

Reproductive biology of captive female southern hairy-nosed wombats (*Lasiorhinus latifrons*).

Part 2: oestrous behaviour

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Abstract. The poor captive breeding success of southern hairy-nosed wombats (SHNWs; *Lasiorhinus latifrons*) has been attributed to the difficulty in accurately characterising oestrous behaviour and their relationship to circulating reproductive hormones. Over two wombat breeding seasons, the use of infrared cameras for 24-h remote behavioural monitoring and the analysis of urine samples collected from seven captive females, were investigated to determine the relationship between behaviour and changes in urinary progesterone metabolites (P4M). Urinary P4M was divided into two concentrations: (1) \leq baseline P4M values and (2) $>$ baseline P4M values and evaluated against urine volume (mL) and the duration (s) and frequency of 23 behaviours recorded for 8 days surrounding D₀ of the luteal phase (D₀: a sustained increase in P4M for three or more consecutive days). When P4M was \leq baseline, the duration of urination and volume both decreased, whereas the duration and frequency of both pacing and rump bites by the female towards the male increased. These results suggest that there were detectable behavioural changes that can be mapped to the changes in the SHNW oestrous cycle, which may be used as behavioural indicators to identify the reproductive status of females.

Additional keywords: hormone, marsupial, progesterone, reproduction, urine.

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Introduction

The southern hairy-nosed wombat (SHNW; *Lasiorhinus latifrons*) is a large nocturnal and fossorial species found in five fragmented populations across southern Australia (Swinbourne *et al.* 2017a) that is currently listed as 'near threatened' by the International Union for Conservation of Nature (IUCN; Woinarski and Burbidge 2016). Despite the SHNW being represented in many zoos and wildlife parks across Australia, the species fails to breed regularly in captivity and the current captive population is not sustainable. The closely related northern hairy-nosed wombat (NHNW; *Lasiorhinus krefftii*) is critically endangered (<250 individuals), with most of the wild population located in Epping Forest National Park, central Queensland (Department of Environment and Heritage Protection 2015); currently, there are no NHNWs in captivity. A better understanding of SHNW breeding behaviour and physiology in captivity should lead to improved captive breeding outcomes within this species and serve as a reproductive model to better understand the reproductive biology and captive propagation of the NHNW, should the need arise.

The use of non-invasive methodologies, such as remote behavioural monitoring and faecal hormone analysis, have proven to be useful tools for characterising the reproductive behaviour and physiology of many wildlife species (for reviews, see Monfort 2003; Schwarzenberger 2007; Schwarzenberger and Brown 2013). Despite significant investment in developing these tools to better understand the reproductive behaviour and physiology of the SHNW (Hogan *et al.* 2009, 2010a, 2010b, 2010c, 2011a, 2011b; Descovich *et al.* 2012a, 2012b; Swinbourne *et al.* 2015, 2017b; Du *et al.* 2017, 2018), there is still considerable difficulty in accurately identifying the reproductive status of females in captivity (Hogan *et al.* 2010a, 2010c).

Although the analysis of progesterone metabolites (P4M) in both urine and faeces accurately reflects the longitudinal progesterone profiles of captive females (Paris *et al.* 2002; Hogan *et al.* 2010c; Swinbourne *et al.* 2017c), changes in courtship and mating behaviours have not consistently correlated with changes in reproductive hormones (Hogan *et al.* 2010c). This lack of agreement may be attributed to a range factors, some of

Table 1. Bodyweight and housing configuration of nine captive female southern hairy-nosed wombats during two consecutive wombat breeding seasons (July–December 2013 and August–December 2014)
Reproduced from Swinbourne *et al.* (2018)

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	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
	F9 ^E	5	22.7	Breeding pair	M1	36.4
				Female pair	F10	23.6
	F10 ^E	4	23.6	Breeding pair	M1	36.4
			Female pair	F9	22.7	

^AFemale was successfully mated in August 2013.

^BVasectomised male.

^CHip injury as a joey limited reproductive behaviours as an adult.

