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RESEARCH ARTICLE

Comparison of Different Enzymeimmunoassays for Assessment of Adrenocortical Activity in Primates Based on Fecal Analysis

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Most studies published to date that used fecal glucocorticoid measurements to assess adrenocortical activity in primate (and many nonprimate) species applied a specific cortisol or corticosterone assay. However, since these native glucocorticoids are virtually absent in the feces of most vertebrates, including primates, the validity of this approach has recently been questioned. Therefore, the overall aim of the present study was to assess the validity of four enzymeimmunoassays (EIAs) using antibodies raised against cortisol, corticosterone, and reduced cortisol metabolites (two group-specific antibodies) for assessing adrenocortical activity using fecal glucocorticoid metabolite (GCM) measurements in selected primate species (marmoset, long-tailed macaque, Barbary macaque, chimpanzee, and gorilla). Using physiological stimulation of the hypothalamo-pituitary-adrenocortical (HPA) axis by administering exogenous ACTH or anesthesia, we demonstrated that at least two assays detected the predicted increase in fecal GCM levels in response to treatment in each species. However, the magnitude of response varied between assays and species, and no one assay was applicable to all species. While the corticosterone assay generally was of only limited suitability for assessing glucocorticoid output, the specific cortisol assay was valuable for those species that (according to high-performance liquid chromatography (HPLC) analysis data) excreted clearly detectable amounts of authentic cortisol into the feces. In contrast, in species in which cortisol was virtually absent in the feces, group-specific assays provided a much stronger signal, and these assays also performed well in the other primate species tested (except the marmoset). Collectively, the data suggest that the reliability of a given fecal glucocorticoid assay in reflecting activity of the HPA axis in primates clearly depends on the species in question. Although to date there is no single assay system that can be used successfully across species, our data suggest that group-specific assays have a high potential for cross-species application. Nevertheless, regardless of which GC antibody is chosen, our study

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clearly reinforces the necessity of appropriately validating the respective assay system before it is used. Am. J. Primatol. 68:257–273, 2006.

Key words: glucocorticoids; fecal hormones; cortisol; corticosterone; adrenocortical activity; ACTH

INTRODUCTION

It is well established that severe stress can exert potentially deleterious effects on a variety of physiological, psychosocial, and behavioral parameters in many vertebrate species. For example, it has been shown that chronic stress can disrupt immune function and increase susceptibility to disease [e.g., Cohen & Crnic, 1983; Munck et al., 1984], suppress reproductive function [e.g., Ferin, 1999; Wingfield & Sapolsky, 2003], impair cognitive abilities [e.g., McEwen & Sapolsky, 1995; Ohl & Fuchs, 1999], and influence behavior [e.g., Carlstead et al., 1993; Wielebnowski et al., 2002]. Given the marked impact of stress on animal well-being, health, and reproduction, there is increasing interest in different fields of biomedical and biological research, including conservation, in assessing an animal's stress physiology under both laboratory and wildlife conditions [Romero, 2004].

Since one of the main reactions to a stressor is a marked increase in glucocorticoid release from the adrenal cortex, glucocorticoid levels in plasma [e.g., Johnson et al., 1996; Morton et al., 1995] or urine [Brown et al., 1995; Crockett et al., 1993; Smith & French, 1997] traditionally have been used as a physiological index of a stress response in many vertebrate species. In more recent years, however, the alternative measurement of glucocorticoid metabolites (GCM) in feces has gained increasing attention, particularly because of its suitability for application in wild populations. Fecal GCM analysis has now been widely applied to monitor adrenocortical activity in a large number of vertebrates [for review see Möstl & Palme, 2002; Touma & Palme, 2005], including primates [e.g., Cavigelli et al., 2003; Lynch et al., 2002; Weingrill et al., 2004; Whitten et al., 1998]. In many of these studies (and almost all studies in which primates were the focus of interest), GCM measurements were performed using a cortisol or corticosterone assay. However, cortisol and corticosterone are heavily catabolized by the liver and intestinal bacteria before they are excreted into the feces [Brownie, 1992; MacDonald et al., 1983], resulting in a large number of metabolites with little native hormone present [Bahr et al., 2000; Möstl et al., 2002; Palme et al., 2005; Wasser et al., 2000]. Thus, immunoassays utilizing antibodies specific to blood glucocorticoids may have relatively little affinity to the fecal GCM and thus may have limited suitability for quantifying fecal GCM excretion [Bahr et al., 2000; Goymann et al., 1999; Palme et al., 2005; Terio et al., 1999; Touma & Palme, 2005; Wasser et al., 2000]. Moreover, despite the wide use of these assays to monitor adrenal endocrine function in vertebrate species, the reliability of the assays for measuring the most abundant GCM present and accurately reflecting glucocorticoid output often has not been adequately tested. Therefore, the validity of these measures and interpretations of the data derived from them have recently been brought into question [Palme et al., 2005; Touma & Palme, 2005]. This general problem applies particularly to studies on primates, since in the nearly 20 studies that applied fecal GCM analysis published to date in peer-reviewed journals, physiological validation based on the

