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SHORT COMMUNICATION

Validation of a non-invasive technique for quantifying a stress-associated biomarker in a southern African hornbill

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Abstract

Quantification of glucocorticoids and their metabolites as a central component of the physiological stress response has many potential applications in ornithological research, but require careful species-specific validations. We established a reliable non-invasive method to measure faecal glucocorticoid metabolites (fGCM) in the southern yellow-billed hornbill (*Tockus leucomelas*). Following an adrenocorticotropic hormone (ACTH) challenge, plasma corticosterone levels increased by 110%, with a tetrahydrocorticosterone enzyme immunoassay (EIA) revealing a ~ fourfold increase in fGCM concentrations approximately 3 h post-injection. These data reveal droppings are a suitable matrix for monitoring stress-associated biomarkers in this species and suggest the tetrahydrocorticosterone EIA may often be appropriate for wild birds.

Keywords Faecal glucocorticoid metabolites · Non-invasive hormone measurement · Enzyme-immunoassay validation · ACTH challenge · Hornbill

Zusammenfassung

Validierung einer nicht-invasiven Methode zur Quantifizierung eines Stress-assoziierten Biomarkers bei einem südafrikanischen Nashornvogel

Die Quantifizierung von Glukokortikoiden und ihren Metaboliten als zentralen Bestandteilen einer physiologischen Stressreaktion hat viele potentielle Anwendungen in der ornithologischen Forschung, erfordert aber eine sorgfältige, artspezifische Validierung. Wir etablierten eine zuverlässige, nicht-invasive Methode zur Messung der Glukokortikoid-Metaboliten (fGCM) aus dem Kot von Rotringtokos (*Tockus leucomelas*). Im Anschluss an eine Ausschüttung von Adrenocorticotropem Hormon (ACTH) stiegen die Plasmakortikosteronspiegel um 110%, wobei ein Tetrahydrocorticosteron-Enzymimmunoassay (EIA) einen ~ 4-fachen Anstieg der fGCM-Konzentrationen etwa 3 Stunden nach der Injektion aufzeigte. Diese Ergebnisse zeigen, dass bei dieser Art der Kot eine geeignete Matrix für die Erfassung von Stress-assoziierten Biomarkern ist und deuten darauf hin, dass der Tetrahydrocorticosteron-Enzymimmunoassay (EIA) oft auch für Wildvögel geeignet sein könnte.

Introduction

Avian stress responses, including the activation of the hypothalamic–pituitary–adrenal (HPA) axis and associated temporal increases in circulating glucocorticoid concentrations (Sapolsky et al. 2000; Touma and Palme 2005), have far-reaching consequences for several fitness components. Over short time scales, activation of the HPA axis increases the likelihood of escaping life-threatening situations such as predation attempts (Wingfield et al. 1998), supports immune system function (Buchanan 2000), and promotes behaviours such as increased feeding urgency (Harvey et al. 1984). Over longer time scales, however, a prolonged increase in

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glucocorticoid output can cause reduced growth and immune function, long-term reproductive suppression, and negative behavioural and cognitive impacts (Harvey et al. 1984).

Non-invasive techniques for quantifying stress-associated biomarkers that obviate the need for capture and blood sampling are increasingly being employed (Touma and Palme 2005), particularly in the burgeoning field of conservation physiology (Wikelski and Cooke 2006). One such technique is the measurement of faecal glucocorticoid metabolite (fGCM) concentrations, which reflect a smoothed pattern of circulating plasma glucocorticoid concentrations (Cyr and Romero 2008). Although fGCM analysis offers a host of advantages over traditional blood-sampling, it requires species-specific validation because of interspecific variation in gut transit times (Möstl and Palme 2002), types and polarities of metabolites present in faeces, and specificity and cross-reactivity of the antibody used (Touma and Palme 2005).

