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Research paper

Can spines tell a story? Investigation of echidna spines as a novel sample type for hormone analysis in monotremes

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ABSTRACT

The short-beaked echidna (*Tachyglossus aculeatus)* is a monotreme endemic to Australia and New Guinea, and is the most widespread native mammal in Australia. Despite its abundance, there are considerable gaps in our understanding of echidna life history such as reproductive cycles in both sexes, patterns of stress physiology, and possible seasonal changes in metabolism. Slow-growing integumentary sample types comprised of keratin (hair, claw, etc.) have been used in other wildlife to assess these questions via analysis of longitudinal patterns in steroid and thyroid hormones that are deposited in these tissues as they grow. Hairs and spines comprise the pelage of echidnas, the spines being keratinized structures homologous to hair. Thus, echidna spines could be a viable sample type for hormone analysis contributing to a better understanding of the biology of echidnas. The aim of this work was to determine whether steroid hormones are detectable in echidna spines, to perform assay validations, and to establish a protocol for extracting and quantifying hormones in echidna spines using commercially available assay kits. We also inspected cross-sectioned spines using light and electron microscopy for any evidence of annual growth markers that might enable inferences about spine growth rate. Corticosterone, progesterone, estradiol, and testosterone were detectable in all samples, and echidna spine extract passed standard assay validations (parallelism and accuracy), indicating that commercially available assay kits can quantify hormones accurately in this sample type. No visible growth marks were identified in the spines and thus spine growth rate is currently unknown. Echidna spines show promise as a novel matrix from which hormones can be quantified; next steps should involve determination of spine annual growth rate, possible seasonal changes in growth rate, and persistence of spines over time in order to perform physiological validations, *i.e.*, relationship between physiological status and hormone concentrations in spines.

1. Introduction

The most widely distributed native mammal in Australia is a monotreme, the short-beaked echidna (*Tachyglossus aculeatus*), which occurs over most of the continent and its offshore islands, as well as in New Guinea. There are five geographic subspecies (Augee et al., 2006) which display climate-related differences in diet, pelage, thermoregulation, hibernation, reproduction, and maternal care (Nicol, 2021; 2017; Nicol et al., 2019; Nicol and Morrow, 2012; Rismiller and McKelvey, 2000). Unique among mammals, monotremes are oviparous: short-beaked echidnas have a ~21-day uterine gestation followed by laying of the egg into a pouch, where it incubates for another 10–11 days before hatching (Morrow et al., 2009).

Despite the importance of understanding the physiology of reproduction in egg-laying mammals, there are considerable gaps in our knowledge of how environmental factors influence echidna reproduction and monotremes in general (McAllan, 2011). The most detailed field studies of echidna reproductive endocrinology have been carried out on readily accessible populations of subspecies in temperate regions: *T. a. setosus* in Tasmania (Morrow et al., 2017; 2016; Nicol, 2021), and *T. a. multiaculeatus* on Kangaroo Island (Dean, 2000). Recent advances in echidna husbandry have facilitated captive breeding of echidnas

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(Wallage et al., 2015) allowing detailed study of hormone profiles (Dutton-Regester et al., 2021). Still, there is no information on the reproductive physiology of echidnas from the arid and semi-arid zones of central Australia, the tropical north, or the wet tropics of northeast Queensland.

Elucidation of temporal patterns of adrenal and gonadal steroid hormones could help fill this knowledge gap, since these hormones play key roles in reproduction and survival in vertebrates. The gonadal steroids (*e.g.,* estradiol, progesterone, testosterone) are critical in the regulation of gametogenesis, reproductive behavior, and other aspects of reproductive cycles. In female mammals, estradiol usually peaks with ovulation, while progesterone rises in the post-ovulatory stage and usually remains elevated during pregnancy (Bronson, 1989). In male mammals, testosterone is usually elevated in mature individuals during the breeding season (Hau, 2007), often concurrent with reproductive behaviors or with post-hibernation recrudescence of testes (Buck and Barnes, 2003; Richter et al., 2017). These patterns appear to hold for short-beaked echidnas, with some variations that may be characteristic of monotremes. In Tasmanian echidnas, males increase testis mass from approximately 32% to 75% of maximum size before entry into hibernation although testosterone levels are low at that time; upon final emergence from hibernation, testosterone rises rapidly, and testes reach their maximum size (Morrow et al., 2016). Testosterone is also linked to reproductive behavior in males (Morrow et al., 2009; Nicol et al., 2005). In female echidnas, plasma progesterone levels only rise above basal shortly before egg-laying (Dean, 2000; Dutton-Regester et al., 2021; Morrow et al., 2017). Hibernating females disturbed by males early in the mating season re-enter hibernation, even if pregnant, and progesterone remains low. On emergence from hibernation, development of the embryo resumes, and progesterone begins to rise, peaking 4–6 days before egg-laying, and then plummets (Morrow et al., 2017).