exogenous administration of adrenocorticotrophic hormone (ACTH) or the well-documented effect of anesthesia to stimulate glucocorticoid output (as recommended by several researchers [e.g., Touma & Palme, 2005; Wasser et al., 2000]) was performed in only three studies (baboons and long-tailed macaques (ACTH) [Wasser et al., 2000], chimpanzees (anesthesia) [Whitten et al., 1998], and douc langur (anesthesia) [Heistermann et al., 2004]). In all other studies, validation ''experiments'' were limited to more indirect approaches, such as testing the patterning of fecal GCM with respect to circadian variation or social behaviors [e.g., Stavisky et al., 2001; Wallner et al., 1999] or were not reported at all (and thus presumably not carried out [e.g., Bales et al., 2002; Bardi et al., 2003]). Thus, the reliability of the different glucocorticoid measures applied (including cortisol) for monitoring adrenocortical activity in primate species remains largely unclear.

The overall aim of the present study therefore was to assess the validity of four different enzymeimmunoassays (EIAs) for monitoring adrenocortical activity using fecal GCM measurements in selected primates. Specifically, using physiological stimulation of the adrenal gland by administering ACTH and/or anesthesia in five species of simian primates of the major primate taxa (Old World monkeys, New World monkey, and great apes), we tested whether the physiologically induced increase in glucocorticoid output could be detected by the different fecal GCM measurements. Furthermore, using high-performance liquid chromatography (HPLC) analysis we investigated the specificity of the different antibodies and their ability to detect immunoreactivity associated with abundant GC metabolites. Finally, by combining these two data sets, we evaluated the relative suitability of the different GCM measures for assessing adrenocortical activity in order to judge which type of assay is most suitable for the species in question, and whether any of the four antibodies tested can be applied across species.

MATERIALS AND METHODS

Animals and Physiological Challenges

This study was performed on adult animals of five primate species: the common marmoset (*Callithrix jacchus*), the Barbary macaque (*Macaca sylvanus*), the long-tailed macaque (*Macaca fascicularis*), the common chimpanzee (*Pan troglodytes*), and the lowland gorilla (*Gorilla gorilla*) (Table I).

Two approaches were used to activate adrenal glucocorticoid output (Table I). In four of the five species (*C. j.*, *M.s.*, *M.f.*, and *P.t.*), a pharmacological challenge with ACTH was performed to stimulate adrenocortical activity [Wasser et al., 2000], whereas the stress-inducing effect of anesthesia [Sapolsky, 1982; Whitten et al., 1998] was used to activate adrenocortical function in the gorilla. The different experiments were carried out between 1998 and 2001.

All animals that received ACTH were captured and injected intramuscularly with a single dose (12.5–75 IU) of a synthetic ACTH preparation (Synacthen®/Synacthen Depot®; Novartis, Basel, Switzerland) (Table I). This was done under sedation in the chimpanzee, while the marmoset and macaque species were manually restrained. Ketamine anesthesia was performed as part of a routine veterinary control in the gorilla. Immediately following treatment, all of the animals were released into their former housing condition (Table I).

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TABLE I. Animals Involved in the Study

Species	Abbreviation	Sex (*N*)	Type of treatment	Dose rate IU/kg (total dose)	Housing	Location[a]
Barbary macaque (*Macaca sylvanus*)	M.s.	Female (2)	ACTH challenge	5.8–6.3 IU/kg (75 IU/per animal)	Social group	Tierpark Gettdorf
Longtailed macaque (*Macaca fasicularis*)	M.f.	Male (2)	ACTH challenge	~1.8 IU/kg (12.5 IU/per animal)	Singly	German Primate Centre
Lowland gorilla (*Gorilla gorilla*)	G.g.	Male (1)	Ketamine anaesthesia	Unknown	Social group	Zoo Duisburg
Common chimpanzee (*Pan troglodytes*)	P.t.	Male (1)	ACTH challenge	~0.45 IU/kg (25 IU total)	Pair	Zoo Halle
Common marmoset (*Callithrix jacchus*)	C.j.	Male (2)	ACTH challenge	~31 IU/kg (12.5 IU/per animal)	Singly	German Primate Centre

[a]All located in Germany.

Sample Collection and Fecal Extractions

Fecal samples (n = 2–6) were collected 2–6 days immediately prior to the experimental procedure to obtain pretreatment control values. Following ACTH or anesthetic drug administration, each sample voided by the animals was collected for up to 4 days postinjection. Individuals were continuously observed and samples were collected within 30 min after defecation. Following collection, the fecal samples were stored frozen at −20°C until they were further processed.

All fecal samples were processed and extracted as described by Heistermann et al. [1995]. Briefly, the fecal samples were lyophilized and pulverized, and, depending on the species, an aliquot representing ~0.05–0.2 g of fecal powder was extracted with 3 ml of 80% methanol by vortexing for 15 min. Following centrifugation of the fecal suspension, the supernatant was recovered and stored at –20°C until a hormone analysis was performed. All hormone concentrations are expressed as mass/g dry weight.

