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TECHNICAL COMMENT

Validation of Noninvasive Monitoring of Adrenocortical Endocrine Activity in Ground-Feeding Aardwolves (*Proteles cristata*): Exemplifying the Influence of Consumption of Inorganic Material for Fecal Steroid Analysis

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ABSTRACT

Biologically inert material in feces may confound interpretations of noninvasive fecal endocrine data, because it may induce variance related to differences in foraging behavior rather than to differences in endocrine activity. We evaluated two different enzyme immunoassays (EIAs) for the noninvasive evaluation of adrenocortical activity in ground-feeding aardwolves (*Proteles cristata*) and tested the influence of soil content in aardwolf feces on the interpretation of fecal glucocorticoid metabolite data. Using adrenocorticotropic hormone (ACTH) challenges for validation, we successfully identified a cortisol EIA suitable for assessing adrenocortical activity in aardwolves. An alternatively tested 11-oxoetiocholanolone EIA failed to detect a biologically relevant signal after ACTH administration. Although the proportion of inorganic content in aardwolf feces did not alter qualitative conclusions from the endocrine data, the data related to mass of organic content had a larger amount of variance attributed to relevant biological contrasts and a lower amount of variance attributed to individual variation, compared with data related to total dry mass of extracted material. Compared with data expressed as dry mass of extracted material, data expressed as mass of organic content may provide a more refined and statistically powerful measure of endocrine activity in species that ingest large amounts of indigestible material.

Introduction

Noninvasive hormone analysis is an established technique for monitoring the functional status of the hypothalamic-pituitary-gonadal as well as hypothalamic-pituitary-adrenal axes (Palme 2005; Schwarzenberger 2007; Hodges et al. 2010). The method has gained popularity among wildlife endocrinologists after recognition of the practical difficulties and welfare implications of collecting blood samples from free-ranging animals, and it is often advantageous because it allows assessments of the endocrine status without perturbing the study animal (Heistermann et al. 1995; Whitten et al. 1998; Monfort 2003; Touma and Palme 2005; Sheriff et al. 2011). Usually, the concentrations of steroid hormone metabolites of gonadal or adrenal origin are measured in excreta such as urine or feces using radio- or enzyme-immunoassay (Hodges et al. 2010; Sheriff et al. 2011). In addition to the standard validation criteria for immunoassays, a physiological validation for each assay, sample material, and species is necessary, because pronounced species-specific differences in hormone metabolite excretion have been reported (Palme 2005; Palme et al. 2005; Touma and Palme 2005; Hodges et al. 2010).

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In fecal steroid analysis, results are generally expressed as amounts per unit of either the wet or dry mass of extracted fecal material (Schwarzenberger et al. 1996; Palme 2005; Lepschy et al. 2010). In both cases, the sample material should be intensively homogenized before collection, because steroids in feces can be unevenly distributed (Wasser et al. 1996; Palme 2005; Hodges et al. 2010). If the fecal material is dried prior to extraction, additional homogenization can be performed by sifting the lyophilized and pulverized fecal samples through a mesh strainer to remove nonhomogenized material, such as bone fragments or undigested fibrous material (Heistermann et al. 1993; Ganswindt et al. 2010*b*). An additional factor that might need to be considered for appropriate expression of results is the amount of physiologically inert material within a sample. For target species that ingest substantial amounts of soil while feeding (e.g., Nelson-Beyer et al. 1994), hormone metabolite concentrations may be more accurately expressed per mass organic compound rather than per total mass of extracted fecal material. Because soil represents physiologically inert material that is not related to the amount of metabolites excreted into the feces, it will dilute the sample and, hence, introduce a variation in the data that is unrelated to endocrine processes.

The aardwolf (*Proteles cristata*) is a small termitivore hyaenid occurring in eastern and southern Africa. Unlike most other myrmecophagus mammals, aardwolves are physically not capable of digging into termite mounds. Therefore, the animals feed on active termites only by licking them directly from the surface of the ground or from their mounds, which results in a large amount of soil being ingested (Koehler and Richardson 1990). Therefore, the aardwolf is an excellent candidate for examining the influence of consumption of intake of inorganic matter on fecal steroid levels. The aims of this study were twofold: (1) to validate noninvasive methods for assessing adrenocortical activity in free-ranging aardwolves and (2) to evaluate the influence of ingestion of inorganic material for interpretation of fecal glucocorticoid metabolite measures.

