



## Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses

K.M. Young,<sup>a</sup> S.L. Walker,<sup>a</sup> C. Lanthier,<sup>b</sup> W.T. Waddell,<sup>c</sup> S.L. Monfort,<sup>a</sup>  
and J.L. Brown<sup>a,\*</sup>

<sup>a</sup> Conservation and Research Center, National Zoological Park, Smithsonian Institution, Front Royal, VA, USA

<sup>b</sup> Le Zoo de Granby, Granby, Quebec, Canada

<sup>c</sup> Point Defiance Zoo and Aquarium, Tacoma, WA, USA

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### Abstract

Measurement of glucocorticoid metabolites in feces has become an accepted method for the noninvasive evaluation of adrenocortical activity. The objective of this study was to determine if a simple cortisol enzyme immunoassay (EIA) was suitable for monitoring adrenocortical activity in a variety of carnivore species. Performance of the cortisol EIA was gauged by comparison to a corticosterone radioimmunoassay (RIA) that has been used for measuring glucocorticoid metabolites in feces of numerous species. Tests for parallelism and extraction efficiency were used to compare the cortisol EIA and corticosterone RIA across eight species of carnivores (Himalayan black bear, sloth bear, domestic cat, cheetah, clouded leopard, black-footed ferret, slender-tailed meerkat, and red wolf). The biological relevance of immunoreactive glucocorticoid metabolites in feces was established for at least one species of each Carnivora family studied with an adrenocorticotrophic hormone (ACTH) challenge. High performance liquid chromatography (HPLC) analysis of fecal extracts for each species revealed (1) the presence of multiple immunoreactive glucocorticoid metabolites in feces, but (2) the two immunoassays measured different metabolites, and (3) there were differences across species in the number and polarities of metabolites identified between assay systems. ACTH challenge studies revealed increases in fecal metabolite concentrations measured by the cortisol EIA and corticosterone RIA of ~228–1145% and ~231–4150% above pre-treatment baseline, respectively, within 1–2 days of injection. Concentrations of fecal glucocorticoid metabolites measured by the cortisol EIA and corticosterone RIA during longitudinal evaluation (i.e., >50 days) of several species were significantly correlated ( $P < 0.0025$ , correlation coefficient range 0.383–0.975). Adrenocortical responses to physical and psychological stressors during longitudinal evaluations varied with the type of stimulus, between episodes of the same stimulus, and among species. Significant elevations of glucocorticoid metabolites were observed following some potentially stressful situations [anesthesia (2 of 3 subjects), restraint and saline injection (2 of 2 subjects), restraint and blood sampling (2 of 6 episodes), medical treatment (1 of 1 subject)], but not in all cases [e.g., gonadotropin injection ( $n = 4$ ), physical restraint only ( $n = 1$ ), mate introduction/breeding ( $n = 1$ ), social tension ( $n = 1$ ), construction ( $n = 2$ ) or relocation ( $n = 1$ )]. Results reinforced the importance of an adequate baseline period of fecal sampling and frequent collections to assess adrenocortical status. The corticosterone RIA detected greater adrenocortical responses to exogenous ACTH and stressful exogenous stimuli in the Himalayan black bear, domestic cat (female), cheetah, clouded leopard, slender-tailed meerkat, and red wolf, whereas the cortisol EIA proved superior to resolving adrenocortical responses in the black-footed ferret and domestic cat (male). Overall results suggest the cortisol EIA tested in this study offers a practical method for laboratories restricted in the usage of radioisotopes (e.g., zoological institutions and field facilities) to integrate noninvasive monitoring of adrenocortical activity into studies of carnivore behavior and physiology.

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### 1. Introduction

The mammalian order Carnivora represents a diverse group of ~274 extant species that vary considerably in

\* Corresponding author. Fax: 1-540-635-6506.  
E-mail address: [jbrown@crc.si.edu](mailto:jbrown@crc.si.edu) (J.L. Brown).

their behavior, ecology, morphology, and physiology (Gittleman, 1989; Macdonald, 1992). Although some debate continues regarding the phylogeny of carnivores, one of the accepted taxonomic arrangements divides the order into 10 families: Canidae (dogs, jackals, foxes, and wolves); Ursidae (bears); Procyonidae (raccoons, coatis, and kinkajou); Mustelidae (badgers, skunks, otters, and weasels); Viverridae (civets, genets); Herpestidae (mongooses, meerkats); Hyaenidae (hyenas, aardwolf); Felidae (cats), Otariidae (walrus, sea lions, and fur seals), and Phocidae (earless seals) (Wozencroft, 1989a,b). In a recent report, the World Conservation Union identified 76 carnivore taxa threatened with extinction globally and four taxa lost to extinction since the mid-nineteenth century (IUCN, 2002).

Captive management of carnivores has afforded conservationists the opportunity to gather detailed scientific information concerning the unique biology of these species. For example, our considerable knowledge of wild felid reproductive endocrinology and gamete biology has been gained from in-depth studies only possible with frequent access to zoo-maintained animals (Brown et al., 1994, 2001; Wildt and Roth, 1997; Wildt et al., 1984, 1986a,b, 1988). However, inappropriate captive environments are known to have deleterious effects on the behavior and physiology of mammals (Carlstead, 1996; Estep and Dewsbury, 1996). Adverse physical, physiologic, and psychogenic stimuli evoke a series of biological responses (behavioral and/or hormonal changes) that help animals cope with stressful stimuli (Moberg, 1985, 1987, 2000), including those imposed by captivity.

In mammals, stress modulates the activities of the hypothalamic-pituitary-adrenal (HPA) axis and sympathoadrenal axis, releasing a variety of hormones to counter aversive stimuli (Axelrod and Reisine, 1984; Breazile, 1987; Melmed and Kleinberg, 2003; Stewart, 2003). Activation of the HPA axis during stress enhances the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, stimulating the synthesis and secretion of glucocorticoids from the adrenal cortices (Axelrod and Reisine, 1984; Melmed and Kleinberg, 2003; Stewart, 2003). Chronic stress and heightened levels of HPA axis hormones (i.e., glucocorticoids, ACTH, and corticotropin releasing hormone) can have detrimental effects, including inhibition of normal reproductive function (deCatanzaro and MacNiven, 1992; Dobson and Smith, 1995; Liptrap, 1993; Rivier and Rivest, 1991), suppression of the immune system and atrophy of tissues (Munck et al., 1984; Stewart, 2003). Elevated levels of glucocorticoids resulting from chronic stress may also cause depression, hypertension, gastrointestinal ulceration, electrolyte imbalances, calcium loss and bone mass reduction, and inhibition of growth (Breazile, 1987; Stewart, 2003). Thus, improving the health and general well-being of

carnivores in captivity requires identifying stressful environmental conditions or management practices and developing mitigating strategies.

While blood glucocorticoid concentrations are accepted indices of stress (Broom and Johnson, 1993), their usefulness in long-term studies with intractable wildlife species is limited due to the circadian rhythm and pulsatile nature of glucocorticoid secretion (Fox et al., 1994; Monfort et al., 1993; Stewart, 2003; Thun et al., 1981) and the possible induction of a stress response during sampling procedures (Cook et al., 2000; Kenagy and Place, 2000; Reinhardt et al., 1990, 1991). Conversely, the excretion of metabolized blood steroids into feces (Macdonald et al., 1983; Taylor, 1971) permits the monitoring of physiological functions without disturbance to animal subjects. Analysis of fecal steroid metabolites also provides a more representative measure of adrenocortical activity over time because the pooling of metabolites during excretion dampens the episodic secretion of blood glucocorticoids.

Measurement of fecal glucocorticoids has been used to investigate adrenocortical activity during exposure to stressful stimuli, such as novel environment, transportation, social tension and aggression, human disturbances, and exposure to predators, in a diverse number of species, including felids (Terio et al., 1999; Wielebnowski et al., 2002), canids (Creel et al., 1996, 1997), hyenas (Goymann et al., 1999, 2001), primates (Wallner et al., 1999), lagomorphs (Teskey-Gerstl et al., 2000), and cervids and bovids (Dehnhard et al., 2001; Millspaugh et al., 2001; Morrow et al., 2002; Palme et al., 2000; Schwarzenberger et al., 1998). Most of the studies in carnivores have successfully evaluated adrenocortical responses to an ACTH challenge by measuring glucocorticoid metabolites in feces using a commercially available corticosterone radioimmunoassay (RIA) (Graham and Brown, 1996; Monfort et al., 1998; Terio et al., 1999; Wasser et al., 2000; Wielebnowski et al., 2002). By contrast, attempts to use antibodies specific to cortisol have produced inconsistent results. Jurke et al. (1997) found differences in basal fecal glucocorticoid metabolite concentrations for cheetahs classified as 'nervous' or 'calm' by zookeepers using a cortisol enzyme immunoassay (EIA), while others were unable to monitor adrenal activity in felids using a commercial RIA specific for cortisol (Graham and Brown, 1996; Terio et al., 1999). Following ACTH stimulation and dexamethasone suppression of the adrenal gland, Schatz and Palme (2001) found adrenocortical activity could be monitored in the domestic dog using a cortisol EIA. They further determined an antibody produced against 11-oxoetiocholanolone and measuring 11,17-dioxoandrostanes was effective for monitoring adrenal function in the domestic cat. However, this antibody was not effective for use in the spotted hyena (Goymann et al., 1999).

