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EVALUATING ADRENAL ACTIVITY IN AFRICAN WILD DOGS (*LYCAON PICTUS*) BY FECAL CORTICOSTEROID ANALYSIS

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Abstract: A noninvasive corticosteroid hormone monitoring technique was validated for use in African wild dogs (*Lycaon pictus*). The double-antibody ^{125}I radioimmunoassay for corticosterone was validated by demonstrating parallelism between serial dilutions of wild dog fecal extracts and the standard curve, recovery of corticosterone added to fecal extracts, and the time course of fecal corticoid excretion after an exogenous adrenocorticotropic hormone (ACTH) challenge. All feces were collected from three female and two male African wild dogs for 72 hr before and 144 hr after i.m. injection of long-acting ACTH (Acthar Gel, 400 IU). Fecal corticosterone immunoreactivity increased 10–30-fold within 24 hr of ACTH administration in all individuals, with peak concentrations from 1,200–8,000 ng/g. High-pressure liquid chromatography analysis revealed that >90% of all corticosterone immunoreactivity was associated with a single peak that exhibited intermediate polarity relative to cortisol and corticosterone reference tracers. Fecal corticosterone immunoreactivity appears to reflect adrenal activity in the African wild dog and, therefore, may be useful for evaluating stress. From a conservation perspective, these techniques can complement in situ and ex situ research studies designed to evaluate how environmental conditions and management strategies affect overall animal health.

Key words: Lycaon pictus, fecal corticosteroids, ACTH, adrenal gland, stress, hormones.

INTRODUCTION

The African wild dog (*Lycaon pictus*) is one of the most endangered canids, with stable populations exceeding 100 individuals in only seven countries.[15–17] It is estimated that 3,000–5,000 adults remain in the wild, but current rates of decline suggest that the species may become extinct within the next 20–40 yr.[15] African wild dogs live, hunt, and reproduce in permanent, obligately cooperative groups in sub-Saharan Africa.[6,8,17] Although only the socially dominant individual of each sex within a group normally reproduces, the mechanisms underlying reproductive suppression are poorly understood.

Because most wildlife species are intractable and highly sensitive to restraint and anesthesia, the conventional approach for assessing endocrine function via blood sampling usually is impractical. Alternative, noninvasive reproductive endocrine monitoring methods already have been useful for examining the mechanisms whereby reproductive hormones modulate reproduction in free-living social carnivores (dwarf mongoose,[10–12] African wild dog[7–9]). As part of an investigation of the modulating effects of stress on reproduction and animal health in African wild dogs, the present study provides detailed extraction and radioimmunoassay (RIA) procedures for assessing fecal corticosteroid metabolites and confirms the physiologic validity of noninvasive corticosteroid hormone monitoring by evaluating adrenal responsiveness to exogenously administered adrenocorticotropin hormone (ACTH).

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MATERIALS AND METHODS

Animals and ACTH challenge

Feces were collected from three adult female and two adult male African wild dogs housed at the Brookfield Zoo (Brookfield, Illinois; 41°N latitude) in indoor–outdoor enclosures and exposed to natural fluctuations in photoperiod. Two 2-yr-old sibling females (females 1 and 2) were housed together, whereas a 3-yr-old female (female 3) was paired with a male that was not studied. Both 8-yr-old males were housed together. Each individual was injected i.m. with 400 IU of long-acting ACTH preparation (Acthar Gel, Rhone-Poulenc Rorer Pharmaceuticals, Inc., Collegeville, Pennsylvania 19034, USA) between 0900 and 1000 hours. For paired individuals, 6 ml green food coloring (McCormick & Co., Hunt Valley, Maryland 21030, USA) was mixed with the normal rations (1,025 gm Nebraska Canine Meat, Animal Spectrums, Inc., North Platte, Nebraska 69103, USA) of one individual per pair to permit individual identification of fecal samples. Entire scats were collected from every defection beginning 72 hr before and for 144 hr after ACTH administration and frozen at −20°C until analysis.

