

Using ACTH Challenges to Validate Techniques for Adrenocortical Activity Analysis in Various African Wildlife Species

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Abstract: Monitoring adrenocortical activity using fecal hormonal analysis can provide information on how environmental changes are affecting non-domestic species health and success in the field; however, this non-invasive method needs proper validation to ensure that analysis reflects true physiological events. Our objectives were to use adrenocorticotrophic hormone (ACTH) challenges as a physiological validation method to test the suitability of a new corticosterone enzyme immunoassay (EIA) to accurately assess the adrenocortical activity using fecal samples in four African wildlife species—the black rhinoceros (rhino; *Diceros bicornis*), African elephant (*Loxodonta africana*), chimpanzee (*Pan troglodytes*) and African lion (*Panthera leo krugeri*). In the rhino and elephant, fecal Glucocorticoid metabolites (GC) surged 75 and 51 h post-ACTH injection, respectively. In the chimpanzee, fecal GC metabolites peaked at 29 h post-injection. And the lion had a peak of fecal GC at 24 h post-ACTH. This study determined that adrenocortical activity was reflected in concentrations of fecal GC metabolites suggesting that this corticosterone EIA is an effective technique for the monitoring stress in four African species.

Key words: ACTH challenge, adrenocortical activity, African wildlife, corticosterone, enzyme immunoassay, fecal glucocorticoid metabolites

INTRODUCTION

Anthropogenic activity and environmental changes have profoundly impacted the health of free-ranging populations as evident by the number of emerging infectious disease outbreaks in recent years (Harper and Austad, 2004). One method to determine the physiological effect of these stressors on the health of an individual and/or population is to monitor Glucocorticoids (GC), which are steroidal hormones released from the adrenal cortex in response to a perceived stress activating the Hypothalamic-Pituitary-Adrenal (HPA) axis (Reeder and Kramer, 2005). These hormones enable an individual to cope with a stressor by providing energy through increased gluconeogenesis and associated decreased glucose use and sensitivity to insulin, along with reduced protein and fat metabolism. However, prolonged stressors can negatively affect the body by suppressing the immune system resulting in increased susceptibility to disease (Reeder and Kramer, 2005). Like other steroids, GCs have anti-inflammatory effects decreasing cytokine production, suppressing white blood cells and several interleukins (IL)

including IL-1 and IL-2 (Welsh *et al.*, 1999). Furthermore, chronic exposure to GCs can cause severe protein loss (muscle wasting), disruption of normal behaviors, neuronal cell malfunction, suppression of growth and various other pathologic conditions in humans and other vertebrates (Boonstra, 2005; Wingfield, 2005; Wingfield and Romero, 2001).

Monitoring the adrenocortical activity of non-domestic species is often challenging because blood glucocorticoid concentrations can increase rapidly, in less than 5 min for birds and for small mammals, upon capture and restraint (Harper and Austad, 2000; Mostl and Palme, 2002; Millspaugh and Washburn, 2004; Touma and Palme, 2005). Additionally, a blood sample represents a brief point in time and can have fluctuations related to the pulsatile nature of hormones and, therefore, may not thoroughly provide a comprehensive view of overall adrenocortical activity in an animal. However, if the individual is trained for collection, blood provides easy interpretation of results. Recently, hormonal analysis using samples collected by non-invasive methods, such as fecal or urine collection, have been developed. Urine

and feces provide more accurate insight into adrenocortical activity by concentrating the constantly changing hormone amounts found in blood prior to excretion (Monfort, 2003). Feces are easy to obtain without disturbing the subject through capture and handling and often provide large quantities of sample. Because the sampling method is non-invasive, frequent collection can provide a longitudinal profile and better interpretation of the hormonal patterns. Due to the period from circulation to excretion, glucocorticoid metabolite values in fecal and urine samples are from several hours or days prior to collection and provide further comprehensive data on adrenocortical activity in an individual.

The drawback to fecal GC metabolite analysis is that the interpretation of the results requires validation. Quantification of GC metabolites is possible in feces because the body (mainly the liver) metabolizes the hydrophobic steroidal hormone into a hydrophilic compound that can be excreted in the feces. However, steroid metabolism can be species-specific causing a wide variety of hormone metabolites with little naïve hormone (Bahr *et al.*, 2000; Wasser *et al.*, 2000). For example, the major GC in most primates, carnivores and ungulates is cortisol, whereas in rodents, birds and reptiles corticosterone is the main GC (Wasser *et al.*, 2000; Touma and Palme, 2005). Therefore to accurately monitor adrenocortical activity via feces, a suitable enzyme immunoassay needs to be validated and no single assay is applicable to all species (Heistermann *et al.*, 2006). Three types of validation methods, biochemical, biological and physiological, are commonly used to determine the reliability of a hormonal assay to quantify fecal GC metabolites and monitor adrenocortical activity in wildlife (Wielebnowski and Watters, 2007).