Recently, a cortisol EIA has proved useful for non-invasively monitoring adrenocortical activity in the black-footed ferret (Young et al., 2001) and red wolf (Walker, 1999). Based on these findings, a study was conducted to determine if the cortisol EIA was suitable for monitoring adrenocortical activity in a variety of carnivore species and how it performed when compared with the corticosterone RIA. The objectives were to: (1) validate each immunoassay system for measuring glucocorticoid metabolites in feces of an array of carnivore species, (2) determine the correspondence in glucocorticoid data generated by the two immunoassays, (3) investigate the potential for fecal glucocorticoid metabolite analysis to detect adrenocortical responses to stressful stimuli, and (4) determine the suitability of each immunoassay for use as a standardized technique for noninvasive monitoring of adrenocortical activity in carnivore species.

## 2. Materials and methods

### 2.1. Study animals

Adult animals from five Carnivora families (Canidae, Felidae, Herpestidae, Mustelidae, and Ursidae) were evaluated in this study. Study subjects were housed under comparable conditions at the following zoological facilities across North America: Himalayan black bear ( $n = 1$ , Le Zoo de Granby, Granby, QC;  $n = 1$ , Little Rock Zoo, Little Rock, AK); sloth bear ( $n = 1$ , Little Rock Zoo, Little Rock, AK); domestic cat ( $n = 2$ , Conservation and Research Center, Front Royal, VA); cheetah ( $n = 2$ , White Oak Conservation Center, Yulee, FL); clouded leopard ( $n = 3$ , Conservation and Research Center, Front Royal, VA); black-footed ferret ( $n = 2$ , Toronto Zoo, Toronto, ON), slender-tailed meerkat ( $n = 2$ , Saint Louis Zoo, Saint Louis, MO); and red wolf ( $n = 1$ , Point Defiance Zoo and Aquarium, Tacoma, WA;  $n = 1$ , Red Wolf Breeding Facility, Graham, WA). The level of visual, auditory, and olfactory exposure to conspecifics and heterospecifics varied for each study subject. In general, study subjects were fed high-quality commercial diets that, depending on the species, were frequently supplemented with other items, including whole animals (e.g., rabbits, mice, mealworms, and crickets), animal parts (e.g., chicken), vegetables (e.g., carrots, potatoes, and yams), and/or fruit (e.g., grapes, apples, oranges, and raisins). All animals had ad libitum access to fresh water.

### 2.2. ACTH challenges

To determine the biological relevance of glucocorticoid metabolites excreted in feces, an ACTH challenge was conducted in at least one species from each Car-

nivora family. The species, number and sex of subjects, ACTH dosages, and methods used to facilitate ACTH administration are presented in Table 1. Most species received a single intramuscular (i.m.) injection of a slow-release ACTH gel (Wedgewood Pharmacy, Sewell, NJ or Medicine Shop, Front Royal, VA). Slender-tailed meerkats received two separate i.m. injections of ACTH gel (10 IU each; 20 IU total) with 2 h between injections. The domestic cats received a series of five 0.125 mg intravenous infusions of ACTH liquid (Cortrosyn, ~62.5 IU total; Organon, West Orange, NJ) into the jugular vein at 1.5 h intervals over 6 h. To facilitate the administration of ACTH, animals were physically restrained (squeeze cage, catchpole or manually) or chemically immobilized. Prior to the ACTH challenge, a surgical plane of anesthesia was induced in the domestic cat (ketamine hydrochloride, 20 mg/kg body weight, i.m.; Vetalar, Parke-Davis, Morris Plains, NJ; and acepromazine maleate, 0.2 mg/kg body weight, i.m.; Ayerst Laboratories, Rouses Point, NY) and cheetah (tiletamine hydrochloride and zolazepam hydrochloride, Telazol, 3.5 mg/kg body weight, i.m.; Fort Dodge Laboratories, Fort Dodge, IA). Anesthesia was maintained during the procedures for both species with isoflurane gas (Aerane, 1–2%; Anaquest, Madison, WI). Animals were fasted for 12–24 h prior to anesthesia.

Feces were collected from each species during their daily husbandry routine. Most animals defecated once per day, but occasionally no defecations or multiple scats were found. Generally, sample collections commenced 1–10 days before ACTH injection (day 0) and continued for 5–10 days following the adrenocortical stimulation. Fecal material was stored frozen ( $-20^{\circ}\text{C}$ ) in plastic bags or tubes until processing and analysis.

### 2.3. Longitudinal evaluations and exogenous stressors

To assess the ability of fecal glucocorticoid analysis to detect acute changes in adrenocortical activity, several species were evaluated over extended periods of time (i.e., >50 days) that included exposure to different types of potentially stressful stimuli. Longitudinal monitoring of fecal glucocorticoids also provided sufficient data for comparisons between concentrations of steroid metabolites measured by the two immunoassay systems.

The longitudinal assessment periods for three felid species encompassed single episodes of chemical immobilization. A female cheetah was immobilized with a tiletamine–zolazepam combination (Telazol, 3.5 mg/kg body weight, i.m.) for a routine physical examination. During an assisted reproduction study, domestic cat ( $n = 1$ ) and clouded leopard ( $n = 1$ ) females were restrained for injection of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) to induce follicular development and ovulation, respec-

Table 1  
ACTH challenges in species from 5 families of carnivores

Species	Sex	ACTH Dose (IU)	Restraint method	Glucocorticoid metabolite	Metabolite concentration (ng/g feces)		Rise (%)
					Baseline ( $\pm$ SEM)	Peak	
Himalayan black bear	M	955	Squeeze cage	Cortisol	107.9 $\pm$ 8.0	432.6	401
				Corticosterone	34.5 $\pm$ 3.9	197.9	573
Domestic cat	M	62.5	Anesthesia	Cortisol	181.0 $\pm$ 40.4	1673.2	925
				Corticosterone	348.1 $\pm$ 70.6	2015.6	579
	F	62.5	Anesthesia	Cortisol	745.1	2068.5	278
				Corticosterone	746.2	2314.6	310
Cheetah	F	400	Anesthesia	Cortisol	628.4 $\pm$ 95.3	7193.2	1145
				Corticosterone	436.6 $\pm$ 107.7	5941.1	1361
Clouded leopard	M	400	Squeeze cage	Cortisol	223.1 $\pm$ 14.8	569.5	255
				Corticosterone	579.2 $\pm$ 68.5	3225.9	557
	M	400	Squeeze cage	Cortisol	176.2 $\pm$ 30.5	1350.1	766
				Corticosterone	85.3 $\pm$ 10.5	3541.1	4150
	F	400	Squeeze cage	Cortisol	183.6 $\pm$ 46.9	431.8	235
				Corticosterone	555.4 $\pm$ 190.3	5664.7	1020
Black-footed ferret	F	2	Squeeze cage	Cortisol	220.2 $\pm$ 19.3	548.6	249
				Corticosterone	114.5 $\pm$ 8.8	264.3	231
Slender-tailed meerkat	M	20	Manual	Cortisol	110.2 $\pm$ 17.5	614.1	557
				Corticosterone	175.0 $\pm$ 32.4	2672.8	1527
	M	20	Manual	Cortisol	169.5 $\pm$ 14.4	386.9	228
				Corticosterone	173.5 $\pm$ 5.3	554.4	320
Red wolf	M	140	Catchpole/ manual	Cortisol	525.6 $\pm$ 94.6	2130.7	405
				Corticosterone	162.1 $\pm$ 22.6	1875.0	1160

With two exceptions, ACTH was administered to subjects by a single intramuscular injection. For the domestic cats, ACTH was administered via five intravenous infusions (12.5 IU each) into the jugular vein over a period of 6 h. Slender-tailed meerkats received two separate ACTH injections (10 IU each) with 2 h between injections. Restraint method refers to the techniques used to facilitate the injection of exogenous ACTH. Manual restraint was defined as the use of hands to restrain the animal. Glucocorticoid metabolites were measured in extracts prepared from fecal samples with the cortisol EIA and corticosterone RIA. Baseline represents the average glucocorticoid metabolite concentrations for all pre-treatment samples. Peak refers to the ACTH-induced peak in metabolites. Rise represents the peak expressed as a percentage of the pre-treatment baseline.

tively. To permit laparoscopic examination of the ovary and confirmation of ovulation, the domestic cat was immobilized with a tiletamine–zolazepam combination (Telazol, 5 mg/kg body weight, i.m.) and the clouded leopard was anesthetized using ketamine hydrochloride (Vetalar, 12 mg/kg body weight, i.m.). Cats were maintained in a surgical plane of anesthesia by delivering isoflurane gas (Aerane, 1–2%; Anaquest, Madison, WI) following intubation. Only the clouded leopard ovulated and was subsequently artificially inseminated. Feces were collected regularly during the range of days (53–56 days) examined, providing a total of 51 samples for the cheetah and 50 samples each for the domestic cat and clouded leopard.

A black-footed ferret and two red wolves were evaluated during periods of physical restraint. Fecal samples were collected from a female ( $n = 70$  samples) and male ( $n = 60$  samples) red wolf during the natural reproductive season. The female was physically restrained with a catchpole on six occasions to collect blood for determination of reproductive status and timing of artificial insemination. The male red wolf experienced several potentially stressful stimuli: (1) restraint with a catch-

pole to facilitate the administration of a saline injection (1.75 ml, i.m.); (2) construction in the adjacent enclosure on two occasions; and (3) relocation to an adjacent enclosure within the zoological facility. Fecal samples ( $n = 53$ ) also were evaluated over a 54-day period from a female black-footed ferret that experienced single episodes of cage restraint and cage restraint combined with saline injection (0.2 ml, i.m.).

