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Measurement of testosterone and cortisol metabolites and luteinising hormone in captive southern hairy-nosed wombat (*Lasiorhinus latifrons*) urine

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Highlights

- This study validated an urinary testosterone and cortisol EIA for southern hairy-nosed wombats.
- Urinary LH changes in male SHNWs could not be detected by the LH EIA.
- Sex-related differences in cortisol secretion were found in captive SHNWs.

Abstract

This study reports the validation and use of enzyme immunoassays (EIA) to measure changes in plasma and urinary luteinizing hormone, testosterone metabolites (UTM) and cortisol metabolites (UCM) in captive southern hairy-nosed wombats (*Lasiorhinus latifrons*). GnRH agonist and ACTH agonist challenges were conducted to validate urinary testosterone (male wombat only) and cortisol (male and female wombats) EIAs. Following intra-muscular injection of 8–12 μg buserelin (n = 4 males), there was a significant increase in both plasma ($P < 0.001$) and urinary testosterone concentrations ($P < 0.001$) 60 min and 21 h after administration, respectively. Plasma LH levels were

elevated ($p < 0.05$) at 20 min but there was no significant increase found in urinary LH concentrations after injection. Intra-muscular injection of Synacthen® Depot (250 µg) ($n = 3$ males, 3 females) resulted in a significant increase ($p < 0.05$) in plasma cortisol secretion 15 min and in urinary cortisol concentrations 3 h post injection, respectively. Sex-related differences in cortisol secretion were also reported in this study. These findings indicate that (1) urinary LH might not be an appropriate index for describing the reproductive status in captive male *L. latifrons*, and (2) the UTM and UCM assays appear to be suitable for the assessment of the testicular steroidogenic capacity and the adrenocortical activity in captive southern hairy-nosed wombats, respectively.

Keywords

GnRH agonist challenge; ACTH agonist challenge; Luteinizing hormone; Testosterone; Cortisol; Enzyme immunoassay

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1 Introduction

In male mammals, episodic release of gonadotrophin-releasing hormone (GnRH) from the hypothalamus results in the corresponding pulsatile secretion of luteinizing hormone (LH) in the pituitary, leading to a similar pattern of testosterone (T) secretion produced in the Leydig cells of the testis (Clarke and Cummins, 1982, Levine et al., 1982 and Caraty and Locatelli, 1988). The hypothalamic-pituitary-gonadal (HPG) axis acts as a negative feedback system for the regulation of male reproductive hormones (Bryant, 1992). In a similar physiological negative feedback system, the hypothalamic–pituitary–adrenal (HPA) axis, corticotrophin-releasing factor (CRF) secreted from the hypothalamus, stimulates the release of adrenocorticotrophic hormone (ACTH) in the anterior pituitary, which further results in the release of glucocorticoids (GC) from the adrenal cortex (Moberg and Mench, 2000, Stewart, 2000, Möstl and Palme, 2002 and Young et al., 2004). GC influences almost all biological processes in mammals, and primarily includes cortisol and corticosterone (McLaren et al., 2007). Altering cortisol secretion can enable the animal to respond appropriately to an acute stressor (e.g. presence of predator) by modulating its underlying metabolism (Wikelski and Cooke, 2006).

Hormones can be detected in a range of biological products such as blood, urine, faeces, saliva, milk and hair (Hodges et al., 2010 and Kersey and Dehnhard, 2014). Circulating hormones typically provide accurate and immediate endocrine information

on an experimental animal, so that analysis of hormone concentration in plasma or serum is often the most reliable method for studying physiological mechanisms in both domestic and laboratory animals (Lasley and Savage, 2007). Nevertheless, repeated blood collection is often associated with confinement, capture and sedation, which can elicit a stress response in animals. This phenomenon can lead to confounding experimental variables that mask or interfere with the very hormone profiles investigators are attempting to document (Reeder and Kramer, 2005). Additionally, regular blood sampling is impractical for field studies especially for studying wild species (Brown et al., 2010 and Kersey and Dehnhard, 2014). Consequently, non-invasive methods are more suitable for studying longitudinal physiology. A non-invasive endocrine method relies on measuring hormones in biological products other than blood such as urine, faeces, hair and saliva, among which urinary and faecal analysis are most widely used (Schwarzenberger, 2007, Heistermann, 2010 and Kersey and Dehnhard, 2014). Urine, is an ideal sample for studying animal physiology, as it not only contains protein (e.g. LH) and steroid hormones (estrogen, androgen and glucocorticoids) (Heistermann, 2010) but is also continuously produced, making it relatively easy to collect (Lasley and Savage, 2007). Swinbourne et al. (2015a) have recently described the non-invasive collection of urine from the captive southern-hairy nosed wombat by means of operant conditioning.

However, before a non-invasive technique can be applied to studying physiology in a specific species, a rigorous biological validation is required and once validated, this technique will become an effective tool for helping investigate the biology of this species both in the wild and in captivity (Schwarzenberger, 2007 and Kersey and Dehnhard, 2014). For physiological validation, a GnRH/ACTH challenge is a common method for confirming whether the technique is appropriate for detecting respective changes in LH, T and GC (e.g. Phillips et al., 2008 and Keeley et al., 2012). Moreover, the lag time of metabolism in a specific species (time between detected hormone change in plasma and its first appearance in excreta) can also be established by means of a hormone challenge (Fanson et al., 2015).

The southern hairy-nosed wombat (SHNW – *Lasiorhinus latifrons*) is a large herbivorous marsupial which is nocturnal, burrowing and endemic to Australia (Gaughwin et al., 1998). Currently, the species is classified as ‘near threatened’ by the International Union for Conservation of Nature and Natural Resources (IUCN; Woinarski and Burbidge, 2016) and the total free range population of SHNW has been predicted

to decline owing to habitat reduction (Alpers, 1998), threats from predators (e.g. dingoes; Wells, 1989), disease (e.g. sarcoptic mange; Ruykys et al., 2009 and Sparrow, 2009), increased road accidents (Ramp et al., 2005) and climate change (Finlayson et al., 2005 and Kellermann et al., 2009). Southern hairy-nosed wombats have been kept in captivity since the 1970s (Jackson, 2003 and Hogan et al., 2013) and several captive breeding programs have already been established in zoos (e.g. Rockhampton Zoo) and purpose built research facilities (e.g. Australian Animal Care and Education; Hogan et al., 2010 and Swinbourne et al., 2015a). However, captive breeding success to date has been limited for this species and the *ex situ* population of *L. latifrons* in Australia has been regarded as unsustainable (Hogan et al., 2010 and Hogan et al., 2013). The lack of basic knowledge (i.e. behavior and physiology) of this species is likely to be a major contributor for the unsuccessful breeding in captivity (Hogan et al., 2013). Due to the nocturnal, burrowing and cryptic nature of *L. latifrons*, it is extremely challenging to monitor their reproduction in captivity (Paris et al., 2002). Non-invasive techniques for studying their basic reproductive knowledge in captivity have been shown to be beneficial but further research in this area is urgently required (Paris et al., 2002 and Hogan et al., 2013). With more information available regarding the endocrinology of this species, not only will poor breeding success in captivity be improved, but the relevant knowledge and techniques may also be further applied to the reproduction and genetic management of their critically endangered cousin, the northern hairy-nosed wombat (*L. krefftii*; Paris et al., 2002 and Hogan et al., 2013).