The biochemical validation takes place in the laboratory and verifies that the assays are accurately measuring the hormone in the sample through two steps, parallelism and percent recovery. Testing the parallelism between binding inhibition curves of the fecal extract dilutions and the assay standards evaluates whether the metabolites in the sample bind to the antibody similarly to the standards (Keay *et al.*, 2006). The recovery determines if the assay is accurately analyzing the amount of hormone metabolite in the fecal extracts by recovering >90% of an exogenous hormone that is added to fecal extracts (Graham *et al.*, 1995; Keay *et al.*, 2006). High Pressure Liquid Chromatography (HPLC) is another biochemical technique that is used to identify hormonal metabolites by separating them by size and polarity (Keay *et al.*, 2006).

Biological validation involves the use of biological events to determine the integrity of the hormone results. For reproductive evaluation, certain events, such as

pregnancy (via ultrasound), birth, menstruation, estrous behavior, mating, egg laying, testes enlargement and vulvar swelling, provide clear evidence of changes in hormone activity. Similarly, for adrenal hormone activity fluctuations that correspond with a perceived stressful situation, such as veterinary procedures (anesthesia, blood collection, vaccination), changes in social structure and situation (separation from group), restraint, temperature extremes and changing the environment (translocation), also may be used to validate the adrenal hormone assay for the given species (Touma and Palme, 2005).

Physiological validations involve the manipulation of the physical state or condition of the individual to stimulate a predictable, measurable hormone response. This can be accomplished by using pharmaceutical drugs such as adrenocorticotropic hormone (ACTH), dexamethasone (Dex) and gonadotrophic releasing hormone (GnRH). Both ACTH and Dex affect adrenocortical activity (ACTH stimulates; Dex suppresses) and GnRH affects gonadal activity. The ACTH, injected into the subject, acts on the adrenal glands and causes the release of GCs into the bloodstream preparing the body to respond to a stressor. The GCs in the blood stream are then metabolized by the liver and kidneys, which add a hydrophilic compound to the steroid; and the GCs are excreted in the feces and urine. Fecal and urine samples are collected prior to the ACTH injection to quantify the individual's normal values for GCs. ACTH challenges have been performed on numerous species from mammals to birds (Touma and Palme, 2005) and even reptiles (Fence lizard, *Sceloporus undulates*, Phillips and Klukowski, 2008). Most responses to the challenges are short-lived and do not impact the individuals. Samples are collected pre- and post-injection and evaluated for hormone activity with the expectation of observing a change (increase or decrease) in hormone concentration post-injection.

These methods of validation assist with determining the suitability of the assays for non-invasive monitoring of adrenocortical activity in wildlife. In the present study, our objectives were to use biochemical and physiological (ACTH challenge) validations to test the suitability of a corticosterone Enzyme Immunoassay (EIA) to accurately assess the adrenocortical activity non-invasively in four African wildlife species: the black rhinoceros (rhino; *Diceros bicornis michaeli*), African elephant (elephant; *Loxodonta africana*), chimpanzee (*Pan troglodytes*) and African lion (lion; *Panthera leo krugeri*). Additionally, we evaluated serum GC concentrations in the elephant and rhino.

MATERIALS AND METHODS

Animals: Animals were housed at various facilities including one captive-born adult (22-years-old) male

black rhino and one captive-born adult (12-years-old) female African lion housed at the Lincoln Park Zoo (LPZ; Chicago, IL, USA), one wild-born adult female African elephant (32-years-old) housed at the Maryland Zoo at Baltimore (Baltimore, MD, USA) and one captive-born chimpanzee adult (21-years-old) male housed at Yerkes National Primate Research Center (Atlanta, GA, USA). The black rhino and the elephant were fed Mazuri Elephant® diet (St. Louis, MO), grass hay, produce and browse. Chimpanzees were fed monkey pellets (Purina LabDiet® 5037, Richmond, IN, USA) twice daily and received fresh fruits and vegetables daily. The lion was fed Natural Balance Carnivore® diet (Pacoima, CA, USA). Water was available *ad libitum* for all individuals.