^DAge is estimated as the animal was rescued and brought into captivity as an adult.

^EAnimal was hand-raised as pouch young or brought into captivity as a juvenile.

which may include females participating in silent oestrus, the female becoming indifferent or habituated to the presence of the male or low levels of libido from the captive male to instigate reproductive behaviours in receptive females (Hogan *et al.* 2010a).

Consequently, rather than primarily focusing on observing specific reproductive behaviours of SHNWs, which are infrequent and hard to detect, it is possible that changes in general behaviours may be used to identify the reproductive status of female SHNWs, as have been used in other captive marsupials. For example, the female Tasmanian devil (*Sarcophilus harrisi*) undergoes periods of decreased appetite during oestrus (Keeley *et al.* 2012) and the female Julia Creek dunnart (*Sminthopsis douglasi*) exhibits 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 or for enhanced breeding technology, such as AI. In addition, physiological changes, such as urination behaviour (micturition), have been linked to changes in reproduction in the house mouse (*Mus domesticus*; Drickamer 1995), and may also potentially serve as biomarkers to identify and monitor the reproductive status in the female wombat.

As the application of behavioural monitoring and faecal or urinary hormone analysis has proven beneficial for the development of *ex situ* breeding programs in other marsupial species (Power *et al.* 2009; Pollock *et al.* 2010; Lambert *et al.* 2011; Keeley *et al.* 2012), the aim of the present study was to identify changes in general and/or reproductive behaviours in the SHNW that could be mapped and validated against changes in urinary

progesterone profiles in an attempt to elucidate non-invasive measures that could be used in the reproductive management of this species or serve as a model for other marsupial species.

Materials and methods

Animals and animal management

The study was conducted over two wombat breeding seasons (August–December 2013 and 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°50′09.8″S 150°58′39.9″E). Table 1 documents the wombat housing arrangements (pairings or single) of nine females over the two breeding seasons. Housing of wombats was consistent with that described in the SHNW husbandry manual (Treby 2005), which recommends pairs or trios. 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 m × 90 m and 4 m × 100 m), 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), 120 g gumnut pellets (Mitavite), 35 g oat chaff (Rich River Chaff and Grain) and 200 g sliced sweet potato (locally farmed produce). Animals had *ad libitum* access to water.

Non-invasive urine collection

Daily urine samples were collected non-invasively from females using the methodology described previously 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) and maintained on ice. The volume of urine (mL) and concentration, as determined by specific gravity measured using a hand-held refractometer (range 1.000–1.080; Bellingham + Stanley), were recorded and the sample divided into 2-mL aliquots and stored at -20°C for subsequent urinary hormone analysis.

Reproductive cycle characterisation

Urine samples, standardised for creatinine (Cr), were analysed for P4M using an enzyme immunoassay (EIA) previously described and validated for SHNW urine (Swinbourne *et al.* 2017b). An iterative process, described by Swinbourne *et al.* (2018), was used to determine 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. An oestrous cycle was defined as the end of one luteal phase to the end of the next (Finlayson *et al.* 2006).

Behavioural monitoring

Remote behavioural observations were conducted using a digital video surveillance system. One infrared (IR) indoor camera (Model CAM35IRHR; SUMO) and one IR weatherproof bullet outdoor camera (Model CAM78IRHR; SUMO) were installed in each of the eight wombat enclosures (total 16 cameras). Cameras were connected to a digital video recorder (DVR; KOBİ 16 channel) surveillance system, and footage was reviewed using XQ Pro Series DVR surveillance software, which allowed 24-h continuous behavioural observations. Animal identification was achieved based on individual coat markings or 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 ($5 \times 4\text{TB}$ Seagate Expansion Desktop) until retrospective behavioural analysis could be conducted.