Fecal samples ( $n = 100$ ) were analyzed from a female sloth bear during a 241-day period that included the introduction of a male and subsequent breeding. This subject also suffered from a chronic nematode (ascarids) infection and frequently defecated abnormal stools. Although treated periodically with deworming medication (mebendazole, Telmin; Pitman-Moore, Mundelein, IL), the nematode infection persisted throughout the study period. Fecal samples ( $n = 65$ ) were evaluated over a 151-day period from a female Himalayan black bear that was housed with a male. An episode of social tension uncharacteristic for the pair of Himalayan black bears occurred on a single day during the study period. During this episode, the bears exhibited a series of aggressive displays, in-

cluding snarling, mouthing, and pounding of the ground.

#### 2.4. Extraction of steroids from feces

Frozen fecal samples were dried using a Savant Instruments Speedvac Rotary Evaporator and then pulverized to a fine powder. Hormones were extracted from dried fecal samples using established methods for extraction of ovarian (Brown et al., 1994, 1996) and adrenocortical (Graham and Brown, 1996) steroids. Briefly, ~0.2 g well-mixed powdered feces was boiled in 5 ml of 90% ethanol for 20 min. After centrifugation (500g, 20 min), the supernatant was transferred into a glass tube and the pellet was resuspended in an additional 5 ml of 90% ethanol, vortexed for 1 min, and recentrifuged for 20 min at 500g. Combined ethanol supernatants were dried under air and resuspended in 1 ml of 100% methanol. Methanol extractants were vortexed (1 min), sonicated (15 min) and revortexed (30 s) prior to decanting into a plastic tube for storage at -20 °C until assayed. The efficiency of steroid extraction from feces of each species was evaluated by adding radiolabeled glucocorticoid (<sup>3</sup>H-cortisol or <sup>3</sup>H-corticosterone; 4000–8000 dpm) to a subset of fecal samples prior to boiling extraction. The mean recoveries of <sup>3</sup>H-cortisol from fecal extracts were: domestic cat, 80.8% (*n* = 50 samples); cheetah, 85.0% (*n* = 71); clouded leopard, 87.1% (*n* = 97) and black-footed ferret 81.5% (*n* = 58). The mean recoveries of <sup>3</sup>H-corticosterone from fecal extracts were: Himalayan black bear, 85.5% (*n* = 21); black-footed ferret, 85.8% (*n* = 58); slender-tailed meerkat, 75.3% (*n* = 46); sloth bear, 85.8% (*n* = 30) and red wolf, 73.7% (*n* = 71).

#### 2.5. Fecal glucocorticoid metabolite analyses

##### 2.5.1. Cortisol enzyme immunoassay

A cortisol EIA was used to analyze fecal extracts by a modification of methods described by Munro and Lasley (1988). The assay employed a cortisol-horseradish peroxidase ligand and antiserum (No. R4866; C.J. Munro, University of California, Davis, CA) and cortisol standards (hydrocortisone; Sigma-Aldrich, St. Louis, MO). The polyclonal antiserum was raised in rabbits against cortisol-3-carboxymethyloxime linked to bovine serum albumin and cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5% and <1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (C.J. Munro, pers. comm.). The EIA was performed in 96-well microtiter plates (Nunc-Immuno, Maxisorp Surface; Fisher Scientific, Pittsburgh, PA) coated 14–18 h previously with cortisol antiserum (50 µl/well; diluted 1:20,000 in

coating buffer; 0.05 M NaHCO<sub>3</sub>, pH 9.6). Fecal extracts evaporated to dryness and diluted (bears 1:10–1:30, felids 1:30–1:115, ferrets 1:20, meerkats 1:15, and wolves 1:60–1:100) in steroid buffer (0.1 M NaPO<sub>4</sub>, 0.149 M NaCl, pH 7.0) were assayed in duplicate. Cortisol standards (50 µl, range 3.9–1000 pg/well, diluted in assay buffer, 0.1 M NaPO<sub>4</sub>, 0.149 M NaCl, 0.1% bovine serum albumin, pH 7.0) and sample (50 µl) were combined with cortisol-horseradish peroxidase (50 µl, 1:8500 dilution in assay buffer). Following incubation at room temperature for 1 h, plates were washed five times before 100 µl substrate buffer [0.4 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, 1.6 mM H<sub>2</sub>O<sub>2</sub>, 0.05 M citrate, pH 4.0] was added to each well. After incubation on a shaker for 10–15 min, the absorbance was measured at 405 nm. Parallel displacement curves were obtained for each species by comparing serial dilutions of pooled fecal extracts and the cortisol standard preparation. Intra- and interassay coefficients of variation were 6.4% (*n* = 26 replicates of a single sample) and 11.0% (*n* = 57 assays), respectively. Assay sensitivity was 3.9 pg/well at maximum binding. Cortisol metabolite concentrations are expressed as nanograms per gram dry fecal matter (ng/g).

##### 2.5.2. Corticosterone radioimmunoassay

Fecal extracts were also analyzed using a double-antibody <sup>125</sup>I RIA (ICN Biomedicals, Costa Mesa, CA) for corticosterone according to the manufacturer's instructions, except all reagent volumes were halved. The polyclonal antiserum was raised in rabbits against corticosterone-3-carboxymethyloxime coupled to bovine serum albumin and cross-reacts with corticosterone 100%, desoxycorticosterone 0.34%, testosterone 0.1%, cortisol 0.05%, aldosterone 0.03%, progesterone 0.02%, androstenedione 0.01%, 5 $\alpha$ -dihydrotestosterone 0.01%, and <0.01% with all other steroids tested (manufacturer's data). For each species, serial dilution of pooled fecal extracts produced a displacement curve parallel to the corticosterone standard preparation. Samples were diluted in steroid buffer (bears 1:10–1:15, felids 1:30–1:1000, ferrets 1:40, meerkats 1:50–1:500, and wolves 1:60–1:200) and analyzed in duplicate. Intra- and interassay coefficients of variation were 5.0% (*n* = 20 replicates of a single sample) and 14.4% (*n* = 33 assays), respectively. Sensitivity of the assay at maximum binding was 12.5 ng/ml. Corticosterone metabolite concentrations are expressed as nanograms per gram dry fecal matter (ng/g).

#### 2.6. High-performance liquid chromatography

The number and relative proportions of immunoreactive glucocorticoid metabolites in feces were determined by reverse-phase high-performance liquid

chromatography (HPLC) as previously described by Monfort et al. (1991). For each species, 4–6 extracts were prepared from the fecal sample containing the peak value of glucocorticoid metabolites following injection of exogenous ACTH. Fecal extracts were pooled, evaporated to dryness, and reconstituted in 0.5 ml phosphate-buffered saline (0.01 M NaPO<sub>4</sub>, 0.14 M NaCl, 0.5% bovine serum albumin, pH 5.0) before loading the total volume on a pre-conditioned C-18 matrix cartridge (Spice Cartridge; Analtech, Newark, DE). The cartridge was washed with 5 ml distilled water and the total steroids eluted with 5 ml of 100% methanol, evaporated to dryness, then reconstituted in 300 µl of 100% methanol containing <sup>3</sup>H-cortisol, <sup>3</sup>H-corticosterone and <sup>3</sup>H-desoxycorticosterone (~4000–8000 dpm for each radiolabeled glucocorticoid). Filtered fecal extracts (55 µl) were separated on a Microsorb C-18 column (Reverse Phase Microsorb MV 100 C18, 5 µm diameter particle size; Varian, Woburn, MA) using a linear gradient of 20–100% methanol in water over 80 min (1 ml/min flow rate, 1 ml fractions). A subsample of each fraction (100 µl) was assayed for radioactivity to determine the retention times for the radiolabeled reference tracers. The remainder of each fraction (900 µl) was evaporated to dryness, reconstituted in 125 µl steroid buffer and an aliquot (50 µl) assayed singly in each immunoassay as described above.

### 2.7. Analysis of data

For the ACTH challenges, individual pre-treatment baselines were calculated as the average of fecal samples collected on all days before the ACTH injection, including the day of treatment (day 0). Adrenocortical responses to stimulation with ACTH are expressed as a percentage of the pre-treatment baseline, with the baseline value being equivalent to 100%.

Several measures were calculated to summarize fecal hormone values during the longitudinal steroid evaluation of each animal: (1) an overall mean of all samples for the collection period; (2) a mean baseline that excludes all values greater than the overall mean plus 1.5 standard deviations (SD); and (3) a peak mean that included all values greater than the overall mean plus 1.5 SD. An increase in glucocorticoid metabolites following exposure to an exogenous stressor was considered significant if the value exceeded the mean baseline plus 3 SD (Goymann et al., 1999). Responses to stressful stimuli (i.e., post-stressor rise in metabolites) are presented as a percentage of the mean baseline, which was designated as 100%.

Data from the longitudinal evaluations were corrected for a nonnormal distribution by performing a common logarithm (log<sub>10</sub>) transformation. Pearson product moment correlation analysis was used to de-

termine the relationship between fecal metabolites (log<sub>10</sub> transformed data) measured by the cortisol EIA and corticosterone RIA.

Average data are presented as mean ± standard error (SEM). The level of significance defined for statistical tests was  $P < 0.05$ .

## 3. Results

### 3.1. ACTH challenges

In all species, fecal glucocorticoid metabolites increased sharply to peak concentrations 1–2 days following ACTH administration and then declined rapidly. Cortisol and corticosterone immunoassays generated temporally similar fecal glucocorticoid metabolite excretion profiles for most species, but measured differing levels of immunoreactivity (Fig. 1). Concentrations of glucocorticoid metabolites during the pre-treatment baseline and at the ACTH-induced peak are shown in Table 1. The corticosterone RIA detected a greater ACTH-induced rise from pre-treatment baseline glucocorticoid metabolite concentrations in the Himalayan black bear, cheetah, clouded leopard, slender-tailed meerkat, red wolf, and female domestic cat, whereas the cortisol EIA measured a greater rise in metabolite concentrations for the black-footed ferret and male domestic cat (see Table 1 for comparison).