ACTH challenge protocols: An ACTH challenge was used as a means of physiological validation for the black rhino, lion, elephant and chimpanzee. The ACTH (corticotrophin; Monument Pharmacy, Monument, CO, USA) dosages were: 1.5 IU/kg IM (800 IU total) for the black rhino (Wasser *et al.*, 2000; Brown *et al.*, 2001) and 5 IU/kg IM (720 IU total) for the lion (Wasser *et al.*, 2000; Wielebnowski *et al.*, 2002). For the elephant, we divided the ACTH (Cortosyn; Organon, West Orange, NJ, USA) dose IM (0.5 IU/kg; 2000 IU total) into three equal injections that were given 2 h apart (Brown *et al.*, 1995; Wasser *et al.*, 2000). For the chimpanzee, the ACTH (Cortosyn; Amphastar Pharmaceuticals, Inc. Rancho Cucamonga, CA) dose was 0.45 IU/kg IM (22 IU total; Heistermann *et al.*, 2006). All research was approved through each facility's IACUC or Research Committee (Yerkes IACUC # 151-2008Y, NZP IACUC; and Lincoln Park Zoo's Research Committee).

Sample collection protocols: Fresh fecal samples were collected daily for approximately 1 week prior to the ACTH injection, and all defecations were collected for 3 days post-injection. Then, we collected daily samples for days 4 to 7 post-injection. All samples were stored at -20°C until processing. For the black rhino, blood was collected 1 and 2 weeks prior to the challenge and 1 h pre- and 3, 24, 48 and 72 h post-injection of ACTH, respectively. For the elephant, daily blood samples were drawn for 1 week prior to the challenge. Then, blood was collected 1 h before the first injection and prior to the other two injections of ACTH. Samples were collected hourly for 3 h and then at 20 and 28 h post-injections of ACTH. Finally, blood samples were collected daily for the next 6 days.

Fecal sample processing: Processing procedures varied due to the future projects that will be conducted on these species. All samples were analyzed from the chimpanzee and elephant as wet weight because future studies will be conducted in the field where equipment and resources (such as electricity) are limited. However, the lion and black rhino samples were dried on a lyophilizer (Thermo

Modulyo Freeze Dryer, Waltham, MA, USA) until the pressure reached between 70 and 90 mbar and then crushed before processing. Each sample was weighed (wet, 0.5±0.02 g; dry, 0.2±0.02 g) prior to the hormone extraction. For the extraction, 5.0 mL of 90% ethanol: distilled water was added to each weighed sample (Brown *et al.*, 1994). The samples were either agitated on a mixer (Glas-col, Terre Haute, IN, USA) on setting 60 for 30 min (elephant, rhino, and chimpanzee) or boiled for 20 min (lion; Brown *et al.*, 1994), centrifuged (500 X g; 20 min), and the supernatant was poured into clean glass tubes. The fecal pellet was re-suspended in 5 mL of 90% ethanol: distilled water, vortexed for 30 sec and re-centrifuged (500 X g, 15 min). Both supernatants were combined and dried under air and heat (60°C). All extracted samples were reconstituted in 1 mL of methanol, vortexed briefly, sonicated for 20 min and diluted (black rhino, 1:64; elephant, neat; lion, 1:60; chimpanzee, 1:40) in phosphate buffered saline (PBS; 0.2M NaH₂PO₄, 0.2M Na₂HPO₄, NaCl) before analysis.

Blood sample processing: Blood was collected from the ear vein in the elephant and the radial vein in the black rhino. Then, the blood was placed in a serum separation vial, allowed to clot at room temperature for 30 min, and centrifuged (500 x g; 20 min). The serum was removed and stored in cryovials at -20°C until analysis at the Lincoln Park Zoo's Endocrinology laboratory. Serum samples were analyzed diluted with PBS (elephant, 1:1-1:15; black rhino, 1:1).

Enzyme immunoassay: The fecal corticosterone metabolites were analyzed using an EIA with previously described methods (Munro and Stabenfeldt, 1984; Santymire and Armstrong, 2010; Narayan *et al.*, 2010). C. Munro (University of California, Davis, CA, USA) provided the horseradish peroxidase (HRP) ligands and polyclonal antiserum (CJM006). The corticosterone antiserum and HRP was diluted to 1:6,000 and 1:20,000, respectively. Antiserum cross-reactivities for corticosterone were: corticosterone, 100%; desoxycorticosterone, 14.25%; tetrahydrocorticosterone, 0.9%; 11-deoxycortisol, 0.03%; prednisone, <0.01%; prednisolone, 0.07%; cortisol, 0.23%; cortisone, <0.01%; progesterone, 2.65%; testosterone 0.64% and estradiol 17β, <0.01% (Santymire and Armstrong, 2010; Narayan *et al.*, 2010). The EIA was validated biochemically for all species by demonstrating:

- Parallelism between binding inhibition curves of fecal extract dilutions and the corticosterone standard;
- Significant recovery (>90%) of exogenous corticosterone (1.95-1000 pg/well) added to fecal extracts. Assay sensitivity was 1.95 pg/well and intra- and inter-assay coefficients of variation were <10%.