Material and Methods

Study Area and Study Animals

We conducted the study at Benfontein Nature Reserve, which is situated approximately 10 km southeast of Kimberley, South Africa (28.80°S, 24.77°E). The reserve covers 11,000 ha of semiarid terrain and lies within a transitional zone between dry Karoo, grassland, and Kalahari thornveld.

We fitted mounted VHF monitor collars to one male and one female aardwolf (Sirtrack; mass, ~60 g). Both animals were habituated to night observation with a spotlight from a vehicle in a concurrent study.

Fecal Sample Collection and Extraction

We collected 10–15 g of feces shortly after a study animal had defecated and had moved away from the defecation site. After the spot of defecation was identified, the fecal sample was collected using rubber gloves, and a well-homogenized aliquot was stored in a glass vial. The samples were placed on ice immediately and frozen at −20°C within 1 h after collection and maintained at that temperature until analysis.

We lyophilized and pulverized the fecal samples and sifted them using a mesh strainer to remove fibrous material (Fieß et al. 1999). We then extracted approximately 0.2 g of the fecal powder with 3 mL of 80% ethanol in water. After vortexing for 15 min and subsequent centrifugation for 10 min at 1,500 *g*, we transferred the supernatants into microcentrifuge tubes and stored them at −20°C until assayed (Ganswindt et al. 2010*b*). We determined the organic content of each sample according to the procedure described by Anestis et al. (2010). After the extracted fecal pellets were air-dried at room temperature under a fume cupboard for at least 48 h, we weighed (AT261 Delta Range; Mettler-Toledo) the dried fecal material again (initial weight). Subsequently, the dried fecal material was ashed in a muffle furnace (Protea Incinerator; Protea Laboratory Equipment) at 460°C for 1 h. After the remains were cooled in a desiccator for approximately 2 h, we weighed them (final weight) to calculate the weight of organic material combusted. Loss on ignition was calculated as the initial weight minus the final weight.

Physiological Validation

To evaluate whether the glucocorticoid metabolites (GCM) measured by our enzyme immunoassays (EIAs) corresponded to pharmacologically induced changes in circulating glucocorticoid levels, we performed an adrenocorticotropic hormone (ACTH) challenge on one male and one female aardwolf. A total of 10 IU of synthetic ACTH (Synacthen depot; Novartis) was injected intramuscularly into each study animal after anesthetizing it with 36 mg of ketamine hydrochloride and 0.6 mg of medetomidine hydrochloride using a CO_2-powered remote injection system (Tel-Inject). The medetomidine was subsequently reversed with 3 mg of atipamezole hydrochloride. Animals were kept under anesthesia for 60 min (for the male) or 50 min (for the female) and were mobile within 10 min after reversal. An initial blood sample was taken before ACTH administration, and a second blood sample was taken 30 min after administration, shortly before the animal was released, to provide a control of the actual effects of the challenge on circulating levels of glucocorticoids. Fecal samples were collected during a 4-d period before ACTH administration to provide a measure of baseline fecal GCM levels. After ACTH administration, the animals were continuously monitored as long as they were aboveground for 4 d, and all fecal samples were collected, stored, and processed as described above. We have no information as to whether animals defecated in their burrows. However, our observations showed that all animals always defecated very soon after emergence, which suggests that they did not defecate below ground. Therefore, we do not believe we have any sampling gaps for the 4-d period after ACTH administration.

Immunoassays

We measured serum cortisol concentrations in duplicates by solid-phase radioimmunoassay (RIA) using a polyclonal antibody supplied by Siemens (Coat-A-Count Cortisol). In brief, 25-μL aliquots of calibrator, control, or sample were pipetted into prepared tubes. Then, 1.0 mL of ^{125}I cortisol was added, and the tubes were incubated for 45 min at 37°C. After decanting all liquid, the tubes were patted dry and subsequently counted for 1 min in a gamma counter (2470 Wizard^2; Perkin Elmer). The analytical sensitivity of the RIA is 5.5 nmol/L, and its intra-assay precision is 3.0%–6.4%, expressed as coefficients of variation. The antibody used shows the following major cross-reactivities: cortisol, 100%; prednisone, 76%; 11-deoxycortisol, 11.4%; and corticosterone and cortisone, <1%.

