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Validation of an enzyme immunoassay for the non-invasive measurement of faecal androgen metabolites in spinifex hopping mice (*Notomys alexis*)

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ABSTRACT

Androgens may play a key role in shaping the unique reproductive traits of male spinifex hopping mice (*Notomys alexis*), but little is known about the reproductive endocrinology of this species. Measurement of faecal androgen metabolites (FAMs) offers a non-invasive tool for monitoring testicular activity. Before applying this tool, physiological validation is required to demonstrate that changes in FAMs reflect changes in circulating testosterone for a given species. The goal of this study was to validate an enzyme immunoassay for monitoring FAMs in *Notomys alexis*. We compared the performance of two different assays (UVM-T and R156/7) for monitoring changes in FAMs following the administration of human chorionic gonadotropin (hCG) to stimulate androgen production by the testes. Both assays detected a significant increase in FAMs and had similar patterns. The UVM-T assay was more sensitive to changes in testicular activity, with a shorter excretion lag time and greater response magnitude. These findings indicate that we can reliably monitor testicular activity using faecal hormone metabolite analysis in *Notomys alexis* and can now utilise FAM measurements to better understand the species' unusual reproductive biology.

Keywords: EIA, faecal metabolites, hormone, Muridae, rodent, sex steroids, testicular activity, testosterone.

Introduction

The spinifex hopping mouse (*Notomys alexis*) is a social, arid-dwelling rodent found throughout central and western Australia, and the males exhibit several unique reproductive traits. They have unusually small testes for their body size, just 0.1–0.2% of body mass compared to the expected 1–3% for a mammal of their size (Bauer and Breed 2008). Unlike other murid rodents, their sperm morphology is highly variable (Bauer and Breed 2006) and they lack the prominent apical hook that is found in murids with high inter-male competition (McLennan *et al.* 2017; Breed *et al.* 2020). The penis is small and has very large spines compared to most other Australian murids, likely to assist with locking during mating (Morrissey and Breed 1982; Breed *et al.* 2020). Males are also smaller (Breed 1983; Berris *et al.* 2020*b*) and less aggressive than females (Stanley 1971). Although the functional significance of these traits is still under investigation, they suggest reduced inter-male competition and potential monogamy (Breed *et al.* 2020).

One of the major challenges to advancing our understanding of the reproductive biology of this species is that it is difficult to identify the reproductive condition of individuals. Although breeding can occur year-round, the reproductive status of individual males within the population is variable (Berris *et al.* 2020*a*) and female reproductive suppression can occur under high densities (Berris *et al.* 2020*b*). External morphological traits (e.g. testes mass, scrotal size and pigmentation), body size and age are not useful indicators of male reproductive condition or sexual maturity, making it difficult to assess the reproductive state of male spinifex hopping mice (Berris *et al.* 2020*a*). Examining the reproductive endocrinology of this species may offer valuable insights into the development of these unique reproductive traits and provide a better indicator of male reproductive condition.

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The principal male androgen, testosterone, contributes to testicular function and spermatogenesis, as well as the development of secondary sex traits such as muscle mass and dominance (Mooradian *et al.* 1987). Testosterone also shapes behaviour and social hierarchies in both sexes (Schradin 2008). To date, only one study has measured testosterone in spinifex hopping mice, using blood samples collected post-mortem (Breed 1983). An individual's sample thereby represented only a single timepoint, which may inadvertently be representative of a diurnal or pulsatile release (Palme 2005). Testosterone levels ranged widely among males of a similar age, with some individuals having concentrations lower than females in the same age group (Breed 1983). However, the biological significance of this is currently unknown. Longitudinal hormone monitoring could provide more insight into the biological role of testosterone in this species, including temporal hormone changes and the significance of individual variation in testosterone levels.