The current study focused on establishing a non-invasive method of hormone analysis that could be applied to investigate both reproductive and stress biology in captive southern hairy-nosed wombats. To achieve this, we conducted separate GnRH agonist (GnRHa) and ACTH agonist (ACTHa) challenges in a captive population and then measured GnRHa-stimulated LH and testosterone changes in plasma and urine as well as ACTHa-induced cortisol changes in plasma and urine, respectively. The aims of the present study were to (1) evaluate the use of urinary LH, testosterone and cortisol to detect responses to the corresponding exogenous hormone challenge, (2) validate the utility of enzyme immunoassay (EIA) for measuring urinary LH, testosterone and cortisol and (3) estimate the lag time of reproductive and stress hormone metabolism in captive *L. latifrons*.

2 Materials and methods

2.1 Animals and study site

Four adult male SHNWs (M1, M3, M4 and M6) were used for the GnRH agonist challenge (February 2016) while three adult females (F1, F2 and F3) and three adult males (M1, M3 and M4) were used for the ACTH agonist challenge (August 2016). M1 was a sexually mature vasectomized male (8 years of age) while the other animals were sexually mature and intact (6–9 years of age). All wombats were clinically healthy throughout the course of the corresponding hormone challenge and were housed in two housing structures located in the Safe Haven Wombat breeding facility – Australian Animal Care and Education (AACE) located at Mount Larcom, Queensland (23.75° S, 151.00° E). Each housing structure was internally air-conditioned (23 °C) and included eight internal enclosures each connecting to a respective outside yard. In each enclosure, wombats were allocated into either pairs (one male and one female) or an individual living chamber but with each animal having its own sleeping den. Each enclosure was connected to a fenced outside yard which contained soil substrate and native grass. Each wombat was fed daily with a mixture of sliced sweet potatoes, rolled oats, oaten chaff and horse pellets; half a corn cob was provided when available and water was supplied *ad libitum*. This study was approved by the University of Queensland's Animal Ethics Committee (Approval Number: SAFS/333/15).

2.2 Anaesthesia

Prior to anaesthesia, wombats received an intramuscular injection of Zoletil (10 mg/kg; VIRBAC, Australia) using a 21 gauge needle. Following recumbency, animals were maintained under anaesthesia by mask on a surgery table with 1–5% isoflurane (Abbott Australasia Pty Ltd, Australia) at a flow rate of 1.5 L/min oxygen (BOC Health Care, England). Following anaesthesia and recovery, wombats were placed back into their den and monitored once an hour until they had fully recovered; all anaesthesia was conducted without incident.

2.3 Experiment 1: GnRH agonist stimulation test

Based on the results of a parallel study on the use of a GnRH agonist challenge on female SHNWs, higher doses of exogenous hormone may be required to stimulate sufficient measurable quantities of LH from the anterior pituitary (Swinbourne et al., 2015b) than those reported for other marsupial species such as the koala (Allen et al., 2008). Therefore, in the current study when under general anaesthesia, M1 and M6 (each body weight <30 kg) were intramuscularly injected with 8 μg GnRH agonist buserelin (Receptal, Intervet, Australia) while M3 and M4 (each body weight >40 kg) received 12 μg buserelin, respectively; this corresponded to dose of approximately

0.27–0.30 μg per kilogram of body weight. Baseline blood samples were taken at 15 and 5 min prior to the GnRHa injection but after anaesthesia and 5, 10, 20, 30, 40, 50, 60, 80, 100 and 120 min post injection. While these wombats were under anaesthesia, urine samples were collected at 0, 15, 30, 60, 90, 120 and 180 min post GnRHa injection. Urine samples were collected approximately 48 h, 24 h and 16 h prior and up to 4 days after the GnRHa challenge using classical conditioning, the procedure for which has been described for captive female *L. latifrons* by Swinbourne et al. (2015a).

2.4 Experiment 2: ACTH agonist stimulation test

While the wombats were under general anaesthesia, each wombat received 250 μg ACTH agonist Synacthen® Depot (Novartis®, Australia) by intramuscular injection. Blood samples were taken at −15, 0, 15, 30, 45, 60, 75, 90, 105 and 120 min with respect to the ACTHa injection. Urine samples were collected from the anesthetised wombats at 0, 30, 60, 90, 120, 150 and 180 min following the ACTHa injection. Urine samples were collected by classical conditioning (Swinbourne et al., 2015a) approximately 48 h and 24 h prior and up to 3 days post the ACTHa challenge.

2.5 Sample collection and storage

2.5.1 Plasma

Blood samples (1 mL) were collected by venipuncture from the cephalic vein using a 23 gauge wing-infuser set (TERUMO, Australia) with a 3 mL syringe. Each sample was transferred into a 1.3 mL lithium-heparin blood tube (Sarstedt, Provet, Australia) immediately after collection and then stored on ice. For the GnRH agonist challenge, once the last sample was collected, all blood tubes were centrifuged at 1800 rpm for 10 min. For the ACTH agonist challenge, hemolysis occurred in all three female blood samples following centrifugation; thereafter static settlement of blood cells (≥30 min) was used instead of centrifugation. Following centrifugation/static settlement, the plasma supernatant was removed from blood sample by a 1 mL pipette and allocated into 2 × 1.5 mL Eppendorf tubes (Eppendorf, Provet, Australia). After labelling, all tubes were maintained at −20 °C until hormone analysis.

2.5.2 Urine

While urine samples were collected by classical conditioning (Swinbourne et al., 2015a) before and after anaesthesia, some modifications were required for the collection of male urine; the primary difference was that urine could not be directly collected under a male by means of holding a collection pan underneath the cloacal area, as males are typically more aggressive. Consequently, the urine sample was recovered directly from the clean floor using a 10 mL syringe (BD, Australia). Given that all the experimental wombats had been conditioned to human approach, a period of two weeks proved sufficient for the training of these animals to urinate by classical conditioning (Z. Du, pers. comm.). Using classical conditioning methods, male wombats were trained to urinate in a common area within their enclosure. Following urination, these males were then locked into their separate sleeping dens and the urine was collected from the floor – the floor was mopped clean with only tap water (Heistermann, 2010) and dried before each urine collection. After urine collection, each sample was transferred into a clean 50 mL specimen container and maintained on ice. Thereafter, 4 mL of urine was pipetted into 2 × 2 mL ProSciTech (PST, Australia) tubes and stored at −20 °C. When wombats were under anaesthesia, urine samples were collected directly from the urogenital sinus (females) or penis (males) by placing gentle pressure on the bladder.

