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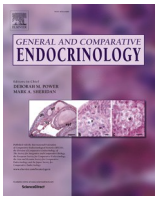

Non-invasive monitoring of adrenocortical activity in the Gould's wattled bat (*Chalinolobus gouldii*)

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ABSTRACT

Although bats are the second most species-rich mammalian order, very little is known about their endocrine physiology. Glucocorticoids (GCs) are commonly associated with the stress response, but also modulate vital physiological functions which help animals adapt to their environment. Understanding normal patterns of adrenocortical activity can provide valuable insights into a species' fitness. Non-invasive hormone monitoring via faecal samples provides an integrated measure of adrenocortical activity while minimising stress on the animal but must be properly validated to ensure reliable results. The goal of this study was to validate an enzyme immunoassay for monitoring faecal glucocorticoid metabolites (FGMs) in a common Australian insectivorous bat species, the Gould's wattled bat (*Chalinolobus gouldii*). We compared the performance of five assays for monitoring changes in FGMs following capture and transfer of *C.gouldii* from the wild to captivity. Four of the five assays detected a significant increase in FGMs following capture, but the magnitude of the increase and consistency across individuals differed considerably. We selected the UVM-69a assay as the best performing assay to then describe normative patterns of adrenocortical activity in the species. Males had higher FGM levels than females, and juveniles had higher FGM levels than adults. Individuals with poorer body condition had higher FGM levels. We also demonstrate seasonal patterns of FGMs with higher levels in March and April corresponding with reproductive up-regulation and lower levels in May and November. Our study is the first of its kind to examine adrenocortical activity in an Australian insectivorous bat and provides a valuable tool for studying this species. Understanding adrenal function in common species such as *C.gouldii* can shed light on the physiological mechanisms facilitating survival and success in changing environments.

1. Introduction

With over 1400 described species (Burgin et al., 2018), bats (Chiroptera) are the second most species-rich mammalian order, comprising nearly one-third of all nocturnal mammals (Mathews et al., 2015). Simultaneously, bats are one of the most understudied mammals, especially pertaining to their physiology. Bats exhibit several unique life history traits compared to other small mammals, such as long lifespan, delayed sexual maturity, and small litter sizes (Racey and Entwistle, 2000). They act as keystone species within ecosystems by dispersing seeds, pollinating flowers, and reducing pest insect abundances (Vanitharani, 2014). A growing number of bat species are facing extinction due to climate change and urbanisation, with the most recent extinction in Australia occurring in 2009 (Mendoza et al., 2019). Whether an individual can adapt to these environmental changes is largely determined by variation in biological fitness. Therefore, understanding the physiological processes that influence reproduction and survival in bats could help inform conservation approaches.

Glucocorticoids (GCs; e.g. cortisol and corticosterone) are key hormones responsible for regulating circadian rhythms, energy allocation, metabolism, immune function, reproduction, and the physiological stress response (Androulakis, 2021; Oster et al., 2017) and are also thought to shape life history traits (Crespi et al., 2013). GCs are secreted by the adrenal gland, modulating behavioural and physiological functions that are crucial for maintaining homeostasis. When an organism experiences a real or perceived threat to homeostasis, GC production

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surges in an attempt to re-establish equilibrium (Sapolsky et al., 2000). GC levels can exhibit considerable inter- and intra-specific variation due to both intrinsic (i.e. age, sex, life history state, body condition, and behaviour) and extrinsic factors (i.e. season, resource availability, population dynamics, and predation; Crespi et al., 2013; Hau et al., 2016). Failing to account for these factors often leads to inaccurate interpretation of physiological stress (Busch and Hayward, 2009). Identifying the factors that influence GC secretion in bats will not only enhance our knowledge of bat physiology but will also allow more accurate interpretation of their physiological response to environmental changes.

Blood is the most common sampling method for monitoring adrenocortical activity in bats (Allen et al., 2011; Edwards et al., 2022b; Gustafson and Belt, 1981; Lewanzik et al., 2012; Meniri et al., 2020; O'Mara et al., 2017; Reeder et al., 2006, 2004; Richardson et al., 2018). While blood is useful in detecting immediate changes in GCs by providing a snapshot of circulating levels, it also has limitations. Blood sampling is generally highly invasive (although see Arnold et al., 2008; Becker et al., 2006 for non-invasive techniques), requiring restraint and/or often anaesthesia; consequently, samples are limited in both volume and frequency. Additionally, blood must be collected quickly to prevent handling-induced changes to circulating GC levels (Touma and Palme, 2005). In contrast, non-invasive sampling techniques such as faeces collection are ideal for minimising stress while monitoring adrenocortical activity across a longer timeframe. GCs circulate in the plasma, are metabolised in the liver and excreted as metabolites in urine and faeces, albeit with a certain delay (Oster et al., 2017; Palme, 2019). Faecal glucocorticoid metabolites (FGMs) provide a pooled measure of circulating GCs, offering an integrated measure of adrenocortical activity (Touma and Palme, 2005; Palme, 2019). Monitoring faecal hormone metabolites allows for repeated sampling to detect longitudinal fluctuations in hormone secretion that influence physiological function. However, few studies to date have utilised non-invasive methods for bats (Edwards et al., 2022a; Freeman et al., 2018; Hernández-Arciga et al., 2020; Kelm et al., 2016; Lobato-Bailón et al., 2023; Parry-Jones et al., 2016; Sandoval-Herrera et al., 2021), among which only Kelm et al. (2016) and Freeman et al. (2018) provided a validation.