Adrenal glucocorticoids (*e.g.,* cortisol and corticosterone) are critical for the coordination of basal metabolic processes and responses to stressors (Bentley, 1998). Corticosterone is considered the dominant glucocorticoid in short-beaked echidnas, due to its higher plasma concentrations (Sernia and McDonald, 1977). Although the stress response is important for survival, long-term activation of the hypothalamo-pituitary-adrenal (HPA) axis can inhibit reproductive behavior and alter aspects of other physiological processes, such as the immune response (Wingfield et al., 1997). Therefore, glucocorticoids can be useful to monitor in populations vulnerable to human disturbance and other stressors, both in the wild and in captivity (Walker et al., 2005; Wingfield et al., 1997).

Retrospective analysis of patterns of reproductive and adrenal steroids over time could be used to reconstruct valuable information on reproductive history, hibernation, and stress in both male and female short-beaked echidnas. To date, serial sampling of reproductive steroids in the field has been limited to easily accessible echidna populations from which plasma samples can be obtained (Dean, 2000; Morrow et al., 2017; 2016). However, it has been demonstrated that reproductive steroids and glucocorticoids are present in cornified epithelial tissue (*e.g.,* baleen, vibrissae, feathers, claws, shed skins, hair and nails) of all vertebrates yet tested (Berkvens et al., 2013; Bortolotti et al., 2008; Dillon et al., 2021; Gleixner and Meyer, 1997; Hunt et al., 2017b; Karpovich et al., 2019; Koren et al., 2002; Warnock et al., 2010). These cornified epithelial tissues offer important advantages for field-based research; samples are often relatively easy to obtain, data is likely not influenced by capture-related stress, and keratinized samples are robust to degradation and can be easily stored in field settings. Further, certain linear and slow-growing keratinized tissues such as claws, nails, hair and other hair-like structures (*e.g.,* pangolin scales, Blecher et al., 2021), could contain longitudinal information on hormones over the time period of tissue growth (months to years), allowing retrospective analysis of hormone concentrations (Hunt et al., 2017a; Pokharel et al., 2021) from which reproductive, metabolic, and stress histories can be inferred (Fernández Ajó et al., 2020; Hunt et al., 2016).

The pelage of the dorsal surface of short-beaked echidnas consists of a mixture of hairs and spines, with the spines randomly shed and replaced (Griffiths, 1978; Rismiller and Grutzner, 2019). Short-beaked echidna spines (Fig. 1) are modified hairs (Griffiths, 1978), but appear to grow over a longer period of time than hairs, and their greater width and mass suggests they may be useful as an endocrine sample type. However, the use of hair-like tissues for hormone assessments requires understanding hormone deposition during the timeframe of growth (Koren et al., 2019), and possible change over time, because hair could lose or potentially gain hormone after growth (Colding-Jørgensen et al., 2020; Hamel et al., 2011). In some cases, mammalian epidermal structures have visible growth lines (due to seasonal changes in diet and activity) that can help resolve some of these questions (see Hemming, 1969).

Two types of validations are necessary when using a novel sample type like echidna spines: assay (technical) and physiological validations. Assay validations assess whether hormones are present and can be reliably quantified, while physiological validations test whether the hormone patterns observed in the sample correlate with the physiological state of the animal. For example, known pregnancies (Hunt et al., 2016), decreased food availability (Bryan et al., 2013), and increased hunting pressure (Bryan et al., 2015) can be correlated with hormone levels found in hair and hair-like samples. Therefore, life history relevant events could be used to perform physiological validations. Collectively, knowledge on growth rate and validations assure that a new sample type provides accurate, repeatable data that is informative of reproduction, stress, and energetics in free-living wildlife (Fernández Ajó et al., 2020; Fourie and Bernstein, 2011; Hunt et al., 2017a).

To date, the utility of mammalian spines (of any species) for endocrine analyses has not been explored. Moreover, no keratin tissue types have yet been tested for any monotreme. Thus, we set out to establish a protocol for extracting and quantifying hormones in spines that could be used with remote populations of *Tachyglossus aculeatus*, long-beaked echidnas (*Zaglossus spp.*), and could be generalized for other spine-bearing mammals. Specific goals were to (1) determine if steroid hormones are detectable in echidna spines using commercial enzyme immunoassay (EIA) kits, including evaluation of minimum sample mass necessary for adequate detectability; (2) perform assay validations (parallelism and accuracy) to assess whether EIAs can reliably quantify steroid hormones in echidna spine extract; (3) investigate spines for visible growth marks that might help elucidate growth rates (*e.g.,* spines

Fig. 1. Echidna spines. Spines from three different short-beaked echidnas. Differences in size are likely due to growth time and location in the body (A, B, C).

may cease or slow growth during annual hibernation); and (4) as a preliminary investigation of possible physiological validation, determine whether spines of males and females have different hormone content for any of the measured steroids. It is beyond the scope of a single study to address all possible validations; most physiological validations are not directly addressed in this study. Rather, our primary aim was to complete sufficient initial validations to evaluate whether echidna spines show potential as an endocrine sample type and warrant further investigation.