2.6 Enzyme immunoassay (EIA)

2.6.1 LH assay – plasma and urine

LH concentration was determined by enzyme immunoassay (EIA) based on the protocols described by Wilson et al. (2013) using a monoclonal anti-bovine LH antibody 518-B7 (1:400, 000; provided by Jan Roser, University of California, Davis, USA). All samples were analyzed in duplicate and the EIA was validated by demonstrating parallelism between serially diluted pooled samples (plasma or urine) and the standard curve. Dilution rates were determined according to pooled samples that resulted in 50% binding on the parallelism curve, which was 1:2 for plasma LH and 1:1 (neat) for urinary LH. The detection limit of the assay was 0.156 ng/mL. The intra- and inter-assay coefficients of variation (CV) for the LH assay were <10% and <15%, respectively. Hormone concentration in plasma was expressed as ng per mL of plasma.

2.6.2 Testosterone assay – plasma and urine

Plasma testosterone and urinary testosterone metabolites (UTM) were measured by

testosterone EIA according to Hogan et al. (2010) with minor modifications, which has previously been validated for measuring faecal testosterone metabolites in captive SHNWs. The assay used a goat *anti*-rabbit IgG immobilizing antibody (Arbor Assays®, USA), a polyclonal anti-testosterone antibody R156/17 (1:120, 000; supplied by C. Munro, University of California, Davis, USA) and a testosterone horseradish peroxidase (HRP) conjugated label (1:400, 000; C. Munro, UC Davis, USA). Major cross-reactivities (>5%) for the antibody were testosterone (100%) and 5α-dihydrotestosterone (29%). Parallelism between serially diluted pooled samples (plasma or urine) and the standard curve was demonstrated. Dilution rates for plasma and urine were based on pooled samples that resulted in 50% binding on the parallelism curve. The dilution of plasma samples was 1:8 for all animals. Urine samples were run at 1:12 for M4 and 1:4 for the others (i.e. M1, M3 and M6). All samples were analyzed in duplicate and the assay sensitivity was 0.039 ng/mL. The intra- and inter-assay CV for testosterone assay were 3.1% and 7.5%, respectively.

2.6.3 Cortisol assay – plasma and urine

Cortisol analyses were conducted using a modified cortisol EIA based on Arbor Assay Mini-Kit ISWE002 (Ann Arbor®, Michigan, USA). The assay recruited a polyclonal anti-rabbit cortisol antibody #C208 (1:50), a cortisol-HRP label #C209 (1:50) and cortisol standards (#C210), which were provided by the manufacturer while the other materials were in-house prepared (e.g. plates coated with anti-rabbit IgG secondary antibody and assay buffer, etc.). In brief, 50 µL of standards (0.05–3.2 ng/mL), high and low controls and diluted samples were dispensed in duplicate into a 96-well microtiter plate (Costar Assay Plates – Corning Inc., USA) coated with goat anti-rabbit IgG. Then 50 µL of cortisol-HRP and 50 µL of cortisol antibody were added into each well. Following a 2-h incubation with shaking at room temperature, 3,3′,5,5′-tetramentylbenzidine (TMB) substrate solution was added for colour reaction. The colour reaction was then stopped by dispensing 50 µL of 4 M H2SO4 into each well. Plates were read at 450 nm (reference filter: 630 nm) on a Biotek microplate reader (Elx808) with Gen5 software (Biotek, USA). Cross-reactivities for the antibody were reported as: cortisol (100%), dehydrocortisol (42.08%), cortisone (26.53%), dexamethasone (4.10%), prednisone (3.37%), corticosterone (0.35%), desoxycorticosterone (0.18%) and tetrahydrocorticosterone (<0.16%). Assay validation was achieved by demonstrating parallelism between serially diluted pooled samples (plasma or urine) and the standard curve. Dilution rates for plasma and urine

were identified based on pooled samples that resulted in 50% binding on the parallelism curve. Plasma samples were run at 1:30 for F2 and 1:20 for the others (i.e. M1, M3, M4, F1 and F3). Urine samples were run at 1:2 for M1, 1:3 for M3, 1:8 for M4, 1:10 for F1 and F2, and 1:5 for F3. The assay sensitivity was 0.05 ng/mL while the intra- and inter-assay CV were 2.7% and 8.7%, respectively.

2.7 Creatinine assay

Creatinine (Cr) is a byproduct of muscle activity and is excreted in urine at a constant rate (Brown, 2008). Therefore, measuring creatinine levels in urine can be used to correct the concentration of urinary hormone metabolites. In the current study, all urinary hormone levels were standardized by the corresponding creatinine concentration and expressed as ng/mg Cr. Urinary creatinine was measured by using a creatinine assay (Cayman Chemicals, USA) based on the reaction with sodium hydroxide and picric acid (Taussky, 1954). In brief, 100 µL of creatinine standards (0.0005–0.03 ng/mL), high and low controls and diluted samples were dispensed in duplicate into a 96-well microtiter plate (Costar Assay Plates, USA). Thereafter, 50 µL of 0.75 M NaOH and 50 µL of 0.04 M picric acid were added into each well. Plates were incubated for 5–10 min at room temperature and then read at 490 nm (reference 650 nm) on a Biotek microplate reader (Elx808). Assay data were analyzed using Gen5 software (Biotek, USA).

