EVALUATION OF FECAL GLUCOCORTICOID METABOLITE ASSAYS FOR SHORT-TERM STRESSORS AND VALIDATION FOR STRESS MONITORING IN AFRICAN HERBIVORES

A Thesis presented to the Faculty of the Graduate School
University of Missouri – Columbia

In Partial Fulfillment
of the Requirements for the Degree

Master of Sciences

---

by

SATHYA K. CHINNADURAI, D.V.M.

Joshua J. Millspaugh, Ph.D., Thesis Supervisor

AUGUST 2006

The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

EVA LUATION OF FECAL GLUCOCORTICOID METABOLITE ASSAYS FOR SHORT-TERM STRESSORS AND VALIDATION FOR STRESS MONITORING IN AFRICAN HERBIVORES

Presented by Sathya K. Chinnadurai

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance.

Dr. Joshua J. Millspaugh

Dr. James Schadt

Dr. Matthew Gompper

Dr. Mark Milanick

ACKNOWLEDGMENTS

First and foremost, I would like to thank my collaborators on both chapters. Chapter 1 was a collaborative project and the co-authors were Dr. J. Schadt, Dr. B. Washburn, T. Mong, Dr. M. Milanik and my advisor, Dr. J. Millspaugh. Financial and logistical support was provided by the University of Missouri (MU) Department of Fisheries and Wildlife Sciences, an MU Life Science Mission Enhancement Postdoctoral Fellowship, an MU Research Board Grant, the Missouri Department of Conservation (Federal Aid in Wildlife Restoration Project W-13-R), and the 2001 Webless Migratory Game Bird Research Program (United State Fish and Wildlife Service and the United States Geological Survey – Biological Resources Division). We thank B. Crampton, S. Kistner, B. Hoenes, T. Bonnot, R. Woeck, and C. Rittenhouse for their assistance in pen construction and/or feces collection for the mourning dove project and J. Ivey and M McKown and C. Lenox for assistance with the NZW rabbit project. Dr. E. Blaine and the Dalton Cardiovascular Research Center provided assistance and funding for the NZW project. The Mourning dove project was approved by the University of Missouri Animal Care and Use Committee under Protocol #3581 and the NZW project was under protocol #595.

Chapter 2 was a joint effort by our laboratory and Dr. R. Slotow at the University of Kwa-Zulu Natal at Durban. Dr. Slotow, Dr. W. Winter at Tembe Elephant Park and their technicians, graduate students, and field biologists dedicated many hours to sample collection, processing and shipment. Financial support for travel and sample collection was provided by the Brown Fellowship for International Agriculture and the University of Missouri College of Agriculture Food and Natural Resources. Fecal glucocorticoid assays were conducted in the Wildlife Stress Physiology Laboratory in the Department of Fisheries and Wildlife Sciences at the University of Missouri-Columbia. I greatly thank R.J. Woods for technical work in sample processing and assaying.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

List of Tables

Table Page

CHAPTER 1

USE OF FECAL AND PLASMA GLUCOCORTICOID ASSAYS TO QUANTIFY THE EFFECTS OF SHORT-TERM STRESSORS

ABSTRACT

During stressful periods, glucocorticoid (cortisol and corticosterone) production and release, by the adrenal glands, is increased. These hormones and their metabolites are found in blood, urine and feces. Previous studies have found measurements of fecal glucocorticoid metabolites (FGM) accurately detect long-term stressors. Our goal was to determine if FGM measurements are a reliable means of detecting short-term (<30 min) stressors in two animal models. We quantified FGM levels after short-term stressors and compared them to plasma glucocorticoid levels in captive raised New Zealand White rabbits (*Oryctolagus cuniculus)* and wild caught Mourning doves (*Zenaida macroura*) held in captivity. We instrumented rabbits with venous catheters and subjected them to three separate stressors: air jet, oscillation, and sham (control) for 20 min each. We drew ten blood samples at 5 min intervals before, during and after the stressor. We collected fecal samples every hour post-treatment for 12 hr and one sample 24 hr post-treatment. We exposed Mourning doves to a brief (≤2 min) capture, handling, and release treatment and a capture stress protocol (i.e., capture, restrain for 30 min, and release). We collected feces every hour for 24 hr pre-treatment and 36 hr post-treatment. We quantified plasma and fecal corticosterone metabolites using a double-antibody radioimmunoassay (ICN). While both species showed increases in plasma corticosterone metabolite levels in response to short-term stressors, we did not detect corresponding changes in FGM levels. The inability to track changes in glucocorticoid production with fecal samples indicates

FGM monitoring is not reliable for detecting short-term stressors. For short-term stressors, plasma corticosterone levels may be a more useful parameter. Thus, FGM assays are best suited to studies investigating the effects of chronic stressors in animals. We discuss the advantages and disadvantages of FGM monitoring in wild animals.

INTRODUCTION

Animals respond to stressors through a variety of mechanisms. Broadly defined, a stressor is anything that causes an animal to stray from homeostasis. Autonomic responses mediate the "fight or flight" response and are beneficial for short activities and include changes in heart rate, blood pressure, and gastrointestinal motility (Hilton, 1982; Yousef, 1988). Neuro-endocrine responses mediated through the Hypothalamic-Pituitary-Adrenal (HPA) Axis have longer lasting effects on a number of functions including immunity, reproduction, and metabolism (Moberg, 2000; Vleck *et al.,* 2000; Sapolsky *et al.,* 2000). During periods of stress, the HPA axis increases its production of stress hormones, cortisol and corticosterone (Harvey, 1984). These hormones and their metabolites can be measured in blood, tissues, urine and feces of animals (Wingfield *et al.,* 1994; Wasser *et al.,* 2000).

To avoid the stress inherent in blood sample collection, measurement of fecal glucocorticoid metabolites (FGM) has gained popularity (Graham and Brown, 1996; Harper and Austad, 2000; Le Maho *et al.,* 1992; Cook *et al.,* 2000; Wasser *et al.,* 2000; Millspaugh *et al.,* 2001; Millspaugh *et al.,* 2002; Millspaugh and Washburn, 2004). Such techniques make it is possible to collect fresh fecal samples in the field without disturbing the animal, allowing for a noninvasive assessment of stress. Glucocorticoids are

metabolized in the liver before passage through the biliary system and gastrointestinal tract before accumulation and excretion in the feces (Bokkenheuser and Winter, 1980; Harper and Austad, 2000). Thus, FGM measurements may show net effects accumulated over time, which may be missed by a single plasma sample. In contrast, plasma hormone measurements taken over a period of time can be highly variable due to the pulsatile release of stored glucocorticoids into circulation and this effect is muted in fecal samples (Harper and Austad, 2000; Montfort *et al.,* 1993; Goymann, 2005).

When interpreting FGM results gathered from an animal or population, it is essential to determine not only the presence of stress, but also, the nature of that stress. Chronic (long-term) and acute (short-term) stressors are of different significance to the well-being of the animal (Moberg, 2000). The response to an acute stressor can be beneficial (e.g. glucocorticoid release can mobilize glucose and provide energy to escape a predator). Chronic stress, on the other hand, can prove maladaptive by decreasing reproductive and immune function over time (Harvey *et al.,* 1984; Harper and Austad, 2000; Sapolsky *et al.,* 2000). Both acute and chronic stressors can cause increases in plasma glucocorticoid levels (Harper and Austad, 2000; Washburn *et al.,* 2003).

Despite the increasing popularity of FGM analysis (Wasser *et al.,* 1997; Wasser *et al.,* 2000; Ludders *et al.,* 2001) for investigating the impacts of long-term stressors, such as social structure, tourist activity and parasitism (Foley *et al.,* 2001; Millspaugh *et al.,* 2001; Creel *et al.,* 2002; Goldstein *et al.,* 2005), it is unknown whether this technique is capable of detecting responses to individual short-term stressors (those lasting less than 30 min) in wild animals. If FGM levels increase due to an acute stressor, it is possible that occasional acute stressors can give a misleading appearance of chronic stress. On the

other hand, an inability to detect short-term stressors in fecal samples might lead to a false conclusion that the animal is under no stress whatsoever.