2. Methods

2.1. Study animals and sample collection

Echidna spines were obtained from either hand-captured or recently road-killed animals (4 male; 5 female) from October 2016 to April 2017 in southern Tasmania, Australia. For hand-captured echidnas, spines were obtained opportunistically during animal handling. Therefore, for hand-captured individuals we had as few as one spine available for some individuals, whereas for road-killed animals we had as many as 47 spines per individual. For some echidnas in this study, spines were harvested on more than one occasion, because these individuals were captured multiple times or were found deceased, which allowed for more complete spine collection. Animal trapping and manipulation was approved by the Animal Ethics Committee of the University of Tasmania (Permit: A0016112) and by the Tasmania Department of Primary Industries, Parks, Water and Environment (Collection permit: FA16336). Spines (Fig. 1) were sent to Northern Arizona University in Flagstaff, Arizona, USA where they were washed with soap and water, freeze-dried overnight, and stored in screw top glass jars at room temperature (Export permit: PWS2017-AU-000676 0; Import declaration: 2017ME2035080).

2.2. Hormone extraction

As spines were obtained opportunistically, they varied considerably in length and weight. Following the methods used in other studies with cornified samples in our laboratory (Dillon et al., 2021; Fernández Ajó et al., 2020; Hunt et al., 2017b), spines were pulverized with a hand-held rotary tool (Dremel Model 395, type 5) fitted with a flexible extension and a tungsten-carbide ball tip. Resultant powder was collected and weighed on a digital scale (±0.0001 g, Ohaus Explorer Pro EP214C, Pine Brook, NJ, USA). To avoid cross-contamination of samples, pulverization of individual spines was conducted in a chemical fume hood, with comprehensive cleaning between samples (washes of 70% ethanol of all tools and surfaces). During handling and weighing of the spine powder, a workstation ionizer (SPI No. 94000, SPIwesstek.com) was used to reduce the effects of static electric charge on apparent sample weight. To create a sample pool for assay validations, powder from entire spines of multiple females was mixed and subsampled, with three different sample masses tested for hormone detectability (100, 50, and 25 mg aliquots of powder). Only female spines were used for validating corticosterone and cortisol due to the small number of spines available from males. For progesterone, estradiol, and testosterone validations, two additional 50 mg pools were made for each sex. Sample masses of 50 mg of spine powder were used for subsequent analyses.

For each sample, entire spines from the same individual were pulverized and combined into the same test tube. Following methods used for extraction of other "firm" keratinized samples (Fernández Ajó et al., 2020; Hunt et al., 2017a; b), individual echidna spine samples and pools were extracted in 1 mL of 100% high performance liquid chromatography grade methanol per 20 mg of sample, shaken for 2 h using a multi-tube vortexer (Glas-Col Large Capacity Mixer, speed set on 65; Glas-Col, Terre Haute, IN, USA), and centrifuged for 15 min at 4 °C at 1056 × g. Supernatant was collected in 12x75 mm borosilicate glass tubes and dried down in a ThermoSavant SpeedVac SPD121P (Thermo Fisher Scientific, Waltham, MA, USA) under vacuum at 35 °C overnight. Dried samples were reconstituted in 500 μL of EIA assay buffer (buffer 'X065,' Arbor Assays, Ann Arbor, MI, USA), vortexed 1 h, and stored at 4 °C overnight. This reconstituted volume was chosen to ensure sufficient concentration to obtain acceptable detectability, while retaining good assay accuracy and adequate volume for multiple immunoassays.

