

A Generalized Fecal Glucocorticoid Assay for Use in a Diverse Array of Nondomestic Mammalian and Avian Species

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Noninvasive fecal glucocorticoid analysis has tremendous potential as a means of assessing stress associated with environmental disturbance in wildlife. However, interspecific variation in excreted glucocorticoid metabolites requires careful selection of the antibody used in their quantification. We compared four antibodies for detecting the major fecal cortisol metabolites in yellow baboons following ³H cortisol administration, ACTH challenge, and HPLC separation of fecal glucocorticoid metabolites. The most effective antibody (ICN corticosterone RIA; Cat. No. 07-120102) demonstrated relatively high cross-reactivities to the major cortisol metabolites present in feces during peak excretion, following both radiolabel infusion and ACTH challenge. This same anti-

body also detected increased fecal glucocorticoid metabolites after ACTH administration in the African elephant, black rhinoceros, Roosevelt elk, gerenuk, scimitar-horned oryx, Alaskan sea otter, Malayan sun bear, cheetah, clouded leopard, longtailed macaque, and northern spotted owl. Results suggest that (1) fecal glucocorticoid assays reliably detect endogenous changes in adrenal activity of a diverse array of species and (2) where comparisons were made, the ICN corticosterone antibody generally was superior to other antibodies for measuring glucocorticoid metabolites in feces. © 2000 Academic Press

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Noninvasive measures of physiological stress have a wide array of applications for conservation biology, wildlife management, animal husbandry, behavioral ecology, and biomedicine. Measurement of fecal glucocorticoid (GC) metabolites may be particularly useful, since samples can be obtained relatively easily in captivity or in the field without disturbing subjects

and may reflect adrenal responsiveness to a wide array of potential stressors (Wingfield, 1994; Creel *et al.*, 1997; Wasser *et al.*, 1997; Wingfield *et al.*, 1997; Mills-paugh, 1999; Goymann *et al.*, 1999; Foley *et al.*, 2000). However, until recently, assays for fecal GC metabolites have proven difficult to develop compared to the reproductive steroids. Feces typically contain multiple GC metabolites with little native hormone (e.g., Eriksson, 1971; Palme *et al.*, 1997; Bahr *et al.*, 2000), because the main GCs (cortisol in most large mammals and corticosterone in birds and many small mammals) are rapidly and extensively metabolized before excretion. Moreover, species-specific differences in steroid metabolism and gut microflora can cause these metabolites to differ between species. Presumably, rapid GC metabolism serves to moderate the strong catabolic effects of GCs and to reduce interference with mineralocorticoid receptors in the gut (Setchell *et al.*, 1975; Munck *et al.*, 1984; Vylitová *et al.*, 1998). As a result, highly specific cortisol antibodies may have relatively little affinity for the fecal GC metabolites in many species.

The potential usefulness of a noninvasive assessment of adrenal activity led us to search for an antibody(s) that can produce biologically relevant fecal GC profiles across a wide array of species. Three different techniques were used to test the validity of several GC assays for fecal analysis in yellow baboons (*Papio cynocephalus*). First, infusion of radiolabeled cortisol was used in conjunction with high-performance liquid chromatography (HPLC) studies to determine the number and predominance of excreted metabolites, their affinities to four separate GC antibodies, and their respective lag times from secretion until excretion. Second, a pharmacological challenge with adrenocorticotrophic hormone (ACTH) established whether fecal assays accurately reflect acute adrenal activation. In vertebrates, ACTH administration mimics a natural adrenal stress response by causing a rapid rise in circulating native GCs (e.g., cortisol or corticosterone), followed by a return to baseline within a few hours (Norris, 1996). The same pattern should also occur in feces, with the onset of the peak excretion delayed by the species-specific excretion lag time (e.g., Palme *et al.*, 1998). Third, parallelism tests were used to demonstrate whether each antibody binds with serially diluted fecal GC metabolite extracts in a dose-dependent manner (producing a displacement curve

parallel to the standard curve; Diamandis and Christopoulos, 1996).

Results from radiolabel infusion and antibody-specific ACTH challenge studies in baboons enabled us to identify antibodies that are particularly useful for accurately assessing adrenal status in a variety of wild-life species. Specifically, the two most promising antibodies in the baboon studies were next examined through ACTH challenges in the longtailed macaque (*Macaca fascicularis*), African elephant (*Loxodonta africana*), Roosevelt elk (*Cervus elaphus roosevelti*), and Alaskan sea otter (*Enhydra lutris kenyoni*). Finally, the most promising antibody was investigated with ACTH challenges in the Malayan sun bear (*Helarctos malayanus*), gerenuk (*Litocranius walleri*), scimitar-horned oryx (*Oryx dammah*), black rhinoceros (*Diceros bicornis*), cheetah (*Acinonyx jubatus*), clouded leopard (*Neofelis nebulosa*), and northern spotted owl (*Strix occidentalis caurina*).

METHODS

Sample Collections

The species, sex, and housing locations of study animals are shown in Table 1. Unless otherwise noted, all injections were given to subjects in deep muscle, under temporary restraint, without anesthesia to maintain normal gastro-intestinal motility. Generally, for radiolabel infusion or ACTH challenge studies, all feces and urine were collected for 0–5 days before and then at regular intervals (e.g., every 2 h) for the next 3–5 days after injection. Every precaution was taken to separate urine and feces at the time of excretion, using a 0.6-cm mesh screen with an underlying urine collection pan for primates and a sloping floor for carnivores, hoofstock, and elephants. Alaskan sea otters defecated directly into the water, after which feces were scooped up using a fine mesh net. Because of the excretion lag time (6–30 h in mammals), sample collections that began at the time of ACTH injection (time 0) were considered equivalent to preinjection samples. All samples were stored frozen (–20°C) within 1 h postcollection until processing.

TABLE 1
ACTH Doses and Fecal Hormone Extraction Methods Used for the 12 Species in This Study

Species	Males/females	ACTH IU/kg (total dose)	Wt wet/dry	Extraction method
Yellow baboon ¹	0/2	0.92 IU/kg (6 IU total)	0.6 g wet	Boil, 90% EtOH
Longtailed macaque ¹	0/10	2.0 IU/kg (6.25 IU total)	0.6 g wet	Boil, 90% EtOH
African elephant ²	0/2	~0.5 IU/kg (~2000 IU total)	0.2 g dry	Boil, 90% EtOH
Alaskan sea otter ³	1/0	0.9 IU/kg (36 IU total)	0.2 g dry	Boil, 90% EtOH
Roosevelt elk ⁴	1/1	Male: 0.65 IU/kg (300 IU total) Female: 1.1 IU/kg (375 IU total)	0.6 g wet 0.6 g wet	Vortex, 90% MeOH Vortex, 90% MeOH
Malayan sun bear ⁴	0/1	1.0 IU/kg (88 IU total)	0.6 g wet	Boil, 90% EtOH
Black rhinoceros ⁵	1/1	1.5 IU/kg (800 IU total)	0.2 g dry	Boil, 90% EtOH
Gerenuk ⁶	2/0	12.5 IU/kg (400 IU total)	0.2 g dry	Boil, 100% EtOH
Scimitar-horned oryx ⁷	2/0	0.81 IU/kg (120 IU total)	0.2 g dry	Boil, 100% EtOH
Cheetah ⁶	2/2	10 IU/kg (400 IU total)	0.2 g dry	Boil, 90% EtOH
Clouded leopard ⁷	3/2	5 IU/kg (80 IU total)	0.2 g dry	Boil, 90% EtOH
Northern spotted owl ⁴	0/1	1.4 IU/kg (1 IU total)	0.05 g dry	Boil, 90% EtOH

Note. Fecal samples were mixed and weighed out in the amount shown, either after freeze-drying ("dry") or without drying ("wet"). Steroids were extracted from weighed feces using boiling or vortexing for 30 min in 90 or 100% ethanol (EtOH) or methanol (MeOH), as shown.