We were interested in evaluating whether or not FGM analyses could detect responses to short-term stressors, such as capture, handling, and restraint commonly associated with techniques to collect biological data (e.g., attach leg bands or radio transmitters). Thus, we examined the effects of short-term (<30 min) stressors on blood corticosterone and FGM levels. We chose two animal models, New Zealand White (NZW) rabbits (*Oryctolagus cuniculus)* and Mourning doves *(Zenaida macroura*) to provide a more comprehensive understanding of short-term stress assessment. The rabbit model allowed us to examine the effects of stressors in a controlled laboratory environment with animals that are accustomed to the laboratory setting and to human contact, while the Mourning doves allowed us a more realistic model applicable to field wildlife studies.

We hypothesized that an acute stressor would cause a rapid increase in plasma corticosterone levels and a smaller, delayed increase in the fecal corticosterone metabolites. To our knowledge, this is the first experiment to correlate plasma corticosterone and fecal corticosterone metabolites levels with a single short-term stressor.

METHODS

NZW Rabbit Experiment

Experimental animals

By using New Zealand White rabbits that were captive bred and acclimated to humans and the laboratory setting, we minimized environmental factors leading to a stress response. We used 7 rabbits for this experiment; 3 male and 4 female. We individually housed the rabbits in stainless steel cages with mesh flooring and a paper lined drop pan to collect feces. We fed rabbits a standard diet of 135 g of rabbit feed and *ad libitum* water, except we removed food for 14 hr pre-surgery and 14 hr before each treatment.

Under halothane anesthesia, we instrumented all 7 rabbits with arterial and venous catheters according to a previous protocol (Schadt and Hasser, 1998). We placed catheters in the abdominal aorta to measure arterial blood pressure and heart rate (part of another experiment) and in the caudal vena cava for withdrawal of blood samples. We tunneled catheters through subcutaneous tissue and exited the skin on the dorsal aspect of the neck. These catheters could then be accessed during the experiment without handling the rabbit. We gave each rabbit 22.7 mg of enrofloxacin subcutaneously, immediately before surgery and 24 hr post-surgery. We gave each rabbit 0.06 mg of buprenorphine subcutaneously immediately post-surgery; the dose was repeated 12 hr later. We allowed animals to recover for at least 14 days after surgery, prior to experimentation.

Experimental procedures

We subjected each rabbit to two 20 min stressor treatments and a sham (control) treatment. No more than one experiment per rabbit was performed per week. For all treatments, we placed the animal in a box measuring 33x15x18cm. Twenty-four hours before each treatment, we placed the animal in the containment box for one hour for acclimation to the laboratory. We fasted the rabbits overnight before each treatment and

placed a fresh liner in the litter pan under the cage. All experiments began at the same time of day to minimize the effects of daily cyclic variations in corticosterone production (Harper and Austad, 2000). Immediately before each treatment, we collected an overnight fecal sample from the drop pan. Before each treatment, the rabbit was left undisturbed for 10 min to acclimate to the laboratory.

The two stressor treatments used to test the rabbits, an air jet and an oscillator, have been shown to induce a stress response in rabbits (Schadt and Hasser, 1998; 2001). The air jet treatment consisted of a constant stream of air from a polyvinyl chloride tube directed at the rabbit's nose through a 6-cm diameter hole in the front of the containment box. The air tube was positioned 4-6 cm from the front of the box. For the oscillator treatment, the rabbit was placed in the containment box on an oscillating shaker that moved counterclockwise in 2 cm circles at a rate of 48 cycles/min. The sham treatment consisted of letting the rabbit sit undisturbed in the same containment box used for the other treatments for the same period of time. We subjected each rabbit to each of the three treatments in a random order.

Plasma sample collection

For each experiment, we collected venous whole blood samples from the catheter in the caudal vena cava, before, during and after the stressor treatment. For each blood draw, 3 mL of blood were drawn into a syringe, to clear any blood or saline in the catheter tube. Once 3 mL was withdrawn, a 1 mL blood sample was drawn into a separate syringe and placed on ice. The 3 mL of blood and saline were returned to the animal followed by 1 mL of heparinized saline flush.

The first sample (t = -15 min) was drawn immediately after placing the animal in the containment box. Three more samples were drawn 5 min apart. Immediately following the fourth sample (t = -0.5 min), we started the treatment (air, oscillation or sham). The next sample (t = 0.5 min) was drawn 30 sec after beginning the treatment. Samples were drawn after 5, 10, 15, and 20 min of treatment. A final sample was drawn 5 min after the treatment was stopped. We stored all blood samples on ice until the end of the experiment and then centrifuged all samples for 5 min. We removed the plasma from each sample and transferred it to a separate tube; we stored all plasma samples at -20°C, until they could be assayed.

Fecal sample collection

After the final blood sample was collected, we returned the rabbit to its cage in the housing facility and fed it. A fecal sample, consisting of all feces in the dropping pan under the cage, was collected 1 hr after the termination of the stressor. No soft cecotropes pellets were collected. Subsequent samples were collected every hour for 12 hr. We collected one overnight sample consisting of feces deposited 12-24 hr post-stress. We immediately froze all fecal samples at -20°C, until they could be processed.

Mourning Dove Experiment

Experimental animals

We captured wild Mourning doves using modified Kniffin traps (Reeves *et al.,* 1968). Upon capture, we banded each bird with an individually numbered metal leg band and assigned age and gender based on plumage characteristics (Mirarchi, 1993; Schulz *et al.,* 1995). The doves were kept individually in outdoor 1.8x1.8x1.8 m wooden framed pens raised 0.6 m above ground.

We randomly assigned individual birds to one of three treatments, a sham (control), a capture, handle and release treatment (CHR) and a capture stress protocol (CSP) treatment (Wingfield *et al.,* 1992). Using a total of 10 birds, we attempted to assign 1 male and 1 female to each treatment, but lack of available individuals precluded equal numbers of males and females in each treatment.

Experimental procedures

The sham treatment was administered in winter and summer, the CHR treatment was conducted in summer, and the CSP treatment was conducted during two experiments in winter (Table 1). For all treatments, Time 0 represents the beginning of stress treatment; control birds were left undisturbed in their pens at Time 0, when other birds were subjected to treatments. We captured birds assigned to the CHR treatment using a mesh net, removed the birds from the net, and held them in our hands for 50 sec. We then released them into their respective pen (total disturbance time of 72 +/-3 SE sec). We repeated this same procedure with each bird 30 min later. We used two separate disturbances in the CHR treatments to extend the length of adrenocortical stimulation (Washburn *et al.,* 2002; Washburn *et al.,* 2003). Similar to the CHR group, those assigned to the CSP group were captured using a net; however, these birds were removed from the net and immediately placed into a breathable cotton sack. We placed the birds in sternal recumbence in the sack for 30 min, at which time we released them back into their respective pens.

Fecal sample collection

We collected droppings, consisting of mixed feces and urates from each individual bird every hour, when available, beginning 24 hr pre-treatment and continuing

for 30-36 hr post-treatment. We froze fecal samples at –20°C within 10 min of collection.

Laboratory Methods

Fecal hormone metabolite extraction-NZW rabbits and Mourning doves

For both experiments, we dried frozen fecal samples in a lyophilizer for 24 hr. Once freeze-dried, we sifted samples through a stainless steel mesh to remove large particles and then thoroughly mixed each sample. We extracted fecal glucocorticoid metabolites from feces using a modification of Schwarzenberger *et al.* (1991). We placed dried feces (0.200-0.220 g for rabbits and 0.016 to 0.120 g for Mourning doves) in a glass test tube with 2.0 mL of 90% methanol and vortexed the sample at high speed in a multi-tube vortexer for 30 min. We centrifuged samples at 2,200 rpm for 20 min, removed the supernatant, and stored it at –84^{o} C until assayed.