Retrospective behavioural analysis

An ethogram (Table 2) was developed based on the general and reproductive behaviours previously described by Hogan *et al.* (2010a, 2010c). Because SHNW are typically only sexually receptive for approximately 13 h (Hogan *et al.* 2010a), both general and reproductive behaviours were analysed for 6 days surrounding Day 0 of the luteal phase and two additional days that 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) less than or equal to baseline values, which included Days -14 , -3 , -2 and -1 ; and (2) greater than baseline values, which included Days 0, 1, 2 and 14. Using CowLog 2.0 software (Hänninen and Pastell 2009), the duration (length of time, measured in seconds) and frequency (number of times behaviour was observed) of a

total of 23 general ($n = 10$), defensive ($n = 3$) and reproductive ($n = 10$) behaviours were analysed for the first 5 min of every 30-min interval throughout each day. All behaviours were observed and analysed by the same investigator (AMS).

Statistical analysis

All statistical analyses were conducted using IBM PASW Statistics GradPack 18. 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 relationships of the two urinary P4M concentrations and the volume of urine and specific gravity with 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 that underwent short or long cycles, with short cycles lasting < 35 days and long cycles lasting > 35 days. For all ANOVAs, a post hoc analysis was conducted which included a least significant difference test and Bonferroni correction to identify individual animal variation between the expression of behaviours. Finally, a principal component analysis (PCA) was conducted to determine behavioural clustering. Data for clustered behaviours were 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 two-tailed $P \leq 0.05$. Unless indicated otherwise, data are presented as the mean \pm s.e.m.

Results

Urine samples were collected from nine females during both sampling periods; however, behavioural analysis was only conducted for seven females (eight individual datasets). Although urinary P4M was evaluated for all mature females within this captive breeding population (Swinbourne *et al.* 2018), behavioural analysis for wombat F11 was not conducted because the animal was not under continual video surveillance in 2013. F6 was blind, and although the urinary P4M profile in this animal was representative of normal reproductive activity, she chose to remain in the indoor enclosure at all times and therefore did not exhibit normal wombat behaviours. Further, the hormone profiles for wombats F10 (2013) and F4 (2014) were not representative of normal reproductive activity (Swinbourne *et al.* 2018). For behavioural analysis, 17 distinct oestrous cycles were able to be paired with daily behavioural footage, which comprised six non-mated oestrous cycles and one mated pregnant cycle in 2013 and nine mated cycles and one non-mated cycle during 2014 (Table 3).

An analysis of toileting behaviour showed that when urinary P4M was less than or equal to baseline, the duration of toileting behaviour was significantly shorter than when urinary P4M was greater than baseline (Table 4); however, there was no difference in the frequency of toileting behaviour over the duration of the analysis period (Table 5). Urinary volume was also significantly lower when P4M was less than or equal to baseline than

Table 2. Ethogram of wombat general and reproductive behaviours
Modified from Hogan *et al.* (2010a, 2010c). IR, infrared

Behaviour	Description
General behaviours (female only)	
Digging	Repeated pawing at the ground or the den walls
Exploring or foraging	Investigation of, or searching for, both edible and inedible objects within indoor and outdoor enclosures
Feeding	Consumption of food and water provided by keepers
Grooming	Scratching of body part with hindlimbs or rubbing against a stationary object
Lying awake	Sternal recumbency; eyes open, ears erect, doing nothing else
Sitting	Sitting on hindquarters and front paws, doing nothing else
Sleeping	Lying with neck recumbent on the ground, curled into a ball or on its back, ears relaxed, eyes closed
Pacing	Traversing the same path three or more times consecutively
Toileting	Stopping to eliminate waste, leaving visible pools of urine on the ground (difficult to determine or observe just defecation behaviour using IR cameras)
Defensive behaviours (female only)	
Defensive	Female uses rump to deter the den mate's approach; usually blocking the entrance of the sleeping chamber or indoor den access
Kicking	Female uses her back legs to repeatedly kick others in the enclosure if they approach
Evading	Female avoids the den mate's approach by walking or running away
Reproductive behaviours (male and female)	
Urogenital sniffing	Male approaches the female and sniffs the urogenital sinus, associated with female's acceptance
Urine smelling	Approaches freshly voided urine and places snout in urine sample
Approach	Either male or female approaches the other to investigate (e.g. sniff, nudge, bite)
Chase	Male approaches the female and follows her at a close distance (<1 m); the female may initiate chase by approaching the male first
Rump bite	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
Grasp/restraint	Male jumps onto the back of the 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 and mating period both animals share a single sleeping chamber

