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Research paper

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

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1. Introduction

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

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

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

Pacific white sided dolphin (*Lagenorhynchus obliquidens*) (Robeck et al., 2009). Therefore, the analysis of urine may provide a more comprehensive evaluation of reproductive hormones in the female SHNW.

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

To date, no study has reported the use of immunoassays for the measurement of urinary reproductive protein hormones in marsupials. Given that it is possible to collect urine from captive female SHNW using either classical conditioning methods and/or direct collection off the floor of their enclosure (Swinbourne et al., 2014), the detection of LH, oestrogen and progesterone metabolites in urine could be highly beneficial for SHNW reproductive assessment. The use of either a synthetic or natural sequence mammalian gonadotrophin releasing hormone (GnRH) to trigger an LH surge, has been successfully used to validate immunoassays for the detection of serum LH in a range of marsupials (Allen et al., 2008; Ballantyne et al., 2016a; 2016b; Bryant, 1992; Fletcher, 1989; Johnston et al., 2004; Matson et al., 2009; Moore et al., 1996; Rudd et al., 1999; Sutherland et al., 1980; Tyndale-Biscoe et al., 1983; Wilson et al., 2013).

To test the efficacy of urine as a suitable biological sample for the measurement of reproductive hormones (LH, oestrogen and progesterone metabolites) in female SHNWs, a series of biological and immunoassay validation trials were conducted using two exogenous hormones. In the first trial, a supermaximal dose of GnRH agonist (GnRH_a) was used to challenge the anterior pituitary to secrete a surge of LH that could then be analysed in both blood and urine. The second trial involved two sequential but increasing

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doses of equine chorionic gonadotrophin (eCG) in order to stimulate gonadal steroid secretion. Further assay validation included urinary steroid extraction, the analysis of longitudinal sample to evaluate the urinary hormone profiles of a pregnant female and a mated non-pregnant female, as well as a comparison of hormone profiles between matched urine and faecal samples.

2. Material and methods

2.1. Animal management

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

Each enclosure consisted of an indoor temperature controlled (air-conditioned) area (5.8 m²) with individual sleeping chambers (3 × 0.6 m² each). The inside enclosure had an adjoining large outdoor yard (between 90 and 100 m²) consisting of soil substrate, partial grass vegetation, and a dirt mound for enrichment. All animals were fed a daily mixed ration of 120 g rolled oats (COLES Smart Buy, Australia), 120 g Gumnut[®] pellets (MITAVITE, Australia), 35 g oaten chaff (Rich River Chaff and Grain, Australia), 200 g sliced sweet potato. Half cob of corn was also provided three times a week when available and water was available *ad libitum*.

Indoor infrared dome cameras (SUMO[®] – Model: CAM351RHR) and weatherproof outdoor infrared cameras (SUMO[®] – Model: CAM781RHR) were installed in all wombat enclosures. Footage

was recorded on to a digital video recorder (DVR – KOBIL[®] 16 channel) surveillance system, and was reviewed daily using XQ Pro Series DVR surveillance software to confirm the occurrence and timing of attempted mating and birthing behaviours.

2.2. Daily non-invasive sample collection

Unless otherwise stated, fresh urine samples were collected daily from female SHNW using the methodology previously described by Swinbourne et al. (2014). Urine samples were kept on ice until they could be divided into 2 mL aliquots (original and duplicate samples) and stored at –20 °C. Where possible, uncontaminated fresh faecal pellets were also collected daily directly off the enclosure floor, sealed in a labelled snaplock bag, immediately placed on ice, then stored at –20 °C until hormone analysis could be conducted.

2.3. Experiment 1: GnRHa challenge to experimentally induce an LH surge

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

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

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

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

Equine chorionic gonadotrophin (eCG) stimulation was conducted during the 2014 wombat breeding season (October – December 2014). Each female was administered two sequential and increasing doses of eCG (Folligon, Intervet, Australia); 150 IU

Table 1

Age, weight, housing, validation method and trial date for female southern hairy-nosed wombats used for validation of urinary luteinizing hormone, estrone-3-glucuronide, oestradiol 17β, and progesterone metabolite enzyme-immunoassays.

Wombat ID	Age (years)	Weight (kg)	Housing
<i>GnRH trial #1: October 2013</i>			
F5	6*	31.1	Female only pair
F6	6*	28.3	Female only pair
F9	4	20.6	Female only pair
F10	3	23.5	Female only pair
<i>eCG trial: October – December 2014</i>			
<i>GnRH trial #2: January 2015</i>			
F6	7	30.8	Female only pair
F7	10*	21.5	Female only pair
F8	10*	22.7	Female only pair
F11	11*	30.8	Female only
<i>Urinary steroid extraction. Samples collected from August – December 2014</i>			
F1	9	24.6	Breeding pair
F2	5	22.0	Breeding pair
F9	5	22.6	Breeding pair
F10	4	23.5	Breeding pair
<i>Biological validation: pregnant cycle in 2013 and mated, non-pregnant cycles in 2014</i>			
F3	4	24.8	Breeding pair
F10	4	23.5	Breeding pair

GnRH#1 and GnRH#2 – wombats used for gonadotrophin releasing hormone agonist.

eCG – wombats injected with equine chorionic gonadotrophin for gonadal steroid stimulation.

