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Research paper

Behavioral and endocrine responses to season and social dynamics of captive male southern hairy-nosed wombats (*Lasiorhinus latifrons*)

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ABSTRACT

Although southern hairy-nosed wombats (SHN wombats; *Lasiorhinus latifrons*) rarely breed in captivity, further knowledge of their reproductive physiology and behavior is likely to improve their breeding potential. This study examined the effect of seasonal variation and changes in social dynamics on the physiology and behavior of a captive population of male SHN wombats (n = 6). Seasonal changes in urinary testosterone metabolites (UTM), urinary cortisol metabolites (UCM), qualitative estimates of spermatorrhoea (QS), aggressive behavior and reproductive behavior were measured over an 11-month period. While there was no effect of month on QS (GLM ANOVA, P = 0.27), reproductive behavior (GLM ANOVA, P = 0.19) or aggressive behavior (Tukey pairwise comparisons), the secretion of UTM (GLM ANOVA, P = 0.051) was only marginally affected by season, compared to that reported for wild male SHN wombats. Mean UCM concentrations of July and August 2016 were significantly higher than those between October 2015 and January 2016 (Tukey pairwise comparisons). To examine social dynamics, two trials of animal positioning exchange with the enclosure system were implemented and behavioral data were examined for each trial over a six week period; UTM, UCM and general behaviors (n = 27) were measured for each trial. Neither UTM nor UCM concentration varied significantly ($P \geq 0.45$) before and after the exchanges. "Scratching" decreased at the group level following the animal exchange in both trials, suggesting reduction in self-grooming may be a behavioral response to novel stimuli. UCM and UTM concentrations were both positively correlated with "standing still" and "body rub" behaviors. This may be evidence of a hormonal control of a "freezing behavioral response" to external stimuli and marking behavior, respectively. As there was no evidence that changing the social dynamics affected reproductive or agonistic behavior or hormone concentrations, it was concluded that captive male wombats in this study showed reduced reproductive seasonality compared to wild wombats and that animal exchange resulted in a behavioral response to novel stimuli but was not sufficient to affect testosterone or cortisol secretion, within the context of our study.

1. Introduction

The southern hairy-nosed wombat (SHNW – *Lasiorhinus latifrons*) is a large, herbivorous, nocturnal, fossorial marsupial that is endemic to Australia. Wild SHNWs are mainly distributed in South Australia and southern Western Australia (Gaughwin et al., 1998). According to the International Union for Conservation of Nature and Natural Resources (IUCN), this species is currently listed as 'near threatened' (Woinarski and Burbidge, 2016) and the population sizes of free-ranging SHNW have been estimated to be declining due to a series of threats that include habitat fragmentation (Alpers et al., 1998), disease (e.g. sarcoptic mange; Ruykys et al., 2009; Sparrow, 2009) and climate change (Finlayson et al., 2005; Kellermann et al., 2009). The SHNW has routinely been kept in captivity since the 1970s (Hogan et al., 2013; Jackson, 2003) but given that no second generation offspring (F2) have been born in captivity, the captive population is incapable of being maintained without recruitment of animals from the wild (Hogan et al., 2013). The limited success in captive breeding has resulted in an unsustainable *ex situ* population (Hogan et al., 2010a, 2013), primarily attributable to a dearth of knowledge with respect to their reproductive physiology and behavior (Hogan et al., 2013). Gaining more information on physiology and behavior of this species will not only improve captive breeding success, but could also help with the management of critically endangered

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northern hairy-nosed wombat (*Lasiorhinus krefftii*; Hogan et al., 2013; Paris et al., 2002).

The SHNW is regarded as a seasonal breeder in the wild, with a breeding period between July and December (Gaughwin et al., 1998; Paris et al., 2002; Taggart et al., 2005). In SHNW, a significant increase in both ejaculate volume and the total sperm number, along with a corresponding higher sperm motility, has been found to occur during the breeding season, and fewer motile spermatozoa and lower sperm motility being observed during the non-breeding season, suggesting that breeding in this species is seasonal (Taggart et al., 2005). Previous studies have also demonstrated seasonal changes in plasma androgen concentrations in wild male SHNWs, with the timing of peak concentration coinciding with female reproductive activity (Gaughwin, 1981). Hamilton et al. (2000) used a non-invasive fecal androgen analysis technique to study seasonal changes in wild SHNW and confirmed that changes in testosterone metabolite levels were a reliable indicator for studying male reproductive capacity. The study also noted that the concentration of the testosterone metabolites was significantly higher during the breeding season (August to October) than during the non-breeding season (February to April). However, due to the implementation of cull management during their field research, Hamilton et al. (2000) were unable to reveal serial seasonal changes in fecal androgen concentration for the same individuals. Contrary to the findings of Hamilton et al. (2000), Hogan et al. (2010a) failed to demonstrate any significant seasonal variation in fecal testosterone metabolite concentrations in four captive male *L. latifrons* housed at Rockhampton Zoo on the central coast of Queensland; nevertheless, this study did report that plasma samples taken 90 min after the gonadotrophin-releasing hormone agonist (GnRHa) challenge showed significant seasonal changes in testosterone secretion response, whereas blood samples collected before the GnRHa stimulation did not. These data suggest that, despite a lack of seasonal change in circulating testosterone, these animals showed a lower capacity for testosterone stimulation outside the breeding season.

Studies of seasonal change in cortisol concentration in marsupials are limited. Stead-Richardson et al. (2010) reported a lack of apparent seasonal variation in fecal cortisol metabolites (FCM) in both captive and wild Gilbert's potoroos (*Potorous gilbertii*). Conversely, seasonal variation in FCM concentrations has been reported in captive numbats (*Myrmecobius fasciatus*), with a higher concentration of FCM being secreted by captive males during the summer mating season (December–February; Hogan et al., 2012).

Urinary hormone analysis as a non-invasive endocrine method has been widely applied to studying animal physiology (Heistermann, 2010; Kersey and Dehnhard, 2014), as urine not only contains a variety of steroid hormone metabolites of androgens, estrogens and glucocorticoids (GCs; Heistermann, 2010) but is also constantly produced and thereby an ideal sample for repeated collection (Lasley and Savage, 2007). Previously, Swinbourne et al. (2015) established a non-invasive urine collection method for captive female *L. latifrons* by means of operant conditioning and recently, Du et al. (2017) biologically validated an enzyme immunoassay (EIA) for urinary testosterone metabolites (UTM) and cortisol metabolites (UCM) in captive SHNWs using exogenous GnRHa and adrenocorticotropic hormone agonist (ACTHa) challenges, respectively. In marsupials, urine is also useful for the measurement of spermatorrhoea as a non-invasive index to assist with studying seasonal variation in spermatogenesis and testicular and epididymal function in marsupials (Fletcher, 1985; Power et al., 2009; Woolley, 1991). Previously, Hogan et al. (2010a) reported that spermatorrhoea occurred throughout the year in captive male SHNW, confirming continuous sperm production in this species in captivity; however, like all studies to date, only the presence/absence of spermatozoa in the urine of captive male wombats was examined without an evaluation of overall levels (e.g. low vs. high sperm excretion). In general, these studies revealed that non-invasive urine samples appear to be suitable for assessment of both reproductive capacity and adrenal response in captive male SHNWs.