2.3. Validations and hormone assays

Undiluted (neat, 1:1) reconstituted extracts were used for all validation assays. For tests of parallelism, neat extracts were serially diluted in EIA assay buffer (X065, Arbor Assays), producing eight dilutions: neat, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128. All diluted extracts were assayed in duplicate with commercially available enzyme immunoassay kits (Arbor Assays, Ann Arbor, MI, USA). The slope of the spine serial dilution binding curve (percentage-bound *vs.* log [relative dose]) was compared to the slope of the linear portion of the standard curve. Parallelism of the two lines demonstrates that the antibody binds well to the hormone of interest and unknowns can be accurately interpolated from the standard curve (Grotjan and Keel, 1996). To test for accuracy (aka "matrix effect"; Grotjan and Keel, 1996), a set of standards of known hormone content in assay buffer (concentration range specific to each assay kit) were spiked with the pooled spine sample (equal parts standard and pooled spine extract) and assayed alongside an unspiked set of standards. The apparent concentrations of the spiked standards were regressed against concentrations of the pure standards. In this study, a regression of pure standards against spiked standards with a slope of 0.7–1.3 (ideal slope = 1) was considered to demonstrate little to no "matrix effect", meaning that the spine extract components (keratin, etc.) are not significantly affecting assay performance (*i.e.*, mathematical accuracy), and that the assay has acceptable accuracy across the desired range of concentrations (Fernández Ajó et al., 2020; Hunt et al., 2017b). Parallelism tests were conducted for testosterone (Arbor Assays kit #K032), estradiol (Arbor Assays kit #KB30), progesterone (Arbor Assays kit #K025), corticosterone (Arbor Assays kit #K014), and cortisol (Arbor Assays kit #K003). Accuracy tests and hormone assays were conducted for testosterone, estradiol, progesterone and corticosterone, but not cortisol, as corticosterone was present in higher concentration in the spines and thus was considered the dominant glucocorticoid and of most interest. From the parallelism results, a dilution was selected for each hormone assay to keep results as near as possible to 50% bound, the area of greatest assay precision. The dilutions selected were 1:4 for progesterone in females and 1:8 in males, while corticosterone, estradiol and testosterone were assayed with undiluted sample extracts (1:1).

2.4. Histological examination of spines

Two methods were used to assess presence of growth lines that could be used to determine temporal linear growth patterns in echidna spines. Firstly, spines were scanned using electron microscopy (SEM) to visualize surface markings. Five whole spines were dehydrated in a graded ethanol series and then dried in a Pelco CPD-2 (Ted Pella Co., Redding, CA, USA) critical point dryer and mounted on aluminum stubs using carbon tape. Samples were then sputter-coated with gold palladium for one minute in a Denton Vacuum Desk II (Denton Vacuum, Moorestown, NJ, USA) and visualized with a Zeiss Supra 40VP SEM (Carl Zeiss SMT, Thornwood, NY, USA) at 5 kV. In addition, we paraffin-embedded and stained 10 spines. Prior to embedding, sectioning and staining, we developed a method to soften the keratinized cuticle using a modified protocol for softening keratin in hooves and nails (Luna, 1992). Briefly, spines were fixed for two days in 10% buffered formalin and transferred to potassium thioglycolate (Nair Body Hair Removal Cream; Church & Dwight Co, Ewing NJ, USA) for 7–10 days (duration depended on size of the spine). Softened spines were embedded in paraffin using a Shandon Citadel 2000 tissue processor (Thermo Shandon, Pittsburgh, PA, USA), sagitally sectioned at 20 μm, and stained using hematoxylin and eosin (H&E). Six sections were created from each spine — three from the cortex and three from the medulla. The sectioned spines were placed on positively charged glass slides and coverslipped. Slides were inspected on a compound microscope at 40x–400x for any repetitive markings. Specifically, we were looking for any indication of reduced spine growth that could be indicative of winter (hibernation period) when growth rates of hair are presumed either stopped or slower than growth rates in summer months (based on patterns seen in other hibernating species).

2.5. *Statistical analysis*

For tests of parallelism, differences between slopes of the binding curve of the serially diluted pool and the standard curve were assessed with an F-test statistic. Slopes not found to be significantly different at a level of $p < 0.05$ were considered sufficiently parallel. Accuracy was evaluated using a linear regression of the observed hormone concentration *vs*. the known standard concentration. In this study, we considered acceptable accuracy results are $r^2 > 0.95$ and slope between 0.7 and 1.3. All plots, parallelism and accuracy statistical analyses were carried out using GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Parallelism and accuracy plots (Figs. 2 and 3) show the data points for each dilution.

Sex differences were investigated with Student's *t*-test or Mann-Whitney *U* test, after assessing normality with Shapiro–Wilk tests in RStudio Version 1.2.1335 (RStudio team, Boston, MA). In the case of individuals that were sampled more than once, spines obtained from different capture events were assayed independently. However, to avoid pseudoreplication, hormone concentrations of spines harvested in different dates from the same individual were averaged. Therefore, for the sex comparisons (Fig. 4) there is only one data point per individual.

3. Results

3.1. *Validations*

Corticosterone, cortisol, progesterone, estradiol and testosterone were detectable in all spine extracts (25 mg, 50 mg and 100 mg). Based on validation results from pooled samples, corticosterone concentration (705.4 pg/g in the pooled extract) was generally higher than cortisol concentration (440.5 pg/g for the same pooled extract). Therefore, although corticosterone and cortisol have the potential to show different patterns (Koren et al., 2012), we decided to perform no further validations for cortisol. Binding curves of serially diluted spine extracts did not differ from the standard curve for any hormone tested, *i.e.*, parallelism was acceptable (Fig. 2, Table 1). Assay accuracy was acceptable for corticosterone, progesterone, estradiol and testosterone (Fig. 3, Table 1).