Hormone Analyses

Fecal extracts (and HPLC fractions; see below) were analyzed for glucocorticoid immunoreactivity by means of four different EIA systems using antibodies that were known or at least expected to differ in their degree of specificity. Two antibodies that were developed to measure 5β-reduced cortisol metabolites with a 3α,11-oxo and 3α,11β-dihydroxy structure, and were previously shown to reliably detect changes in adrenocortical activity in various mammal species [Möstl et al., 2002; Ganswindt et al., 2003; Heistermann et al., 2004] were compared with two commercially available antibodies: one against cortisol (AB 1002; BioClinical Services, Cardiff, UK) and one against corticosterone (#07-120116; MP Biomedicals (formerly ICN), Costa Mesa, CA) (Table II). The latter antibody (ICN-corticosterone) has been shown to reliably detect elevations in GCM in response to ACTH challenge in a wide range of vertebrates [Goymann et al., 1999; Wasser et al., 2000; Young et al., 2004], including baboons [Wasser et al., 2000]. All hormone assays were carried out on microtiter plates according to the procedure described in detail by Möhle et al. [2002]. Data on the assay sensitivities, as well as intra- and interassay coefficients of variation for the different hormone assays, are shown in Table II. The cross-reactivities of the two antibodies measuring 3α,11-oxo-CM and 3α,11β-dihydroxy-CM were described by Möstl et al. [2002] and Ganswindt et al. [2003], respectively. For the ICN-corticosterone antibody, the cross-reactivities relative to corticosterone (100%) are as follows: 5α-dihydrocorticosterone 30.9%, allotetrahydrocorticosterone 5.1%, 5α-pregnan-3β,11β,21-triol-20-one 3.6%, 11-desoxycorticosterone 2.4%, 11-dehydrocorticosterone 0.2%, 20β-dihydrocorticosterone 0.1%, tetrahydrocorticosterone 0.8%, cortisol 0.3%, 5α-dihydrocortisol 0.2%, and <0.1% for allotetrahydrocortisol, 5β-dihydrocortisone, 5α-pregnan-3α,11β-diol-20-one, and 5α-pregnan-3β,11β,20β,21-tetrol. Additional compounds with cross-reactivities <1% were reported by Wasser et al. [2000]. According to the manufacturer, the cortisol antibody showed the following cross-reactions relative to cortisol (100%): prednisolon 45%, 11-deoxycortisol 25%, cortisone 8.5%, fludrocortisone 6.3%, corticosterone 4.5%, 17α-hydroxyprogesterone 2.3%, and progesterone <0.1%.

HPLC Analysis

To assess the pattern of metabolites measured by the different GC assays, reverse-phase (RP)-HPLC was carried out on samples that showed peak GCM

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TABLE II. Characteristics of the Four EIAs Which Were Used to Determine Fecal Glucocorticoid Metabolites

EIA	11β-hydroxy etiocholanolone[a]	11-oxo etiocholanolone[b]	Cortisol[c]	ICN-corticosterone[d]
Immunogen	5β-androstane-3α,11β-diol-17-CMO[e]	5β-androstane-3α-ol-11-one-17-CMO[f]	Cortisol-3-CMO[f]	Corticosterone-3-CMO[f]
Label	5β-androstane-3α,11β-diol-17-CMO[g]	5β-androstane-3α-ol-11-17-CMO[g]	Cortisol-3-CMO[g]	Corticosterone-3-CMO[h]
Standard	5β-androstane-3α-11β-diol-17-one	5β-androstane-3α-ol-11,17-dione	Cortisol	Corticosterone
Specificity[i]	3α,11β-dihydroxy-CM[j]	3α,11oxo-CM[j]	Cortisol (ring A reduced)	Corticosterone (ring A reduced)
Sensitivity[k]	2	3	1.5	1.5
Intraassay CV[l]	6.3, 12.4 ($n = 16$)	2.6, 2.9 ($n = 18$)	4.8, 8.8 ($n = 16$)	5.2, 4.3 ($n = 18$)
Interassay CV[l]	12.0, 17.9 ($n = 32$)	9.8. 7.1 ($n = 32$)	6.9, 8.9 ($n = 26$)	4.5, 5.8 ($n = 21$)

[a]First described by Ganswindt et al. [2003].
[b]First described by Möstl et al. [2002].
[c]First described by Schmid et al. [2001].
[d]First described by Goymann et al. [1999].
[e]Coupled with Bovine serum albumine (BSA) and raised in sheep.
[f]Coupled with BSA and raised in rabbit.
[g]Coupled with N-biotinyl-1,8-diamino-3,6-dioxaoctane (DADOO-biotin).
[h]Coupled with biocytin.
[i]Group of metabolites measured.
[j]3α, 11oxo-corticoid metabolites, 3α, 11β-dihydroxy-corticoid metabolites.
[k]Given in pg/well (determined at 90% binding).
[l]Values represent percentage variance for high and low concentrated quality controls.

