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Evaluation of Glucocorticoid Faecal Monitoring as a Non-Invasive Assessment of Stress in Captive Crab-Eating Fox (*Cerdocyoun thous*) After ACTH Stimulation

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Abstract

Blood sampling is a common method in biological research of domestic animals for studying hormone mechanisms. However, this approach can be stressful for wild species, and compromise research concerning animal welfare. The crab-eating fox (*Cerdocyoun thous*) is a medium-sized canid found in South America and a popular zoo species in that region. However, almost nothing is known of their biology or what factors impact captive welfare. Thus, a non-invasive method to measure adrenal activity and stress was developed for quantifying fecal glucocorticoids non-invasively in this species. The physiological relevance of the cortisol immunoassay was demonstrated by injection of exogenous ACTH into two males and one female, which led to a significant increase in fecal glucocorticoid metabolites within 24-48 h. From these findings we conclude that fecal samples can be used for the non-invasive assessment of adrenocortical status in crab-eating fox.

Keywords: Feces; Cortisol; Corticosterone; Stress; Non-invasively method; Enzyme immunoassay; ACTH challenge

Introduction

The crab-eating fox (*Cerdocyoun thous*) is a medium-sized canid found in Colombia, Venezuela, Guyana, French Guyana, Surinam, eastern Peru, eastern Bolivia, Paraguay, Uruguay, Northern Argentina and in most of Brazil outside the lowlands of the Amazon basin [1]. Their range includes subtropical and tropical savannas as well as riperian forest areas. The species is socially monogomous, and the adult female gives birth to one or two litters per year after a 58-60-day gestation [2]. As the name implies, the diet includes crabs caught on muddy floodplains during the wet season, as well as insects, rodents and birds when available, and fruits, and the species plays an important role as seed dispersers and controlling rodent populations [3-6]. No precise estimates of population sizes are available in Brazil, but populations generally are considered stable and adaptable to deforestation, agricultural and horticultural development (e.g., sugarcane, eucalyptus, melon, pineapples) and habitats in regeneration [3]. However, the fox is perceived as a pest of poultry throughout much of its range (and in Uruguay as a predator of lambs), and they are thus shot, trapped, and poisoned indiscriminately.

Measures of circulating cortisol have been widely used to evaluate stress responses in domestic animals. However, blood sampling techniques often are not applicable to studies of wildlife species. For example, capture and handling of animals for venipuncture can induce a stress response, with increased peripheral glucocorticoid concentrations observed within minutes [7]. Due to the increasing demand for methods to quantify adrenal activity in zoo and wild animals in response to mate introduction and transportation, after pharmacological and management precautions to minimize stress, and the necessity to investigate stress impacts on animal welfare, non-invasively methods for measuring adrenal activity have been developed for a variety of species: primates [8,9]; ruminants [10,11]; several felids [12-14]; African wild dog [15].

Glucocorticoids are extensively metabolized by the liver, and excretions of glucocorticoid metabolites can differ among species, and sometimes even between sexes and individuals within a given species [16]. The lag time between glucocorticoid secretion from the adrenal gland and excretion into feces or urine also tends to be fairly species-specific [16-19]. Therefore, it is not always possible to draw on data from other species to develop non-invasively approaches for assessing adrenal function in an unstudied species. Thus, the aims of this study were to validate an enzyme immunoassay to quantify glucocorticoid metabolites in feces of crab-eating fox. The first goal was to show that fecal glucocorticoid metabolite concentrations increase after an ACTH challenge [19].

Materials and Methods

Animals

Two female (F4 and F5) and two males (M1 and M2) crab-eating foxes were used for ACTH challenges. Permission for animal use was

approved by the SISBIO/IBAMA/Brazil (n^0 11167-1) and CEPA/UFMT Bioetical Committee (n^0 23108.002900/09-3).

Animals were housed at the Universidade Federal de Mato Grosso Zoo, Pantanal/MT, Brazil, (186 m and lat - 15°05'S, long - 56°36'W), which has a tropical climate [20].

The animals were fed twice a daily (morning and afternoon), a diet of fruits (papaya, banana), fish or meat, commercial dog food and a food coloring dye added to a ground meat once a daily (morning) to identify individual feces. Water was provided ad libitum. Individual fecals were identified by visualization with tail tricotomy and feeding dye.

Experimental Design

During the ACTH challenge the animals were housed in two separate enclosures. Three animals (M1, M2, F5) were injected with 0.2 mL of adrenocorticotropic hormone (ACTH; Synacthen; Novartis Pharma AG, Basel, Switzerland, 1 mg/mL) intra-muscularly. A control animal (F4) was injected with 0.2 mL saline, intra-muscularly. Fecals samples were collected for 6 days before and 6 days after injection. All samples were immediately stored at −22°C until analyses.

Fecal Steroid Analyses

Extraction of fecal samples followed the procedure described by Schwarzenberger et al. [21], with minor modifications. Fecal samples were thawed and mixed. For each sample, 0.5 g of homogenized wet feces was suspended in 5 mL of 80% methanol and homogenized. Tubes were vortexed (30 s) and gently homogenized for 15 h. Subsequently, samples were centrifuged (1,300 g for 15 min) and the supernatant kept in a water bath at 60°C until total evaporation of the 80% methanol. The samples were suspended in 1 mL dilution buffer [NaH_2PO_4 (5.4 g), Na_2HPO_4 (8.66 g), NaCl (8.7 g), H_2O Mili-Q (1 L), pH (7.0)], pH (7.0)] and vortexed for about 1 min. An aliquot of the supernatant was diluted with dilution buffer (final dilution: 1:30) and frozen at −22°C until EIA analysis.