Table 1: Biochemical validation to analyze adrenocortical activity in all four African species via fecal glucocorticoid (GC) metabolite concentration analysis on a corticosterone EIA

Species	Relationship between fecal extracts and standards	Fecal extract dilutions for parallelism	Recovery linear regression	Percentage of recovered added exogenous hormone
Black rhino	r = 0.998	1:8-1:2048	y = 0.86x-5.83 R ² = 0.994	134.2±7.5%
Elephant	r = 0.917	4xNeat-1:80	y = 1.05x-6.45 R ² = 0.992	91.5±10.7%
Lion	r = 0.969	1:2-1:512	y = 0.95x-3.40 R ² = 0.995	108.0±8.2%
Chimpanzee	r = 0.934	1:10-1:640	y = 1.12x+0.24 R ² = 0.997	90.8±6.4%

Data analyses: For the biochemical validation, Pearson's Product Moment correlation was used to compare the relationship of the parallelism between the corticosterone standards and serially diluted fecal extracts for all species separately. For the percent recovery, we graphed a scatterplot of the observed over expected values of samples spiked with the corticosterone standards and did linear regression to get the equation of the best fit line and R² value for each species. For the physiological validation, all pre-ACTH fecal GC metabolite values were averaged and compared to values post-ACTH injection to determine its effects on adrenocortical activity and the time between HPA axis stimulation, steroid metabolism and excretion of steroid metabolites in the feces of these four African species. All statistical analyses were performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and SIGMAPLOT (2008, v 11; Systat Software, Inc., San Jose, CA, USA). Values are presented as mean±SE. For all analyses, p<0.05 was considered significant.

RESULTS

Biochemical validation: The EIA was validated for all species by demonstrating parallelism and significant recovery of exogenous corticosterone added to fecal extracts (Table 1).

Physiological validation: For the black rhino, mean fecal GC metabolite value prior to the ACTH challenge was 760.8±30.8 µg/g dry feces with peaked values (n = 2; 1251.6±180.6 µg/g dry feces) occurring approximately 75 to 93 h post-injection of ACTH (Fig. 1). In the elephant, mean pre-ACTH fecal GC metabolite concentration was 1.1±0.2 µg/g wet feces (Fig. 2). Elevated values above the mean (n = 10; 1.6±0.1 µg/g wet feces) occurred from 51 to 146 h post-ACTH injection with the peak (2.3 µg/g wet feces) occurring at 75 h (Fig. 2). Mean pre-ACTH fecal GC metabolite value for the chimpanzee was 43.8±6.7 µg/g dry feces with an elevated value of 54.2±4.3 µg/g dry feces (n = 3) occurring 21-29 h after the ACTH injection (Fig. 3). Finally, for the lion, fecal GC metabolite value increased above pre-ACTH mean (407.9±103.7 µg/g dry feces) at 24 h ACTH post-injection with an elevated value of 637.7 µg/g dry feces (Fig. 4).

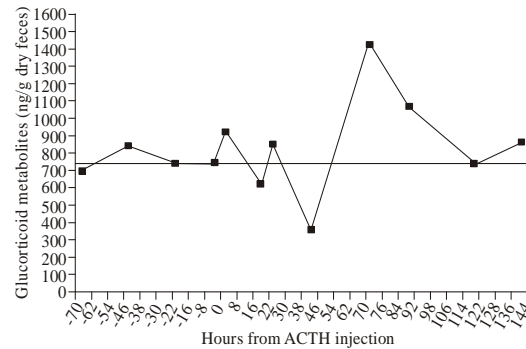


Fig. 1: Fecal Glucocorticoid (GC) metabolite concentration (µg/g dry feces) profile from an adrenocorticotrophic hormone (ACTH) challenge in a male black rhino using a corticosterone Enzyme Immunoassay (EIA). The ACTH injection (800 IU total) was administered at 9:00 am on September 2 (h 0) with sample collection beginning August 26 and continuing until September 12. Solid line represents the mean of pre-ACTH fecal GC metabolite concentration (µg/g dry feces)