levels in the assay with the greatest response to the physiological challenge in a given species. HPLC was also carried out on peak radioactive fecal samples derived from a previous radiolabel infusion study of ^{3}H-cortisol [Bahr et al., 2000] in three of the five species (*M.f.*, *C.j.*, and *P.t.*). Steroids were separated using a Nova Pak C 18 column (3.9 × 300 mm; Milipore, Milford, MA) and an isocratic solvent system of acetonitrile : water (ACN:H_2O, 40:60, v:v) at a flow rate of 0.3 ml/min [Möhle et al., 2002]. This system also allowed us to evaluate whether the GC antibodies tested showed a comeasurement of fecal androgens that could potentially be detected by antibodies raised against cortisol metabolites [Ganswindt et al., 2003; Möstl et al., 2002]. Prior to HPLC, fecal extracts (1 ml for *M.s.*, *C.j.,* and *G.g.*; 2 ml for *M.f.* and *P.t.*) were cleaned up using SepPak C18 columns according to the method described by Teskey-Gerstl et al. [2000]. In brief, 3 ml (*M.s.*, *C.j.,* and *G.g.*) or 6 ml (*M.f.* and *P.t.*) of sodium acetate buffer (0.2 M, pH 4.2) were added to the extract before the total volume was passed through the SepPak cartridge. Steroids were eluted with 10 ml of absolute methanol, which was subsequently evaporated to dryness. The extract was then reconstituted in 150 µl ACN:H_2O (40:60, v:v). An aliquot of 100 µl was then subjected to HPLC and 100 fractions of 0.3 ml were collected. Each fraction was evaporated to dryness, steroids were reconstituted in assay buffer (500 µl), and an aliquot was measured in the four GC EIAs to generate the profiles of immunoreactivity. To check for HPLC consistency between sample runs, immediately before the samples were run on a given day, a test run was performed in which the elution positions of radioactive labeled cortisol, corticosterone, and testosterone standards were determined. All test runs were consistent in that the elution positions of the three standards differed by no more than one fraction between runs.

Biological Validation

In addition to the physiological validation, whenever possible we tested the biological validity of the fecal GCM measurements [Touma & Palme, 2005]. In this respect, we used specific ''stressful situations'' known to elicit increased glucocorticoid output, such as transportation/translocation [e.g., Möstl et al., 2002; Terio et al., 1999] and change in housing conditions [e.g., Wielebnowski et al., 2002] to evaluate whether the fecal GCM measurement is also capable of detecting ''naturally'' occurring changes in adrenocortical activity. The samples used for these biological validation tests were not collected specifically for this purpose, but were available from other studies carried out in the past 5 years. Fecal samples were analyzed as described above and tested in only those assays that, based on the findings of the physiological challenge test and HPLC analysis, were considered suitable for monitoring glucocorticoid output in the respective study species (Tables III and IV).

RESULTS

Physiological Challenge Tests

All of the animals responded to ACTH or anesthesia with an increase in fecal GCM levels. The profiles measured by each of the assay systems tested are shown for one individual per species in Fig. 1.

At least two of the four assays detected a clear (>2.5-fold) elevation in immunoreactive GCM levels following stimulation of the HPA axis in each species (Table III). The assay(s) that responded best, however, differed among the

TABLE III. Fecal GCM Increases in Response to Physiological Stimulation as Detected by Four Enzymeimmunoassays*

	11ß-hydroxy-etiocholanolone	11-oxo-etiocholanolone	Cortisol	Corticosterone
Barbary macaque	**7.4**	**5.1**	**20.3**	**11.7**
Longtailed macaque	**3.2**	**2.6**	2.0	2.5
Lowland gorilla	1.6	**3.0**	**8.1**	1.3
Common chimpanzee	**6.0**	3.3	2.7	3.8
Common marmoset	1.8	1.5	**5.4**	3.1

*Numbers represent magnitude of GCM increase (fold above baseline) following pharmacological stimulation. Numbers in bold indicate assays in which the following criteria were all fulfilled: i) substantial amounts of immunoreactivity found after HPLC; ii) no indication of co-measurement of fecal androgens; and iii) low variation in pre-treatment baseline levels.

species. For example, only the cortisol and ICN-corticosterone assay measured increases in immunoreactivity following ACTH administration in the marmoset, whereas in the gorilla a marked elevation in GCM levels was detected only by the cortisol and 11-oxoetiocholanolone assays (Fig. 1). Furthermore, within a given species, the magnitude of the GCM elevation also differed among the assays. This is best illustrated by the profiles of the Barbary macaque (Fig. 1), in which the increase in fecal GCM was substantially higher in the cortisol assay (20-fold) than in any of the others. In absolute terms, the highest levels of fecal GCM were measured by the 11-oxo- and 11β-hydroxyetiocholanolone EIA (peak value range: 2–10 μg/g), with those measured by the cortisol and ICN-corticosterone antibodies being generally much lower (peak value range: 0.01–1.4 μg/g). Measures of cortisol also showed a marked interspecies variation, with levels in *M.s.*, *C.j.* and *G.g.* (peak value range: 0.35–1.4 μg/g) being substantially higher than those in *M.f.* and *P. t.* (peak value range: 0.015–0.08 μg/g).

The timing of the GCM elevation also differed between species from 7 hr post-ACTH in the marmoset to 21 and 46 hr in the chimpanzee and Barbary macaque, respectively (Fig. 1).