Steroid extraction – wombats used for additional oestrogen extraction studies.

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

was administered on day 1 and another 300 IU was injected 53 days later. For each injection, females ($n = 4$; Table 1) were confined to their sleeping chamber and received an intramuscular injection in their hind quarter/rump. Each female was monitored for approximately one hour after the injection, then released. Fresh, non-invasive urine samples were collected daily using the methodology previously described by Swinbourne et al. (2014). All urine samples were stored at $-20\text{ }^{\circ}\text{C}$ until hormone analysis could be conducted. No sedation or anaesthetic was used in this stimulation trial.

2.5. Enzyme-immunoassay validation for urinary reproductive hormones

2.5.1. Urinary creatinine

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

2.5.2. Urinary luteinizing hormone (LH)

Serum and urinary LH was quantified using a monoclonal anti-bovine LH antibody (518-B7 – provided by Janet Roser from the UC Davis, USA) EIA previously validated for serum LH in eastern grey kangaroos (*Macropus giganteus*) (Wilson et al., 2013). Briefly, 50 μL of undiluted serum samples and diluted urine samples (1:2 in assay buffer), standards (0.156–20 ng/mL) and high and low controls were dispensed in duplicate into goat anti-mouse gamma globulin (GAMG) pre-coated 96-well microtiter plate (Corning Inc., USA). LH antibody (final LH antibody dilution in assay buffer was 1:400,000) was added into each well (except blank or zero well) and the EIA was incubated in the dark overnight at room temperature (approximately $24\text{ }^{\circ}\text{C}$). Biotinylated labelled conjugate (final dilution 1:300,000) was added to each well and incubated for 3.5 h at room temperature before utilising streptavidin and a 3,3', 5,5'-tetramethylbenzidine (TBM, Sigma Aldrich, Australia, catalogue # T2885) colour reaction steps to evaluate hormone concentrations.

2.5.3. Urinary estrone-3-glucuronide (E1C)

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

2.5.4. Urinary oestradiol-17 β (E2)

Urine samples were analysed for E2 (antibody R4972 provided by Coralie Munro, UC Davis, USA) using a modified EIA previously

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

2.5.5. Urinary and faecal progesterone metabolites (P4M)

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

2.5.6. Enzyme-immunoassay validation

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

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

2.6. Urinary oestrogen sample preparation and steroid extraction

To determine the optimal urine handling and analysis process for the detection of oestrogen metabolites, different extraction or processing methods were trialled for four females (Table 1) for one complete oestrous cycle each. Urine samples were divided into four treatment groups; (1) analysis of estrone-3-glucuronide (E1C) from untreated samples, (2) the analysis of oestradiol 17 β (E2) from untreated samples, (3) steroid extraction with an enzyme

hydrolysis buffer, then analysis of E2 (E2 + EHB), and (4) double extraction using an enzyme hydrolysis buffer, followed by liquid extraction with diethyl ether, then analysed for E2 (E2 + EHB/DE).

2.6.1. Enzyme hydrolysis

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

2.6.2. Liquid extraction

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

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

2.7. Reproductive hormone characterisation

Baseline values for all urinary hormone profiles were calculated using the iterative process described in Steinman et al. (2012). All values >2 SD from the mean were temporarily removed from the data set and the mean recalculated. This process was repeated until no more values >2 SD could be removed. The remaining mean was then adopted as the baseline hormone concentration. Values ≥2 SD were considered as a significant increase in hormone concentration and peak hormone concentrations were classified as the maximal hormone concentration before returning to baseline values. To characterise reproductive cycles, a sustained increase in P4M concentration above baseline for ≥3 consecutive days was defined as the onset of the luteal phase which concluded when P4M concentration returned to baseline values. The period between the end of one luteal phase and the beginning of the next was classified as either the follicular phase or inter-oestrous period (Hogan et al., 2010a). To confirm biological validation, the hormone profiles from challenged females ($n = 4$) were compared to two natural reproductive events; an oestrous cycle with a successful mating and parturition, and a three consecutive non-pregnant oestrous cycles. Faecal samples from the same females were analysed and compared to urinary P4M profiles.

2.8. Data analysis

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

3. Results

The pooled serum samples showed a stronger parallelism to the bovine LH standard ($R^2 = 0.99$, $P = 0.001$) compared to the pooled urine samples ($R^2 = 0.69$, $P = 0.01$, Fig. 1A). Parallelism for each urinary steroid hormone analysed (E1C, E2 and P4M) was confirmed for individual females (parallelism graphs are not shown). The mean recovery of spiked urine pools for LH, P4M and E1C was 112%, 102% and 101% (Fig. 1B), and revealed little matrix interference during the analysis of urine samples.