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Radiolabel Infusion in β -Methasone-Suppressed Baboons and in Two Owl Species

Baboons were given daily i.m. injections of 3 mg β -methasone (Celestone Solvspan; Schering Corp., NJ) for three consecutive mornings immediately before the radiolabel infusion. β -Methasone is a long-acting form of dexamethasone and was given at a dose expected to result in adrenal deactivation prior to, and for at least 24 h following, radiolabel infusion (G. J. Pepe, pers. comm.). This was employed to ensure that the majority of GC metabolites recovered in feces were derived from the infused radiolabeled cortisol. Thirty minutes following the last β -methasone injection, each baboon was infused i.v. with 50 μ Ci of [¹⁴C]cortisol (4-¹⁴C-hydrocortisone, 2.0 GBq/mmol, NEN-163; NEN Life Science Products, Boston, MA), delivered in a 2-cc saline infusion. At a concentration of 7000 ng/ μ Ci, each baboon received 350,000 ng of [¹⁴C]cortisol, which should have produced blood levels of approximately 500 ng/ml. This dose was calculated to produce a level of circulating cortisol similar to that observed in an animal experiencing moderate adrenal activation (G. J. Pepe, pers. comm.). All urine and feces were collected as described above.

To address excretion lag time in the endangered northern spotted owl (730 g), 13.2 μ Ci [³H]corticosterone was injected i.v. into two nonendangered surrogate species, a barred owl (*Strix varia*; 800 g) and a

great horned owl (*Bubo virginianus*; 1400 g). Each individual was then checked hourly and all urine and feces collected from time 0 until 60 h postinfusion.

Radioactive Counting of ¹⁴C Infusion Samples

Calculation of ¹⁴C excretion following infusion was conducted using the method described in Wasser *et al.* (1994). A 1-ml aliquot of urine was counted in 20 ml Ultima Gold scintillation fluid (Packard Instrument Co., Meriden, CT) using the ¹⁴C channel on a Beckman LS6500 liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA). For feces, a 1-g aliquot of each well-mixed sample was homogenized in 0.5 ml 90% ethanol to liberate the steroids. To minimize sample quench, each 1 g fecal homogenate was subdivided into 10 or more scintillation vials (each containing 20 ml Ultima Gold) until the "H" number of each vial was <120. The dpm per vial generated by the ¹⁴C channel of our Beckman counter were then summed to generate the total counts per gram fecal sample. Counting of dual-labeled materials involving ¹⁴C-excreted metabolites in a ³H RIA was conducted using the dual channel on our Beckman LS6500, according to manufacturer specifications. In such cases, a >10:1 ratio of ³H:¹⁴C was maintained to accommodate the overlap of ¹⁴C in the ³H window. Similar precautions were unnecessary when conducting ¹²⁵I-based RIAs of

^{14}C metabolites, since the β -emissions of ^{14}C are unable to penetrate glass and thus do not reach the gamma detector.

ACTH Challenges

ACTH dosages used for each species are given in Table 1. All mammalian species received ACTH administered i.m. in a slow-release gel to cause sustained adrenal corticoid secretion (ACTHAR gel; Rhone-Poulenc Rorer Pharmaceuticals, Inc., Collegetown, PA; or a more highly concentrated version synthesized by Hadfield's Pharmacy, Edmonds, WA). Sustained adrenal activation is especially important in species that excrete a large fecal mass (e.g., African elephant) to prevent short-lived changes in adrenal status from becoming too diluted in the gut for fecal detection. The northern spotted owl received an i.v. infusion (0.1 mg ACTH, Cortrosyn; Organon, West Orange, CA) into the jugular vein. Most animals received a single injection at ~0800 h. However, the baboons received two injections, each of 6 IU ACTHAR gel, the first at 0800 h and the second at 1400 h. The elephants and elk received a more highly concentrated, slow-release ACTH gel (500 IU/ml) into the deep muscle of the hindquarter.

Steroid and Creatinine Analyses from Urine

After centrifugation to remove particulates, steroids were extracted from 200 μl of clean baboon urine with 1 ml dichloromethane (see Brown *et al.*, 1995); the dichloromethane fraction was removed, dried under air, and resuspended in assay buffer. Samples were then analyzed with a ^3H radioimmunoassay using the CSU cortisol antibody (see below). Urinary cortisol concentrations were divided by creatinine concentrations to correct for variations in fluid intake (Tausky, 1954). Urinary creatinine was measured using a modified Jaffé reaction with a 20-min incubation and colorimetric analysis with an automated microplate reader (Tietz, 1976).

Extraction of Steroids from Feces

Fecal samples from all species except the Roosevelt elk were extracted using the method described in Wasser *et al.* (1994). Briefly, depending on the species

(Table 1), ~0.2 g fully lyophilized, well-mixed powdered feces or ~0.6 g well-mixed wet feces was weighed and boiled (20 min) in 5 ml 90% ethanol in water or 100% ethanol. Extracts were centrifuged (500g for 10 min), the supernatant was recovered, and the pellet was resuspended in 5 ml 90–100% ethanol (Table 1), vortexed (1 min), and recentrifuged. The combined supernatants were dried under air, redissolved in 1.00 ml methanol, and diluted (1:8–1:400, depending on species) in phosphate-buffered saline (PBS) (0.01 M PO_4 , 0.14 M NaCl, 0.5% bovine serum albumin 0.01 M NaN_3 , pH 7.4). This boiling method produces excellent steroid extraction recoveries without cleaving conjugates from the parent steroid (Wasser *et al.*, 1994).

More recently, we began using a vortexing (nonboiling) extraction method modified from Schwarzenberger *et al.* (1991). Briefly, ~0.2 g dry sifted feces or ~0.6 g well-mixed wet feces was placed in a capped tube containing 2.00 ml 90% methanol, vortexed (30 min) using a multitube, pulsing vortexer (Glas-Col, Terre Haute, IN; pulse rate 1/s, speed 70), and then centrifuged for 20 min at 500g. The supernatant was diluted in either PBS (for ^3H RIA) or ICN diluent (for ^{125}I RIA; ICN Biomedicals, Costa Mesa, CA) for assay.

To compare the two extraction methods, ^3H -labeled cortisol, progesterone, and testosterone were each added in triplicate to weighed aliquots of a macaque fecal pool and recovered radioactivity was assessed after extraction by each method. In a separate trial, we measured concentrations of immunoreactive macaque fecal metabolites of endogenous cortisol, progesterone, and testosterone after extraction in triplicate by each method. Immunoreactivity was assessed with the ICN corticosterone ^{125}I assay and with in-house testosterone and progesterone ^3H assays. The boiling and vortexing extractions produced similar recoveries of radioactive labeled steroids (~90–100%, depending on steroid and species) and of immunoreactive metabolites of endogenous steroids (e.g., average of 9.0 ± 0.5 ng/g of immunoreactive cortisol metabolites recovered with boiling method vs 9.2 ± 0.1 ng/g with vortexing method). This indicates that the two methods are comparable and further supports our conclusion that boiling in ethanol does not appreciably alter steroid immunoreactivity.

Tests of all extraction methods used in this paper, across species, produced consistent and high (>85%)

recoveries of 16,000 dpm [^3H]cortisol added to feces prior to extraction or of endogenously produced [^{14}C]cortisol fecal metabolites excreted in the sample following radiolabel infusion (data not shown; see also Table 2). The vortexing method generally recovers ~85–100% of most radiolabeled and endogenous fecal steroids in species such as grizzly bear, elk, and elephant (data not shown). The vortexing method was used on the Roosevelt elk samples in the present study and is now the preferred method in our laboratory due to time savings.