For the cortisol EIA, 50 µl of cortisol R4866 antibody (Coralie Munro, UC Davis, California) in coating buffer [Na_2CO_3(1.59 g), $NaHCO_3$ (2.93 g), H_2O Mili-Q (1 L), pH (9.6)] (1:8500) was pipetted onto NUNC microtitre plates and incubated overnight at 5°C. Plates were washed five times with MilliQH2O/Tween 20 (1:500,000),then standards (3.9 – 1000 pg/well), low and high controls, each sample in duplicate and cortisol HRP (Coralie Munro) in assay buffer [Tris(2.42 g), NaCl (17.9 g), BSA (1 g), Tween 80 (1 mL) H2O Mili-Q (1 L), pH (7.5)] (1:20,000) was pipetted into the microtiter plate wells and incubated for 1 h at room temperature. Plates were washed again with MilliQH_2O/Tween 20 and 100 µl of 40 µl 0.5H_2O_2, 125µl 40mM ABTS and 12.5 ml substrate buffer was added to each well. The absorbance was measured at 450 nm/540 nm, using a DYNEX MRX reader (Dynex Technologies, Chantilly, VA, USA).

Results and Discussion

Administration of ACTH resulted in a 10 to 45-fold increase in fecal glucocorticoid metabolite concentrations in all animals, which peaked within a day of injection (Figure 1, Table 1). Following the ACTH injection, fecal metabolite concentrations peaked after 24-48 h and then returned to baseline within 24 h of the peak. Concentrations were unchanged in the saline-treated control animal (Figure 1).

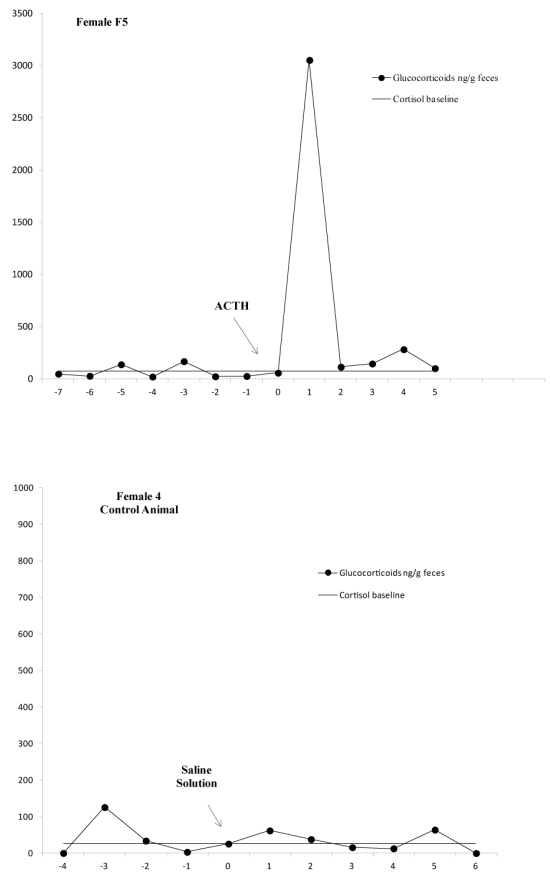

Figure 1: The circles indicate fecal glucocorticoids metabolite concentrations (ng/g feces) in crab-eating foxes before, during and after an ACTH challenge (F5) or saline injection (F4). The line indicates cortisol baseline concentrations for crab-eating foxes F5 and F4.

Concentrations of fecal glucocorticoid metabolites were significantly increased within a day of an ACTH challenge, indicating that adrenocortical activity can be monitored via fecal sample analyses in crab-eating fox using the cortisol R4866 antibody. Responses were robust in all treated individuals, representing a 10- to 40-fold increase over pre-injection concentrations within 24-48 h, with concentrations returning to baseline within a day of the peak. Palme et al. [22] suggested that the delay of fecal glucocorticoid excretion in sheep, ponies, and pigs roughly corresponds with gut transit time from duodenum to rectum. In crab-eating fox, the time lag of glucocorticoid excretion in feces appears to correspond with a food transit time of 24-48 h based on dye feeding of animals (Paz, unpublished).

ANIMALS	Mean (± SEM) glucocorticoid concentrations (ng/g feces)		
	Before ACTH or saline injection	After ACTH or saline injection	Peak1
Control F4	37.8 ± 51.3 (5)	28.6 ± 22.6 (5)	61.9(1)
Female F5	66.6 ± 57.2 (8)	164.0 ± 83.2 (4)	3055.7 (1)

Male M[1]	63.9 ± 66.9 (8)	46.2 ± 11.2 (4)	681.5 (1)
Male M[2]	123.0 ± 122.2 (8)	55.99 ± 30.5 (5)	1178.4 (1)

Table 1: Mean ± SEM fecal glucocorticoids concentrations (ng/g feces) before (days 1-8) and after (days 10-14) an ACTH or saline challenge in crab-eating fox males and females.

[1]Peak concentrations occurred on Day 9.

[2]Numbers in parentheses refer to number of fecal samples.

In summary, the physiological relevance of our cortisol EIA to assess adrenal activity in crab-eating foxes was proven valid by demonstrating that concentrations of fecal glucocorticoid metabolites were significantly increased following an ACTH challenge. From these findings we conclude that fecal samples can be used for the non-invasive assessment of adrenocortical status in crab-eating fox.

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