3.2. *Individual samples*

There were no visible growth lines either externally (SEM) or internally (H&E). Nevertheless, hormone concentrations were obtained for whole spines of individual echidnas. No significant differences were found between males and females for any of the hormones assayed (estradiol, testosterone, progesterone and corticosterone; Fig. 4).

4. Discussion

The use of mammalian spines as a sample for endocrine assessments could be a powerful resource to increase our understanding of monotreme endocrinology. Endocrine data could aid conservation by identifying populations vulnerable to stressors, assessing breeding cycles in the wild within and between populations, and improving captive

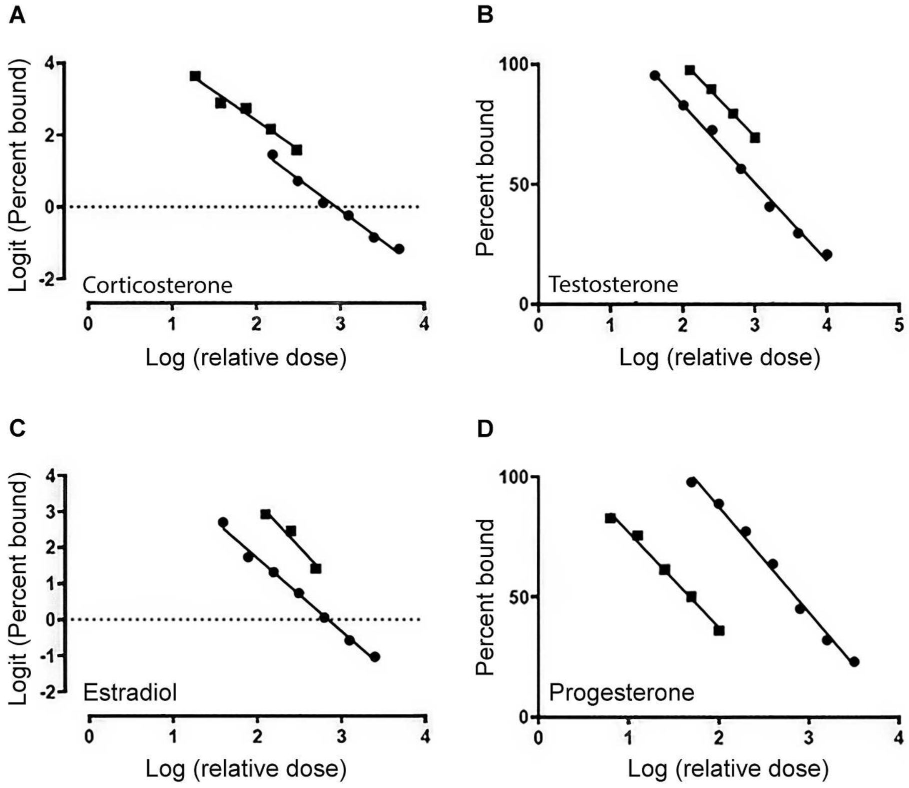

Fig. 2. Parallelism validation results for corticosterone (A), testosterone (B), estradiol (C) and progesterone (D) immunoassays tested with short-beaked echidna spines. Individual F-tests for parallelism are shown in table 1. All hormone assays tested demonstrate good parallelism, *i.e.*, the slopes of the binding curves of pure hormone standards (circles) and serial dilutions of spine extracts (squares) are not significantly different ($p > 0.05$)..