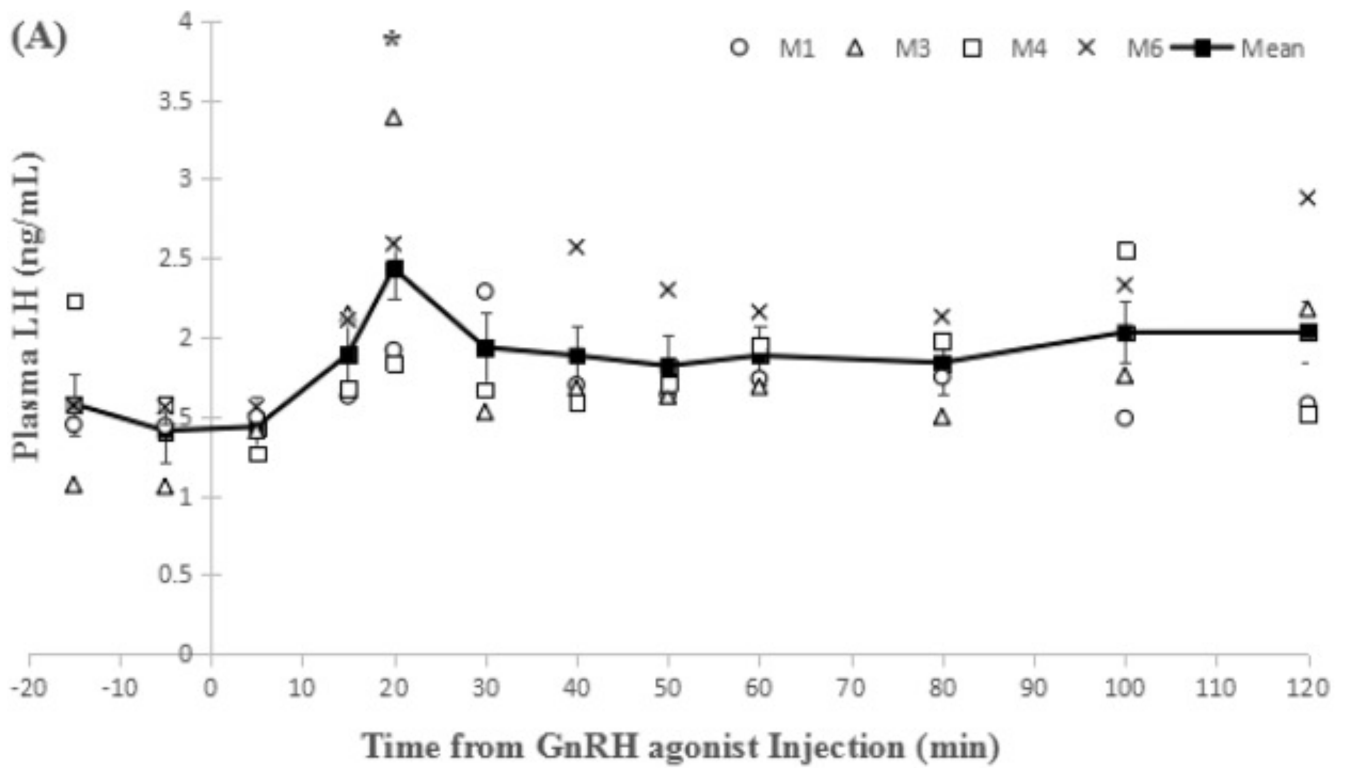
2.8 Statistical analysis

Statistical analysis was performed with Minitab (Version 17.3, 2013) using Dunnett's multiple comparison tests following a General Linear Model (GLM) ANOVA. Significance levels were set at $p < 0.05$ for all tests and means were reported with standard errors (SE). For the GnRH agonist challenge, plasma LH, urinary LH, plasma testosterone and UTM levels were treated as the response variable with animal ID and sample time as the fixed factors, respectively. For the ACTH agonist challenge, plasma cortisol and urinary cortisol metabolite (UCM) levels were set as the response variable with sex, animal ID and sample time as the fixed factors and sex X sample time interaction terms, respectively. Dunnett's multiple comparison tests were used for post hoc analysis to compare the differences between baseline (T-5 min for GnRHa challenge and T0 for ACTHa challenge) and all other times.

3 Results

3.1 Experiment 1: GnRHa challenge

Changes in plasma LH concentrations (ng/mL) in four male SHNWs following the GnRHa challenge are shown in Fig. 1A. The results from the GLM ANOVA demonstrated significant changes in mean plasma LH levels under the influence of both subject (i.e. significant differences in overall mean hormone values among experimental animals; $F_{3,32} = 3.58$, $p < 0.05$) and the time of sample collection ($F_{11,32} = 2.13$, $P < 0.05$). Post hoc analyses revealed that only the mean LH concentration 20 min after the GnRHa injection (2.435 ± 0.192 ng/mL) was significantly higher (Dunnett's Multiple Comparison tests, T = 3.78, $P < 0.01$, n = 4) than mean pre-stimulation values (T-5 min – 1.410 ± 0.192 ng/mL). Urinary LH concentrations of three male wombats in relation to the GnRH agonist administration are shown in Fig. 1B. It was not possible to detect any changes in urinary LH levels in M6, as nearly all sample values were below the assay sensitivity. The results from the GLM ANOVA showed that urinary LH levels were significantly affected by subject ($F_{2,28} = 7.19$, $P < 0.01$) but not by sample time ($F_{14,28} = 0.90$, $P = 0.569$). Therefore, no further post hoc analysis was conducted.



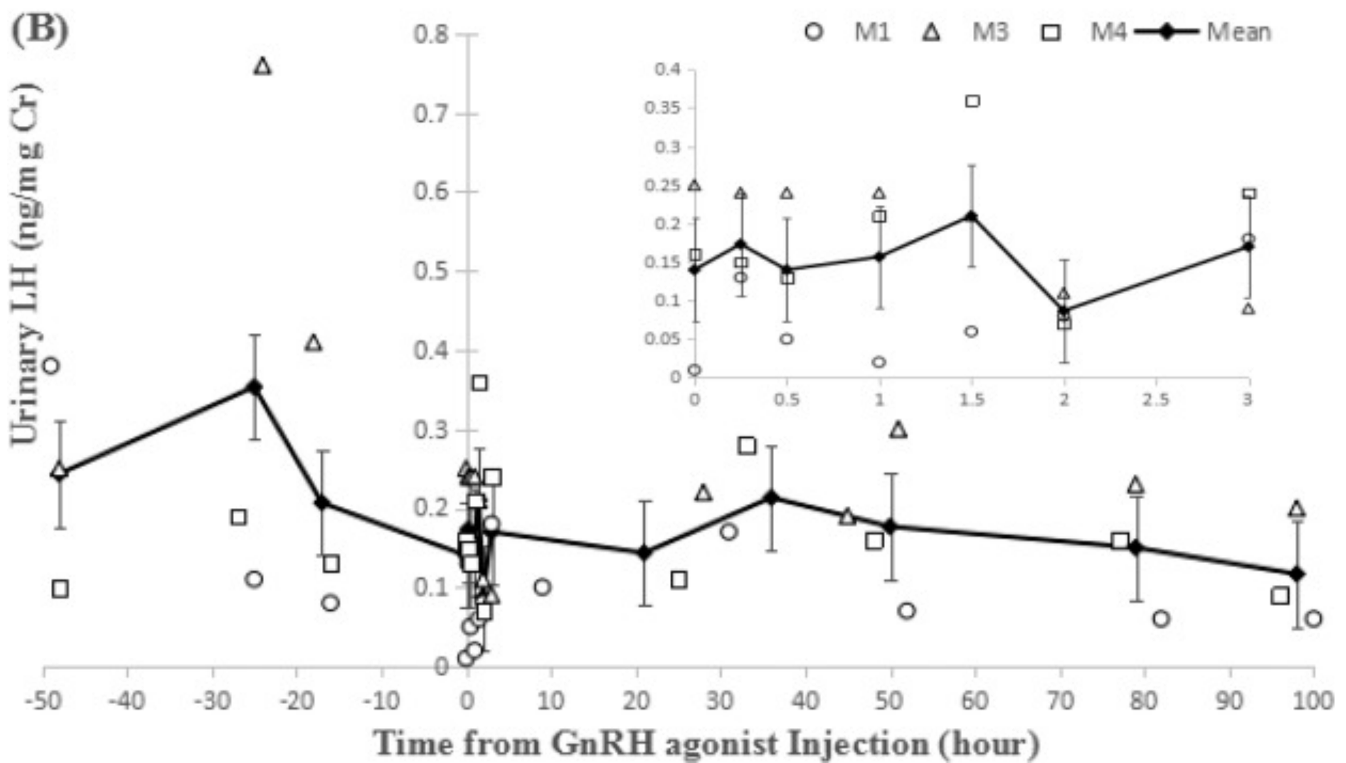


Fig. 1. Mean ± SEM and individual male wombat changes in (A) plasma (n = 4) and (B) urinary LH (n = 3) concentration following a GnRH agonist challenge (T0). Urinary LH results from M6 are not shown due to undetectable levels of LH. *Indicates a statistically significant increase in plasma LH levels. The inset within (B) illustrates Mean ± SEM and individual male wombat changes in urinary LH (n = 3) concentration from 0 to 3 h in relation to the GnRH agonist challenge (T0).