Table 3. Reproductive cycle dynamics of eight 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) concentrations 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

Season	Animal	No. cycles analysed	Luteal phase (days)		Oestrous cycle (days)	
			Mean	Range	Mean	Range
2013	F3 ^A	1			~21-day gestation period	
	F4	3	13.6	12–15	28	26–30
	F5	1	24.0		31	
	F9	2	26.5	26–27	33	
2014	F1	3	23.3	18–30	41	39–43
	F2	2	23.5	23–24	42	38–46
	F9	2	27	20–33	43.5	40–47
	F10	3	22.3	22–23	32.3	29–35

^AF3 was successfully mated during the 2013 breeding season and information on previous oestrous cycles was unavailable.

when it was greater than baseline (22.8 ± 2.8 vs 30.4 ± 2.4 mL respectively; $F_{1,100} = 4.434$, $P = 0.04$), and urine concentration (evaluated using specific gravity and creatinine) was significantly higher when P4M was less than or equal to baseline than

when it was greater than baseline (specific gravity 1.036 ± 0.001 vs 1.035 ± 0.001 respectively ($F_{1,1174} = 12.545$ $P = 0.001$); creatinine 6.28 ± 0.18 vs 5.43 ± 0.15 ng mg^{-1} respectively ($F_{1,1174} = 44.111$, $P = 0.001$)).

Table 4. Mean logarithmic duration (\log_{10} seconds for the first 5 min of each 30-min interval throughout the day) of behaviours recorded from eight female southern hairy-nosed wombat datasets when urinary progesterone metabolites (P4M) were \leq or $>$ individual baseline values
 Bolded values indicate significant behaviour–urinary P4M interaction

Behaviour	\leq baseline urinary P4M		$>$ baseline urinary P4M		$F_{1,117}$	P -value
	Mean \pm s.e.m.	Range	Mean \pm s.e.m.	Range		
Digging	2.6 \pm 0.1	0.0–4.0	2.8 \pm 0.2	0.0–4.03	0.056	0.81
Exploring	3.9 \pm 0.1	1.9–4.5	4.0 \pm 0.0	2.3–4.47	0.005	0.95
Feeding	2.9 \pm 0.1	0.0–3.9	3.2 \pm 0.1	0.0–3.87	0.005	0.96
Grooming	2.1 \pm 0.8	0.0–4.0	2.2 \pm 0.9	0.0–3.56	0.554	0.46
Lying awake	3.7 \pm 0.1	0.0–4.4	3.7 \pm 0.1	0.0–4.34	0.003	0.96
Sitting	3.5 \pm 0.1	1.9–4.6	3.4 \pm 0.1	2.3–4.29	0.505	0.48
Sleeping	4.7 \pm 0.1	4.3–4.8	4.7 \pm 0.1	4.3–4.86	2.034	0.16
Pacing	1.1 \pm 0.2	0.0–4.0	0.3 \pm 0.1	0.0–3.57	3.899	0.05
Sniffing	1.1 \pm 0.1	0.0–3.5	1.2 \pm 0.1	0.0–3.52	0.539	0.46
Toilet	0.6 \pm 0.1	0.0–3.2	0.9 \pm 0.1	0.0–3.19	5.183	0.03
Defensive	2.0 \pm 0.2	0.0–4.4	1.8 \pm 0.2	0.0–4.41	0.162	0.69
Approach	0.5 \pm 0.1	0.0–3.3	0.5 \pm 0.1	0.0–3.25	0.001	0.98
Evading	0.9 \pm 0.1	0.0–3.3	0.5 \pm 0.1	0.0–3.24	0.651	0.42
Nudging	0.1 \pm 0.0	0.0–2.2	0.0 \pm 0.0	0.0–2.15	0.324	0.57
Chase	$<0.1 \pm 0.1$	0.0–1.1	0.1 \pm 0.7	0.0–2.13	0.930	0.34
Urogenital sniffing	$<0.1 \pm 0.0$	0.0–0.7	0.1 \pm 0.0	0.0–1.36	2.668	0.11
Urine smelling	0.1 \pm 0.0	0.0–1.8	0.2 \pm 0.1	0.0–1.81	1.838	0.18
Rump bite	0.4 \pm 0.1	0.0–3.2	0.1 \pm 0.0	0.0–1.74	4.518	0.04
Grasp/restraint	0.5 \pm 0.1	0.0–3.3	0.2 \pm 0.1	0.0–3.01	1.905	0.17
Turning	0.1 \pm 0.0	0.0–2.2	0.1 \pm 0.0	0.0–2.28	0.045	0.83
Coitus	0.3 \pm 0.1	0.0–3.2	0.1 \pm 0.1	0.0–2.66	1.595	0.21
Break	0.2 \pm 0.1	0.0–3.6	0.0 \pm 0.0	0.0–1.97	4.248	0.04
Cohabitation	0.1 \pm 0.1	0.0–3.3	0.0 \pm 0.0	0.0–1.08	0.167	0.68
Guarding		0.0–1.3			0.158	0.69

