Physiological validation of the use of faecal glucocorticoid metabolites as a measure of stress in a passerine and a columbid from southern Africa

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Abstract

Faecal glucocorticoid metabolite (fGCM) analysis provides a non-invasive, feedback-free approach for monitoring adrenocortical responses to natural and anthropogenic stressors. The use of enzyme-immunoassays (EIAs) to quantify immunoreactive fGCMs has gained popularity in recent years but requires species-specific validations prior to first use. We conducted a pharmacological challenge with adrenocorticotropic hormone (ACTH) to determine whether changes in circulating glucocorticoids are reflected in fGCM, concentrations and therefore to validate excreta as a matrix for monitoring endocrine status in a southern African passerine, the White-browed Sparrow-weaver (*Plocepasser mahali*) and a columbid, the Laughing Dove (*Spilopelia capensis*). We tested the suitability of four EIAs to quantify fGCMs in ten individuals of each species. Two of the EIAs, tetrahydrocorticosterone and 11-Oxoetiocholanolone II, detected significant elevations and were therefore most suitable for quantifying fGCMs in the White-browed Sparrow-weavers. In contrast, the 5α-pregnane-3β, 11β, 21-triol-20-one EIA detected the highest elevations in fGCM concentrations in the Laughing Doves. The lag time between stressor initiation (ACTH injection) and resulting peak fGCM concentrations was ~2 h in both species. The validations presented here open opportunities for monitoring physiological responses in free-ranging individuals and contribute to our knowledge of the EIAs suitable for non-invasive quantification of avian fGCM concentrations.

Keywords: ACTH challenge, assay validation, enzyme-immunoassay, faecal glucocorticoid metabolites, physiological stress

Introduction

Wild bird populations are declining rapidly due to anthropogenic impacts such as habitat destruction and climate change (Crick 2004; Riddell *et al*. 2019). Successful conservation efforts often require an understanding of the physiological processes and constraints underlying these declines (Wikelski and Cooke 2006). The use of faecal glucocorticoid metabolites (fGCM) as a biomarker for adrenocortical responses to stressful stimuli provides diverse opportunities for understanding and improving the welfare, health, and reproduction of birds (Möstl *et al*. 2005; Touma and Palme 2005). Unlike plasma GC quantification which is often limited by the frequency and volume of blood samples that can be collected from small animals such as birds, fGCM analysis allows for collection of samples without interfering with the animal's behaviour or welfare (Goymann 2005; Goymann *et al*. 2002; Touma and Palme 2005). Additionally, this approach does not require the capture and handling of animals, making it stress-free and convenient for use in small, evasive animals like birds. Faecal GCM analysis is particularly useful when working with habituated populations, where repeated samples can be collected from the same, individually identifiable birds (see Hirschenhauser *et al*. 2005; Jepsen *et al*. 2019; Moagi *et al*. 2021).

However, the analysis of fGCMs requires species-specific validations (Touma and Palme 2005; Palme 2019), because of inter- and intraspecific variations in the metabolism and excretion of fGCMs (Möstl *et al*. 2005; Touma and Palme 2005). These validations may be physiological or biological (Touma and Palme 2005). Physiological validations involve the use of pharmacological adrenocorticotropic hormone (ACTH) to induce changes in circulating GCs, whereas biological validations would involve monitoring a presumed stressful situation; in both cases to establish whether these changes/alterations are reflected in alterations of faecal GC metabolite concentrations (Möstl *et al*. 2005; Touma and Palme 2005). The suitability of an assay for fGCM quantification depends on the ability of specific antibodies to detect GC

metabolites in excreta of the investigated species (Touma and Palme 2005). Therefore, higher increase of fGCM concentrations following administration of ACTH indicates a better-suited assay (Möstl *et al*. 2005). Unlike circulating GCs, fGCM concentrations represent metabolized GCs over a species-specific lag period influenced in part by gut passage time (Goymann 2005; Palme 2019). Here, we aimed to 1) identify suitable enzyme-immunoassays (EIAs - assays that use enzyme labelled antibodies and antigens to detect biological molecules) for quantifying fGCM concentrations in two phylogenetically distant southern African bird species, White-browed Sparrow-weavers (*Plocepasser mahali;* Ploceidae: Passeriformes) and Laughing Doves (*Spilopelia capensis;* Columbidae: Columbiformes), and 2) estimate the lag period between the time of initiation of stress reaction (ACTH injection) and peak fGCM concentration response. Both species are considered common (not threatened), with Laughing Dove numbers increasing because of habitat disturbance (Hockey *et al*. 2005). This validation study provides biological support for using fGCMs to understand how environmental variables affect these birds.

