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Non-invasive evaluation of stress hormone responses in a captive population of sugar gliders (*Petaurus breviceps*)

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Abstract. Faecal hormone monitoring offers a robust tool to non-invasively determine the physiological stress experienced by an individual when faced with natural or human-driven stressors. Although already quantified for several species, the method needs to be validated for each new species to ensure reliable quantification of the respective glucocorticoids. Here we investigated whether measurement of faecal glucocorticoid metabolite (fGCM) provides a feasible and non-invasive way to assess the physiological state of sugar gliders (*Petaurus breviceps*), an arboreal marsupial native to Australia, by using both a biological and physiological validation. Our analysis confirmed that the cortisol enzyme immunoassay (EIA) was the most appropriate assay for monitoring fGCM concentrations in sugar gliders. Comparing the fGCM response to the physiological and the biological validation, we found that while the administration of ACTH led to a significant increase in fGCM concentration in all individuals, only six of eight individuals showed a considerable fGCM response following the biological validation. Our study identified the most appropriate immunoassay for monitoring fGCM concentrations as an indicator of physiological stress in sugar gliders, but also supports recent suggestions that, if possible, both biological and physiological stressors should be used when testing the suitability of an EIA for a species.

Additional keywords: ACTH challenge, faecal glucocorticoid metabolites, individual variation, physiological stress, separation.

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Introduction

Monitoring adrenocortical activity in wild animal populations is critical given the well-documented relationship between stress, health and reproduction (Tilbrook *et al.* 2000; Romano *et al.* 2010). When an animal is experiencing stress such as unpredictable environmental changes, a main component of the body's response is the activation of the hypothalamic–pituitary–adrenal (HPA) axis, which results in the increased production and secretion of glucocorticoids (GCs) into the bloodstream (Sapolsky *et al.* 2000).

Based on the 'reactive scope model' the increase in GCs (cortisol or corticosterone) can be seen as a mediator of the allostatic load and are a way for the individual to achieve homeostasis again, often through adjustments in metabolism, energy availability, cardiovascular activity and behaviour (Moberg 2000; Romero 2002; Romero *et al.* 2009). Although this response can be beneficial when it comes to circadian or seasonal variations (predictive homeostasis) as well as short-term disturbances (reactive homeostasis), chronically elevated GC secretion, also described as 'homeostatic overload', may lead to a suppression of the immune system and reproductive activities, muscle atrophy and a shortened life span (Sapolsky 2002; Charmandari *et al.* 2005; Cohen *et al.* 2007; Romero *et al.* 2009).

Due to the role GCs play in this response, and the numerous deleterious effects that homeostatic overload, i.e. chronically elevated GCs, can have on an individual, they are often used as a physiological marker for the level of stress experienced and welfare of an individual. Thus, physiological measurements of stress hormones are often used to estimate the consequences of natural or human-induced change in ecological studies of various animals. Non-invasive hormone monitoring has become a reliable technique for assessing physiological stress in a range of wildlife species (Creel *et al.* 2013). Because glucocorticoids (active molecule) circulating in the bloodstream are processed by the liver and excreted via the bile as GC metabolites (Touma and Palme 2005; Sheriff *et al.* 2011), GCs can be monitored non-invasively by collecting excreted faecal material (Hodges *et al.* 2010). Although non-invasive faecal glucocorticoid metabolite (fGCM) monitoring has some shortcomings, such as the inability to monitor short-term stressors or the need to determine the time of fGCM excretion relative to the applicable stressor (Touma and Palme 2005; Heistermann 2010), it is often chosen above invasive blood collection techniques for several reasons. For example, there is little to no need for animal capture, restraint or anaesthesia to collect faeces, which decreases animal contact and potentially dangerous consequences to animal or collector health (Behringer and Deschner 2017). As a result of the ease of collection, longitudinal sampling can be conducted from captive and free-ranging animals. Another inherent advantage of using faecal material to monitor adrenocortical function is the ability to monitor free (non-protein bound) GCs that are excreted via faeces. This method is often classified as more relevant than looking into the amount of total GC level in blood samples, as only free GCs are able to reach the target organs and invoke the necessary physiological changes in response to a stressor (Palme *et al.* 2005; Sheriff *et al.* 2011).

Before a specific assay can be used to monitor fGCM concentrations in a particular species, it is important that the method has been carefully validated either physiologically or biologically to ensure that the assay can monitor biologically meaningful differences (Palme 2005). Physiological validation refers to the artificial activation, through the injection of synthetic adrenocorticotrophic hormone (ACTH), of the HPA axis and the ability to monitor the resulting change in fGCM concentrations (ACTH challenge test). Where a physiological validation cannot be performed (e.g. when working with critically endangered or intractable species), biological validations (e.g. handling, constraint, blood collection, transportation and/or agonistic interactions) should be conducted (Bosson *et al.* 2009; Rimbach *et al.* 2013). Although biological validations are often employed as part of the validation process, individual variation in the stress response towards specific stressors may lead to inconsistent and varying results (Koolhaas *et al.* 2010). Thus, to ensure the most appropriate enzyme immunoassay is used to quantify physiological stress in a species, many authors highlight the need to conduct both a physiological and biological validation on the chosen study species (Goymann *et al.* 1999; Sheriff *et al.* 2011).

Recent studies have demonstrated a dramatic decline in Australian wildlife as a result of anthropogenic activities such as the introduction of exotic species, the reduction or fragmentation of vegetative cover, as well as a change in fire regimes and climatic variables (Burbidge and McKenzie 1989; McKenzie *et al.* 2007; Hing *et al.* 2014). Despite evidence that chronic stress has significant welfare implications, studies focusing on the possible effects of such stressors on the adrenocortical activity have been conducted on only a few Australian marsupials (Hing *et al.* 2014). In this regard, non-invasive hormone monitoring techniques using hair as hormone matrix have been successfully applied to determine adrenocortical function in squirrel gliders (*Petaurus norfolcensis*) faced with anthropogenic disturbances (Brearley *et al.* 2012).