To ascertain the portion of hydrolyzable conjugates, extractants from the 26-h baboon fecal sample containing the peak radioactivity in animal 1 were first subjected to an ether:water extraction to determine the percentage of polar and nonpolar metabolites. The sample was dried and then rehydrated in 1 ml dH $_2\text{O}$ and 10 ml ether, capped, and vortexed for 1 min. After snap-freezing (10 min at -80°C), the ether portion was poured off into a separate tube; 0.5 ml of both the ether and the aqueous solutions were dried separately. The ether portion was rehydrated in 1 ml methanol and the aqueous solution in 1 ml PBS, with 50 μl of each being placed in scintillation vials containing 20 ml Ultima Gold and counted for radioactivity. Acid solvolysis was carried out to determine the portion of hydrolyzable conjugates in the remaining 0.5 ml of the aqueous solution. We added 1 ml PBS, 1 ml saturated NaCl, 1 ml 5 N H $_2\text{SO}_4$, and 7 ml ethyl acetate to the 0.5 ml aqueous solution, vortexed (1 min), and then incubated (37° for 48 h) the Parafilm-covered tube in a shaking water bath. The ethyl acetate fraction was removed and placed in a separate tube. An additional 6 ml ethyl acetate was added to the aqueous portion, vortexed, allowed to separate, removed, and pooled with the first ethyl acetate fraction. After drying the ethyl acetate, samples were again ether:water-extracted and then half of each fraction was counted for radioactivity, as described above. The remaining half was analyzed by HPLC as described below.

HPLC

The [^{14}C]cortisol metabolites, extracted from baboon feces as described above, were analyzed by HPLC. Extractants were first cleaned by passing them through a 0.2- μm filter followed by a C-18 matrix column ("Spice cartridge," Varian Instruments, Wal-

nut Creek, CA) and eluted with 5 ml of 80% methanol as described by Shackleton (1986). Fecal samples from two different time points were analyzed separately for each baboon: (1) the sample with the first detectable radioactivity, ~6 h postinfusion, and (2) the sample that contained peak concentrations of radioactivity, ~26 h postinfusion. Fecal steroids were separated using a reverse-phase C-18 column (Varian Instruments) with a gradient solvent system (1 ml/min) as follows: 20–30% methanol (0–10 min), 30–40% (10–40 min), 40–50% (40–55 min), 50–80% (55–80 min), 80–100% (80–85 min), and 100% (85–120 min). All eluates were assessed for radioactivity and immunoreactivity.

Radioimmunoassay

The three cortisol antibodies used to examine fecal GC metabolites in the yellow baboon and longtailed macaque were: Pantex 031 (Pantex, Santa Monica, CA), Incstar CA-1529 (Incstar Corp., Stillwater, MN), and CSU R1222 (G. D. Niswender lab, Colorado State University, Fort Collins, CO). A corticosterone antibody was also tested (07-120102; ICN Biomedicals Inc., Costa Mesa, CA). The latter two antibodies (see below) were used to analyze ACTH challenge samples from African elephant, Roosevelt elk, and sea otter, whereas only the ICN antibody was used to analyze ACTH challenge samples from Malayan sun bear, gerenuk, scimitar-horned oryx, black rhinoceros, cheetah, clouded leopard, and northern spotted owl. Urine samples from the baboon ACTH challenge were assayed with the CSU antibody.

All four antibodies were raised in rabbits. The Incstar, Pantex, and ICN antibodies were used in ^{125}I RIA kits, using the manufacturers' assay protocols. The CSU antibody was used in a ^3H RIA with an overnight incubation and separation with dextran-coated charcoal. The manufacturers' reported cross-reactivities, coefficients of variation, and assay sensitivities are shown in Table 2.

Ultimately, all fecal samples were assayed using the ICN [^{125}I]corticosterone antibody. All antisera exhibited both parallelism and accuracy for feces of all species on which they were tested (data not shown). For all species, inter- and intraassay variations were similar to the manufacturer's reported values (generally <7%).

TABLE 2

Source and Cross-Reactivity Data for the Four Antibodies Used in This Study, with Assay Sensitivities and Variations

	Manufacturer, Catalog No., antigen			
	Pantex 031 cortisol	Incstar CA-1529 cortisol	CSU-Niswender R1222 cortisol	ICN 07-120102 corticosterone
Crossreactivities				
Cortisol	100%	100%	100%	<1%
Corticosterone	35%	<1%	<0.03%	100%
11-Desoxycortisol	17.5%	6.3%	NR	<0.01%
Desoxycorticosterone	NR	<1%	<0.01%	<1%
Cortisone	<0.01%	<0.1%	<0.05%	NR
Prednisolone	58%	77%	NR	NR
Progesterone	2.9%	<0.1%	NR	<1%
Prednisone	1.2%	<1%	NR	NR
Assay specifications				
Assay type	¹²⁵ I RIA	¹²⁵ I RIA	³ H RIA	¹²⁵ I RIA
Intraassay variation	10.4%	7.0%	4.5%	7.2%
Interassay variation	11.3%	9.2%	3.0%	6.9%
Assay sensitivity	0.2 ng	0.21 ng	0.1 ng	0.2 ng

Note. Additional cross-reactivity data: Pantex 031: 30% for 21-desoxycortisol; less than 0.01% with androstenedione, androsterone, cholesterol, DHEA, dexamethasone, dihydrotestosterone, α - and β -estradiol, estriol, estrone, 17 α -hydroxypregnenolone, pregnenolone, and testosterone. Incstar CA-1529: 43% for 6-methylprednisolone; 1.2% for 17-hydroxyprogesterone; less than 1% for 17-hydroxyprogesterone, dexamethasone, tetrahydrocortisone; less than 0.1% for aldosterone, β -cortol, β -cortolone, dihydrocortisone, spironolactone, tetrahydrocortisol, and 6 β -hydrocortisone. ICN 07-120102: less than 1% for testosterone, aldosterone, androstenedione, and 5 α -dihydrotestosterone; less than 0.01% for cholesterol, DHEA, DHEA-sulfate, 20 α -dihydroprogesterone, estrone, 17- α and - β estradiol, estriol, pregnenolone, 17 α -hydroxypregnenolone, 17 α -hydroxyprogesterone. All data provided by manufacturers except assay variations for ³H assay (CSU antibody). Antigen, antigen used to raise antibody in rabbit; NR, manufacturer did not report cross-reactivity data for that steroid. For steroids tested with only one antibody, cross-reactivity data are shown at bottom.

RESULTS

Yellow Baboon Radiolabel Infusion and ACTH Challenge

Serial dilutions of baboon fecal extracts yielded displacement curves parallel to standard hormone (cortisol or corticosterone) preparations using all four antibodies (data not shown). All three cortisol antibodies exhibited comparable relative affinities for each of the radioactive cortisol metabolites. Temporal immunoreactive excretion profiles after ACTH administration were also qualitatively similar across the three cortisol antibodies. However, only the CSU antibody was chosen for subsequent comparisons to the ICN corticosterone antibody because it exhibited the highest cross-reactivities of the three cortisol antibodies with each of the radioactive cortisol metabolites detected in baboons (and longtailed macaques).