### 3.2. Longitudinal evaluations and exogenous stressors

Figs. 2–4 depict longitudinal profiles of cortisol and corticosterone metabolites excreted in feces of seven different carnivore species. Mean overall, mean baseline, and mean peak concentrations of glucocorticoid metabolites are presented in Table 2. Significant correlations were found between glucocorticoid metabolites quantified by the cortisol EIA and corticosterone RIA in feces of all carnivores (Table 3).

#### 3.2.1. Physical restraint, nonanesthetic injections, and blood sampling

Considerable intra- and interindividual variation was observed in adrenocortical responses to manipulations involving physical restraint (Figs. 2 and 3). Cage restraint of a black-footed ferret (Fig. 2A), gonadotropin injection of a domestic cat (Fig. 3A), and clouded leopard (Fig. 3B), and most episodes of blood sampling for a female red wolf (Fig. 2B) did not elevate fecal glucocorticoid concentrations above the set level of significance (mean baseline + 3 SD). In contrast, saline injection combined with physical restraint increased fecal glucocorticoid metabolite concentrations above mean baseline in the black-footed

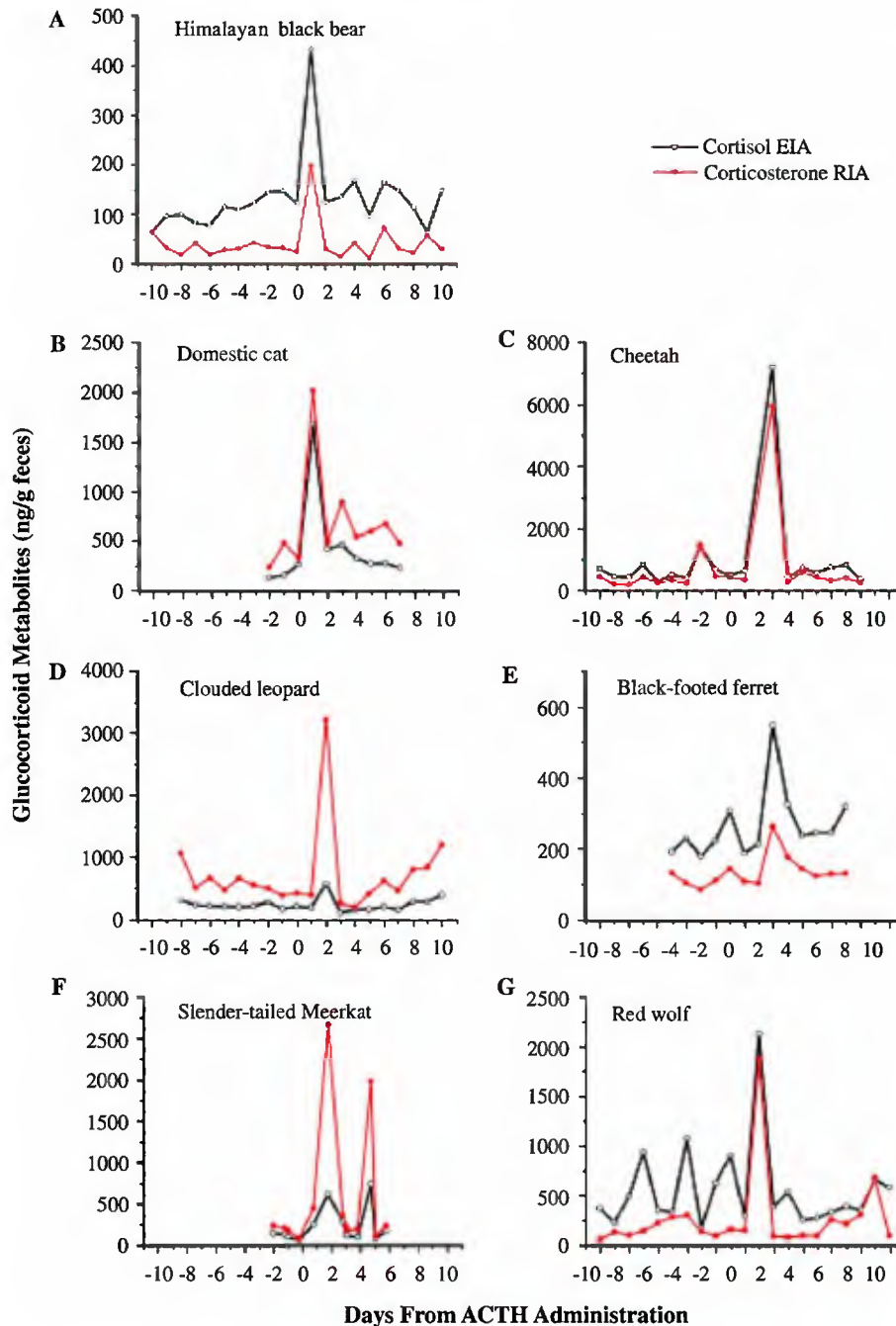


Fig. 1. Glucocorticoid metabolites in feces of a Himalayan black bear (A), domestic cat (B), cheetah (C), clouded leopard (D), black-footed ferret (E), slender-tailed meerkat (F), and red wolf (G) before and after administration of exogenous ACTH (day 0). The dosages of ACTH administered to each species are presented in Table 1. Immunoreactive metabolites in extracts prepared from fecal samples were measured with a cortisol EIA (open circles) and corticosterone RIA (closed circles).

ferret (Fig. 2A) and potentially in the male red wolf (Fig. 2C). One day following the saline injection in the black-footed ferret, fecal cortisol and corticosterone metabolite concentrations were 199% (338.0 ng/g) and 189% (192.8 ng/g) above mean baseline, respectively. Three and four days after saline injection in the male red wolf, fecal cortisol metabolites were 264% (936.0 ng/g) and 304% (1078.8 ng/g) above baseline,

respectively. Restraint of the female red wolf for blood sampling on day 23 was followed by a significant increase in cortisol metabolites (301% above mean baseline; 657.0 ng/g) on day 25, whereas blood sampling on day 30 was followed by a significant increase in cortisol (298% above mean baseline; 805.2 ng/g) and corticosterone metabolites (409% above mean baseline; 896.3 ng/g) on day 31.

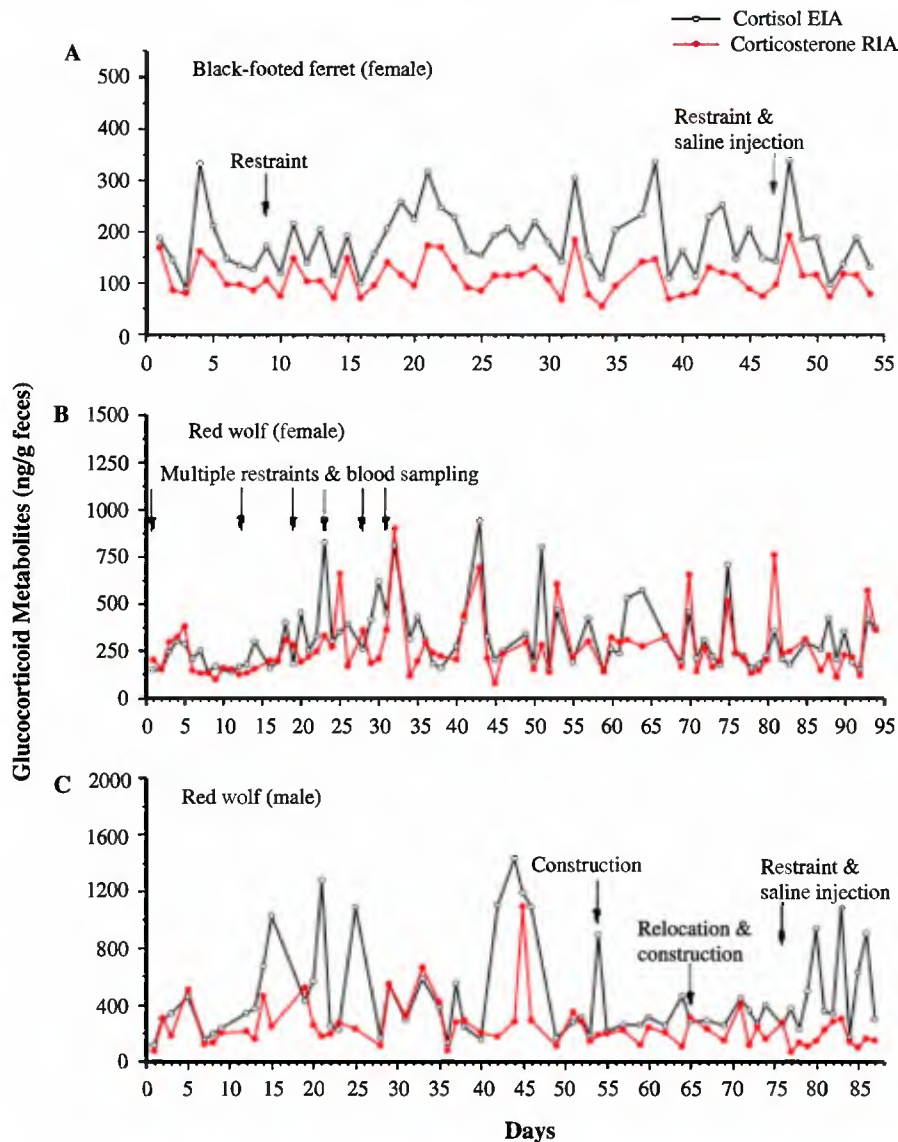


Fig. 2. Longitudinal profiles of fecal cortisol (open circles) and corticosterone (closed circles) metabolites for a female black-footed ferret (A), female red wolf (B), and male red wolf (C). All three animals experienced procedures that involved physical restraint. The male red wolf also experienced minor construction in an adjacent enclosure and relocation to an adjacent enclosure.