We used two EIAs to determine GCM levels in the fecal samples collected during the experiment (table 1). The two evaluated EIAs were a cortisol and an 11-oxoetiocholanolone EIA, respectively. Sensitivity and accuracy of the assays as well as intra- and interassay coefficients of variation, determined by repeated measurements of pool samples, are given in table 1. Serial dilutions of extracted fecal samples gave displacement curves that were parallel to the respective standard curves in both assays. The cross-reactivities of the two antibodies are described by Palme and Möstl (1997) for cortisol and by Möstl et al. (2002) for 3α,11oxo-CM. Assays were performed on microtiter plates according to the procedure described by Ganswindt et al. (2002).

Statistical Analysis

We used mixed linear models to test for differences in fecal GCM levels between samples collected before and after ACTH administration and for differences in the proportion of inorganic material in feces between the male and the female. In the models for the ACTH challenge, we used fecal GCM levels as response and used a categorical variable classing each sample as having been collected before or after administration as fixed effect. We added hours from ACTH administration nested within each individual as a random effect structure. For post-administration samples, we included only samples collected within 24 h of ACTH administration. In the model evaluating differences in organic content, we used arcsine- and square root–transformed percentages of dry mass of organic content as response variable and each individual as fixed effect. In this model, we added collection date as a random effect structure. We used a likelihood ratio test to assess the significance of the main effect in each model. For all validation comparisons, we expressed hormone concentrations as mass of hormone excreted per unit mass of dry fecal material.

To evaluate the influence of inorganic material on interpretations of fecal steroid data, we used a variance component analysis to partition the amount of explained variance in fecal hormone data between the contrast of concern (i.e., before or after administration for the ACTH challenge), between-individual variation, temporal variation (i.e., between hours from injection; see random effect structure above), and between-sample variation (i.e., the residuals).

Results

Circulating cortisol concentrations increased nine- and fourfold by 30 min after ACTH administration in the male (before ACTH administration, 56 nmol/L; after ACTH administration, 512 nmol/L) and the female (before ACTH administration, 155 nmol/L; after ACTH administration, 631 nmol/L), respectively. The 11-oxoetiocholanolone EIA did not detect a significant increase in fecal GCM concentrations up to 24 h after ACTH administration ($\chi^2 = 1.72$, df = 1, $P = 0.19$), for either the male or the female (fig. 1*A*, 1*B*). However, the cortisol EIA detected a significant increase in fecal GCM levels after ACTH

Table 1: Characteristics of the two enzyme immunoassays used to examine glucocorticoid metabolite levels in aardwolf feces

Variable	Cortisol[a]	11-oxoetiocholanolone[b]
Antibody against	Cortisol-3-CMO:BSA	5β-androstan-3α-ol-11,17-dione-17-CMO:BSA
Label	Cortisol-3-CMO[c]	5β-androstan-3α-ol-11,17-dione-17-CMO[d]
Standard	Cortisol	11-oxoetiocholanolone
Sensitivity (pg/well)	1.5	3
Structure of measured metabolites	11β,17α,21-triol-20-one	5β-3α-ol-11-one
Accuracy (%)[e]	108.1 ± 10.2	118.1 ± 5.2
Intraassay coefficient of variation[f]	7.0–11.6	6.6–11.0
Interassay coefficient of variation[f]	5.4–8.4	4.0–7.1
Applicability verified	Yes	No

[a]Palme and Möstl 1997.

[b]Möstl et al. 2002.

[c]Coupled with N-biotinyl-1,8-diamino-3,6-dioxaoctane.

[d]Coupled with biotinyl-3,6,9-trioxaundecanediamine.

[e]Mean ± standard deviation (recovery of known steroid concentrations from fecal extracts).

[f]Values represent intra- and interassay coefficients of variation, determined by repeated measurements of high- and low-value quality controls.