Characterization of GC Metabolites by HPLC Analysis

An HPLC analysis was conducted to obtain information on the specificity of the different antibodies used and the characteristics of metabolites measured by each assay. As can be seen in Fig. 2, the highest levels of immunoreactivity that were associated with the presence of several peaks were revealed by the 11β-hydroxy- and 11-oxoetiocholanolone assays. In both assays and all species, almost all immunoreactivity peaks eluted between fractions 9 and 31 (Fig. 2), at the same positions at which the major radioactivity peaks of in vivo metabolized ^{3}H-cortisol were also detected (Fig. 3). In the chimpanzee, substantial additional amounts of immunoreactivity were found in fractions 46–50 in the 11-oxoetiocholanolone EIA (Fig. 2) at a position at which no radioactivity was detected following HPLC of ^{3}H cortisol metabolites (Fig. 3).

The elution positions of the major immunoreactivity peaks detected by the two group-specific assays differed notably: the 11β-hydroxyetiocholanolone assay mainly detected immunoreactivity at positions 16–18 and 24–26 (coeluting with 11β-hydroxyetiocholanolone), whereas the 11-oxoetiocholanolone EIA detected

TABLE IV. Relative Change in Fecal Glucocorticoid Levels in Response to Different Stressful Situations

Species	Type of validation (number of cases/animals)	Assay	Factorial increase (+) or decrease(−)
Barbary macaque	Escape/re-capture ($n = 2$)[a]	Cortisol	× 12.6+[e]
		11β-hydroxyetiocholanolone	× 4.2+[e]
		11-oxoetiocholanolone	× 3.5+[e]
Common marmoset	Translocation to other institute ($n = 2$)[b]	Cortisol	× 10.8+[e]
Common chimpanzee	Eviction from group and housed as "pair" ($n = 1$)[c]	11β-hydroxyetiocholanolone	× 2.8+
	Transport and group integration ($n = 1$)[d]		× 6.2–

[a]Comparison of levels 1–2 days before vs the day after the event.
[b]Comparison of levels 3–10 days before vs 1–5 days after translocation.
[c]Comparison of levels 2–4 weeks before eviction vs 0–2 weeks housed outside group.
[d]Comparison of levels immediately (1–3 days) following transport vs week 1–4 after transport.
[e]Mean value.

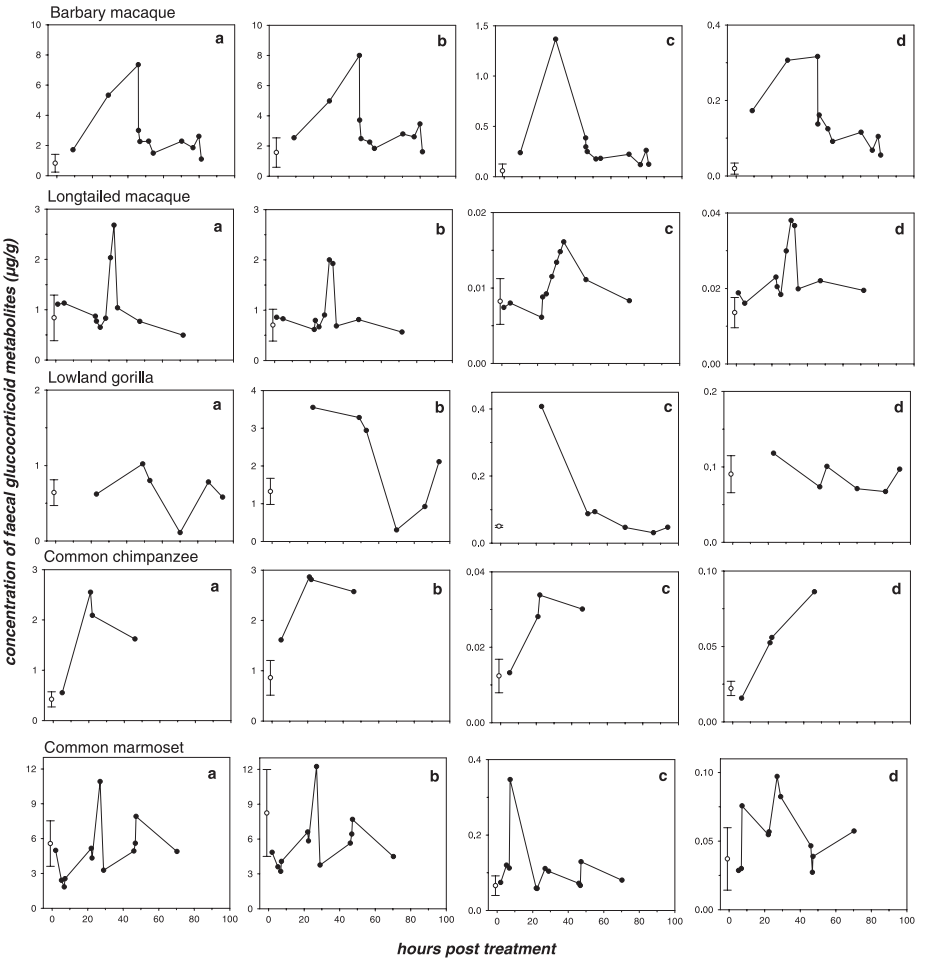

Fig. 1. Immunoreactive GCM concentrations measured in the (**a**) 11β-hydroxyetiocholanolone, (**b**) 11-oxoetiocholanolone, (**c**) cortisol, and (**d**) corticosterone EIA in feces before and after ACTH challenges. Pretreatment values are given as mean ± SD. Note that in the gorilla adrenocortical activity was stimulated by anesthesia rather than ACTH (see Materials and Methods), and samples were not available for the first 20 hr post treatment.