RIA validation for NZW plasma corticosterone and FGM measurements

We used an I^{125} corticosterone radioimmunoassay (RIA) kit (ICN #07-120103, ICN Biomedicals, Costa Mesa, CA, USA), which was previously validated for use in Mourning doves to quantify plasma corticosterone (Washburn *et al.,* 2002) and fecal glucocorticoid metabolite concentrations (Washburn *et al.,* 2003). We followed the ICN protocol, except that we halved the volume of all reagents. For fecal measurements, the methanol-hormone mixture was used instead of plasma. While this kit is designed to quantify the amount of native corticosterone in plasma, it has been validated for FGM monitoring in a wide range of species (Wasser, 2000). In our laboratory, we have been able to use it successfully in multiple mammals and birds (Millspaugh *et al.,* 2001; Millspaugh *et al.,* 2002; Washburn *et al.,* 2002; Washburn *et al.,* 2003).

We conducted a standard laboratory validation for NZW plasma and fecal samples (Jeffcoate, 1981; O'Fegan, 2000). We tested samples for parallelism by comparing serial dilutions (1:2 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) to a corticosterone standard curve. On a log-transformed scale, we plotted percent binding vs. concentration of standard and percent binding vs. concentration of sample. Parallelism of the sample dilution curve with the standard curve was determined using SAS software. We used the sample dilution that resulted in a percent binding closest to 50% for all future assays (1:8 for NZW plasma and 1:8 for NZW feces). Accuracy was measured by recovery of a known amount of standard from spiked samples. We mixed the diluted sample 1:1 with each of the 3 standards supplied with the RIA kits (50, 100 and 250 ng/mL). We calculated a mean and standard deviation for recovery of exogenous hormone for both plasma and fecal samples; acceptable recovery was determined to be between 90-110% (unpublished data).

RIA quantification of plasma corticosterone and FGM

Once validated, we ran all NZW plasma samples according to the standard ICN protocol, with halved volume of reagents (Wasser *et al.,* 2000), using a 1:8 dilution of plasma to steroid diluent (supplied with RIA kit). For fecal assays, we used the methanol extract instead of plasma and samples were diluted 1:8 for NZW and 1:4 for Mourning doves (Washburn *et al.,* 2003). We ran all samples in duplicate and reran any samples with a coefficient of variation (CV) greater than 15%.

Statistical Analyses

For the NZW experiment, we compared plasma sample data from pre-stressor (samples from time -15, -10, -5, -0.5 min) and during/post-stressor sets (samples from

time 0.5, 5, 10, 15, 20, 25 min). For each treatment, we performed a repeated measures one-way ANOVA to compare pre-treatment and during/post-treatment plasma sample sets (Zar, 1996).

We combined rabbit fecal sample data into five time periods for each treatment. Period 1 included data from feces deposited the night before the procedure (14 hr pre-treatment). Period 2 included samples collected between 1-4 hr post-treatment, Period 3 included 5-8 hr, Period 4 included 9-12 hr and Period 5 included the overnight samples from 12-24 hr post-treatment. We performed a repeated measures one-way ANOVA to determine whether FGM levels differed by time periods and an LSD post-hoc comparison to determine difference between individual time periods (Zar, 1996). We used a repeated measures one-way ANOVA to determine if Mourning dove FGM levels differed pre- and post-treatment. All analyses were considered significant at $P < 0.05$.

RESULTS

NZW Rabbit Experiment

We collected 10 plasma samples per rabbit during each experiment, for a total of 30 samples per rabbit. We collected an average of 9.5 fecal samples per rabbit per experiment (SE = 2.560, Range = 2–13, $n = 7$). One rabbit died of unknown causes after only undergoing the sham treatment.

NZW rabbit plasma corticosterone data

For the sham stress treatment (Figure 1A), there was no significant difference in plasma corticosterone levels between the pre and post-treatment sample sets ($F = 0.183$, df = 1, $P = 0.670$). For the air jet and oscillator stressors (Figures 1B, 1C) post-stressor

plasma corticosterone metabolites levels were significantly elevated compared to pre-stressor levels ($F = 12.447$, df = 1, $P<0.001$ for air jet and $F = 5.149$, df = 1, $P = 0.029$ for oscillator).

NZW rabbit fecal corticosterone metabolite data

We found no significant difference in fecal corticosterone metabolite levels between the three treatments during any time period. For the sham treatment, the pre-stressor FGM levels (Period 1) were significantly higher ($F = 3.806$, df = 4, $P<0.015$) than the daytime post-stressor (Period 2, 3, 4) samples (Figure 2A). There was no significant difference ($P = 0.063$) between overnight samples collected the night before and the night after treatment (Periods 1 and 5). There were no significant differences in FGM between Periods 2, 3 and 4 ($P = 0.181$ between Periods 2 and 3; $P = 0.801$ between Periods 3 and 4; $P = 0.128$ between Periods 2 and 4).

For the air jet treatment, the Period 1 FGM level was significantly higher ($F = 5.256$, df = 4, $P<0.023$) than the Period 2, 3 and 4 levels (Figure 2B). Again, there was no significant difference ($P = 0.622$) between overnight samples collected the night before and the night after each treatment (Periods 1 and 5). There were no significant differences between Periods 2, 3 and 4 ($P = 0.282$ between Periods 2 and 3; $P = 0.546$ between Periods 3 and 4; $P = 0.617$ between Periods 2 and 4).

We found no significant differences in FGM levels between any of the treatment periods in the oscillator treatment ($F = 0.836$, df = 4, $P = 0.516$ for difference between groups) (Figure 2C).

Mourning Dove Experiment

We collected and assayed an average of 53.5 samples/bird (SE = 2.33, Range =

47–60, n = 10) during this study (total of 535 fecal samples). Mourning doves defecated at a rate of 0.9 samples/bird/hour during our experiments (SE = 0.04, Range =0.8–1, n = 10). In total, we had two control groups, one CHR group and two CSP groups (Table 1).

Both control groups showed significant elevations in FGM levels after Time 0 (for summer 2001 control, F = 43.34, df = 122, P = <0.001; for winter 2002 control, F=9.61, df=99, P = 0.01) (Figures 3 and 4). Although FGM concentrations varied greatly for the two females receiving the CHR treatment (Figure 5), there was no significant elevation in FGM levels post-treatment (F = 0.31, df = 121, P = 0.693). FGM concentrations in CHR birds were similar to the levels of the control group birds collected at the same time (Figure 3). The maximum FGM level observed post-treatment in the first CHR female (55 ng/g at 25 hr) and second CHR female (46 ng/g at 16 hr) did not occur within the expected 4 hr range observed by Washburn *et al.* (2003). Consequently, we did not attribute these pulses to the CHR treatment.

The winter 2001 CSP group showed significant elevations in FGM levels following treatment (F=18.23, df=95, P < 0.001), while the winter 2002 CSP group did not (F=1.96, df=100, P = 0.148) (Figures 6 and 7). The maximum FGM level observed post-treatment in CSP birds did not occur within the expected 4 hr range observed by Washburn *et al.* (2003). Fecal glucocorticoid concentrations in capture stress protocol birds in winter 2002 were similar to the levels of the winter control group birds (Figure 4).

We observed the greatest variation and highest fecal glucocorticoid levels for the two females in the winter 2001 CSP group; these birds were held for six weeks in captivity prior to the start of experiments (Table 1). In contrast, we observed the least

amount of variability and the lowest overall fecal glucocorticoid concentrations in control birds during winter 2002. These birds were held in captivity for 20 weeks prior to the experiment, the longest of any birds (Table 1).

DISCUSSION

Our study evaluated changes in fecal glucocorticoid metabolite levels in New Zealand White rabbits and Mourning doves after short-term stressors and compared these changes to those found in plasma corticosterone levels. We found that while plasma corticosterone levels may be a useful indicator of short-term stress, FGM measurement was not.