Sixty (animal 2) to seventy percent (animal 1) of the [¹⁴C]cortisol metabolites were recovered, with 13% (animal 2) to 15% (animal 1) in feces and 85–87% in

urine. Excretion of [¹⁴C]cortisol metabolites were detected in the first urine sample, collected ~1 h postinfusion. Animal 1 voided one third the amount of urine (3274 ml) as did animal 2 (10,856 ml) over the 78-h study period at a substantially and consistently slower rate. Animal 1 also voided 20% less radioactivity in her urine (71.6 million dpm) than did animal 2 (89.3 million dpm). However, peak excretion in animal 1 was 713,459 dpm/ml at 7.6 h postinfusion, whereas peak excretion in animal 2 was 201,977 dpm/ml and occurred at 4 h postinfusion. Animal 1 shed 37% of all her urinary ¹⁴C metabolites within 10 h postinfusion, compared to 58% in animal 2 over that same time period. Excretion of [¹⁴C]cortisol metabolites in feces were first apparent within 6–8 h postinfusion. Peak excretion in feces occurred at ~26 h postinfusion and continued for an additional 24 h (baboon 1, Fig. 1A) to 48 h (baboon 2, Fig. 1B) postinfusion. Animal 1 voided somewhat more fecal mass (706 g) than did animal 2 (562 g), but at comparable rates, over the 78-h sampling period. Animals 1 and 2 also shed comparable amounts of radioactivity in their feces over the 78-h

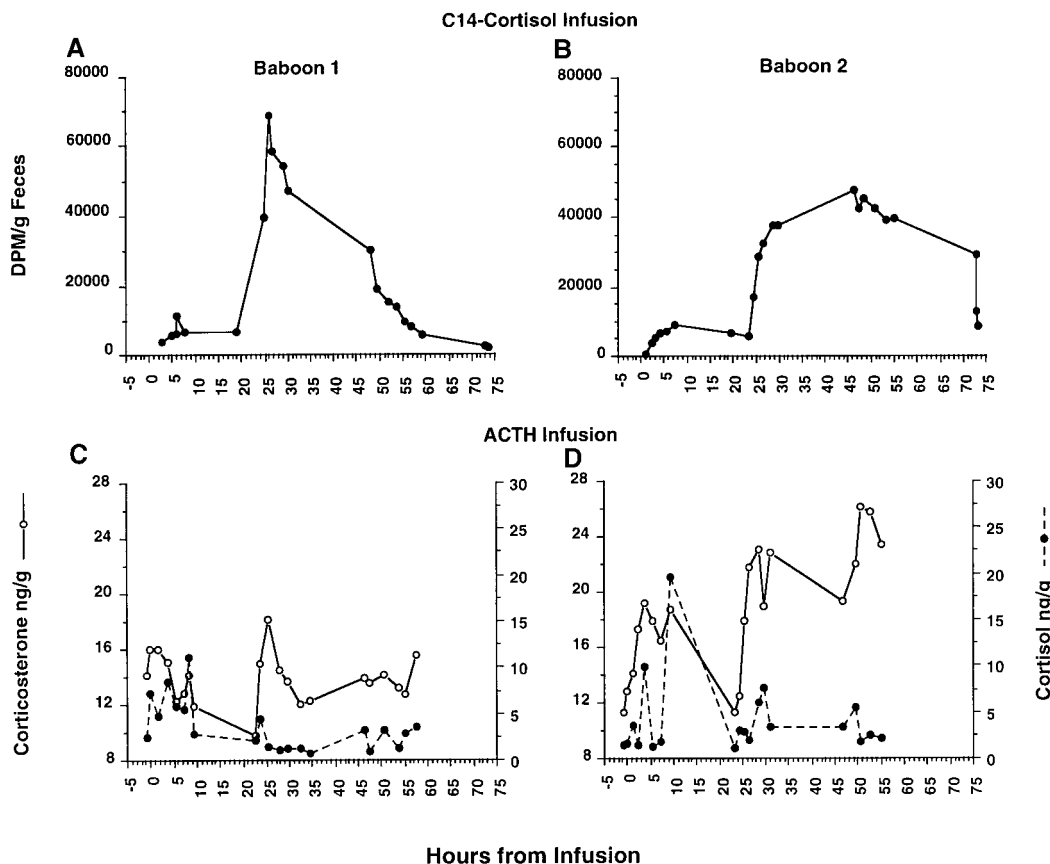


FIG. 1. Results of [^{14}C]cortisol infusion and ACTH challenge in two yellow baboons, baboon 1 (A, C) and baboon 2 (B, D). (A, B) Excretion rate of radioactivity in feces after [^{14}C]cortisol infusion. (C, D) Steroid extracts of feces collected after ACTH challenge were tested for immunoreactivity with CSU cortisol antibody (solid circles) and ICN corticosterone antibody (open circles).

period (13.0 million dpm vs 12.9 million dpm, respectively).

An ether:water extraction followed by acid solvolysis to determine the percentage of hydrolyzable conjugates was conducted on the fecal sample containing the radioactive excretion peak (26 h). Fifty-seven percent of the ^{14}C metabolites in the peak (26 h) fecal sample were highly polar and recovered in the water portion following ether:water extraction. However, less than 20% of these were hydrolyzable by acid solvolysis. These results were also confirmed by comparison of HPLC analyses before and after each extraction.

HPLC analyses also revealed that different fecal metabolites of [^{14}C]cortisol were present in baboon feces at excretion onset (6 h) compared to those present at peak excretion (\sim 26 h postinfusion) in both animals. Analysis of the 6-h postinfusion sample

(when ^{14}C metabolite excretion was first detected) revealed one predominant radioactive peak (fractions 78–82; Fig. 2, closed circles). In contrast, the peak excretion fecal sample (26 h postinfusion) included at least six distinct radioactive metabolites over a wide range of polarities, with the largest peak falling between fractions 72 and 82 (Fig. 3, closed circles). Most metabolites in the 26-h sample (Fig. 3) were generally more polar than the predominant ^{14}C metabolites in the 6-h sample (Fig. 2). None of the major 6-h or 26-h ^{14}C metabolites eluted with cortisol (fraction 67). However, some ^{14}C metabolites in both the 6-h and the 26-h samples did coelute with corticosterone (fraction 75).

HPLC-separated fractions from early (6 h) and peak (26 h) ^{14}C metabolite excretion samples were tested for immunoreactivity with the ICN and CSU antibodies. Both the CSU cortisol and the ICN corticosterone antibodies cross-reacted with relatively few of the

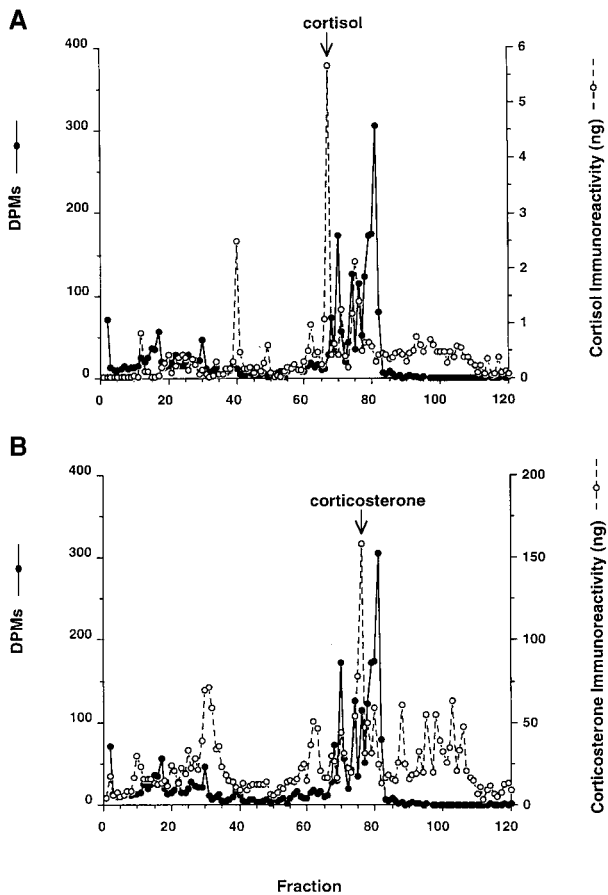


FIG. 2. Results of HPLC analyses of steroid extract from baboon feces excreted 6 h after [^{14}C]cortisol infusion (baboon 1), the time of the first noticeable increase in radioactivity. (A) ^{14}C dpm content of HPLC fractions (solid circles) compared with immunoreactivity with CSU cortisol antibody (open circles). (B) ^{14}C dpm content of HPLC fractions (solid circles) compared with immunoreactivity with ICN corticosterone antibody (open circles). The fractions in which cortisol and corticosterone elute are demarcated with an arrow in Figs. A and B, respectively.