Previous studies have found that wild SHNWs inhabit small but extensively overlapping home ranges that are closely associated with their warren system, and have also been reported to simultaneously share warrens (Finlayson et al., 2005; Gaughwin, 1981; Wells, 1973). When combined with the observation that males tend to establish individual territories that overlap considerably with the home ranges of several females and minimally with other males (Gaughwin et al., 1998), one could speculate that SHNW might possibly establish some type of social structure in the wild. As warren systems are extensive and have multiple burrow entrances, the extent of social organization, interactions and affiliations in this species is still unclear; consequently, it is also uncertain as to whether social structure has an effect on mating in the wild (Sparrow, 2009; Walker, 2004). A better understanding of wombat social structure and its influence on mating and breeding success could provide insight into whether or not forced social interactions (e.g. group housing) in captive SHNWs influence captive breeding success. In captivity, this can be achieved through the use of a digital video recording system such as the one used in a previous study by Hogan et al. (2009) to reveal daily behavior patterns in a captive SHNW population by calculating time budgets. This non-invasive approach provides researchers with high animal visibility with low disturbance, allowing reliable animal identification and concurrent documentation of various real-time behaviors.

The present study focused on investigating the influence of season and a forced change in social dynamics of captive male SHNW on reproductive and adrenal physiology. The aims of the present study were (1) to provide insight into seasonal changes in testicular and adrenal physiology with respect to UTM concentration, UCM concentration, aggressive behavior, reproductive behavior and a qualitative index of sperm output based on spermatorrhoea (QS), (2) to measure changes in UTM and UCM secretion following a forced change in social dynamics within a captive enclosure system, in order to determine if there exists a social hierarchy in captive male SHNWs, (3) to explore the influence of changes in social dynamics on general behaviors of captive male SHNW and (4) to determine any correlation between hormone (i.e. UTM or UCM) concentration and general behaviors for captive male SHNWs.

2. Materials and methods

2.1. Animals and study site

Six captive male SHNWs were used in this study (Table 1) and all animals were originally wild-sourced from South Australia. All wombats were kept within two adjacent breeding facilities at Australian Animal Care and Education (AACE) located at Mount Larcom, Queensland (23.75° S, 151.00° E). Each facility was fitted with 24-h air-conditioning (set at 23 °C) and included eight internal enclosures, with each enclosure being connected to a respective fenced outside yard that contained native grasses and soil substrate. Wombats were kept either individually or in pairs (one male and one female) in each enclosure, while each individual animal had its own sleeping den. All six experimental males were monitored inside their enclosure by a 24-h digital video surveillance recording system (XQ, Australia). Daily husbandry occurred between 6:30 am and 9:00 am and daily feeding between 3:00 pm and 5:30 pm. Each animal was fed a mixture of oaten chaff, horse pellets, rolled oats and sliced sweet potatoes while water

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Table 1
Husbandry and reproductive information of the six male *L. latifrons*.

Wombat ID	M1	M2	M3	M4	M5	M6
Estimated age in 2016 (years)	11	2	6	11	6	6
During which year the animal was introduced into AACE	2008	2013	2009	2007	2009	2009
Mean weight during the sampling period (kg)	26.7	27.2	43.5	39.6	35.8	27.5
Paired or single	Single	Paired	Only paired during the breeding season	Only paired during the breeding season	Paired	Paired
If the wombat was a successful breeder	No	No	Yes	Yes	No	No
Location of wombat's enclosure	From 3rd January 2016 to 12th June 2016: Facility 1 Other time: Facility 2	Facility 1	Facility 2	From 3rd January 2016 to 12th June 2016: Facility 2 Other time: Facility 1	Facility 1	Facility 1

was available *ad libitum*. This study was approved by the University of Queensland's Animal Ethics Committee (SAFS/333/15).

2.2. Urine collection, storage and sperm observation

Captive SHNW urine collection via classical conditioning has previously been described for both females (Swinbourne et al., 2015) and males (Du et al., 2017). As all six experimental animals had been habituated to human approach, a period of two weeks was sufficient for these males to be trained to urinate in a common area within their enclosure by classical conditioning (Du et al., 2017). Urine samples were collected either in the late morning (i.e. after husbandry) or in the early afternoon (i.e. before feeding). The floor was mopped clean with tap water (without any detergent) and thoroughly dried before each collection; after urination, the animals were then locked into their own sleeping dens and the urine was recovered from the clean floor with a 10 mL syringe (BD, Australia). Following collection, the urine sample was pooled into a 50 mL specimen container and then maintained on ice (for 1–2 h). Animal identification (ID), collection time, volume and pH were recorded for each sample; 4 mL of urine were pipetted into 2 × 2 mL ProSciTech tubes (PST, Australia) and stored at −20 °C until analysis. The remaining urine was transferred into 2 × 15 mL Falcon tubes (BD, Australia) and centrifuged (Eppendorf, Germany) at 1500 rpm for 10 min. After removing the supernatant, the sample was standardized by resuspending the pellet in 0.5 mL of the remaining urine sample; 10 μL of this sperm mixture was then pipetted onto a microscope slide (Fronine, Australia) and covered with a coverslip and observed under a phase contrast microscope (Olympus, Tokyo) using the 40× objective. Ten fields of view were randomly selected on the slide and the number of spermatozoa in each view was recorded; the average number of spermatozoa in ten views was calculated and recorded as a qualitative index of spermatorrhoea. Spermatorrhoea (QS) was calculated monthly (i.e. monthly average spermatorrhoea index) for each male and reported as an index of sperm number per field of view. In addition, the presence/absence of motile spermatozoa was also determined and recorded during each observation. Notably, M1 was vasectomized previously so that it was not possible to detect any sperm in his urine.

2.3. Hormone analysis

2.3.1. Testosterone

UTM was measured by a testosterone EIA based on the methodology described in Du et al. (2017); this assay recruited a polyclonal anti-testosterone antibody R156/17 (C. Munro, UC Davis, USA), a goat anti-rabbit IgG secondary antibody (Arbor Assays®, USA) and a testosterone horseradish peroxidase (HRP) label (C. Munro, UC Davis, USA). Major cross-reactivities (>5%) for the antibody were testosterone (100%) and 5α-dihydrotestosterone (29%). Parallelism between the standard curve and serially diluted pooled samples was demonstrated; dilution rates were identified according to pooled urine samples that led to 50% binding on the parallelism curve. The assay sensitivity was 0.039 ng/mL and all samples were analyzed in duplicate. The inter- and intra-assay coefficients of variation (CV) were 9.8% and 2.9%, respectively.

2.3.2. Cortisol

UCM was measured by the Arbor Assay Mini-Kit ISWE002 (Ann Arbor®, USA) based on the methodology described by Du et al. (2017). The manufacturer supplied a polyclonal anti-rabbit cortisol antibody #C208, a cortisol-HRP conjugated label #C209 and cortisol standards #C210, while the assay buffer and plates were coated "in-house" with anti-rabbit IgG immobilizing antibody and run according to the manufacturer's specifications. Major cross-reactivities for the antibody (>5%) were cortisol (100%), dehydrocortisol (42.08%) and cortisone (26.53%). Parallelism between the standard curve and serially diluted pooled samples was demonstrated; dilution rates were identified according to pooled urine samples that led to 50% binding on the parallelism curve. The assay sensitivity was 0.05 ng/mL and all samples were analyzed in duplicate. The inter- and intra-assay CV were 11.6% and 2.7%, respectively.