Fecal sample extraction and processing

Steroids were extracted from feces by modifications of a previously reported method.[2,30,31] Each scat was thoroughly mixed, and a subsample (2–5 g) from each defecation was dried with a Savant Instruments Speedvac Rotary Evaporator (Forma Scientific, Inc., Marietta, Ohio 45750, USA). Dried feces were pulverized, and 0.1–0.2 g of powder was boiled in 10 ml of 100% ethanol for 20 min. After centrifugation (500 *g* for 15 min), the supernatant was decanted into a clean tube (16 × 125 mm) and dried under a stream of compressed air. As samples evaporated, vessel walls were rinsed twice with ethanol (4 ml per rinse) before the supernatant was evaporated completely and redissolved in 1 ml methanol. To complete the extraction procedure, tubes were vortexed (1 min), placed in an ultrasonic glass cleaner for 30 sec to free particulates adhering to the vessel wall, and then vortexed for 15 sec. A total of 3,000–5,000 d.p.m. of ^{3}H-cortisol (New England Nuclear, Wilmington, Delaware 19860, USA) was added to each fecal sample before extraction to monitor recovery by a quench curve compensation program.

Fecal radioimmunoassays

A double-antibody ^{125}I RIA for corticosterone (ICN Biomedicals, Inc., Costa Mesa, California 92626, USA) was validated for extracted wild dog feces. According to the manufacturer, the antisera cross-reacts with corticosterone (100%), desoxycorticosterone (0.34%), testosterone (0.10%), cortisol (0.05%), aldosterone (0.03%), progesterone (0.02%), and less than 0.01% for all other steroids tested. Assays were used according to the instructions provided except that all reagent volumes were halved.

High-pressure liquid chromatography (HPLC)

The number and relative proportions of fecal corticosteroid metabolites were determined by reverse-phase HPLC (Microsorb RP C-18, Rainen Instruments, Woburn, Massachusetts 01801, USA).[25] Before HPLC, fecal extracts were preprocessed with reverse-phase C18 cartridges (Spice™, Analtech, Newark, Delaware 19720, USA).[19,25] A 55-µl portion of fecal extract, combined with ^{3}H-cortisol (2,500 d.p.m.) and ^{3}H-corticosterone (2,500 d.p.m.), was separated with a linear gradient of 20–100% methanol in water within 80 mm (1 ml/min flow rate, 1.0 ml fractions). Separate aliquots of each eluate were counted directly to determine recovery and/or analyzed to identify immunoreactive corticosteroid metabolites.

Statistical analyses

Summary data are presented as mean ± SEM. Pre- and post-ACTH corticosterone immunoreactivity was examined by repeated measures analysis of variance. All statistical comparisons were performed with Statview® 512+ (BrainPower, Inc., Calabasas, California 91302, USA) on a Macintosh computer.

RESULTS

Serial dilutions (1:32 to 1:512) of wild dog fecal extracts yielded displacement curves parallel to standard corticosterone, and the mean recovery of added cortisol (range, 25–500 ng/ml) was 102.6 ± 4.5% ($y = 1.02x - 1.78$, $r^2 = 0.99$). Assay sensitivity was 25 ng/ml. Interassay coefficients of variation for two separate internal controls ($n = 2$ assays for all samples) were 8.0% (55–60% binding) and 13.7% (25–30% binding), respectively, and intraassay variation was <5% ($n = 20$).

Fecal corticosteroid excretory profiles for three females are depicted in Figure 1A. Fecal corticosteroid metabolites increased approximately ninefold (female 2, from 169 to 2,845 ng/g feces; female 3, from 583 to 3,704 ng/g feces) in two of three females that defecated within 5 hr of ACTH administration (Fig. 1A). Mean fecal corticosteroid metabolites increased ($P < 0.05$) more than tenfold above pre-ACTH concentrations to 4,446 ± 766 ng/g feces during the first 24 hr post-ACTH administration and returned to baseline by 48 hr postinjection.

Both males excreted increased fecal corticosteroid metabolites within 30 hr post-ACTH administration (Fig. 1B). Pre-ACTH (time 0) fecal corticoid concentrations in both males (male 1, 80 ng/g feces; male 2, 39 ng/g feces) were lower than levels in females. Mean 24-hr postinjection fecal corticoid concentrations in males peaked ~25-fold (male 1, 2,047 ng/g) and ~13-fold (male 2, 490 ng/g) higher than basal concentrations and returned to baseline 48 hr post-ACTH injection.

RIA of HPLC-separated fecal extracts from females (Fig. 2A) and males (Fig. 2B) revealed that >90% of immunoreactivity was associated with a single peak that exhibited intermediate polarity between cortisol and corticosterone reference tracers, whereas only a minor portion of immunoreactivity (<5%) coeluted with radiolabeled cortisol.