The sugar glider (*Petaurus breviceps*) is a small arboreal marsupial native to Australia and currently listed as of least concern by the International Union for Conservation of Nature (Salas *et al.* 2016). Sugar gliders are a social species known to form groups consisting of several individuals and are frequently found in large huddling groups (Suckling 1984; Nowack and Geiser 2016). They are well adapted to survive short-term changes in their environment (Henry and Suckling 1984; Körtner and Geiser 2000; Parmesan *et al.* 2000; Christian and Geiser 2007). However, chronic or extreme changes in temperature, food availability and habitat loss may lead to energetic bottlenecks as well as changes in foraging behaviour and reproduction. Validating a method for monitoring physiological stress in the species may assist in determining sugar glider health and survivability throughout its natural distribution during such periods of change. Here we used both a biological (separation) as well as physiological (ACTH administration) validation to assess the suitability of five enzyme immunoassays (EIAs) that would allow non-invasive monitoring of physiological stress of captive and free-ranging sugar glider populations via the collection of faecal samples.

Material and methods

Ethical note

Approval to conduct this study was granted by the University of New England Animal Ethics Committee and the New South Wales National Parks and Wildlife Service (AEC14-108).

Capture and housing

The experiment was performed in February 2014 on eight sugar glider individuals (five adult females, two adult males, one subadult male) originally retrieved from wooden nest boxes near Dorrigo (30°22′S, 152°34′E) and within Imbota Nature Reserve (30°35′S, 151°45′E, Australia) (a group of four animals per location). The individuals were transferred to the University of New England, Armidale, Australia, where they were used to establish a breeding colony, which was used during this study. All individuals were weighed to the nearest 0.1 g, sexed and aged according to Suckling (1984), before being micro-chipped for individual recognition (PIT tags, Destron Technologies, South St Paul, MN, USA). Animals were kept in their capture groups and housed in two outdoor enclosures (3.6 × 1.8 × 2 m), each fitted with branches, two feeding platforms and three wooden nest boxes per group. All individuals of one group usually shared one nest box (Nowack and Geiser 2016). Following a physical evaluation, all animals were deemed healthy at the start of the study. Individuals were removed from their group housing in the late afternoon (start of active period) on the first day of the study and placed into individual enclosures (0.7 × 1 × 2 m) for the study period: individuals were able to have visual and olfactory contact with one or two other members of their family group situated in close-by aviaries. Each individual enclosure was equipped with a wooden nest box and branches; the floor of the enclosure was lined with shade cloth to captured faeces while allowing urine to drain off. Animals were fed daily with a mixture of high protein baby cereal, egg, honey and water, to which a high protein supplement (Wombaroo Food Products, Glen Osmond, SA) was added. This food was supplemented with a dish of fresh fruits. Water was provided *ad libitum*.

Separation, ACTH challenge and faecal sample collection

In total, faecal samples were collected for eleven nights including separation (Day 1), five nights where no animal manipulation occurred, ACTH administration on Day 7, and for four nights after the treatment. After both separation and ACTH injection, enclosures were checked for faecal samples at 2-h intervals from 2100 hours to 0600 hours. The freshest sample was collected and all other faecal samples were removed from the enclosure and discarded. For all other nights, enclosures were checked at the start and end of the active period (2100 hours through to 0600 hours the next day; the same sampling procedure as described above was used). Samples were marked according to the date and time of collection to allow for longitudinal fGCM monitoring. On Day 7, all eight individuals were injected intramuscularly with 0.1 mL of synthetic ACTH (1–2 IU/kg of Synacten Depot, Novartis, Auckland, NZ) at the start of the active phase between 1925 and 2000 hours then released back into their individual enclosures. This ACTH dose was chosen as it has been used successfully in several studies to invoke a stress response, such as the African lesser bushbaby (*Galago moholi*, Scheun *et al.* 2015), yellow baboons (*Papio cynocephalus*, Wasser *et al.* 2000) and the black-footed ferret (*Mustela nigripes*, Young *et al.* 2001). All faecal samples were stored in 1.5 mL Eppendorf tubes and frozen at –20°C within 20 min of collection. At the end of the experiment, all individuals were relocated into their original groups.

Faecal sample extraction

Faecal samples were lyophilised, pulverised and sieved through a thin mesh to remove any undigested material (Fieß *et al.* 1999). Following this, 0.050–0.055 g of faecal powder were extracted by adding 1.5 mL 80% ethanol before vortexing for 10 min. Suspensions were then centrifuged for 10 min at 1500*g* and the supernatants transferred into a new microcentrifuge tube. Centrifugation of the supernatants was repeated at 1500*g* for 5 min and the resulting supernatants transferred into new microcentrifuge tube. Subsequently, 1 mL of supernatant was dried in an oven at 50°C overnight; the dried product was sent to the Endocrine Research Laboratory (ERL), University of Pretoria, South Africa, for EIA analysis. At the ERL, dried samples were reconstituted with 1 ml assay buffer and stored at –20°C until EIA analysis.

Enzyme immunoassay analysis

To determine an appropriate EIA for measuring alterations in fGCM concentrations in sugar gliders, a subset of faecal extracts from two males (Male1, Male2) and two females (Female1, Female2), injected with synthetic ACTH, were measured for immunoreactive fGCMs using five EIAs, namely: cortisol, corticosterone, 11-oxoetiocholanolone I (measuring 11,17 dioxoandrostanes), 11-oxoaetiocholanalone II (detecting fGCMs with a 5β-3α-ol-11-one structure), and 5α-pregnane-3β,11β,21-triol-20-one (measuring 3β,11β-diol-CM). The choice of enzyme immunoassays included assays that were specifically designed to target cortisol or corticosterone, but also widely used group specific assays (Palme 2019). The number of individuals that we used for the evaluation of a suitable EIA has been based on previous studies that have successfully validated assays by using between 2 to 4 individuals (Wielebnowski *et al.* 2002 ($N = 4$); Fichtel *et al.* 2007 ($N = 4$); Laver *et al.* 2012 ($N = 2$); Young *et al.* 2017 ($N = 4$); Scheun *et al.* 2018 ($N = 3$)). Details of the five EIAs including cross-reactivities are described by Palme and Möstl (1997) for 11-oxoetiocholanolone I and cortisol, Möstl *et al.* (2002) for 11-oxoaetiocholanalone II, and Touma *et al.* (2003) for 5α-pregnane-3β,11β,21-triol-20-one and corticosterone. Assay sensitivity was 0.6 ng/g for cortisol, 11-oxoetiocholanolone I and 11-oxoaetiocholanalone II, 1.8 ng/g for corticosterone, and 2.4 ng/g for 5α-pregnane-3β,11β,21-triol-20-one EIA. Intra-assay coefficients of variation, of high- and low-value quality controls, were 4.17 and 4.67% for cortisol, 6.87 and 8.22% for corticosterone, 3.05 and 5.71% for 11-oxoetiocholanolone I, 5.27 and 5.76% for 11-oxoaetiocholanalone II and 3.81 and 4.19% for 5α-pregnane-3β,11β,21-triol-20-one. Inter-assay coefficients of variation, of high- and low-value quality controls, were 8.11 and 11.68% for cortisol, 13.46 and 16.88% for corticosterone, 1.80 and 6.38% for 11-oxoetiocholanolone I, 5.74 and 11.68% for 11-oxoaetiocholanalone II and 8.22 and 11.36% for 5α-pregnane-3β,11β,21-triol-20-one.