Our rabbit model suggests that a brief (<30min) stressor can appreciably change plasma corticosterone levels over a 20 min sampling period. Circulating glucocorticoids have been used to quantify the effects of short-term stressors in multiple species including: captive Mourning doves (Roy and Woolf, 2001; Washburn *et al.,* 2002), other avian species (Romero and Romero, 2002; LeMaho *et al.,* 1992), roan antelope *(Hippotragus equines)* and impala *(Aepyceros melampus)* (Hattingh, 1998), oldfield mice (*Peromyscus polionotus*) (Good *et al.,* 2003) and domestic cattle (Earley and Crowe, 2002). Our experimental stressors caused a rapid increase in circulating corticosterone levels within the 25 min post-stressor sampling period. Given the rapid increase in corticosterone levels after the onset stress in our rabbits and in the other species listed above, we believe this measure would be most useful in controlled situations, in which the stressor has a well defined starting and ending point. This would allow for accurate pre and post-stressor sampling. While plasma corticosterone measurements are useful for

measuring the effects of short-term stressors, the method is still limited by its invasiveness and is highly influenced by the stress of sample collection.

Non-invasive assessment of stress using FGM can avoid sampling induced stress, but both of our models showed that short-term stressors do not change FGM levels reliably enough to make meaningful conclusions about the animal's stress level. We did not detect significant differences in the pre and post-stressor FGM levels in the NZW rabbit model. The difference detected in the control and 2002 CSP groups of Mourning doves was likely a type 1 statistical error due to low number of birds. We did not detect a reliable difference between the control and treatment groups in either model. Thus, FGM monitoring may not be a useful tool in short-term stress assessment.

Fecal glucocorticoid metabolite monitoring will likely miss isolated peaks in glucocorticoid production, which may be detectable in serial plasma samples (Goymann, 2005; Touma and Palme, 2005; Mateo and Cavigelli, 2005). Fecal glucocorticoid metabolite levels represent the cumulative amount of metabolized and excreted hormones of a period of hours in mammals or shorter in birds depending on intestinal transit time. (Harper and Austad, 2000; Palme *et al.,* 2005). As FGM analysis reflects adrenocortical activity over an extended time period (Harper and Austad, 2000), a short period of increased corticosterone production may be undetectable in the long-term integrated measure of fecal glucocorticoid metabolite levels. While this limits the use of FGMs for short-term stress assessment, it suggests that short-term stressors do not cause a change in FGM levels, which could be mistaken for the effects of a chronic stressor. This is of special importance in wildlife studies, in which animals may be subject to chronic stress, such as climate or decreased food availability, but also, to acute stressors such as contact

with humans or predators. Short interaction with humans, including capture and banding or sample collection for research, should not elevate FGM levels enough to give a false impression of chronic stress.

Possible confounding factors in this project include the influence of circadian rhythms and individual variation on FGM levels. For the sham and air stressor treatments, the rabbits showed significantly elevated FGM levels during the overnight sampling periods. Rabbits are crepuscular animals that are most active during the early evening. The circadian rhythm results in peak glucocorticoid production when the animal is most active and the individual variation is greatest at the nadir of production (Whitten *et al.,* 1998), which would be during the day time sampling periods (Period 2-4). Circadian variation could result in an increase in overnight glucocorticoid production documented here. It was not possible for us to determine if there was an isolated peak in FGM levels during the night, as the samples were pooled samples of all the feces deposited overnight. Fecal glucocorticoid metabolite analysis is sensitive enough to detect circadian rhythms in adrenal activity in laboratory rats (Cavigelli *et al.,* 2005). In our model, it is likely that changes in glucocorticoid production related to normal circadian rhythms are large enough to mask the relatively minor increases in FGM due to our short-term stressors.

One possible flaw of our experimental design was a short sampling period for both blood and fecal samples. It is possible that the increased level of circulating corticosterone in these rabbits could have continued longer than the sampling period of 20 min post-treatment. Given our short sampling period, we were unable to determine when the circulating corticosterone levels returned to baseline after the termination of the

stressor. Our plasma samples may also have been heavily influenced by the pulsatile release of corticosterone into circulation, as evidenced by high variability in corticosterone levels between samples within individual rabbits. We limited the effect of this problem by collecting multiple pre and post-treatment samples and comparing pre and post-treatment sets, instead of individual sample times, allowing for an average assessment of pre and post-treatment stress level. Another possible limitation of this study stems from the unique gastro-intestinal physiology of rabbits. Lagomorphs ingest soft, partially digested excreta, termed cecotropes, after the first pass through the digestive tract (Cheeke, 1994). Ceoctropes are then redigested and nutrients are reabsorbed and feces are produced. This unique type of coprophagy may delay the time of detection of FGM, or may result in erroneous FGM levels due to dual metabolism (Bokkenheuser, 1980; Teskey–Gerstl *et al.,* 2000). For this study, only the final fecal pellets were collected. The results of this experiment should be corroborated using other mammalian laboratory species.

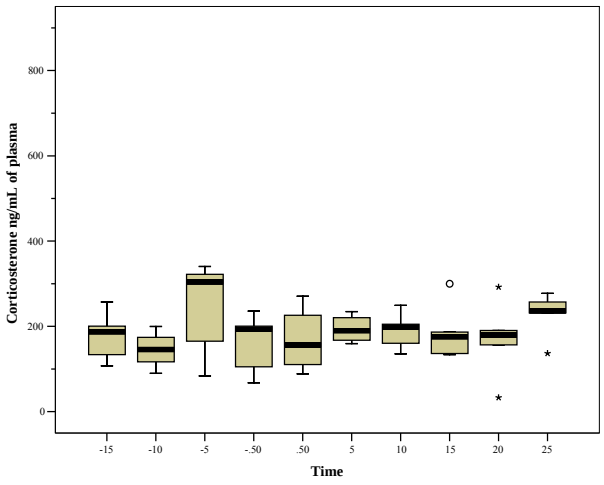
While our findings suggest limiting the use of the FGM measurements in short-term stress studies, we do not discourage the use of FGM for wildlife stress assessment and we make the following suggestions, for future studies:

1) Fecal glucocorticoid measurements are best used for the study of chronic stress. For wildlife management, chronic stressors are often the most important. Response to acute stressors usually serves to maintain homeostasis and is essential to survival. Management may not be able to control each individual stressor, but policy can be enacted, or management practices changed, when it is evident that there is a chronic stress on the population.

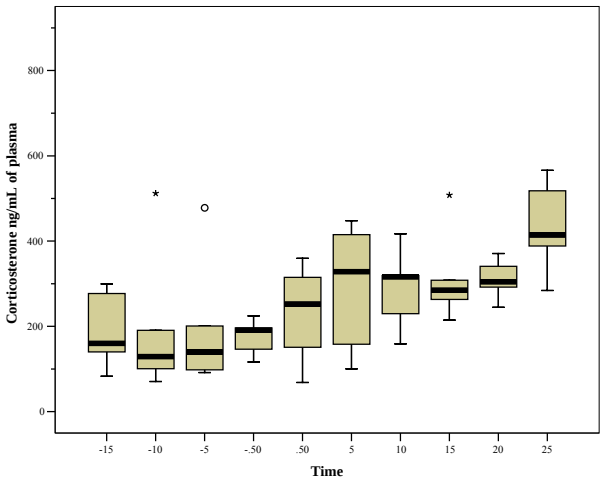
2) Investigators should be able to conduct capture and release type experiments, while still accurately monitoring chronic stressors. Short research associated stressors, such as capture and radio-tagging do not appear to cause a significant change in the FGM levels. Chronic stress studies involving captive and free-ranging wildlife can be conducted with minimal concern that brief encounters with predators or humans will affect FGM levels, thus clouding interpretation.