Faecal hormone metabolite assays must be properly validated to ensure reliable results (Palme, 2019). Patterns of steroid metabolism can vary by species and sexes, resulting in different GC metabolites being excreted (Palme et al., 2005). Because different assays recognise different groups of metabolites, it is important to compare multiple assays to identify the most suitable one for detecting biologically relevant changes in GCs for each species (Touma and Palme, 2005). Physiological validation is achieved by stimulating the adrenal glands to produce elevated GC levels through an adrenocorticotropic hormone (ACTH) challenge, or a biological validation is performed with exposure to a stressful event (i.e. capture, transport, and handling; Touma and Palme, 2005; Palme, 2019). The most suitable enzyme immunoassay (EIA) for monitoring FGMs will show the greatest magnitude of response, and therefore the greatest sensitivity for detecting adrenocortical changes in a species (Fanson et al., 2017).

The aim of this study was to establish foundational knowledge of adrenal physiology in an ecologically significant bat species, the Gould's wattled bat (*Chalinolobus gouldii*). *C. gouldii* is a common insectivorous bat species widespread throughout most Australian landscapes, including urban environments (Lumsden et al., 2023). As a disturbance-adapted generalist, *C. gouldii* dominates artificial roost box occupancy (Bender and Irvine, 2001; Evans and Lumsden, 2011; Griffiths et al., 2017; Rueegger et al., 2019), enabling accessible faecal sample collection for non-invasive monitoring. We compared the performance of five EIAs for monitoring adrenocortical activity non-invasively in *C. gouldii* by measuring the biological response of wild bats to capture. We then characterised normative patterns of adrenocortical activity using the most sensitive assay for *C. gouldii* across factors previously documented to influence GC secretion in other species: age, sex, season, body condition, and reproductive status. Understanding adrenal function in *C. gouldii* may help explain the physiological processes that support their ability to thrive and adapt in changing habitats.

2. Materials and methods

2.1. Biological validation of an enzyme immunoassay

2.1.1. Study animals

To biologically validate an EIA for monitoring adrenocortical activity non-invasively, we utilised *C. gouldii* that were captured from the wild, transported, and temporarily held in captivity for another study. A total of 27 individuals were collected from artificial roost boxes in the Nangak Tamboree Wildlife Sanctuary (NTWS; Bundoora, Victoria, Australia) and individually placed into calico bags shortly (65 ± 35 min) after sunrise. All animals were collected on the same morning in May 2021.

Bats were transported a short distance (1–2 km) to the La Trobe University Zoology Reserve (Bundoora, Victoria, Australia) for processing. They were checked for an existing microchip or wing band from a long-term capture-mark-recapture program (Griffiths et al., 2018). Those without bands were banded with a metal-alloy bat-band (Australian Bird and Bat Banding Scheme) containing a unique identification number. Using standard methods (Churchill, 2008), the following information was recorded for each individual: sex, age (adult or juvenile), reproductive status (testes morphology for males and teat morphology for females), weight, and forearm length. For reproductive status, female bats were defined as either preparous (having not previously reared young) if the teats were very small with no bare patches of fur, or post-lactating (previously weaned young) if teats were elongated and contained bare skin around the nipple. Male reproductive status was based on testes morphology: testes internalised (non-reproductive & pre-sperm production), testes enlarged (sperm production), testes enlarged and epididymis distended (sperm production & storage), and testes regressed and epididymis distended (sperm storage). Bats were held in individual calico bags for the duration of the validation study. Animals were hand-fed mealworms and water until satiated before release at the start of the active period the following evening.

Approval to conduct this study was granted by the La Trobe University Animals Ethics Committee (AEC-20036) and the Department of Environment, Land, Water and Planning (permit number 10009920).

2.1.2. Faecal sample collection and steroid extraction

The physiological response of *C. gouldii* to capture was measured by collecting faecal samples excreted during three different timeframes post-capture: 0–4 hrs (baseline), 4–10 hrs, and 10–16.5 hrs. At the conclusion of each timeframe, calico holding bags were checked and all faecal material was collected from the bag. Therefore, faecal samples were collected 0–6.5 hrs after defecation. Gut passage time in this species ranges from 103 to 172 mins (Walker et al., 2019), consequently the first timeframe (0 – 4 hrs) was considered baseline. However, samples produced later in this timeframe may not represent true baseline (Kelm et al., 2016), therefore the actual increase from true baseline may be higher than calculated here.

