (July–December) and/or 2014 breeding season (August–December): (*a*) F3, with successful mating (broken line box) and gestation, (*b*) F11, (*c*) F4, (*d*) F9, (*e*) F10, (*f*) F1, (*g*) F2, (*h*) F4, (*i*) F9 and (*j*) F10. The grey boxes indicate periods when urinary P4M was above individual baseline (BL) values. Cr, creatinine.

mating confirmed the presence of a pouch young. During the same breeding season (2013), F4 was housed with a vasectomised male (M6), and although no mating behaviour was ever observed between the two animals, hormone analysis showed that F4 had three complete oestrous cycles (Fig. 2*f*). The oestrous cycle lengths for F4 increased over the course of the 2013 sampling period (23 days, 25 days and 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 with the other females (0.85 and

Table 2. Reproductive cycle dynamics of 10 captive female southern hairy-nosed wombat datasets during the 2013 and 2014 wombat breeding seasons

The luteal phase was classified as the beginning of a sustained increase in urinary progesterone metabolites (P4M) above baseline values to when urinary P4M returned to baseline values. An oestrous cycle was from the end of one luteal phase to the end of the subsequent luteal phase. Cr, creatinine

Season	Animal	Urinary P4M (ng mg^{-1} Cr)		Luteal phase length (days)			Oestrous cycle length (days)		
		Baseline	Range	No. sampling periods	Mean	Range	No. cycles analysed	Mean	Range
2013	F3[A]	1.72	0.14–7.03	~21-day gestation period; no luteal phase identified					
	F4	1.37	0.11–5.00	3	13.6	12–15	3	28	26–30
	F9	0.85	0.03–5.89	2	26.5	26–27	1	33	
	F10	1.01	0.17–7.62	1	19		1	29	
	F11	1.56	0.34–12.32	3	18.3	14–24	2	30	22–38
2014	F1	1.63	0.82–12.88	3	23.3	18–30	2	41	39–43
	F2	1.94	0.73–8.17	2	23.5	23–24	2	42	38–46
	F4	4.04	0.87–12.96	1	18		1	23	
	F9	1.33	0.54–7.99	2	27	20–33	1	43.5	40–47
	F10	2.26	0.11–13.46	3	22.3	22–23	3	32.3	29–35

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

1.01 ng mg^{-1} Cr). F9 was in a luteal phase at the beginning of the 2013 sampling period that lasted 30 days before returning to baseline values briefly for 6 days and then increasing significantly again for 33 days. Urinary P4M returned to baseline levels again for 21 days, indicating an extended period of baseline P4M before the next luteal phase (Fig. 2*d*). For F10, urinary P4M remained below baseline values until Day 68 of urine collection, when urinary P4M increased significantly for 17 days, returning to baseline values for 4 days, then remained significantly elevated for the remainder of the 2013 sampling period (Fig. 2*e*). Urinary P4M for F11 (Fig. 2*b*) exhibited 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) that 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 with 2013. In 2014, luteal phases for F4 were difficult to identify accurately due to elevated urinary P4M during the first half of the sampling period (Fig. 2*h*), with the only notable difference being that she was housed with a larger male (M5) who exhibited persistent mating behaviour (chasing, rump bites and grasp/restraint, occurring between 10 and 33 days apart over 5–9 consecutive days). Two complete oestrous cycles were measured for F9 (2014), and although the variability in luteal phase length between the two seasons was greater in 2014 (20 and 33 days) compared with 2013 (26 and 27 days), the mean luteal phase length was similar for both sampling periods (25.5 vs 26.5 days in 2013 and 2014 respectively). 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 (Fig. 2*i*). F10 had three distinct oestrous cycles throughout the 2014 sampling period, with the last oestrous cycle having an extended period before the commencement of the following luteal phase (Fig. 2*j*).