### 3.2.2. Chemical immobilization

Fecal glucocorticoid metabolite concentrations in the clouded leopard and cheetah, but not the domestic cat, increased after chemical immobilization (Fig. 3). In the clouded leopard, fecal glucocorticoid concentrations increased 472% (cortisol metabolites) and 666% (corticosterone metabolites) in response to anesthesia combined with laparoscopic artificial insemination, reaching maximum values 3 days (1175.1 ng/g) and 2 days (2013.5 ng/g) after anesthesia, respectively. Fecal cortisol and corticosterone metabolite concentrations 2 days (1026.4 ng/g feces; 412% above mean baseline) and 3 days (1230.0 ng/g; 407% above mean baseline) following anesthesia, respectively, were also significantly elevated. Two days after chemical immobilization of the cheetah, fecal metabolites of cortisol

and corticosterone increased 390% (2434.3 ng/g) and 1540% (3578.0 ng/g) above baseline, respectively. Because all felids were fasted for 12–24 h prior to anesthesia, the animals failed to defecate either on the day of (domestic cat) or following (clouded leopard, cheetah) immobilization. Therefore, fecal samples collected from nondomestic felids on days 2 and 3 following anesthesia represent the first and second defecations after the immobilization event, respectively.

### 3.2.3. Environmental disturbances

For the male red wolf, fecal glucocorticoid metabolites were not elevated or did not surpass the set level of significance (mean baseline + 3 SD) during construction or following relocation (Fig. 2C).



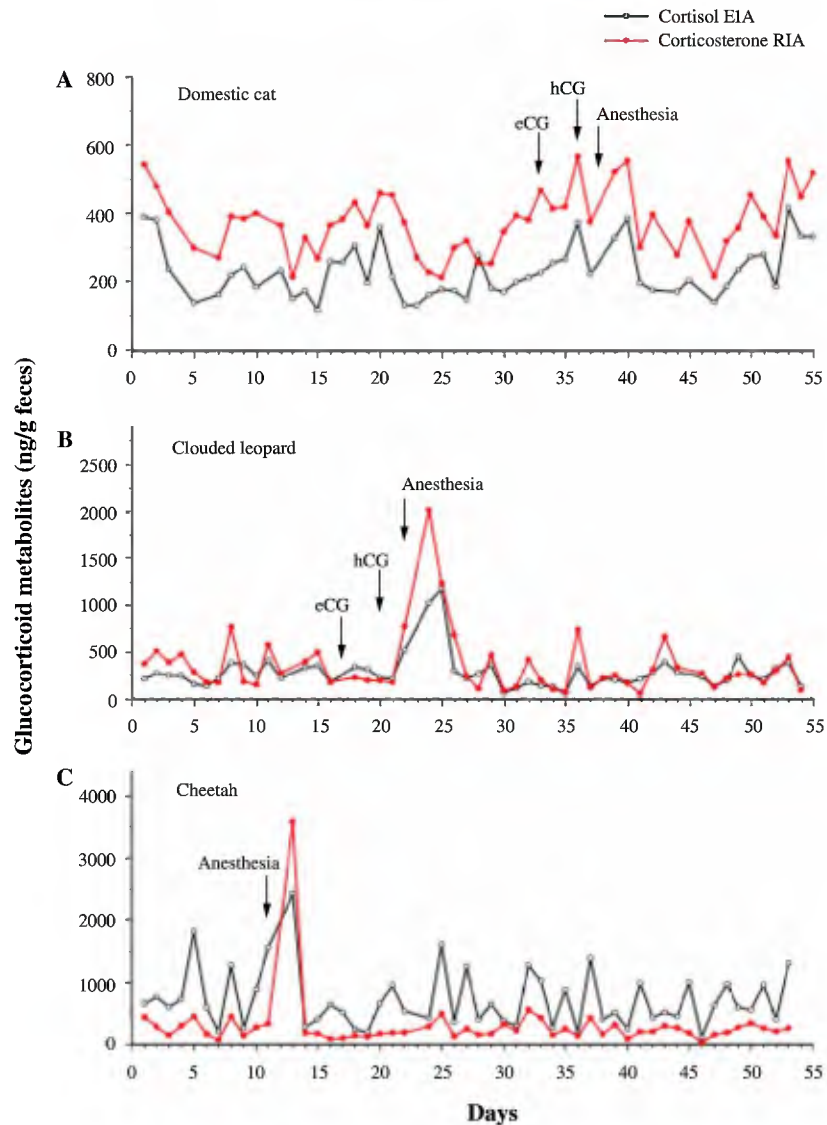


Fig. 3. Longitudinal profiles of fecal cortisol (open circles) metabolites and corticosterone (closed circles) metabolites for three felid species subjected to anesthesia. A domestic cat (A) and clouded leopard (B) were treated with gonadotropins before laparoscopic examination of the ovaries for evidence of ovulation. Only the clouded leopard was artificially inseminated. A cheetah (C) was anesthetized to facilitate a routine physical examination.

#### 3.2.4. Social tension

Concentrations of fecal glucocorticoid metabolites were not elevated in the female Himalayan black bear following a series of aggressive displays between this study subject and a co-housed male (Fig. 4A).

#### 3.2.5. Mate introduction

Fecal glucocorticoid metabolite excretion did not increase in the female sloth bear following mate introduction or breeding. Instead, significantly elevated concentrations were associated with abnormal defecations and treatment with deworming medication (Fig. 4B). Peaks in fecal glucocorticoid metabolite concentrations following day 210 coincide with the rise and sustained elevation in fecal progestins during pregnancy (data not shown).

### 3.3. HPLC

#### 3.3.1. Felidae

The majority of cortisol and corticosterone immunoreactivity in eluates from the domestic cat was associated with polar peaks eluting in fractions 7–23 (86% of total immunoreactivity) and fractions 12–26 (89%), respectively (Fig. 5A). A minor portion of the immunoreactivity (<4%) detected by the cortisol EIA eluted in fractions slightly more polar (37–40) than the  $^3\text{H}$ -cortisol reference tracer.

Analysis of HPLC-purified fecal eluates from the cheetah revealed that 47% and 89% of the cortisol EIA and corticosterone RIA immunoreactivity, respectively, corresponded to single polar peaks (fractions 8–18 for the EIA and 9–18 for the RIA) (Fig. 5B). Both immu-

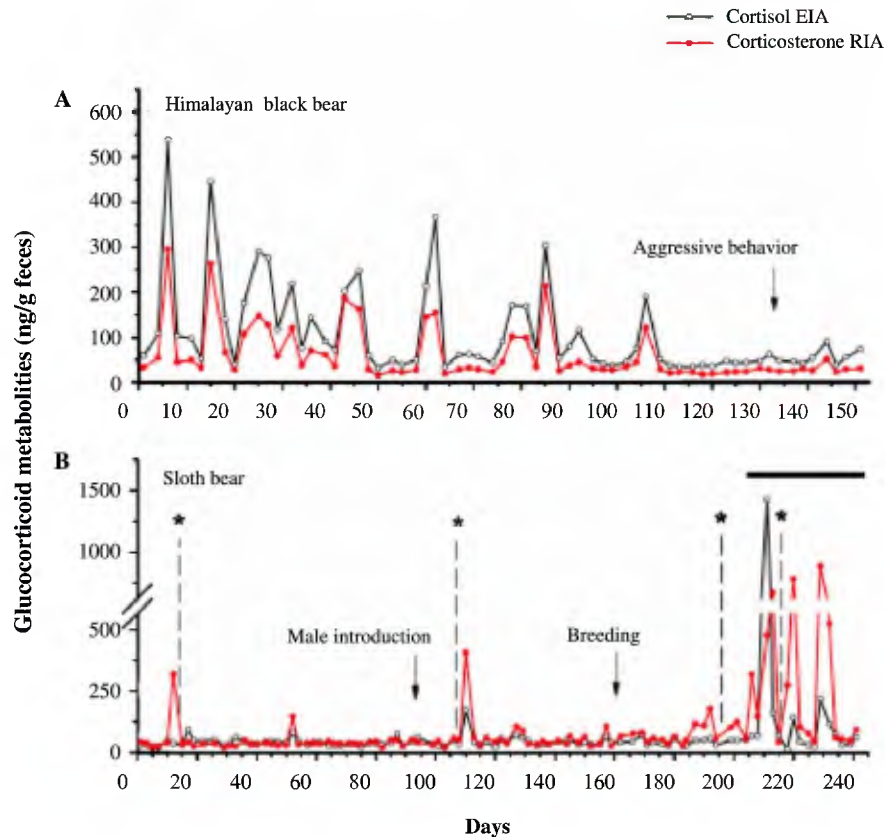


Fig. 4. Longitudinal profiles of fecal cortisol (open circles) and corticosterone (closed circles) for a female Himalayan black bear (A) and sloth bear (B) during the presence of conspecific males. A period of social tension involving aggressive displays between the female and male Himalayan black bears was observed on a single day (A). Introduction of a male to the female sloth bear was followed by breeding activity (B). The female sloth bear had a persistent nematode infection during the period of evaluation. Asterisks indicate abnormal defecations and treatments with deworming medication. The horizontal bar denotes the rise and sustained elevation of fecal progestins during pregnancy (B).