immunoreactivity mainly in fractions 10–13, 19–20, and 29–31 (coeluting with 11-oxoetiocholanolone). This pattern of immunoreactivity measured by the two assays was similar among species; however, the relative abundance of the different immunoreactivity peaks differed (Fig. 2).

Although levels of cortisol immunoreactivity were lower than those measured by the group-specific assays, substantial amounts eluting at the position of authentic cortisol (fractions 14–15) were detected in the Barbary macaque, gorilla, and marmoset (Fig. 2). In contrast, cortisol immunoreactivity was virtually absent in the long-tailed macaque and chimpanzee (Fig. 2). Corticosterone immunoreactivity was present in the smallest amounts in all species, and clearly measurable quantities were found only in the Barbary macaque and marmoset, in which they eluted at positions 10–11 and 21–23 (elution position of authentic corticosterone).

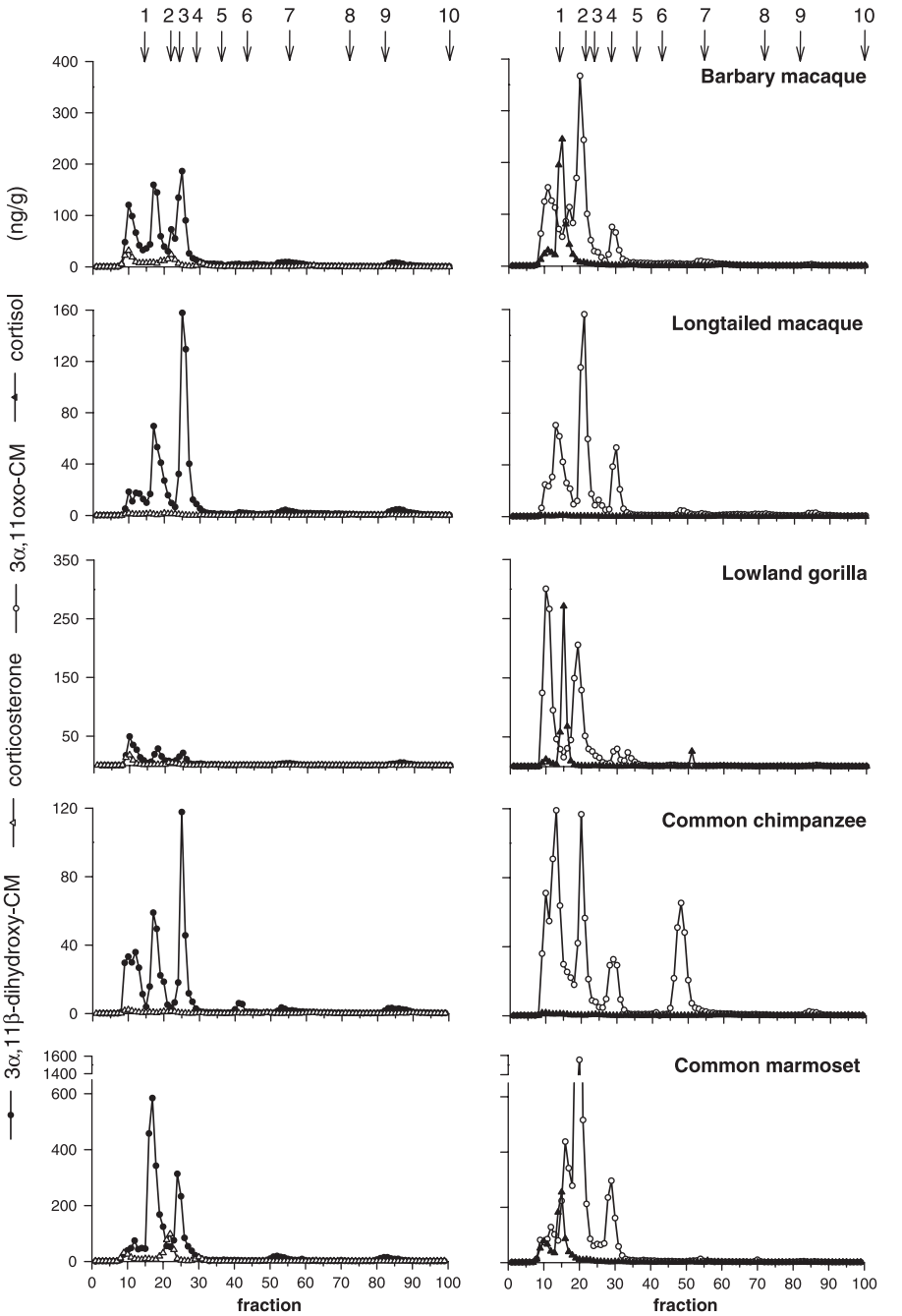

Fig. 2. HPLC profiles of immunoreactivity detected with the 11β-hydroxyetiocholanolone, 11-oxoetiocholanolone, cortisol, and corticosterone EIA in peak samples following adrenocortical stimulation in the study species. Associate elution positions of reference standards: 1) cortisol (fractions 14–15), 2) corticosterone (22), 3) 11β-hydroxyetiocholanolone (24), 4) 11-oxoetiocholanolone (29), 5) 5β-androstane-3,11,17-trione (36), 6) testosterone (43), 7) androstendione, dehydroepiandrosterone (55-56), 8) epiandrosterone, 5β-DHT, 5β-androstane-3β-ol-17-one (72), 9) 5β-androstane-3α-ol-17-one (82), and 10) androsterone (100).

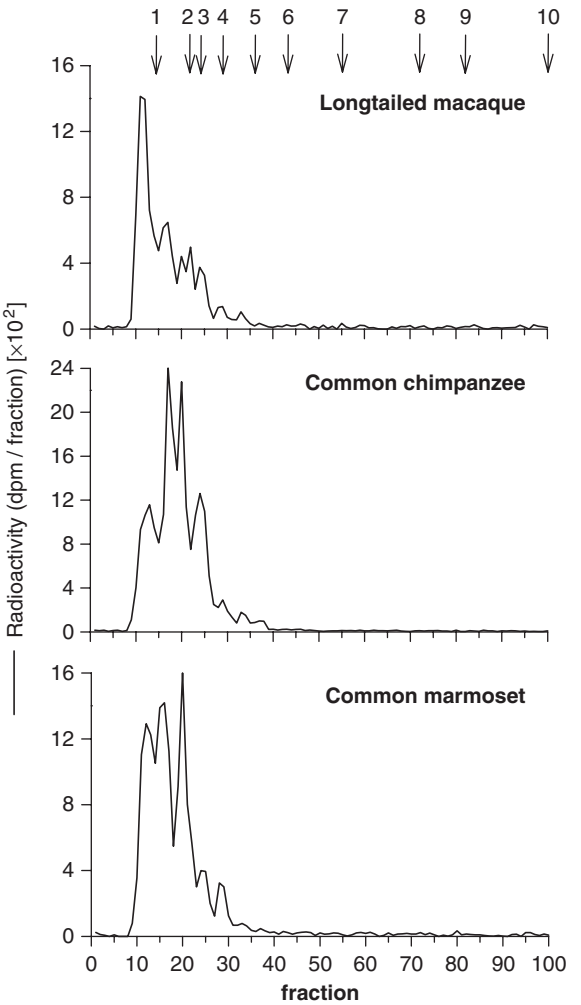

Fig. 3. HPLC profiles of radioactivity in fecal extracts of peak radioactive samples after in vivo metabolism of ^{3}H-cortisol in an individual long-tailed macaque, chimpanzee, and marmoset, respectively [c.f., Bahr et al., 2000]. Arrows indicate the elution positions of the reference standards (see Fig. 2).

Evaluation of Assay Suitability

To evaluate the degree of suitability of the four different assays for monitoring adrenocortical activity in each species, the results of the physiological challenge test and the data on HPLC immunoreactivity profiles were considered together. The following criteria were used: 1) magnitude of peak response to treatment, 2) amount of immunoreactivity detected following HPLC, 3) extent of comeasurement of androgens, and 4) degree of variation in pretreatment GCM. Table III summarizes these findings and indicates the assays that were considered suitable for each species.