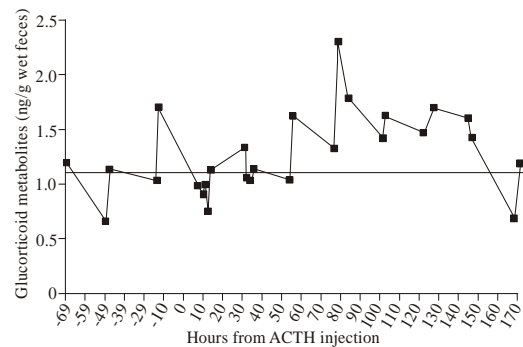


Fig. 2: Fecal Glucocorticoid (GC) metabolite concentration (µg/g wet feces) profile from an ACTH challenge in a female elephant using a corticosterone EIA. The ACTH injections (2000 IU total divided in three injections given 1 h apart) were administered at 9:30, 10:30 and 11:30 am, respectively on November 16 with sample collection beginning November 12 and continuing through November 25. Solid line represents the mean of pre-ACTH fecal GC metabolite concentration (µg/g dry feces)

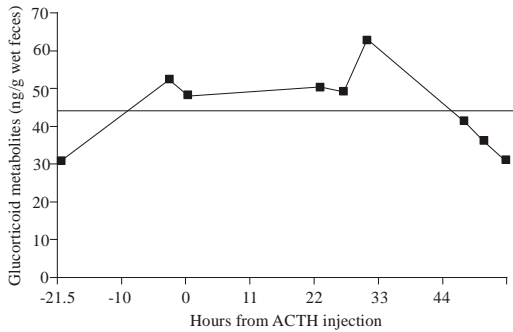


Fig. 3: Fecal Glucocorticoid (GC) metabolite concentration ($\mu\text{g/g}$ wet feces) profile from an ACTH challenge in a male chimpanzee using a corticosterone EIA. The ACTH injection (22 IU total) was administered 11 am on August 22 with sample collection beginning August 21 and continuing through August 24. Solid line represents the mean of pre-ACTH fecal GC metabolite concentration ($\mu\text{g/g}$ dry feces)

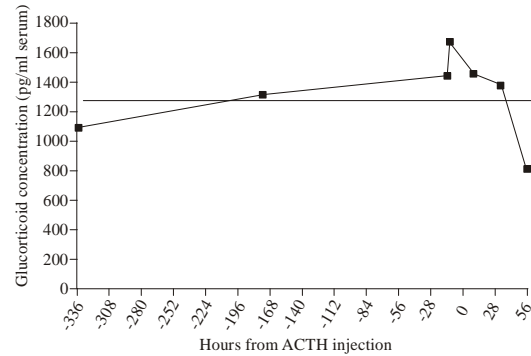


Fig. 5: Serum Glucocorticoid (GC) concentration (pg/ml) profile from an ACTH challenge in a male black rhino using a corticosterone enzyme immunoassay (EIA). The ACTH injection (800 IU total) was administered at 9:00 am on September 2 (h 0) with sample collection beginning August 19 and continuing until September 5. Solid line represents the mean of pre-ACTH serum GC metabolite concentration (pg/mL)

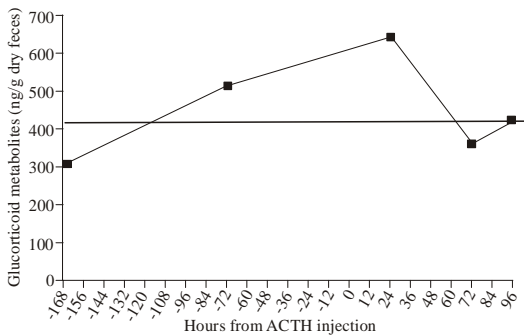


Fig. 4: Fecal Glucocorticoid (GC) metabolite concentration ($\mu\text{g/g}$ wet feces) profile from an ACTH challenge in a female lion using a corticosterone EIA. The ACTH injection (720 IU total) was administered 9 am on June 20 with sample collection beginning June 13 and continuing through June 24. Solid line represents the mean of pre-ACTH fecal GC metabolite concentration ($\mu\text{g/g}$ dry feces)

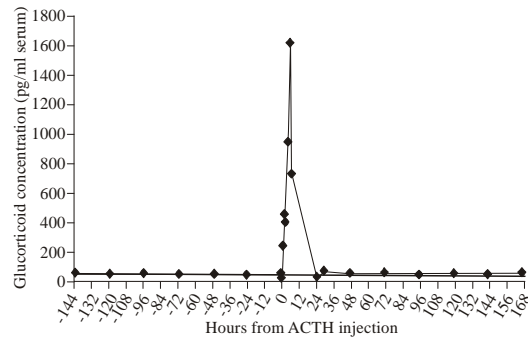


Fig. 6: Serum Glucocorticoid (GC) concentration (pg/mL) profile from an ACTH challenge in a female elephant using a corticosterone EIA. The ACTH injections (2000 IU total divided in three injections given 1 h apart) were administered at 9:30, 10:30 and 11:30 am, respectively on November 16 with sample collection beginning November 11 and continuing through November 25. Solid line represents the mean of pre-ACTH serum GC metabolite concentration (pg/mL)