3.1. Experiment 1: measurement of luteinizing hormone

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

For the second GnRH challenge (10 µg dose), serum samples were analysed as neat and urine samples were analysed at a 1:2 dilution. The mean (±SEM) pre-GnRH serum LH was 1.20 ± 0.19 ng/mL which increased to 1.8 and 4.2 ng/mL approximately ten minutes following GnRH_a injection (Fig. 3A). The mean (±SEM) pre-GnRH urinary LH was 0.06 ± 0.01 ng/mL Cr and peaked between 30 and 60 min following the GnRH_a injection (peak range 0.12–0.23 ng/mg Cr, Fig. 3B). It was not possible to collect urine from F11 between T0 and T60, nor was it possible to collect urine from anaesthetised F7 (T-5 to T120) by means of palpation of the bladder.

3.2. Experiment 2: measurement of urinary reproductive steroid hormones

Baseline (± SEM) hormone concentration for urinary E1C for F6, F7, F8 and F11 were 0.50 ± 0.02 , 0.39 ± 0.02 , 0.61 ± 0.03 and 0.33 ± 0.03 ng/mg Cr, respectively. Baseline hormone concentration for urinary P4M for F6, F7, F8 and F11 were 2.87 ± 0.17 ,

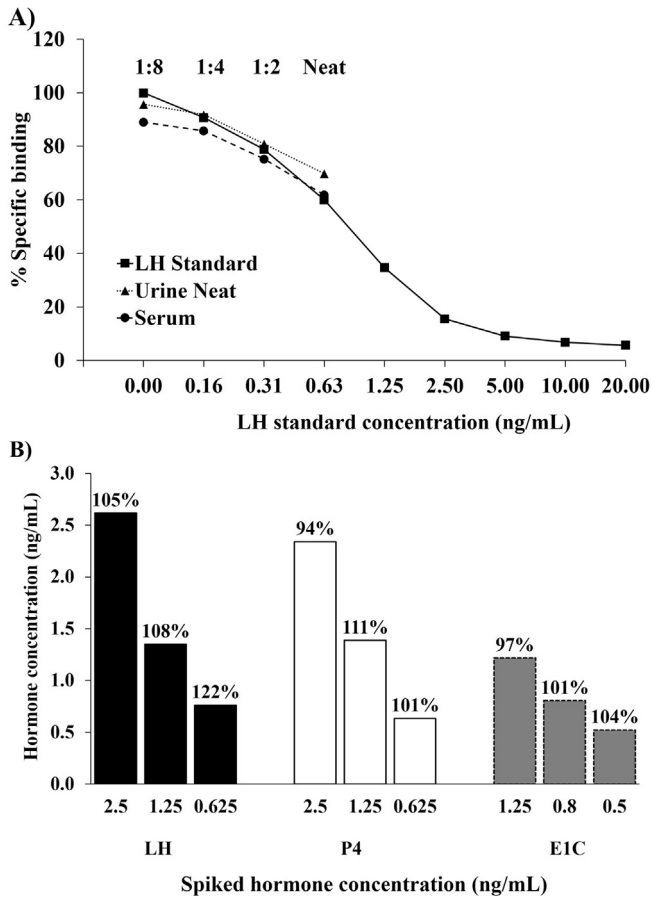


Fig. 1. A) Serial dilution (neat to 1:8) of pooled serum (circles) and pooled urine (triangles) samples compared against eight bovine luteinizing hormone (LH) concentrations (squares) from 20.0 to 0.16 ng/mL. 0.0 ng/mL was assay buffer only (zero well). B) Hormone recovery assays of individual animal urine pools spiked with 2.5, 1.25 or 0.625 mg/mL LH standard, 2.5, 1.25 or 0.625 mg/mL progesterone standard (P4), or 1.25, 0.8, or 0.5 ng/mL estrone-3-glucuronide (EIC) standard.

2.22 ± 0.09, 4.32 ± 0.16, and 1.34 ± 0.27 ng/mg Cr, respectively. The urinary steroid hormone analysis showed within and between animal variation (Fig. 4A-B) in hormone concentrations or patterns of hormone response. Females responded to one or both eCG injections which resulted in a significant increase in E1C followed by a subsequent rise in P4M between ten and 15 days following the

injection. F6 appeared to be in the peak of a luteal phase when administered 150 IU eCG which resulted in peak E1C approximately seven days post-injection, followed by a rise in P4M from day 17 post-injection. F6 did not respond to the second, higher eCG injection as both urinary hormones remained around baseline values for the remainder of the 2014 breeding season. F7 appeared to be in anoestrus prior to the hormone stimulation as changes in urinary hormones only occurred after both eCG injections. The 150 IU eCG injection resulted in a sustained increase in both urinary E1C and P4M for F7; however, the second higher eCG injection (300 IU) resulted in a stronger response, as peak hormone concentrations more than doubled in value compared to the first injection. F8 was at the end of the luteal phase when stimulated with 150 IU eCG resulting in a slow rise in E1C, which was then followed by a sustained increase in P4M. Once again, F8 appeared to be at the end of the luteal phase when administered the second higher eCG; while there was no significant rise in E1C, P4M peaked eight days following the injection. F11 was in an inter-oestrous period when injected with the 150 IU. There was no significant rise in E1C after the 150 IU injection; however, there was a sustained P4M increase 14 days post-injection. Based on urinary P4M, the second eCG injection for F11 was administered during the mid-luteal phase. Two days following the injection, E1C significantly increased for seven days and P4M continued to rise, peaking 13 days following the 300 IU injection.