[^{14}C]cortisol metabolites in the early excretion samples (Figs. 2A and 2B, respectively). The CSU antibody appeared to detect a relatively sharp immunoreactive peak in the 6-h sample, which corresponded with cortisol (fraction 67). However, this probably has minimal biological significance since the scale of the immunoreactive cortisol axis is small (0–6 ng, Fig. 2A, compared to 0–200 ng for corticosterone, Fig. 2B) and there is virtually no corresponding peak in radioactive metabolites in this fraction of the 6-h sample. Similarly, the ICN antibody detected a sharp immunore-

active peak in the 6-h sample that corresponded with corticosterone (fraction 75).

In the peak excretion samples (26 h), the CSU and ICN antibodies both detected an immunoreactive peak around fraction 75, consistent with a major radioactive peak that corresponded with corticosterone (Figs. 3A and 3B, respectively). However, again, the immunoreactivity of the CSU antibody was small (<3 ng) in comparison to that of the ICN antibody (100 ng; Fig. 3B) and comprised only a narrow portion (fraction 75) of the relatively broad radioactive peak in the 26-h sample. In contrast, the ICN antibody cross-reacted with virtually the entire broad radioactive peak from fractions 75–80 (Fig. 3B). The ICN antibody also cross-

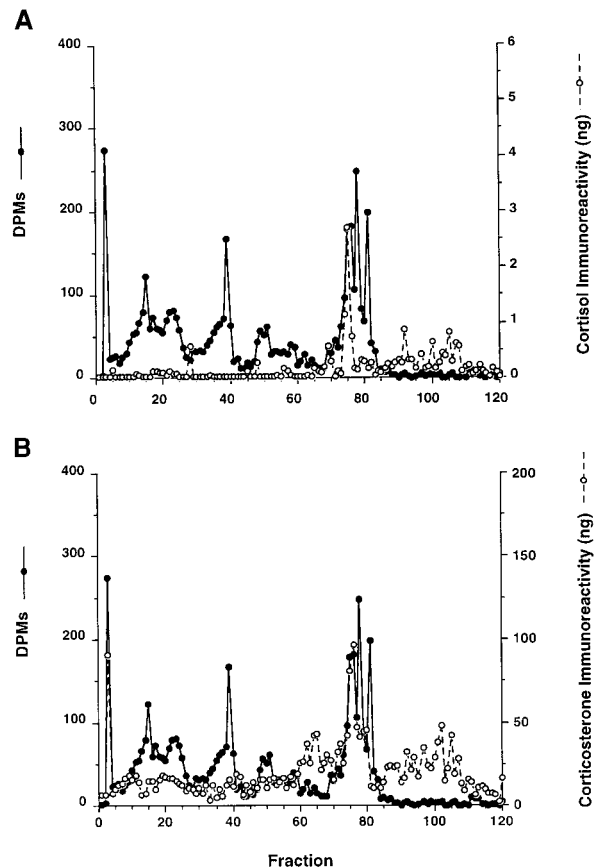


FIG. 3. Results of HPLC analyses of steroid extract from baboon feces excreted 26 h after [^{14}C]cortisol infusion, the time of peak excretion of radioactivity. (A) ^{14}C dpm content of HPLC fractions (solid circles) compared with immunoreactivity with CSU cortisol antibody (open circles). (B) ^{14}C dpm content of HPLC fractions (solid circles) compared with immunoreactivity with ICN corticosterone antibody (open circles).

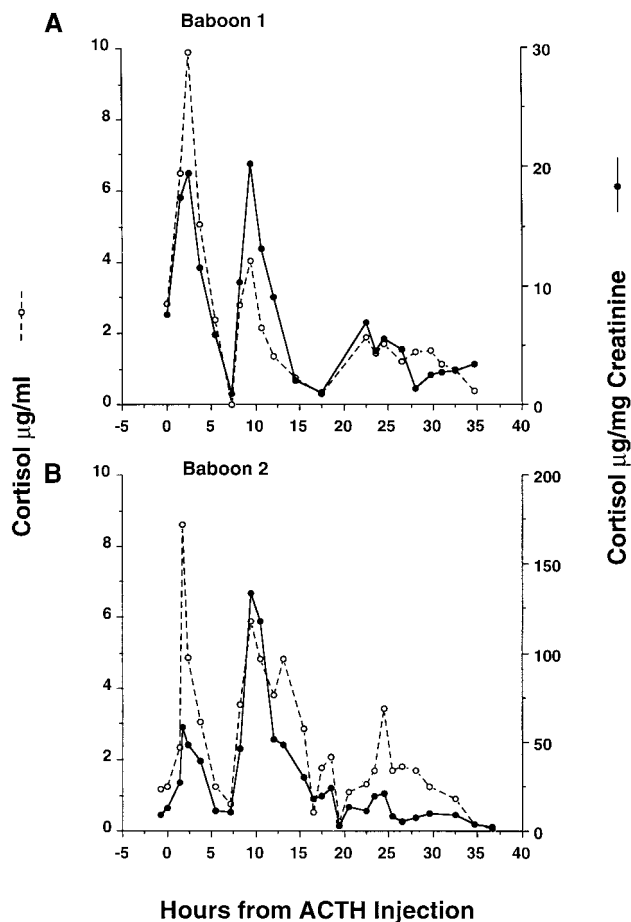


FIG. 4. Urinary cortisol levels after ACTH challenge, expressed as $\mu\text{g}/\text{ml}$ urine (open circles) and $\mu\text{g}/\text{mg}$ creatinine (solid circles). Urine samples were assayed using the CSU cortisol antibody following dichloromethane extraction.

reacted considerably with the sharp, highly polar radioactive peak at fraction 3 (Fig. 3B).

Differences in immunoreactivities to fecal GC metabolites in the ICN and CSU antibodies were even more marked in response to the ACTH challenge. Specifically, both antibodies showed similar reactivities to fecal GCs during the first 20 h post-ACTH challenge, with brief, moderate-sized peaks at 4 and 10 h that declined thereafter (Figs. 1C and 1D). The timing of these peaks also paralleled those of urinary cortisol at 2 h (animal 2) to 3 h (animal 1) and at 10 h, measured using the CSU antibody after dichloromethane extraction (Figs. 4A and 4B). Moreover, the mean creatinine levels for animal 2 (0.04 mg/ml) were consistently 10-fold lower than those for animal 1 (0.4 mg/ml), reflecting once again that animal 2 voided

substantially more urine volume over the study period than did animal 1. Correcting for creatinine levels accordingly resulted in a dramatic increase in the measures of the amount of cortisol shed in urine by animal 2 versus animal 1.

Only the ICN antibody showed the predicted marked and extended fecal GC excretion profile at ~ 26 h post-ACTH challenge (Figs. 1C and 1D). These 26-h peaks were temporally consistent with the excretion lag time observed after [^{14}C]cortisol administration in the same animal (26 h; compare Fig. 1A with 1C and 1B with 1D). Excretion of ICN-immunoreactive metabolites continued to parallel the respective radiolabel excretion profiles for an additional 24 h (Fig. 1C) to 48 h (Fig. 1D) after the 26-h excretion peak in both animals. It is also noteworthy that baboon 1 started with a relatively high level of immunoreactive fecal and urinary GCs around time 0, presumably a result of stress associated with moving the baboons into a new cage just prior to the procedure.