In conclusion, our results are significant for two reasons. First, those wishing to study acute stressors may be best served monitoring plasma glucocorticoid levels or parameters such as heart rate and blood pressure (Schadt and Hasser, 1998; Schadt and Hasser, 2001). Second, those monitoring chronic stressors with FGM levels can do so without concern that acute stressors, such as a capture and restraint, would mask the presence or absence of chronic stressors.

(1A)



(1B)



(1C)

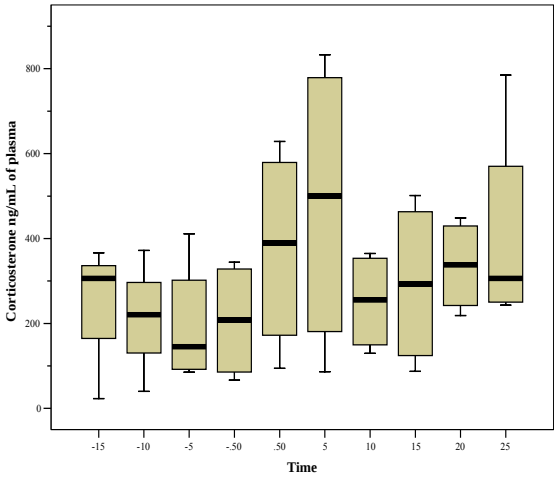


Figure 1. Plasma corticosterone levels (ng/mL) before, during and after stressor treatment. Stressors were initiated between t = -0.50 and t = 0.50 and discontinued at t = 20. The three treatments were sham (1A), air jet (1B) and oscillator (1C). Statistical comparisons were made between pre-stressor sample sets (-15, -10, -5, -0.5) and post-stressor sample sets (0.5, 5, 10, 15, 20, 25)

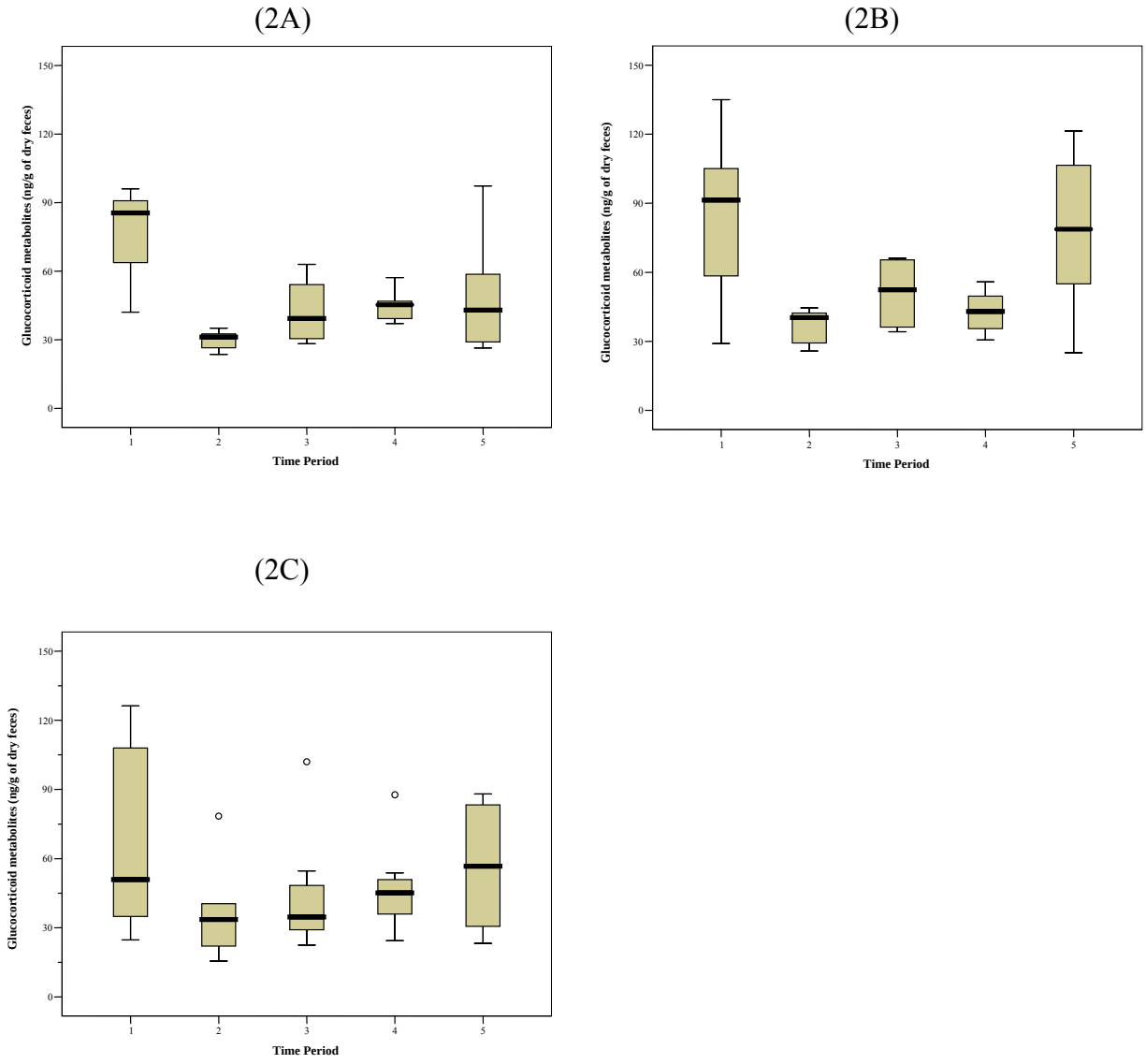


Figure 2. Fecal glucocorticoid metabolite concentrations (ng/g of dry feces) for New Zealand White rabbits. We administered three treatments: sham (2A), air jet (2B) and oscillator (2C). We combined fecal sample data into five time periods for each treatment. Period 1 included data from feces deposited the night before the procedure (14 hr pre-treatment). Period 2 included samples collected between 1-4 hr post-treatment, Period 3 included 5-8 hr, Period 4 included 9-12 hr and Period 5 included the overnight samples from 12-24 hr post-treatment.

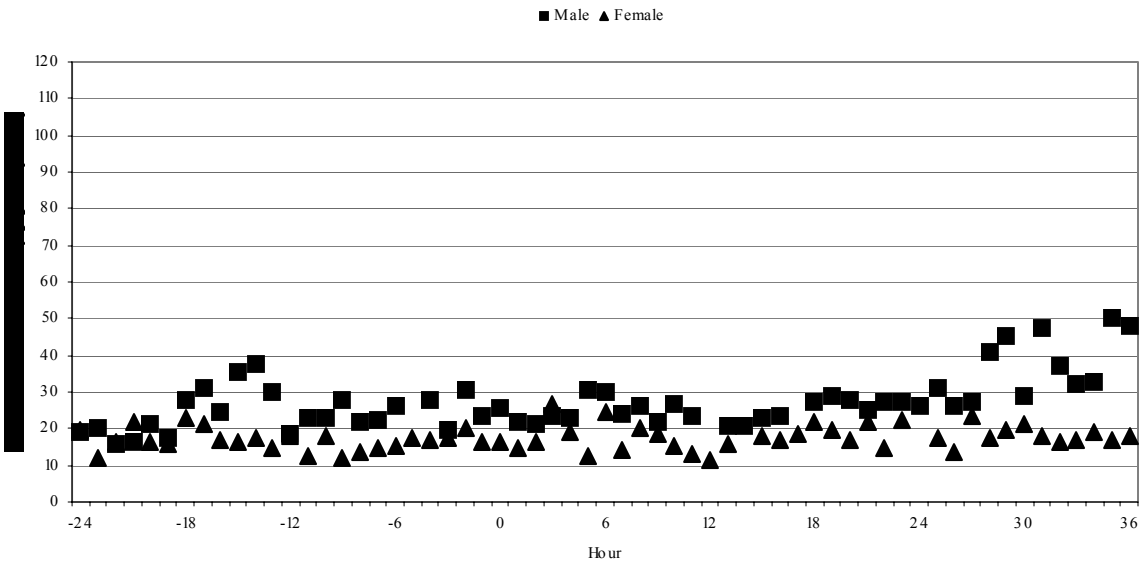


Figure 3. Fecal glucocorticoid concentrations (ng/g) of two control Mourning doves in summer 2001 (n = 1 male, n = 1 female) over 60 h. Time 0 represents the time when treatments were administered to treatment group birds. Study began at 08:00 h. Post-treatment FGM were significantly higher (F = 43.34, df = 122, P = <0.001)