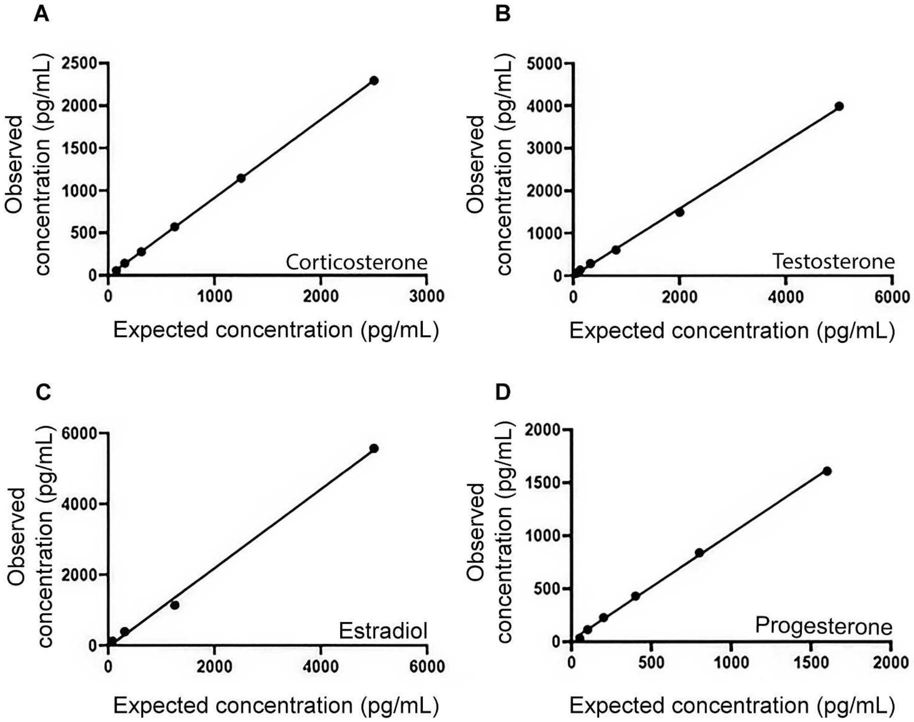

Fig. 3. Accuracy validation results for corticosterone (A), testosterone (B), estradiol (C) and progesterone (D) immunoassays tested with short-beaked echidna spines. Slope of observed dose (y-axis) *vs.* known standard dose (x-axis) is shown along with the r^2 of the linear regression line. All four immunoassays demonstrate good accuracy with spine extracts, *i.e.,* slope is close to 1.0 (acceptable range = 0.7–1.3) and r^2 is close to 1.00, indicating that the assay can quantify hormones in the spine extract with acceptable mathematical accuracy.

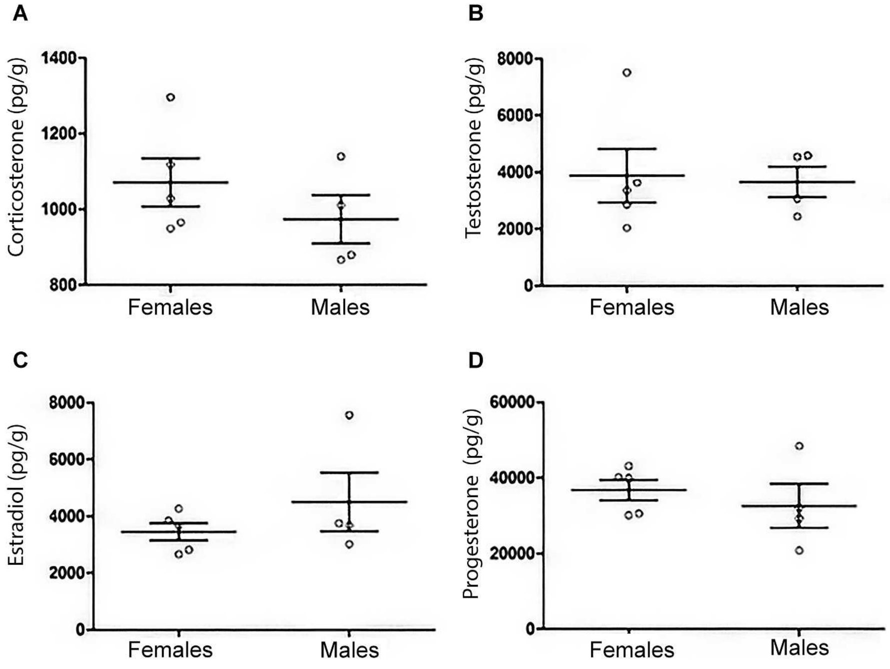

Fig. 4. Hormone concentrations in samples of each individual echidna expressed as pg of hormone per g of powdered spine. Inter-individual variation of average spine hormone content is evident for all four hormones. No significant differences were found between males and females for corticosterone (A), testosterone (B), estradiol (C) and progesterone (D) ($p > 0.05$).

breeding (Wingfield et al., 1997). However, when conducting investigations with a novel sample type, it is necessary to assure that hormones can be reliably quantified and that the patterns of deposition correlate with life history events (Koren et al., 2019). Our research team has previously performed some assay validations (*e.g.,* detectability, parallelism) for corticosterone in echidna spines (Dillon et al., 2021) in the context of a test of keratinase as potential method of extraction; to that initial analysis we now add validations for estradiol, progesterone, testosterone and corticosterone in echidna spines using a simpler methanol extraction, and we have also presented the first comparison of hormone content between the sexes. To our knowledge, these are the first data published on gonadal steroid hormones of any keratinized sample type from monotremes.

