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Sex and dose-dependent responses of urinary and faecal glucocorticoid metabolite concentrations following an ACTH challenge in eastern rock sengis (*Elephantulus myurus*)

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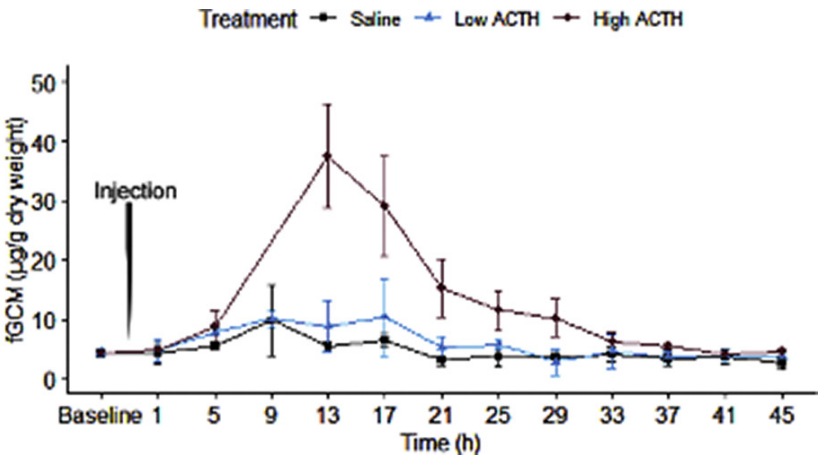
Highlights

- Non-invasive monitoring of adrenocortical activity in eastern rock sengis.
- We determined urinary and faecal glucocorticoid metabolite concentrations in sengis.
- An ACTH dose-dependence could be seen in glucocorticoid output in sengis.
- Females showed a more pronounced response than males.
- No effect of the injection procedure on glucocorticoid output was found in sengis.

Graphical abstract



Abstract

Non-invasive methods for measuring glucocorticoids and their metabolites are frequently used in ecological, behavioural and physiological studies of mammals. Using faeces, urine and other matrices for such a measurement has considerable advantages in comparison to more traditional methods, but also requires thorough validation of the methods used. Eastern rock sengis (*Elephantulus myurus*) are fascinating African mammals and the non-invasive monitoring of the adrenocortical activity opens up new opportunities to study their biology. We were able to validate two assays for measuring urinary (uGCM) and faecal glucocorticoid metabolite (fGCM) concentrations in this species using a dose-dependent challenge with adrenocorticotropic hormone (ACTH). A higher concentration of ACTH elicited higher uGCM and fGCM concentrations in both males and females. Interestingly, uGCM and fGCM concentrations and the responses to ACTH were higher in females than in males and small changes in faecal glucocorticoid metabolites could not be reliably detected in males. In contrast to ACTH, a saline injection did not result in an increase in uGCM or fGCM concentrations. The study also provided insight into when responses to a stressor are likely to be detected in the urine and faeces of sengis and opens up new opportunities to study the stress physiology of this and other sengi species. It further emphasises the importance of thoroughly validating non-invasive methods for measuring hormones in both sexes of a species and for incorporating dose-dependent approaches.

Key Words: non-invasive hormone monitoring, steroid metabolites, adrenocortical activity, sexual dimorphism, Macroscelidea

Introduction

Studies that investigate interactions between the ecology and behaviour of vertebrates and their endocrine correlates have been increasing over recent years. A distinct number of these studies have been focussing on glucocorticoids due to their importance for a variety of physiological and behavioural processes. For example, glucocorticoids are important metabolic mediators (Rose et al., 2010) and they are imperative for the processes related to homeostasis recovery by stimulating glycogenolysis, gluconeogenesis, and the suppression of immune function and secretion of hormones and neuropeptides (Munck et al., 1984). Prolonged exposure to elevated glucocorticoid levels caused by frequent or prolonged stressful events can, however, have detrimental effects on the health, reproduction, and even memory of an individual (Conrad, 2010; Dobson and Smith, 2000; Martin, 2009; Martin et al., 2012; Roozendaal, 2002). Consequently, the quantification of glucocorticoids has been widely employed to investigate their role in, for example, social systems (Avitsur et al., 2001; Hardy et al., 2002; Hill et al., 2015), reproduction (Ganswindt et al., 2005; Schoech et al., 2009; Tilbrook et al., 2000), parental care (Bales et al., 2006; Carlson et al., 2006), annual activity cycles (Boonstra et al., 2001; Reeder et al., 2004), and infections and immune function (O'Dwyer et al., 2019). The measurement of glucocorticoids has been particularly used in conservation biology to monitor the wellbeing of animal populations in the wild and to ascertain the impact of environmental change and human disturbance (Hunninck et al. 2020; Majelantle et al., 2020; Scheun et al., 2015; Thiel et al., 2008; Wingfield, 2013, 1997). Further, glucocorticoid monitoring is often used to ensure the welfare of animals in captivity and assist captive breeding projects (Dehnhard et al., 2008; Terio et al., 2004).

Many studies on wild animals and also an increasing number of studies using animals in captive settings turn to minimally and non-invasive methods for measuring glucocorticoid concentrations by using urine and faeces as steroid matrices (Cook, 2012; Sheriff et al., 2011). Advantages of using non-invasive methods include that these samples are easy to obtain and allow for repeated sampling especially in smaller mammals and birds in which repeated blood sampling would not be possible (Medger et al., 2018; Palme, 2019; Touma et al., 2004). Furthermore, capture and repeated handling of animals, which in itself can result in an increase of glucocorticoid concentrations, is usually not necessary when applying non-invasive methods (Small et al., 2017). Steroid concentrations measured in urine and faeces

are integrated over longer periods of time, thus reducing the effect of episodic fluctuations and pulsatile hormone secretions and allowing a better picture of long-term changes in glucocorticoid concentrations (Cook, 2012; Touma and Palme, 2005). In addition, most of the glucocorticoids in the blood are bound to carrier proteins and measures of its free equivalents in matrices such as urine and faeces may provide a better assessment of biologically active hormone concentrations (Cook, 2012).