2.3.3. Creatinine

Creatinine (Cr), as a byproduct of muscle activity, is excreted into urine at a constant rate (Brown, 2008). Therefore, urinary creatinine level can be used to standardize urinary hormone concentration. In the current study, urinary creatinine was measured using a creatinine assay (Taussky, 1954; Cayman Chemicals, USA), for which the protocol has been described elsewhere (Du et al., 2017). All urinary hormone levels in the present study were corrected by the corresponding creatinine concentration and reported as ng/mg Cr.

2.4. Recording of daily aggressive and reproductive behavior

Daily behavioral characteristics of each animal were assessed by means of daily digital video recording (XQ, Australia) and the review of 24-h video footage; there were no gaps in the data and 24-h daily observations were conducted for the entire sampling period. Daily behavioral features of each individual were classified into three categories: (1) presence of reproductive behavior with aggressiveness, (2) presence of aggressive behavior only and (3) absence of reproductive or aggressive behavior. The classification and description of subclass behaviors are described in Table 2 and were based on those described by Hogan et al. (2011b) with some modification. As the performance of reproductive behavior in captive male SHNW was always accompanied by aggressiveness,

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Table 2
Classification and descriptions of aggressive and reproductive behaviors.

Behavior classification	Agonistic behavior		Reproductive behavior	
Category	Chase and bite	Aggression towards human	Restraint	Coitus
Description	Male chases paired female and attempts to or delivers bite	Male attempts to bite his human keeper during routine husbandry	Male mounts paired female, grasps female around the hips with his forelimbs and rolls onto one side, causing the female to be positioned into lateral recumbency suitable for coitus	Coitus is achieved and is characterized by rhythmic pelvic thrusting of the male

a subclass of reproductive behavior (i.e. restraint, coitus) was identified as a type of aggressive behavior but not vice versa. Hence, when a male wombat exhibited aggressive or reproductive behavior for a consecutive period of 3 min or more, the video recording of this event was analyzed to determine the intent. A consecutive period of 3 min was chosen as, on occasions, a male wombat would perform a shorter period of aggressive/reproductive behavior without any need for clarification of intent (Z Du, personal observations).

2.5. General behavior recording and processing

Twenty-four hour video footage of behavior from all six male wombats was stored and backed up to a 5 TB hard disk drive (WD, Australia) for the first trial and a 4 TB hard disk drive (Seagate, Australia) for the second trial. However, only 18 individual days within each trial period (i.e. −13 d, −11 d, −9 d, −7 d, −5 d, −3 d, −1 d, 1 d, 2 d, 3 d, 5 d, 7 d, 9 d, 12 d, 15 d, 19 d, 23 d and 28 d in relation to the animal exchange day – 0 d) were selected for general behavioral analysis. Behavioral analysis was based on the protocols and ethograms described by Hogan et al. (2011b) and Descovich et al. (2012) but with modifications (Table 3). Behavioral observations and processing were conducted manually using the XQ player (XQ, Australia) by the same person (ZD) for the selected sampling days. Individual daily patterns for each behavior were identified for a 24 h period. Each day included 24 periods of 1 h duration, starting from 0000-h duration and ending at 2300-h duration. A 5-min period within each hour (XX00-h to XX05-h with XX ranging from 00 to 23 h) was selected to represent the behavioral pattern during this hour (Martin et al., 1993). Using the standard approach for focal time sampling, the estimated percentage of time spent on each behavior during each hour of the day was first calculated [i.e. (Y/300) × 100, Y being the total time (s) spent in a specific behavior within the 5 min duration of a certain hour]. The daily mean percentage of a behavior was then determined by the formula "$(Y_1 + Y_2 + \ldots + Y_{24})/24$"; where Y_1 indicated the estimated percentage of time spent in a behavior during the first hour of the day (i.e. 0000-h duration).

Table 3
General behavior categories.

Behavior category	Description
Air smelling	Smelling of the air, usually accompanied by a head movement up and down
Approach	Male (at a walk) frequently and repeatedly approaches to the paired female from behind
Body rub	A body part rubbed against an inanimate object
Break	Cessation of a coitus bout, indicated by the release of the paired female by the male
Chase	Male runs after the paired female
Coitus	Coitus is achieved dorsoventrally, while both animals lie on their sides, and is characterized by rhythmic pelvis thrusting of the male
Defecating	Defecating on the floor
Den check	Entering the paired female's den with sniffing
Drinking	Drinking of water from a provided bowl
Exploring	Investigation or examination of inedible objects
Feeding	Consumption of food items
Outside activity	Male staying within fenced outside yard area; out of vision from inside cameras
Pacing	Repetitive pacing, usually along the enclosure boundary
Restraint	Male jumps onto the back of the paired female, grasps around the hips with his forelimbs and rolls onto one side, flipping the female onto her side
Retreat	Male retreating from the paired female
Scratching	Vigorous back and forth motion of foot claws across an area of the body
Sitting rest	Sitting on hind quarters and front paws, while doing nothing else
Sleeping	Lying with neck recumbent on the ground
Standing still	Standing on four feet without any locomotion
Stereotypic behavior	Repetitive digging-like behavior (i.e. male using a front paw to scratch the concrete floor hard and frequently)
Urinating	Urinating on the floor
Walk	A slow gait using four limbs
Wall biting	Biting the wall with vigorous head movements
Wall climbing	Climbing action performed at the walls of a den
Wall scratching	Scratching the wall with a front claw
Wombat biting	Male biting the paired female
Wombat smelling	Projecting the head towards the paired female and smelling

2.6. Experiment 1: seasonal changes in captive male SHNW physiology and behavior

Sample and data collection for studying captive male wombat seasonal change was conducted for 11 months (October 2015–August 2016). This included weekly urine collection from each male – leading to a qualitative index of spermatorrhoea – and documentation of daily aggressive and reproductive behaviors by evaluating digital video recording of each individual animal. For urine samples, 433 samples in total were successfully collected.