Materials and methods

Adrenocorticotropin hormone (ACTH) stimulation/challenge tests were conducted on ten individuals of each of two species, White-browed Sparrow-weavers (blood samples: n = 18; dropping samples: n = 68) and Laughing Doves (blood samples: n = 18; dropping samples: n = 49). Experiments on White-browed Sparrow-weavers and Laughing Doves were conducted at the University of Pretoria Small Animal Physiological Research Facility (25°45′ S, 28°15′ E), in March 2019 and August 2020, respectively. White-browed Sparrow-weavers were caught at night using two small cloth bags mounted on aluminium poles and placed over both entrances of roost nests at the Barberspan Bird Sanctuary (26°33′ S, 25°36′ E) and Laughing Doves were caught in mist nets at the University of Pretoria's Experimental Farm. At the capture site, all birds were weighed and individually marked with plastic colour rings. Whereas

Laughing Doves were kept at the site of capture, White-browed Sparrow-weavers were transported to the experimental facility by road (3 h), in modified pet carriers. All birds were kept in individual cages (61 cm long × 43 cm high × 51 cm wide), with food (bird seed for Laughing Doves, and super worms (*Zophobas morio*) and bird seed for White-browed Sparrow-weavers) and water provided *ad libitum*.

Individuals of each species were allowed a 14-day habituation period prior to ACTH administration. On the day of injection, cage floors were lined with wax paper to facilitate the collection of droppings, conducted hourly starting 3 h pre-injection. We followed a previously used protocol (Dehnhard *et al*. 2003; Jepsen *et al*. 2019) using a ~2 IU kg^{-1} dose of Synacthen® Depot (Novartis) mixed with saline solution per bird. Each injection was administered using a 29-gauge needle to the *pectoralis* muscle (IM) to initiate an increase in GC production. Samples were collected for a further 5 h (White-browed Sparrow-weavers) and 4 h (Laughing Doves), respectively. Hourly collection of faecal samples was done to ensure that all excreta was collected and therefore changes and peak-fGCMs were not missed (as per Palme 2019). Collected samples were immediately frozen at -20 °C to minimise fGCM degradation at room temperature. Dropping samples were analysed as collected, with no separation of urine from faecal matter.

Additionally, we collected two blood samples per bird; the first immediately before ACTH injection and the second 30 min post-injection, to confirm increases in serum GC concentrations. Blood samples (maximum 200 µL) were collected via brachial venepuncture using 26-gauge needles and 100 µL non-heparinized capillary tubes (Hirschmann Laborgerate, Germany). Blood samples (in Eppendorf tubes) were chilled on ice for no more than 4 h to allow clotting, and then centrifuged at 1500 *g* for 15 min. The collected serum was then stored at -20 °C until subsequent analysis. Pre-injection concentration data from one individual per species were each > 2 SD above the mean value and excluded from further analysis as outliers.

After data collection, the birds temporarily remained in captivity for another study, whereafter they were released at their capture sites.

Circulating glucocorticoid analysis

To determine serum GC concentrations, samples were analysed using an enzyme-immunoassay (EIA), utilizing antibodies against corticosterone-3-CMO:BSA (Palme and Möstl 1997) . For analyses, untreated sera were diluted between 1:20 and 1:2000 for Laughing Doves, and 1:100 and 1:2000 for White-browed Sparrow-weavers. Sensitivity for the Corticosterone EIA was 80 pg mL^{-1}. Assay details including components, and antibody cross-reactivities are provided by Palme and Möstl (1997).

Faecal steroid extraction and fGCM analysis

The frozen faecal samples were lyophilized and pulverized before adding 1.5 mL of 80% ethanol in distilled water to ~0.05 g of faecal powder. The suspension was then vortexed for 15 min to facilitate steroid extraction following Ganswindt *et al*. (2002). After centrifuging for 10 min at 1500 *g*, the supernatant was transferred into micro centrifuge tubes and frozen at -20 °C until analysis.