Methods for the quantification of glucocorticoids and their metabolites using urine and especially faeces need to be thoroughly validated to ensure that the steroid concentrations obtained reflect the activity of the hypothalamic–pituitary–adrenal (HPA) axis (Cook, 2012; Palme, 2019; Sheriff et al., 2011). Glucocorticoids and other steroid hormones are extensively metabolized by the liver and little to no native, unmetabolized glucocorticoids remain in faeces (Touma and Palme, 2005), whereas some native hormones may still be found in urine (Bahr et al., 2000). These metabolic processes as well as the route of excretion of hormones vary considerably between species and between the sexes (Sipari et al., 2017; Touma et al., 2003; Touma and Palme, 2005). Although sex-related quantitative differences in immunoreactive faecal glucocorticoid metabolite (fGCM) concentrations are not always detected (Arias et al., 2013; Hammond et al., 2015; Mendonça-Furtado et al., 2017), some studies demonstrated considerable differences in baseline and peak concentrations of fGCMs between females and males. Male Syrian hamsters (*Mesocricetus auratus*) and bank voles (*Myodes glareolus*), for example, have higher baseline and peak fGCM concentrations than females (Chelini et al., 2010; Sipari et al., 2017). In contrast, fGCM concentrations are higher in female than in male coyotes (*Canis latrans*; Stevenson et al. 2018) and the same was found for two house mouse subspecies (*Mus musculus musculus* and *M. m. domesticus*; Daniszová et al. 2017).

The subjects of this study were sengis, also known as elephant-shrews. Sengis are unique African mammals belonging to the superorder Afrotheria, a mammalian clade that also includes hyraxes (Hyracoidea) and elephants (Proboscidea). Within the superorder, sengis are most closely related to the tenrecs of Madagascar and the golden moles (Afrosoricida), which, together with the aardvark (*Orycteropus afer*), belong to one clade, the Afroinsectiphilia (Seiffert, 2007; Springer et al., 2004). Sengis are highly cursorial enabling them to readily escape

predators (Lovegrove and Mowoe, 2014; Rathbun, 2009). However, the natural histories of the smaller species of the genera *Elephantulus* and *Macroscelides* make them particularly vulnerable to predation and disturbances. Eastern rock sengis (*Elephantulus myurus*) prefer very rocky habitats in areas of southern Africa that are characterized by very dry winter and wet and hot summer months (Skinner and Chimimba, 2005). They are crepuscular with the main activity occurring during the early morning and another smaller activity period during the late afternoon and early evening (van der Merwe et al., 2012). During this time, eastern rock sengis forage for insects in the open relying primarily on their speed to escape predators (Rathbun 2009; Lovegrove and Mowoe 2014; K Medger, personal observation). Furthermore, they only find shelter under rocks where they also leave their highly precocial young as they do not build nests (Rathbun 2009). Interestingly, eastern rock sengis are able to carry a large burden of parasites, especially ticks, without showing obvious signs of physiological distress (Lutermann et al., 2015, 2012). The unique biology of a mammal of this size makes the eastern rock sengi an interesting study subject to investigate how natural and human induced changes affect animals that live in challenging habitats. In addition, sengis are ideal to investigate the evolution of, for example, unique reproductive strategies (i.e. precociality) and host-parasite interactions. In some of our previous studies, we have also observed remarkable high glucocorticoid concentrations in the urine of eastern rock sengis that did not appear to be stressed (K Medger, unpublished results). We, therefore, designed this study to validate methods that enable the non-invasive measurement of glucocorticoids in the eastern rock sengi, which can then be used to investigate the ecology, behaviour and physiology of this fascinating mammal species and to determine the response of this species to a variety of natural and artificial stressors. This study may also pave the way for similar studies on other sengi species, some of which are threatened (e.g. the endangered golden-rumped sengis (*Rhynchocyon chrysopygus*), FitzGibbon & Rathbun 2015).

The aim of the present study was to examine the suitability of different immunoassays for determining stress-related physiological responses in the urine (radioimmunoassay, RIA) and faeces (enzyme immunoassay, EIA) of male and female eastern rock sengis (*Elephantulus myurus*) by performing an adrenocorticotropic hormone stimulation test (ACTH challenge test). We used two different doses of adrenocorticotrophic hormone (ACTH) to stimulate the adrenal

cortex into releasing glucocorticoids to additionally examine the influence of a dose-dependent response. So far, the use of different doses of ACTH has only been done in a limited number of validation studies for establishing non-invasive methods for quantifying glucocorticoids and their metabolites (e.g. Touma et al. 2004; reviewed in Palme 2019). Such information can be very valuable to determine how the intensity of a stressor affects the measurement of glucocorticoids in different matrices. Further, we included a treatment with saline to investigate if handling and the injection procedure alone were perceived as a stressor and result in an increase in glucocorticoid concentrations in the urine and faeces of this species (Palme, 2019).

Materials and Methods

Eastern rock sengis originated from Welgevonden Private Game reserve (24° 18'S, 27° 53'E) in the Limpopo Province, South Africa. The five females and six males were in captivity for about a year before being used for the current experiments and were housed in large polyurethane cages (33 cm × 33 cm × 21 cm) between trials. The cages were provided with wood shavings, a small rock as raised seating area as well as a refuge. They were fed with canned dog food (Promeal Ltd., Dassenberg, South Africa), Pronutro (high protein cereal; Pioneer Foods Ltd., Bokomo Foods, Cape Town, South Africa) and grated apples and carrots. Water was provided in open dishes and exchanged once a day. For urine and faeces collection, animals were kept in plastic crates (60 cm × 40 cm × 35 cm) with a wire-mesh bottom, which separated the animals from the collection sheets underneath that could be slid out without opening up the crates, reducing disturbance of the animals. A small house was provided as a shelter during the experiments. The same climate controlled room with a temperature of 25°C and a day-night cycle of 12 h light and 12 h dark was used for housing and conducting the experiments. Lights were set to go on at 6:00 and off at 18:00. The necessary permits by the South African nature conservation authorities were obtained (capture permit 001-CPM402-00001) and the study was approved by the animal ethics committee of the University of Pretoria (EC012-12).

Sample collection

Animals were placed in the collection chambers three days prior to the start of sample collection to allow habituation to the new environment. Sengis received the

same food during sample collection as during housing. Any leftover food was replaced at 17:00 every day during collections. Water was provided twice a day (7:00 am and 17:00) for two hours each to avoid contamination of the samples and to ensure sufficient water intake. Sample collection commenced at 11:00 am on the fourth day and chambers were checked for urine and faeces every two hours for a total of 70 hours (23 h baseline and 47 h after treatment). The collection sheet was carefully removed from the chambers to keep disturbances to the animals minimal. All urine and faeces were collected with pipettes and tweezers, respectively. A new pipette was used for every sample and the tweezers and sheets were cleaned with moist tissue paper between collections. Urine and faeces were separately placed into Eppendorf tubes and immediately frozen and stored at -20°C.