The duration (Table 4) and frequency (Table 5) of pacing behaviour increased significantly by up to fourfold when urinary P4M was less than or equal to baseline values. However, there was considerable individual animal variability in this behaviour ($F_{7,110} = 5.629$, $P < 0.01$), because pacing was only observed in six of the eight datasets analysed, with F4 and F5 not observed pacing during each of the study periods. When comparing behaviours between females with short (<35 days) and long (>35 days) cycles, the duration of evading behaviour was significantly longer for females with short than long cycles (0.8 ± 0.2 vs 0.7 ± 0.1 s respectively; $F_{1,117} = 4.292$; $P = 0.04$). The frequency of sitting decreased significantly in females who underwent short cycles compared with those undergoing long cycles (0.9 ± 0.01 vs 1.1 ± 0.01 times respectively; $F_{1,117} = 5.424$; $P = 0.02$). Duration of pacing was increased significantly in females who had long cycles when urinary P4M was less than or equal to baseline values than when P4M was greater than baseline (1.3 ± 0.2 vs 0.29 ± 0.17 respectively; $F_{1,72} = 7.108$; $P = 0.01$). There were no significant behavioural relationships found for short cycling females when urinary P4M was less than or equal to or greater than baseline values ($P > 0.05$).

The frequency and duration of rump bites exhibited by the female towards the male when in breeding pairs or to a female den mate when housed in female-only pairs was significantly higher when urinary P4M was less than or equal to baseline

(Tables 4, 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, because 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 per each first 5-min period of the 30-min blocks respectively when urinary P4M was \leq baseline compared with 3, 4, 0 and 0 times respectively when urinary P4M was $>$ baseline. The duration of female rump biting also increased when urinary P4M was \leq baseline (65.1 ± 31.6 vs 1.4 ± 0.9 s per each first 5-min period of the 30-min blocks). Similarly, the mean frequency of rump bites exhibited by males towards females was higher when urinary P4M was \leq baseline (8.5 ± 2.7 vs 2.3 ± 1.1 times per each first 5-min period of the 30-min blocks respectively; $F_{1,104} = 6.901$, $P = 0.01$).

The duration of courtship and mating behaviours exhibited by the male (grasping and restraining the female and guarding behaviour) increased significantly when urinary P4M was \leq baseline values (Table 4). In addition, the mean frequency of males grasping and restraining the female was sixfold higher when urinary P4M was \leq baseline compared with when it was $>$ baseline (Table 5). In contrast, the frequency of urogenital sniffing by the male was lower when urinary P4M was \leq baseline (Table 5).

Coitus was observed in only four females (F2, F3, F9 and F10) on seven different days during the behavioural analysis period.