We found that adrenal and gonadal steroid hormones are present and

Table 1

Parallelism and accuracy results for echidna spines. F-statistics and p-values indicate no significant difference between the slope of the standard curve and the serial dilution from the echidna pool sample ($p > 0.05$). The slopes of the linear regressions (standards spiked with echidna spine extract *vs.* unspiked standards) fall within the acceptable range (0.7–1.3) and r^2 is close to 1.00 for all hormones tested. These results show that the protocols used in this study can reliably assess concentrations of corticosterone, estradiol, progesterone, and testosterone in echidna spine extract.

	Parallelism	Accuracy
Corticosterone	$F_{1,7} = 0.38$ $p = 0.56$	Slope = 0.92 $r^2 = 1.00$
Testosterone	$F_{1,7} = 0.13$ $p = 0.72$	Slope = 0.79 $r^2 = 0.99$
Estradiol	$F_{1,6} = 1.75$ $p = 0.23$	Slope = 1.14 $r^2 = 0.99$
Progesterone	$F_{1,8} = 1.82$ $p = 0.21$	Slope = 1.00 $r^2 = 0.99$

detectable in echidna spines extracted with a simple methanol extraction method, and that corticosterone, estradiol, progesterone, and testosterone can be reliably measured in echidna spines from both males and females using commercially available EIAs. Hormones were detectable in all sample masses tested, and in a single 500 μL extract produced from 50 mg of powder concentrations were high enough to enable assays of all four hormones. Using this extraction protocol, no samples were below the detectability limit of any assay, and no samples fell outside the range of the standards of any assay (Table 2). Although 25 mg is a viable sample mass for assay of one or two hormones, our results suggest that that the lower hormone concentration in the resulting extract may preclude assay of all four hormones.

Parallelism and accuracy tests indicate that EIAs can reliably quantify concentrations of corticosterone, estradiol, progesterone, and testosterone in echidna spine extract with acceptable binding affinity (indicated by good parallelism results) and accurate discrimination between low and high concentrations (indicated by good accuracy results) with minimal matrix effects. Although cortisol was also detectable in echidna spines, validations were only completed for corticosterone, as the latter was found in higher concentrations.

As observed in other non-plasma vertebrate sample types, gonadal steroids were found in higher concentrations than adrenal steroids in echidna spines (Hunt et al., 2018). Irrespective of sex, progesterone was found in highest concentrations, followed by estradiol, testosterone, corticosterone and cortisol; this general pattern matches that observed in baleen (Hunt et al., 2017a). Further, these differences in relative concentration match general patterns observed in other sample types from echidnas. For example, in echidna feces, fecal metabolites of progesterone are higher in concentration than fecal metabolites of other gonadal hormones (Dean, 2000), and in echidna plasma, corticosterone is higher in concentration than cortisol (Sernia and McDonald, 1977). Thus, hormone content of spines might indeed be reflective of concentrations of hormones circulating in plasma, although direct comparisons of plasma to spine remain to be performed.

No visible growth lines were evident in our histological examinations of echidna spines. This finding suggests either that growth is continuous, without seasonal cessation or slowing of growth, or that growth rate for echidna spines varies seasonally, but changes in growth rate fail to leave visible marks in the keratin. Because of the apparent lack of annual or seasonal growth marks on the spine, estimation of the timeline represented in each spine will likely have to rely on extrapolations from an average spine growth rate; however, growth rate of echidna spines has not yet been precisely determined. Based on the regrowth of spines trimmed for attachment of tracking transmitters, the growth rate appears to be approximately 3–6 mm a year (Nicol pers. obs.), but this estimate needs to be confirmed by spine-marking techniques. Nonetheless, since the longest spines on the Tasmanian subspecies are ~ 60 mm, this field-based estimate suggests that one spine may contain roughly 10–20 years of growth. Given this rough estimate and our finding that 25 mg of powder is a viable sample mass, in a spine of typical width and density it is possible that annual increments of spine growth could be tested as separate samples. Future efforts should be made to determine growth rate using other methods, such as marking spines of individually known echidnas and monitoring spine growth over time. Further adjustment of extraction techniques may also improve hormone extraction, enabling analysis of reduced sample mass and thus finer temporal resolution.