Control and experimental treatments

All individuals were subjected to two treatments that differed in concentrations of synthetic ACTH administered. For the low-dose ACTH treatment (low-dose treatment), the individuals were injected with 0.2 µg ACTH/g body weight while a concentration of 0.6 µg ACTH/g body weight was used for the high-dose ACTH treatment (high-dose treatment). The respective ACTH doses were chosen according to Touma et al. (2004). Synthetic ACTH (Synacthen® depot, Novartis, South Africa (Pty) Ltd) was dissolved in a sterile isotonic saline solution and individuals were injected subcutaneously with 1 ml of the solution. Injections were done at 10:00 am after the initial 23 h of urine and faeces collections (baseline). Urine and faeces collections continued one hour after the injections at 11:00 am and thereafter took place every two hours for another 46 h (collections for a total of 47 h after injection). The capture, injection, and release of the animals back into the collection cages took less than 3 min in each case. To control for possible effects of these procedures, we added a control treatment for which sengis were injected with 1 ml of a saline solution only. The handling and injection procedures were similar to those of the ACTH treatments. The saline and the two ACTH treatments were performed on each individual, while the animals were allowed to rest for two weeks between treatments. At any injection time, one third of the animals each received the control, low-dose, or high-dose injections and individuals were randomly assigned to those groups to maintain repeatability and avoid a bias in the time the injections were performed.

Quantification of urinary glucocorticoids

Urinary glucocorticoid (uGCM) concentrations were determined using a Coat-a-Count cortisol RIA (Diagnostic Products Corporation, Los Angeles, California, USA). Urine samples were defrosted at room temperature, thoroughly vortexed and subsequently assayed according to manufacturer's guidelines. Sensitivity of the assay was 0.36 nmol/l and a serial dilution of a urine sample gave a displacement curve that was parallel to the respective standard curve with a relative variation of the slope of respective trend lines of <6% (see also Hoffmann, 2014). Intra-assay coefficients of variation of high- and low-value quality controls were 7.9% and 5.3%, respectively. Inter-assay coefficients of variation of high- and low-value quality controls were 10.5% and 12.7%, respectively. To control for variation in water content of samples, uGCM concentrations were standardized against creatinine concentrations. A modified Jaffe reaction (Folin 1914) was used to determine the creatinine concentration for each of the urine samples. All uGCM concentrations were corrected with their respective creatinine concentrations and are thus expressed in µg uGCM/mg creatinine (Cr).

Faecal glucocorticoid metabolite extraction and assay

All faecal samples were lyophilized and pulverized using a mortar and pestle. Hair and other large undigested material were removed manually. Between 0.05 - 0.06 g of the dry faecal powder was weighed per sample for extraction. Some samples (n = 193 of 772) weighed less than the required amount, but at least 0.025 g dry faecal mass or more and in these cases, the entire sample was used for steroid extraction. Steroids were extracted by adding 1.5 ml of 80% ethanol to the faecal powder. The mixture was shaken for 15 min on a multi-vortex and then centrifuged for 10 min at 1500 × *g.* The supernatant was transferred into Eppendorf tubes and stored at -20°C.

We used five different enzyme immunoassays (EIAs): (i) a Cortisol; (ii) a 11-oxoaetiocholanolone I (detecting 11,17 dioxoandrostanes); (iii) a 11-oxoaetiocholanolone II (detecting fGCMs with a 5β- 3α-ol-11-one structure), (iv) a Corticosterone, and (v) a 5α-pregnane-3β,11β,21-triol-20-one EIA (detecting fGCMs with a 5α-3β-11β-diol structure) to measure immunoreactive fGCM concentrations in a sub-set of faecal samples (n = 43) from one female and two male sengis treated with the high ACTH concentration. Detailed assay characteristics, including full

descriptions of the assay components and cross reactivities have been provided for the 11-oxoaetiocholanolone I, cortisol, and corticosterone EIAs by Palme and Möstl (1997), 11-oxoaetiocholanolone II EIA by Möstl et al. (2002) and for the 5α-pregnane-3β,11β,21-triol-20-one EIA by Touma et al. (2003). Serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curve with relative variations of the slope of respective trend lines of <5% for the 11-oxoaetiocholanolone II EIA, <4% for the 5α-pregnane-3β,11β,21-triol-20-one EIA, <3% for the 11-oxoaetiocholanolone I and corticosterone EIAs, and < 1% for the cortisol EIA. Three of the five EIAs tested (11-oxoaetiocholanolone I, 11-oxoaetiocholanolone II, and 5α-pregnane-3β,11β,21-triol-20-one) showed an overall median increase above 100% post ACTH administration for all individuals. The 5α-pregnane-3β,11β,21-triol-20-one EIA performed best showing a distinct increase after the challenge for all three individuals and a median increase above 300% (see supplementary figures for individual profiles of ES04, ES18 and ES22). Subsequently, fGCM concentrations (µg/g dry weight) were determined for the entire sample set using the 5α-pregnane-3β,11β,21-triol-20-one EIA. The sensitivity of the EIA was 2.4 ng/g faecal dry weight. Intra-assay coefficients of variation of high- and low-value quality controls were 5.8% and 6.2%, and inter-assay coefficients of variation of high- and low-value quality controls were 8.0% and 10.3%. Faecal steroid concentrations are given as µg/g faecal dry weight (DW). EIAs were performed at the Endocrine Research Laboratory, University of Pretoria, as described previously (Ganswindt et al., 2012, 2014).

Data analysis

For both uGCMs and fGCMs, concentrations were pooled into 4 h intervals (average of two consecutive samples) to reduce the number of comparisons. Further, we calculated the average for all samples obtained over the 23 h before the injection of saline or ACTH and used this as individual baseline values in all subsequent analyses. Therefore, times compared were baseline and every 4 h from 1 h until 45 h post treatment (the 45 h data point reflects average of concentrations at 45 h and 47 h).