3.3. Experiment 3: urinary oestrogens – sample preparation and steroid extraction

After extraction, there was a significant treatment effect on absolute urinary oestrogen metabolite concentrations ($F_3, 591.7 = 265.3; P < 0.001$). The mean hormone concentrations for E1C, E2, E2 + EHB and E2 + EHB/DE were 0.42 ± 0.02, 0.77 ± 0.02, 0.88 ± 0.02 and 0.35 ± 0.02 ng/mg Cr, respectively. Urine samples extracted using only the enzyme hydrolysis buffer (E2 + EHB) had consistently higher hormone concentrations compared to the other three treatments, with the double extraction method using diethyl ether (E2 + EHB/DE) having the lowest mean concentration of urinary oestrogens. However, despite the significant extraction treatment effects on oestrogen metabolite concentrations, when compared to changes in urinary P4M concentrations, there was no significant difference found in the pattern or magnitude of changes between each treatment group that could be associated with the follicular phase, oestrus or ovulation in this species (Fig. 5).

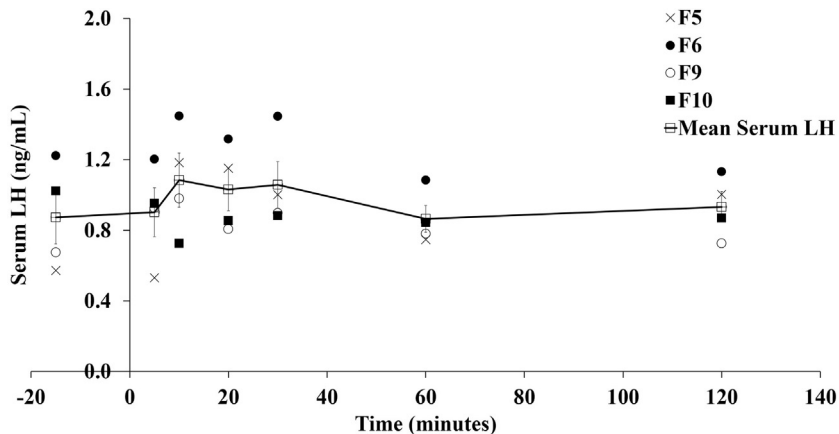


Fig. 2. GnRH challenge 1 (2013). Mean (± SEM) serum luteinizing hormone concentration (ng/mL) of serial blood collections from four captive female southern hairy-nosed wombats injected with 4 µg GnRH (Buserelin; Intervet).

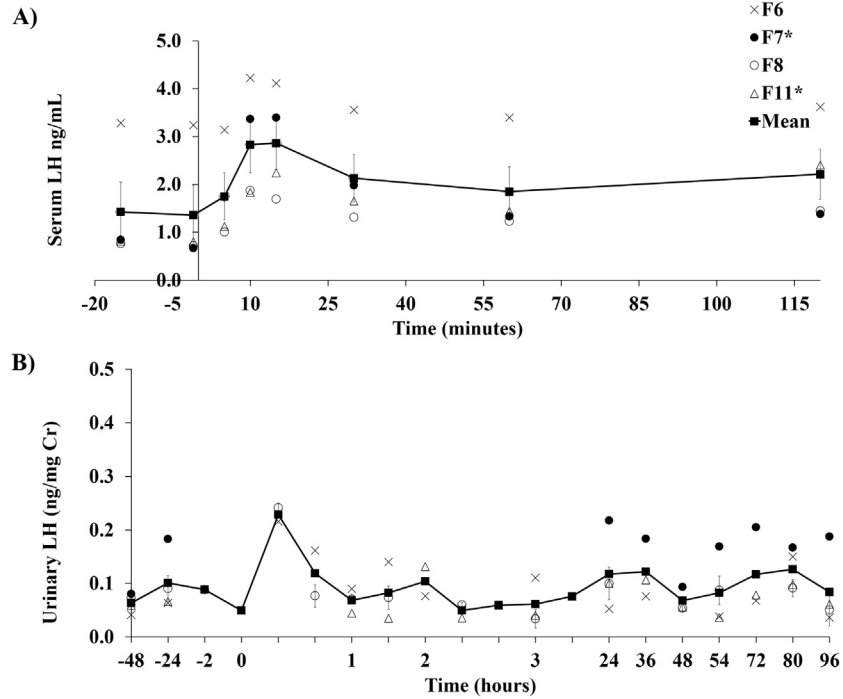


Fig. 3. GnRH challenge 2 (2014) from four captive female southern hairy-nosed wombats (SHNW) injected with 10 µg GnRHa (Buserelin; Intervet). *F7 and *F11 were administered with an additional 5 µg GnRHa 30 mins following first injection. A) Individual and mean serial serum luteinizing hormone (LH) concentrations 15 min prior and 120 min post GnRHa injection. B) Individual and mean serial urinary LH concentrations 48 h prior to 96 h following GnRHa injection. Please note, it was not possible to collect urine from F11 (open triangle) between T0 and T1h, or from F7 (closed circle) while under anaesthesia (T-5 to T120).