Data analysis

Choice of enzyme immunoassay

To determine EIA suitability, individual baseline and peak fGCM concentrations were identified for each of the EIAs tested, using a subset of samples collected two days prior and following ACTH administration. Individual baseline fGCM concentration was determined for the respective datasets, using an iterative process (Brown *et al.* 1994; Scheun *et al.* 2016). Here, the mean and standard deviation (s.d.) value for each individual was calculated. Subsequently, all data points higher than the mean + 1.5 s.d. were removed and the mean and s.d. recalculated. This process was repeated until no value exceeded the mean + 1.5 s.d., thus yielding the individual baseline value. To determine the effect of a stressor (ACTH/Separation) on the HPA axis, the absolute fGCM change was determined, defined as percentage fGCM response, by calculating the quotient of baseline and fGCM samples. An average increase of ≥100% was considered a significant rise in fGCM levels (e.g. Young *et al.* 2017; Jepsen *et al.* 2019). To identify the most suitable EIA, we then chose the commonly used approach to select the EIA with the highest percentage increase for all individuals (e.g. Ludwig *et al.* 2013; Young *et al.* 2017, and see Touma and Palme 2005 for a list of studies). The cortisol EIA showed the largest peak fGCM response of the five EIAs tested, exceeding the 100% average response (range: 100–2155.30%, Table 1) post-injection for the four study animals (Fig. 1). The lack of a response in one study animal (Female2) is not uncommon during a physiological validation via ACTH administration (see Touma and Palme 2005), and does not lower the reliability of the assay. Subsequently, the cortisol EIA was used to assess fGCM concentrations in the samples from the remaining four ACTH administered individuals, as well as in the samples linked to separation from all eight animals. However, we note that despite the lack of an average increase exceeding 100%, the corticosterone assay produced fGCM responses that were comparable between the four individuals, which is another favourable indicator for assay suitability, and as such, the tested corticosterone EIA may also be suitable to monitor fGCM in sugar gliders. For the assay of

Table 1. Comparison of five enzyme immunoassays

The faecal glucocorticoid metabolites (fGCM) results were obtained from the ACTH challenge using on two male and two female sugar gliders. Shown here are baseline fGCM concentrations before injection, as well as peak fGCM values and the percentage response from baseline values. ID, identification: M1, Male1; M2, Male2; F1, Female1; F2, Female2

ID	Enzyme immunoassay														
	Cortisol			Corticosterone			5α-pregnane-3β,11β,21-triol-20-one			11-oxoaetiocholanolone I			11-oxoaetiocholanolone II		
	Baseline (µg/g)	Peak (µg/g)	% response	Baseline (µg/g)	Peak (µg/g)	% response	Baseline (µg/g)	Peak (µg/g)	% response	Baseline (µg/g)	Peak (µg/g)	% response	Baseline (µg/g)	Peak (µg/g)	% response
M1	0.14	0.69	398	0.74	1.08	46	8.06	12.77	58	29.51	39.38	33	5.27	9.28	76
M2	0.15	3.07	2055	0.67	1.04	55	4.48	6.09	36	6.94	12.39	79	2.63	2.60	−1
F1	0.18	0.47	157	0.62	0.99	61	5.89	14.71	150	18.14	17.26	−5	2.86	3.90	37
F2	1.50	1.50	0	1.01	1.45	43	12.99	16.02	23	52.71	73.28	36	9.63	17.92	86

choice (cortisol EIA), serial dilutions of extracted samples gave displacements curves, which were parallel to the respective standard curves (the relative variation of the slopes of the trend lines was <5%). Faecal glucocorticoid metabolite concentrations are given as µg/g dry weight (DW). All EIAs used throughout the study were performed on microtiter plates as described by Ganswindt *et al.* (2012).

ACTH administration and separation

After deciding on an appropriate EIA for monitoring fGCM concentrations in the sugar glider, the entire sample set was analysed using the cortisol EIA. Individual baseline fGCM concentration was calculated from the entire dataset using the iterative process as described above. The production of GCs from the adrenal gland can fluctuate daily (Peter *et al.* 1978; Lincoln *et al.* 1982). In order to determine whether natural daily fluctuations are apparent in sugar gliders, fGCM concentrations from the unmanipulated period preceding the ACTH injection were compared to the calculated baseline value (as above) for each individual. The deviation from the calculated baseline level was expressed as a percentage deviation value and ranged from 14–29% (Table 2). Thus, daily variation in fGCM excretion is negligible for sugar gliders.

Results

ACTH challenge

Seven of the eight animals exhibited a pronounced increase in fGCM concentrations, following ACTH administration, when using the cortisol EIA (range: 69–1566%, Table 2). Both adult males as well as the subadult male showed a considerable increase in fGCM response (206–1566%) 4.5–8 h following ACTH administration (Table 2). The fGCM concentrations returned to baseline levels for all three individuals between 6.5 and 25 h following ACTH administration (Table 2). Four of the five females injected with ACTH showed an increase in fGCM response (69–1290%) 1.5 to 10.5 h following ACTH administration (2–6 samples post-injection, Table 2). The fGCM concentrations of all four females returned to baseline levels between 6.5 and 49 h following ACTH administration (Table 2).

Biological validation via separation

Six of the eight individuals showed a considerable increase in fGCM response after separation (range: 62–2413%, Table 2; Fig. 2), but two females did not show an acute fGCM response above 50% (Table 2). Both adult males, the subadult male and two adult females showed a peak fGCM response between the first and third collected faecal sample post-separation, with fGCM concentrations returning to baseline levels on the subsequently collected sample for each individual. Additionally, one adult female showed a prolonged, elevated fGCM response following the separation event, with the fGCM response exceeding 125% from the first to the fifth collected faecal sample before returning to baseline level.