Faecal hormone metabolite monitoring provides a valuable tool for studying endocrine physiology, with the added benefit of being (largely) non-invasive (Kersey and Dehnhard 2014). Circulating androgens are excreted in faeces and reflect changes in testicular activity (Busso *et al.* 2005; Sipari *et al.* 2017). Faecal collection also enables repeated sampling, which can offer valuable insights about changes in testicular activity over time (Palme 2005). In contrast to blood sampling, which provides a single snapshot in time, faeces reflect a pooled estimate of circulating hormone levels, thereby allowing broader insights into endocrine physiology (Palme 2019). However, circulating androgens are metabolised prior to their excretion in faeces, so assays must be validated for each new target species and hormone (Möhle *et al.* 2002). Validation is achieved by demonstrating that immunoreactive faecal androgen metabolites (FAMs) reflect changes in circulating androgen levels (Touma and Palme 2005). Different assays can cross-react with different FAMs and yield different signals of testicular activity. Therefore, assay comparison is an important part of the physiological validation process to ensure selection of an assay that is most sensitive to changes in testicular activity (Fanson *et al.* 2017).

This study aimed to physiologically validate an assay for the measurement of FAMs in spinifex hopping mice. We compared the performance of two different testosterone enzyme immunoassays (EIAs) to monitor changes in testicular activity in response to a human chorionic gonadotropin (hCG) challenge (see details below). Once validated, this assay could help advance our understanding of the unusual reproductive biology of this native Australian rodent.

Material and methods

Study animals

Four sexually mature, virgin male spinifex hopping mice (83–106 days old, 30–33 g) were obtained from captive collections and held at La Trobe University, Bundoora, Australia. Individuals were singly housed in enclosures (39.5 W × 57 L × 30.5 H cm) containing a water bottle and nest box. Substrate consisted of layered whole paper sheets to allow for easy collection of all faecal material. As spinifex hopping mice live in social groups, enclosures were semi-transparent and situated adjacently so that visual and olfactory contact was maintained with at least one other mouse. The room housed only males and was maintained at 20–24°C on a light cycle of 12L:12D, with natural light available. Individuals were fed seeds and fresh fruit/vegetables daily and three live mealworms (*Tenebrio molitor*) every 3 days, with water provided *ad libitum*. The research was approved by the La Trobe University Ethics Committee (AEC 18051) and Department of Environment, Land, Water and Planning Wildlife Research Permit (DELWP 10008843).

Physiological validation

A human chorionic gonadotropin (hCG) challenge was used to determine whether the assays detected biologically relevant changes in testicular activity (Billitti *et al.* 1998; Busso *et al.* 2005; Allen *et al.* 2006). hCG is structurally similar to luteinising hormone (LH) and binds to the same receptor, which stimulates the Leydig cells in the testes to produce testosterone (Auer *et al.* 2020). FAMs were monitored from 3 days prior to the hCG challenge and until 3.5 days after.

At 19:00 (start of active period), each mouse was hand-caught from its nest box, manually restrained, and given a single intraperitoneal injection of 20 IU hCG (Chorulon® 1500 IU, Merck Animal Health Intervet Inc., Madison, NJ, USA) to stimulate testosterone secretion (Jean-Faucher *et al.* 1985). Mice were released back into their home cages immediately after the injection and visually monitored for any adverse side-effects.

Sample collection

During the baseline period, prior to hCG injection, faecal samples were collected twice daily (09:00 and 15:00) for 3 days. Following the injection, samples were collected every 3 h for 12 h (22:00, 01:00, 04:00, 07:00), and then three times daily for an additional 3 days (morning, midday, night). During each sample collection time, the paper substrate was removed and replaced with fresh paper. All faecal pellets were collected from the old paper and stored in 1.5 mL tubes. Any samples in close proximity to urine were not collected. When there were multiple pellets for an individual during any given check, they were all combined in the same tube. If no pellets had been produced by an individual, then they were left undisturbed until the next sample time. Between 6 and 7 pre-injection samples and 11–12 post-injection samples were collected per individual. Samples were stored at −20°C until steroid extraction.