Urine concentration, evaluated as both specific gravity ($F_{3,1097} = 8.532$; $P = 0.001$) and urinary Cr concentrations ($F_{3,1097} = 17.735$; $P = 0.001$), was highest during the late follicular phase compared with the other three stages of the oestrous cycle (Table 3). There was also a significant correlation between the two urine standardisation methods ($P = 0.001$; Fig. 3); however, when analysing individual hormone profiles, the correlation between urinary progesterone values standardised for Cr and urinary progesterone standardised for specific gravity varied between females, demonstrated both weak and strong relationships (Table 4). Both standardisation methods produced similar urinary progesterone profiles as there was no observable difference in the pattern of the urinary progesterone profiles (Fig. 4). Although urine volume was significantly lower ($P = 0.007$) during the late follicular phase compared with the early follicular phase or during both luteal phase stages (Table 3), there were no significant changes in urinary pH between each of the oestrous cycle stages ($P = 0.114$; Table 3). Regarding urinary cytology, urogenital epithelial cells increased significantly during the late follicular phase ($P = 0.001$; Fig. 5) and decreased during the luteal phase; however, there was no significant relationship between changes in urinary leucocyte concentration during each stage of the oestrous cycle ($P = 0.334$; Fig. 5). No spermatozoa were found in any of the urine samples collected following bouts of mating, including the female that had a successful mating and gave birth in 2013.

Discussion

The present 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 concentrations across cycles), the results of the present study reveal that analysis of urine samples could be used to identify the length and frequency of oestrous cycles in captive female SHNWs, with

Table 3. Urinary characteristics and cytology parameters of daily urine samples collected from nine different captive female southern hairy-nosed wombats

Volume, specific gravity (SG), pH and urinary creatinine concentration (Cr) during the early and late follicular phase (FP) and early and late luteal phase (LP) based on concentrations of urinary progesterone metabolites. Bolded values indicate significant changes in the urine characteristic at different stages of the oestrous cycle. Within rows, different superscript letters indicate significant differences between each stage of the oestrous cycle ($P < 005$)

Parameter	Early FP		Late FP		Early LP		Late LP		$F_{3,1027}$	*P*-value
	Mean ± s.e.m.	Range	Mean ± s.e.m.	Range	Mean ± s.e.m.	Range	Mean ± s.e.m.	Range		
Volume (mL)	**26 ± 1^{a}**	**1–142**	**25 ± 1^{ab}**	**1–100**	**31 ± 2^{c}**	**1–122**	**30 ± 2^{c}**	**2–156**	**4.071**	**0.007**
SG	**1.036 ± 0.001^{a}**	**1.00–1.07**	**1.039 ± 0.001^{b}**	**1.00–1.07**	**1.033 ± 0.001^{c}**	**1.00–1.08**	**1.033 ± 0.001^{c}**	**1.00–1.08**	**6.758**	**0.001**
pH	6.5 ± 0.1	4.0–9.0	6.4 ± 0.1	5.0–9.0	6.3 ± 0.1	4.0–9.0	6.4 ± 0.04	4.0–9.0	1.990	0.114
Urinary Cr (ng mg^{-1})	**5.6 ± 0.2^{a}**	**0.35–14.4**	**6.07 ± 0.30^{b}**	**0.3–18.1**	**4.8 ± 0.3^{c}**	**0.2–15.2**	**4.3 ± 0.2^{c}**	**0.3–14.72**	**18.412**	**0.001**

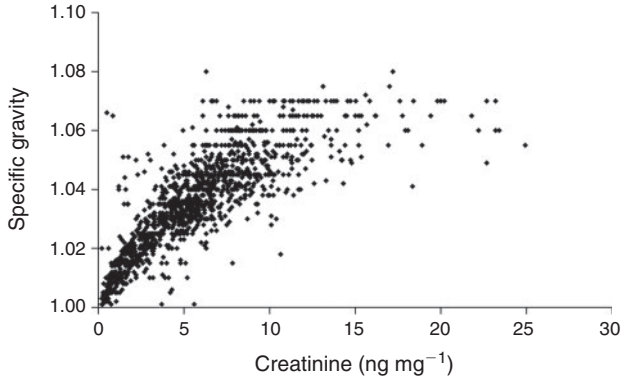

Fig. 3. Correlation between two urine standardisation methods: specific gravity and creatinine concentration ($R^2 = 0.66$; $r = 0.810$; $P = 0.001$).