2.7. Experiment 2: reallocation of male SNHW in captivity

The wombat enclosure systems within two separate facilities (i.e. facility 1 and 2) at AACE and the corresponding locations of each male are reported in Fig. 1. Given that wombats M3 and M4 had previously been the only successful male breeders at AACE and that they were the only wombats weighing over 39 kg in this captive population (Table 1), it was postulated that both animals were the dominant individuals within their enclosure systems. Consequently, two trials with respect to 'social dynamic change' were conducted. These aimed to test if there was a potential social hierarchy within this captive male population, if the general behavior pattern of male SHNW at the group level would be altered, and if this was reflected in urinary steroid secretion. Regarding the first trial, M1 from facility 2 was exchanged with M4 from facility 1 on January 3, 2016, so that the two supposedly dominant males (i.e. M3 and M4) now occupied the same facility (i.e. facility 2), while the remaining males were all grouped within facility 1. With respect to the second trial (the retrospective trial), M4 from facility 2 was exchanged with M1 from facility 1 on June

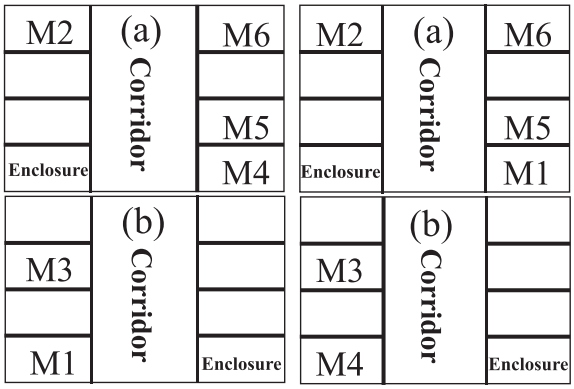

Fig. 1. The wombat enclosure system at AACE [(a) facility 1; (b) facility 2] showing individual male location (left side - before 3rd January 2016 and after 12th June 2016; right side - after 3rd January 2016 and before 12th June 2016).

12, 2016 and consequently M3 and M4 were assigned back to their original facilities (M3 – facility 2 and M4 – facility 1). Data and sample collection for these two trials are shown in Table 4. Notably, the period of data and sample collection for the first trial fell within the apparent ("local") breeding season of these captive wombats, while the second trial was conducted in the non-breeding season (T. Janssen, pers. comm.).

2.8. Statistical analysis

2.8.1. Data processing

A qualitative daily presence or absence of reproductive behavior was integrated into a weekly report (e.g. as long as a male showed reproductive behavior during the week, it would be noted as "1 point" for that week; otherwise it scored "0 point") (Martin et al., 1993); the same method was applied to recording weekly aggressive behavior for each male. With respect to identifying the impacts of animal exchange on general behavior, owing to the limited statistical power for the majority of behavioral categories (i.e. only a few males displayed these behaviors during the sampling periods), only eight behavior subtypes were included for statistical analyses; these were "scratching", "outside activity", "sitting rest", "walk", "feeding", "standing still", "exploring" and "sleeping". Notably, 189 days of behavioral data were successfully collected and some general behavioral data were lost due to logistical problems (e.g. camera malfunction); several urine samples were not collected as sometimes the males did not provide sufficient urine for analysis. Statistical analyses were carried out using Minitab (Version 17, 2016) and SAS (Version 9.3, 2012). Residual plots were used for testing if data sets were normally distributed but it was determined that no logarithmic transformations were necessary.

Table 4
Data and sample collection for Experiment 2.

Reallocation of male SNHW in captivity		
Trial	1	2
Exchange day	January 3 2016	June 12 2016
Durations of sample/data collection	2 weeks prior to the exchange day + 4 weeks following the exchange day	
Urine collection (with observations of QS)	Three times a week	
Behavioral data collection	During each trial (six weeks), 24-h video footage of behavior was stored and backed up for each male	

Significance levels were set at $P < 0.05$ for all tests and means are reported with standard errors (SE) unless otherwise noted.

2.8.2. Experiment 1: seasonal changes in captive male SHNW physiology and behavior

For each response variable (i.e. UTM, UCM, QS, aggressive behavior and reproductive behavior), a general linear model (GLM) analysis of variance (ANOVA) was used with animal ID and month being the fixed factors; Tukey pairwise comparison tests were used for post hoc analysis. Notably, weekly hormone (i.e. UTM and UCM) and behavior (i.e. aggressive and reproductive behavior) data were integrated into monthly information during the statistical analysis.

2.8.3. Experiment 2: reallocation of male SHNW in captivity

For hormonal data (i.e. UTM and UCM), a GLM ANOVA was used with trial (i.e. first, second), animal ID and week being the fixed factors and trial × week interaction terms, respectively. To determine the effects of animal exchange on eight major behaviors, a repeated-measures ANOVA (rANOVA) was constructed with compound symmetry covariance structure, using individual (i.e. animal ID) as a subject factor. Within subjects, the effects of trial, period (i.e. before vs. after the exchange date), date within each period, trial × period and date within each period × trial interaction terms were treated as fixed effects. Spearman correlations were conducted for evaluating the associations between hormone (i.e. UTM or UCM) level and 27 general behaviors (Table 3) for trial 1 and 2 respectively, using individual weekly mean values across available animals. Moreover, in order to avoid chance-created significance, the Benjamini-Hochberg procedure was used to control for false discovery with the rate being set as 0.05 (Benjamini and Hochberg, 1995).

3. Results

3.1. Experiment 1: seasonal changes in captive male SHNW physiology and behavior

Monthly means (±SE) of urinary testosterone and cortisol metabolite concentrations (ng/mg Cr) from six captive male wombats over the 11-month period are reported in Fig. 2A and B, respectively. For UTM, the results from a GLM ANOVA revealed that there was a significant effect of individual ($F_{5,50} = 5.75$, $P < 0.001$) but the month effect was not significant, although this did come very close to reaching significance ($F_{10,50} = 2.02$, $P = 0.051$). Regarding UCM, the results from GLM ANOVA demonstrated there were significant effects of both individual ($F_{5,50} = 3.62$, $P = 0.007$) and month ($F_{10,50} = 4.75$, $P < 0.001$). The results from post hoc analysis (Tukey pairwise comparisons; Fig. 2B) revealed that (1) mean UCM concentrations of June 2016 were significantly higher than the mean values of December 2015, (2) mean UCM concentrations of July 2016 were significantly higher than the mean values of October 2015, November 2015, December 2015, January 2016 and February 2016, respectively and (3) mean UCM concentrations of August 2016 were significantly higher than the mean values of October 2015, November 2015, December 2015 and January 2016, respectively. Notably, mean UCM concentrations were highest in August (0.941 ± 0.0876 ng/mg Cr) and lowest in November (0.528 ± 0.0729 ng/mg Cr).

Mean ± SE (n = 4) and individual male wombat changes in the monthly urinary spermatorrhoea index over the 11-month period are presented in Fig. 3. The results from GLM ANOVA showed that QS was significantly affected by individual ($F_{3,30} = 5.32$, $P = 0.005$) but not by month ($F_{10,30} = 1.30$, $P = 0.27$); therefore, no further post hoc testing was performed. With regards to individual variation,

