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Determination of Fecal Glucocorticoid Metabolites to Evaluate Stress Response in *Alouatta pigra*

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Abstract Measuring fecal glucocorticoid metabolites is now a common practice to assess the stress response in primates. Nevertheless, it is important to validate the utilized immunoassay for each primate species before the technique is applied to populations in the wild. We determined the stress response of black howlers (*Alouatta pigra*) via 2 different group-specific enzyme immunoassays (EIAs). 11-oxoetiocholanolone EIAs are suited to assess the stress response of black howlers via fecal glucocorticoid metabolites. Levels of fecal glucocorticoid metabolites increased after we applied a stressor, i.e. anesthesia, reaching peak concentrations 24–96 h poststressor. Both basal and stress-induced fecal glucocorticoid metabolite levels showed individual variations. The increase of fecal glucocorticoid metabolites after the stressor (paralleling increases in serum) indicates that one can effectively measure adrenocortical activity in *Alouatta pigra* via these 2 enzyme immunoassays. However, it is important to consider individual variations in the excretion of fecal glucocorticoid metabolites when planning field endocrinological research on *Alouatta pigra*. Fecal glucocorticoid metabolite excretion takes 1–3 d poststressor depending on the individual. Further, there is an important individual variability in the concentrations of glucocorticoid metabolites, which might reflect differences in stress reactivity or fecal glucocorticoid metabolite metabolism and excretion.

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Introduction

Hormonal studies are currently being incorporated in wildlife research as a means to evaluate the health and physiology of individuals (Tarlow and Blumstein 2007). Because stressful events have potential deleterious effects on animal reproduction and immune systems (Sapolsky *et al*. 2000), it is of special concern to monitor the stress response in free-ranging animals. After the organism has been exposed to a stressful event, the adrenal cortex releases glucocorticoids into circulation, and their concentrations in the blood increase as part of the stress response (Sapolsky *et al*. 2000). Glucocorticoids are also involved in metabolic regulations and may vary according to reproductive state and seasonal fluctuations adapting the organism to changing conditions (Romero 2002; Sapolsky *et al*. 2000). Because glucocorticoids—either cortisol or corticosterone—are released during stressful situations, they can serve as an index of the stress response, and the development of noninvasive techniques to measure glucocorticoid metabolites in feces or urine has received increasing attention in field research (Schwarzenberger 2007; Ziegler and Wittwer 2005). Such a technique has the advantage of keeping subjects undisturbed during collection of samples (Möstl and Palme 2002). However, the stress response and the metabolism and excretion of glucocorticoids may vary among species, or even between sexes of the same species (Palme *et al*. 2005). Therefore, it is very important to conduct validations for each species under investigation (Palme 2005; Touma and Palme 2005).

Physiological or biological validation is a crucial issue to demonstrate that a technique can detect changes in adrenocortical activity and thus provides meaningful results (Touma and Palme 2005). A pharmacological stimulation of the adrenal cortex via administration of adrenocorticotropic hormone (ACTH) has proven a useful method for such a validation, but its practice is not always feasible because of policies of zoological institutions and animal handling restrictions (Palme 2005; Touma and Palme 2005). As an alternative to stimulate adrenal activity, it is possible to take advantage of periodical medical routines or anesthesia, which act as a stressor in primates (Heistermann *et al*. 2006; Whitten *et al*. 1998).

Some authors have reported fecal cortisol levels in primates: tufted capuchins (Lynch *et al*. 2002) and ring-tailed lemurs (Pride 2005), but other researchers demonstrated that native cortisol is excreted in low quantities in primate feces or that it is even absent (Bahr *et al*. 2000; Heistermann *et al*. 2006), in which cases cortisol-specific assays may result in spurious values. In contrast, glucocorticoid metabolites are always excreted in feces, but the amount of different glucocorticoid metabolites varies according to species (Palme *et al*. 2005). Therefore, it is impossible to know *a priori* which fecal glucocorticoid metabolites might represent the circulating hormonal status of a subject more accurately, and which antibody will detect the majority of the formed metabolites (Touma and Palme 2005).

Commercial antibodies, manufactured to measure specific circulating hormones in blood, are frequently used to determine hormone concentrations. However,

sometimes they cannot quantify hormone metabolites excreted in feces because their chemical structures changed when they were metabolized (Palme *et al*. 2005). Based on several studies, Möstl and Palme (2002) and Möstl *et al*. (2005) suggested that antibodies capable of detecting a group of fecal glucocorticoid metabolites are more useful to determine adrenocortical activity.