We tested the suitability of four EIAs for quantifying fGCMs in the two avian species: 1) Corticosterone, 2) Tetrahydrocorticosterone (TH-CC; detecting fGCMs with 5β-pregnane-3α, 11β, 21-triol-20-one structure), 3) 11-Oxoetiocholanolone II (detecting fGCMs with a 5β-3α-ol-11-one structure) and 4) 5α-pregnane-3β, 11β, 21-triol-20-one (measuring 3β,11β-diol-cortisol metabolites). All four EIAs were used to measure fGCM concentrations in a subset of the faecal steroid extracts. The subsets (n = 33 for Laughing Doves and n = 31 for White-browed Sparrow-weavers) were from the five birds with the highest post-injection serum GC concentrations i.e., individuals showing greater responses to ACTH injection. Each EIA was tested for its suitability to detect fGCM concentrations in each species' droppings, using a set

minimum increase of 100% post-ACTH injection. Faecal GCM concentrations in the remainder of the samples were then measured using the best performing assay(s) for each species. Detailed assay characteristics have been provided by Quillfeldt and Möstl (2003) for TH-CC, by Palme and Möstl (1997) for corticosterone, by Möstl *et al*. (2002) for 11-Oxoetiocholanalone II, and by Touma *et al*. (2003) for the 5α-pregnane3β, 11β, 21-triol-20-one EIA. All analyses were performed at the Endocrine Research Laboratory, University of Pretoria, as described by Ganswindt *et al*. (2002).

The inter- and intra-assay coefficients of variation (CV) were determined by repeated measurements of high- and low-quality controls, and sensitivities of each assay for each species sample set are provided in Table 1. Additionally, parallelism tests were conducted for the best performing assay(s) in each species; serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curve (relative variation of the slope of respective trend lines; Table 1).

All statistical analyses were conducted in R version 4.0.4 (R Core Team 2021). Normality was confirmed using the Shapiro-Wilk goodness of fit test and paired t-tests were used to evaluate differences in circulating GC (log transformed, for Laughing Doves) between pre- and post-injection samples using the R package *dplyr* (Wickham and Francois 2015). The effect of lag time (predictor) on fGCM concentrations (response variable) was tested with a linear mixed-effects model run using the package *lmne* (Pinheiro and Bates 2000). Tukey's post hoc tests were also conducted, using package *emmeans* (Russell 2018) to test the difference between each time lag. If the p-value was less than 0.05 then the variables were considered to show a significant difference.

Table 1: Coefficients of variation, sensitivity, and parallelism (for the best performing assay for each species) of EIAs used for fGCM analysis in Laughing Doves (*Spilopelia capensis*) and White-browed Sparrow-weavers (*Plocepasser mahali*) in an ACTH challenge test.

EIA		**Laughing Dove**	**White-browed Sparrow-weaver**
Tetrahydrocorticosterone	Inter-assay CV	11.9 and 13.1%	13.6 and 14.4%
	Intra-assay CV	5.3 and 7.8%	5.3 and 7.8%
	Sensitivity	9.6 ng g^{-1} DW	12 ng g^{-1} DW
	Parallelism		<4%
Corticosterone	Inter-assay CV	10.1% and 14.4%	9.3% and 12.2%
	Intra-assay CV	5.5% and 6.0%	5.5% and 6.0%
	Sensitivity	1.4 ng g^{-1} DW	1.8 ng g^{-1} DW
	Parallelism		
11-Oxoaetiocholanolone II	Inter-assay CV	11.7% and 12.0%	11.1% and 13.7%
	Intra-assay CV	4.9% and 6.6%	5.3% and 6.8%
	Sensitivity	0.8 ng g^{-1} DW	0.6 ng g^{-1} DW
	Parallelism		
5α-pregnane-3β, 11β, 21-triol-20-one	Inter-assay CV	9.9% and 9.8%	13.0% and 13.1%
	Intra-assay CV	5.1% and 5.6%	4.9% and 7.3%
	Sensitivity	2.4 ng g^{-1} DW	2.4 ng g^{-1} DW
	Parallelism	<1%	

