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RESEARCH ARTICLE

Non-Invasive Urine Collection in The Female Southern Hairy-Nosed Wombat (*Lasiorhinus latifrons*) With the Aid of Classical Conditioning

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We propose that regular urine samples can be used to monitor and characterize 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 in captive female 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 urinated in response 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 more rapidly to the stimulus than juvenile females with no prior conditioning. This study is the first description of a successful method of urine collection by classical conditioning in a marsupial. Zoo Biol. XX:XX–XX, 2014.

Keywords: captive; marsupial; reproduction; training

STATEMENT OF THE PROBLEM

The development of a successful captive breeding program for the southern hairy-nosed (SHN) wombat (*Lasiorhinus latifrons*) has been difficult to achieve, as the relationship between reproductive endocrinology and estrus behavior is still poorly understood (Hogan et al., 2010a). In addition, the closely related northern hairy-nosed (NHN) wombat (*Lasiorhinus krefftii*) 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* spp. 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 SHN wombat prior to and during estrus, 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; these methods can be highly stressful, costly, 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 fecal 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 fecal samples (Hamilton et al., 2000; Paris et al., 2002; Hogan

Conflict of interest: None.

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et al., 2010b, 2010c). Thus far, it has been difficult to accurately detect estrogen metabolites throughout the estrous 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 fecal analysis in the SHN wombat from being a useful means of either estrus detection or measurement of reproductive protein hormones. Therefore, an alternative biological sample is required. Similar to feces, urine is produced constantly and contains various classes of hormones and their metabolites, in particular both reproductive protein and steroid hormones. While Peters (1977) 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 kuhli*; Smith and French, 1997). A range 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 off the ground (Collins et al., 2011), thereby removing the need for metabolic cages, capture, restraint or sedation. In marsupials, 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.

DESCRIPTION OF THE PROCESS

Animals

All female SHN wombats 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 SHN wombats 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 (~200 g) at the AACE facility (F1-2006; F2-2009; F3-2009) and as part of this care had been previously conditioned to eliminate after bottle-feeding. This involved initially involved the animal being placed in lateral recumbence, and then the pericloacal region was lightly stimulated until elimination occurred. The other two females had been brought into the facility as juveniles (less than 2 years of age; F9-2011 and F10-2012) and had not been exposed to any elimination conditioning.

Conditioning and Collection

There were three stages of conditioning for urine collection. Stage 1 consisted of a period of habituation and general observation and was conducted over 7 days as part of the daily husbandry routine. Habituation involved the researcher being introduced to the wombats via regular cleaning of the dens and yard enclosures, which was conducted daily between 0700 and 0900 hr, with feeding at 1,600 hr. During this time, observations were made of the animals' natural elimination times and locations by means of direct or digital video surveillance (Fig. 1A–C).