From the 27 individuals, 11 individuals did not provide samples during all three collection windows and were excluded from the biological validation. Samples from the remaining 16 individuals (8 males, 8 females) were analysed. Faecal samples were collected in 1.5 ml centrifuge tubes labelled with each animal's unique microchip or bat-band ID, sample number, and collection time and immediately stored at −20 °C.

To extract the steroids from the faeces, 0.03 ± 0.001 g of wet faecal material was weighed into a 1.5 ml centrifuge tube and mixed with 1 ml of 80% ethanol. Samples were vortexed and placed on a rocking shaker overnight (20 ± 1 hrs). The following day, samples were centrifuged for 5 mins at 5,000 RCF and the supernatant was decanted into a new tube

and stored at −20 °C until analysis (Palme et al., 2013).

2.1.3. Glucocorticoid metabolite assays

Five EIAs were compared to determine the most suitable assay for detecting biologically relevant changes in adrenocortical activity in *C. gouldii* (Table 1). Each assay procedure followed previously published protocols.

Assays were biochemically validated in our lab by demonstrating parallelism between serial dilutions of a faecal extract pool and the standard curve. Separate sample pools were run for males and females alongside respective standard curves. The average intra-assay precision for each assay (based on low and high controls; $n = 12$ each) was as follows: CJM006 = 8.1%; ISWE002 = 3.23%; UVM-F = 6.2%; UVM-69a = 5.6%; UVM-72a = 5.8%. All statistical comparison were made between samples run on the same plate.

2.1.4. Statistical analysis

All statistical analyses were performed using R v. 4.1.0 (R Development Core Team, 2014).

To identify the most suitable EIA for adrenocortical monitoring in *C. gouldii,* we compared FGM concentrations in samples provided during the three different timeframes post-capture: (1) 0–4 hrs, (2) 4–10 hrs, and (3) 10–16.5 hrs. To determine whether capture had a significant effect on FGM levels, we fitted a linear mixed model using the R package *lme4* (Bates et al., 2015). For each assay, log-transformed FGM concentrations were modelled as a function of sample number. Separate models were run for males and females, and animal ID was included as a random effect to account for repeated sampling from individuals. Assumptions of normality and heterogeneity of variance were checked. Dunnett's tests were used for post hoc pairwise comparisons between baseline (sample 1) and stress-induced (samples 2 and 3) FGM levels using the R package *emmeans* (Lenth et al., 2018).

To further evaluate assay performance and account for individual variation, we also assessed individual-level metrics. Assay sensitivity was calculated as the magnitude of the adrenal response (fold increase from baseline (sample 1) to stress-induced (samples 2 and 3) FGM levels). Assay consistency was determined as the number of individuals that exhibited >3-fold increase in FGM concentration for at least one of the stress-induced samples.

2.2. Characterisation of adrenocortical activity patterns

2.2.1. Study animals

To characterise normative patterns of adrenocortical activity in *C. gouldii*, faecal samples were collected opportunistically from bats during routine roost box checks at adjacent reserves (<1.5 km apart): NTWS and Gresswell Nature Conservation Reserve (GNCR; Macleod, Victoria, Australia). All samples were collected on the same day during November 2013 and April 2014 at GNCR and March 2014 at NTWS. Samples collected in May 2021 for the biological validation previously outlined (see section 2.1) were also used in the seasonal comparison. At each sampling event, bats were collected from existing roost boxes and placed in calico bags. Bats were processed as described above (see section 2.1.1), including checking for an existing microchip or bat-band, sexing, aging, weighing, measuring and recording reproductive status (Churchill, 2008; Griffiths et al., 2018).

Approval to conduct this study was granted by the La Trobe University Animals Ethics Committee (AEC-13–30) and the Department of Environment, Land, Water and Planning (permit number 10006790).

Table 1
Assay details for the five enzyme immunoassays compared in the biological validation.

Source	Assay code	Immunogen	Reference/ link
Coralie Munro (*University of California, Davis, California, USA)*	CJM006	Corticosterone-3-CMO:BSA	Watson et al., 2013
Arbor Assays (*Ann Arbor, Michigan, USA)*	ISWE002	Cortisol-3-CMO:BSA	https://www.arborassays.com/product/cortisol-iswe-mini-kit/
Rupert Palme (*University of Veterinary Medicine, Vienna, Austria*)	UVM-F	Cortisol-3-CMO:BSA	Palme and Möstl, 1997
	UVM-69a	11-β-hydroxyaetiocholanolone-17-CMO:BSA	Frigerio et al., 2004
	UVM-72a	11-oxoetiocholanolone-3-HS:BSA	Palme and Möstl, 1997

2.2.2. Faecal sample collection and steroid extraction

At each sampling event, faecal samples were collected from individual calico holding bags 0–7 hrs after defecation and stored at −20 °C. A total of 176 faecal samples were opportunistically collected. Of these, 7 samples were collected from GNCR during November 2013, 90 samples from GNCR in April 2014, and 79 from NTWS in March 2014. All samples were extracted using the previously described methodology (see section 2.1.2).

