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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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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.



## 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; 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; 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; 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). Addition-

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ally, regular blood sampling is impractical for field studies especially for studying wild species (Brown et al., 2010; 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; 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; 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; 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; Sparrow, 2009), increased road accidents (Ramp et al., 2005) and climate change (Finlayson et al., 2005; Kellermann et al., 2009). Southern hairy-nosed wombats have been kept in captivity since the 1970s (Jackson, 2003; 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; 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, 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; 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; 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