Data were analysed using the R statistical software (version 3.5.1, R Core Team 2018, https://www.R-project.org/). Males and females were analysed separately. Subsequently, we compared males and females separately for the control

and the high-dose treatments. We were missing samples for some treatments at specific times for females and excluded those times from the analyses when necessary. For uGCM concentrations, time 29 h was removed from all comparisons involving females. Similarly, time 9 h was omitted for fGCM concentrations from females. After log-transformation, only uGCM concentrations for the control treatment (male-female comparison) were normally distributed and analysed with a linear mixed model (LMM). All other dependent variables were not normally distributed and were analysed using generalized linear mixed models fitted with gamma distributions with log-link function (GLMM). All analyses were performed with the R package lme4 (Bates et al. 2015). *P*-values were subsequently generated using the R package car (Fox and Weisberg 2011, http://socserv.socsci.mcmaster.ca/jfox/Books/Companion). For the comparisons between treatments, the fixed factors included in the models were treatment, time and their interaction. Sex, time and their interaction were used for comparisons between the sexes. Animal ID was included as a random factor to account for repeated measurements. The R package emmeans (Lenth 2019, https://CRAN.R-project.org/package=emmeans) was used *for post hoc* multiple comparisons with Tukey's HSD. *P*-values of ≤ 0.05 were considered to be significant and results are presented as mean ± standard error (SE) of the raw data.

Results

uGCM concentrations in females

Mean female uGCM concentrations were significantly lower during the control treatment (18.41± 3.11 µg/mg Cr; Tukey's HSD: $p < 0.001$) compared to the low-dose (71.45 ± 21.21 µg/mg Cr) and the high-dose treatments (119.61 ± 46.58 µg/mg Cr; Wald $\chi^2 = 31.16$, df = 2, $p < 0.001$). They were not significantly different between the low-dose and high-dose treatments (Tukey's HSD: $p = 0.99$). There was also a significant difference in mean female uGCM concentrations between the times (Wald $\chi^2 = 111.42$, df = 11, $p < 0.001$), which was influenced by treatment (Time*Treatment: Wald $\chi^2 = 136.86$, df = 22, $p < 0.001$). When looking at the main effect of time only (treatments combined), uGCM concentrations were significantly higher at 5 h and 9 h post treatment in comparison to baseline (Tukey's HSD: $p \leq 0.002$; baseline: 26.55± 8.11 µg/mg Cr, 5 h: 192.62 ± 121.38 µg/mg Cr and 9 h: 279.06 ± 173.84 µg/mg Cr). However, female uGCM concentrations did not increase during the control treatment and all uGCM concentrations post treatment were similar to baseline (Tukey's HSD:

$p \geq 0.07$, Fig. 1a). During the low-dose treatment, uGCM concentrations were higher at 1h and 5h post treatment in comparison to baseline ($p \leq 0.03$, Fig. 1a) and the concentrations at these times (1 h and 5 h) were also significantly higher than the concentrations determined for the same time points for the control treatment (Tukey's HSD: $p \leq 0.005$, Fig. 1a). The highest uGCM concentrations during the low-dose treatment were measured at 5 h post treatment and concentrations dropped rapidly thereafter reaching significantly lower levels at 13 h post treatment (Tukey's HSD: $p = 0.005$, Fig. 1a). During the high-dose treatment, concentrations at 5 h to 17 h post treatment were significantly higher than baseline uGCM concentrations (Tukey's HSD: $p \leq 0.04$, Fig. 1a). Concentrations of uGCM peaked only at 9 h post high-dose treatment, four hours after we recorded the peak during the low-dose treatment (Fig. 1a). Thereafter, we observed a slow decrease in female uGCM concentrations with levels still being high at 13 h but rapidly decreasing at 17 h post treatment (Fig. 1a), resulting in significantly lower concentrations at 21 h post treatment compared to peak concentrations determined at 9 h and 13 h post treatment (Tukey's HSD: $p < 0.001$). Peak uGCM concentrations at 9 h and 13 h during the high-dose treatment were significantly higher than concentrations measured at the same times during the control treatment (Tukey's HSD: $p < 0.001$, Fig. 1a). Also concentrations of uGCMs were higher during the high-dose than the low-dose treatment at 13 h post treatment (Tukey's HSD: $p < 0.001$, Fig. 1a). Baseline uGCM values were similar during the three treatments (Tukey's HSD: $p \geq 0.97$, Fig. 1a).