The collection of faecal material is often more challenging for birds compared to mammals, but researchers working with habituated study populations can gain detailed insights into intra- and inter-individual variation in fGCM levels (e.g., Jepsen et al. 2019). Here we examine the suitability of five enzyme immunoassays for determining stress-related physiological responses in southern Yellow-billed Hornbills (*Tockus leucomelas*, Bucerotidae) by performing an adrenocorticotropic hormone (ACTH) challenge test. The species is widespread in southern Africa (Kemp 2005), but much of its breeding range will become unsuitable under business-as-usual climate change scenarios (Conradie et al. 2019). A non-invasive technique for quantifying stress-associated fGCM levels will facilitate studies of how endocrine stress pathways modulate responses to acute or chronic heat exposure.

Materials and methods

Five pairs of adult southern Yellow-billed Hornbills (hereafter, hornbills) were captured in the Kalahari Desert at Radnor farm (S 26°11′ E 22°88′), South Africa (see Jepsen et al. 2019 for site description) using springtraps baited with superworms (*Zophobas morio*). Following capture, each pair was temporarily housed in an aviary (3.5 m long × 1.7 m wide × 1.7 m high) for 1–3 days before transport in individual cloth bags suspended in an air-conditioned vehicle to the University of Pretoria. Upon arrival, each pair was transferred into one of five outdoor aviaries, four 5.5 m long × 1.8 m high × 1.8 m wide and one 5.0 m long × 2.5 m high × 2.0 m wide, for a two-week habituation period. The aviaries were outfitted with perches, with water and food (superworms, boiled eggs and chicken hearts) available ad libitum. Mean ± SD body mass was 259.0 ± 13.60 g for males and 199.3 ± 10.0 g for females, with body mass maintained throughout the period in captivity.

Following initial habituation, each bird was transferred to an individual cage (61 cm long × 42 cm high × 51 cm wide) in temperature-controlled rooms (4 m long × 3 m wide × 2.5 m high) in the University of Pretoria's Small Animal Physiological Research Facility, maintained at 20 °C with a photoperiod of 12 h light (06:00- 18:00), 12 h dark (18:00- 06:00). The following day, we conducted an ACTH challenge using methods identical to those described by Jepsen et al. (2019). Each bird received an injection of 2 IU/kg Synacthen® Depot (Novartis), with faecal samples collected hourly from 3 h preceding to 5 h following injection (Online Resource 1). With one exception, two blood samples were obtained from each individual, one immediately before and the second 30 min post-injection. Thereafter, birds were returned to the outside aviaries for a 2-week recovery period. After a subsequent separate study, each pair was released close to their original territories.

Plasma glucocorticoid and fGCM analyses were performed using EIA techniques, following Jepsen et al. (2019). References describing assay characteristics and components including cross-reactivities of the polyclonal antibodies are provided in Jepsen et al. (2019). Inter-assay CVs were determined for the two EIAs selected for analyzing the complete ACTH sample set ($n = 45$) by conducting repeated measurements of high- and low-value quality controls (12.62% and 15.13% for the 11-oxoaetiocholanolone II, and 10.87% and 14.90% for tetrahydrocorticosterone). Serial dilutions of faecal extracts gave displacement curves that were parallel to the standard curve for the best performing assay (relative variation of the slope of the trend lines < 2%). Sensitivities were 0.6 ng/g dry weight (DW) for 11-oxoaetiocholanolone I, 0.6 ng/g DW for 11-oxoaetiocholanolone II, 2.4 ng/g DW for 5α-pregane-3β,11β,21-triol-20-one, 9.0 ng/g DW for tetrahydrocorticosterone and 1.8 ng/g DW for corticosterone.

All statistical analyses were conducted in R 3.4.3 (2016, R Foundation for Statistical Computing, Vienna, Austria). Assumptions of normality were checked using a Shapiro–Wilk goodness of fit test, followed by Wilcoxon rank-sum tests to quantify concentration differences in both plasma glucocorticoid and fGCM measurements pre- and post-injection.