Changes in plasma testosterone concentrations (ng/mL) in four male SHNWs following injection of the GnRH agonist are shown in Fig. 2A. According to the results from GLM ANOVA, there was a significant effect of sample time on plasma testosterone levels ($F_{11,32} = 6.20$, $P < 0.001$) but the subject effect was not significant ($F_{3,32} = 2.08$, $P = 0.122$). Post hoc tests revealed that compared to mean pre-injection values (T-5 min – 2.277 ± 0.822 ng/mL), administration of the GnRH agonist induced a significant rise in the mean plasma testosterone concentrations 60 min, 80 min, 100 min and 120 min after injection (Dunnett's Multiple Comparison tests, $T \geq 3.24$, $P < 0.05$, $n = 4$) with maximum secretion occurring 120 min after injection (7.390 ± 0.822 ng/mL). Urinary testosterone metabolite concentrations of four male wombats in relation to the injection of the GnRH agonist are shown in Fig. 2B. The results from GLM ANOVA indicated that there were significant effects of both subject ($F_{3,40} = 3.88$, $p = 0.016$) and time of sample collection ($F_{14,40} = 5.07$, $p < 0.001$) on UTM levels. Post hoc tests revealed that the GnRH agonist injection resulted in a significant increase (Dunnett's Multiple Comparison tests, $T \geq 4.34$, $P < 0.01$, $n = 4$) in the mean UTM concentrations 21 (1.258 ± 0.112 ng/mg Cr) and 50 h (1.210 ± 0.112 ng/mg Cr) after injection compared to mean pre-stimulation values (T0 – 0.520 ± 0.112 ng/mg Cr). However, mean UTM concentrations appeared to decrease between 21 and 36 h after the challenge and the mean concentration of UTM 36 h after the injection (0.967 ± 0.131 ng/mg Cr) was not significantly higher than the pre-injection value ($T = 2.59$, $P = 0.114$, $n = 3$). Within 79 h post-stimulation, mean UTM concentrations returned to pre-injection levels.

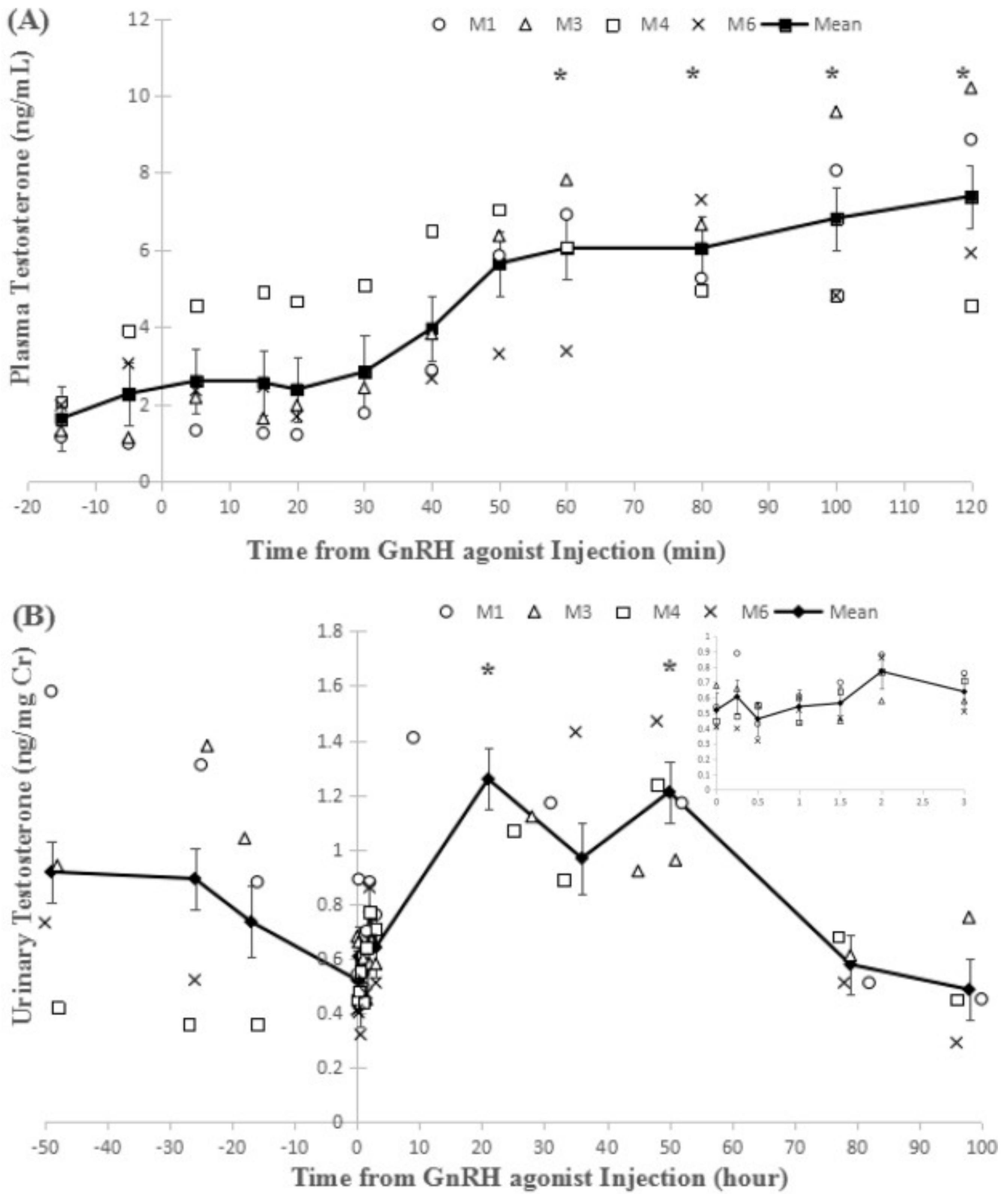
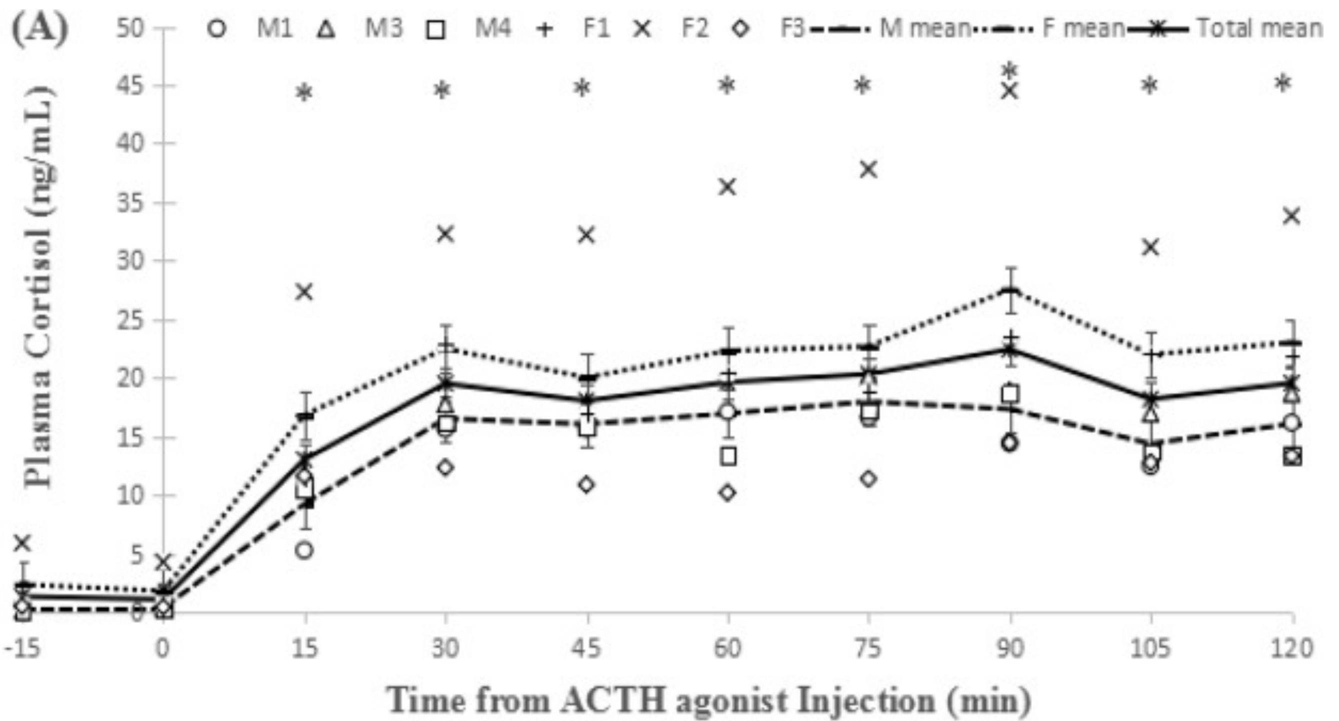