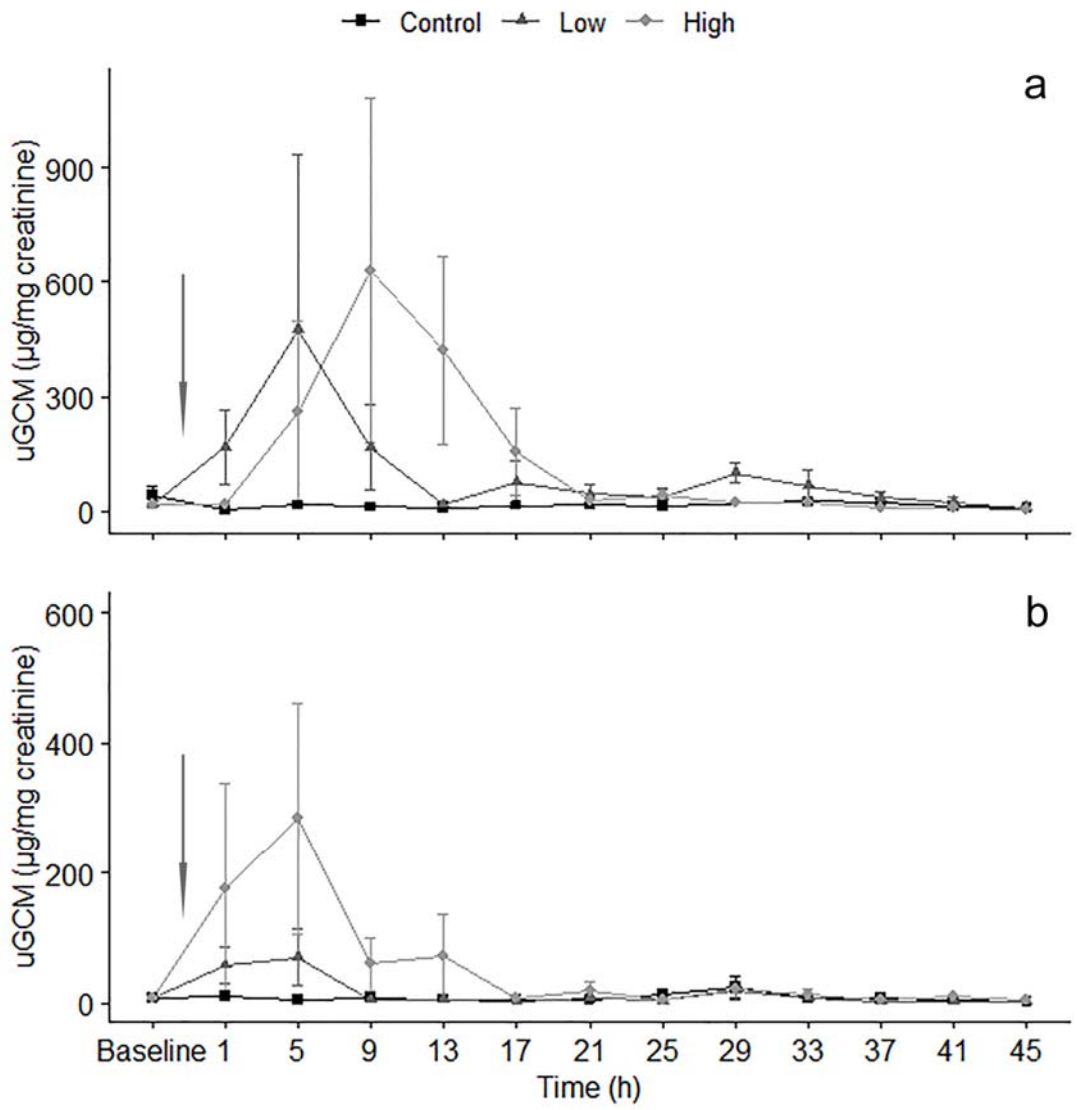


Figure 1. Urinary glucocorticoid concentrations (uGCM, µg/mg Cr) of female (a) and male (b) eastern rock sengis (Elephantulus myurus) before (baseline) and after the injection (indicated by an arrow) of saline (Control), a low dose of ACTH (Low - 0.2 µg ACTH/g body weight) and a high dose of ACTH (High - 0.6 µg ACTH/g body weight). All data are shown as mean ± standard error.

uGCM concentrations in males

Mean male uGCM concentrations were significantly higher during the high-dose treatment (48.08± 18.29 µg/mg Cr; Tukey's HSD: both $p < 0.001$, Wald $\chi^2 = 54.39$, df = 2, $p < 0.001$) than during the control (6.80 ± 1.20 µg/mg Cr) and low-dose treatments (12.43 ± 4.01 µg/mg Cr). The comparison between the control and low-dose treatment was not significant (Tukey: $p = 0.22$). There was also a significant difference in mean male uGCM concentrations between the times (main effect of

time: Wald χ^2 = 158.88, df = 12, p < 0.001), with mean uGCM concentrations being significantly higher at 1 h and 5 h post treatment in comparison to baseline ($p \leq$ 0.001; baseline: 6.85± 0.92 µg/mg Cr, 1 h: 68.48 ± 44.13 µg/mg Cr and 5 h: 127.05 ± 69.01 µg/mg Cr). The interaction between treatment and time was significant (Wald χ^2 = 87.75, df = 24, p < 0.001). Baseline values were similar between the three treatments (p = 1.00, Fig. 1b). We found no significant increase in male uGCM concentrations after the injection of saline (control treatment) with uGCM concentrations during any of the times being similar to baseline concentrations ($p \geq$ 0.40, Fig. 1b). For the low-dose treatment, mean uGCM concentrations increased by about 800% already 1 h post treatment although not significantly (p = 0.49, Fig. 1b). The highest uGCM concentrations determined at 5 h during the low-dose treatment differed significantly compared to baseline (p = 0.01, Fig. 1b) and it was also higher than the uGCM concentrations measured at the same time during the control treatment (p = 0.001, Fig. 1b). Concentrations of uGCM decreased rapidly thereafter reaching significantly lower values at 13 h post treatment (p = 0.002, Fig. 1b). Similar to the low-dose treatment, uGCM concentrations of males who received the high ACTH dose increased already 1 h post treatment and peaked at 5 h post treatment (comparison to baseline: $p \leq$ 0.02). Respective uGCM concentrations decreased slower during the high-dose compared to the low-dose treatment, and they were still significantly higher than baseline at 13 h post treatment (p = 0.005), but were significantly lower than peak concentrations four hours later (p < 0.001, Fig. 1b). Peak uGCM concentrations at 5 h were significantly higher in animals treated with the high dose of ACTH compared to uGCM concentrations measured at the same time in control animals (p < 0.001, Fig. 1b). Male uGCM concentrations were not different between the low-dose and high-dose treatments during peak concentrations (p = 1.0), but they were higher during the high-dose compared to low-dose treatment at 9 h and 13 h post treatment (p < 0.05, Fig. 1b).

fGCM concentrations in females

Mean female fGCM concentrations were significantly higher during the high-dose treatment (11.70 ± 1.79 µg/g DW, Tukey's HSD: p < 0.001) than during the control (4.11 ± 0.34 µg/g DW) and low-dose treatments (5.49 ± 0.76 µg/g DW; Wald χ^2 = 43.66, df = 2, p < 0.001), but there was no difference between the control and low-dose treatments (Tukey's HSD: p = 0.22). We also found a significant difference

in mean female fGCM concentrations between the times (Wald χ^2 = 71.68, df = 11, p < 0.001). Mean fGCM concentrations were significantly higher at 13 h and 17 h post treatment in comparison to baseline (Tukey's HSD: $p \leq 0.001$; baseline: 4.31 ± 0.34 µg/g DW, 13 h: 16.34 ± 4.93 µg/g DW and 17 h: 15.31 ± 4.20 µg/g DW). The interaction between treatment and time only showed a trend (Wald χ^2 = 32.54, df = 22, p = 0.07). Nevertheless, the overall effects as well as Figure 2a suggest an increase in fGCM concentration post high-dose treatment. The *post hoc* results for the interaction reveal a significant increase in fGCM concentrations 13 h and 17 h post treatment in comparison to baseline (Tukey's HSD: $p < 0.001$) with the highest fGCM concentration at 13 h post high-dose treatment (Fig. 2a). At 13 h post treatment, fGCM concentrations were also significantly higher during the high-dose than the control treatment (Tukey's HSD: $p < 0.001$, Fig. 2a). Respective fGCM concentrations decreased relatively slowly and were significantly lower only at 33 h in comparison to 13 h and 17 h post treatment (Tukey's HSD: $p \leq 0.02$). In contrast to the high-dose treatment, we found no significant increase in female fGCM concentrations during the control and low-dose treatments (Tukey's HSD: $p \geq 0.98$, Fig. 2a), and baseline fGCM concentrations were similar between the three treatments (Tukey's HSD: $p = 1.00$, Fig. 2a). In addition, there was a large amount of individual variation in fGCM concentrations in the response to the treatments (Supplementary Figure 3).