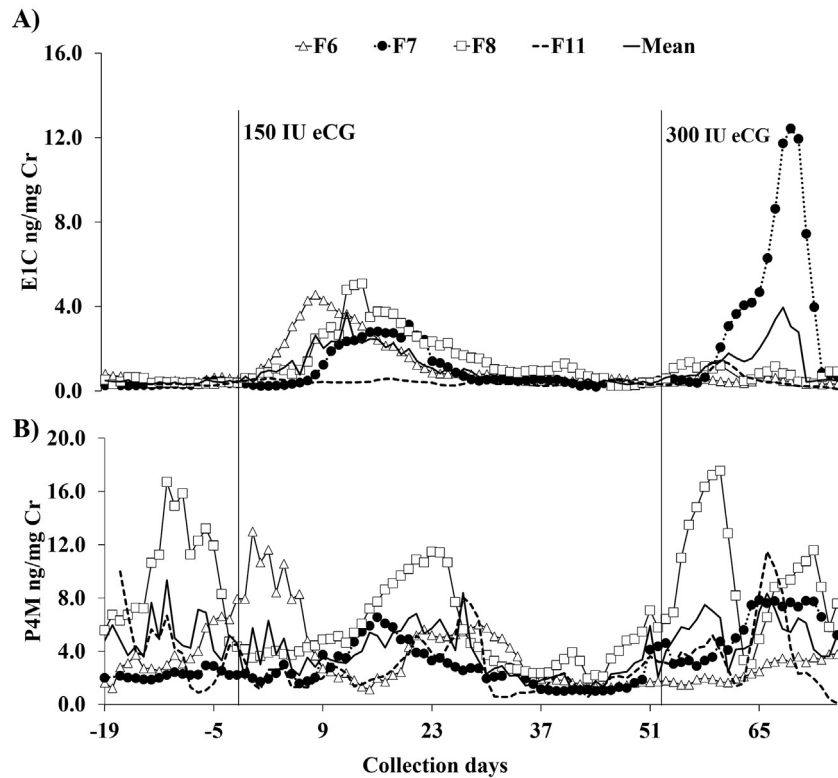


Fig. 4. Results of hormone analysis from daily urine samples collected from four captive female southern hairy-nosed wombats injected with equine chorionic gonadotrophin (eCG) during the 2014 wombat breeding season. Vertical lines indicate day of eCG injection: d0 = 150 IU; d53 = 300 IU. A) Urinary oestrogen metabolites (E1C). B) Urinary progesterone metabolite (P4M).

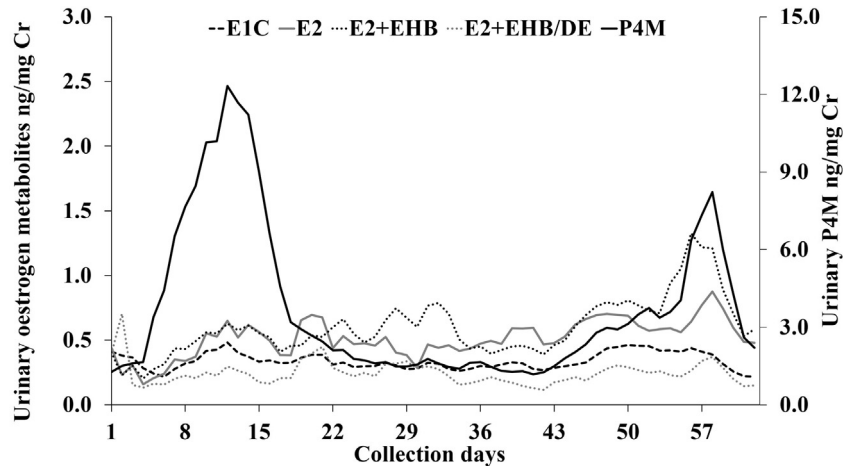


Fig. 5. The use of different oestrogen analysis techniques to characterise the female southern hairy-nosed wombat (SHNW) oestrous cycle. Urine samples were analysed for oestrogen metabolites (E1C), oestradiol-17 β (E2), and E2 after two different steroid extraction methods; enzyme hydrolysis buffer (E2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

3.4. The application of the urinary hormone EIAs during reproductive activity of two female wombats

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

Daily urinary LH concentrations for the pregnant female ranged from undetectable to 0.38 ng/mg Cr (Fig. 6). While there was a significant single day spike (>2 SD above baseline) in urinary LH two days following the last mating bout, the fluctuation of urinary LH surrounding mating was at a similar magnitude and concentration, so that a distinct pre-ovulatory LH surge could not be detected. E1C was significantly above baseline values for the first ten days of urine collection (during the luteal phase), returning to baseline values and remained relatively constant during oestrus, mating period and early gestation (collection days 11–42; Fig. 6). E1C then

increased significantly above baseline two days prior to partition and remained elevated throughout early lactation.