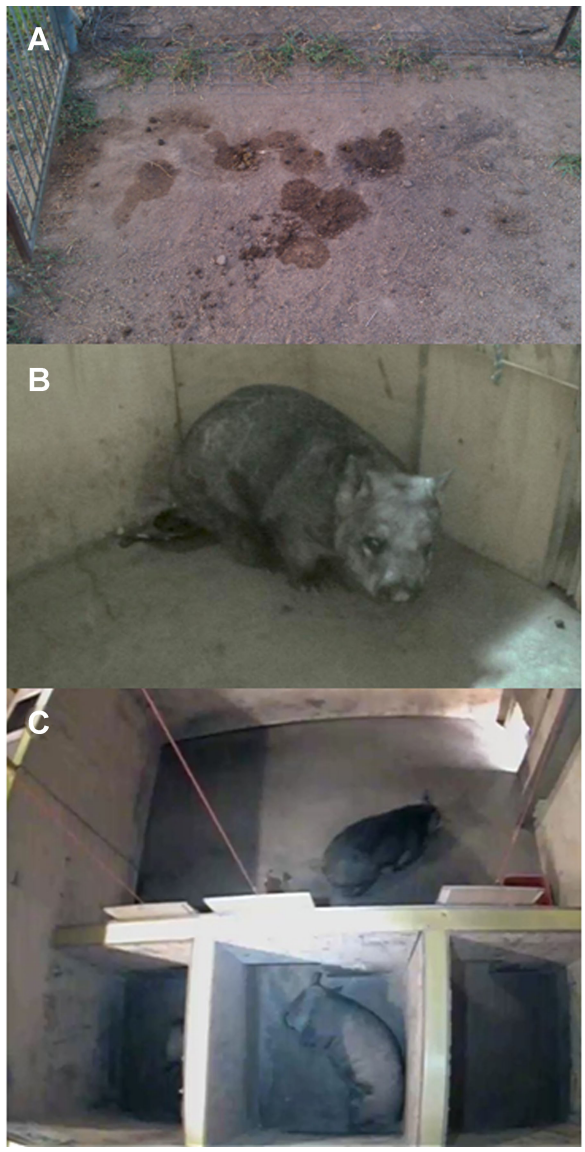

Fig. 1. Stage 1-observation of preferred elimination area (PEA). (**A**) Outdoor PEA, direct observation. (**B**) Indoor PEA, direct observation. (**C**) Indoor PEA, digital video surveillance.

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 hr (AM) and 1,530 hr (PM), just prior to husbandry and feeding. The animal was approached and, if asleep, was awoken, and a small collection tray [120 mm diameter frying pan (Tefal Australia)] placed under the urogenital region (Fig. 2A–C). A tactile stimulation, consisting of a light scratching in the general proximity of the pericloacal 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 a maximum of 25 min, after which time the test was deemed to have failed.

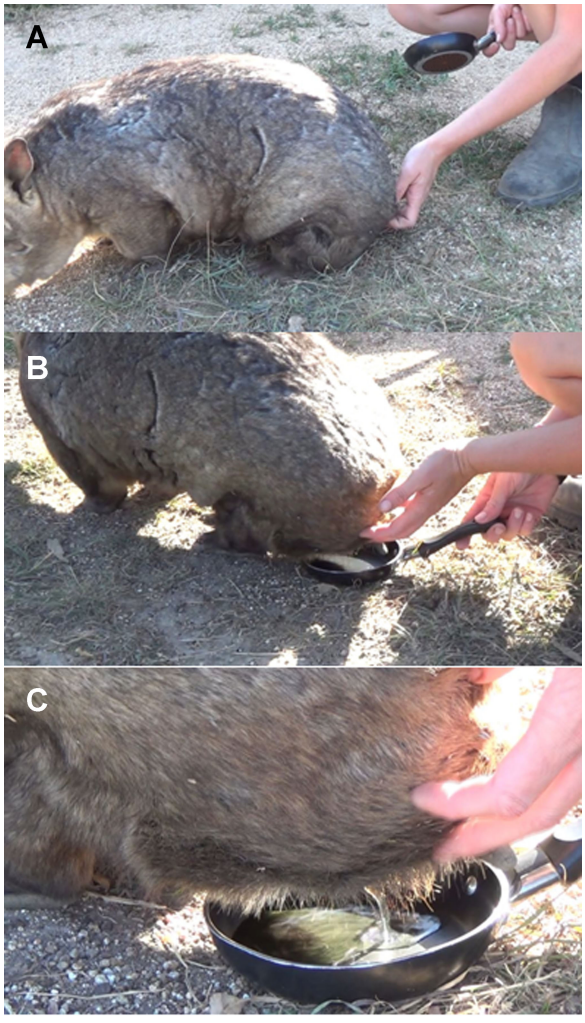

Fig. 2. Stage 3-collection. (**A**) Applying tactile stimulus—scratching around the pericloacal region. (**B**) Collection tray placed under the urogenital region. (**C**) Fresh, clean catch sample is collected into tray.

Stage 3 was the physical 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.

DEMONSTRATION OF EFFICACY

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, so that both stage 2 and stage 3 of the trial were conducted in the specific PEA for each animal. For example, one wombat used the spare sleeping chamber in her den system as her PEA, so that 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 (AMS) 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 the four animals were 255.8 ± 10.71 sec during the morning and 314.5 ± 20.19 sec in the afternoon. Table 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) tended to be shorter than that for the non hand-raised animals (F9 and F10) (AM: 226.8 ± 158.38 vs. 284.9 ± 66.7 sec; PM: 142.2 ± 47.41, vs. 486.8 ± 112.59 sec).