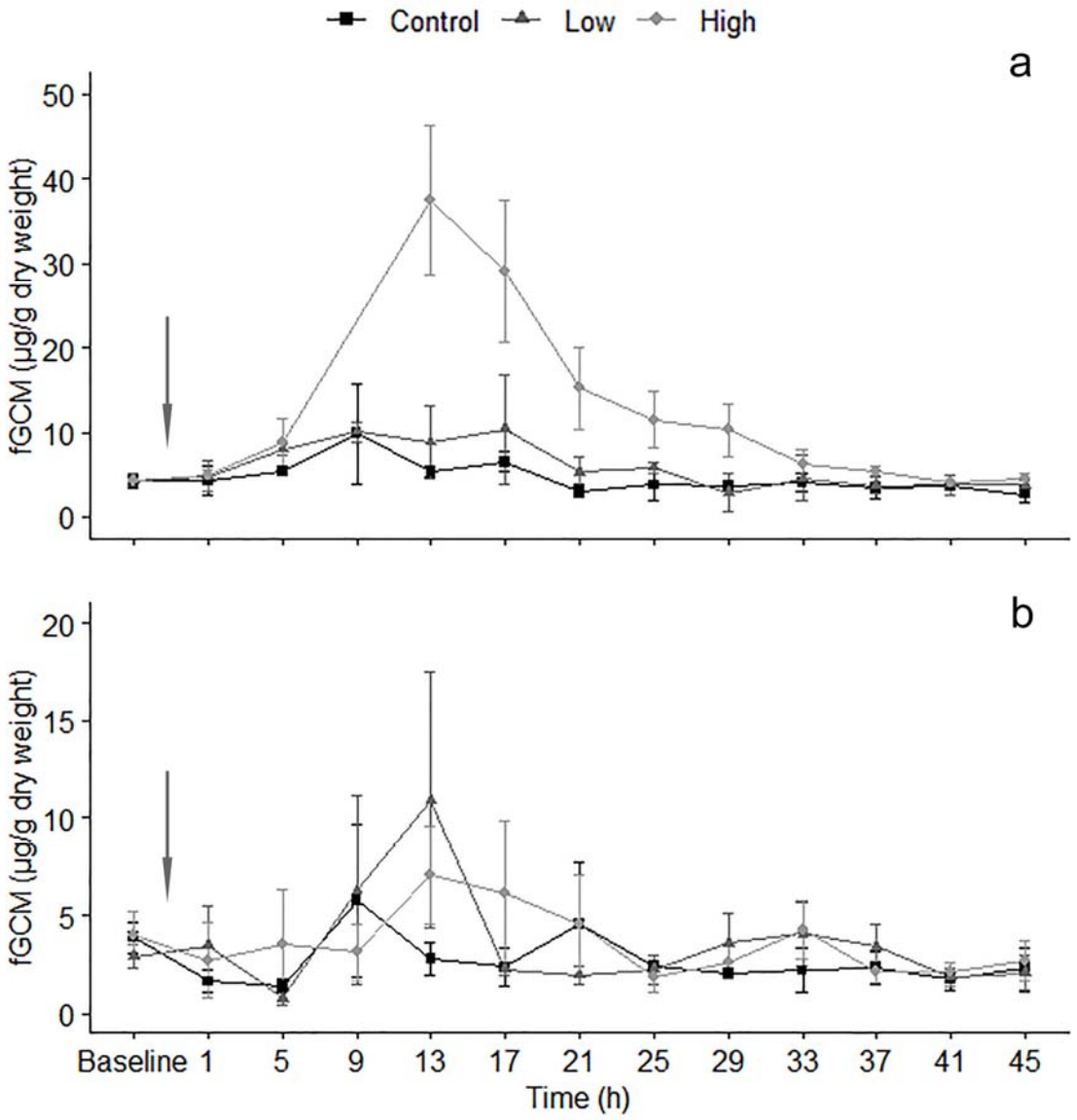


Figure 2. Faecal glucocorticoid metabolite concentrations (fGCM, µg/g DW) of female (a) and male (b) eastern rock sengis (Elephantulus myurus) before (baseline) and after the injection (indicated by an arrow) of saline (Control), a low dose of ACTH (Low - 0.2 µg ACTH/g body weight) and a high dose of ACTH (High - 0.6 µg ACTH/g body weight). All data are shown as mean ± standard error.

fGCM concentration in males

Male fGCM concentrations were not significantly different between the treatments (Wald $\chi^2 = 2.45$, df = 2, $p = 0.29$) and also the interaction of treatment and time was not significant (Wald $\chi^2 = 30.78$, df = 24, $p = 0.16$). However, we found a significant difference in male fGCM concentrations between the times (Wald $\chi^2 = 51.04$, df = 12, $p < 0.001$). Concentrations of fGCMs (mean for treatments) dropped significantly below baseline at 5 h post treatment (Tukey's HSD: $p = 0.03$; baseline:

3.58 ± 0.51 µg/g DW, 5 h: 1.77 ± 0.68 µg/g DW) but fGCM concentrations increased again thereafter and they were higher at 9 h (Tukey's HSD: $p = 0.008$; 4.93 ± 1.84 µg/g DW) and 13 h (Tukey's HSD: $p < 0.001$; 6.92 ± 2.36 µg/g DW) than at 5 h post treatment. Nevertheless, the highest fGCM concentrations measured in males at 13 h post treatment (showing an overall increase of 190% compared to baseline values) were not significantly different to baseline values (Tukey's HSD: $p = 0.62$). This increase in fGCM concentrations at 13 h post treatment is also evident in Figure 2b and appears only in the low-dose treatment, but there was no significant difference between baseline and post treatment concentrations and between control concentrations and the other two treatments at specific times (see the interaction of treatment and time above). Overall, only few males showed a response in fGCM concentrations following an ACTH injection as can be seen in the individual profiles (Supplementary Figure 4).