Steroid extraction

Steroids were extracted by adding 1 mL of 80% ethanol to 0.02 ± 0.001 g of wet faecal material in a 5 mL polypropylene tube. It was difficult to homogenise the faeces, so sub-samples were taken from several different pellets to ensure measurements were not biased by a single pellet. Tubes were vortexed and shaken on an orbital shaker overnight. Samples were then centrifuged for 5 min at 2380 RCF. The supernatant was decanted into a 1.5 mL tube and stored at −20°C until analysis.

Assay procedure

Faecal androgen metabolites (FAMs) were assayed using two different double-antibody enzyme immunoassays. The first assay was directed at testosterone-3-CMO:BSA (antibody and corresponding biotin-labelled testosterone obtained from University of Veterinary Medicine, Vienna, Austria; hereafter referred to as UVM-T). The second assay used a polyclonal testosterone antibody directed at testosterone-6-CMO:BSA (produced at University of California Davis, Davis, USA; antibody and corresponding testosterone horseradish peroxidase (HRP) conjugate obtained from Smithsonian Conservation Biology Institute, Virginia, USA; hereafter referred to by its given antibody code R156/7). The EIAs were biochemically validated in our lab by demonstrating (1) parallelism between serial dilutions of faecal extract and the standard curve, and (2) significant recovery of exogenous testosterone added to extracts.

EIA procedures were similar to those previously described (Palme and Möstl 1994; Freeman *et al.* 2018). Briefly, 96-well microtiter plates were coated with 150 µL of goat anti-rabbit IgG solution (2 µg/mL; A009, Arbor Assays, USA) and incubated overnight at 4°C. Wells were then emptied and blocked with 200 µL of Trizma buffer solution rich in bovine serum albumin.

For the UVM-T assay, plates were washed and loaded with 50 µL of standard, control, or sample, followed by 100 µL of biotin-labelled testosterone (1:5 000 000) and 100 µL antibody solution (1:60 000). Plates were shaken overnight at 4°C. The following day, plates were washed and incubated for 45 min with 250 µL streptavidin-POD conjugate solution. After a final wash, 250 µL of tetramethylbenzidine solution was added to each well. The reaction was stopped after 1 h using 50 µL of sulfuric acid (2 M) and the optical density was read using a 450 nm measurement filter (plate reader from Anthos Labtec Instruments, Salzburg, Austria).

For the R156/7 assay, plates were washed and loaded with 50 µL of standard, control, or sample, followed by 50 µL of testosterone HRP conjugate (1:100 000) and 50 µL of antibody solution (1:100 000). Plates were shaken for 2 h at room temperature, washed, and loaded with 150 µL ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) solution. The optical density was read after 1 h using a 405 nm measurement filter.

All samples were assayed in duplicate, and samples from each individual were assayed on the same plate. FAM concentrations are expressed as ng/g wet faeces. For UVM-T, the inter-assay coefficients of variation (CVs) for low and high controls (~25 and 75% binding) were 5.81 and 3.25%, respectively (n = 3 plates), and the intra-assay CVs were 2.02 and 3.21% (n = 20 control replicates). For R156/7, the inter-assay CVs were 7.25 and 16.34%, respectively (n = 5 plates) and the intra-assay CVs were 5.47 and 7.15%, respectively (n = 24 control replicates).

Statistical analysis

Data were analysed using R 4.1.1 (R Core Team 2021). To test whether there was a significant change in FAMs following hCG injection, we used a linear mixed model (LMM). Data and model residuals met the assumptions of normal distribution and homoscedasticity. FAM concentration was modelled as a function of experimental period and time of day, with animal ID included as a random effect. Experimental period was defined as pre-injection control (all samples collected prior to the hCG injection) followed by 3–12-h intervals post-injection (depending on sample collection frequency). We compared each post-injection interval with the pre-injection mean using a Dunnett's test for multiple comparisons to identify significant increases in FAMs post-injection. Residuals were checked to confirm model assumptions were met.