Table 2

Mean overall, baseline, and peak concentrations of fecal glucocorticoid metabolites for individuals of several carnivore species examined by longitudinal steroid evaluation

Species	Sex	Samples ( <i>n</i> )	Glucocorticoid metabolite	Mean overall ( $\pm$ SEM)	Mean baseline ( $\pm$ SEM)	Mean Peak ( $\pm$ SEM)
Himalayan black bear	F	65	Cortisol	107.3 $\pm$ 12.8	80.7 $\pm$ 7.1	369.6 $\pm$ 42.3
			Corticosterone	60.6 $\pm$ 7.5	45.1 $\pm$ 4.3	212.8 $\pm$ 23.0
Sloth bear	F	100	Cortisol	76.9 $\pm$ 23.8	48.7 $\pm$ 3.1	1428.8
			Corticosterone	127.7 $\pm$ 24.0	64.1 $\pm$ 6.5	666.5 $\pm$ 76.6
Domestic cat	F	50	Cortisol	234.1 $\pm$ 11.1	213.6 $\pm$ 8.8	384.6 $\pm$ 7.8
			Corticosterone	377.4 $\pm$ 13.2	354.7 $\pm$ 11.2	544.5 $\pm$ 7.7
Cheetah	F	51	Cortisol	751.1 $\pm$ 66.8	618.5 $\pm$ 49.5	1850.3 $\pm$ 202.6
			Corticosterone	298.5 $\pm$ 67.5	232.9 $\pm$ 16.4	3578.0
Clouded leopard	F	50	Cortisol	282.9 $\pm$ 27.8	248.8 $\pm$ 14.7	1100.7 $\pm$ 74.3
			Corticosterone	355.0 $\pm$ 47.1	302.2 $\pm$ 28.1	1621.8 $\pm$ 391.7
Black-footed ferret	F	53	Cortisol	184.7 $\pm$ 8.3	170.1 $\pm$ 6.4	324.6 $\pm$ 6.5
			Corticosterone	110.2 $\pm$ 4.5	101.9 $\pm$ 3.6	174.7 $\pm$ 4.6
Red wolf	M	60	Cortisol	462.1 $\pm$ 42.8	354.8 $\pm$ 26.5	1159.4 $\pm$ 47.6
			Corticosterone	245.3 $\pm$ 21.5	212.7 $\pm$ 13.0	702.5 $\pm$ 133.6
	F	78	Cortisol	309.6 $\pm$ 19.6	270.3 $\pm$ 12.6	780.3 $\pm$ 44.8
			Corticosterone	264.9 $\pm$ 18.1	218.9 $\pm$ 9.4	667.1 $\pm$ 42.1

Glucocorticoid metabolite refers to the immunoassay, cortisol EIA or corticosterone RIA, used to measure steroids in fecal samples. Mean overall represents the average concentration of metabolites (ng/g feces) in all fecal samples (*n*) from the collection period. Mean baseline represents the average metabolite concentrations for all samples that fall below the overall mean + 1.5 SD. Mean peak represents the average metabolite concentration for all samples that are greater than the overall mean + 1.5 standard deviations. All values are expressed as means  $\pm$  standard error unless there was only one value in the category.

Table 3

Correlation matrix describing the relationship between glucocorticoid metabolites measured with the cortisol enzyme immunoassay and corticosterone radioimmunoassay in feces of seven different carnivore species

Species	Sex	Samples ( <i>n</i> )	Correlation coefficient ( <i>r</i> )	<i>P</i> value
Himalayan black bear	F	65	0.975	<0.0001
Sloth bear	F	100	0.660	<0.0001
Domestic cat	F	50	0.786	<0.0001
Cheetah	F	51	0.728	<0.0001
Clouded leopard	F	50	0.794	<0.0001
Black-footed ferret	F	53	0.830	<0.0001
Red wolf	M	60	0.383	0.0025
	F	78	0.652	<0.0001

Pearson product moment correlation analyses were performed on data transformed with a common logarithm ( $\log_{10}$ ).

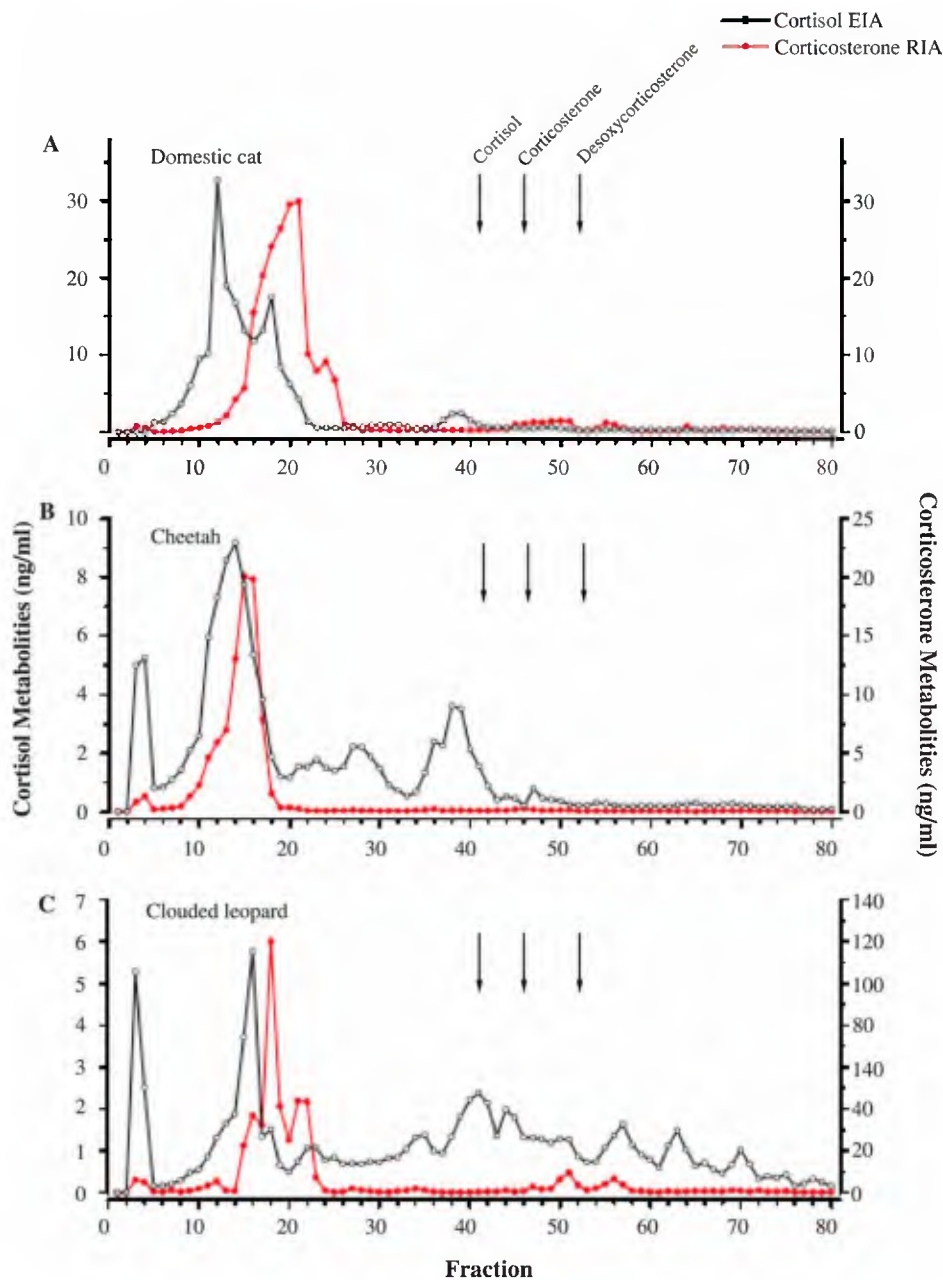


Fig. 5. Reverse-phase HPLC separation of immunoreactive glucocorticoid metabolites in feces of domestic cat (A), cheetah (B), and clouded leopard (C). Immunoreactivity in each fraction was measured with a cortisol EIA (open circles) and corticosterone RIA (closed circles). Retention times for co-eluted tritiated reference tracers are indicated by arrows.

noassays cross-reacted with the metabolite(s) eluting in fractions 3–4 (9% and 2% of EIA and RIA total immunoreactivity, respectively). Additional cortisol EIA immunoreactivity was associated with smaller peaks at fractions 27–29 (5% of total immunoreactivity) and 35–42 (14%).

Corticosterone RIA of clouded leopard fecal extracts separated by HPLC determined that 80% of the total immunoreactivity coincided with two or more peaks (fractions 15–24) (Fig. 5C). Minor corticosterone immunoreactivity eluted in fractions 3–4 (2% of total immunoreactivity), 50–52 (4%), and 55–57 (3%). Cortisol

EIA immunoreactivity corresponded to numerous immunoreactive substances (at least 9) in clouded leopard fecal extracts (Fig. 5C). Four metabolites more polar than  $^3\text{H}$ -cortisol were found to elute in fractions 3–4 (9% of total immunoreactivity), 10–19 (22%), 21–23 (3%), and 33–36 (5%). Three metabolites also reacting with the cortisol EIA eluted in fractions less polar than  $^3\text{H}$ -desoxycorticosterone (fractions 43–46, 7% of total immunoreactivity; 62–63, 4%; 68–71, 3%), while one was more polar than  $^3\text{H}$ -corticosterone (fractions 43–46, 7%). Eleven percent of the total cortisol immunoreactivity was found to co-elute with  $^3\text{H}$ -cortisol.