The fGCM response to the separation event was considerably stronger than the response determined following ACTH administration in sugar gliders (Table 2: not statistically tested due to small and inhomogeneous sample size).

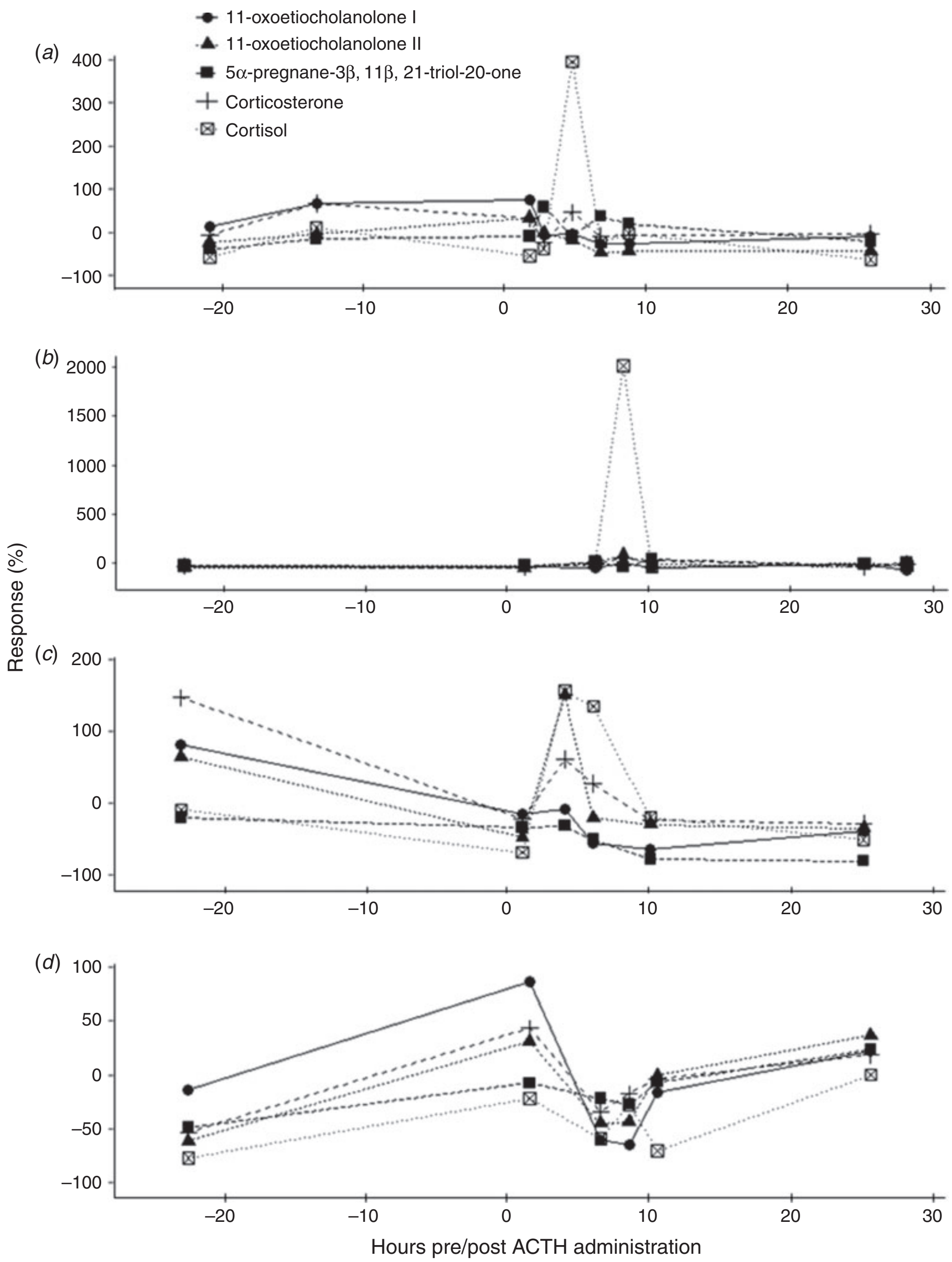

Fig. 1. Relative change (%) of faecal glucocorticoid metabolites (fGCMs) following ACTH administration observed in two male (*a*, *b*) and two female (*c*, *d*) sugar gliders using five different enzyme immunoassays.

Discussion

Our study shows that fGCM changes induced by both physiological stimulation (ACTH) and a behavioural event (separation) can be reliably monitored in faecal samples from sugar gliders using a cortisol EIA. In addition to confirming the ability to non-invasively monitor stress responses in sugar gliders using faecal samples, the measured response to separation further proves the ability of the chosen assay for monitoring biological relevant changes in the stress response.

Sugar gliders are a highly social species, commonly found nesting together throughout the year (Suckling 1984) even though energy savings achieved via torpor expression during winter can be reduced by the presence of normothermic nest mates (Nowack and Geiser 2016). In fact, sugar glider groups are quite stable and although groups occasionally split up when changing nests, they usually re-join after a few days (Körtner and Geiser 2000). Separation of individuals of a highly social species such as sugar gliders can result in the increased

Table 2. Time and intensity of peak faecal glucocorticoid metabolites (fGCM) response for each of the eight study animals following ACTH administration and a separation event

Individual sample numbers are given (N) as well as total numbers of males and females monitored

Sex	Deviation from baseline unmanipulated period (%)	Time to peak response post ACTH administration (h)	Peak fGCM response after ACTH injection (%)	Peak fGCM response after separation (%)
Subadult male	20	6.50 (N = 2)	206	168
Adult male1	19	4.5 (N = 3)	497	1248
Adult male2	19	8.0 (N = 3)	1566	2413
Mean ± s.d.	19 ± 1 (*n* = 3)	6.3 ± 1.8 (*n* = 3)	756 ± 716 (*n* = 3)	1276 ± 1123 (*n* = 3)
Female1	23	1.5 (N = 1)	91	1655
Female2	29	4.0 (N = 2)	32	−17
Female3	18	10.5 (N = 5)	69	2090
Female4	14	4.0 (N = 2)	1290	26
Female5	25	2.0 (N = 2)	681	862
Mean ± s.d.	22 ± 6 (*n* = 5)	4.4 ± 3.6 (*n* = 5)	433 ± 549 (*n* = 5)	923 ± 947 (*n* = 5)