Urinary P4M was above baseline values for the first 17 days of urine collection, dropping below baseline from day 18–27 (Fig. 6). Mating bouts were observed from day 23 to day 25 of urine collection. A sustained increase in urinary P4M started two days following mating indicated the onset of a luteal phase. Urinary P4M peaked on day 46 (7.026 ng/mg Cr), then returned to baseline levels from day 54 of urine collection. Based on the recorded behavioural video, parturition was observed on day 46 of urine collection, suggesting an approximate 21-day gestation from the last mating bout.

Female SHNW, F10, was mated multiple times during the 2014 wombat breeding season, and urinary P4M profiles show three distinct luteal phases. Therefore, it was assumed that this female was cycling normally (Fig. 7). After bouts of mating and the detection of a retrograde seminal plug on the enclosure floor, the female was removed from the male and monitored for birth; however, no pouch young was ever discovered. Urinary LH showed similar patterns to F3, whereby LH was significantly increased sporadically above baseline levels before and after mating; however, a distinct pre-ovulatory LH surge was not detected in daily urine samples from F10 (Fig. 7). Similarly, there were no distinct differences in

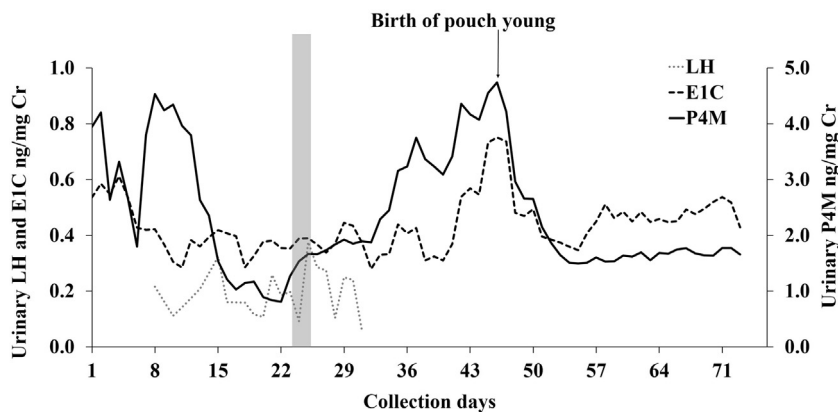


Fig. 6. Urinary reproductive hormone profile of a female southern hairy-nosed wombat (F3) prior to and including a successful mating and gestation. The grey box indicates a three-day mating period. Baseline urinary LH was 0.21 ± 0.02 ng/mg Cr and increased to 0.376 ng/mg Cr two days following the last bout of mating. Baseline E1C and P4M were 0.38 ± 0.03 and 1.72 ± 0.14 ng/mg Cr, respectively.

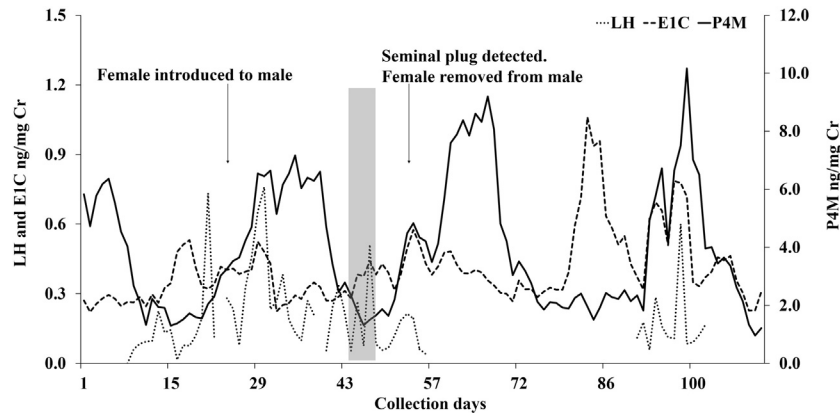


Fig. 7. Urinary reproductive hormone profile from a cycling female southern hairy-nosed wombat (F10) during the 2014 breeding season. The grey box indicates a four-day mating period. Baseline urinary LH was 0.09 ± 0.02 ng/mg Cr and peaked at 0.76 ng/mg Cr on day 30 of urine collection, during the mid-luteal phase of the first oestrous cycle. Baseline urinary E1C and P4M were 0.34 ± 0.06 and 2.26 ± 0.23 ng/mg Cr, respectively.