Fig. 2. Mean ± SEM (n = 4) and individual male wombat changes in (A) plasma and (B) urinary testosterone concentration following a GnRH agonist challenge (T0); *Indicates a statistically significant increase in plasma and urinary testosterone levels. The inset within (B) illustrates Mean ± SEM (n = 4) and individual male wombat changes in urinary testosterone concentration from 0 to 3 h in relation to the GnRH agonist challenge (T0).

3.2 Experiment 2: ACTHa challenge

Changes in plasma cortisol concentrations (ng/mL) in three male and three female SHNWs following the ACTH agonist challenge are shown in Fig. 3A. Hemolysis appeared in plasma samples from three females but did not appear to affect the cortisol measurement using EIA. The results from GLM ANOVA revealed that there was a significant effect of sex ($F_{1,36} = 39.11$, $P < 0.001$), sample time ($F_{9,36} = 30.60$, $P < 0.001$) and subject ($F_{4,36} = 39.18$, $P < 0.001$) on plasma cortisol levels, respectively. However, there was no significant relationship between sex and sample time for plasma cortisol levels ($F_{9,36} = 0.90$, $P = 0.536$), which suggests that the sex difference remained relatively constant over time (i.e. female mean plasma cortisol levels were significantly higher than males). In detail, mean pre-injection plasma cortisol values were 0.307 ± 0.070 ng/mL for males and 2.055 ± 0.977 ng/mL for females; mean post-injection plasma cortisol values were 15.54 ± 0.682 ng/mL for males and 22.09 ± 2.073 ng/mL for females. Consequently, the mean (±SEM) values of all six animals were used for post hoc analyses instead of testing for males and females separately. The results from post hoc analysis revealed that compared to the mean pre-stimulation values (T0; 1.06 ± 1.41 ng/mL), the injection of Synacthen® Depot induced a significant increase in the mean plasma cortisol concentrations from 15 min to 120 min after injection (Dunnett's Multiple Comparison tests, $T \geq 5.98$, $P < 0.001$, $n = 6$) with maximum secretion occurring 90 min after injection (22.42 ± 1.41 ng/mL). Urinary cortisol metabolite concentrations of six wombats in relation to the administration of Synacthen® Depot are shown in Fig. 3B. The results from GLM ANOVA demonstrated that there were significant effects of sex ($F_{1,43} = 11.80$, $P < 0.01$), subject ($F_{4,43} = 4.00$, $p < 0.01$) and time of sample collection ($F_{11,43} = 4.06$, $p < 0.001$) on UCM levels, respectively. However, the effects of the interaction between sex and sample time on UCM concentrations were not significant ($F_{11,43} = 1.60$, $P = 0.132$); hence sex difference remained relatively constant over time (i.e. female mean UCM levels were significantly higher than males). In detail, mean pre-injection UCM levels were 0.714 ± 0.086 ng/mL Cr for males and 1.044 ± 0.122 ng/mL Cr for females while mean post-injection UCM levels were 0.873 ± 0.095 ng/mL Cr for males and 1.634 ± 0.307 ng/mL Cr for females; mean peak values at T3 h for males were 1.580 ± 0.686 ng/mL Cr and were 4.340 ± 1.670 ng/mL Cr for females. Consequently, the mean (±SEM) values of all six animals were tested for post hoc analysis. Post hoc analyses revealed that only the mean UCM concentration 3 h after the ACTHa injection (2.960 ± 0.324 ng/mg Cr) was significantly higher (Dunnett's Multiple Comparison tests, $T = 4.74$, $P < 0.001$, $n = 6$) than the mean pre-injection values (T0; 0.787 ± 0.324 ng/mg Cr). There were large

variations among animals in the UCM concentration at T3 h, with F2 having a much higher UCM level (7.68 ng/mg Cr) than the other wombats (M1: 2.82 ng/mg Cr; M3: 1.47 ng/mg Cr; M4: 0.45 ng/mg Cr; F1: 2.65 ng/mg Cr; F3: 2.69 ng/mg Cr). Within 27 h post-injection, mean UCM concentrations were comparable to pre-stimulation levels.