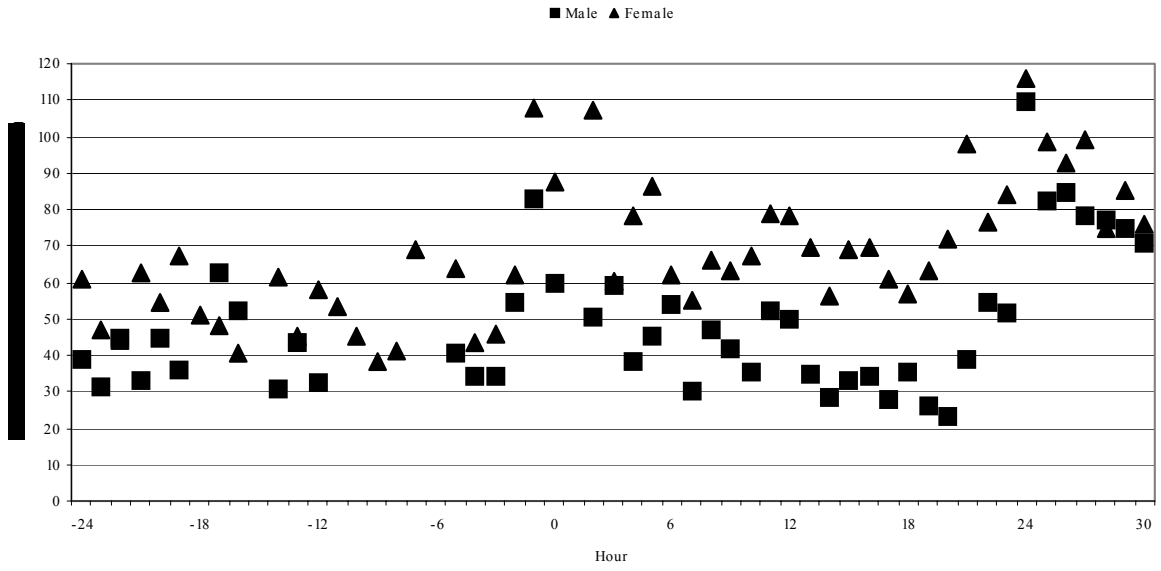


Figure 4. Fecal glucocorticoid concentrations (ng/g) of two control Mourning doves in winter 2002 (n = 1 male, n = 1 female) over 54 h. Time 0 represents the time when treatments were administered to treatment group birds. Study began at 16:00 h. Post-treatment FGM levels were higher (F=9.61, df=99, P = 0.01)

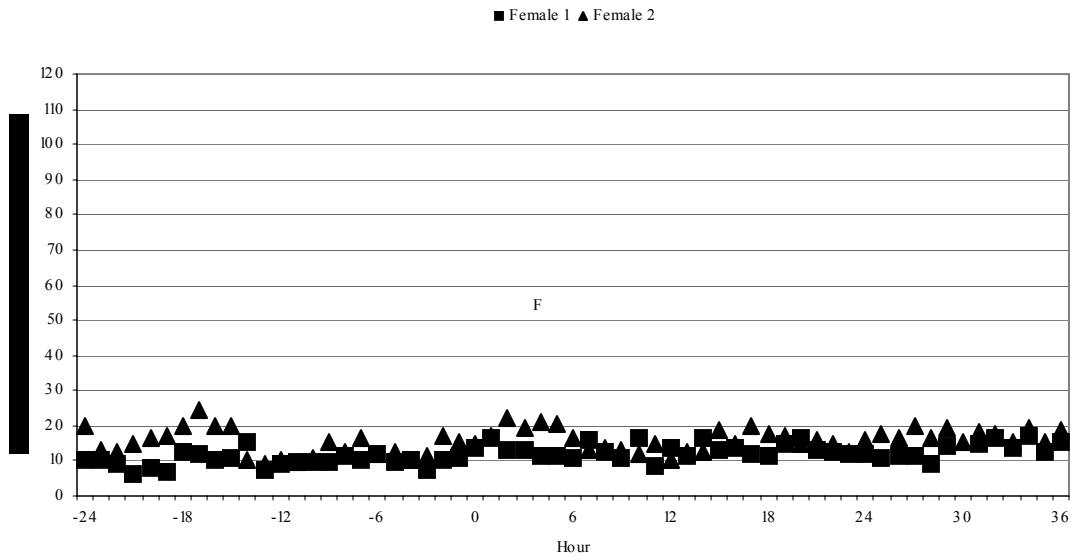


Figure 5. Fecal glucocorticoid concentrations (ng/g) of two Mourning doves assigned to the capture, handling, and release (CHR) treatment group in summer 2001 (n = 2 females) over 60 h. Time 0 represents the time when treatments were administered. Study began at 08:00 h. We found no significant difference pre- and post-treatment (F = 0.31, df = 121, P = 0.693)

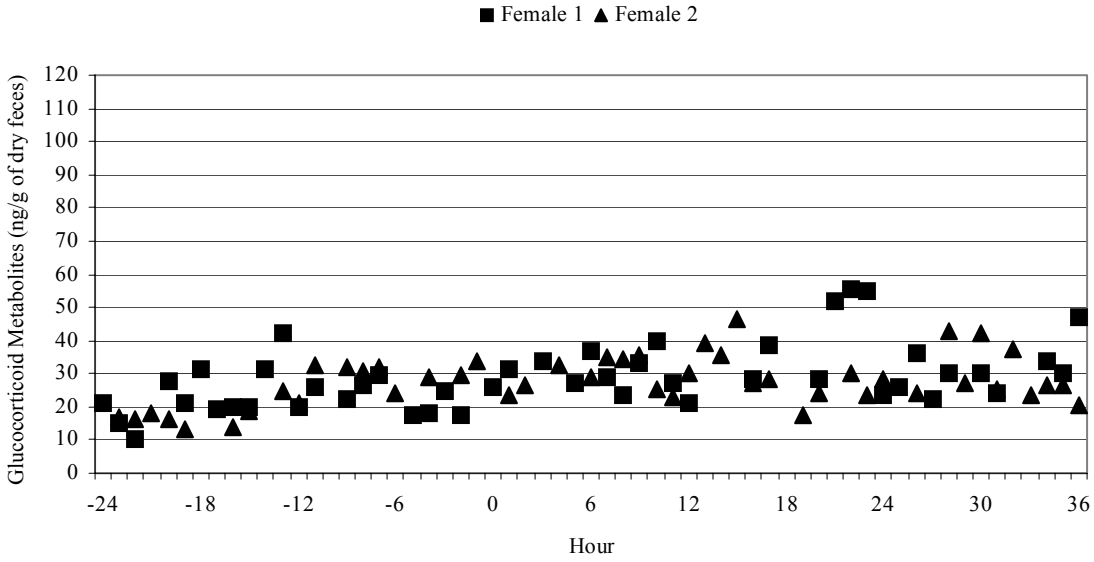


Figure 6. Fecal glucocorticoid concentrations (ng/g) of two Mourning doves assigned to the stress protocol (CSP) treatment group in winter 2001 (n = 2 females) over 60 h. Time 0 represents the time when treatments were administered. Study began at 12:00 h. Post-treatment FGM levels were higher (F=18.23, df=95, P < 0.001)