2.2.2.1. Glucocorticoid metabolite assays. The biological validation identified UVM-69a as the most suitable assay for monitoring adrenocortical activity in male and female *C. gouldii* and was used to analyse the remainder of the samples. Final FGM concentrations are expressed as nanogram of steroids per gram of wet faeces (ng/g). Recovery for UVM-69a was greater than 90% across all concentrations with a strong positive relationship between observed and expected concentrations in both sexes (male: $r^2 = 0.997$; female: $r^2 = 0.998$). Inter-assay coefficients of variation for UVM-69a were 7.27% and 3.86% for high and low controls respectively.

2.2.2.2. Statistical analysis. To characterise normative patterns of adrenocortical activity in *C. gouldii* we fitted a linear model using the R package *lme4* (Bates et al., 2015). We modelled FGM concentration as a function of sex, age (adult or juvenile), month of sample collection and body condition. This analysis included all samples collected in 2013 and 2014 ($n = 176$), as well as baseline samples collected during the biological validation in 2021 ($n = 22$). Body condition scores were calculated for males and females separately using the residuals of an ordinary least squares regression of individual body weight and forearm length (Eastick et al., 2020; Schulte-Hostedde et al., 2001).

To examine the effect of male reproductive status on FGMs we fitted a linear model using the R package *lme4* (Bates et al., 2015). FGM concentrations were modelled as a function of testes morphology (internal testes, enlarged testes, enlarged testes & epididymis distended, testes regressed & epididymis distended) and month of sample collection. Age was confounded with reproductive status (all individuals with testes internalised were juveniles, and all with testes regressed were adults) and was therefore excluded from the model. A similar analysis could not be run for females because reproductive status was confounded with age (only adults were post-lactating).

Assumptions of normality and heterogeneity of variance were checked for each model. FGM concentrations were log-transformed prior to analysis to meet model assumptions. Post hoc comparisons were made using the R package *emmeans* (Lenth et al., 2018).

3. Results

3.1. Biological validation of an enzyme immunoassay

There was a significant increase in FGMs from baseline to stress-

induced samples following capture for all assays except CJM006 (Fig. 1; Table 2). However, the magnitude of increase and consistency of response varied between assays and sexes. For females, UVM-F had the highest average magnitude increase (4.5-fold), followed by ISWE002, UVM-69a and UVM-72a, which all had similar sensitivity (~3.6-fold increase). The UVM-69a assay showed the most consistent performance (7 of the 8 females had a >3-fold increase), followed by ISWE002 and UVM-F (6 of 8 females). For males, ISWE002 had the highest average magnitude increase (4.99) followed by UVM-69a (4.61). Peaks >3-fold higher than baseline were detected in six of eight males using the UVM-F assay and five males using the UVM-69a assay.

None of the assays emerged as the consistent winner across all three metrics for both sexes. Integrating all the results, both UVM-F and UVM-69a were the best assays for monitoring FGMs in *C. gouldii*. The UVM-F assay was more sensitive for females but was less consistent at detecting responses across all females. Conversely, UVM-69a was more sensitive for males, but UVM-F was more consistent across individual males. Since UVM-69a demonstrated high magnitude increases and detected peaks in most individuals for both sexes, this assay was selected for all further analyses. However, UVM-F and ISWE002 would also be suitable assays for analysing FGMs in *C. gouldii*, while CJM006 assay was the least sensitive and is therefore not suitable.

3.2. Characterisation of adrenocortical activity patterns

3.2.1. Population level patterns

FGM concentrations varied significantly with sex, age, time of year (Fig. 2) and body condition (Fig. 3). Males had significantly higher FGMs than females, and juveniles had significantly higher FGM concentrations than adults (sex: $F_{1,191} = 13.87$, $p = <0.001$; age: $F_{1,191} = 32.54$, $p = <0.001$). FGMs also varied significantly across the year ($F_{3,191} = 41.85$, $p = <0.001$). Adrenocortical activity was significantly higher for both sexes in March and April (breeding season) compared to May and November. FGM concentrations showed a significant negative correlation with body condition in which individuals with the lowest body condition scores had the highest FGMs ($F_{1,191} = 4.1$, $p = 0.044$).