Results

Plasma glucocorticoid concentrations

There was a significant difference in plasma GC concentrations between pre- and post-ACTH injection samples in Laughing Doves ($t = 2.65$, $df = 8$, $p = 0.029$) but not in White-browed Sparrow-weavers ($t = 1.29$, $df = 8$, $p = 0.236$). However, in both species, circulating GC concentrations were higher 30 min post-ACTH injection, with 136% increase from 34.44 ± 13.84 ng mL^{-1} (pre-injection mean ± SE) to 42.27 ± 11.33 ng mL^{-1} (post-injection) in Laughing Doves (Fig 1A) and a 50% increase from 32.71 ± 7.79 ng mL^{-1} to 43.15 ± 8.53 ng mL^{-1} in White-browed Sparrow-weavers (Fig 1B).

Faecal glucocorticoid metabolite concentrations

When the subset of samples was analysed for EIA selection, the TH-CC EIA showed an overall 58.6% (from 0.51 ± 0.03 to 0.80 ±0.09 µg/g DW) increase in fGCM concentrations in the Laughing Doves and 388% (from 0.33 ± 0.08 to 1.62 ± 0.29 µg/g DW) increase in White-browed Sparrow-weaver fGCM concentrations post-injection. The 11-Oxoaetiocholanolone II EIA revealed increases above 100% in fGCM concentrations in both Laughing Doves (101%) and White-browed Sparrow-weavers (103%). Further, the 5α-pregnane-3β, 11β, 21-triol-20-one EIA showed a 196% (from 0.15 ± 0.02 to 0.46 ± 0.09 µg/g DW) overall increase in Laughing Doves and 53% (from 0.12 ± 0.02 to 0.18 ± 0.03 µg/g DW) increase in the White-browed Sparrow-weavers. The Corticosterone EIA showed fGCM concentration elevations below the minimum set increase of 100% in both species (Supplementary Table 1).

When the complete sample set was analysed using the selected EIAs, there was significant variation in fGCM concentrations over the 6-7 h sampling period in both species. In Laughing Doves, the 5α-pregnane-3β, 11β, 21-triol-20-one EIA revealed a 235% (from 0.15 ± 0.02 to 0.45 ± 0.09 µg/g DW) increase in fGCM concentrations ($F_{6,35}$ =10.68, n = 49, p <

0.001), with the lowest concentrations recorded 2 h pre- and the highest fGCM concentrations 2 h post-ACTH injection (Fig 1C). In White-browed Sparrow-weavers, the TH-CC and the 11-Oxoaetiocholanolone II assays revealed a 433% (from 0.37 ± 0.07 to 1.99 ± 0.37 µg/g DW; $F_{7,51} = 7.27$, n = 68, $p < 0.001$) and a 284% (from 0.42 ± 0.13 to 1.61 ± 0.25 µg/g DW; $F_{7,51} = 5.62$, n = 68, $p = 0.001$; Fig 1D) increase in fGCM concentrations post-injection, respectively, the lowest fGCM concentrations observed 1 h pre-ACTH injection and highest 2 h post injection.