TABLE 1. Individual variation of urination behavior and collection success of four female captive SHN wombats during stages 2 and 3 of conditioning.

Wombat ID	Collection time	Time to urination (mean ± SEM)	Collection attempt	Urinated prior to collection (*n*)	Collection success (%)
Stage 2-conditioning					
F1[a]	AM	273.5 ± 136.75	4/29	2	14
	PM	0.0 ± 0.00	0/23	2	0
F3[a]	AM	180.0 ± 180.00	1/29	1	3
	PM	284.4 ± 94.81	9/21	2	43
F9	AM	412.6 ± 103.16	16/29	0	55
	PM	534.9 ± 133.7	16/24	0	67
F10	AM	157.2 ± 30.24	27/29	2	93
	PM	438.6 ± 91.45	23/24	0	96
Stage 3-collection					
F1[a]	AM	275.1 ± 37.39	40/60	2	67
	PM	83.0 ± 0.00	1/16	0	6
F3[a]	AM	79.7 ± 9.71	77/93	4	83
	PM	135.0 ± 20.30	29/34	1	85
F9	AM	222.5 ± 25.15	72/91	8	79
	PM	374.2 ± 38.9	55/63	2	87
F10	AM	90.6 ± 7.69	91/94	3	97
	PM	155.6 ± 11.31	34/34	0	100

Time expressed in seconds ± standard error of mean (SEM).
[a]Indicates hand-raised females.

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 and PM collections (280/338–82% vs. 119/147–81%). The mean times to urination were 167.0 ± 9.70 sec in the morning and 187.0 ± 10.20 sec 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 (94.8 ± 9.17 sec) followed by F10 who was not hand-raised (108.3 ± 6.87 sec). F10 had a higher collection success (98%) compared to the other three females (F1: 54%; F3: 83%; F9: 82%), despite her not receiving previous elimination conditioning.

Figure 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 sec. For the females used in this study, 35 days of conditioning appeared to be sufficient to associate the tactile stimulus with the elimination behavior for both hand-raised and non hand-raised females, resulting in the expression of a classical conditioning paradigm for this methodology.

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; the animal associating a tactile stimulus to the desired behavior. Females without previous elimination conditioning successfully 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 not eaten. As these captive SHN wombats were able to associate the tactile stimulus with the desired behavior, 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.

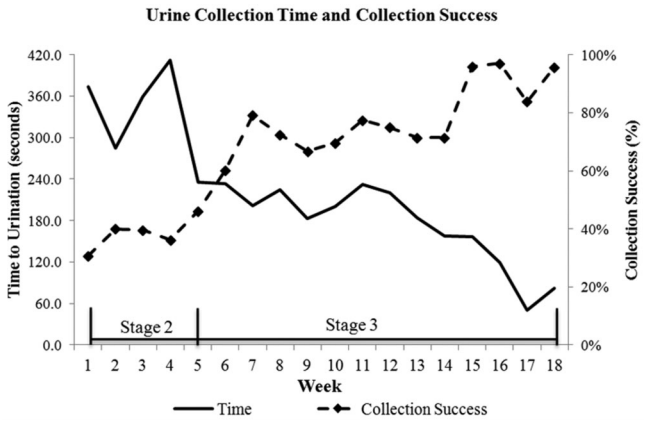

Fig. 3. Mean time to urination and collection success during stages 2 (weeks 1–5) and 3 (weeks 6–18) of conditioning of four captive female SHN wombats.

It should be noted that this method of conditioning was only suitable for 4 of the 11 wombats in our 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 animals' sleeping chambers to collect urine into an underlying tray. Nevertheless, care needs to be taken with this approach, as samples collected in this manner may be prone to contamination with conspecific urine or micro-organisms.

CONCLUSION

To our knowledge this is the first study to describe successful collection of urine by classical conditioning in any marsupial and was achieved for wombats entering captivity both as pouch young to be hand-raised and 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 of our 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.

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