Many forests in tropical regions are being transformed at increasing rates, and the consequent perturbations can impose stressful situations to animals inhabiting the disturbed areas (Wingfield *et al*. 1997). Among New World primates, some populations of Mexican black howlers (*Alouatta pigra*) are under anthropogenic pressures as a result of forest loss (Van Belle and Estrada 2006). Using commercial kits for cortisol quantification, Martínez-Mota *et al.* (2007) previously reported that populations of black howlers in disturbed areas show higher fecal cortisol metabolite concentrations than those from undisturbed forests, probably owing to stress. Because of the low native cortisol concentrations excreted in feces, certainty of results calls for the selection of specific fecal glucocorticoid metabolite assays to determine accurately the physiological status of potentially disturbed populations. Early diagnosis of physiological changes may allow conservation management strategies before extinction takes place. Noninvasive monitoring of the stress response of black howlers might help to evaluate their health. We aimed to validate physiologically 2 group-specific enzyme immunoassays (EIAs) for evaluating the stress response of black howlers. We were particularly interested in determining whether fecal glucocorticoid metabolites increased after anesthesia, using cortisol blood levels as reference.

Methods

Subjects

We used 3 adult male and 1 adult female black howlers (*Alouatta pigra*) at the Chapultepec Zoo in Mexico City (Table I). Howlers were in outdoor enclosures (2.80 m×2.80 m×3.5 m) from *ca*. 0830 h to 1600 h, the period when zoo policy allowed observation and sample collection. One male and the female were in 1 enclosure and each of the other 2 males were in individual ones. In the evening, we moved subjects to night indoor enclosures. This was a daily routine and the male-female pair was maintained together during the night. A keeper fed howlers twice a day with a diet based on a mixture of Mazuri Leaf Ester® monkey chow, lettuce, apple, orange, banana, papaya, watermelon, rice cereal, and yogurt; alfalfa and water were available *ad libitum*. During the study, we kept howlers off display.

Anesthesia and Blood Collection

To stimulate adrenocortical activity in the monkeys, we physically restrained and immediately anesthetized them with 10 mg/kg body mass of ketamine (Ketaset®). We kept them anesthetized for 30 min. We took 2 blood samples from each individual at different times: an initial sample 7–12 min after injection of ketamine and the second sample after 15–20 min. In the female there was a shorter interval

Table I Identification, sex, body mass, number of fecal samples before and after stressor, maximum-fold increase, and poststressor peak concentrations of fecal glucocorticoid metabolites of black howlers at Chapultepec Zoo included in a stress response experiment using anesthesia as stressor

ID	Sex	Body mass (kg)	Fecal samples before treatment	Fecal samples after treatment	11,17-Dioxoandrostanes[a]	Poststressor peak concentrations reached at different hours[b]	5β-3α-ol-11-one corticoid metabolites[a]	Poststressor peak concentrations reached at different hours[c]
M1	Male	9.6	4	4	2.3	2881 ng/g at 96 h	2.2	2859 ng/g at 24 h
M2	Male	7.7	5	4	5.6	775 ng/g at 96 h	13.1	1532 ng/g at 96 h
M3	Male	7.5	5	3	1.4	1152 ng/g at 24 h	1.8	1400 ng/g at 24 h
F1	Female	7.3	4	4	7.1	1792 ng/g at 72 h	6.2	2286 ng/g at 72 h

[a] Numbers represent x-fold increase above the median baseline values.
[b] Concentrations measured via the 11,17-dioxoandrostanes enzyme immunoassay.
[c] Concentrations measured via the 5β-3α-ol-11-one corticoid metabolites enzyme immunoassay.

between the first and the second blood sample. We collected whole blood samples in Vacutainer tubes and separated 1 ml of serum via centrifugation (1600 *g* for 10 min). We stored the serum at –70°C until assay.

Collection of Feces

We collected feces from the 4 individuals during 8–12 d: from 4–8 d before anesthesia until 4 d after anesthesia. We collected feces between 1400 and 1600 h, as monkeys defecated then. We took feces immediately after defecation, stored them in plastic bags labeled with individual identification, and kept them in a freezer (–20°C) until assay. The total numbers of fecal samples per subject are in Table I.

Steroid Analysis

For serum cortisol concentration determinations we used a cortisol EIA after extraction with diethyl ether (Palme and Möstl 1997). Sensitivity of the assay was 0.3 pg/well, and intra- and interassay coefficients of variation were 8.9% and 11.1%, respectively.