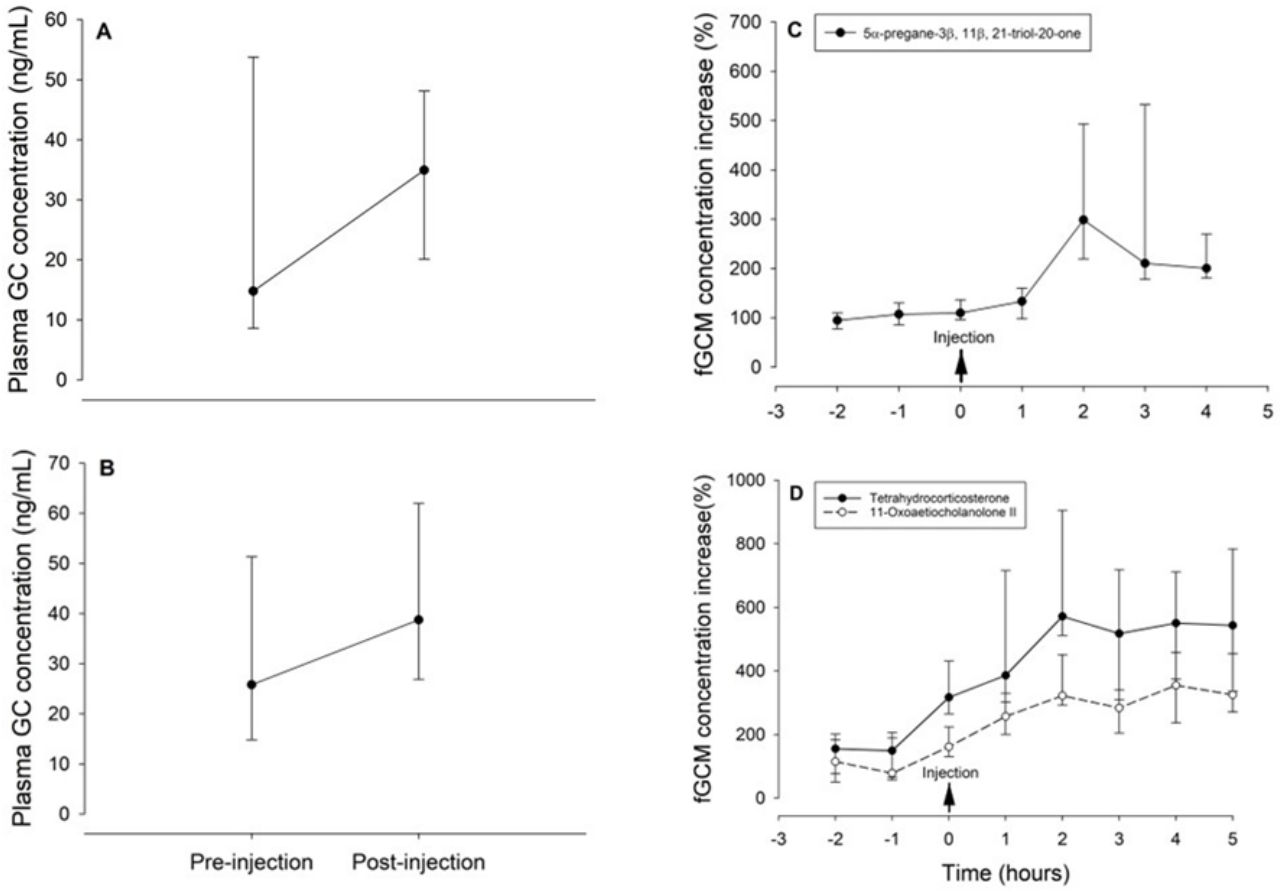

Figure 1: Circulating glucocorticoid (GC) concentrations (A and B) and faecal glucocorticoid metabolite (fGCM) concentrations (C and D) before and after ACTH injection in A) Laughing Doves (*Spilopelia capensis*, n = 9) and B) White-browed Sparrow-weavers (*Plocepasser mahali*, n = 9). Faecal GCM concentrations were higher post-ACTH injection in Laughing Doves evaluated with 5α-pregnane-3β, 11β, 21-triol-20-one (C), and White-browed Sparrow-weavers evaluated with both tetrahydrocorticosterone and 11-Oxoaetiocholanolone II (D). 100% concentration measured at 2 h (doves) and 1 h (sparrow-weavers) pre-injection was used as the baseline fGCM concentration from which the % increase resulting from ACTH challenge could be calculated. Data are presented as medians with the 75th percentile plotted above and the 25th percentile below each median point.

Discussion

Synacthen (ACTH) administration resulted in significant elevations in circulating glucocorticoid (GC) concentrations in Laughing Doves, but non-significant increases in White-browed Sparrow-weavers. Faecal GCM concentrations, on the other hand, increased significantly following injection in both species. The 5α-pregnane-3β, 11β, 21-triol-20-one EIA emerged as suitable (i.e., showed significant increases in fGCMs post-ACTH injection) for quantifying fGCM concentrations in Laughing Doves. Two EIAs, tetrahydrocorticosterone (TH-CC) and 11-Oxoaetiocholanolone II emerged as suitable for fGCM quantification in White-browed Sparrow-weavers.