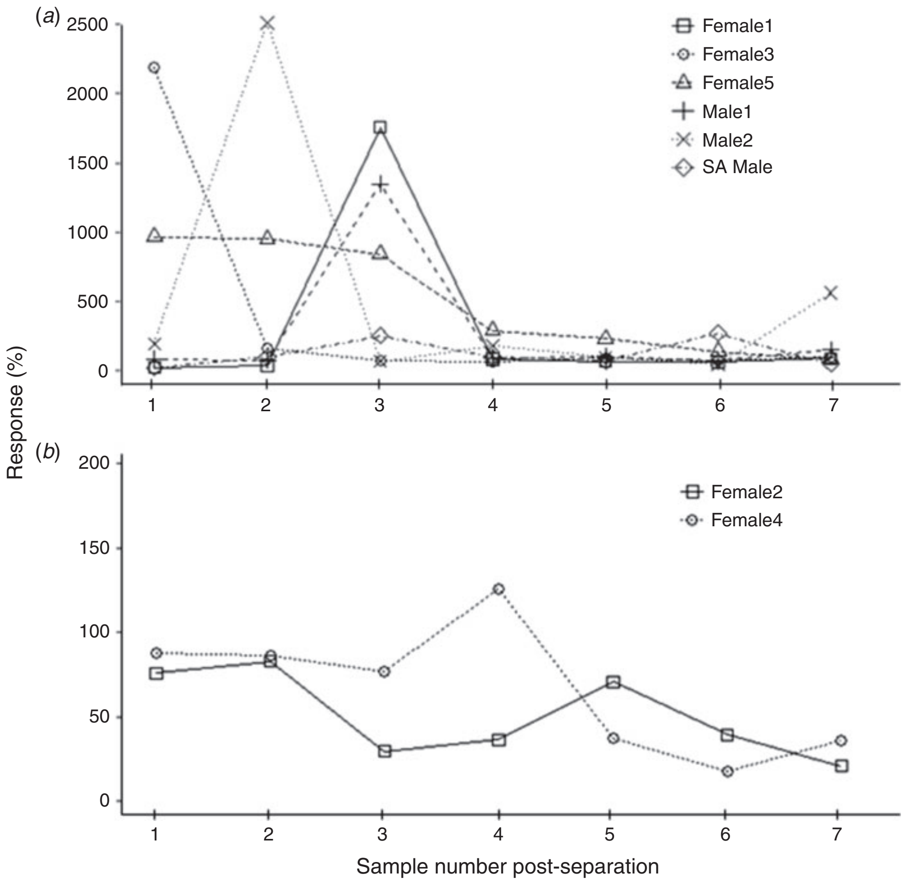

Fig. 2. Relative change (%) of faecal glucocorticoid metabolites (fGCMs) following the separation event in all eight study animals using the cortisol enzyme immunoassay.

production of GCs and shift the HPA response into the 'reactive homeostasis range' in order to facilitate physiological and behavioural changes that promote a return to homeostasis. A similar response has also been shown for several other social species, such as the domestic guinea-pig (*Cavia porcellus*, Hennessy *et al.* 2008), pied babbler (*Turdoides bicolor*, Jepsen *et al.* 2019), African buffalo (*Syncerus caffer*, Ganswindt *et al.* 2012), the common prairie vole (*Microtus ochrogaster*, Ruscio *et al.* 2007), the common squirrel monkey (*Saimiri sciureus*, Hennessy *et al.* 1982) and the black tufted-ear marmoset (*Callithrix kuhlii*, Smith and French 1997).

The time lag between elevated circulating GCs from ACTH administration to the excretion of GCs in sugar glider faeces was around 4–6 h post-injection. This is similar to other small-bodied mammals, such as the degu (6 h, *Octodon degus*, Soto-Gamboa *et al.* 2009), mice (8–10 h, *Mus musculus f. domesticus*, Touma *et al.* 2004), African lesser bushbaby (14 h, *Galago moholi*, Scheun *et al.* 2015) and eastern chipmunks (8 h, *Tamias striatus*, Montiglio *et al.* 2012). However, following both biological and physiological stressors, a considerable amount of individual variability for the tested males and females have been observed in terms of peak fGCM response, time to peak response, and return to fGCM baseline levels. The time span from injection of ACTH to the observed peak response varied by up to 8.5 h between individuals. Furthermore, only three of five female individuals showed an increase in fGCM levels in response to the separation event. Our data also suggest differences between the sexes, as males had a considerably higher average fGCM response to both ACTH administration and handling compared with their female counterparts. Although biological stressors (e.g. animal handling, separation, constraint, blood collection, transportation and/or agonistic interactions; Goymann *et al.* 1999; Bosson *et al.* 2009; Rimbach *et al.* 2013) have been used successfully in several validation studies to increase GC production (Touma and Palme 2005), numerous instances exist where individual variation in the stress response to biological validation has led to inconsistent validation results. The ability of an event to act as a stressor and activate the stress response is based on individual perception; that is, specific biological stressors may not be recognised as such by an individual (Reeder and Kramer 2005). Furthermore, individual and sex-related variations in the stress response can also be caused by the time of year, reproductive status, body condition and the animal's developmental history (Yoshimura *et al.* 2003; Kudielka and Kirschbaum 2005; Cockrem 2013). Individual variation in response to a stressor has been reported in several studies. For example, Smith *et al.* (2012) showed that the stress response to capture in yellow-bellied marmots (*Marmota flavivetris*) were specific to individuals, with several individuals failing to show a significant fGCM increase. Similarly, dwarf hamsters (*Phodopus campbelli*) exposed to a subordinate 'on-back' position showed a large degree of individual variation, ranging from a large to no response (Guimont and Wynne-Edwards 2006), whereas Narayan *et al.* (2012) showed that greater bilby (*Macrotis lagotis*) held in captivity displayed individual variation in the stress response to anthropogenic activities.

Although both physiological and biological validation techniques were largely successful in this study, both can have shortcomings. The injection of ACTH can lead to the overstimulation of the adrenal gland, resulting in a less sensitive EIA being chosen as an ideal assay for fGCM monitoring in a species (Young *et al.* 2017). In contrast to this, the response to a biological stressor is individual specific and may result in the under stimulation of the adrenal gland (Koolhaas *et al.* 2007). As such we agree with previous researchers that, when possible, both a physiological and biological validation should be conducted to ensure the most appropriate EIA is chosen for monitoring fGCM patterns in a particular species.