3.2.2. Patterns in males

FGMs in male *C. gouldii* varied significantly with time of year ($F_{3,82} = 39.6$, $p = <0.001$) but not reproductive status ($F_{3,82} = 1.12$, $p = 0.35$; Fig. 4). The highest average (± SEM) FGM concentrations were observed when males were non-reproductive and had internalised testes (1768.76 ± 316.44 ng/g), while the lowest concentrations occurred when males were storing sperm and had regressed testes & epididymis distended (1303.45 ± 288.27 ng/g).

4. Discussion

GCs modulate vital physiological processes that facilitate survival and success. Understanding normative adrenal function can provide valuable insights into a species' biology and provide an essential foundation for stress physiology studies. In this study, we validated a non-invasive tool for faecal glucocorticoid metabolites in *C. gouldii* and characterised patterns of adrenocortical activity with respect to sex, age, month, body condition, and male reproductive state. This study is the first of its kind to examine adrenocortical activity in an Australian insectivorous bat, and one of a small number of studies monitoring GCs non-invasively in bats.

4.1. Biological validation of an enzyme immunoassay

It is imperative that non-invasive methods of hormone measurement be rigorously validated before application in each species (Palme, 2019). However, only two previous studies monitoring GCs non-invasively in bats have included a validation (Freeman et al., 2018; Kelm et al., 2016). Therefore, we exposed wild-caught *C. gouldii* to an environmental stressor to compare the suitability of five EIAs in detecting biologically relevant changes in FGMs. Four of the five assays (all except CJM006) detected a notable increase in FGMs in response to capture. However, further analyses revealed sex-based discrepancies in assay performance. Differences in GC metabolism, excretion and the types of metabolites formed are common and well described, often resulting from diet, individual or sex differences (Palme, 2019; Palme et al., 2005; Touma et al., 2003). To combat these differences, group-specific EIAs (such as UVM-69a used here) which detect a variety of faecal GC metabolites can

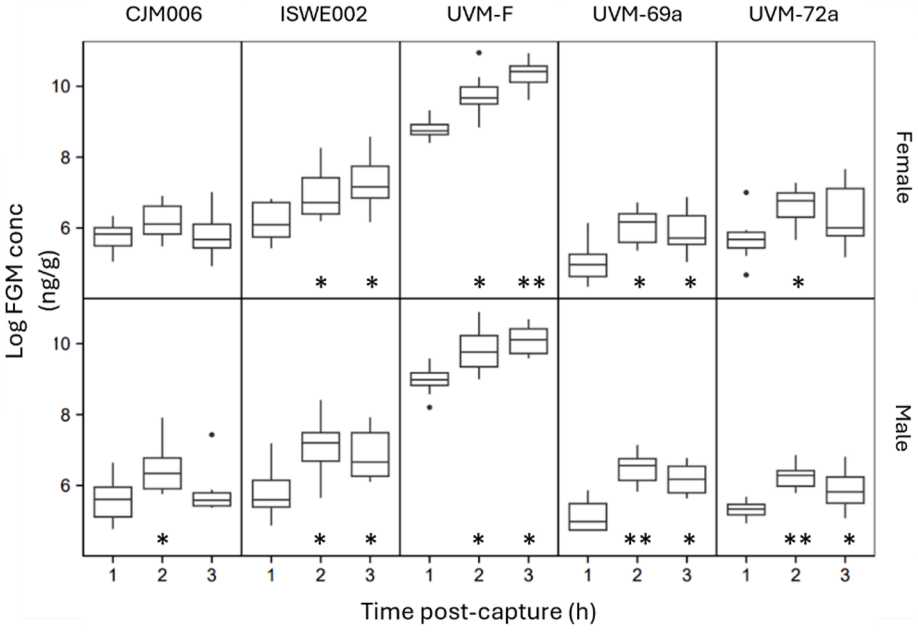

Fig. 1. Comparison of assay performance for monitoring adrenocortical response to capture in female ($n = 8$) and male ($n = 8$) *C. gouldii*. Samples were collected at the conclusion of three excretion windows post-capture: (1) 0–4 hrs (baseline), (2) 4–10 hrs, and (3) 10–16.5 hrs. Asterisks indicate a significant increase in FGMs from baseline levels based on Dunnett's post-hoc tests (* = $p < 0.05$; ** = $p < 0.001$).

Table 2
Performance of enzyme immunoassays used to monitor FGMs in female ($n = 8$) and male ($n = 8$) *C. gouldii* in response to capture and temporary captivity. *F*-values were obtained from the linear mixed model (* = $p < 0.05$, ** = $p < 0.001$; $df = 2$, 21 females and 20 males). "Magnitude" shows fold increase from baseline to stress-induced samples. "Consistency" provides the number of individuals with a >3-fold increase for at least one stress-induced sample. The selected assay is indicated in bold.