To examine individual responses to the hCG challenge, base and peak FAM values were identified for each individual on each assay using the iterative approach with the R package hormLong (Fanson and Fanson 2015). The iterative calculation excluded all values greater than the mean + 1.70 s.d. until no points exceeded the threshold (Brown *et al.* 1996). The values remaining below the threshold were considered 'base FAMs' and values exceeding the threshold were considered 'peak FAMs.' We used this information to calculate the following information for each individual: (1) average baseline = mean of all points remaining below the threshold; (2) maximum peak = highest post-injection value; (3) response magnitude = max peak divided by average baseline (fold-increase); and (4) excretion lag time = time from injection to first peak (measured in hours).

Results

Overall model

FAMs varied significantly across experimental periods for both assays (UVM-T: $F_{12,\ 96.04} = 18.41$, $P < 0.0001$); R156/7: $F_{12,\ 96.15} = 7.69$, $P < 0.0001$). Further examination using Dunnett's *post hoc* comparisons showed both assays detected a significant increase in FAM levels after the hCG injection. However, their patterns were slightly different (Fig. 1). For UVM-T, there was a large initial peak (3–38 h post-injection), followed by a smaller peak (49–62 h post-injection; Fig. 1*a*).

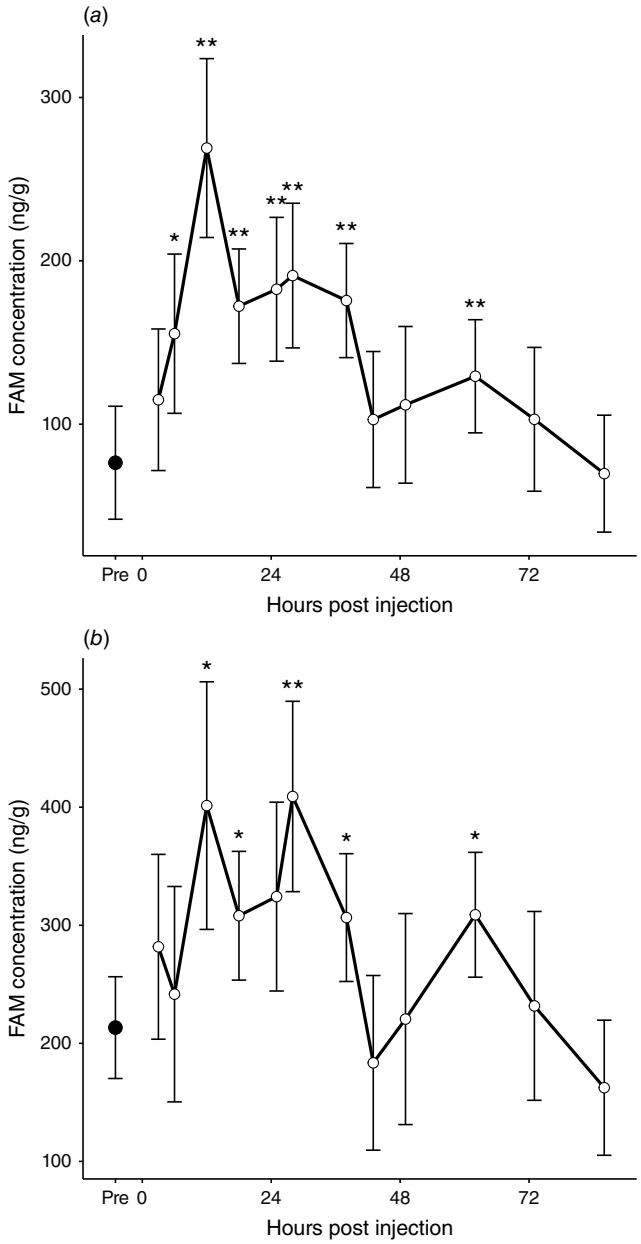

Fig. 1. Changes in FAM concentration (ng/g wet faeces) relative to pre-injection baseline following hCG injection in male spinifex hopping mice ($n = 4$). FAMs from the same faecal extracts were measured using two different assays: (*a*) UVM-T assay and (*b*) R156/7 assay. 'Pre' includes all samples collected prior to the injection. All points represent least square mean ± 95% confidence interval. Asterisks indicate points that are significantly different from the pre-injection period (*, $P < 0.05$; **, $P < 0.001$).