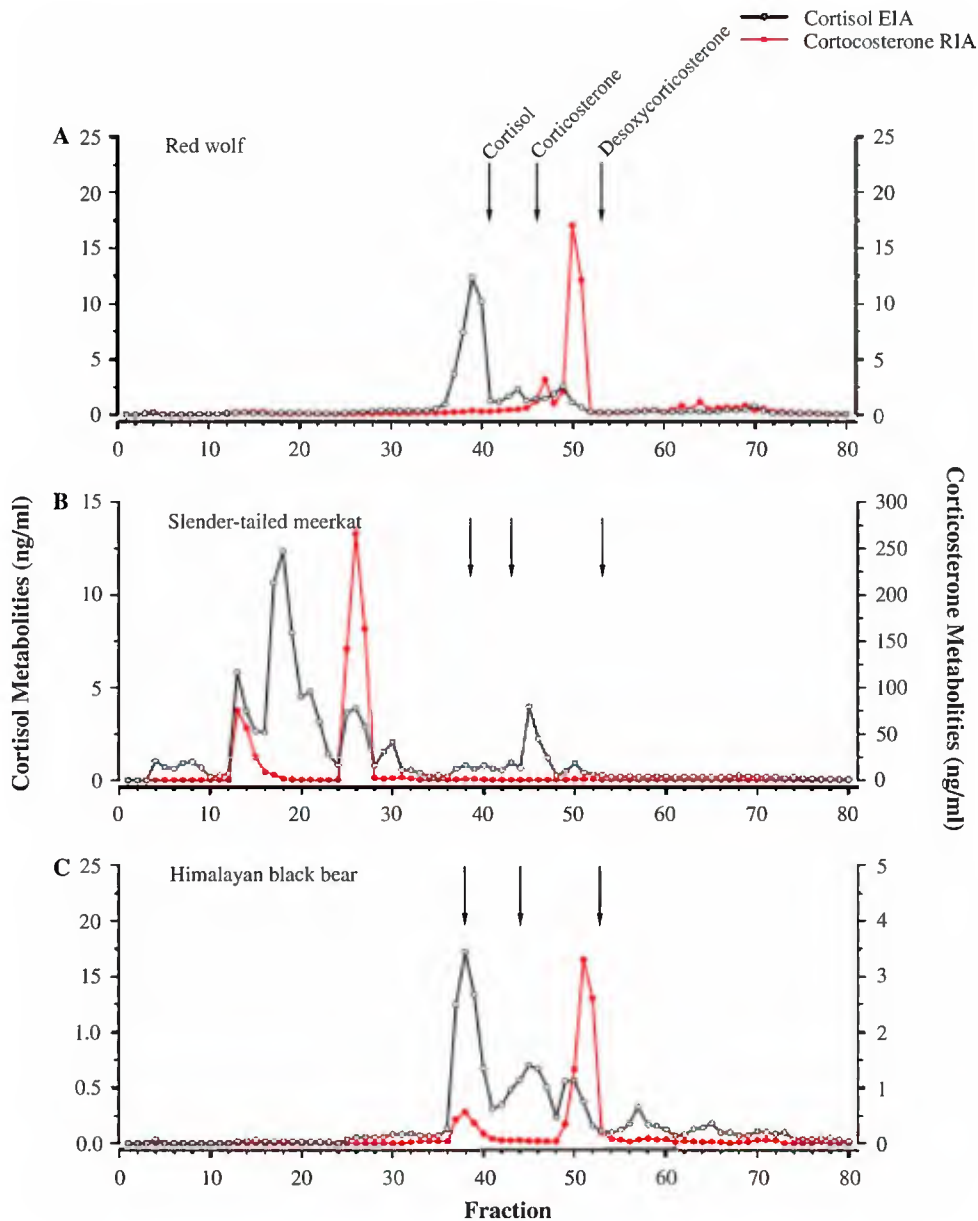


Fig. 6. Reverse-phase HPLC separation of immunoreactive glucocorticoid metabolites in feces of the red wolf (A), slender-tailed meerkat (B), and Himalayan black bear (C). Immunoreactivity in each fraction was measured with a cortisol EIA (open circles) and corticosterone RIA (closed circles). Retention times for co-eluted tritiated reference tracers are indicated by arrows.

### 3.3.2. *Canidae*

The corticosterone RIA measured two immunoreactive substances in HPLC-separated red wolf fecal extracts, which eluted in fractions 46–48 (10% of total immunoreactivity) and 49–51 (59%) (Fig. 6A). The majority of cortisol EIA immunoreactivity (56% of total immunoreactivity) corresponded to a peak (fractions 36–41) slightly more polar than  $^3\text{H}$ -cortisol (Fig. 6A). Additional cortisol EIA immunoreactivity was associated with metabolites more (fractions 43–45; 8% of total immunoreactivity) and less (fraction 47–50; 11%) polar than  $^3\text{H}$ -corticosterone.

### 3.3.3. *Herpestidae*

Cortisol EIA immunoreactivity corresponded to multiple immunoreactive substances in fecal extracts from the slender-tailed meerkat (Fig. 6B). At least five metabolites more polar than  $^3\text{H}$ -cortisol were found to elute in fractions 4–9 (5% of total immunoreactivity), 13–15 (12%), 16–23 (46%), 25–27 (10%), and 29–30 (4%). One metabolite (fractions 45–47; 7% of total immunoreactivity) also detected with the cortisol EIA was less polar than  $^3\text{H}$ -corticosterone. The corticosterone RIA measured two immunoreactive metabolites more polar than  $^3\text{H}$ -cortisol in HPLC-separated fecal extracts, which eluted in fractions 13–17 (22% of total immunoreactivity) and 25–27 (74%) (Fig. 6B).

### 3.3.4. *Ursidae*

Analysis of HPLC-purified fecal eluates from the Himalayan black bear determined that 28% and 15% of cortisol EIA and corticosterone RIA immunoreactivity, respectively, co-eluted with  $^3\text{H}$ -cortisol (fractions 38–41) (Fig. 6C). Additional cortisol EIA immunoreactivity was associated with peaks at fractions 42–47 (23% of total immunoreactivity), 49–52 (11%) and 56–58 (3%). The majority of corticosterone RIA immunoreactivity was associated with a metabolite that eluted in fractions 25–27 (74% of total immunoreactivity).

## 4. Discussion

In recent years there has been growing concern for the welfare of animals in captivity and increasing demand for the development of noninvasive methods to measure the stress associated with management practices and environmental conditions. The aim of this study was to determine if a cortisol EIA was suitable for noninvasively monitoring adrenocortical activity in a variety of carnivore species. The performance of this EIA was gauged by comparison with a corticosterone RIA, an assay currently favored for monitoring fecal corticoids in wildlife species.

Standardized procedures (i.e., tests for parallelism and mass recovery) were used to validate the cortisol EIA and corticosterone RIA for measurement of glucocorticoids in feces of each species. HPLC analyses revealed that the majority of glucocorticoid immunoreactivity in fecal extracts corresponded with two or more peaks, indicating the presence of multiple metabolites. The two assay systems appeared to be measuring different glucocorticoid metabolites between the different Carnivora suborders. Major immunoreactive metabolites in felids and meerkats were more polar than those identified in the red wolf and Himalayan black bear, and previously reported for the African wild dog (Monfort et al., 1998) and black-footed ferret (Young et al., 2001). In contrast, similarities between HPLC profiles for domestic and nondomestic cats suggest that like ovarian steroids (Brown et al., 1994), glucocorticoid metabolism may be conserved across felid species.

For most species, only a minor portion of the immunoreactivity detected in HPLC-separated fecal extracts co-eluted with radiolabeled cortisol or corticosterone. These results are consistent with radiometabolism studies that demonstrated the near absence of authentic radiolabeled cortisol and corticosterone from feces of carnivores (Graham and Brown, 1996; Schatz and Palme, 2001), lagomorphs (Teskey-Gerstl et al., 2000), domestic livestock (Palme and Möstl, 1997; Möstl et al., 1999) and primates (Bahr et al., 2000). By contrast, immunoreactive substances in feces of the Himalayan black bear and clouded leopard co-eluted with  $^3\text{H}$ -cortisol, suggesting these species may excrete native cortisol in variable amounts.

Although the identity of most glucocorticoid metabolites in HPLC-purified fecal eluates remains unknown, their biological relevance as indices of adrenocortical activity in carnivores was demonstrated by a transient increase in excretion following administration of exogenous ACTH as determined by both the cortisol EIA and corticosterone RIA. In the present study, ACTH induced a ~228–1145% and ~231–4150% rise above pre-treatment baseline concentrations of cortisol and corticosterone metabolites across all species of carnivores, respectively. These results were similar to other studies utilizing the corticosterone RIA (or its antibody) in carnivores, such as the cheetah (range ~690–4294% above baseline, 400 IU ACTH, Terio et al., 1999), clouded leopard (mean ~1400%, 500 IU ACTH, Wielebnowski et al., 2002), African wild dog (range ~1000–3000%, 400 IU, Monfort et al., 1998), spotted hyena (range ~1300–4500% as calculated from data, 200 IU ACTH, Goymann et al., 1999), black-footed ferret (mean ~208% as calculated from data, 2 IU ACTH, Young et al., 2001), and Malaysian sun bear (~300% as calculated from graph, 88 IU, Wasser et al., 2000).