Commercially available EIAs offer sufficient resolution to capture differences between individuals. Still, no differences in hormone concentration between sexes were detected (Fig. 4). Although this is not unexpected for adrenal steroids, lack of sex differences in gonadal steroids is perplexing given that sex differences are typically detected for these hormones in mammals. The small sample size likely resulted in low statistical power for this comparison (*i.e.,* possible Type II error) but, even in cases of Type II error, there are typically non-significant trends that are apparent for gonadal steroid concentrations between sexes, which is not the case for this dataset (Fig. 4). Therefore, our study provides no evidence that the hormone concentrations in echidna spines are biologically relevant. It is possible that kit antibodies may be detecting other hormones or metabolites present in the spines (*i.e.*, cross-reactivity). We suggest future studies use mass spectrometry to verify the chemical identity of immunoreactive hormones and metabolites present in spine powder. Lack of sex differences could also be due, in part, by the timeframe our samples represent. For this study, entire spines were pulverized and extracted. Because we lack biological validations, we are unable to determine the timeframe for hormone deposition and thus the extract could contain the average hormone content of the entire spine growth-period, perhaps as long as 10 years. It is possible that during the growth period of an entire spine, the sexes may have similar overall average concentrations for gonadal steroids. For example, progesterone is only above basal for the luteal phase of pregnancy, which is 15 days long (Dutton-Regester et al., 2021), and females

Table 2

Specifications of immunoassay kits (Arbor Assays, Ann Arbor, MI, USA) used to quantify hormones in echidna spines, and range of detected concentrations for diluted extracts of 50 mg samples tested in this study. Note that all sample concentrations fall within the ranges of the standards. Standard range, detection limit and cross-reactivities are provided by the manufacturer.

	Standard range (pg/mL)	Detection limit (pg/mL)	Range detected in samples (pg/mL)[a]	Cross-reactivities
Progesterone (K025)	50–3200	52.9	256.97–1001.5	Hydroxyprogesterones 3α 188%, 3β 172%, 11α 147%; Other steroids < 7%
Testosterone (K032)	41–10000	30.6	183.22–1870.16	Dihydrotestosterone 56.8%; other steroids < 5%
17β-Estradiol (K030)	39–10000	26.5	184.34–748.53	Other steroids < 5%
Corticosterone (K014)	39–10000	17.5	66.85–157.71	1-dehydrocorticosterone 18.9%, Desoxycorticosterone 12.3%; Other steroids < 5%

[a] Values interpolated for each sample from the standard curve, reported here as pg of hormone per mL of diluted extract to enable comparison to standard concentrations. Further calculations were done to convert to pg of hormone per g of spine powder (pg/g) in Fig. 4.

do not breed every year, so the overall effect of a pregnancy on the average progesterone concentration for an entire spine may be too small to detect. Moreover, progesterone is the precursor of all steroid hormones and may be elevated in males when undergoing reproductive maturation or activation, and some studies indicate that progesterone may have important functions of its own in male mammals (Andersen and Tufik, 2006; Luetjens et al., 2006; Schumacher et al., 2014). With respect to testosterone, adult males breed nearly every year and plasma testosterone is above basal for about 10 weeks per year (Morrow et al., 2016), making it more likely that a sex difference would be detectable. However, circulating androgens can be aromatized to estrogens (Ishikawa et al., 2006) and thus it is possible that some amount of testosterone is converted to estradiol before or as it is incorporated into the spine. This would result in decreased average testosterone concentration and increased estradiol in male spines and possibly eliminate sex differences in content of these hormones. Another possible explanation for the lack of differences between sexes is the presence of germinative tissue in the bulb of the spines which could affect the hormone concentrations measured, since in some mammals, epithelial tissues have steroidogenic capacity (Ito et al., 2005; Nikolakis et al., 2016). Analysis of serial sections of individual spines along with studies of spine growth rate should indicate whether echidna spines can provide biologically relevant information on hormones.

5. Conclusions

Our work shows that steroid hormones are detectable in echidna spines using commercially available EIAs. All assays passed standard assay validations for parallelism and accuracy with echidna spine extract. However, we have no evidence that steroid hormone levels in echidna spines are biologically relevant, which can only be shown through physiological validations. Future studies should seek to determine spine growth rates, followed by comparisons of year-long or half-year increments of spine growth between sexes and across time within individuals. These questions could be pursued both in the wild, *e.g.,* marking of spines and recapture to measure spine growth, as well in captive studies. In this way, captive studies could be especially valuable for elucidating rate of spine growth, stability of growth rate across seasons, and persistence of the spines over time, as well as to investigate correlations of reproductive hormones and glucocorticoids with reproductive state and stress history of known individuals. This knowledge will allow future studies to perform physiological validations, *i.e.,* matching parts of a spine or complete spines with biologically relevant events (*e.g.,* hibernation, reproduction, stress), thus facilitating retrospective elucidation of life histories of this species and potentially other cryptic, threatened, or even extinct species. A rich and valuable source of samples of keratinized tissues exists in museums, at universities, and in private collections, which could be used for both validations and for addressing conservation and other biological questions.

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CRediT authorship contribution statement

Fabio Braga Ana: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **E. Hunt Kathleen:** Writing – review & editing. **Dillon Danielle:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. **Minicozzi Michael:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. **C. Nicol Stewart:** Resources, Writing – review & editing. **Buck C. Loren:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2022.114053.

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