Biological Validation

With the exception of the gorilla and long-tailed macaque, case studies were available to examine the biological validity of the selected fecal GC measure

(Table IV). Although the small sample size prevented a statistical analysis, in all cases the appearance or disappearance of the given stressful situation was associated with a marked (usually two- to 10-fold) change in GCM concentrations. Moreover, the response to the tested situation was in every case in the predicted direction, i.e., GCM levels increased in response to potentially stressful situations (capture, translocation, and social challenge) and decreased as a result of habituation following a stressor (Table IV).

DISCUSSION

In the present study we compared the suitability of four antibodies (raised against cortisol, corticosterone, and reduced cortisol metabolites) for their ability to reliably detect changes in fecal glucocorticoid metabolites in response to adrenocortical activation in five selected primate species.

As indicated by the HPLC data, considerably higher amounts of immunoreactivity, associated with the presence of multiple relatively polar compounds, were detected by the two group-specific antibodies (developed to measure 3α, 11β-dihydroxy and 3α,11-oxo cortisol metabolites) compared to the two more specific ones designed to measure cortisol or corticosterone in blood. The retention times of the substances that yielded immunoreactivity in the two group-specific assays were identical to those of the major radioactive peaks following radiolabel infusion of ^{3}H-cortisol, providing circumstantial evidence that they represent metabolites of cortisol. Moreover, coelution of major radioactivity and immunoreactivity peaks with 11β-hydroxyetiocholanolone and 11-oxoetiocholanolone standards indicated the presence of 3α,11β-dihydroxylated GCMs and 11,17-dioxoandrostanes, both of which have also been reported as abundant fecal cortisol metabolites in nonprimate species [e.g., Ganswindt et al., 2003; Palme & Möstl, 1997].