Being able to use fGCM to non-invasively assess the physiological state of sugar gliders will be beneficial to determine the health status of sugar glider populations and may be especially useful to investigate the impact of anthropogenic disturbance and climate change on this species. A study on the closely related squirrel gliders showed that reduced availability of nesting sites in highly fragmented habitats leads to elevated cortisol levels, i.e. a homeostatic overload, in squirrel gliders (Brearley *et al.* 2012); the study utilised hair as a sample matrix for monitoring GC metabolites, which gives a seasonal GC metabolite pattern. In contrast to the seasonal patterns observed in hair, the use of fGCM monitoring, as used in our study, can give a more acute (1 h to 2 days) description of the adrenal activity of a species or population, allowing for an almost real-time assessment of physiological stress experienced in a population. This will provide conservationists and researchers with an accurate, real-time pattern of the physiological stress experienced by populations within altered habitats, leading to the development of more robust conservation programs.

Conclusion

Results from the present study have confirmed the ability to monitor biologically relevant changes in the adrenal function of sugar glider, using faeces as a matrix. The aim of this study was to determine the suitability of the tested EIAs for monitoring fGCM concentrations in the sugar glider; in this regard, only the cortisol assay showed an overall response exceeding 100% of the calculated baseline level and seems to be the most suited out of the five EIAs tested. This validated technique can now be employed to determine the physiological stress experienced by free-ranging populations faced with a range of natural and anthropogenic stressors.

Conflict of interest

The authors declare no conflicts of interest.

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References

Behringer, V., and Deschner, T. (2017). Non-invasive monitoring of physiological markers in primates. *Hormones and Behavior* **91**, 3–18. doi:10.1016/J.YHBEH.2017.02.001

Bosson, C. O., Palme, R., and Boonstra, R. (2009). Assessment of the stress response in Columbian ground squirrels: laboratory and field validation of an enzyme immunoassay for fecal cortisol metabolites. *Physiological and Biochemical Zoology* **82**, 291–301. doi:10.1086/597530

Brearley, G., McAlpine, C., Bell, S., and Bradley, A. (2012). Influence of urban edges on stress in an arboreal mammal: a case study of squirrel gliders in southeast Queensland, Australia. *Landscape Ecology* **27**, 1407–1419. doi:10.1007/S10980-012-9790-8

Brown, J. L., Wasser, S. K., Wildt, D. E., and Graham, L. H. (1994). Comparative aspects of steriod-hormone metabolism and ovarian activity in felids, measured noninvasively in feces. *Biology of Reproduction* **51**, 776–786. doi:10.1095/BIOLREPROD51.4.776

Burbidge, A. A., and McKenzie, N. L. (1989). Patterns in the modern decline of western Australia's vertebrate fauna: causes and conservation implications. *Biological Conservation* **50**, 143–198. doi:10.1016/0006-3207(89)90009-8

Charmandari, E., Tsigos, C., and Chrousos, G. (2005). Endocrinology of the stress response. *Annual Review of Physiology* **67**, 259–284. doi:10.1146/ANNUREV.PHYSIOL.67.040403.120816

Christian, N., and Geiser, F. (2007). To use or not to use torpor? Activity and body temperature as predictors. *Naturwissenschaften* **94**, 483–487. doi:10.1007/S00114-007-0215-5

Cockrem, J. F. (2013). Individual variation in glucocorticoid stress responses in animals. *General and Comparative Endocrinology* **181**, 45–58. doi:10.1016/J.YGCEN.2012.11.025

Cohen, S., Janicki-Deverts, D., and Miller, G. E. (2007). Psychological stress and disease. *Journal of the American Medical Association* **298**, 1685–1687. doi:10.1001/JAMA.298.14.1685

Creel, S., Dantzer, B., Goymann, W., and Rubenstein, D. R. (2013). The ecology of stress: effects of the social environment. *Functional Ecology* **27**, 66–80. doi:10.1111/J.1365-2435.2012.02029.X

Fichtel, C., Kraus, C., Ganswindt, A., and Heistermann, M. (2007). Influence of reproductive season and rank on fecal glucocorticoid levels in free-ranging male Verreaux's sifakas (*Propithecus verreauxi*). *Hormones and Behavior* **51**, 640–648. doi:10.1016/J.YHBEH.2007.03.005

Fieß, M., Heistermann, M., and Hodges, J. K. (1999). Patterns of urinary and fecal steroid excretion during the ovarian cycle and pregnancy in the African elephant (*Loxodonta africana*). *General and Comparative Endocrinology* **115**, 76–89. doi:10.1006/GCEN.1999.7287

Ganswindt, A., Tordiffe, A. S. W., Stam, E., Howitt, M. J., and Jori, F. (2012). Determining adrenocortical activity as a measure of stress in African buffalo (*Syncerus caffer*) based on faecal analysis. *African Zoology* **47**, 261–269. doi:10.3377/004.047.0211

Goymann, W., Möstl, E., Van't Hof, T., East, M. L., and Hofer, H. (1999). Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta*. *General and Comparative Endocrinology* **114**, 340–348. doi:10.1006/GCEN.1999.7268

Guimont, F. S., and Wynne-Edwards, K. E. (2006). Individual variation in cortisol responses to acute 'on-back' restraint in an outbred hamster. *Hormones and Behavior* **50**, 252–260. doi:10.1016/J.YHBEH.2006.03.008

Heistermann, M. (2010). Non-invasive monitoring of endocrine status in laboratory primates: methods, guidelines and applications. *Advances in Science and Research* **5**, 1–9. doi:10.5194/ASR-5-1-2010

Hennessy, M. B., Mendoza, S. P., and Kaplan, J. N. (1982). Behavior and plasma cortisol following brief peer separation in juvenile squirrel monkeys. *American Journal of Primatology* **3**, 143–151. doi:10.1002/AJP.1350030113

Hennessy, M. B., Zate, R., and Maken, D. S. (2008). Social buffering of the cortisol response of adult female guinea pigs. *Physiology & Behavior* **93**, 883–888. doi:10.1016/J.PHYSBEH.2007.12.005

Henry, S., and Suckling, G. (1984). A review of the ecology of the sugar glider. In 'Possums and gliders'. (Eds A. Smith and I. Hume.) pp. 355–358. (Australian Mammal Society: Sydney, NSW.)