Table 5. Mean logarithmic frequency (\log_{10} number for the first 5 min of each 30-min interval throughout the day) of behaviours recorded from eight female southern hairy-nosed wombat datasets when urinary progesterone metabolites (P4M) were \leq or $>$ individual baseline values
Bolded values indicate significant behaviour–urinary P4M interaction

Behaviour	\leq baseline urinary P4M		$>$ baseline urinary P4M		$F_{1,117}$	P -value
	Mean \pm s.e.m.	Range	Mean \pm s.e.m.	Range		
Digging	0.7 \pm 0.0	0.0–1.5	0.7 \pm 0.1	0.0–1.2	0.056	0.81
Exploring	1.3 \pm 0.0	0.6–1.9	1.4 \pm 0.0	1.0–1.7	1.205	0.28
Feeding	0.6 \pm 0.0	0.0–1.3	0.7 \pm 0.3	0.3–1.1	0.015	0.90
Grooming	0.8 \pm 0.0	0.0–1.3	0.9 \pm 0.0	0.0–1.4	0.091	0.76
Lying awake	0.9 \pm 0.0	0.0–1.3	1.0 \pm 0.2	0.6–1.2	0.260	0.61
Sitting	1.1 \pm 0.0	0.3–1.6	1.1 \pm 0.0	0.6–1.6	0.434	0.51
Sleeping	0.9 \pm 0.0	0.6–1.2	0.9 \pm 0.2	0.7–1.2	0.022	0.88
Pacing	0.2 \pm 0.0	0.0–1.3	0.1 \pm 0.0	0.0–0.7	6.210	0.01
Sniffing	0.3 \pm 0.0	0.0–1.0	0.4 \pm 0.0	0.0–1.0	2.643	0.11
Toilet	0.2 \pm 0.0	0.0–0.8	0.1 \pm 0.0	0.0–0.7	2.840	0.10
Defensive	0.6 \pm 0.5	0.0–1.7	0.6 \pm 0.6	0.0–1.7	1.722	0.19
Approach	0.2 \pm 0.0	0.0–1.0	0.2 \pm 0.0	0.0–0.9	0.003	0.96
Evading	0.3 \pm 0.1	0.0–1.6	0.3 \pm 0.1	0.0–1.6	0.039	0.84
Nudging	0.0 \pm 0.0	0.0–0.8	0.0 \pm 0.0	0.0–0.3	0.099	0.75
Chase	0.0 \pm 0.0	0.0–0.8	0.0 \pm 0.0	0.0–0.8	1.307	0.25
Urogenital sniffing	0.0 \pm 0.0	0.0–0.8	0.0 \pm 0.0	0.0–0.3	4.131	0.04
Urine smelling	0.0 \pm 0.0	0.0–0.8	0.0 \pm 0.0	0.0–0.3	1.423	0.24
Rump bite	0.2 \pm 0.0	0.0–1.2	0.1 \pm 0.0	0.0–0.9	4.316	0.04
Grasp/restraint	0.5 \pm 0.1	0.0–0.9	0.1 \pm 0.0	0.0–1.2	4.291	0.04
Turning	0.1 \pm 0.0	0.0–0.7	0.0 \pm 0.2	0.0–0.5	0.350	0.56
Coitus	0.5 \pm 0.0	0.0–0.7	0.0 \pm 0.0	0.0–1.0	0.159	0.69
Break	0.1 \pm 0.2	0.0–0.9	0.0 \pm 0.2	0.0–0.8	0.350	0.56
Cohabitation	0.0 \pm 0.0	0.0–0.8	0.0 \pm 0.0	0.0–0.3	0.159	0.69
Guarding	0.0 \pm 0.0	0.0–0.3			6.239	0.01

In 2013, following the mating of F3 and M6, a retrograde seminal plug was found on Day 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 Day 1, when urinary P4M was $>$ baseline; however, this mating failed to result in a pouch young.

Although the PCA conducted for behavioural clustering showed three distinct behavioural clusters (i.e. reproductive, defensive and general behaviours), there was no correlation found for either the duration or frequency of these clustered behaviours (as distinct from individual behaviours) with changes in urinary P4M ($P > 0.05$; Table 6).