Our finding that only relatively small amounts of authentic cortisol and corticosterone are excreted into the feces of the primate species studied is consistent with data from radioinfusion studies in other vertebrate species [e.g., Graham & Brown, 1996; Palme et al., 1996; Teskey-Gerst et al., 2000] (for literature review see Palme et al. [2005]), including primates [Bahr et al., 2000; Wasser et al., 2000]. However, differences were also detected among the primate species studied here. Whereas cortisol was virtually absent in the long-tailed macaque and chimpanzee, clearly detectable amounts were found after HPLC in the marmoset, Barbary macaque, and gorilla (see also Bahr et al. [2000]). Thus, even closely related species can differ markedly in terms of fecal excretion of native cortisol. This is an interesting finding from an evolutionary perspective, which might stimulate studies to explore in more detail the excretion of glucocorticoids as a function of phylogenetic relatedness [Pryce et al., 1995]. In more practical terms, this finding clearly demonstrates that it is not possible to predict in advance whether cortisol is excreted in clearly measurable amounts in any one species. This, in turn, has important implications for the choice of antibody and reliability of assay system used to monitor adrenocortical activity in primates via fecal analysis (see below).

Irrespective of the number and identity of the individual GCM being measured, the suitability of the assay systems being tested depends on their ability to reliably track alterations in adrenocortical activity [Touma & Palme, 2005; Wasser et al., 2000]. A clear increase in fecal GCM levels following adrenal stimulation was detected with at least two of the four glucocorticoid assays in each of the five species studied. The characteristics in terms of magnitude of

response and time course were within the range of those reported in other studies on primate [Wasser et al., 2000; Whitten et al., 1998] and nonprimate species [e.g., Young et al., 2004] (for a review of ACTH studies see Touma and Palme [2005]). However, the suitability of the assays for monitoring adrenocortical activity varied among species, and no one assay was applicable to all species. Specifically, whereas a cortisol assay is useful for tracking changes in glucocorticoid output in primates that excrete cortisol in clearly detectable amounts into the feces, our data also indicate that in species in which cortisol is nearly absent in the feces, group-specific assays provide a better option because they show a stronger signal response to treatment and thus have a higher biological sensitivity for detecting changes in glucocorticoid production [c.f., Frigerio et al., 2004; Möstl et al., 2005]. Since the group-specific assays also performed well in the other cercopithecoid and hominoid primate species tested, our data suggest that, at least in Old World primates, assays utilizing group-specific GC antibodies have a higher potential for cross-species application than more specific assays using antibodies designed to measure cortisol.

However, since group-specific glucocorticoid assays measure a broad spectrum of steroids, there is also a higher risk (compared to more specific assays) of comeasurement of androgen metabolites [Ganswindt et al., 2003; Schatz & Palme, 2001]. This is presumably the case with the chimpanzee, in which, following HPLC, the 11-oxoetiocholanolone assay detected substantial amounts of immunoreactivity at a position where (according to the radiolabel infusion data [Möhle et al., 2002]) a major fecal metabolite of testosterone (but not of cortisol) elutes. A potential comeasurement of metabolites that do not originate from cortisol should therefore generally be taken into account when fecal glucocorticoid assays are selected for use. In this regard, our cross-reactivity tests suggest that the ICN-corticosterone antibody mainly measures metabolites of corticosterone and not of cortisol. Since corticosterone is not the major glucocorticoid secreted by the primate adrenal cortex, and, moreover, may have a different biological function (mainly acting within the brain [Zanella et al., 2003]), this assay is probably less suitable for noninvasive assessments of stress-induced glucocorticoid output in primates. Our data on the physiological challenge tests together with the low amounts of corticosterone immunoreactivity measured in HPLC fractions support this contention. Although Wasser et al. [2000] demonstrated that the ICN-corticosterone antibody was superior to different cortisol antibodies in detecting adrenal activation in the baboon, they did not test whether the corticosterone antibody would also outperform group-specific assays in that species. Based on our present findings, we would predict that in baboons (and presumably the majority of other primate species) the application of an assay that is capable of detecting a family of cortisol metabolites is most appropriate for assessing adrenocortical activity from fecal samples.

In conclusion, since one cannot predict which GC metabolites might predominate in feces and which assay system might work best in any given primate species, regardless of which GC antibody is chosen, our study and those of others [e.g., Bahr et al., 2000; Goymann et al., 1999; Wasser et al., 2000] clearly reinforce the necessity of appropriately validating the respective assay system before use [Palme, 2005; Touma & Palme, 2005]. This will help to ensure that the fecal GC measurements will provide biologically meaningful data and can thus be successfully applied to noninvasively assess adrenocortical status in both captive and free-ranging primates under a variety of conditions.

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