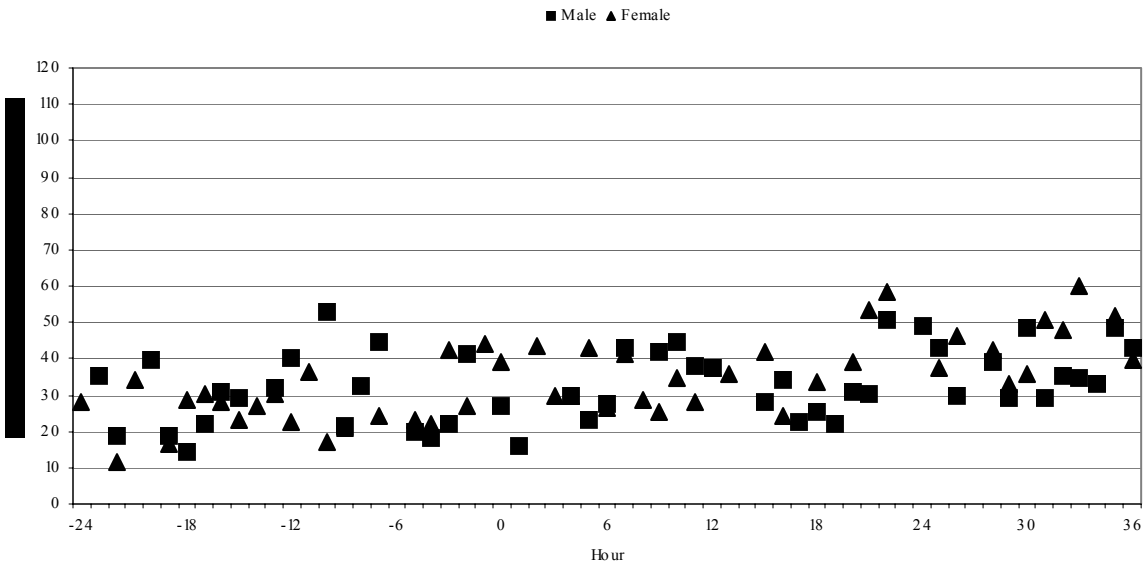


Figure 7. Fecal glucocorticoid concentrations (ng/g) of two Mourning Doves assigned to the stress protocol (CSP) treatment group in winter 2002 (n = 1 male, n = 1 female) over 60 h. Time 0 represents the time when treatments were administered. Study began at 16:00 h. We found no significant difference pre- and post-treatment (F=1.96, df=100, P = 0.148)

Table 1. Summary of experimental treatments used to evaluate short-term stress events in wild Mourning doves held in captivity.

Treatment	Season	Year	Gender M[1],F[2]	Start Time	Captivity[3]
Control	Summer	2001	1, 1	N / A	12 weeks
Control	Winter	2002	1, 1	N / A	20 weeks
CHR [4]	Summer	2001	0, 2	0800	12 weeks
CSP [5]	Winter	2001	0, 2	1200	6 weeks
CSP	Winter	2002	1, 1	1600	20 weeks

[1]Number of male Mourning doves in experiment.

[2]Number of female Mourning doves in experiment.

[3] Length of time wild Mourning doves were held in captivity prior to the beginning of the experiments.

[4]Capture, handling, and restraint treatment. Birds assigned to this treatment were captured using a mesh net, removed from the net, held in our hands for 50 sec, and released into their respective pen. Total disturbance time was 72 +/-3 SE sec. This same procedure was repeated with each bird 30 min later.

[5] Capture stress protocol (Wingfield *et al.,* 1992). Birds assigned to the stress protocol group were captured using a net, removed, and immediately placed into a breathable cotton sack. We placed the birds in a sternal position in the sack for 30 min, at which time we released them back into their respective pens. Total disturbance time of 30 min 47 sec +/- 16 SE sec.

CHAPTER 2

VALIDATION OF FECAL GLUCOCORTICOID ASSAYS FOR MULTIPLE SOUTH AFRICAN HERBIVORES

ABSTRACT

Fecal glucocorticoid metabolites (FGM) have gained popularity as a means of monitoring chronic stress in a wide range of wildlife species. Species-specific differences in the metabolism and excretion of glucocorticoids require thorough, systematic validation of assay methodology before use in a new species. Standard validation includes both laboratory (biochemical) and biological validation. Laboratory validation involves parallelism and exogenous hormone recovery tests, to evaluate whether the assay antibody can reliably detect the appropriate glucocorticoid metabolites in a fecal sample. A biological validation involves comparison of two groups that are producing different levels of glucocorticoids, either due to natural stimulation (a significant stressor) or pharmacological stimulation (ACTH administration). Our goal was to validate a commercially available radioimmunoassay (MP I^{125} corticosterone RIA) for measuring FGM from multiple herbivore species found in Tembe Elephant Park and Thanda Game Reserve, both located in KwaZulu-Natal, South Africa. We collected samples from giraffe *(Giraffa camelopardalis)*, impala *(Aepyceros melampus),* nyala *(Tragelaphus buxtoni)*, kudu (*Tragelaphus strepsiceros)*, wildebeest (*Connochaetes taurinus)* and zebra *(Equus burchelli).* These species represent the wide taxonomic diversity found in both parks, including browsers and grazers, and may serve as indicators of stress levels among other prey species. Our laboratory validation produced

sample dilution curves that were parallel to the standard curve and 90-110% exogenous recovery for all species. Our biological validation compared samples collected during the wet and dry seasons and showed that the technique was sensitive enough to detect biologically significant changes in FGM production associated with seasonal change. Samples collected during the dry season (June-August) were significantly higher in FGM than those collected in the wet season (December-February) in all species except wildebeest and zebra. For each species, we established optimal sample dilutions and baseline fecal corticosterone metabolite levels for the wet and dry season. Others can use this information for future studies monitoring the effects of manipulation of the ecosystem for management and tourism purposes.

INTRODUCTION

In South African parks, officials have to make decisions about the management of their wildlife based on the well-being of the animals, the interests of park visitors, and the needs of local communities. Managers must attend to the interests of tourists, by providing roads, lodging and vehicles in natural habitat and assuring that animals are visible. Increased vehicle and human activity can stress wildlife, causing them to deviate from normal habitats, diets and behaviors (Creel *et al.,* 2002). Chronic stress can lead to decreased immune function, loss of reproductive capacity and diminished survival (Sapolsky *et al.,* 2000). If tourist activity and wildlife health are incompatible, then wildlife conservation will suffer. The key to successful management of stress is accurate detection and monitoring of the effects of stressors.

Animals respond to stressful conditions through multiple mechanisms. Autonomic responses such as the "fight or flight" response are beneficial for short activities and include changes in heart rate, blood pressure, and gastrointestinal motility (Hilton, 1982). Neuro-endocrine responses mediated through the Hypothalamic-Pituitary-Adrenal (HPA) Axis can have longer lasting effects on a number of functions including immunity, reproduction, and metabolism (Jurke, 1997; Moberg, 2000; Sapolsky *et al.,* 2000). Stress hormones, such as cortisol and corticosterone, are indicative of HPA activity and can be detected in blood, tissues, urine and feces (Harvey *et al.,* 1984; Wingfield *et al.,* 1994; Wasser *et al.,* 2000). In humans and animals accustomed to human contact, blood samples can be collected with minimal stress on the subject, making plasma glucocorticoid levels a potentially meaningful measure of stress (Sapolsky *et al.,* 2000).

With free-ranging animals, the stress of capture, handling and blood collection may endanger the animal and complicate data interpretation by causing stress itself (Le Maho *et al.,* 1992; Cook *et al.,* 2000). Procedures have been developed to collect stress hormone data non-invasively, by measuring fecal glucocorticoid metabolite (FGM) levels (Graham and Brown, 1996; Harper and Austad, 2000; Wasser *et al.,* 2000; Millspaugh *et al.,* 2002). Fecal studies have the added benefit of showing cumulative effects of stressors as they contain metabolites accumulated over a period of time, while plasma samples only detect the hormone levels at one point in time (Harper and Austad, 2000; Wasser *et al.,* 2000; Millspaugh *et al.,* 2001). Detailed discussion of the benefits of FGM monitoring can be found in Millspaugh and Washburn (2004) and Touma and Palme (2005).