The adrenal cortex rapidly synthesizes and secretes glucocorticoids in response to ACTH. Although serum cortisol concentrations in mammals rise within 10–30 min (Brown et al., 1995; Carlstead et al., 1992; Graham and Brown, 1996; Terio et al., 1999), the appearance of elevated concentrations of glucocorticoid metabolites in feces following ACTH injection is much slower. The delay between circulating steroid hormones and detection of their metabolites in feces is approximately equivalent to the time required for digesta to pass from the duodenum to the rectum (Palme et al., 1996). In the present study, the delays between ACTH administration and the appearance of peak glucocorticoid metabolite concentrations in feces (1–2 days post-ACTH treatment) were within the range previously reported for other carnivores, including the domestic cat (24–28 h, Graham and Brown, 1996; 24–49 h, Schatz and Palme, 2001), domestic dog (8–71 h, Schatz and Palme, 2001), African wild dog (24–30 h, Monfort et al., 1998), black-footed ferret (~20–44 h, Young et al., 2001), and spotted hyena (16–50 h, Goymann et al., 1999).

Capture and physical restraint elevate plasma glucocorticoid concentrations in wildlife species (Kenagy and Place, 2000; Morton et al., 1995; Wesson et al., 1979). Handling and mild restraint in unfamiliar surroundings for serial blood sampling also increases circulating cortisol levels in animals more habituated to these procedures and/or the presence of human investigators (Fox et al., 1994; Reinhardt et al., 1990, 1991). In domesticated and nondomesticated mammalian species, acute elevations in fecal glucocorticoid metabolites also have been observed following restraint in a squeeze cage (Jurke et al., 1997; Terio et al., 1999), brief handling and blood sampling (Harper and Austad, 2000), anesthesia (Terio et al., 1999; Whitten et al., 1998), relocation within an institution (Terio et al., 1999), translocation between facilities (Goymann et al., 1999; Morrow et al., 2002; Palme et al., 2000; Schwarzenberger et al., 1998), mate introduction and breeding (Terio et al., 1999), exposure to a novel environment (Morrow et al., 2002), and aggressive social interactions (Goymann et al., 1999). Despite monitoring biologically relevant metabolites in feces, our ability to detect adrenocortical responses to acute physical and psychological stressors using the described immunoassay methods varied with the type of stimulus, between episodes of the same stimulus, and among species. A stress response was elicited in 2 of 3 felid species experiencing anesthesia. Significantly elevated concentrations of fecal glucocorticoid metabolites also were observed following saline injections in the black-footed ferret and red wolf, but not after gonadotropin injections in the domestic cat and clouded leopard. In the red wolf, only 2 of 6 episodes of blood sampling were followed by a significant rise in excreted glucocorticoid metabolites. Surgery and

anesthesia are known to increase plasma or serum cortisol concentrations in dogs (Church et al., 1994; Fox et al., 1994), cats (Smith et al., 1996, 1999), horses (Taylor, 1989), rhesus monkeys (Puri et al., 1981), and humans (Bozkurt et al., 2000; Oyama and Wakayama, 1988). The dramatic rise in fecal glucocorticoid metabolites following procedures involving anesthesia in the cheetah and clouded leopard were consistent with previous reports for the cheetah (mean ~475% rise above baseline, Terio et al., 1999) and chimpanzee (mean ~300% rise above baseline, Whitten et al., 1998).

Although no increases of fecal glucocorticoid metabolites were associated with courtship or breeding activity in the female sloth bear, there was a close association between enhanced excretion of metabolites and medicinal treatment/abnormal defecations. Because the female suffered from a persistent nematode infection, the data suggest that factors associated with this medical condition may have induced an adrenal response. Higher plasma levels of cortisol are found in dogs (Church et al., 1994) and horses (Santschi et al., 1991) requiring surgery for abdominal illnesses. In horses, concentrations of fecal 11,17-dioxoandrostanones have been used as indices of pain experienced during colic and after castration (Merl et al., 2000). Alternatively, the deworming process and diarrhea could have increased steroid excretion independent of the stress associated with the nematode infestation.

In some instances, it would appear that accurate identification of a true adrenocortical response to an exogenous stimulus may have been hindered by the high day-to-day variation in concentrations of fecal metabolites. For example, metabolite concentrations following saline injections of the black-footed ferret and male red wolf, and after several episodes of blood sampling in the female red wolf exceeded values required for significance (i.e., mean baseline + 3 SD), suggesting that these procedures were stressful. However, it was difficult to determine if these elevations were true stress responses because a number of unexplained peaks in metabolites during the period of longitudinal evaluation also reached the threshold value. Because our behavioral and environmental effect data were collected opportunistically and based primarily on keeper observations, it is possible that many situations were not adequately documented. Other studies have also had varied success in detecting adrenocortical responses to acute exogenous stressors. Möstl et al. (2002) measured increased excretion of fecal cortisol metabolites in cows following transportation to a novel environment, but not after use for practicing invasive sampling techniques for blood and rumen contents.

The absence of significant elevations in glucocorticoid metabolites following relocation, construction, social tension/minor aggression, mate introduction/breeding, and some procedures involving restraint suggests that

these stimuli may not have been stressful for the individuals examined in this study. However, adrenocortical responses are known to reflect interanimal variation in the perception of a stimulus, and the biological responses evoked to cope with a stressor depend on factors such as previous experience, genetics, age, and physiological state (Moberg, 1985, 2000). Alternately, it is plausible that the stimuli were stressful, but the acute adrenocortical rise was undetectable. Brief or small increases in circulating levels of glucocorticoids are likely to be masked by the pooling of metabolites in bile and feces. Also, fecal samples containing stressor-induced peaks in metabolites may have been missed due to infrequent sample collection (e.g., Himalayan black bear and sloth bear, ~3 fecal collections per week) or failure to collect all scats defecated each day.

Rather than studying brief events that enhance adrenocortical activity, future efforts should focus on the more physiologically detrimental state of chronic stress by diagnosing its associated persistent elevation of fecal glucocorticoids and identifying possible causative factors. Recently, Wielebnowski et al. (2002) determined that higher mean concentrations of fecal glucocorticoids were present in clouded leopards on public display and in close proximity to potential predators. In addition, a positive association was identified between glucocorticoid levels and detrimental behaviors (e.g., fur-plucking, tail chewing, excessive pacing, and hiding).

Steroids circulating in the blood are catabolized in the liver before excretion in urine and bile (Brownie, 1992; Macdonald et al., 1983; Taylor, 1971). Further changes to steroid metabolites are facilitated by the enzymatic activities of bacterial flora during transit through the intestinal tract (Macdonald et al., 1983; Taylor, 1971). Because steroid hormones are extensively metabolized prior to excretion, it is not surprising that immunoassays featuring antibodies specific to blood glucocorticoids may not be suitable for quantifying fecal glucocorticoid metabolites (Goymann et al., 1999; Graham and Brown, 1996; Palme and Möstl, 1997; Schatz and Palme, 2001; Terio et al., 1999). As such, group-specific antibodies cross-reacting with a family of metabolites derived from a single parent steroid are preferred over more specific antibodies when using fecal steroid analysis to characterize physiological functions such as reproductive cycles and status or adrenocortical activation (Brown et al., 2001; Palme and Möstl, 1997; Palme et al., 1997; Schwarzenberger et al., 1996).

Although the corticosterone RIA was specifically developed to measure corticosterone levels in serum or plasma of rodents, its antibody cross-reacts well with glucocorticoid metabolites in feces and has proved useful for evaluating adrenal function in numerous species, including carnivores (domestic cat, spotted hyena, African wild dog, clouded leopard, cheetah, Alaskan sea

otter, and Malayan sun bear), primates (yellow baboon), birds (northern spotted owl, chicken), herbivores (African elephant, black rhinoceros, gerenuk, elk, scimitar-horned oryx, and dairy cattle), and rodents (house mouse, deer mouse, and red-back voles) (Dehnhard et al., 2003; Goymann et al., 1999, 2001; Graham and Brown, 1996; Harper and Austad, 2000; Millspaugh et al., 2001; Monfort et al., 1998; Morrow et al., 2002; Terio et al., 1999; Wasser et al., 1997, 2000; Wielebnowski et al., 2002). In the spotted hyena, an EIA using the same corticosterone antibody outperformed three other EIAs (cortisol, corticosterone, and 11-oxoetiocholanolone) when resolving changes in adrenocortical activity after an ACTH challenge (Goymann et al., 1999). The affinity of the corticosterone antibody for fecal glucocorticoid metabolites in such a diverse array of species thus supports the assertion by Wasser et al. (2000) that the antibody is group-specific.

In the present study, a cortisol EIA generated immunoreactive fecal metabolite profiles in carnivores that were temporally similar to the corticosterone RIA. Although the polyclonal cortisol antibody was developed to measure serum or plasma cortisol, results from our study suggest that it may also behave as a group-specific antibody. HPLC analyses further confirm that the cortisol antibody cross-reacts with a number of glucocorticoid metabolites in feces of a variety of carnivores. Still, there were examples where the two assays did not agree, especially in several of the longitudinal profiles. It remains to be determined whether combining the two assays may be of some benefit, since it might quantify a greater majority of the biologically significant metabolites.

In conclusion, the present study suggests that both a cortisol EIA and corticosterone RIA measure immunoreactive substances in feces reflective of adrenocortical activation in carnivores. Enzyme immunoassays often are preferred by laboratories at zoological institutions and field facilities because they lack the restrictive radioisotope licensing associated with RIAs. The described cortisol EIA offers a practical alternative for investigators wishing to noninvasively monitor adrenocortical activity for improving the health and well-being of carnivores.

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