	Female			Male		
Assay	*F*-value	Magnitude	Consistency	*F*-value	Magnitude	Consistency
CJM006	2.08	1.52	0	3.41	3.37	4
ISWE002	6.77*	3.65	6	7.19*	4.99	4
UVM-F	20.59**	4.51	6	9.29*	3.15	6
UVM-69a	**7.42***	**3.53**	**7**	**19.88****	**4.61**	**5**
UVM-72a	3.65*	3.60	5	13.84**	2.37	3

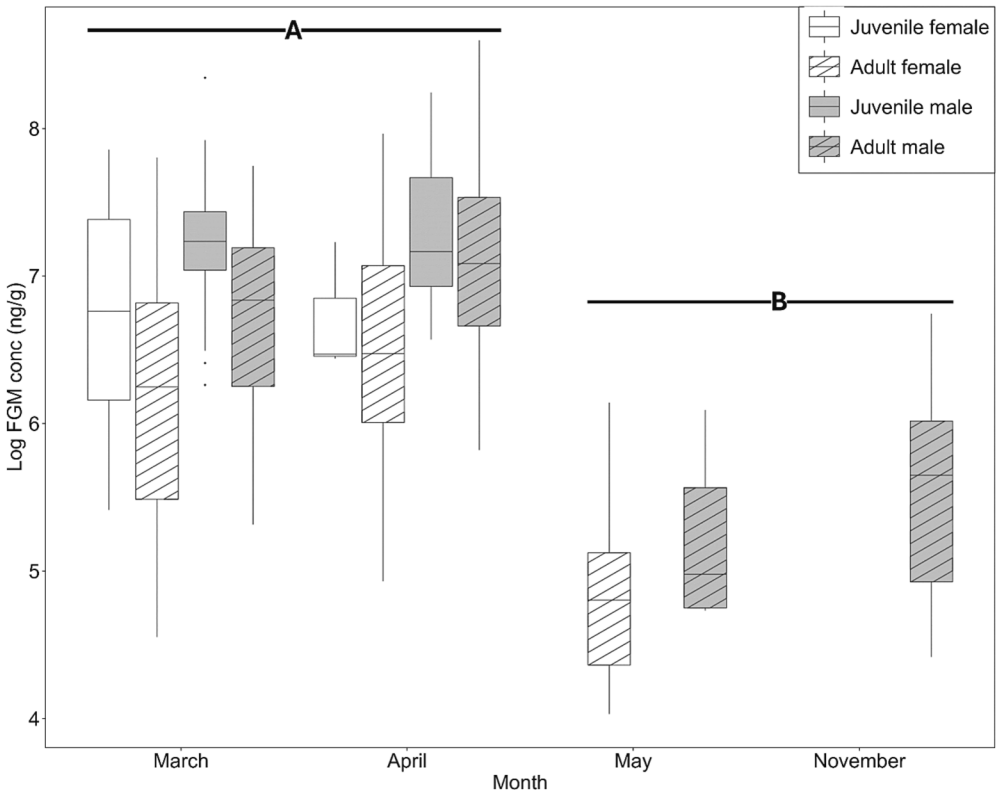

Fig. 2. Seasonal patterns of faecal glucocorticoid metabolite (FGM) concentrations in female (juvenile: $n = 25$; adult: $n = 84$) and male (juvenile: $n = 30$; adult: $n = 59$) *C. gouldii*. Letters indicate a significant difference between groups ($p < 0.01$).

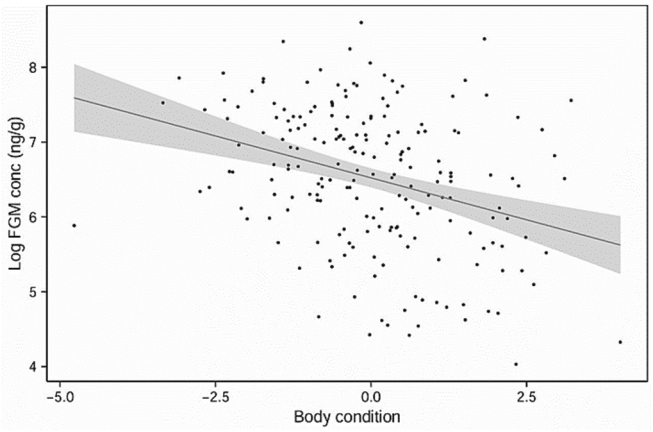

Fig. 3. Relationship between body condition and faecal glucocorticoid metabolite (FGM) concentrations in *C. gouldii*. Each point indicates an individual bat. Grey shading represents 95% confidence intervals.

be used (Palme, 2019). Group-specific EIAs generally provide superior performance and have been successfully validated for a number of mammal species (Palme, 2019). Here, UVM-69a was the only assay to have consistently high sensitivity and consistency between individuals for both sexes. Consequently, UVM-69a was identified as the most suitable assay to monitor adrenocortical activity in male and female *C. gouldii*. However, if only one sex is being monitored, then our results suggest that the assay with the highest sensitivity for the sex of interest can be used. Our results further highlight the importance of comparing several EIAs to account for individual and sex-based differences and ensure adequate validation of non-invasive hormone monitoring techniques.