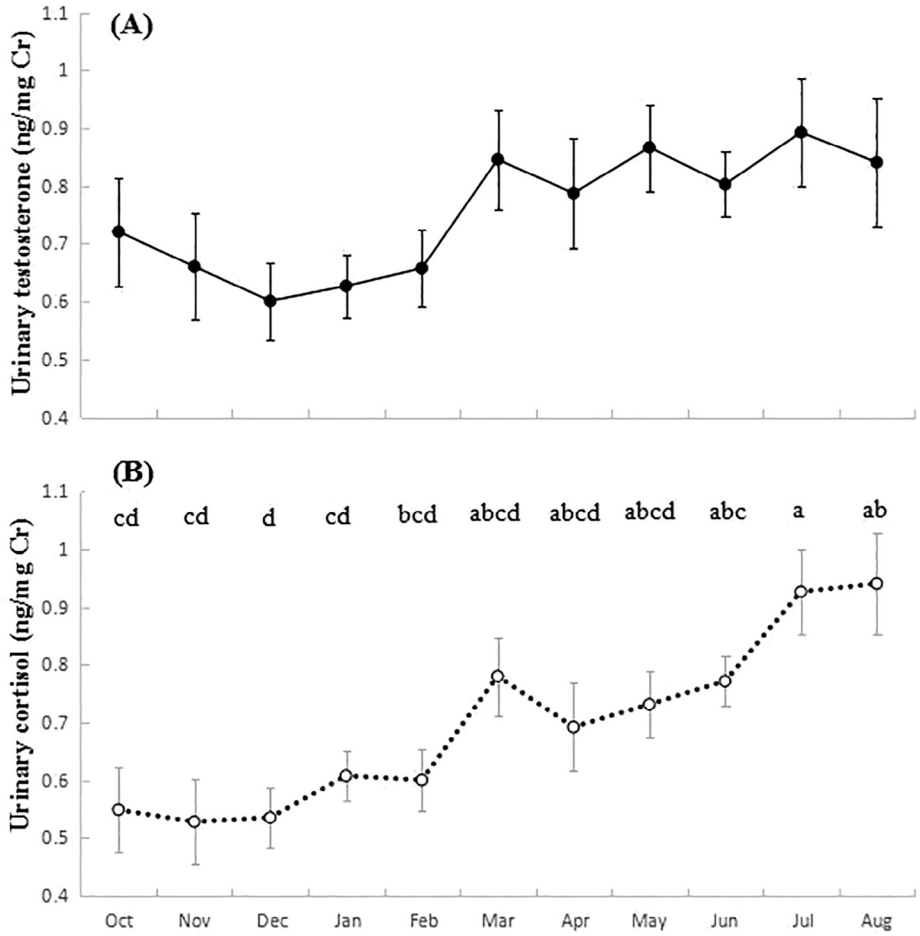

Fig. 2. Mean (±SE) changes in monthly (October 2015 to August 2016) urinary (A) testosterone (UTM) and (B) cortisol (UCM) metabolite concentrations for captive male *L. latifrons* (n = 6). The mean UTM concentrations over time were not significantly different between months (P > 0.05). The UCM concentrations were significantly different between months (P < 0.001), which has been designated by different letters (i.e. means that do not share a letter are significantly different).

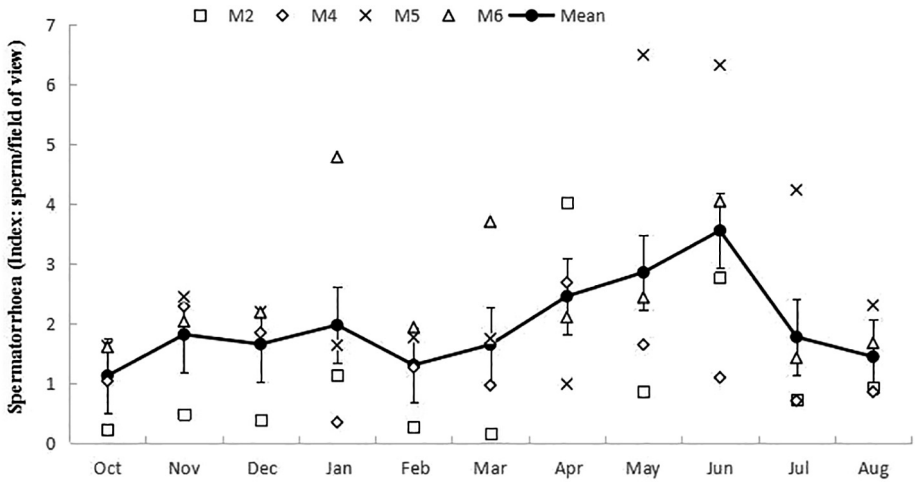

Fig. 3. Mean ± SE (n = 4) and individual male *L. latifrons* changes in monthly (October 2015 to August 2016) urinary spermatorrhoea index (sperm number per field of view). No sperm was present for M1 (vasectomized) or M3. Individual variation between males that exhibited spermatorrhoea was evident over time (P = 0.005).

QS was only observed in the urine of four male wombats (i.e. M2, M4, M5 and M6), while no spermatozoa were found in the urine of M1 or M3. Individual spermatorrhoea information is presented in Table 5.

Monthly means of the proportion of aggressive behavior and reproductive behavior (%) from six captive male wombats over the 11-month period are shown in Fig. 4. Based on the results from a GLM ANOVA, the amount of aggressive behavior was significantly

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Table 5
Individual spermatorrhoea information of the six male *L. latifrons*.

Animal ID	M1	M2	M3	M4	M5	M6
Mean QS (sperm/field of view)	Lack of sperm in urine	1.09 ± 0.37	Lack of sperm in urine	1.33 ± 0.21	2.88 ± 0.58	2.54 ± 0.34
When motile spermatozoa were found	N/A	October 2015, January 2016, April 2016, May 2016, June 2016, July 2016, August 2016	N/A	December 2015, January 2016, March 2016, June 2016, July 2016, August 2016	December 2015, May 2016, June 2016, July 2016	Throughout the sampling period – from October 2015 to August 2016
Average pH of urine	6.8	6.2	7.1	6.7	6.2	6.9

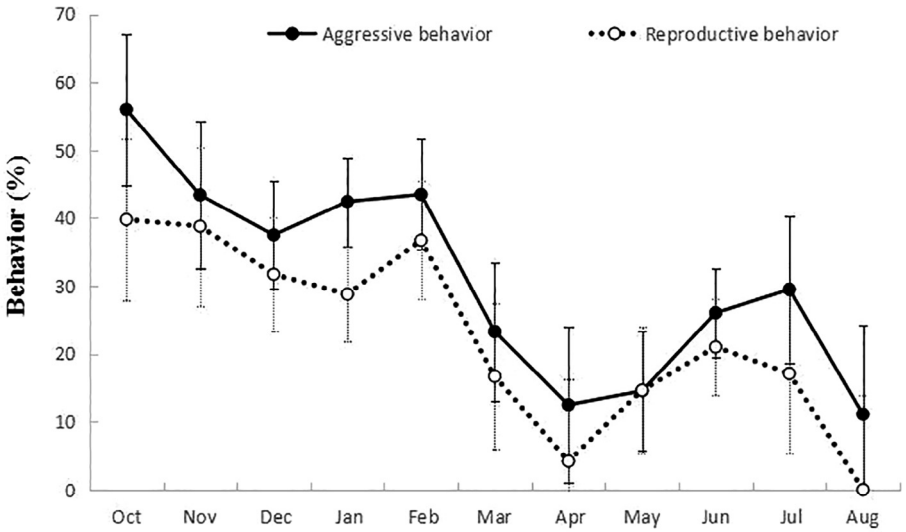

Fig. 4. Mean (±SE) changes in the monthly (October 2015 to August 2016) proportion of aggressive and reproductive behaviors for captive male *L. latifrons* (n = 6). For both aggressive and reproductive behaviors, the month effect was not significant (aggressive behavior: Tukey pairwise comparisons; reproductive behavior: P = 0.19) but the individual effect was significant (P < 0.001) for both behaviors.

affected by individual ($F_{5,50} = 13.15$, $P < 0.001$) and month ($F_{10,50} = 2.18$, $P = 0.035$). However, post hoc analysis (Tukey pairwise comparisons) did not reveal a monthly mean that was significantly different from the others. With respect to reproductive behavior, the individual animal effect was significant ($F_{5,50} = 6.01$, $P < 0.001$) but the month effect ($F_{10,50} = 1.44$, $P = 0.19$) was not.