E1C profiles during the first two oestrous cycles as E1C fluctuated around baseline values from day 12 and 29, both prior to and after the onset of the luteal phase (day 21 of urine collection). While there was a distinct increase in E1C prior to the onset of the third luteal phase, E1C was also significantly increased throughout the duration of the third luteal phase (Fig. 7). Similar to F3, there were no consistent discernible changes in urinary E1C during the first two oestrous cycles for F9. While E1C increased significantly prior to the onset of the final luteal phase, it was significantly above baseline for 19 days (collection day 80–98), including six days into the luteal phase. As a result, the inconsistencies in the E1C concentrations could not provide valuable information regarding the fol-

licular phase, oestrus or ovulation for this female. Peak urinary P4M concentrations progressively increased for each subsequent luteal phase (Fig. 7). For example, during the first luteal phase, peak urinary P4M was 7.17 ng/mg Cr, 9.20 ng/mg Cr during the second luteal phase, and 10.16 ng/mg Cr during the third luteal phase.

There were individual differences between urine and faecal P4M concentrations and secretory patterns observed for F3 and F10 (Fig. 8). F3 had a lower peak P4M concentrations compared to F10 (peak urinary P4M: 5.31 versus 8.54 ng/mg Cr, respectively; peak faecal P4M: 159.18 versus 348.14 ng/g). With respect to P4M secretory patterns, the correlation coefficient between the

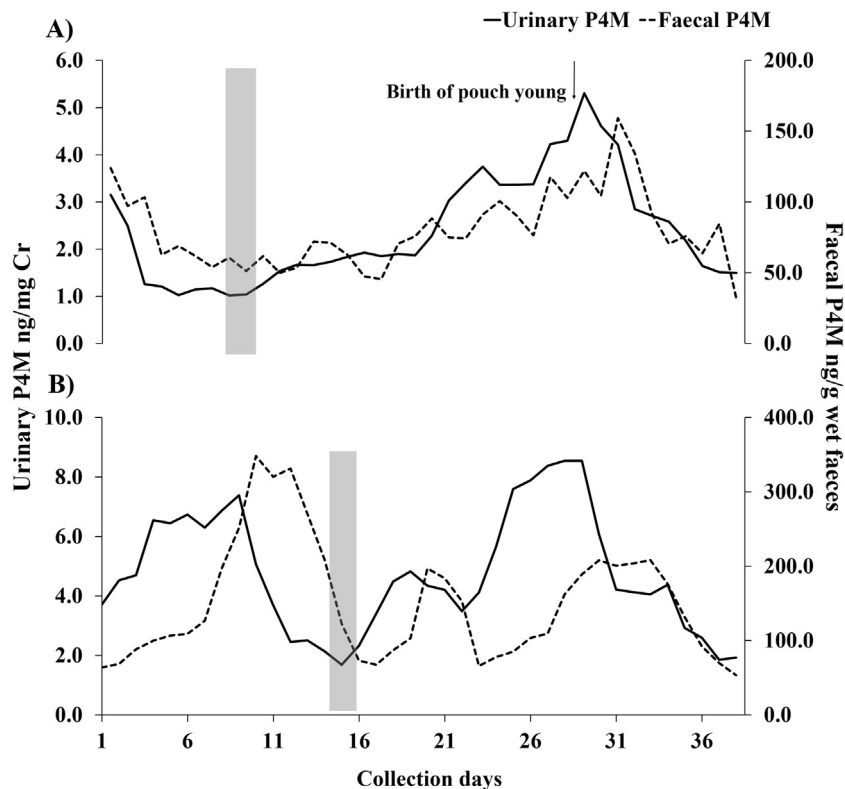


Fig. 8. Unadjusted urinary progesterone metabolite (P4M) profiles from matched non-invasive urine samples and uncontaminated faecal samples from two female southern hairy-nosed wombats at different stages of the reproductive cycle. The grey box indicates period of mating. A) P4M profile of a successful mating and gestation of female F3. B) P4M profile of a cycling female during the 2014 wombat breeding season of female F10.

unadjusted urinary and faecal P4M profiles for F3 was 0.52 ($P > 0.001$), which increased to a correlation coefficient of 0.80 ($P > 0.001$) when adjusted for the three-day faecal lag time. For F10, the correlation coefficient between the unadjusted urinary and faecal P4M was lower compared to F3; 0.40 ($P > 0.001$), and increased to 0.64 ($P > 0.001$) when adjusted for the faecal time lag. While both females were mated, only F3 produced a pouch young during the research period.

4. Discussion

This is the first study to evaluate and validate EIAs for the detection and analysis of urinary reproductive hormones in female SHNWs, as well as the first to report a biological challenge to detect luteinizing hormone excreted in marsupial urine. Despite positive detection of changes in urinary hormone patterns after exogenous hormone injections, a lack of detection of expected hormone of LH and oestrogen patterns in natural oestrous cycles suggest that further research is warranted to refine detection techniques or find more suitable hormone metabolite specific EIA systems.