ACTH Challenges in Other Mammalian Species

The ICN and CSU antibodies were also compared after ACTH challenge in the African elephant, Roosevelt elk, and sea otter (Fig. 5) and in the longtailed macaque (Fig. 6). The ICN corticosterone antibody was superior to the CSU cortisol antibody for detecting immunoreactive fecal GC metabolites after ACTH administration in the elephant and elk. Peak immunoreactive metabolite excretion in the African elephant (as measured using the ICN antibody) occurred at 36 h (Fig. 5A), consistent with the ~ 30 -h excretion lag time for radiolabeled steroids in this species (Wasser *et al.*, 1996). The CSU antibody was essentially ineffective for elephant feces. In elk, the two antibodies produced similar temporal profiles, but the ICN corticosterone antibody provided improved resolution and detected greater concentrations of immunoreactive GC metabolites (Fig. 5B). Peak excretion time in elk was 22 h for both antibodies, consistent with gut passage time in this species (Warner, 1981). In the Alaskan sea otter, peak excretion time of immunoreactive steroids was 6 h for both antibodies (Fig. 5C), and whereas the temporal patterns of fecal GC excretion were similar for both antibodies, the CSU antibody detected greater concentrations of GC metabolites.

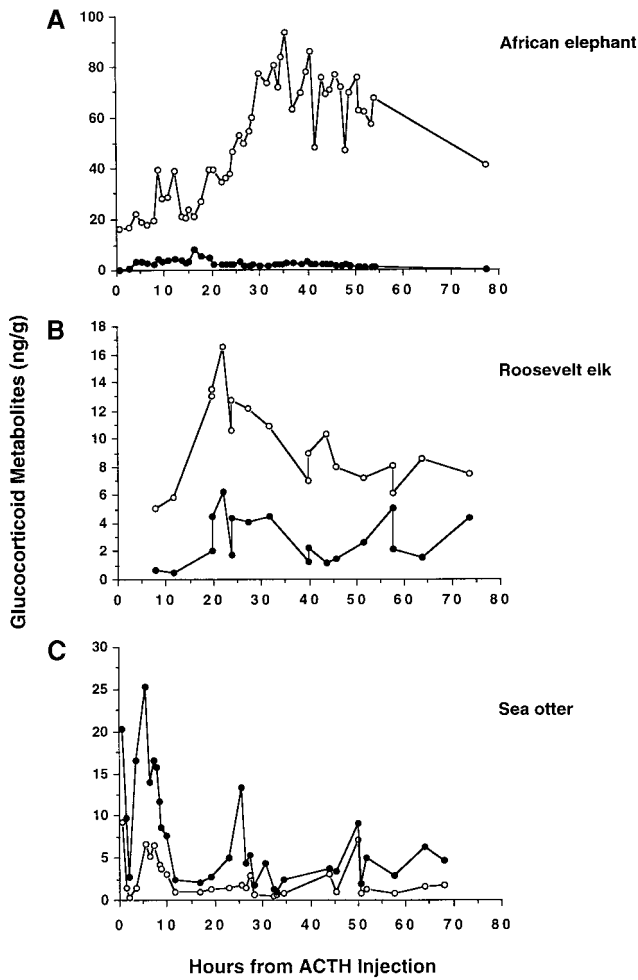


FIG. 5. Immunoreactive glucocorticoid metabolites (ng/g mixed feces) of fecal samples collected after ACTH challenges in (A) African elephant, (B) Roosevelt elk, and (C) Alaskan sea otter. For elk and elephants, in which multiple animals were studied, data shown are from one representative individual. Fecal extracts were assayed with the ICN 07-120102 corticosterone antibody (open circles) and the CSU R1222 cortisol antibody (closed circles) and expressed as ng/g feces.

In longtailed macaques, the ICN corticosterone antibody generally measured higher concentrations of GC metabolites than did the CSU antibody (Figs. 6A–6E). However, excretion profiles were highly variable from individual to individual for both antibodies. Five of 10 macaques showed peaks in fecal GC excretion between 26 and 32 h post-ACTH infusion using the ICN antibody (Figs. 6A–6E), coinciding with the 26-h peak GC excretion time in feces measured by Bahr *et al.* (2000). The remaining 4 females showed either no peak (e.g., Fig. 6F) or a small to moderate peak in fecal

GCs at ~5 h post-ACTH infusion using the ICN antibody (data not shown). When a moderate peak was shown at ~5 h, it was generally sustained or slowly declined until 25 h post-ACTH infusion (data not shown). Fecal GC peaks were also detected in the macaques using the CSU antibody, but their timing relative to the ACTH infusion and amplitude tended to be much more variable between individuals (e.g., Figs. 6A–6F). Two to three of the early fecal peaks measured by the ICN or the CSU antibodies roughly corresponded with the time of the urinary cortisol peak (Figs. 6A–6F). Overall, the GC profiles after ACTH challenge in longtailed macaques were not as distinct as those in the other species tested.

The ICN corticosterone antibody was also used to quantify ACTH challenges in Malayan sun bear, ger-

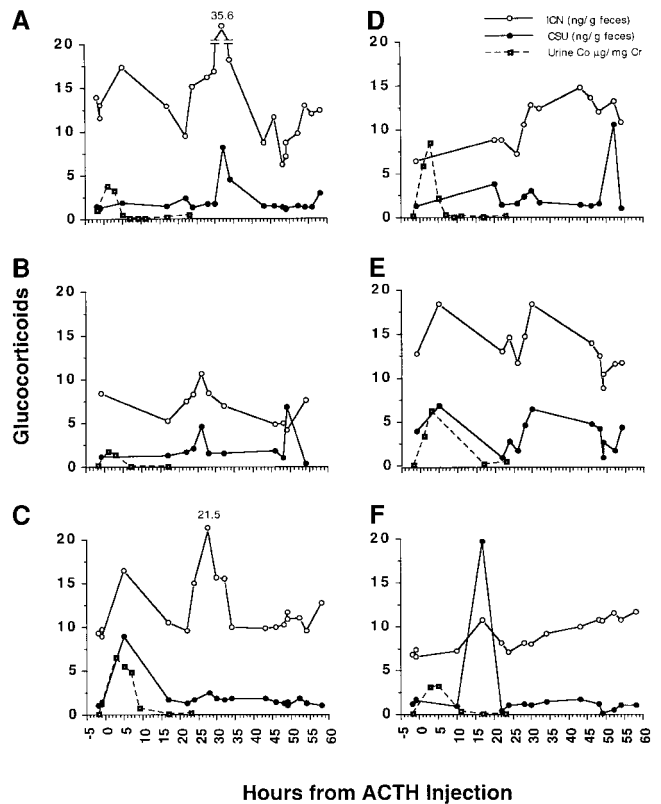


FIG. 6. Immunoreactive glucocorticoid metabolites in urine and feces of six (A–F) longtailed macaques, collected after ACTH challenges. Fecal extracts were assayed with the ICN 07-120102 corticosterone antibody (open circles) and the CSU R1222 cortisol antibody (closed circles) and expressed as ng/g feces. Urinary extracts were assayed with the CSU R1222 cortisol antibody following dichloromethane extraction and expressed as µg cortisol/mg creatinine (closed squares).

enuk, scimitar-horned oryx, black rhinoceros, cheetah, and clouded leopard (Fig. 7). This antibody revealed a clear peak in fecal GC metabolite excretion after ACTH challenge in all species, and the timing of the peak was consistent with known or estimated gut passage time in the respective species (e.g., Warner, 1981; Graham and Brown, 1997; Palme *et al.*, 1997; Heistermann *et al.*, 1998). The Malayan sun bear had substantially higher concentrations of immunoreactive GC's/g feces than other species, both pre- and post-ACTH (note y axes of Fig. 7).