Hing, S., Narayan, E., Thompson, R. C. A., and Godfrey, S. (2014). A review of factors influencing the stress response in Australian marsupials. *Conservation Physiology* **2**, cou027. doi:10.1093/CONPHYS/COU027

Hodges, K., Brown, J., and Heistermann, M. (2010). Endocrine monitoring of reproduction and stress. In 'Wild mammals in captivity: principles and techniques for zoo management'. (Eds D.G. Kleiman, K.V. Thompson and C.K. Baer.) pp. 447–468. (University of Chicago: Chicago, IL, USA.)

Jepsen, E. M., Ganswindt, A., Ngcamphalala, C. A., Bourne, A. R., Ridley, A. R., and McKechnie, A. E. (2019). Non-invasive monitoring of physiological stress in an afrotropical arid-zone passerine bird, the southern pied babbler. *General and Comparative Endocrinology* **276**, 60–68. doi:10.1016/J.YGCEN.2019.03.002

Koolhaas, J. M., de Boer, S. F., Buwalda, B., and van Reenen, K. (2007). Individual variation in coping with stress: a multidimensional approach of ultimate and proximate mechanisms. *Brain, Behavior and Evolution* **70**, 218–226. doi:10.1159/000105485

Koolhaas, J., De Boer, S., Coppens, C., and Buwalda, B. (2010). Neuroendocrinology of coping styles: towards understanding the biology of individual variation. *Frontiers in Neuroendocrinology* **31**, 307–321. doi:10.1016/J.YFRNE.2010.04.001

Körtner, G., and Geiser, F. (2000). Torpor and activity patterns in free-ranging sugar gliders *Petaurus breviceps* (Marsupialia). *Oecologia* **123**, 350–357. doi:10.1007/S004420051021

Kudielka, B. M., and Kirschbaum, C. (2005). Sex differences in HPA axis responses to stress: a review. *Biological Psychology* **69**, 113–132. doi:10.1016/J.BIOPSYCHO.2004.11.009

Laver, P. N., Ganswindt, A., Ganswindt, S. B., and Alexander, K. A. (2012). Non-invasive monitoring of glucocorticoid metabolites in banded mongooses (*Mungos mungo*) in response to physiological and biological challenges. *General and Comparative Endocrinology* **179**, 178–183. doi:10.1016/J.YGCEN.2012.08.011

Lincoln, G. A., Almeida, O. F. X., Klandorf, H., and Cunningham, R. A. (1982). Hourly fluctuations in the blood levels of melatonin, prolactin, luteinizing hormone, follicle-stimulating hormone, testosterone, tri-iodothyronine, thyroxine and cortisol in rams under artificial photoperiods, and the effects of cranial sympathectomy. *The Journal of Endocrinology* **92**, 237–250. doi:10.1677/JOE.0.0920237

Ludwig, C., Wachter, B., Silinski-Mehr, S., Ganswindt, A., Bertschinger, H., Hofer, H., and Dehnhard, M. (2013). Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*). *General and Comparative Endocrinology* **180**, 15–23. doi:10.1016/J.YGCEN.2012.10.005

McKenzie, N. L., Burbidge, A. A., Baynes, A., Brereton, R. N., Dickman, C. R., Gordon, G., Gibson, L. A., Menkhorst, P. W., Robinson, A. C., Williams, M. R., and Woinarski, J. C. Z. (2007). Analysis of factors implicated in the recent decline of Australia's mammal fauna. *Journal of Biogeography* **34**, 597–611. doi:10.1111/J.1365-2699.2006.01639.X

Moberg, G. (2000). Biological response to stress: implications for animal welfare. In 'The biology of animal stress: basic principles and implications for animal welfare'. (Eds G.P. Moberg and J.A. Mench.) pp. 1–21. (CABI Publishing: Oxon, UK.)

Montiglio, P. O., Pelletier, F., Palme, R., Garant, D., Réale, D., and Boonstra, R. (2012). Noninvasive monitoring of fecal cortisol metabolites in the eastern chipmunk (*Tamias striatus*): validation and comparison of two enzyme immunoassays. *Physiological and Biochemical Zoology* **85**, 183–193. doi:10.1086/664592

Möstl, E., Maggs, J. L., Schrötter, G., Besenfelder, U., and Palme, R. (2002). Measurement of cortisol metabolites in faeces of ruminants. *Veterinary Research Communications* **26**, 127–139. doi:10.1023/A:1014095618125

Narayan, E. J., Molinia, F. C., Cockrem, J. F., and Hero, J.-M. (2012). Individual variation and repeatability in urinary corticosterone metabolite responses to capture in the cane toad (*Rhinella marina*). *General and Comparative Endocrinology* **175**, 284–289. doi:10.1016/J.YGCEN.2011.11.023

Nowack, J., and Geiser, F. (2016). Friends with benefits: the role of huddling in mixed groups of torpid and normothermic animals. *The Journal of Experimental Biology* **219**(4), 590–596. doi:10.1242/JEB.128926

Palme, R. (2005). Measuring fecal steroids: guidelines for practical application. *Annals of the New York Academy of Sciences* **1046**, 75–80. doi:10.1196/ANNALS.1343.007

Palme, R. (2019). Non-invasive measurement of glucocorticoids: advances and problems. *Physiology & Behavior* **199**, 229–243. doi:10.1016/J.PHYSBEH.2018.11.021

Palme, R., and Möstl, E. (1997). Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *International Journal of Mammalian Biology* **62**, 192–197.

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S. M., and Möstl, E. (2005). Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. *Annals of the New York Academy of Sciences* **1040**, 162–171. doi:10.1196/ANNALS.1327.021

Parmesan, C., Root, T. L., and Willig, M. R. (2000). Impacts of extreme weather and climate on terrestrial biota. *Bulletin of the American Meteorological Society* **81**, 443–450. doi:10.1175/1520-0477(2000)081<0443:IOEWAC>2.3.CO;2

Peter, R. E., Hontela, A., Cook, A. F., and Paulencu, C. R. (1978). Daily cycles in serum cortisol levels in the goldfish: effects of photoperiod, temperature, and sexual condition. *Canadian Journal of Zoology* **56**, 2443–2448. doi:10.1139/Z78-329

Reeder, D. M., and Kramer, K. M. (2005). Stress in free-ranging mammals: integrating physiology, ecology, and natural history. *Journal of Mammalogy* **86**, 225–235. doi:10.1644/BHE-003.1