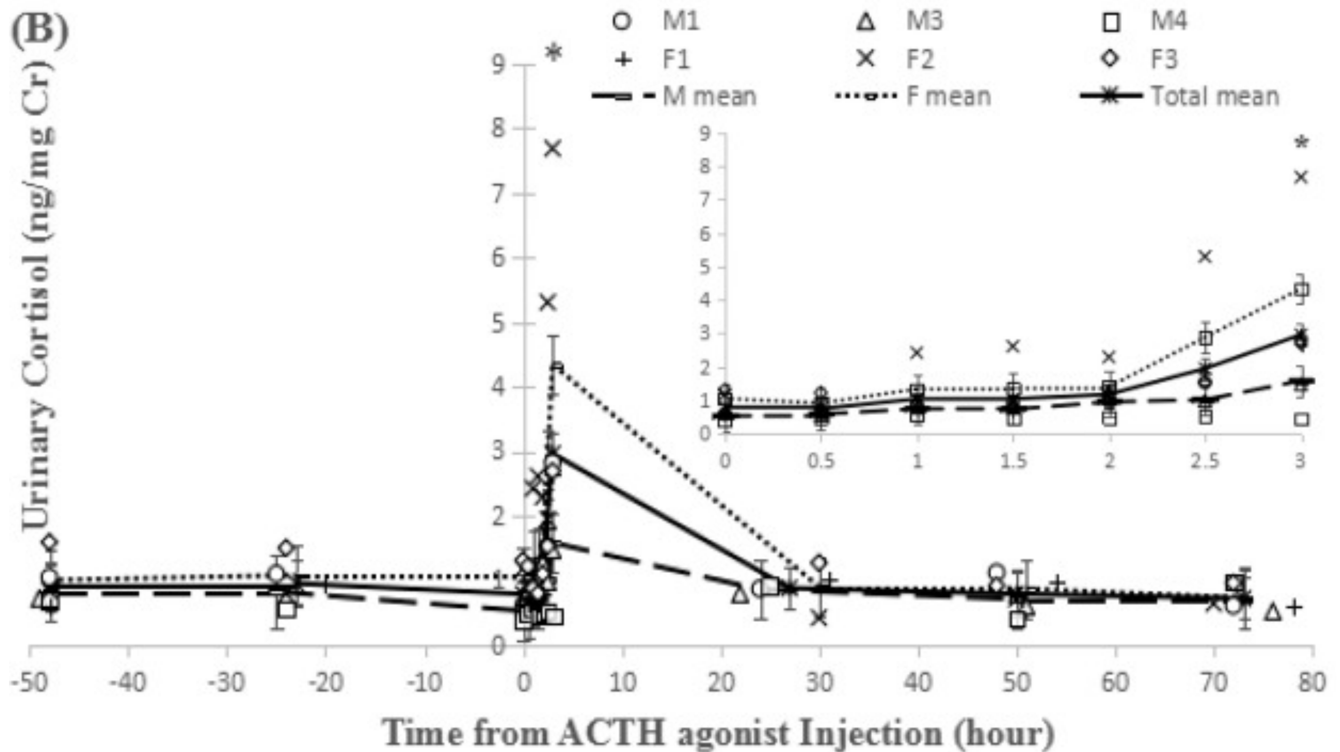


Fig. 3. Mean ± SEM and individual wombat (male – n = 3; female – n = 3) changes in (A) plasma and (B) urinary cortisol concentration following an ACTH agonist challenge (T0). *Indicates a statistically significant increase in total mean (i.e. The mean values of all six animals) plasma and urinary cortisol levels. The inset within (B) illustrates Mean ± SEM and individual wombat (male–n = 3; female–n = 3) changes in urinary cortisol concentration from 0 to 3 h in relation to the ACTH agonist challenge (T0).

4 Discussion

The first aim of this study was to validate and apply an EIA assay for measuring changes in urinary LH changes in four male SHNWs following GnRH agonist stimulation. Despite the LH EIA being used successfully for the measurement of male (current study) and female (Swinbourne et al., 2015b) SHNW plasma LH following GnRHa stimulation, we failed to detect any corresponding change in LH concentration in the urine of the same male wombats; a similar finding has also been reported in female wombats (Swinbourne et al., 2015b). While Allen et al. (2008) succeeded in detecting a significant rise in plasma LH in five captive female koalas (*Phascolarctos cinereus*) following a 4 μg buserelin administration, the same dose in female SHNWs was not sufficient to induce a significant increase in LH (Swinbourne et al., 2015b); these authors determined that 10 μg of GnRH agonist (buserelin) was in fact required to elicit a detectable significant increase in both plasma and urinary LH concentrations. Using this information, we challenged four male SHNWs with two different dosages of GnRHa (buserelin – i.e. 8 μg for M1 and M6 and 12 μg for M3 and M4) based on body mass in order to ensure administration of a supramaximal dose as well as to keep the dose of GnRHa per kg of body weight approximately within the same range (i.e. 0.27–0.30 μg/kg).

The discordance between the present study and Swinbourne et al. (2015b) in captive SHNW LH detection might be related to the inherent difference between sexes with respect to LH secretion. Typically, there are two patterns of LH secretion: the tonic mode and the surge mode. The tonic mode is controlled by the inhibitory effect of gonadal steroids whereas the surge mode is mainly dependent on the positive feedback action of oestradiol. Hence, the tonic mode of LH release is operative in both sexes but the surge mode is normally only exhibited by females (Norman and Spies, 1986 and Herbosa et al., 1996). Additionally, the surge mode of LH secretion is stimulated by the surge-like release of GnRH (Moenter et al., 1991), which is elicited by the Kisspeptin (Kiss1) neurons (Smith et al., 2005); Kiss1 neurons are regarded as major gatekeepers to transmit key information for reproductive viability to the GnRH

neurons by releasing kisspeptin (Navarro and Tenasempere, 2012). Notably, sexual differentiation with respect to the population of Kiss1 neurons has been reported, with greater density of Kiss1 neurons being found at the anteroventral periventricular area (AVPV/PeN) in females than in males, which could therefore facilitate the potential for surge release of GnRH/LH through positive feedback mechanisms in females (Smith et al., 2005). Irrespective of differences between male and female LH secretion following GnRH agonist stimulation, Swinbourne et al. (2015b) were also unable to detect any LH surge in female urine in relation to their natural reproductive cycles, which suggests that urinary LH level might not be an appropriate reproductive index for this species. The antibody 518-B7 recruited for this study has been confirmed to cross react with circulating LH in a number of species (Matteri et al., 1987) and the LH EIA system originally established for measuring LH in elephant serum (Graham et al., 2002). However, to date, biological validation for urinary LH measurement using this system has only been achieved in the Indian rhinoceros (*Rhinoceros unicornis*; Stoops et al., 2004), bottlenose dolphin (*Tursiops truncates*; Robeck et al., 2005) and Amazonian manatee (T*richechus inunguis*; Amaral et al., 2014).

The present study also confirmed the validity of the testosterone EIA to detect changes in urinary testosterone metabolites using the R156/17 antibody (Coralie Munro, UC Davis, USA) following a GnRHa challenge and this was the first study to report a successful biological validation for UTM measurement in marsupials. Previously Hogan et al. (2010) used 4 μg buserelin for GnRHa administration in four captive male SHNWs, which resulted in a significant elevation in plasma testosterone after 45 min of the GnRHa injection (i.e. significant rise was reported from T45 min to T150 min), peaking after 90 min of hormone injection, and a significant elevation in faecal testosterone metabolites 2–4 days following the injection. As 4 μg buserelin was proven to be sufficient for eliciting significant changes in both plasma and faecal testosterone levels in captive male SHNWs, it is reasonable to consider that the dosages provided in this study should be supra-maximal for each animal. The present study showed that plasma testosterone was significantly elevated from 60 min to 120 min after the GnRHa injection, with a peak at T120 min; these results were comparable to those of Hogan et al. (2010).