Radiolabel Infusions and ACTH Challenge in Spotted, Barred, and Great Horned Owls

Excretion of [^3H]corticosterone in the two surrogate owl species (barred and great horned) revealed two peaks, one at 2 h and a second at around 12 h postinfusion. However, the 12-h peak was over twofold greater in the great horned owl than in the barred owl (Fig. 8). After ACTH infusion in the spotted owl, the ICN corticosterone antibody revealed a rapid and significant increase within 2 h postinfusion that sharply increased an additional threefold at 12 h postinfusion (Fig. 8). Thus, the ICN antibody produced results consistent with excretion lag times in related owl species.

DISCUSSION

The studies described in this paper suggest that the ICN corticosterone antibody reliably detects adrenal activity in feces of a wide range of mammalian and avian species.

Yellow Baboon

Sixty to seventy percent of the infused [^{14}C]cortisol was recovered in animals 1 and 2. Thirteen to fifteen percent of the recovered [^{14}C]cortisol metabolites was found in feces compared to 85–87% in urine. Peak excretion of [^{14}C]cortisol metabolites in feces occurred at ~26 h following the radiolabel infusion in both animal 1 and animal 2. However, animals 1 and 2 differed markedly in the temporal excretion patterns. Specifically, excretion of the [^{14}C]cortisol metabolites was more sustained at peak levels in animal 2 vs

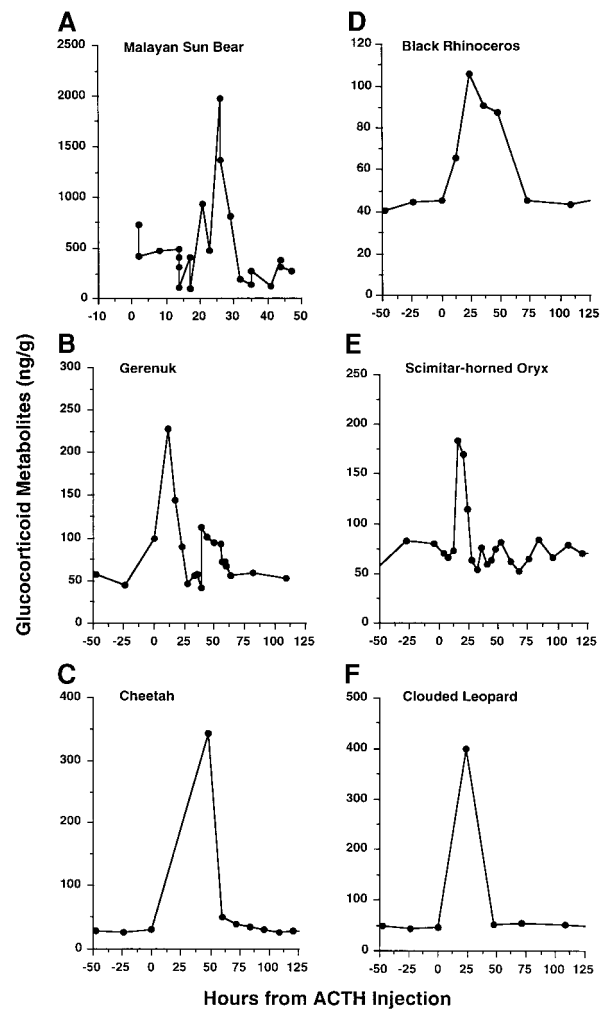


FIG. 7. Immunoreactive glucocorticoid metabolites (ng/g mixed feces), as measured by the ICN 07-120102 corticosterone antibody, of fecal samples collected after ACTH challenge in Malayan sun bear, black rhinoceros, gerenuk, scimitar-horned oryx, cheetah, and clouded leopard.

animal 1. These same individual-specific patterns of cortisol metabolite excretion in feces were observed in response to ACTH challenges in animals 1 and 2. Whereas more data are needed, comparison between urinary and fecal metabolite excretion profiles offers some preliminary explanation for these individual differences.

From the outset of the [^{14}C]cortisol infusion and ACTH challenge experiments, animal 1 voided substantially less urine volume and GC metabolites in her urine, all at a slower rate, than animal 2. Differences in voiding these metabolites were especially dramatic in

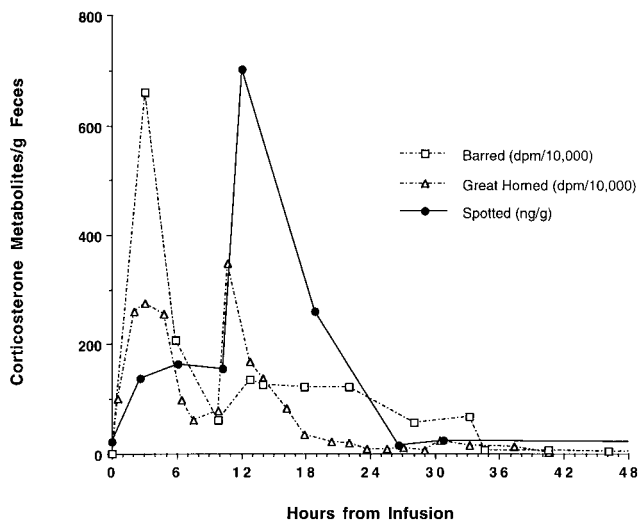


FIG. 8. Corticosterone metabolite excretion in feces following [^3H]corticosterone infusion in the barred and great horned owls and ACTH challenge in the northern spotted owl.

the first 10–13 h postinfusion, whereas urinary excretion of GC metabolites was minimal beyond 20 h postinfusion in both animal 1 and animal 2. It is noteworthy that animal 1 also voided considerably more fecal mass during the study period (probably in part because urine loss made animal 2 relatively dehydrated compared to animal 1). We, accordingly, suspect that this greater urinary water loss in animal 2 was associated with slower enterohepatic recycling and a more sustained excretion of GC metabolites at peak levels in feces of animal 2 after the initial 26-h peak in both the radiolabel infusion and the ACTH challenge studies.

Little native cortisol was excreted in baboon feces, most appearing as metabolites whose relative abundance differed depending on time of excretion relative to infusion. Less than 20% of the [^{14}C]cortisol metabolites found in feces during peak excretion (26 h) consisted of hydrolyzable conjugates.

All three cortisol antibodies tested exhibited some cross-reactivity for one or more of the radiolabeled cortisol metabolite(s) present at the onset (6 h) and peak (26 h) stages of fecal excretion. The ICN corticosterone antibody also exhibited some affinities for the radiolabeled cortisol metabolites present at the early stages of excretion. In addition, the ICN antibody uniquely exhibited relatively high affinity for the predominant cortisol metabolite(s) present at the time of

peak excretion after radiolabel infusion. Similar results were found in response to the ACTH challenge: the three cortisol antibodies detected a small elevation at 6 h, well before peak ACTH-stimulated excretion was expected, but little immunoreactivity thereafter. In contrast, the ICN antibody consistently detected a substantial immunoreactive peak at 26 h, the time when peak ACTH-stimulated excretion was expected based on the excretion lag time. These combined results demonstrate that the predominant GC metabolites present in feces vary depending on the time since their secretion into the bloodstream. It is, accordingly, important to use an antibody that has the greatest affinity for the major metabolite(s) present during peak excretion. The ICN corticosterone antibody best fulfilled these criteria in the present study.