Male-female comparison

Females had significantly higher overall uGCM concentrations than males during both the control and high-dose treatments (concentrations are comparable to those shown earlier in the results; Wald $\chi^2 \geq 5.14$, df = 1, $p \leq 0.02$). The interaction of sex and time was not significant for the control treatment (Wald $\chi^2 = 8.36$, df = 11, $p = 0.68$), but was significant for the high-dose treatment (Wald $\chi^2 = 42.88$, df = 12, $p < 0.001$). Specifically, female uGCM concentrations were significantly higher at 17 h (Tukey's HSD: $p = 0.045$), and the comparison approached significance at 13 h (Tukey's HSD: $p = 0.06$; Figure 1) for the high-dose treatment. In contrast, the comparison between male and female fGCM concentrations revealed no significant differences for animals of the control treatment group (Sex: Wald $\chi^2 = 2.94$, df = 1, $p = 0.09$, Sex*Time: Wald $\chi^2 = 15.57$, df = 12, $p = 0.21$). We, however, observed a marked difference in overall response to ACTH treatment between males and females of the high-dose treatment group (see Figure 2) and that is also mirrored in the results for the statistical comparison between the sexes. Overall, fGCM concentrations during the high-dose treatment were higher in females (see results above) than in males (3.74 ± 0.58 µg/g DW; Wald $\chi^2 = 8.78$, df = 1, $p = 0.003$). The differences between the sexes were especially obvious 13 h and 17 h after the high-dose treatment (Tukey's HSD: $p < 0.01$, Wald $\chi^2 = 51.99$, df = 11, $p < 0.001$) when

females but not males showed a response to the treatment (see above results, Figure 2).

Discussion

Non-invasive methods for the quantification of glucocorticoids and their metabolites need to be validated to ensure that the results reflect an activation of the HPA-axis and are indeed related to stressful situations (Palme, 2019). Although there are many advantages for using these methods in comparison to that of blood collection, determined absolute concentrations vary between different matrices, extraction and analytical protocols, species, and even sexes (Cook, 2012; Palme, 2019; Sheriff et al., 2011). The results of the current study highlights some of this by finding considerable differences in the responses to two different doses of ACTH and the significant differences in uGCM and fGCM concentrations between female and male eastern rock sengis. In addition, the performed control experiment to investigate the effect of handling showed, contrary to the expectations, no related alterations in glucocorticoid concentration to the injection of saline.

ACTH regulates the release of glucocorticoids from the adrenal cortex (Axelrod and Reisine, 1984; Engelmann et al., 2004). The injection of ACTH is commonly used to stimulate the HPA-axis and causes an increase in glucocorticoids and their metabolites in urine, faeces, and other matrices well above baseline levels (Sheriff et al., 2011). Both of our ACTH treatments resulted in a dosage-dependent increase in uGCM concentrations above baseline for both female and male sengis. Concentrations of uGCM already started to increase at 1 h post injection and were highest 4 h later for males receiving both a low and a high dose of ACTH and for females receiving the low ACTH dose. Females injected with a high dose of ACTH showed a delay in response of about 4 h in comparison to the low-dose ACTH treatment, but this was not observed in males and may be an artefact of the sampling and pooling of data for analysis as well as the large individual variations (also see supplementary figures). Palme et al. (1999), however, observed a delay in fGCM increase following higher ACTH doses in cattle. The response of uGCM to ACTH in the sengis followed a similar curve as in other species in which urinary glucocorticoids following an ACTH challenge were measured. Touma et al. (2003) found that peak radioactivity could be detected within 2 h after injection of radiolabelled corticosterone in the urine of mice. In North American ground squirrels

(*Tamiasciurus hudsonicus*), peak radioactivity was measured in urine about 7 h after the injection of radiolabelled cortisol (Dantzer et al., 2010). In Damaraland mole-rats (*Fukomys damarensis*) (Medger et al., 2018), uGCMs were already considerably increased 1 h after the injection of ACTH, but the peak was observed much later (~ 12 h after treatment) than in eastern rock sengis. The presented pattern in uGCM concentrations following an ACTH challenge in sengis is thus comparable to the findings in these studies and time-related differences in peak presence could be related to differences in urine production and excretion between these species. We also observed marked differences in uGCM concentrations between the sexes. Both baseline and elevated uGCM concentrations measured during the high-dose ACTH treatment were comparatively higher in females than in males. Sex differences in glucocorticoid concentrations are relatively common in mammals and may be related to the female oestrous cycle. Although this cannot be excluded in our study, it is unlikely that the higher uGCM concentrations in females could be explained through synchronised oestrous cycles as all females were kept separately. It is more likely that females may have higher plasma glucocorticoid concentrations and/or that there are sex-related differences in the metabolism and/or way of excretion of glucocorticoids and their metabolites. Female mice, for example, excrete a higher proportion of radiolabelled corticosterone via the urine, whereas males excrete more over the faeces (Touma et al., 2003) and similar differences have also been observed in other small mammals (e.g. Sipari et al., 2017).

With regards to uGCM concentrations, we could also confirm preliminary findings of previous studies (Medger, unpublished results; Hoffmann, 2014) indicating generally high concentrations of uGCMs in eastern rock sengis when applying the methods described in this study. These concentrations seem biologically relevant and can reach exceptional concentrations when the HPA-axis is pharmacologically stimulated. The maximum concentrations reached during the present study was 629 ± 450 µg/mg Cr in females and 283 ± 178 µg/mg Cr in males, with baseline concentrations of 27 ± 8 µg/mg Cr for females and 7 ± 1 µg/mg Cr for males, respectively. Although a cross-species comparison of uGCM concentrations provides no body of proof due to the impacting factors mentioned above, uGCM concentrations measured in humans are usually well below 1 ng/mg Cr (Butts et al., 2014) and maximum concentrations reached after an injection of ACTH in Damaraland mole-rats were less than 250 ng/mg Cr (Medger et al., 2018). The

reasons for the presumed high uGCM concentrations in the eastern rock sengi are currently unknown and studies evaluating the effect of biological stressors are required to better understand the origin of these high glucocorticoid concentrations in eastern rock sengis. An unpublished study by our group found a large range of plasma cortisol concentrations in wild-caught eastern rock sengis with some individuals showing concentrations above 0.5 µg/ml. This would indicate that at least some sengis exhibit very high cortisol plasma levels and seemingly with no apparent detrimental effects (unpublished data). Considering the low concentrations of glucocorticoids measured in the faeces, it is, therefore, possible that glucocorticoids are mostly excreted via the urine in this species. Further studies are required to confirm this hypothesis.