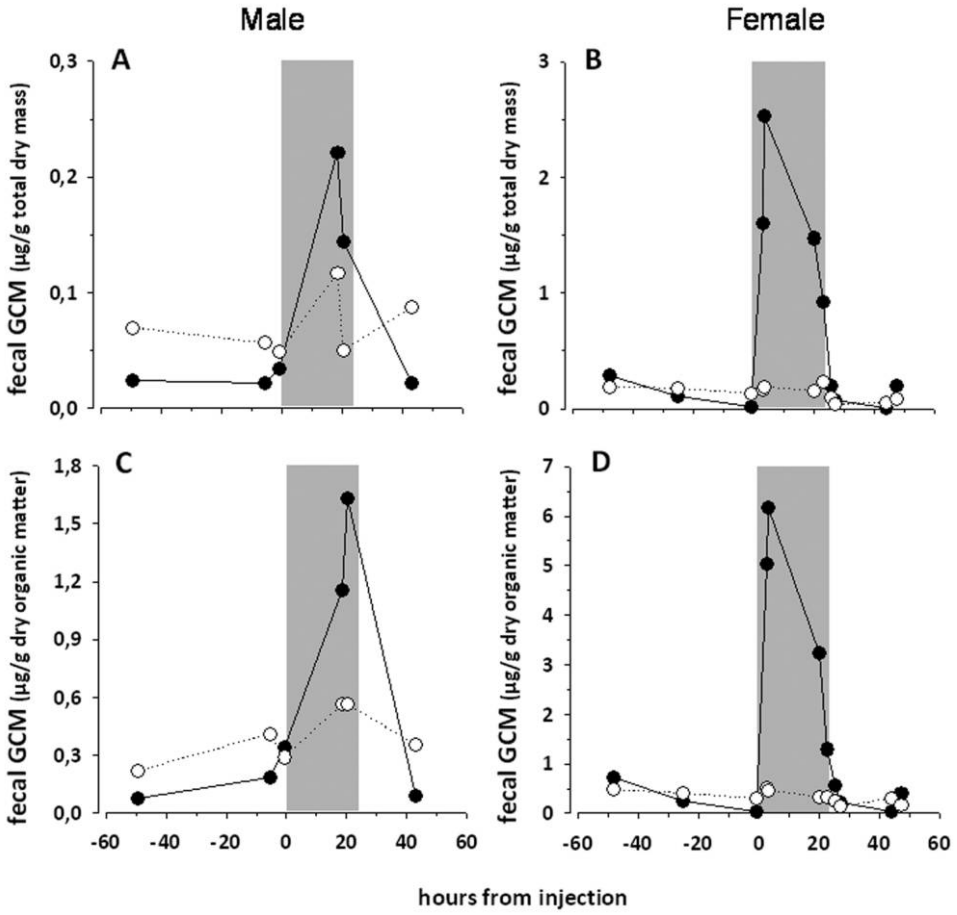

Figure 1. Fecal glucocorticoid metabolite levels measured by a cortisol enzyme immunoassay (EIA; black circles) and an 11-oxoetiocholanolone EIA (white circles) in one male (*A, C*) and one female (*B, D*) aardwolf before and after intramuscular injections of 10 IU of synthetic adrenocorticotropic hormone (ACTH) expressed as micrograms per gram of total dry mass of extracted fecal material (*A, B*) and as micrograms per gram of dry mass of organic material (*C, D*). We used different scales for the male (*A, C*) and female (*B, D*) and between samples expressed as micrograms per gram of total dry mass of extracted fecal material (*A, B*) and as micrograms per gram of dry mass of organic material (*C, D*) to facilitate comparisons between before and after samples within individuals for each data type. The shaded area depicts the time period of 24 h after administration that was used for statistical evaluation of the effect of the ACTH administration on detected glucocorticoid metabolite (GCM) levels.

administration ($\chi^2 = 7.28$, df = 1, $P < 0.01$), with peak GCM levels within 24 h after ACTH administration being 8 times higher for the male and 18 times higher for the female, compared with average baseline values before ACTH administration (fig. 1*A*, 1*B*).