4.2. Characterisation of adrenocortical activity patterns

Males consistently had higher FGM levels than females, indicating sex differences in GC production and/or metabolism in *C. gouldii*. Sex differences can arise in both the circulating levels of GCs and the metabolism of circulating GCs into faecal metabolites (Touma and Palme, 2005; Palme et al., 2005). Sex differences in GC levels are

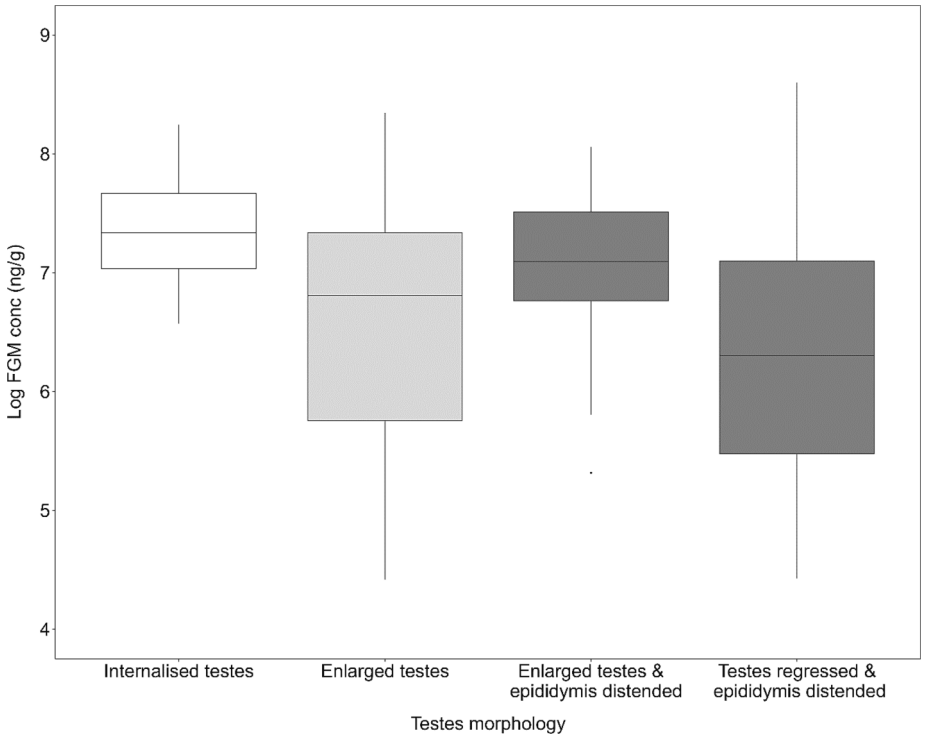

Fig. 4. Faecal glucocorticoid metabolite (FGM) concentrations in male *C. gouldii* at different testes morphology stages (internalised testes: $n = 9$; enlarged testes: $n = 18$, enlarged testes & epididymis distended: $n = 29$; testes regressed & epididymis distended: $n = 21$).

common across taxa, however, there is no consensus about the functional explanation for these differences. One possible explanation is that it is driven by sex differences in metabolism (Jimeno et al., 2018). In *C. gouldii*, female bats roost communally in colonies while males are typically solitary (Godinho et al., 2019; Lumsden et al., 2023). These differences in roosting behaviours could lead to males allocating more energy to thermoregulation and thus having higher FGMs compared to females who thermally benefit from communal roosting (Godinho et al., 2015, 2019). Future studies investigating roost size as well as metabolism could provide insights into the drivers of the observed sex differences in FGMs.

In addition to sex differences, we also observed age related differences in FGMs. Juveniles of both sexes had higher FGMs than adults. These findings are consistent with marmoset monkeys which show elevated cortisol in early infancy compared to other life stages (Pryce et al., 2002). GCs are known to play a key role in age-dependent development (Crespi et al., 2013). However, changes in adrenocortical activity across life history stages are complex and poorly understood (Crespi et al., 2013). The juvenile samples in this study were collected during March and April, which aligns with the time *C. gouldii* become independent (Department of Energy and Zoos Victoria, 2023; Lumsden et al., 2023). In birds and mammals, increased glucocorticoid levels enhance fledging and dispersal (reviewed in Crespi et al., 2013; Wada, 2008). In birds, increased adrenocortical activity is associated with decreased food intake and increased muscle growth to enable fledglings to fly and independently forage (Crespi et al., 2013). As such, the observed higher FGMs in juveniles may be correlated with physiological and behavioural changes associated with the timing of independence and dispersal. These observed age- and sex-dependent differences should be taken into account when monitoring adrenocortical activity in wild *C.gouldii*. Failing to do so may result in misinterpretation of GC metabolite values when collecting "anonymous" samples (Coppes et al., 2018; Rehnus and Palme, 2017). Genotyping offers a valuable tool for field sampling by providing sufficient information from unknown individuals to facilitate analyses of sex-based and individual differences (Coppes et al., 2018; Rehnus and Palme, 2017).