Results

Plasma corticosterone concentrations in pre- (range 8.18–90.22 ng/ml) and post-injection samples (range 22.16–187.06 ng/ml) increased significantly ($Z = -2.52$, $p < 0.05$) by 110% in response to ACTH injection (Fig. 1). By analyzing a sample subset ($n = 30$; 4 animals), the corticosterone EIA measured an overall individual median

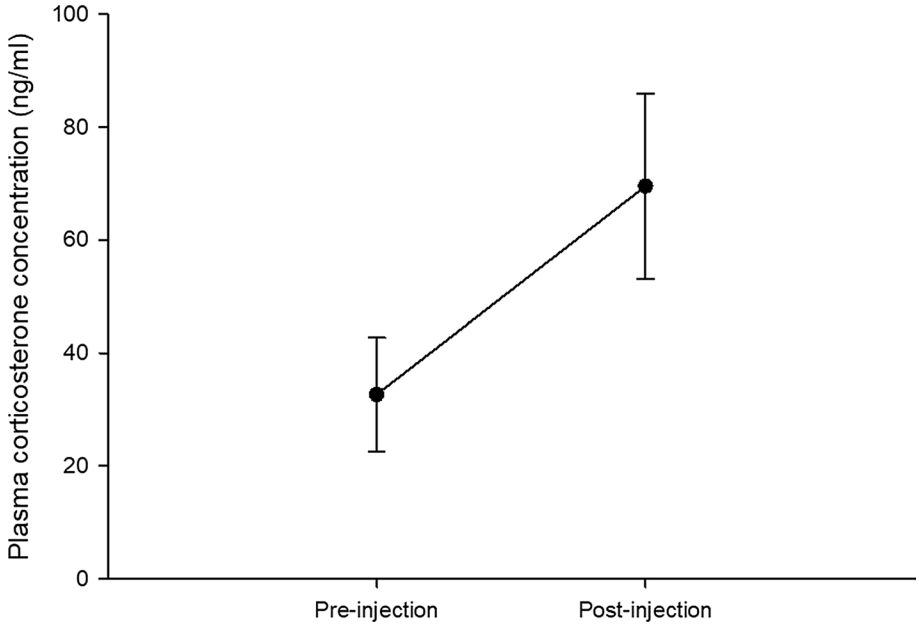

Fig. 1 Mean (±SE) plasma corticosterone concentrations in southern Yellow-billed Hornbills (*Tockus leucomelas*) pre- and post-ACTH injection

increase in fGCM concentrations of 158% post ACTH-injection, whereas the 5α-pregane-3β,11β,21-triol-20-one EIA revealed an increase of 154%. The 11-oxoaetiocholanolone I EIA showed a 17% increase and the 11-oxoaetiocholanolone II EIA an increase of 87%. The tetrahydrocorticosterone EIA performed best, measuring an increase of 372%. The two EIAs selected for analysis of the complete ACTH sample set were tetrahydrocorticosterone and 11-oxoaetiocholanolone II, because only those EIAs revealed responses for all four individuals tested. After analyzing the entire sample set, the tetrahydrocorticosterone EIA demonstrated an overall 322% increase in fGCM concentrations following ACTH injection and the 11-oxoaetiocholanolone II EIA an increase of 192%, with both assays revealing very similar fGCM profiles.

Profiles of fGCM concentrations determined with the tetrahydrocorticosterone or 11-oxoaetiocholanolone II EIA were similar, with the former EIA yielding a more distinct pattern (Fig. 2). Both assays revealed fGCM concentration minima at the time of injection ($t=0$) and maxima 3 h post-injection. Peak fGCM concentrations following ACTH