There are a few aspects of the baboon study that deserve comment. First, the CSU antibody appears to have detected an immunoreactive peak coeluting with cortisol in the early (6-h) excretion sample, despite there being little corresponding radioactivity in this fraction (Fig. 2A). However, this was not very pronounced compared to that of the ICN antibody, given the nearly 50-fold greater scale of the latter (Figs. 2A and 2B). Second, at a calculated dose of 3.2 pg/dpm, there appeared to be more immunoreactive metabolites detected by the ICN antibody than by radioactive counting (Fig. 3). This suggests that the β -methasone suppression may have been incomplete. Thus, these samples probably still contained endogenous cortisol metabolites at later stages of metabolism, some of which may have been secreted prior to the radiolabel infusion. Third, the cortisol metabolite concentrations were relatively high at time 0 of the ACTH challenge in at least one of the baboons (Fig. 1C). We suspect this to be due to stress associated with the anticipation of the treatment; the baboons were placed in new cages 2 days prior to ACTH administration to allow better separation of urine from feces. Finally, dividing the ACTH infusion into two half doses, separated by 6 h, may have reduced the magnitude of the 26-h peak.

Other Species

Overall, the ICN antibody detected clear elevations of GC metabolites after ACTH administration in 11 of 12 species examined in this study—the exception be-

ing the longtailed macaque. With the exception of the sea otter, the ICN corticosterone antibody consistently exhibited higher cross-reactivity with the major fecal GC metabolites across species than did the other antibodies tested in this study. The ICN antibody, accordingly, proved superior to these other antibodies for documenting ACTH-induced adrenal activation in feces. Detection of the GC metabolite peak reflecting adrenal activation across species was also consistent with the respective excretion lag times in those species for which lag time data were available. The ICN corticosterone antibody additionally outperformed other antibodies in fecal-based ACTH challenge studies of domestic cats (e.g., Graham and Brown, 1996) and spotted hyenas (Goymann *et al.*, 1999). This same ICN antibody has also been shown to be effective for detecting fecal GC elevations in response to environmental stress in African wild dogs (*Lycaon pictus*; Creel *et al.*, 1997), northern spotted owls (Wasser *et al.*, 1997), Rocky Mountain elk (Millsbaugh, 1999), African elephant (Foley *et al.*, 2000), spotted hyenas (Goymann *et al.*, 1999), pronghorns (*Antilocapra americana*; J. M. Harper, pers. comm.), and four species of mice and voles (Harper and Austad, 2000).

Comparative Aspects of GC Metabolism in Feces and Choice of Antibody

It is notable that one antibody can perform particularly well across a wide range of species, since fecal GC metabolites appear to vary widely among species. For example, among primates the parent hormone cortisol is present in feces of the common marmoset (*Callithrix jacchus*), but is negligible in feces of the baboon (this study), longtailed macaque, and common chimpanzee (Bahr *et al.*, 2000). Chimpanzees (*Pan troglodytes*) and humans (*Homo sapiens*) predominantly metabolize cortisol to tetra-hydroxylated steroids and, to a lesser extent, cortolic and cortolonic acids (Lowy *et al.*, 1969; Setchell and Shackleton, 1973). The baboon exhibits 20 β -reduction and side-chain cleavage, producing two major C-21 metabolites (Setchell *et al.*, 1975). In contrast, longtailed macaques preferentially exhibit 20 β -reduction and efficient side-chain cleavage, producing 11,17-dioxoandrostanes along with some unusual metabolites not observed in the human, chimpanzee, or baboon (Setchell and Shackleton, 1973). The unusual metabolites in the macaque may be

the cause of the poor ACTH profiles produced with the ICN and CSU antibodies in this species. [Macaques are also known to produce atypical metabolites of progesterone in feces (Shideler *et al.*, 1993a, b)]. E. Möstl, R. Palme and colleagues have developed an 11,17-oxoetiocholanolone assay for fecal GCs that is group specific for 11,17-dioxoandrostanes (11,17-DOAs) and works well in a variety of ruminants, as well as in the domestic cat, hare, horse and pig (e.g., Palme *et al.*, 1998, 1999). Bahr *et al.* (2000) also showed that the 11,17-DOA antibody works well for longtailed macaques.

GC metabolism in the liver is poorly studied in other mammals, but may include oxidation at C-11, oxidation or reduction at C-20 and C-21, which may result in 20-oxo-21-oic acids, reduction of the A-ring, and side-chain cleavage to form a keto group at C-17 (e.g., rabbit, *Oryctolagus cuniculus*, Senciall *et al.*, 1992; rat, *Rattus norvegicus*, Lowy *et al.*, 1969; mouse, *Mus musculus*, Marandici and Monder, 1985; Han *et al.*, 1983; guinea pig, *Cavia aperea*, Quinkler *et al.*, 1997; sheep, *Ovis aries*, Palme and Möstl, 1997). These examples show that it is not possible to predict in advance which fecal metabolites may predominate in any one species, and development of specific antibodies for the excreted metabolites of each species may be impractical. Instead, as Palme *et al.* (1998) have suggested, it is more feasible to develop group-specific antibodies that recognize a family of metabolites rather than a specific steroid. From a practical perspective, it is not necessary to know the chemical identity of the fecal metabolites in each species. Rather, it is more important to demonstrate that an antibody can track fluctuations in metabolites that provide biologically relevant information regarding adrenal status. ACTH challenge studies are ideally suited to this task.

The ICN corticosterone antibody probably acts as a group-specific antibody, with cross-reactivities to multiple GC metabolites excreted in feces of a variety of species in response to adrenal activation. However, at present it is not known what group(s) this antibody recognizes. The ICN antibody has a minimal cross-reactivity with cortisol (hydroxylated at C-17), yet works well for detecting fecal metabolites of cortisol in numerous species. This suggests that some cortisol metabolites may be dehydroxylated during metabolism to a form that increases their relative affinity for the ICN antibody. Given the antibody's reported

cross-reactivities (see Table 2), it is probably particularly sensitive to changes at sites C-11 and C-21.

Overall, the results of the ACTH challenges presented here show that fecal GC assays using the ICN corticosterone antibody can effectively detect changes in endogenous adrenal activity in a wide range of mammalian and avian species, including proboscideans, three classes of carnivores (mustelid, bear, and felid), two classes of ruminants (cervid and bovids), a perissodactyl (rhinoceros), some Old World primates, and owls. (The ICN corticosterone antibody is also available in an EIA developed by E. Möstl and R. Palme.) However, it is important to note that other corticosterone-derived antibodies may not necessarily be equally effective; the cross-reactivities of an antibody to steroids or metabolites other than the steroid used to derive the antibody are often unique to each antibody (e.g., Wasser *et al.*, 1994). For example, in the spotted hyena, a different corticosterone antibody revealed a less distinct ACTH challenge profile than did the ICN corticosterone antibody (Goymann *et al.*, 1999).

Fecal GC assays have been found to have predictive and explanatory value, consistently showing elevations in adrenal activity after times of presumed physiological or psychosocial stress, such as translocation to unfamiliar environments (domestic cattle, C. J. Morrow *et al.*, unpublished data; Palme *et al.*, 2000; scimitar-horned oryx, Morrow, 1999; cheetah, Terio and Brown, 2000; spotted hyena, Goymann *et al.*, 1999), drought or social stress (African elephant, Foley *et al.*, 2000), recovery from anesthesia (common chimpanzee, Whitten *et al.*, 1998; scimitar-horned oryx, Morrow, 1999), environmental degradation (spotted owl, Wasser *et al.*, 1997), aggressive interactions (spotted hyena, Goymann *et al.*, 1999), human activity (elk, Millspaugh, 1999), and other social stressors (greylag geese, Kotrschal *et al.*, 1998; African wild dog, Monfort *et al.*, 1998). These patterns indicate that elevations in fecal glucocorticoids may reasonably be regarded as indicative of the physiological stress response and hence useful for a wide variety of conservation, management, and biomedical investigations, all conducted in a noninvasive manner. Nevertheless, whatever GC antibody one chooses, our study and others (e.g., Goymann *et al.*, 1999; Wallner *et al.*, 1999; Bahr *et al.*, 2000) clearly reinforce the necessity of conducting well-de-

signed species-specific validations before they are utilized to monitor biological responses.

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