For R156/7, there were three shorter peak periods, occurring between 6–18, 25–38, and 49–62 h post-injection (Fig. 1*b*). The maximum peak occurred at 12 h for UVM-T, while R156/7 displayed two maximum peaks of similar size at 12 and 28 h.

Time of day also had a significant effect on measured FAMs for both assays (UVM-T: $F_{1,\ 96.02} = 3.97$, $P = 0.05$; R156/7: $F_{1,\ 96.09} = 6.73$, $P = 0.01$). Both assays showed a similar pattern, with FAMs rapidly increasing from 20:00 and peaking after midnight, then gradually decreasing during daylight hours.

Individual-level analysis

Peaks above the baseline threshold were detected in all individuals for both assays. However, the excretion lag time (first detected peak), number of peak samples, and magnitude of response varied between individuals and between assays (Table 1). The average excretion lag time was 9 h for UVM-T and 19 h for R156/7 (median 6 and 18 h, respectively). The excretion lag time occurred within 6 h for three out of four individuals and by 18 h for the remaining individual with the UVM-T assay. R156/7 excretion lag time was more variable between individuals, ranging between 3 and 38 h.

The UVM-T assay was slightly more sensitive to changes in testicular activity as demonstrated by the greater response magnitude (mean = 2.77-fold-increase) compared to R156/7 (2.20-fold-increase). The UVM-T assay also detected more post-injection peaks (mean = 5.50 peaks) compared to R156/7 (4.25 peaks). One individual (ID #33) had a lower response to the hCG injection than the others, showing fewer peak samples, a smaller response magnitude and a much longer excretion lag time in both assays.

Discussion

Validation helps ensure that measured faecal hormone metabolites reflect physiologically relevant changes in circulating hormone concentrations and must be conducted for each species and hormone of interest. In this study, we used an hCG injection to stimulate production of testosterone in spinifex hopping mice and compared the performance of two assays for monitoring changes in circulating androgens via FAMs. Overall, both assays (UVM-T and R156/7) produced very similar FAM patterns. Both showed an increase in FAM levels after males were physiologically stimulated by an hCG injection, providing evidence that testicular activity could be measured. However, there were some differences between the assays for each of the three performance metrics: (1) lag time (when FAMs start to increase), (2) response magnitude (how much FAMs increase), and (3) response duration (how long FAMs stay elevated).

One male (#33) had a muted response compared to the other three individuals (see Table 1). He had a similar body weight, was sourced from the same place as (and is likely related to) another male (#31), and had no obvious health issues. The idea that #33 could have been seasonally non-breeding (causing a lowered response) is also unlikely as testes mass and sperm production and quality have been found to be comparable between individuals in non-breeding and breeding periods (Bauer and Breed 2008).

Table 1. Faecal testosterone metabolite (FAM) concentrations in response to a human chorionic gonadotropin (hCG) injection in male spinifex hopping mice ($n = 4$).