Serum ACTH results: In the black rhino, mean pre-ACTH serum corticosterone value was 1286.8 ± 104.1 pg/mL (Fig. 5). Serum corticosterone peaked (1674.6 pg/mL) 2 h post-injection (Fig. 5). For the elephant, serum samples were elevated above the pre-ACTH mean (41.2 ± 5.9 pg/ml) 1 h post-injection (Fig. 6). Serum samples (856.4 ± 182.6 pg/mL serum) remained elevated for 7 h prior to returning to mean levels (Fig. 6).

DISCUSSION

This study was the first to determine the suitability of this corticosterone EIA to monitor adrenocortical activity

for an array of African species. Specifically, we determined that adrenocortical activity was reflected in concentrations of fecal GC metabolites suggesting that this EIA is an effective technique for four diverse (herbivores, omnivore and carnivore) African species including elephants, black rhinos, chimpanzees and lions. As with previous reports, we demonstrated that, with proper sample collection and minimal changes to daily routines, ACTH challenges can be used to effectively determine if a hormone assay is appropriate for monitoring adrenocortical activity. Although ACTH challenges have been documented in the other species,

this is the first time using this corticosterone EIA in all species and the first time an ACTH challenge has been documented in a lion.

Stress, a disruption of homeostasis (Ulrich-Lai and Herman, 2009), is a phenomenon that has been studied over time due to its involvement with the myriad of stimuli that threaten an animal's survival and success in life (Touma and Palme, 2005). In recent years techniques that measure an animal's response to stress have been developed to monitor the health and welfare of both zoo-housed and free-ranging animals. These methods can determine the relationship among an animal's hormone values, its environment and how it reacts to changes or fluctuations in its environment. In the present study, we validated the use of non-invasive methods to monitor the stress response via fecal GC metabolite values in an array of species. Potential stressors in an animal's captive environment such as presence/absence of critical sensory stimuli, as in light, sound, odor, space, social group dynamics and resource limitations (Davies and Retani, 2006), can be evaluated using this assay. For wild animals, understanding the effects of environmental pressures (e.g., human encroachment, habitat loss and/or climate change) on an animal's stress response has been complicated by issues of sample transportation logistics, permitting, sample collection, storage and processing. However, several recent field-friendly fecal hormone extraction methods (Whitten *et al.*, 1998a; Khan *et al.*, 2002; Beehner and Whitten, 2004; Ziegler and Wittwer, 2005; MacDonald *et al.*, 2008; Freeman *et al.*, 2010; Santymire and Armstrong, 2010) have been reported that facilitate the accurate application of endocrine analysis to free-ranging wildlife.

With the recent advancement in field endocrine techniques, more opportunities exist to gain a broad understanding of wildlife health and conservation. However, misinterpretation is a problem with fecal hormonal analysis because of several biological and technical confounds. Biological factors, including sex, age, metabolism rate, social pressures, diet, circadian and circannual rhythms and individual differences, can affect successful evaluation of fecal hormone values in wildlife (Wasser *et al.*, 1993; Whitten *et al.*, 1998a; Whitten *et al.*, 1998b; Millspaugh and Washburn, 2004; Reeder and Kramer, 2005; Touma and Palme, 2005). For example, in some social animals dominance rank has an inverse relationship with circulating levels of cortisol (Creel, 2001). With the expansion of field endocrinology studies there is an increasing need to have properly validated hormonal assays so that adrenocortical activity can be accurately evaluated. Validation begins in the laboratory using biochemical means to ensure that the hormonal assays are accurately measuring the hormonal values in the sample. These steps are usually completed, but many

studies fail to conduct biological and physiological means to validate the results. Here we used an ACTH challenge to confirm the reliability of a corticosterone EIA for measuring adrenocortical activity in the lion, elephant, black rhino and chimpanzee.