3.2. Experiment 2: reallocation of male SHNW in captivity

Mean (±SE) changes in weekly urinary testosterone and cortisol metabolite concentrations (ng/mg Cr) over a 6-week period for trial 1 and 2 are provided (Fig. 5A and B). According to the results from a GLM ANOVA, both UTM and UCM levels were affected by trial (UTM: $F_{1,55} = 19.86$, $P < 0.001$; UCM: $F_{1,55} = 29.43$, $P < 0.001$) and individual (UTM: $F_{5,55} = 5.42$, $P < 0.001$; UCM: $F_{5,55} = 4.37$, $P = 0.002$) but not by week (UTM: $F_{5,55} = 0.36$, $P = 0.88$; UCM: $F_{5,55} = 0.67$, $P = 0.65$) or trial × week (UTM: $F_{5,55} = 0.56$, $P = 0.73$; UCM: $F_{5,55} = 0.96$, $P = 0.45$).

With regards to major behaviors, the results from rANOVA demonstrated that "scratching" was only significantly affected by period (i.e. reduced after the animal exchange; $F_{1,9} = 7.90$, $P = 0.02$) and not by trial ($F_{1,9} = 0.01$, $P = 0.93$), date within each period ($F_{16,135} = 1.37$, $P = 0.17$), trial × period ($F_{1,9} = 0.07$, $P = 0.80$) or date within each period × trial ($F_{16,135} = 0.87$, $P = 0.61$) (Fig. 6A). "Outside activity" was significantly affected by date within each period (i.e. increased after the animal exchange in trial 1 and decreased after the animal exchange in trial 2; $F_{16,135} = 4.43$, $P < 0.0001$), trial × period ($F_{1,9} = 13.64$, $P = 0.005$) and date within each period × trial ($F_{16,135} = 4.98$, $P < 0.0001$) but not by trial ($F_{1,9} = 0.63$, $P = 0.45$) or period ($F_{1,9} = 0.87$, $P = 0.38$) (Fig. 6B). "Standing still" was significantly affected by date within each period, being increased after the animal exchange in trial 1 and decreased after the animal exchange in trial 2 ($F_{16,135} = 2.95$, $P = 0.0003$) and trial × period ($F_{1,9} = 8.26$, $P = 0.018$) but not by trial ($F_{1,9} = 0.01$, $P = 0.92$), period ($F_{1,9} = 1.03$, $P = 0.34$) or date within each period × trial ($F_{16,135} = 0.97$, $P = 0.50$) (Fig. 6C). "Sleeping" was significantly affected by date within each period, being decreased after the animal exchange in trial 1 and increased after the animal exchange in trial 2 ($F_{16,135} = 3.37$, $P < 0.0001$), trial × period ($F_{1,9} = 12.88$, $P = 0.0058$) and date within each period × trial ($F_{16,135} = 4.06$, $P < 0.0001$) but not by trial ($F_{1,9} = 0.21$, $P = 0.66$) or period ($F_{1,9} = 0.09$, $P = 0.77$) (Fig. 6D). None of these within-subject factors (i.e. trial, period, date within each period or trial × period or date within each period × trial) significantly affected the frequency of "sitting rest", "walk", "feeding" or "exploring" behaviors ($P \geq 0.071$). Notably, M6's behavioral data for trial 2 were unavailable due to camera malfunction.

The results from the combination of a Spearman correlation and Benjamini-Hochberg procedure indicated that (1) UTM level was positively and significantly correlated with "standing still" ($r = 0.395$, $P = 0.017$, Benjamini-Hochberg $P = 0.031$) and "wall biting" ($r = 0.395$, $P = 0.017$, Benjamini-Hochberg $P = 0.031$) during the time period of trial 1, (2) UCM level was positively and significantly correlated with "standing still" ($r = 0.491$, $P = 0.0023$, Benjamini-Hochberg $P = 0.004$) during the time period of trial 1, (3) UTM level was significantly and positively correlated with "body rub" ($r = 0.611$, $P = 0.0004$, Benjamini-Hochberg $P = 0.0005$) but negatively correlated with "approach" ($r = -0.482$, $P = 0.0081$, Benjamini-

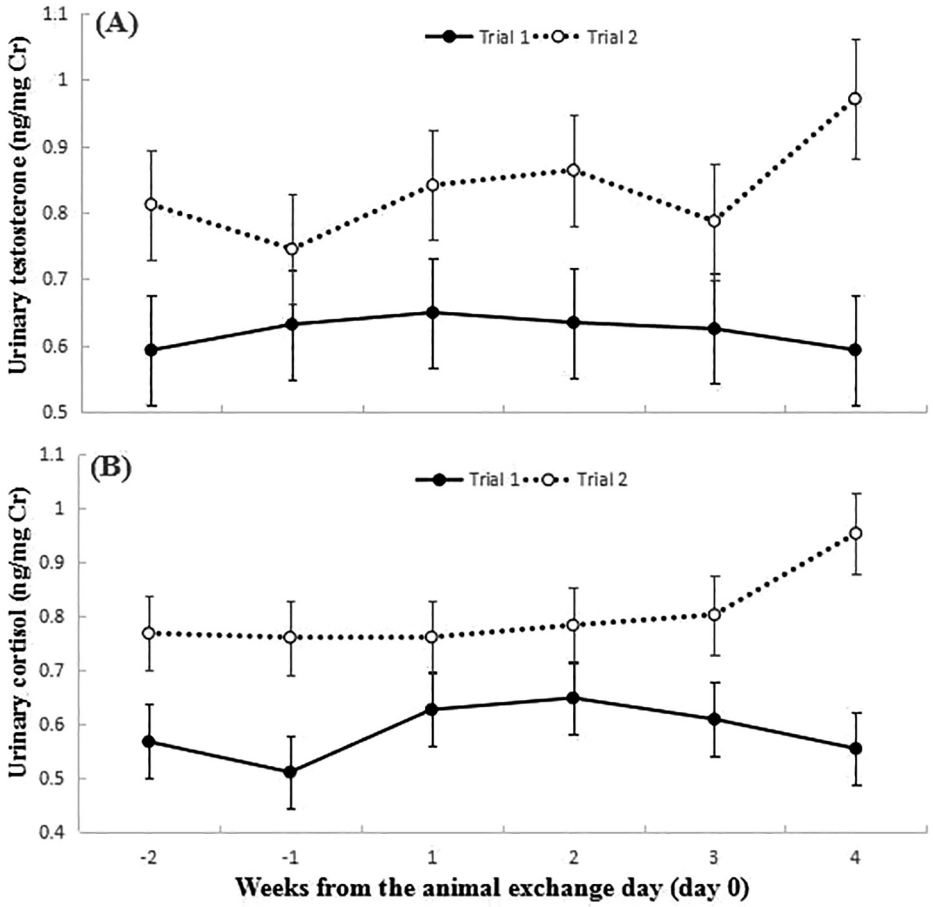

Fig. 5. Mean (±SE) changes in weekly urinary (A) testosterone and (B) cortisol metabolite concentrations for captive male *L. latifrons* (n = 6) following the animal exchange day (0), over a 6-week period (−2 to 4 week) for trial 1 and trial 2, respectively.