Assay	Animal ID	Mean baseline ± SEM (ng/g)	Maximum peak (ng/g)	Response magnitude (fold-increase)	Excretion lag time (h)	Number of peak samples
UVM-T	29	88.52 ± 13.10	264.23	2.98	6	7
	30	101.78 ± 13.78	365.33	3.59	6	4
	31	83.12 ± 10.04	221.05	2.65	6	8
	33	77.76 ± 6.24	144.59	1.86	18	3
	Mean	87.80 ± 5.15	248.80 ± 46.05	2.77 ± 0.36	9 ± 3.00	5.5 ± 1.19
R156/7	29	214.35 ± 21.17	478.05	2.23	18	4
	30	176.04 ± 19.39	527.78	3.00	3	7
	31	260.57 ± 15.74	469.19	1.80	18	4
	33	212.28 ± 14.00	374.37	1.76	38	2
	Mean	222.56 ± 17.32	462.35 ± 32.03	2.20 ± 0.29	19.25 ± 7.18	4.25 ± 1.03

FAM values are expressed as nanograms per gram of wet faeces (ng/g).

The only known difference was that #33 was the youngest individual by 12–22 days. All males were thought to be reproductively mature based on weight (Breed and Leigh 2011), but there is the possibility that #33 was not yet sexually mature and thus had a dampened response to hCG. Therefore, we discuss results below both with and without this male included.

Excretion lag time was defined as the interval between the time of injection and the first sample that was significantly higher than baseline. When averaged across all males, the excretion lag time was 6 h for UVM-T and 12 h for R156/7 (Fig. 1). However, at the individual level, excretion lag time was more variable (Table 1). Excluding male #33, the excretion lag time was much more uniform for UVM-T (6 h) compared to R156/7 (3–18 h). One reason the lag times differed between assays may be the result of R156/7 being less sensitive, thus taking longer for the post-injection increase in FAMs to become significantly different from base FAM levels. The assays could also be detecting different metabolites that are excreted at different times. Assays targeting the same hormone can cross-react differently with the numerous metabolites formed, thus presenting different results (Fanson *et al.* 2017). While it was not practical for our study, a more reliable method to determine excretion lag time is to inject individuals with radiolabelled testosterone (^{3}H- or ^{14}C-testosterone) and measure the time-course for recovering the labelled metabolites after excretion (Auer *et al.* 2020). Using this approach, other rodent studies found that radioactivity in the faeces peaked between 4 and 10 h in *Mus musculus f. domesticus* (C57BL/6JRj strain house mouse; Auer *et al.* 2020), 12 h in *Peromyscus maniculatus* (deer mouse; Billitti *et al.* 1998) and *Chinchilla lanigera* (chinchilla; Busso *et al.* 2005) and between 4 and 16 h (median 8 h) in *Myodes glareolus* (bank vole; Sipari *et al.* 2017), with the majority of radioactivity excreted within 24 h. Therefore, the excretion lag time we observed for *N. alexis* is comparable to other rodent species.

Response magnitude, calculated for each individual as maximum FAM level divided by average baseline FAM levels, was greater with the UVM-T assay than the R156/7 assay (average fold increase = 2.77 and 2.20, respectively; Table 1). Excluding male #33, the response magnitude was 3.07 and 2.34 for UVM-T and R156/7, respectively. UVM-T peaks were also more statistically different to pre-injection FAM levels, indicating it was more sensitive to testicular changes than R156/7. This response magnitude is similar to that observed in another rodent species following an hCG injection (3-fold increase in *M. musculus domesticus*; Auer *et al.* 2020), although stronger responses have been observed in other rodents [8-fold increase in *P. maniculatus* (Billitti *et al.* 1998) and 9-fold in *C. lanigura* (Busso *et al.* 2005)].

While the FAM patterns of both assays were similar, the duration of the response was greater for UVM-T. Based on the overall model, the initial response duration for UVM-T was 32 h (significantly elevated points from 6 to 38 h; Fig. 1). For R156/7, the initial response was technically 6 h (significantly elevated points from 12 to 18 h; Fig. 1). However, although the point at 25 h was not significantly greater than pre-injection levels, visual inspection of the plot suggests that the initial response for R156/7 lasted from 12 to 38 h (26 h). Overall, this highlights that there was more noise and less sensitivity in the R156/7 data. This compares with two other rodent species which both had response durations just under 40 h (Billitti *et al.* 1998). Both assays also detected a second peak around 62 h. While FAMs had returned to baseline levels for both assays by the conclusion of the study (86 h or 3.5 days), longer sampling post-injection would have provided a better estimate of baseline.