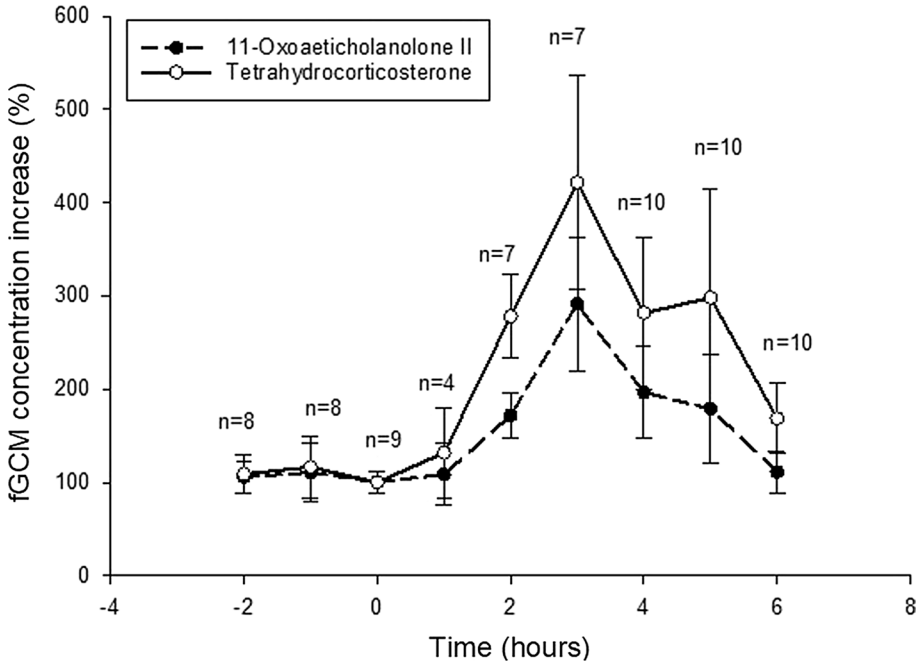

Fig. 2 The mean (±SE) change in faecal glucocorticoid metabolite (fGCM) concentrations (%) in southern Yellow-billed Hornbills (*Tockus leucomelas*) pre- and post-ACTH injection as measured by 11-oxoaetiocholanolone I and tetrahydrocorticosterone EIAs. Concentrations measured at the time of injection were considered as baseline fGCM concentrations (100%) from which the % increase in response to the injection could be calculated. Sample sizes were the same for both EIAs

administration were significantly higher than respective baseline values measured using the tetrahydrocorticosterone ($Z = -2.15$, $p < 0.05$) and 11-oxoaetiocholanolone I ($Z = -2.42$, $p < 0.05$) EIAs.

Discussion

Increases in hornbills' plasma glucocorticoid concentrations following ACTH administration were mirrored by elevated fGCM concentrations in excreta, confirming droppings are a suitable matrix for monitoring HPA axis activity. The magnitude of increases in plasma glucocorticoid concentrations were comparable to those observed in domesticated (e.g., Moneva et al. 2008) and non-domesticated species (e.g., Jepsen et al. 2019). Much larger increases have also been documented, including a ~ 40-fold increase in Greater Rheas (*Rhea americana*; Lèche et al. 2009). Variation also exists among studies of particular species: Dehnhard et al. (2003) reported a 16-fold increase 90 min post-injection in chickens, an increase more than twice that reported 60 min post-injection by Moneva et al. (2008). Sources of among-study variation, apart from assay specificity, are thought to include sex, age, and sensitivity to the injection (Touma and Palme 2005; Lèche et al. 2009).

Similarly to Jepsen's et al. (2019) study of Southern Pied Babblers (*Turdoides bicolor*), tetrahydrocorticosterone emerged as the best-performing EIA for quantifying fGCMs in hornbill droppings. That tetrahydrocorticosterone was the best for members of two phylogenetically-distant taxa suggests it may also prove suitable for non-invasively monitoring stress responses in other taxa.

For both EIAs, hornbill fGCM concentrations peaked ~ 3 h following ACTH injection (Fig. 2), a delay similar to that reported for southern pied babblers (75–95 g) inhabiting the same area (Jepsen et al. 2019). For both species, the lag times are substantially longer than allometrically-predicted gut passage times (hornbills: 92–97 min; babblers: 74–78 min) (Karasov 1990). Jepsen et al. (2019) attributed the later-than-expected peak in fGCMs to the intensity of the stress response caused by the ACTH injection or a greater-than-normal amount of food present in the gut. These factors could also apply to the present study, as the hornbills were fed superworms regularly throughout the ACTH challenge to facilitate faecal sampling, and a positive relationship exists between the amount of food in the gut and transit time (Afik and Karasov 1995).

In conclusion, non-invasive quantification of fGCM in individual wild birds will often be feasible only for habituated populations in which faecal samples can be reliably collected from focal individuals. In situations where this approach is feasible, however, it holds substantial promise for quantifying the effects of anthropogenic stressors on wild populations, as well as creating opportunities to evaluate the physiological roles of stress pathways in mediating the effects of higher maximum temperatures and more frequent heat waves in a rapidly warming world.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10336-021-01861-5.

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