Many factors can impact the reliability of the ACTH challenge results including environmental changes, dosage and drug issues and injection procedures. For example, any change to an animals' environment or routine (i.e., squeeze chute or injection training) can increase the fecal GC values and lessen the impact of the ACTH injection. Another important factor is use of the correct dosage, which is often difficult to determine in unstudied species. For the elephant, chimpanzee and rhino, we used the dose that was documented in previous literature (elephant, Brown *et al.*, 1995; Wasser *et al.*, 2000; black rhino, Wasser *et al.*, 2000; Brown *et al.*, 2001; chimpanzee, Heistermann *et al.*, 2006). The lion ACTH dose determination was based on the clouded leopard (*Neofelis nebulosa*) dose (Wasser *et al.*, 2000; Wielebnowski *et al.*, 2002). Additionally, if the injection procedure invokes too much fear, which may result just from the presence of a veterinarian, a stress response may be initiated that could reduce the response to the exogenous ACTH or distort the lag-time of GCs from blood to its metabolism and excretion. For accurate testing, the animal's normal routine should not be altered from the time of baseline sample collection up through post-injection collection which should be at least 1 week after the challenge. Finally, the brand of ACTH may also vary the response. Unfortunately, the cost of some of the ACTH gels (such as Acthar Gel; quoted >\$20,000 for black rhino dose) are not feasible. However, the Cortrosyn used in this study was more reasonably priced, although switching the brand of ACTH may also mandate a change in the dose.

In addition to environmental changes, individual animals may respond differently to the exogenous ACTH (Touma and Palme, 2005) depending on subject-specific factors. Generally, males may have a higher metabolic rate resulting in a different rate of hormonal conjugation and excretion. Additionally, the concentration of metabolites excreted via urine or feces depends on the sex of the individual (Touma and Palme, 2005). For example, male mice (*Mus musculus*) excreted a higher proportion of radio-labeled corticosterone metabolites in feces compared to females (Touma *et al.*, 2004). Male flying foxes (*Pteropus hypomelanus*) have higher baseline GC concentrations than females (Reeder and Kramer, 2005). Dietary differences (i.e., more or less fiber) can alter the excretion rate (Wasser *et al.*, 1993). Also, age and health of the individual can affect the ACTH results. Or if the individual is already chronically stressed and has desensitized the GC receptors in the body, then he or she

may not respond at all to the exogenous ACTH. Despite these methodological concerns, we documented a response from all individuals to the ACTH as described below.

Serum GC values were measured in a black rhino during an ACTH challenge; however, serial blood samples were not taken to reduce the chances of inducing a stress response in addition to the exogenous ACTH. Therefore, we did not get an hourly profile, but nonetheless still observed the increase of corticosterone values but may have missed the peak of serum GC. Others have reported a ten-fold increase in serum cortisol but the time of the peak was not reported (Brown *et al.*, 2001). For fecal GC analysis, the lag-time from the time of injection until the surge of fecal GCs was later (~ 75 h post-injection) than what was previously published (25-50 h, Wasser *et al.*, 2000; 48 h, Brown *et al.*, 2001), which may have been attributed to one or two missing sample(s) that were not collected by animal care staff. Our sample collection went from 4 September 2008 0900 to 5 September 2008 1400, which was ~ 24-72 h post-injection, the timeframe when previous studies detected the peak of GC (Brown *et al.*, 2001; Wasser *et al.*, 2000). Even though all three studies (including the present study) used similar extraction procedures, we observed a ~ 2-fold increase in fecal GCs, which was lower to previous reports (~ 2.5-fold, Wasser *et al.*, 2000; 4-fold, Brown *et al.*, 2001) and somewhat suppressed compared to similar species (our elephant response in Fig. 6; previous elephant response, Brown *et al.*, 1995). This decreased response could be attributed to the age and sex of the black rhino (~ 20 years and male) in the present study. Wasser *et al.* (2000) conducted the ACTH challenge on a male and female black rhino and Brown *et al.* (2001) conducted their challenge on four males. Another explanation for the differences among these studies is that the others used a similar radio immunoassay (RIA) and we used a new EIA. Previous studies that used a corticosterone RIA (Wasser *et al.*, 2000; Brown *et al.*, 2001) diluted their black rhino samples 1:10 while we needed a 1:64, meaning that our corticosterone EIA was able to detect more corticosterone metabolites.

In the elephant, we observed elevated fecal GC ~ 50 h post-challenge. This response was delayed compared to previous reports (~ 36 h, Wasser *et al.*, 2000). Additionally, we observed a 2-fold increase above mean pre-ACTH fecal GCs, which was lower than the 4-fold (Wasser *et al.*, 2000) and 6-fold (Stead *et al.*, 2000) increase observed previously. In the present study, the elephant's GC metabolites were analyzed at very concentrated levels (analyzed neat on the assay) suggesting that corticosterone may not be the major GC metabolite in feces, which was previously reported using a different corticosterone EIA (Stead *et al.*, 2000). Although we were able to detect an increase in fecal GC

metabolites, there may be a more sensitive assay that has different cross-reactivities or a more specific assay that can detect more fecal GC metabolites in the elephant (Stead *et al.*, 2000; Ganswindt *et al.*, 2003).