Hochberg P = 0.011), "retreat" (r = −0.441, P = 0.017, Benjamini-Hochberg P = 0.022) and "den check" (r = −0.386, P = 0.039, Benjamini-Hochberg P = 0.049) during the time period of trial 2.

4. Discussion

The changes in mean UTM level of six captive male *L. latifrons* over the study period did not quite reach significance but there was a trend in seasonal variation (i.e. mean UTM concentrations appear to be higher between March and August 2016 than those between October 2015 and February 2016) in the current study. However, this trend is somewhat inconsistent with previous findings in terms of seasonal change in plasma testosterone concentrations (Hamilton et al., 2000; Hogan et al., 2010a). Hamilton et al. (2000) reported the concentrations of both plasma and fecal testosterone metabolites (FTM) were significantly elevated during the breeding season (August to October) compared to the non-breeding season (February to April) for wild SHNWs living at the Swan Reach region of South Australia. Meanwhile, Hogan et al. (2010a) found that in captive SHNW at Rockhampton Zoo, there were seasonal changes in stimulated testosterone secretion in blood samples that were taken 90 min after a GnRHa challenge, but no seasonal changes in circulating testosterone levels before GnRHa injection, nor in excreted testosterone levels over time. We therefore speculate that a less pronounced natural seasonal variation in testosterone secretion in captive male SHNW may be contributing to a lack of breeding success in some males; future studies should further investigate seasonal testosterone secretion in captive male SHNW housed in South Australia, within their natural range, to identify the ubiquitousness of this phenomenon. It is possible that in captive male SHNWs, the lack of seasonal testosterone secretion may be due to an alteration of the wombat's physiology that is itself potentially linked to environmental or captive management artefacts. Alterations in photoperiod could have potentially contributed to the altered testosterone production pattern observed in captive male SHNWs as there was a significant difference in latitude between where the captive wombats are presently located and where they were sourced (Swan Reach, Murraylands region, South Australia). Previous studies have demonstrated that altering photoperiod can have a profound impact on the number of neurons that express kisspeptin (Kiss) in seasonal-breeding species (Chalivoix et al., 2010; Revel et al., 2006). Kiss neurons deliver essential information on reproductive viability to GnRH neurons by secreting kisspeptin (Navarro and Tenasempere, 2012) so changes in photoperiod associated with a more northerly latitude could influence the pattern of GnRH secretion, leading to changes in testosterone secretion and altered patterns of normal reproductive function.

The mean UCM level of six captive male wombats increased gradually from October 2015 to August 2016, reaching a maximum in August. It was somewhat unusual that the mean UCM concentrations peaked in July and August 2016, while the mean UCM concentrations in October–December 2015 were relatively low. It would be more reasonable to expect that the mean UCM level would decrease smoothly in July and August 2016 in order to reach a level comparable to those in October–December 2015. One possible explanation for this pattern is a change in captive

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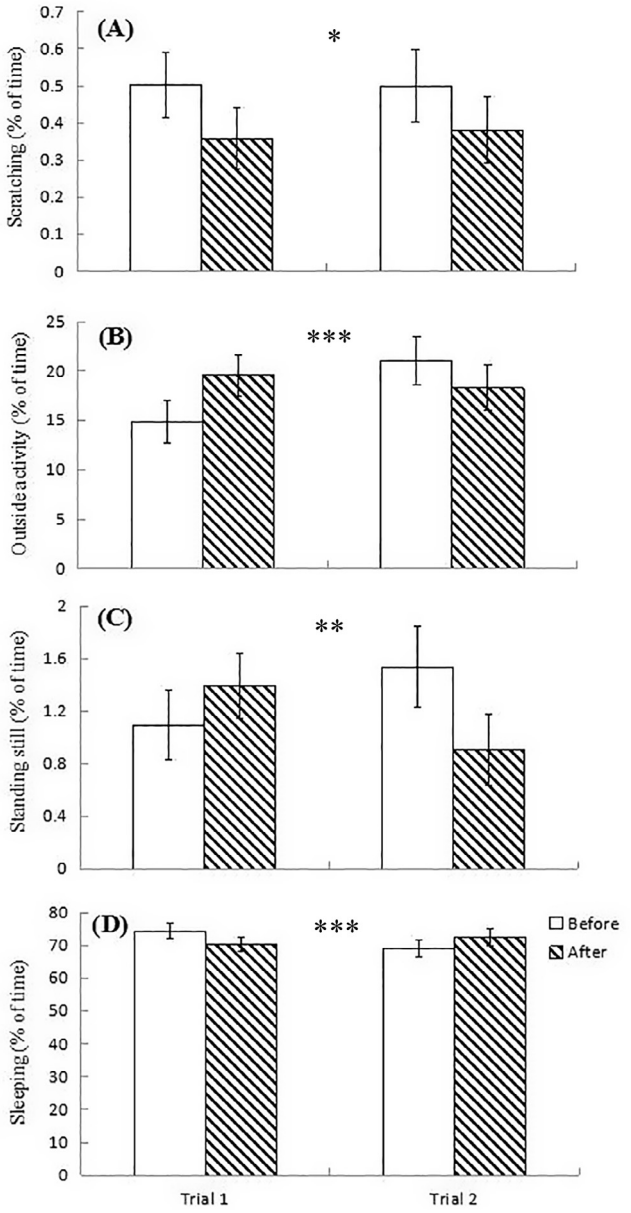

Fig. 6. Mean (±SE) percentage time of (A) "scratching", (B) "outside activity", (C) "standing still" and (D) "sleeping" behaviors for captive male *L. latifrons* before [an average of 7 days (i.e. −13 d, −11 d, −9 d, −7 d, −5 d, −3 d and −1 d) - white bar] and after [an average of 11 days (i.e. 1 d, 2 d, 3 d, 5 d, 7 d, 9 d, 12 d, 15 d,19 d, 23 d and 28 d) - striped bar] the animal exchange for trial 1 (n = 6) and trial 2 (n = 5). For Fig. 6A, * ($P < 0.05$) indicates a statistically significant decrease in "scratching" after the animal exchange; for Fig. 6B, *** ($P < 0.0001$) indicates "outside activity" was increased after the animal exchange in trial 1and decreased after the animal exchange in trial 2; for Fig. 6C, ** ($P < 0.01$) indicates "standing still" was increased after the animal exchange in trial 1 and decreased after the animal exchange in trial 2; for Fig. 6D, *** ($P < 0.0001$) indicates "sleeping" was decreased after the animal exchange in trial 1 and increased after the animal exchange in trial 2.