Time of day significantly affected FAM levels, with FAMs at their highest after midnight for both assays. Accounting for

an excretion lag time of 6 h (UVM-T), circulating androgens are estimated to peak around the onset of darkness. Hopping mice are nocturnal (Stanley 1971), so this peak coincides with the beginning of their active phase. This contrasts with *M. musculus domesticus*, in which a peak in circulating testosterone levels occurred at the end of their active phase (Auer *et al.* 2020). If the excretion lag time of *N. alexis* is closer to 12 h (R156/7), then the peak in circulating androgens would occur at midday. *M. musculus domesticus* exhibits two peaks in circulating testosterone levels, estimated to occur around 07:00 (end of their active phase) and 12:00. The authors hypothesise the midday peak may be from laboratory mice being less strictly nocturnal, with activity also occurring during the day (Auer *et al.* 2020). Two similarly timed peaks were seen in bank voles, which also display daytime activity despite being nocturnal (Sipari *et al.* 2017). The hopping mice in our study may have had active bouts during the day, although the higher sensitivity of UVM-T indicates that their excretion lag time is more likely closer to 6 h than the 12 h shown by R156/7.

Both assays in this study have been extensively used to monitor FAMs in other species [UVM-T: (Ninnes *et al.* 2010; de Bruin *et al.* 2014; Sipari *et al.* 2017; Auer *et al.* 2020); R156/7: (Billitti *et al.* 1998; Walker *et al.* 2002; Curry *et al.* 2012; Umapathy *et al.* 2015)]. However, it is less common for studies to compare assays. In a study conducted on *Crocuta crocuta* (spotted hyena), both assays produced similar FAM profiles, but the antibody that targeted testosterone-3-CMO (the same ligand as UVM-T) was more sensitive to changes in circulating androgens than that of R156/7 (Pribbenow *et al.* 2017). This correlates with our own findings. Another study comparing three different testosterone/androgen assays found that two were highly correlated, while the third was unreliable at monitoring gonadal function (Ganswindt *et al.* 2002). Evidently, the difference between assays can be significant. While it has become increasingly recognised that assays need to be compared for reliable monitoring of glucocorticoid metabolites (Fanson *et al.* 2017), this has not become standard for other hormones, yet. The variability between and within species demonstrates why assay validation is so important.

The subtle but significant differences between the two assays in our study illustrate why it is important to compare assay performance in validation studies. These distinctions are also important for accurately monitoring testicular activity, as changes can be small or large according to several factors, such as sex, age, size, and time of day (Palme 2005). This is particularly relevant for spinifex hopping mice, in which there have been no simple methods to measure reproductive condition. Analysis of faecal hormone metabolites via EIA is a valuable method, which can now be used to non-invasively monitor androgen levels and testicular activity in this species. Most hopping mice reproductive literature has been male-focused, so future work should incorporate females. One study found that some females had equal, if not higher, testosterone than males of the same age (Breed 1983). Female spinifex hopping mice are the larger and more aggressive of the sexes (Stanley 1971), so it would be interesting to examine the role testosterone plays in female behaviour and morphology. This would require biological validation for females because steroid metabolism can differ between the sexes (Touma and Palme 2005). Finally, there is ample opportunity to apply our knowledge to the remaining species in the *Notomys* genus, so that we can fill the large gaps in our understanding and improve conservation measures. While *N. alexis* is considered common, 60% of *Notomys* species have a threatened conservation status. Knowledge about the biology of *N. alexis* can offer valuable insights into the biology of threatened *Notomys* species, which are much more difficult to study. Applicable knowledge about their reproduction and biology would thus be invaluable in the conservation of some of Australia's unique, native mammals.

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Data availability. Raw data is available from Figshare data repository at doi:10.26181/21375996.

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