The corticosterone EIA was able to detect increases in serum GCs in the elephant. Values peaked at 39-fold higher than the pre-ACTH mean value and were elevated for 7 h, which is similar to what was previously reported using serum RIA cortisol analysis (Brown *et al.*, 1995). For both the elephant and black rhino, the serum results demonstrated that the ACTH injections were successful and that the individuals were responding to the exogenous ACTH and not the blood collection, since we collected blood samples prior to and after the challenge.

We observed a response in fecal GC metabolites ~ 25 h post-challenge in the chimpanzee with peaks 1.5-fold over the mean of pre-ACTH samples. Heistermann *et al.* (2006) reported a 3.8-fold increase above baseline corticosterone values using a RIA. These authors determined that 11 β -hydroxyetiocholanolone is a more suitable assay for detection of changes in fecal GC metabolites in response to adrenocortical stimulation than cortisol, corticosterone and 11-oxo-etiocholanolone (Heistermann *et al.*, 2006). However, others have determined a 4-fold increase in fecal cortisol metabolites 48 h post-anesthesia (Whitten *et al.*, 1998b). Using radio-labeled cortisol (^3H), a 22.2 h post-injection lag-time was observed in a male chimpanzee (Bahr *et al.*, 2000). Interestingly, during this chimpanzee ACTH challenge, saliva samples were collected (Heintz *et al.*, 2011). Results demonstrated an immediate response with elevated salivary cortisol concentrations increasing from 15 min through 3 h with a peak of 8-fold over the baseline occurring 45 min post-ACTH injection (Heintz *et al.*, 2011).

In contrast to the other species in this study, there are no published results to compare the results of the ACTH challenge conducted in the lion. Although only one lion was used on the study, adrenocortical activity was observed with a 1.5-fold increase in fecal GC metabolites occurring ~ 24 h post-injection. ACTH challenges have been conducted on other feline species including cheetah (*Acinonyx jubatus*, Terio *et al.*, 1999; Wasser *et al.*, 2000) which had a ~ 14-fold increase in fecal corticosterone metabolites 48 h post-injection. In clouded leopards, Wasser *et al.* (2000) and Wielebnowski *et al.* (2002) both observed an 8-fold increase in fecal GCs ~ 25 h post-ACTH challenge over baseline. Additionally, ACTH challenges have been conducted in the domestic cat (*Felis catus*), using serum, urine and feces (Carlstead *et al.*, 1992; Chatdarong *et al.*, 2006; Graham and Brown, 1996; Middleton *et al.*, 1987; Scatz and Palme, 2001). The GC values from the serum and urine results cannot be directly compared to the fecal analysis (Middleton *et al.*, 1987; Carlstead *et al.*, 1992; Chatdarong *et al.*, 2006). However,

a 2 to 8-fold increase in fecal cortisol metabolites was observed 24 to 48 h post-ACTH injection in male and female intact and gonadectomized domestic cats (Graham and Brown, 1996). An additional domestic cat study observed a 24 h surge of fecal GC metabolites (using different assays: cortisol and 11-oxoetiocholanolone) that were 1 to 9-fold greater than basal concentrations (Schatz and Palme, 2001). Interestingly, Schatz and Palme (2001) determined that the HPA stimulation was not detected using corticosterone EIA. This study observed a similar single point 2-fold increase in fecal GC metabolites 24 h post-ACTH using the corticosterone EIA.

CONCLUSION

Our ACTH challenge results demonstrate this corticosterone EIA could be used to monitor adrenocortical activity in four African species—elephant, lion, black rhino and chimpanzee, although there are other hormonal assays that may be more sensitive and, therefore, may be more suitable for adrenocortical activity analysis. However, using this EIA technique is advantageous because it does not use radioactive material and is user-friendly making it is applicable to conducting field research in developing countries in Africa and elsewhere. For example, a similar EIA also developed by C. Munro has been converted to a simple field pregnancy kit for elephants (Freeman *et al.*, 2010, 2011). Additionally, coupled with new field extraction methods (MacDonald *et al.*, 2008; Freeman *et al.*, 2010, 2011; Santymire and Armstrong, 2010), samples can now be processed and analyzed in the field eliminating logistical constraints associated with sample shipping, resources and disease risk.

Further research into the effects of HPA stimulation on sex and age of these species is important to obtain an improved understanding on how stressors are impacting these populations. As conservation becomes more multidisciplinary, it is important to develop new non-invasive tools that provide a more comprehensive picture of wildlife health. Future development and validation of new techniques is a necessity to advance the field of wildlife and comparative endocrinology and determine how environmental changes affect wildlife populations.

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