management (i.e. reallocation of female wombats; Z. Du, personal observations) in preparation for the upcoming mating season; during July and August 2016, many female wombats were removed from their original enclosures and paired with new males for captive breeding purposes, while males remained in their enclosures (Fig. 1A). Specifically, if a female wombat was found to be 'incompatible' with her new paired male (e.g. the male showed intensive aggression towards the female without any reproductive purposes), then the female would be removed from this male's enclosure and paired with another male or assigned back to her original enclosure. The implementation of this management strategy during July and August 2016 may have contributed to the "unexpected" increase in cortisol secretion in these captive male wombats for two reasons; firstly, the frequent introduction/removal of females may have resulted in an increase in cortisol secretion in response to these novel pairings. Secondly, given the removal process required extensive human intervention (e.g. disinfection of enclosures and temporarily locking the paired male within his sleeping den in order to allow the newly introduced female to habituate to her new environment or to prevent the male from attacking/biting the female), this interruption could have been perceived as a negative stimulus by the wombats. Although it was not possible to quantify the effect of human intervention on cortisol secretion in the male wombats in this study, Hogan et al. (2011a) have suggested that forced human approach/contact could significantly elevate cortisol secretion in captive SHNWs and that the GC response was not attenuated by regular handling. Future studies should investigate the influence of season on adrenal physiology in free-ranging SHNWs, as well as those in more southern captive-breeding facilities. This would then elucidate the significance of the current findings to wild populations.

Consistent with the findings of Hogan et al. (2010a) and Taggart et al. (2005), spermatorrhoea was observed in four males throughout the 11-month sampling period, indicating that both captive and wild male SHNWs maintain a pattern of continuous sperm production over the whole year. The reason for the absence of spermatorrhoea in M3 is not clear, especially since this male had previously successfully sired an offspring at AACE. The lack of noted seasonal variation in mean QS in this study is consistent with the observations of Taggart et al. (2005), who reported that the sperm concentration of electro-ejaculate (expressed as sperm number per mL of ejaculate) did not undergo significant seasonal changes for wild male SHNW but that it was seminal fluid and sperm quality that were instead affected by season. Neither the mean proportion of aggressive behavior nor the mean proportion of reproductive behavior of six captive male wombats exhibited pronounced seasonal changes, even though the proportion of both behaviors appeared to be elevated between October and February. The lack of significance of these changes may be related to individual variation but the trend suggests an association with the breeding season. Previous studies have defined the breeding season for SHNWs as occurring between July and December in both wild (Gaughwin et al., 1998; Paris et al., 2002; Taggart et al., 2005, 2008) and captive populations (Hogan et al., 2010a,b, 2011b). The less pronounced seasonality displayed by captive male SHNW housed in central Queensland might be due in part to changes in photoperiod and differences in environmental conditions (e.g. elevated ambient temperature, humidity). These altered environmental cues might have shifted or extended the breeding season of these captive male SHNWs beyond that reported previously, as supported by a successful birth at this facility in March 2016. This finding is also in line with a shift or extension of breeding season in mainland captive female Tasmanian devil (*Sarcophilus harrisii*) held in institutions at greater latitudes when compared to their native habitat of Tasmania (Keeley et al., 2017).

Previous studies have demonstrated a decrease in plasma testosterone during a period of hierarchical instability for both captive male bare-tailed woolly opossums (*Caluromys philander*; Guillemin et al., 2000) and the wild male brush-tail possums (*Trichosurus vulpecula*; Wehi et al., 2006), which has been suggested to be primarily associated with stress (Guillemin et al., 2000; Holst, 1977). However, neither the mean UTM concentration nor the mean UCM concentration of six captive male wombats varied significantly after the forced change in location and individual wombat proximity for both trials. Given their polygynous mating system, the male's tendency to establish and maintain territories,

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the territories of individual males showing extensive overlap with the home ranges of females and minimal overlap with other males, and simultaneous sharing of warren systems (Finlayson et al., 2005; Gaughwin, 1981; Gaughwin et al., 1998; Wells, 1973), SHNW may establish some form of social structure in the wild. Thus the results from the current study may suggest a level of tolerance of conspecifics even in captive settings. The present study appears to be the first to investigate the social dynamics of male SHNW in captivity; future research may be useful for establishing the extent of social organization in this species. In turn, this could optimize captive husbandry and breeding techniques for both SHNW and, potentially, northern hairy-nosed wombats.

In the current study, the mean percentage of "scratching" decreased significantly at the group level following the animal exchange for both trials. Descovich et al. (2012) reported this behavior to be associated with space allowance, with captive SHNWs living in large enclosures 'scratching' significantly less when compared to those living in medium or small enclosures, suggesting that such self-grooming had an appeasing function in the small enclosures. In the present study, a lack of enclosure size difference between facility 1 and facility 2 and lack of significant change in UCM concentrations following the animal exchange for both trials, it might be more parsimonious to regard "scratching" as a behavioral response to novel stimuli, rather than a direct correlate of stress *per se*.

The moderate and positive correlation between UCM concentration and "standing still" behavior during the time period of trial 1 may support the theory that a stress-stimulated rise in GC release results in an increase in the amplitude of behaviors that enhance emotions and motivation (Dallman et al., 2005, 2006; Smith et al., 2009). Hence, "standing still" may serve as a behavioral response to threatening and/or cognitively meaningful stimuli for captive male SHNW. The moderate and positive relationship between UTM concentration and "body rub" during trial 2 might infer an interplay between testosterone secretion and marking behavior for captive male SHNWs, which supports results of previous studies (Fadem, 1990; Fadem et al., 1989) that have indicated a close association between testosterone stimulation and the performance of various types of marking behavior in gray short-tailed opossums (*Monodelphis domestica*). Notably, M6's behavioral data for trial 2 were missed owing to camera malfunction, so that the difference in sample size, together with the season effect (two trials were conducted in different months of the year), might account for the inconsistency of the results between the two trials (i.e. significant hormone-behavior correlations emerged during the time period of trial 1 that subsequently could not be detected during trial 2 and vice versa). Additionally, the moderate and negative relationship between UTM concentration and "approach/retreat" during the time period of trial 2 could also be attributed to the reduced statistical power of our analysis, as these two types of male-female interaction could have been extensively displayed by M6.

5. Conclusion

This study has provided novel insight into captive male SHNW seasonality and appears to be the first to investigate physiological changes associated with social dynamics. Collectively, the results of the current study support the claim that captive male SHNWs exhibit less pronounced seasonality compared to their wild conspecifics and suggest that (1) captive management may affect seasonal cortisol secretion pattern in these captive male SHNWs and (2) social hierarchies within this captive male population might not be present. The lack of a strong seasonal influence on male reproduction in captive male SHNWs implicates this phenomenon as a factor that may be related to the relatively low rate of captive breeding success in this species. In particular, given the possibility that photoperiod is likely to play a crucial role in regulating captive SHNW reproduction, future research should further investigate this effect by artificially manipulating photoperiod for captive SHNW.

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Conflicts of interest

None.

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