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

Season	Animal ID	No. samples	R^2	*r*	*P*-value
2013	F3	76	0.22	0.452	0.001
	F4	103	0.48	0.694	0.001
	F5	38	0.74	0.861	0.001
	F6	55	0.16	0.395	0.002
	F9	95	0.54	0.734	0.001
	F10	93	0.21	0.460	0.001
	F11	104	0.09	0.304	0.002
2014	F1	111	0.52	0.718	0.001
	F2	120	0.44	0.666	0.001
	F4	124	0.52	0.719	0.001
	F9	117	0.54	0.736	0.001
	F10	125	0.43	0.655	0.001

significant changes in the volume of urine recovered, urine concentration (urinary specific gravity and creatinine concentration), as well as the concentration of urogenital epithelial cells throughout the oestrous cycle. Therefore, we suggest that the analysis of urine samples has the potential to be used as a biomarker for monitoring reproduction in captive SHNWs.

The longitudinal urinary P4M profiles analysed in the present 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*c*). In the present 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 before the sampling period (T. Janssen, pers. obs.) 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 and December (Gaughwin *et al.* 1998).

Although mean oestrous cycle length was similar to previous reports, the variability in the oestrous cycle length of the females in the present study (range 23–47 days) was greater than the range reported previously, for example 41.1 days ($n = 2$; Paris *et al.* 2002), 35–38 days ($n = 8$; Finlayson *et al.* 2006) and 27–38 days ($n = 8$; Hogan *et al.* 2010*c*). Similarly, the variability of luteal phase length between females within the present study (12–33 days) was slightly greater than the variability previously reported for captive females SHNW, namely 27.6 days (Paris *et al.* 2002), 16–26 days (Finlayson *et al.* 2006) and 15–31 days (Hogan *et al.* 2010*c*). Interestingly, Hogan *et al.* (2010*c*) also reported luteal phases during March and April in 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, currently we cannot accurately determine what may be considered normal oestrous cycle variability within this species and therefore are uncertain whether our results are reflective of normal SHNW reproductive biology or an artefact of captivity.

Although 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 exhibited by some female captive SHNWs in the present 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*c*) without the accompanying analysis of meaningful changes in oestrogen and luteinizing

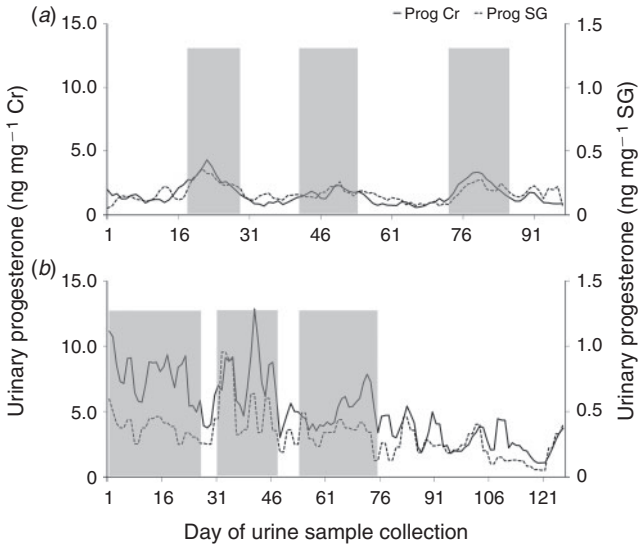

Fig. 4. Comparison of urinary progesterone metabolites adjusted using two different urinary standardisation methods, namely creatinine (Cr) and specific gravity (SG). Urinary P4M profiles in F4 during (*a*) 2013 and (*b*) 2014. The grey boxes indicate periods when urinary P4M was above baseline for the SG method.