DISCUSSION

These data suggest that monitoring fecal corticosteroid metabolites can be a useful noninvasive tool for tracing adrenal activity in African wild dogs. Combined with standardized validation pro-

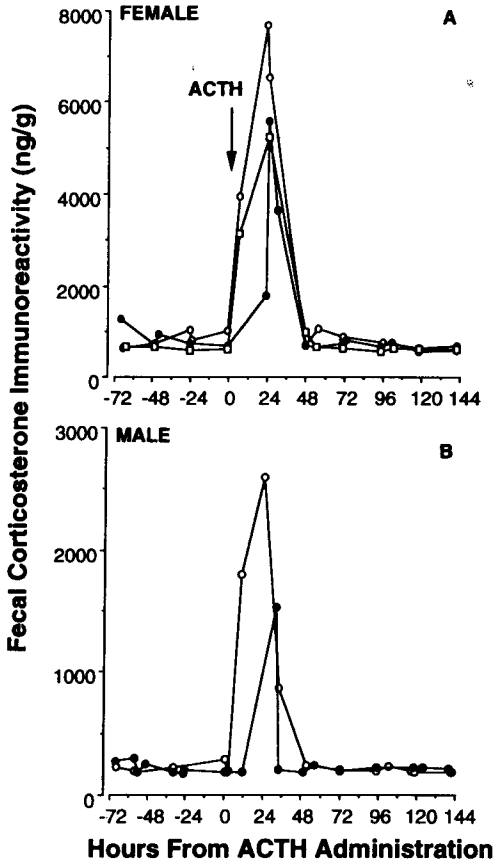

Figure 1. Immunoreactive fecal corticosteroid metabolites during the 72 hr before and 96 hr after an exogenous ACTH challenge in female (**A:** female 1, closed circles; female 2, open squares; female 3, open circles) and male (**B:** male 1, open circles; male 2, closed circles) African wild dogs.

cedures, HPLC analysis confirmed that the corticosterone RIA detected a single immunoreactive metabolite. Although the identity of this metabolite remains unknown, its physiologic relevance as an indicator of adrenal activity was confirmed by demonstration of a temporal association between the administration of exogenous ACTH and its appearance in feces.

Although there is no single definition or physiologic measure of "stress,"[24] blood cortisol measures have been useful for evaluating adrenal function in primates exposed to a variety of stressors.[14,20] Similarly, urinary cortisol determinations have been used to evaluate adrenal status in primates,[13,21] felids,[4,5] mule deer,[28,29] bighorn sheep,[22,23] domestic sheep,[1] and elephants.[3]

Monitoring techniques for fecal corticoid metabolites also have been used successfully in bighorn sheep[22] and domestic cats.[18] In both these species, fecal corticoids increased significantly within 24 hr of ACTH administration. Whereas free cortisol was detected in bighorn sheep, unidentified, highly polar, immunoreactive corticoid metabolites were detected in the domestic cat. Despite HPLC data confirming that domestic cats[18] and wild dogs excrete different corticosteroid metabolites, the same RIA detects fecal corticoids in both species that reflect ACTH-induced adrenal activity, suggesting that the corticosterone RIA may be useful for assessing fecal corticoids in other carnivore species.

Blood sampling of wild animals requires restraint and/or anesthesia, and this approach can itself elicit an adrenal "stress" response.[27] Furthermore, corticosteroid secretion is pulsatile, and failure to collect sequential samples in unanesthetized individuals can lead to erroneous estimations of adrenal status.[26] Monitoring fecal or urinary corticosteroid metabolites is noninvasive, and this approach also provides an integrated measure per unit time of adrenal status because episodic secretory patterns are dampened by the "pooling" of adrenocorticosteroids in urine or feces. Although fecal sample collections are more adaptable for field sampling free-living species, excretory lag times (the time from appearance in blood until excretion) may be extended in feces compared with urine.