There was no significant effect of subject on plasma testosterone levels found in this study, indicating that all four challenged male wombats had a similar testicular steroidogenic response. However, our urinary testosterone results showed some

individual difference in terms of UTM levels, which contrasted with the plasma testosterone results, suggesting that differential metabolism might account for this variation. Previously, Vermeulen et al. (1972) reported the decrease in testosterone metabolic clearance rate (MCR) was related to senescence in adult men, indicating that a series of factors (e.g. relevant enzyme activity) would influence the MCR of testosterone during the aging process. Hence, for this study, it is also possible that the age difference of male SHNWs might contribute to the individual variation in UTM levels (e.g. M4 was older than both M3 and M6 but the overall mean UTM level of M4 was lower than M3 and M6).

Notably, significant rises in UTM reported in this study were not successive; UTM levels were significantly elevated 21 h and 50 h but not 36 h following the buserelin injection. This result may be associated with the fact that a urine sample from M6 was missed during this time point. While the period of elevated UTM appears to have lasted for 50 h following GnRHa stimulation, it is difficult to conclude whether this secretory pattern was associated primarily with GnRHa administration or natural diurnal variation in UTM secretion. While it is possible that natural diurnal variation could account for endogenous patterns of testosterone secretion (Heistermann, 2010), it was not possible in the current study to account for time of day given the intensity of the sampling procedure and given the logistical limitation of only anaesthetizing one animal at a time. A prolonged period of elevation in faceal testosterone metabolites was also reported by Hogan et al. (2010), which was attributed to the longer half-life of buserelin compared with natural-sequence GnRH (Johnston et al., 2007 and Allen et al., 2008). Additionally, some pre-stimulation UTM values were comparable to the maximal UTM concentrations after the buserelin administration in M1 and M3, which might be explained by the coincidence that an endogenous pulse secretion of testosterone occurred in these two animals several days before the GnRHa challenge.

The current study appears to be the first to biologically validate the measurement of urinary cortisol metabolites in marsupials using an enzyme-immunoassay (Arbor Assays®, USA). Although hemolysis appeared in plasma samples from three females, it seemed to have no effect on cortisol measurement in this study and this is consistent with the findings of Lucena et al. (1998) who also reported hemolysis did not significantly affect the serum cortisol detection for dogs using EIA. Following the injection of 250 μg ACTH agonist Synacthen® Depot, there was a rapid elevation (T15 min) in plasma cortisol concentrations with a peak at T90 min and a subsequent rise in

UCM detected at T3 h in six captive *L. latifrons* (3♂, 3♀). These results were essentially consistent with Hogan et al. (2011), which validated the detection of faecal cortisol metabolites (FCM) using an ACTHa stimulation, resulting in a similar increase at T15 min in plasma cortisol levels in four captive SHNWs (2♂, 2♀); however, compared to the delayed elevation in FCM (3 days after the injection of Synacthen®) reported by Hogan et al. (2011), mean UCM values were elevated almost 'immediately' (only 3 h) after the ACTHa administration in this study. Notably, as there was a lack of samples between 3 and 27 h post injection, we are not able to confirm if the mean UCM level continued to increase after T3 h. Additionally, the fact that the mean UCM level returned to baseline within 27 h post injection might indicate the short life span of the induced ACTHa stimulation. Therefore, based on our results, when attempting to describe changes in stress hormone release with UCM levels in captive SHNWs, urine samples should be obtained between 3 and 12 h in relation to a stress-related event. This short period actually provides an extremely narrow window for capturing subtle changes in cortisol secretion and will need to be accounted for when designing future experiments or assessments that are interested in documenting acute stress responses (e.g. human approach or handling). The mean baseline plasma cortisol values (T0; 1.06 ± 1.41 ng/mL) of captive *L. latifrons* found in this study were generally comparable to Tasmania devils (*Sarcophilus harrisii*; Keeley et al., 2012), koalas (basal 1.101 ± 0.53 ng/mL; Davies et al., 2013) and results from the other observations of captive SHNWs (basal 0.40 ± 0.71 ng/mL; Hogan et al., 2011). Sex differences in plasma and UCM concentrations were also noted in the current study and both the differences remained relatively constant over time (i.e. female mean cortisol levels were significantly higher than males). However, Hogan et al. (2011) reported there were no significant sex effects detected in either plasma or faecal cortisol levels in captive SHNWs. Owing to the small sample size (2 males and 2 females), it is possible that the sex difference in cortisol secretion might be masked in their study. Previously, Narayan et al. (2012) reported sex variation in FCM levels in the semi-free ranging Eastern Greater Bilby (*Macrotis lagotis*), with female FCM concentrations being constantly higher than males for over 21 days. Moreover, there was some evidence that the sex-related differences existed in both plasma and FCM levels in captive koalas (Davies et al., 2013 and Narayan et al., 2013). In addition to these findings, different patterns of GC secretion due to sex differences have been reported previously (Stead-Richardson et al., 2010), indicating the variation of reproductive hormones may alter the capacity of HPA axis (von der Ohe and Servheen, 2002). Results of Owen et al. (2004) showed testosterone elevation in male giant pandas

(*Ailuropoda melanoleuca*) would result in an increase in GC secretion whereas progesterone would inhibit GC secretion during oestrus, pregnancy and lactation in females. It was suggested that sexual dimorphism was associated with an interplay and connection between HPA and HPG axes (Chand and Lovejoy, 2011) and oestrogens displayed a significant role leading to different stress responses between the sexes (Magiakou et al., 1997). It has been suggested that estrogens may influence the differential sex response by regulating CRF gene expression in the hypothalamus in a sex-dependent manner, which further impacts both HPA and HPG axes (Vamvakopoulos and Chrousos, 1993, Dibbs et al., 1997 and Kageyama, 2013).

There may be some differences in the secretory patterns of urinary hormones collected by classical conditioning and those collected under general anaesthesia. However, our experimental design allowed us to document parallel changes in hormones in both plasma and urine to ensure that a physiological response to challenge was induced.

5 Conclusion

In summary, this study has biologically validated the EIA measurement of UTM and UCM in captive *L. latifrons* using exogenous GnRH agonist and ACTH agonist stimulation, respectively. Hence, reliable estimates of the testicular steroidogenic capacity and the adrenocortical activity have now been provided for captive SHNWs. The LH EIA used in the current study is able to detect changes of LH concentrations in plasma but not in urine, suggesting that captive male SHNWs may not produce appreciable amounts of LH in urine or that their metabolism affects excretion of this hormone. The utility of measuring steroid hormones in captive SHNW urine will make endocrine monitoring and reproductive management more accessible to institutions which manage this species. Based on results from the present study, future research may apply UTM/UCM analysis into (1) investigating seasonal changes and acute or chronic stress responses in captive *L. latifrons*, and (2) studying other marsupial species.

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