Of the four published studies that have investigated reproductive steroid hormones in marsupial urine, two were validation studies associated with numbat urine, but reproductive hormone profiles were not assessed in detail (Ditcham et al., 2009; Matson et al., 2008). Urinary oestrogens were analysed in a single female koala (Takahashi et al., 2009), and changes in both urinary oestrogen and progesterone were detected in samples collected from two aged Tasmanian devils (Crichton et al., 2003). For all these studies, while validation of the assay was conducted through parallelism of the standard curve, limited biological validation was performed to determine if the immunoassay was appropriate for the hormone of interest or the species in question. For example, in the koala, changes in urinary reproductive hormones could not be correlated to changes in reproductive behaviour (Takahashi et al., 2009). This may be due to the female not provided with access to a male or similarly to the results of this study, the EIA used to measure urinary oestrogen may not have been appropriate for the matrix analysed – marsupial urine.

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

The 518-B7 antibody (Jan Roser, UC Davis, USA) was chosen for the LH urinary assay as it cross-reacts with circulating and urinary LH in a diverse range of mammalian species (Matteri et al., 1987), including the successful detection of serum LH surge in the Sumatran rhinoceros (*Dicerohinus sumatrensis*; Roth et al., 2001), Asian (*Elephas maximus*) and African elephants (*Loxodonta africana*; Brown et al., 2004; Brown et al., 2010; Czekala et al., 2003; Graham et al., 2002;) as well as the detection of urinary LH surges in species such as the bottlenose dolphin (*Tursiops truncatus*; Robeck et al., 2005), and beluga (*Delphinapterus leucas*; Steinman et al., 2012). It has also been used to detect natural and artificially

induced LH activity in marsupials, such as the koala (Ballantyne et al., 2016a, 2016b), tammar wallaby (*M. eugenii*) (Sutherland et al., 1980; Rudd et al., 1999), western grey kangaroo (*M. fuliginosus ocydromus*), black-flanked rock wallaby (*Petrogale lateralis lateralis*) (Matson et al., 2009), and the eastern grey kangaroo (Wilson et al., 2013), but it's potential to detect LH in marsupial urine had yet to be tested.

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

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

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

6 am (Crawford et al., 1999). If the SHNW is similar to the tammar wallaby and the brushtail possum, it is likely that the ovulatory LH surge could easily be missed if samples were collected only once per day or if the pooling of hormones within the urine masked the LH surge if urination was infrequent.

Additionally, during the second GnRH challenge, urinary LH more than doubled 30 min after GnRHa was administered, returning to around baseline levels between one to two hours post-injection. This suggests that the clearance rate of LH may be rapid in female SHNW and was only detectable during the challenge where urine collections were 15–60 min apart. A study conducted on male rats showed that labelled ovine LH had a half-life of 5 min in circulation, and degraded labelled LH was present in the tissue of the liver, testicle, kidneys and excreted in the urine 60–90 min after injection (Ascoli et al., 1975). The circulatory half-life of human LH occurs between 30 and 60 min in middle-aged women, and the LH found in the urine 24 h later was mostly degraded (Kohler et al., 1968).

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

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

The E1C antibody, R522-2, (Coralie Munro, UC Davis, USA) has been shown to be effective in the longitudinal reproductive assess-

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

A single extraction method with an enzyme hydrolysis buffer resulted in higher E2 concentrations (0.108–3.350 ng/mg Cr) compared to the double extraction with diethyl ether (0.023–1.309 ng/mg Cr), untreated E1C (0.139–0.957) and untreated E2 (0.034–1.998 ng/mg Cr) methods. However, while extraction with just the enzyme hydrolysis buffer appeared to be effective for E2 extraction, significant changes in urinary oestrogens over time were still not detectable in biologically relevant patterns during natural reproductive cycles. It may, therefore, be the case that oestrogens are heavily metabolised and/or are degraded during metabolism (Hogan et al., 2010a), and were only measurable from increased artificial stimulation. Once again, the use of a hormone tracer would enable the accurate mapping of the oestrogen metabolic and excretion pathway, which would enable the development of the most appropriate oestrogen metabolite antibody and analysis technique for SHNW urine.

The progesterone antibody, CL425, chosen for this study cross reacts with a number of progestogens and has previously been used for the detection of P4M in marsupial urine (Ditcham et al., 2009), and faeces (Ballantyne et al., 2009; Descovich et al., 2012; Hogan et al., 2012; Keeley et al., 2012; North and Harder, 2008; Pollock et al., 2010). The CL425 antibody has also been validated for SHNW faeces and was effective for the reproductive assessment of female wombats in captivity (Hogan et al., 2010a). The validation of the CL425 assay for SHNW urine was supported by the hormone profiles observed from the eCG treated females, the natural cycling females and the matched urine and faecal P4M profiles. The secretory patterns of the urinary P4M between the naturally cycling and hormone challenged females were consistent with progesterone profiles previously described in female SHNW (Finlayson et al., 2006; Hogan et al., 2010a; Paris et al., 2002), showing distinct and regular progesterone cycles throughout the wombat breeding season. Additionally, while there were individual differences regarding excretion lag time in urinary versus faecal P4M between F3 and F10, changes in urinary P4M were reflected in faecal P4M profiles two to three days later.

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

5. Conclusion

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

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

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