Discussion

The present study represents an attempt to use non-invasive tools for monitoring reproduction in a captive nocturnal and fossorial marsupial. The use of IR cameras and non-invasive methodology for the collection and analysis of daily urine samples was effective for the analysis of general and reproductive behaviours during different stages of the oestrous cycle in the captive female SHNW. The results from the present study showed that there was a high level of individual animal variation between the reproductive cycle dynamics based on urinary progesterone (Table 3),

which may have also contributed to the individual variation observed in the general and reproductive behaviours exhibited by each of the captive females (Tables 4, 5). Based on urinary P4M analysis, there were specific behavioural changes in pacing, aggression or agitation and toileting behaviours, which have the potential to be used as non-invasive markers to identify the reproductive status of females in captivity.

Both the duration of toileting and urinary volume (mL) decreased during periods when urinary P4M was \leq baseline values. Because there was no difference observed in the frequency of urination or in the frequency or duration of feeding, this evidence suggests that female SHNW may be retaining water before 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 coincided with the decline in progesterone levels (Armstrong 1968; Nequin *et al.* 1979). In Merino ewes (*Ovis aries*), oviductal fluid volume was threefold higher around the time of oestrus compared with 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 exogenous 17β -oestradiol for 14 days had lower urine output than control females injected with saline (Stachenfeld *et al.* 1998). Therefore, the phenomenon of decreased urine output and duration observed in the

Table 6. Mean logarithmic duration (\log_{10} s for the first 5 min of each 30-min interval throughout the day) and frequency (\log_{10} number for the first 5 min of each 30-min interval throughout the day) of clustered behaviours expressed by captive southern hairy-nosed wombats when urinary progesterone metabolites (P4M) were \leq or $>$ individual baseline values

Behaviour	Analysis	\leq Baseline P4M		$>$ Baseline P4M		$F_{1,127}$	P -value
		Mean \pm s.e.m.	Range	Mean \pm s.e.m.	Range		
Group 1: reproductive behaviours							
Chasing	Duration	0.3 \pm 0.1	0–3.7	0.4 \pm 0.1	0–4.3	3.308	0.07
Urine smelling	Frequency	0.2 \pm 0.1	0–3.7	0.1 \pm 0.1	0–1.9	1.803	0.18
Urogenital sniffing							
Nudging							
Chin resting							
Cohabitation							
Group 2: defensive behaviours							
Defensive	Duration	3.2 \pm 0.3	0–8.8	2.4 \pm 0.2	0–9.3	0.009	0.92
Evading	Frequency	0.2 \pm 0.1	0–2.3	0.2 \pm 0.1	0–1.1	0.063	0.80
Kicking							
Group 3: general behaviours							
Exploring/foraging	Duration	12.2 \pm 0.2	7.9–15.5	12.3 \pm 0.2	7.5–15.5	0.002	0.97
Sitting	Frequency	3.8 \pm 0.1	1.9–5.9	4.0 \pm 0.1	2.0–5.7	0.661	0.42
Digging							
Grooming							

female SHNW may be an important non-invasive behavioural and physiological link to reproductive hormones, such that monitoring individual animal changes in urination behaviour in breeding females could potentially be used to identify 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, especially in females who underwent long oestrous cycles. Because 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 the present study may be overlooked. For example, F1, F2 and F3 demonstrated the most significant changes in pacing behaviour associated with changes in reproductive hormones, but 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 before this study resulted in her demonstrating slower and/or limited mobility. For this reason, she was not used as a breeding female, but it may have also contributed to her not exhibiting 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 variety of captive wildlife species, such as female giant pandas (*Ailuropoda melanoleuca*; Owen *et al.* 2016) and a female Malaysian sun bear (*Helarctos malayanus*; Rog *et al.* 2015). Increased activity has also been reported in marsupials, with the stripe-faced dunnart (*Sminthopsis macroura*; Francis and Coleman 1990), the Julia Creek dunnart (Pollock *et al.* 2010) and the female common wombat (*Vombatus ursinus*; Peters and Rose 1979) exhibiting periods of increased activity either before the onset of or during oestrus. However, in a previous study on the SHNW, no such relationship was found (Hogan *et al.* 2010c). The lack of a significant observable increase in activity in female SHNW may be due to high levels of between-animal variation, as seen in other variables in the present study, differences in the captive environment or to the fact that the device used for monitoring activity levels in female SHNW in the study of Hogan *et al.* (2010c) was attached by a collar to the neck of the female and was not a pedometer *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 with males (Walker *et al.* 2008); however, the link between locomotion and reproductive hormones in wild female SHNW has yet to be investigated. Although such a task would be difficult logistically, it is important to investigate whether this increased exploratory behaviour in wild females is 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 whether 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 were true, then this behaviour would be less variable during the oestrous cycle; an increased number of study animals may be able to confirm this.