From a conservation perspective, noninvasive monitoring of fecal corticosterone metabolites already has been applied to the study of dominance and stress in free-living African wild dogs.[7,8] Dominant individuals have been shown to excrete higher corticosteroid concentrations than subordinate pack members. Because dominant wild dogs are involved in more aggressive interactions, presumably to maintain dominance, chronic stress may be a cost of social dominance.[8] Fecal corticosterone metabolites also have been used to evaluate the adrenal status of radiocollared wild dogs.[9] No differences in fecal corticoid concentrations were detected between radiocollared and uncollared wild dogs, and fecal corticoids did not differ in wild dogs sampled before and after radiocollaring, suggesting that short-term anesthesia and radiocollaring procedures do not evoke a chronic "stress" response. This finding illustrates the power of noninvasive endocrine assessments of adrenal status for studying the relationships between environmental stressors and animal well-being in African wild dogs.

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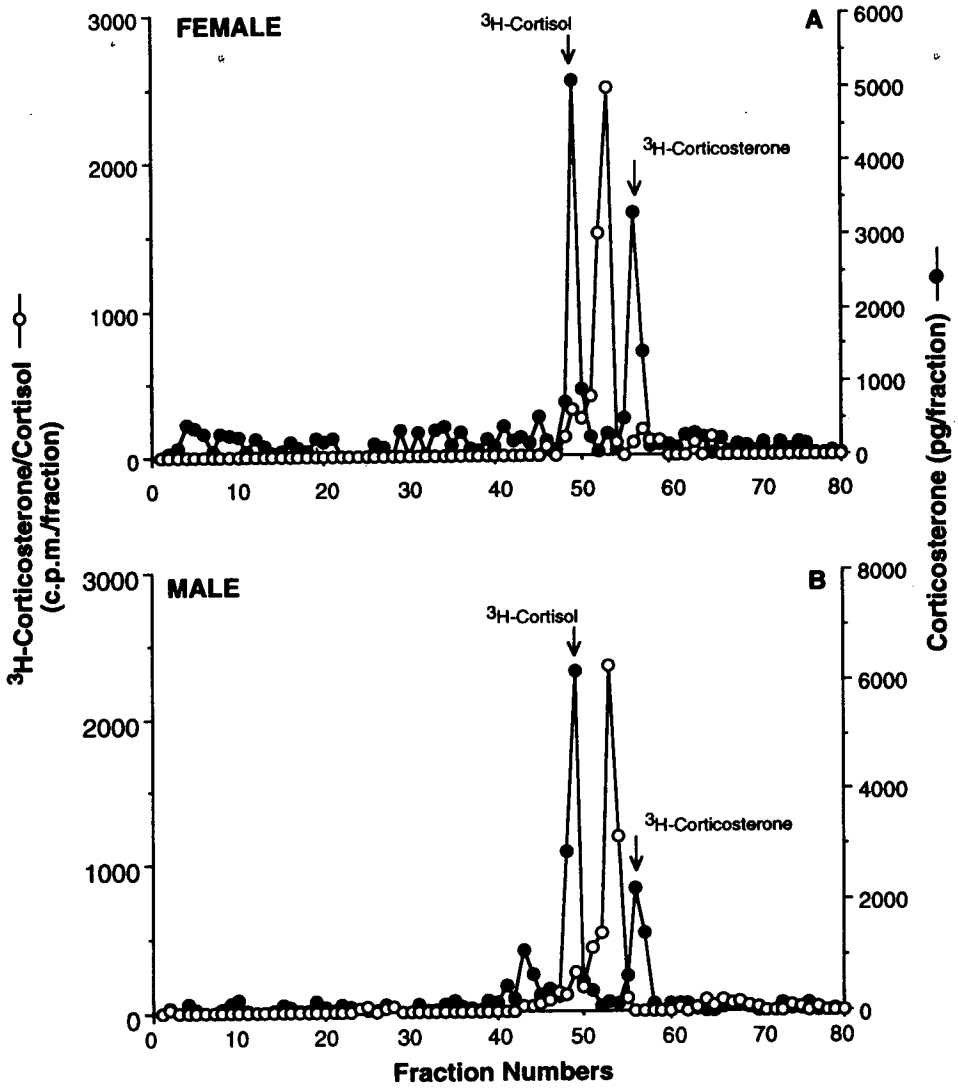

Figure 2. Immunoreactive corticoids after HPLC of pooled fecal extracts from female (**A**) and male (**B**) African wild dogs sampled 24 hr after ACTH administration. Tritiated cortisol and corticosterone (open circles) were added before HPLC (arrows) as reference standards.

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