We extracted glucocorticoid metabolites from the fecal matrix per the procedure of Palme (2005). In brief, we added 5 ml of methanol (80%) to 0.5 g of the fecal sample (wet mass) and vortex-mixed the suspended sample for 2 min. We centrifuged fecal suspensions (2500 *g* for 15 min) and analyzed an aliquot of the supernatant after a 1:10 dilution with buffer assay. We measured fecal glucocorticoid metabolites with 2 group-specific 11-oxoetiocholanolone EIAs. One EIA measures 11,17-dioxoandrostanes and the other one fecal corticoid metabolites with a 5β-3α-ol-11-one structure. Heistermann *et al*. (2006) and Ostner *et al*. (2008) used assays successfully in primate species, and Möstl *et al*. (2002) and Palme and Möstl (1997) provided a

detailed description of the EIAs and their cross-reactivities. We checked both EIAs for parallelism by diluting 2 high-concentration samples each in the same steps as the standard, resulting in perfectly parallel curves. Sensitivity of both assays was 0.8 pg/well. Intra- and interassay coefficients of variation are 9.5% and 11.3%, and 8.9% and 12.5%, respectively. Concentrations are in ng/g wet feces.

Statistical Analysis

Data were not normally distributed and our sample size was small; therefore we used nonparametric analyses. To determine if fecal glucocorticoid metabolites measured via the 2 EIAs differed before and after the stressor, we calculated median values per individual before anesthesia and matched them with poststressor peak concentrations, i.e., the highest poststressor values that were≥2 standard deviations above the mean concentration before stress, using a Wilcoxon signed-rank test. We performed a Spearman correlation analysis to compare concentrations of fecal glucocorticoid metabolites detected by the 2 EIAs.

Results

Serum cortisol concentrations increased after anesthesia at least during 20 min. In the 3 males, cortisol levels were higher in the second blood sample (taken 15–20 min after the injection of ketamine) compared to the first blood sample (taken 7–12 min after the injection of ketamine). However, concentrations varied among individuals (Fig. 1). Female cortisol levels showed no variation between the first and second sample.

Fecal glucocorticoid metabolites increased after the stressful event, but differences with respect to prior-stressor levels are not significant (Fig. 2 a, b; $Z=1.82$, $n=4$, $p=0.06$ in both cases). Fecal glucocorticoid metabolite levels varied among individuals during the days after the stressful event. Maximum-fold increase differed between them and reached a peak after 72–96 h depending on the individual (Table 1). There is a significant correlation ($r_s=0.88$, $p<0.001$) between fecal glucocorticoid metabolite levels determined via the 2 EIAs.

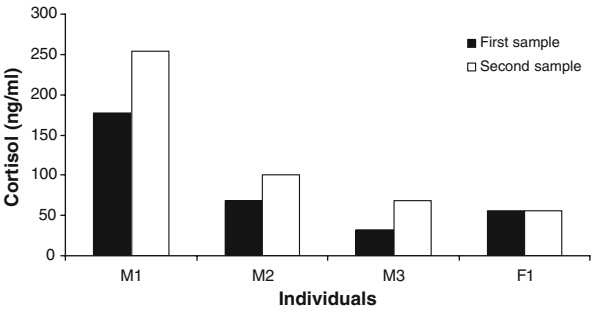

Fig. 1 Serum cortisol concentrations (ng/ml) of 4 black howlers measured in 2 consecutive samples during anesthesia. M1 = male 1, M2 = male 2, M3 = male 3, F1 = female 1.