We did not detect any significant differences in the proportion of inorganic material in feces between the male and the female ($\chi^2 = 3.06$, df = 1, $P = 0.08$). The qualitative patterns of detected steroid metabolites were similar when expressed as amount of metabolites per total dry mass of extracted sample (fig. 1*A*, 1*B*) and as amount of metabolites per amount of mass of organic material (fig. 1*C*, 1*D*). However, fecal hormone data expressed as metabolites per total dry mass consistently had a lower amount of explained variance attributed to the experimental contrasts and a larger amount of variance attributed to between-individual variation, compared with data presented as metabolites per mass of organic content (table 2).

Discussion

Our results provide the necessary physiological validations for establishing noninvasive analytical tools to monitor changes in GCM levels in aardwolf feces. We measured a substantial increase in circulating glucocorticoid levels after administration and detected the corresponding increase in fecal GCM levels with the cortisol EIA. Because the tested 11-oxoetiocholanolone EIA could not detect an increase in GCM after ACTH administration, we assume that this EIA failed to recognize relevant GCM in aardwolf feces. Interestingly, the same cortisol EIA was found to be suited for noninvasive evaluation of adrenocortical activity in domestic dogs (*Canis familiaris*), whereas another 11-oxoetiocholanolone EIA was not (Schatz and Palme 2001). In the spotted hyaena (*Crocuta crocutta*), which is a closer taxonomic relative to the aardwolf than is the dog, neither of the two evaluated EIAs was successfully validated for measuring

Table 2: Results from a variance component analysis of variance in measured glucocorticoid metabolites

Variable	Total dry mass (%)	Dry mass organic material (%)
Period[a]	21.42	79.64
Individual	72.17	4.27
Time[b]	<.01	.09
Residual	6.36	15.18

Note. Variance component analysis decomposing variance in measured glucocorticoid metabolites into experimental contrast of concern (i.e., before or after administration of adrenocorticotropic hormone [ACTH]), between-individual variation, time elapsed between samples, and between-sample variation (residual). Results are given for two ways of presenting fecal hormone metabolite data: total dry mass and dry mass of organic material. Total dry mass is the amount of measured steroid in the sample divided by total mass of dry extracted fecal material. Dry mass of organic material is the amount of measured steroid in the sample divided by the mass of the organic material in the extracted fecal material.

[a]Period refers to before or 24 h after ACTH administration, which was compared to evaluate the effect of ACTH administration on fecal glucocorticoid metabolite levels.

[b]Time refers to the effect of time elapsed between samples measured in hours.

GCMs, although a corticosterone RIA was (Goymann et al. 1999). However, the tested 11-oxoetiocholanolone EIA has successfully been used to detect hormonal changes in other mammal species (Ganswindt et al. 2010*a*, 2010*b*; Kleinsasser et al. 2010; Hulsman et al. 2011). These results highlight the necessity for species-specific validation of every assay used to detect GCM in feces (Palme 2005; Touma and Palme 2005).

Although the amount of inorganic material did not affect our qualitative interpretations of the fecal hormone data, it did influence the variance structure. Data normalized by total dry mass consistently had a larger amount of the variance attributed to within-individual variation, compared with data normalized by dry mass of organic material, whereas data normalized by dry mass of organic content consistently had a higher proportion of variance explained by the experimental contrast (i.e., sample period). We suggest that these results could have been caused by the removal of confounding variance related to intraindividual variation in the amount of ingested inorganic material. We therefore believe that data presented in this way could be a closer representation of the circulating hormone levels and also have a higher statistical power to detect biologically relevant differences in endocrine parameters. Although our study had low samples sizes and is preliminary in nature, it presents a novel method to control for variation in foraging behavior on fecal steroid monitoring. We believe it could be a useful approach for many study systems, in particular ones in which animals frequently ingest large amounts of biologically inert material.

To conclude, we successfully identified a noninvasive assay system suitable for measuring glucocorticoid metabolites in aardwolf feces. Although the variable amount of inorganic material in aardwolf feces did not substantially affect the qualitative interpretations of our endocrine data, our analyses suggest that fecal endocrine data related to mass of organic content will provide a more refined measure of endocrine activity, with a higher statistical power to detect biologically relevant differences.

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