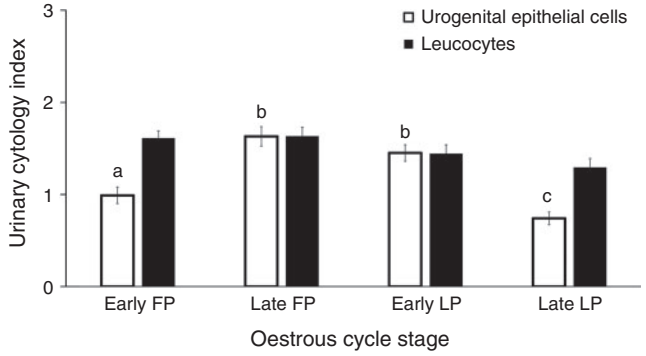

Fig. 5. Changes in urogenital epithelial cells and leucocytes across the different stages of the captive female southern hairy-nosed wombat oestrous cycle. Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between the stages of the oestrous cycle. Urinary cytology index as described in methods: 0, none or clean urine; 1, low level; 2, moderate number of cells; 3, high number of cells. FP, follicular phase; LP, luteal phase.

hormone (LH), which, so far, have been difficult to accurately measure in non-invasively obtained samples (Hogan *et al.* 2010*c*; Swinbourne *et al.* 2017*b*). In the present study, 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 SHNWs. 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 1–4 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 the present 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 an over- or underestimation of the length of the luteal phase 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 which 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 ±1.5 s.d. from the mean (Keeley *et al.* 2012) or ±1.75 s.d. from the mean (Oates *et al.* 2004; Hogan *et al.* 2010*c*, 2012; Mills *et al.* 2012). Although Moreira *et al.* (2001) initially used the same methodology, they reported more biologically relevant profiles when baseline values were calculated using the mean ± 2 s.d. This was also the case for the profiles reported in the present study. For example, when baseline values greater than the mean ± 1.75 s.d. were removed during the process, urinary P4M for F11 and F4 remained above baseline for 47 and 85 days respectively. Similar to the outcomes reported by Moreira *et al.* (2001), using the mean ± 2 s.d. resulted in more biologically relevant hormone profiles compared with the mean ± 1.5 s.d. (Keeley *et al.* 2012) or the mean ± 1.75 s.d. (Oates *et al.* 2004; Hogan *et al.* 2010*c*, 2012; Mills *et al.* 2012), which have 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, whether the iterative process needs to be evaluated and adjusted for each female.

Although 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 luteinised unruptured follicles, a persistent CL or the formation of an ovarian cyst, can result in prolonged, erratic and infertile anovulatory cycles and has been previously reported in a variety of eutherian mammals, including some deer (*Cervinae*) species (Adam *et al.* 1985; Curlewis *et al.* 1988), domestic cattle (Sheldon *et al.* 2006), breeding mares (King *et al.* 2010) and African black (*Diceros bicornis*), white (*Ceratotherium simum*; Brown *et al.* 2001) and Indian rhinoceros (*R. unicornis*; Stoops *et al.* 2004). Another possibility worth investigating are the effects of stress and increased adrenal response, which have been shown to disrupt or reduce reproductive fitness (Bonier *et al.* 2009). Persistent mating behaviour exhibited from the males towards the female regardless of her receptivity may be a contributing factor to the prolonged periods of progesterone secretion. More research needs to be conducted to determine whether the long luteal phases reported in some females in the present study are the result of abnormal ovarian function or a response to the intrinsic factors within the captive environment. This will help 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 $F_{2\alpha}$-induced luteolysis, CL regulation in marsupial appears to be programmed by intrinsic factors. Once formed, the CL, influenced by LH (Stewert 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 fetus 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 exhibited by some captive female SHNWs in the present 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.* 2005). 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 present study (F2, F4, F9, F10 and F11), the increase in days between luteal phases coincided with increased day length during the sampling period. The effect of photoperiod on reproduction has been investigated in the brushtail possum (*Trichosurus vulpecula*; Gemmell and Sernia 1995) and honey possum (*Tarsipes rostratus*; Oates *et al.* 2007), as well as some *Antechinus* species (Dickman 1985; McAllan and Dickman 1986; McAllan and Geiser 2006); these studies have revealed that an increase in day length has a negative effect on reproduction, resulting in increased oestrous cycle length or reduced fertility. Although data on wild female SHNWs is extremely limited, a long-term study on wild male SHNW reproduction showed that significant reductions in ejaculate volume, sperm number and motility and the size of sex accessory glands occur from November to January (Taggart *et al.* 2005); this time period coincides with increased photoperiod in South Australia (Geoscience Australia 2017). It is currently unknown what effect photoperiod may have on SHNW reproduction, and further research could include the investigation of the use of artificial light schedules to identify the role that photoperiod may have in SHNW reproduction or as a means of altering the captive environment to optimise reproduction.