In contrast to uGCM, fGCM concentrations were only significantly increased in females receiving a high dose of ACTH. Peak fGCM concentrations following stimulation were observed about 4 h (13 h post treatment), later than in urine. Peak fGCM concentrations observed in female sengis occurred slightly later than in laboratory mice (8-10 h, Touma et al., 2003, 2004), bank voles (6-8 h, Sipari et al., 2017) and North American red squirrels (~8 h, Dantzer et al., 2010), but were observed much earlier than in other small mammals such as golden hamsters (20-28 h, Chelini et al., 2010), Damaraland mole-rats (~24 h, Medger et al., 2018), and meerkats (*Suricata suricatta*) (~25 h, Dantzer et al., 2017). These differences are probably related to differences in gut passage times (Palme et al., 1996). The gut passage time is currently unknown for the eastern rock sengi, but defecation rate appears to be higher during the night than during the day (K Medger, personal observation). During the night, these higher defecation rates could result in a shortening of the time until peak fGCM concentrations are observed. Conducting radiometabolism experiments, Touma et al. (2003) found a shortening of the time of peak concentrations in faeces from 10 h during the day to 4 h during the night. The group-specific antibody used in the present study was not able to show a consistent increase in fGCM concentrations after ACTH injection in male sengis although two animals exhibited an increase in fGCM after ACTH injection (see supplementary figures). As the composition of fGCMs can differ between the sexes of a species (Sipari et al., 2017; Touma et al., 2003), the antibody used in the present study may detect predominantly fGCMs of the females, but to a lesser extend biologically relevant metabolites present in the faeces of males. Consequently, the EIA may not

be sensitive enough to detect the comparatively lower changes in fGCM concentrations following ACTH administration in males. In mice, for which the EIA was developed, the chosen assay was able to detect a fGCM response following the same ACTH doses used in the present study in both males and females (Touma et al. 2003, 2004). However, sengis are phylogenetically ancient mammals and are not closely related to rodents but insectivorous (Churchfield, 1987), which may explain the different results observed in the present study in comparison to mice. Further studies would be required to determine the presence and relative abundance of respective immunoreactive steroids in male and female sengi excreta and if required a suit of additional assays would need to be tested to identify a potentially better performing assay for determining fGCM concentrations in male sengi.

Both uGCM and fGCM (in females only) concentrations remained elevated for between 4 h and 12 h post treatment in the eastern rock sengi, which is relatively short for a small mammal species, but similar to the durations observed in mice (Touma et al., 2004) and bank voles (Sipari et al., 2017). Many species, however, exhibit elevated fGCM concentrations following an ACTH challenge for more than one day (Chelini et al., 2010; Dantzer et al., 2010; Medger et al., 2018). Surprisingly few studies in small mammals give a more detailed description of the duration of the response to a stressor or specifically ACTH although such knowledge is of importance for future study designs such as those using repeated sampling and attempting to measure repeated exposure to stressors. This is even more important considering the large variation in responses between different species that are of similar size and between the sexes. In bank voles, for example, fGCM concentrations are elevated for about 6 h longer in males than in females following an ACTH injection (Sipari et al., 2017). The opposite was found for golden hamsters, in which the fGCM response to ACTH stimulation lasts 8 h longer in females than in males (Chelini et al., 2010). In addition, the intensity of a stressor can also play a role in the duration of the elevated glucocorticoid concentrations. Touma et al. (2004) found that the injection of a low dose of ACTH resulted in a shorter elevation of fGCM concentration in mice than a higher dose of ACTH. We were able to observe a similar dose-dependent response in the eastern rock sengi with uGCM concentrations being elevated for about 4 h during the low-dose and for about 12 h during the high-dose ACTH treatment. Overall, the responses to the two doses of ACTH were markedly different with higher uGCM concentrations measured after the high ACTH dose than

the low dose indicating that the method is sensitive enough to distinguish dose-dependent responses. In contrast, a response of the faecal metabolites was only observed in females after treatment with a high ACTH dose. Although studies using a dose-dependent approach would be advantageous in understanding if stressors of different intensity do indeed result in a measurable increase in glucocorticoids in different matrices, few studies have used such an approach (Crill et al., 2019; Palme et al., 1999; Touma et al., 2004). Distinct dose-dependent responses in fGCM concentrations were only observed in cattle by Palme et al. (1999) and in mice by Touma et al. (2004). In black-tailed prairie dogs (*Cynomys ludovicianus*), plasma cortisol concentrations were higher after a high dose of ACTH than a low dose, but this difference was not obvious when they measured faecal metabolites (Crill et al., 2019). These and our results indicate the importance of careful selection of matrix for the measurement of glucocorticoids for studies investigating stress-related hormone alterations and even more so, the necessity of a reliable validation of the applied method(s) and cautious interpretation of results.

Besides for the injection of ACTH, we also injected a saline solution to determine the effect of the procedure itself on the sengis. We observed no increase in uGCM or fGCM concentrations in response to the handling and subsequent saline injection, which was surprising as these animals show distinct behaviour changes upon small disturbances (usually they freeze when e.g. entering the room; K. Medger, personal observation). However, the sengis used in the present study had been in captivity for more than a year before the start of the experiments and may have become used to short-term handling and other disturbances associated with laboratory life. In general, laboratory animals or animals in zoological institutions, that are exposed to routine handling and examination, do not appear to show marked increases in glucocorticoids following the injection of saline (Bauer et al., 2008; Chelini et al., 2010; Mendonça-Furtado et al., 2017). Even some wild animals, such as black tailed prairie dogs, do not respond to a saline injection with an increase in glucocorticoid output (Crill et al., 2019). The lack of response in this and our study may be a result of a low sensitivity of the methods applied; however, contrary to Crill et al. (2019), we did observe distinct dose-dependent responses indicating that our method can detect small differences in at least uGCM concentrations in response to physiological stressors. Further studies need to investigate if the lack of a response to the injection procedure is due to the time in captivity and presumed habituation to laboratory

procedures of the sengis. In addition, the method should be tested using biological stressors to evaluate if it is sensitive enough to detect the effect of natural occurring stressors. Overall, uGCM concentrations were much higher after ACTH treatment than saline treatment in the sengis, which further confirms that the higher measurements are a true reflection of an activation of the adrenal cortex and not an effect of circadian changes in glucocorticoids. The same is true for the fGCM concentrations measured in females after the treatment with a high dose of ACTH.

The present study was able to validate a commercial RIA for the measurement of urinary glucocorticoid concentrations for both female and male eastern rock sengis. The assay used distinguished reliably between different strengths of a stressor as distinct differences of uGCM concentrations were detected for different doses of ACTH. Furthermore, we were able to confirm that a 5α-pregnane-3β,11β,21-triol-20-one EIA is suitable for the measurement of glucocorticoid metabolite concentrations in the faeces of females of this species. However, this EIA does not appear to be able to reliably detect small changes in faecal glucocorticoid metabolites following minor stressors and thus might not be optimal when monitoring male sengis. This study clearly underlines the importance of a thorough validation of methods for the non-invasive measurement of glucocorticoids in different matrices. It further emphasises the need to include both sexes and a dose-dependent approach in such studies if at all logistically and ethically possible.

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