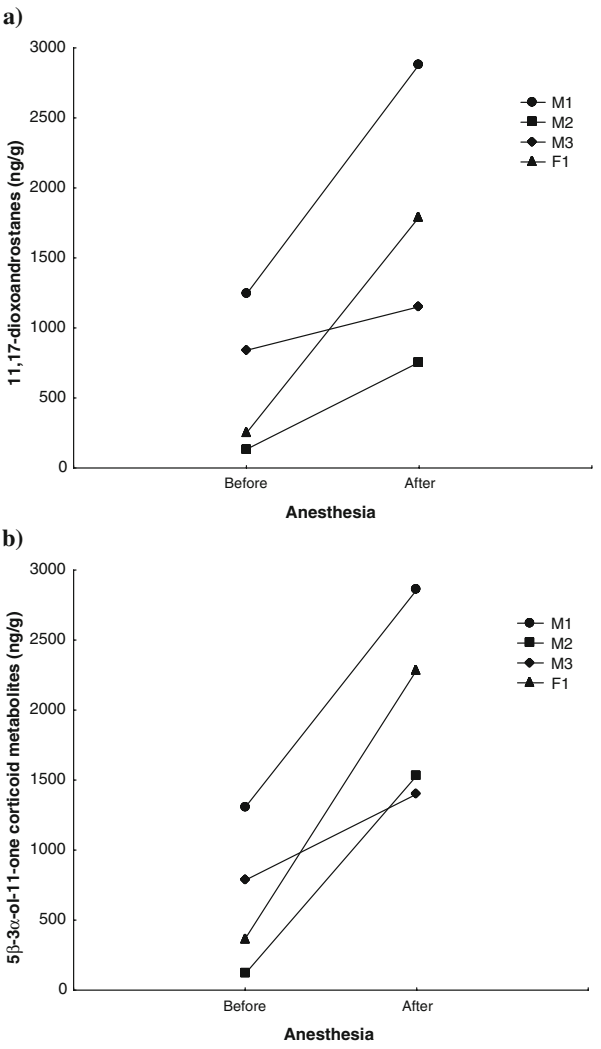

Fig. 2 Fecal glucocorticoid metabolite levels of black howlers before (individual median values) and after (individual peak-concentrations) anesthesia measured via (a) 11,17-dioxoandrostanes and (b) 5β-3α-ol-11-one corticoid metabolites enzyme immunoassays. M1 = male 1, M2 = male 2, M3 = male 3, F1 = female 1.

Discussion

We successfully validated the emulation of the physiological stress response in excreted fecal glucocorticoid metabolites of 4 black howlers via 2 group-specific EIAs. First, we found that circulating cortisol concentrations were higher 15–20 min after the stressor. Because of zoo handling limitations, we do not have baseline values to compare with those reflecting induced stress, because we took first blood samples 7–12 min after commencement of the anesthesia protocol. Romero and Reed (2005) mentioned that baseline levels are reliably obtained during the first 2–3 min poststressor. Although first serum cortisol measurements do not reflect baseline levels, they are a useful comparison point for contrasting with second

cortisol measurements, which were higher than the first ones for the males. We consider this proof that blood cortisol concentrations increase throughout time after the occurrence of a stressor in black howlers, and it was also reflected by increased concentrations of fecal glucocorticoid metabolites. However, the female did not show variation between the first and second serum sampling measurement. The last finding may be the result of the narrow interval between the 2 blood samples, or that the female's concentrations reflect an earlier onset of the stress response than that of the males.

Fecal glucocorticoid metabolite levels increased after the application of the stressor, though differences between glucocorticoid metabolites before and after anesthesia are only marginally significant, probably owing to a small sample size. Working with captive animals limits sample sizes to a few individuals, but our findings suggest that an increase in the number of subjects sampled would result in a statistically significant difference. Our results are in agreement with those of Heistermann *et al*. (2006), who found that fecal glucocorticoid metabolites in lowland gorillas and in long-tailed and Barbary macaques, increase after anesthesia or a pharmacological challenge, e.g., ACTH injection, as detected via the 11-oxoetiocholanolone EIA.

The fecal glucocorticoid metabolite poststressor increment differed among individuals. There was a general rise after 24 h, and highest peaks were reached during the subsequent 72–96 h. The time lag that exists between the stress-induced circulating glucocorticoids and the appearance of their metabolites in the feces is related to gut passage time (Palme *et al*. 2005). Howler gut passage time ranges from 23–30 h to a maximum of 72 h (Milton 1984), in agreement with the first rise of excreted glucocorticoid metabolites in all monkeys, which occurred 24 h post-stressor, but reached maximum levels after 3–4 d.

Though each individual showed increased fecal glucocorticoid metabolite levels after the stressor, their magnitude and increase varied among individuals in the course of time. For example, the maximum poststressor concentration detected via the 11,17-dioxoandrostanes EIA for male M1 was 2881 ng/g, whereas there was a maximum of only 755 ng/g for male M2. Thus, as shown in almost all other species investigated (Touma and Palme 2005), there is notable individual variation in fecal glucocorticoid metabolites excretion that one should take into account when evaluating the stress responses of wild primates.

The 2 group-specific antibodies, known to detect a family of cortisol metabolites in feces (Möstl *et al*. 2002; Palme and Möstl 1997), were suitable to assess the stress response of black howlers. Both assays quantified similar amounts of excreted fecal glucocorticoid metabolites. The 11-oxoetiocholanolone EIA, which measures 11,17-dioxoandrostanes, detects adrenal activity in other New World monkeys (common marmoset, *Callithrix jacchus*: Bahr *et al*. 2000), in Old World monkeys (Barbary macaque, *Macaca sylvanus*: Wallner *et al*. 1999), and in great apes (chimpanzee, *Pan troglodytes*: Bahr *et al*. 2000). Researchers have also successfully used the other EIA, which measures fecal corticoid metabolites with a 5β-3α-ol-11-one structure, to evaluate stress responses in several primate species (Barbary macaque [*Macaca sylvanus*], long-tailed macaque [*M. fascicularis*], and lowland gorilla [*Gorilla gorilla*]: Heistermann *et al*. 2006). Thus, we recommend application of the EIAs to the study of wild populations of black howlers.

Finally, our results provide baseline information showing that fecal glucocorticoid metabolites in *Alouatta pigra* increase after exposure to a stressor, and also that there are individual-related differences in excretion time and concentration: an important fact deserving special consideration when planning field studies.

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