Although urinary P4M was an effective tool for identifying and evaluating the reproductive activity of captive female SHNW, the urinary characteristics of volume, specific gravity and urinary cytology also appear to be useful and non-invasive tools that captive wombat managers could use to identify females in different stages of the oestrous cycle. First, when urinary P4M was below baseline values, the volume of the urine collected decreased, which coincided with decreased toileting behaviour observed during the same sampling period (Swinbourne *et al.* 2018). Because urine can be easily collected from the concrete floor of the enclosure and transferred into a measuring cylinder, this may be a useful tool for potential oestrous detection; however, the collection of urine from wombats in more naturalistic enclosures could present some logistical challenges. Second, the concentration of urine (both specific gravity and Cr values) was significantly higher when urinary P4M was less than 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 vs urinary Cr 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). Although there was a strong correlation between the two urine standardisation methods in the present study, the analysis of individual female profiles demonstrated a strong correlation between the two methods for some (e.g. F9 (2014): $R^2 = 0.583$), but a weak correlation for others (F11: $R^2 = 0.09$). When urinary hormone profiles were reanalysed for urinary progesterone standardised for specific gravity, the profiles themselves did not appear to vary significantly (Fig. 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 Cr, demonstrating slightly shorter or slightly longer luteal phase lengths. Unfortunately, because the correlation between the two methods appeared variable and weak for some females, we cannot be certain which standardisation method (specific gravity or Cr) is the most appropriate for evaluating urinary hormone concentration. The use of specific gravity may be more beneficial for assessing urinary concentration in the field and may also help determine the appropriate dilution for hormone analysis in the laboratory.

Although 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 present study showed that the relative density 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 or captive breeding facilities) to use a combination of the urinary characteristics (changes in urine volume, urinary 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, hand-held refractometer and a ×40 light microscope) to facilitate identification of the reproductive status (cyclicity) of female SHNWs without the need for a detailed hormonal analysis, which, in contrast, requires specialised equipment, training and laboratory facilities.

Conclusion

Although there was individual variation in the urinary P4M concentrations, oestrous cycle length and frequency between and within females and breeding seasons, we conclude that the analysis of urine has the potential to be used as an effective, non-invasive biomarker to identify the reproductive status of captive SHNW. The urinary P4M assays used in the present study provided strong evidence of cyclicity in captive female SHNWs. However, it may be prudent to first evaluate each of the urinary physiochemical and cytology characteristics 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 biomarker compared with changes in the urinary hormone. Once these parameters have been established for each female, the collection and analysis of urine may prove beneficial for monitoring the reproductive status of captive female SHNWs, techniques that may also be applied to other captive marsupial breeding programs where appropriate. Nevertheless, it is still unclear as to why mated oestrous cycles of many SHNWs in the present 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 map the urinary hormone profiles of the follicular phase and to confirm the timing of oestrus and ovulation in order to have a complete understanding of female SHNW reproduction.

Conflicts of interest

The authors declare no conflicts of interest.

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