In addition to pacing behaviour, the increase in evading behaviour exhibited by short cycling females, and the increased frequency in rump bites exhibited by the female when urinary P4M was \leq individual baseline values 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 decreased frequency of 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. Although 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 have prevented mating attempts (grasp/restraint behaviour becoming successful mating bouts).

Although most mating behaviours observed in the present study occurred during the normal breeding season in the wild (July–December; Gaughwin *et al.* 1998), males in this captive population have also been observed attempting to mate females during March (T. Janssen, unpubl. obs.), which is outside the normal SHNW breeding season, with one pouch young produced in February 2015. In addition, males in the present study were observed to mate females during the luteal phase. 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.* 2017b). Forced cohabitation in captivity affects 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. Although he did attempt to grasp, restrain and turn F2, she did not appear to be receptive to him despite being at the optimum time for mating. F2 was aggressive towards M5 and exhibited grasp/restraint behaviour towards the male. 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 the present study were not receptive to their male den mate at the optimum time for mating. It is possible that group-housed individuals become incompatible by being

indifferent to each other (habituation) or overly aggressive (Zhang *et al.* 2004). With regard to wombats, long-term housing of breeding pairs may not be appropriate for successful mating as the phenomenon of increased pacing observed before the onset of the luteal phase in captive females in the present study, as well as the increased burrow visits reported in wild females (Walker *et al.* 2008), possibly indicates that females may be seeking an unfamiliar male to mate.

For those females that were receptive to the male, it is difficult to accurately determine why the coital bouts observed just before the onset of the luteal phase did not result in a pouch young, including the mating bouts that resulted in a seminal plug in 2014. Although the social structure of this species is not clearly understood, the number of burrow visits (Walker *et al.* 2008) suggests that conspecifics do not cohabitate for long, if at all. This is in direct contrast with the current captive management strategy, where individuals cohabitate throughout the year, a housing strategy that 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 that 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 the present study (Table 3) may be an indication of such 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 pairing during breeding seasons, to determine the effects of stress in females during the mating seasons in an attempt to identify any negative effects that unwanted mating attempts may have on female reproduction and the successful production of viable offspring.

Conclusion

The results from the present study demonstrate that the application of non-invasive remote behavioural monitoring and the collection and analysis of urinary hormones appear to be effective tools for monitoring reproduction in the SHNW. Although there was individual variation in the presentation of these behaviours, as well as their corresponding urinary hormone profiles, we suggest that changes in pacing, aggression and toileting behaviours are potential behavioural markers that could be used for long-term monitoring of reproduction in this species.

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. Therefore, we conclude that a thorough monitoring and evaluation of these behaviours on an individual animal basis may be useful in helping identify reproductive status and that this approach may be more profitable rather than

relying on clearly defined behaviours that are contiguous across the population. Our present inability to use urinary oestrogens and LH to define oestrus and ovulation (Swinbourne *et al.* 2017c) or to identify strongly expressed specific reproductive behaviours means that 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 to better understand the social structure and reproductive strategy of this species in the wild so that we can better evaluate the potential effect of social structure in the reproductive management of captive SHNWs.

Conflicts of interest

The authors declare no conflicts of interest.

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