Season (month) had a significant effect on FGM concentration, with levels highest in March and April and significantly lower in May and November. *C. gouldii* mate from April to July, females use sperm storage over winter with pregnancies occurring from September to November, followed by lactation from October to January (Eastick et al., 2022). The observed seasonal patterns of adrenocortical activity may be correlated with shifts in reproductive activity. Higher FGMs were observed during mating, with lower levels observed during sperm storage and pregnancy. Shifts in prey availability and utilisation of torpor may also be a driver of changes in adrenocortical activity. In the southern part of their range, decreased temperatures during cooler months (May to September) lead to a decrease in insect abundance, resulting in *C. gouldii* utilising torpor more frequently to conserve energy (Stawski and Currie, 2016). Adrenocortical activity is lower during torpor than periods of activity due to decreased metabolic rates triggered by decreased body temperature (Giroud et al., 2021). GC levels of other bat species have shown similar seasonal fluctuations with resource abundance and foraging conditions (Lewanzik et al., 2012). Consequently, the observed seasonal patterns of adrenocortical activity may result from different reproductive stages, changes in food availability and increased utilisation of torpor, or a combination of both.

GCs play a key role in modulating physiological functions crucial for maintaining homeostasis including energy allocation and metabolism. Here we show that FGMs were negatively correlated with body condition in *C. gouldii,* indicating individuals with poorer body conditions may be experiencing greater physiological stress. Our findings reflect those commonly found in other mammals (Cabezas et al., 2007; Hing et al., 2017). As GCs play a large role in metabolism, food intake and activity, declining body condition elevates GCs since there is more stress on the body to maintain homeostasis. However, elevated GCs from stressors (e.g., reduced resource availability, predation and poor habitat quality) can also in turn reduce body condition.

FGMs in male *C. gouldii* were influenced by time of year, while differences between testes morphology were not strong enough to be

detected. This is likely driven by reproductive status and month being partially confounded, and a low sample size for some testes stages. The highest FGMs were detected in juveniles (testes internalised – non-reproductive) and during peak breeding (enlarged testes & distended epididymis – sperm production and storage). Evidence has suggested male *C. gouldii* undergo energy-intensive efforts such as roost guarding to facilitate mating with multiple females during the breeding season (Godinho et al., 2015). The observed decline in FGMs between March and April (sperm production) and May (sperm storage) could result from a reduction in the physiological costs associated with production of reproductive hormones (i.e. testosterone). However, larger and more consistent sample sizes are needed to further explore the effect of reproductive status on FGM concentrations in *C. gouldii*. Samples taken at least monthly within the same year could reveal clearer seasonal changes in FGMs that may be aligned with reproductive cycles of *C. gouldii*. Our opportunistic seasonal samples were collected over several years (2013 – November; 2014 – March and April; 2021 – May). Since reproduction in insectivorous bats typically fluctuates with resource availability (Racey and Entwistle, 2000), differences in climatic conditions and therefore insect abundances by year may have also influenced the observed differences in FGM concentrations between seasons.

4.3. Conclusions

Understanding normal patterns of adrenocortical activity can provide valuable insights into a species' fitness. Here we successfully validated an assay to monitor FGMs in *C. gouldii* and demonstrate that sex, age, time of year, and body condition influence FGM excretion. Our findings highlight the need to incorporate individual differences in basal GCs to accurately interpret physiological stress. There remains a lack of information about the life history strategies and physiology of Australian insectivorous bat species, making it difficult to fully explain the observed patterns of GC secretion. Future studies are required to investigate the drivers of the observed sex and age differences as well as the influence of climatic conditions and reproductive status on the observed changes in FGMs across the year. Knowledge about normal physiological function can offer valuable insights into how bats respond to environmental stressors such as urbanisation. This knowledge can be used to characterise endocrine function in common species which can reveal the mechanisms facilitating survival and success, thus providing a model for threatened species.

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CRediT authorship contribution statement

Lauren K. Sandy: Writing – original draft, Investigation, Formal analysis, Data curation. **Kerry V. Fanson:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Stephen R. Griffiths:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Kylie A. Robert:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Rupert Palme:** Writing – review & editing, Resources. **Alicia M. Dimovski:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

Data availability

All supporting data will be made available on the Figshare data repository upon acceptance of the manuscript.

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