Rimbach, R., Heymann, E. W., Link, A., and Heistermann, M. (2013). Validation of an enzyme immunoassay for assessing adrenocortical activity and evaluation of factors that affect levels of fecal glucocorticoid metabolites in two New World primates. *General and Comparative Endocrinology* **191**, 13–23. doi:10.1016/J.YGCEN.2013.05.010

Romano, M. C., Rodas, A. Z., Valdez, R. A., Hernández, S. E., Galindo, F., Canales, D., and Brousset, D. M. (2010). Stress in wildlife species: noninvasive monitoring of glucocorticoids. *Neuroimmunomodulation* **17**(3), 209–212. doi:10.1159/000258726

Romero, L. M. (2002). Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *General and Comparative Endocrinology* **128**, 1–24. doi:10.1016/S0016-6480(02)00064-3

Romero, L. M., Dickens, M. J., and Cyr, N. E. (2009). The reactive scope model – a new model integrating homeostasis, allostasis, and stress. *Hormones and Behavior* **55**, 375–389. doi:10.1016/J.YHBEH.2008.12.009

Ruscio, M. G., Sweeny, T., Hazelton, J., Suppatkul, P., and Carter, C. S. (2007). Social environment regulates corticotropin releasing factor, corticosterone and vasopressin in juvenile prairie voles. *Hormones and Behavior* **51**, 54–61. doi:10.1016/J.YHBEH.2006.08.004

Salas, L., Dickman, C., Helgen, K., Winter, J., Ellis, M., Denny, M., Woinarski, J., Lunney, D., Oakwood, M., Menkhorst, P., and Strahan, R. (2016). *Petaurus breviceps*. The IUCN Red List of Threatened Species 2016: e.T16731A21959798. doi:10.2305/IUCN.UK.2016-2.RLTS.T16731A21959798.EN

Sapolsky, R. M. (2002). Endocrinology of the stress-response. In 'Behavioral endocrinology'. (Eds J.B. Becker, S.M. Breedlove, D. Crews and M.M. McCarthy.) pp. 409–450. (MIT Press: Cambridge, MA, USA.)

Sapolsky, R. M., Romero, L. M., and Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews* **21**, 55–89.

Scheun, J., Bennett, N., Ganswindt, A., and Nowack, J. (2015). The hustle and bustle of city life: monitoring the effects of urbanisation in the African lesser bushbaby. *Naturwissenschaften* **102**, 1–11.

Scheun, J., Nowack, J., Bennett, N., and Ganswindt, A. (2016). Female reproductive activity and its endocrine correlates in the African lesser bushbaby, *Galago moholi. Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology* **186**, 255–264. doi:10.1007/S00360-015-0947-Z

Scheun, J., Greeff, D., and Ganswindt, A. (2018). Non-invasive monitoring of glucocorticoid metabolite concentrations in urine and faeces of the sungazer (*Smaug giganteus*). *PeerJ* **6**, e6132. doi:10.7717/PEERJ.6132

Sheriff, M., Dantzer, B., Delehanty, B., Palme, R., and Boonstra, R. (2011). Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia* **166**, 869–887. doi:10.1007/S00442-011-1943-Y

Smith, T. E., and French, J. A. (1997). Psychosocial stress and urinary cortisol excretion in marmoset monkeys. *Physiology & Behavior* **62**, 225–232. doi:10.1016/S0031-9384(97)00103-0

Smith, J. E., Monclús, R., Wantuck, D., Florant, G. L., and Blumstein, D. T. (2012). Fecal glucocorticoid metabolites in wild yellow-bellied marmots: experimental validation, individual differences and ecological correlates. *General and Comparative Endocrinology* **178**, 417–426. doi:10.1016/J.YGCEN.2012.06.015

Soto-Gamboa, M., Gonzalez, S., Hayes, L. D., and Ebensperger, L. A. (2009). Validation of a radioimmunoassay for measuring fecal cortisol metabolites in the hystricomorph rodent, *Octodon degus. Journal of Experimental Zoology. Part A, Ecological Genetics and Physiology* **311A**, 496–503. doi:10.1002/JEZ.546

Suckling, G. C. (1984). Population ecology of the sugar glider, *Petaurus breviceps*, in a system of fragmented habitats. *Wildlife Research* **11**, 49–75. doi:10.1071/WR9840049

Tilbrook, A., Turner, A., and Clarke, I. (2000). Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Reviews of Reproduction* **5**, 105–113. doi:10.1530/ROR.0.0050105

Touma, C., and Palme, R. (2005). Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. *Annals of the New York Academy of Sciences* **1046**, 54–74. doi:10.1196/ANNALS.1343.006

Touma, C., Sachser, N., Möstl, E., and Palme, R. (2003). Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. *General and Comparative Endocrinology* **130**, 267–278. doi:10.1016/S0016-6480(02)00620-2

Touma, C., Palme, R., and Sachser, N. (2004). Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. *Hormones and Behavior* **45**, 10–22. doi:10.1016/J.YHBEH.2003.07.002

Wasser, S. K., Hunt, K. E., Brown, J. L., Cooper, K., Crockett, C. M., Bechert, U., Millspaugh, J. J., Larson, S., and Monfort, S. L. (2000). A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. *General and Comparative Endocrinology* **120**, 260–275. doi:10.1006/GCEN.2000.7557

Wielebnowski, N. C., Fletchall, N., Carlstead, K., Busso, J. M., and Brown, J. L. (2002). Noninvasive assessment of adrenal activity associated with husbandry and behavioral factors in the North American clouded leopard population. *Zoo Biology* **21**, 77–98. doi:10.1002/ZOO.10005

Yoshimura, S., Sakamoto, S., Kudo, H., Sassa, S., Kumai, A., and Okamoto, R. (2003). Sex-differences in adrenocortical responsiveness during development in rats. *Steroids* **68**, 439–445. doi:10.1016/S0039-128X(03)00045-X

Young, K., Brown, J., and Goodrowe, K. (2001). Characterization of reproductive cycles and adrenal activity in the black-footed ferret (*Mustela nigripes*) by fecal hormone analysis. *Zoo Biology* **20**, 517–536. doi:10.1002/ZOO.10001

Young, C., Ganswindt, A., McFarland, R., de Villiers, C., van Heerden, J., Ganswindt, S., Barrett, L., and Henzi, S. P. (2017). Faecal glucocorticoid metabolite monitoring as a measure of physiological stress in captive and wild vervet monkeys. *General and Comparative Endocrinology* **253**, 53–59. doi:10.1016/J.YGCEN.2017.08.025