The lack of a significant increase in circulating GC concentrations in the White-browed Sparrow-weavers was unexpected as Synacthen ACTH solution at the same dosage (~2IU kg^{-1}) significantly increased circulating GC concentrations in the Laughing Doves involved in the present study, as well as in Southern Pied Babblers (*Turdoides bicolor*) and Southern Yellow-billed Hornbills (*Tockus leucomelas*) in recent similar ACTH challenge experiments (Jepsen *et al*. 2019; Bouwer *et al*. 2021). This unexpected observation may reflect individual variation in adrenocortical responses. For both species, baseline GC concentrations of some individuals were 2- to 3-fold higher than ACTH-induced GC concentrations. Considerable individual variation in avian circulating GC concentrations attributed to genetic variation, prior exposure to stressors and among-individual variation in Hypothalamic Pituitary Adrenal (HPA) axis sensitivity has been observed in both baseline and stress-induced GC, as well as in the GC response magnitude (Wingfield *et al*. 1994; Cockrem and Silverin 2002). It is also possible that pre-ACTH injection GC levels were already elevated in response to the presence of researchers. Southern Pied Babblers increased GC concentrations (reflected in fGCM concentrations) prior to the ACTH injection in response to being separated from their social groups (Jepsen *et al.* 2019). This may also explain the slight increase in fGCM concentrations 1 h before the

injection was administered (Fig 1D). Additionally, plasma GC concentrations in the White-browed Sparrow-weavers may have peaked before or after the 30 min at which the post-injection samples were collected. Similar sized Dark-capped Bulbuls (*Pycnonotus tricolor*) showed peak GC concentrations at 20 min (in winter) and closely related Southern-masked Weavers (*Ploceus velatus*) had peak GC concentrations at 60 min post capture in a capture and restraint study (Ngcamphalala *et al*. *unpublished data*). Thus, the concentrations observed here may reflect GC levels already declining to baseline concentrations or still rising to peak concentrations. This may explain why there was no significant difference in plasma GC concentrations but significant increases in fGCM levels post-ACTH injection.

In this study, we validated the 5α-pregnane-3β, 11β, 21-triol-20-one EIA as the best performing assay out of four tested for quantifying fGCMs in Laughing Doves. We also validated two EIAs; TH-CC and 11-Oxoaetiocholanolone II as suitable for fGCM analysis in the White-browed Sparrow-weavers. All three assays have been successfully used for monitoring adrenocortical responses to a wide range of stressors in several vertebrate groups (Quillfeldt and Möstl 2003; Jepsen *et al*. 2019; Palme 2019; Moagi *et al*. 2021). The 2 h lag period between ACTH injection and peak fGCM concentrations observed in the Laughing Doves [91.78 ± 2.28 g (mean ± SE)] and White-browed Sparrow-weavers (44.88 ± 0.43 g) is comparable to that of similar-sized birds such as the Southern Pied Babblers (~75 g) investigated by Jepsen *et al*. (2019). However, the lag period in all these species is longer than the allometrically predicted mean digesta retention time (Y = 77.6 min in Laughing Doves, and Y = 66.5 min in White-browed Sparrow-weavers) calculated following Karasov (1990). These observations and additional observations on much smaller birds, European Stonechats (*Saxicola torquata rubicola;* ~14.7 g) with lag periods of ~4 h (Goymann *et al*. 2002), suggest that lag period is also influenced by factors other than gut passage time. One such factor is diet, which can affect both the concentrations of excreted GC metabolites and excreta mass

(Goymann 2005). Additionally, the administration of exogenous ACTH may have altered the gut microbiome, resulting in a longer lag period than expected. House Sparrows (*Passer domesticus*) exposed to mild chronic stress showed significantly reduced diversity in cloacal microbiota (Madden *et al*. 2022), suggesting that changes in the HPA axis may have implications for GC metabolism.

In conclusion, this study provides further support for the use of excreta as a matrix for quantifying changes in avian GC concentrations, and the use of fGCMs as a biomarker for physiological stress in birds. Our observations further emphasize the need for the species-specific validation of an assay prior to first use. The successful validation of EIAs for the two species in this study opens opportunities for monitoring their stress-related adrenocortical responses.

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Disclosure statement

The authors have no competing interests.

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