In plasma studies, most of the hormones detected will be the parent hormone, corticosterone or cortisol. In fecal samples, after extensive hepatic and gastrointestinal metabolism (Schwarzenberger *et al*., 1996; Bokkenheuser and Winter, 1980), there is little parent hormone left and what is detected is a variety of glucocorticoid metabolites (Palme *et al*., 2005). The wide distribution of FGMs found in individual samples, has been demonstrated through high performance liquid chromatography (HPLC) (Palme *et al*., 2005; Goymann, 2005). The assortment of individual metabolites can be different for each species (Touma and Palme, 2005; Goymann, 2005; Mateo and Cavigelli, 2005). As we are not measuring native hormone, it is essential to validate the assay methodology to assure that the species-specific suite of hormone metabolites is detectable and that the level detected is indicative of the level of adrenal activity in the species studied. This is often accomplished first, through a standardized laboratory validation, involving

parallelism and exogenous recovery tests (Harper and Austad, 2000). Secondly, we must determine if the assay is sensitive enough to detect biologically significant changes in hormone production, using a biological validation (Washburn *et al.,* 2002; Touma and Palme, 2005).

The goal of biological validation is to compare FGM levels in two groups of animals that are producing different levels of adrenal hormones. This can be accomplished pharmacologically by administering ACTH to stimulate adrenal hormone production, or administering dexamethasone to suppress adrenal function (Palme *et al.,* 2005; Millspaugh and Washburn, 2004). In wild animals, pharmacologic manipulation is often not feasible, and biological validation can be accomplished by comparing groups with different levels of adrenocortical activity. In these validations, samples are collected from groups of animals after exposure to a stressor, such as capture, human contact, or immobilization. Many bird and mammal species show seasonal variation in glucocorticoid production, which can be used in biological validation (Harper and Austad, 2001; Kenagay *et al., 1999;* Kenagay and Place, 2000; Strier *et al.,* 1999; Millspaugh *et al.,* 2001; Huber *et al.,* 2003; Temple and Gutíerrez, 2004). Seasonal variation in glucocorticoid levels may be due to varying food availability, temperature, rainfall, and tourist activity (Romero and Healy, 2004; Temple and Gutierrez, 2004; Creel *et al.,* 2002). One intrinsic drawback to this type of biological validation compared to an ACTH challenge is that we do not know *a priori* what type of response an animal should have to seasonal change. With an ACTH challenge, adrenal function is expected to increase resulting in increased glucocorticoid production that can be detected by FGM assays.

The purpose of this study was to assess the feasibility of using a commercially available radioimmunoassay to quantify fecal glucocorticoid metabolites as an indicator of stress in multiple herbivore species in two South African wildlife parks, through laboratory and biological validations. By performing validations, determining optimal sample dilutions and establishing baseline data for herbivore species in these parks, we have established groundwork for future projects in this area monitoring the effects of introduced stressors, especially introduction of large carnivores, on a wide range of prey species.

METHODS

We studied six species: giraffe *(Giraffa camelopardalis)*, impala *(Aepyceros melampus),* nyala *(Tragelaphus buxtoni)*, kudu (*Tragelaphus strepsiceros)*, wildebeest (*Connochaetes taurinus)* and zebra *(Equus burchelli)*. These species represent a wide taxonomic range, including both browsers and grazers, and account for several important large herbivores in the study areas. Some of these species share habitat and resources with more rare herbivores, such as the suni (*Neotragus moschatus kirchenpaueri*).

Study sites

We collected samples in Tembe Elephant Park during July and August 2003 and Thanda Game Reserve between May 2004 and July 2005. Both parks lie in northeastern KwaZulu-Natal, South Africa in a geographic area known as Maputaland. The main tourist draws to both of these parks are the "Big Five" game species: elephant *(Loxodonta africana)*, leopard *(Panthera pardus)*, lion *(Panthera leo)*, rhino *(Ceratotheruim simum)* and buffalo *(Syncerus caffer)*. Though these animals may be the main attraction, both

parks sustain populations of many herbivore species, which are integral parts of the ecosystem.

Sample collection and processing

We collected dry season samples from June through August and collected wet season samples between December and February. We collected fecal samples at gathering places, such as watering holes or clearings, where park employees frequently observed large groups of animals. We attempted to observe the animals without disruption by remaining in a vehicle on the road, away from the group. For all species, we observed animals gathering and defecating and collected samples from those areas when the animals left. If there was possibility of multiple similar species (e.g., nyala and impala) in the same area, we did not collect samples to avoid misidentification of samples. Whenever possible, we collected samples from individuals which we witnessed defecating; when this was not possible we collected only fresh (moist) fecal pellets and avoided any large clusters of pellets, which appeared to come from multiple individuals. We collected representative sub-samples from different parts of each fecal pile, to minimize variability of FGM levels within a pile or fecal bolus (Millspaugh and Washburn, 2003). We did not collect dried pellets or those with urine contamination. We stored samples in plastic scintillation vials at -20ºC until processing.

Before shipment to the United States, we thawed each sample and thoroughly mixed it to account for uneven distribution of hormones in the fecal samples (Millspaugh and Washburn, 2003). To comply with USDA regulations for importation of ungulate fecal material, we submerged each sample in 2% acetic acid to minimize viable viral particles (Millspaugh *et al*., 2003). After 30 minutes, we drained the acetic acid from

each sample vial and refroze the sample at -20ºC. We shipped frozen samples to the Wildlife Physiology Laboratory at the University of Missouri – Columbia for further processing.

Laboratory methods

We dried fecal samples in a lyophilizer for 24 hr. Once freeze-dried, we sifted samples through a stainless steel mesh to remove large particles and each sample was then thoroughly mixed. We extracted glucocorticoid metabolites from the feces using a modification of Schwarzenberger *et al.,* (1991). We placed 0.200-0.220g of dried feces in a glass test tube with 2.0 mL of 90% methanol and vortexed the sample at high speed in a multi-tube vortexer for 30 min. We centrifuged samples at 2,200 rpm for 20 min, removed the supernatant, and stored it at -84^{o}C until assayed.

We used I^{125} corticosterone radioimmunoassay (RIA) kits (MP Biomedicals, Solon, OH, USA). We followed the MP protocol for the I^{125} corticosterone RIA, except that we halved the volume of all reagents (Wasser *et al.,* 2000) and used the methanol fecal hormone extract instead of plasma. We assayed all samples in duplicate and if the coefficient of variation (CV) was greater than 15%, we reran the samples in the next assay.

Laboratory validation

We pooled aliquots from 6 individual samples per species collected during the dry season for standard laboratory validation (Jeffcoate, 1981; O'Fegan, 2000). For each species, we performed parallelism and exogenous recovery tests on the pooled samples. We used a parallelism test (Harper and Austad 2000) to determine if the assay is capable of producing linear results with serial dilutions of the sample. We compared serial

dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) of a pooled sample from each species with a curve produced by dilutions of the corticosterone standard stock solutions. On a log-transformed scale, we plotted percent binding vs. concentration of standard and percent binding vs. concentration of sample (Figures 1A-1F).

We measured accuracy by recovery of a known amount of standard from spiked samples. The optimum sample dilution from the parallelism test was determined and the diluted sample was mixed 1:1 with each of the 3 standards supplied with the RIA kits (0.25, 0.50, and 1.25 ng/mL). Sample recovery was calculated for each spiked sample and a mean and standard deviation was calculated for recovery of exogenous hormone. This allowed us to determine if the assay could accurately detect FGM levels throughout the working range of the assay.

Biological validation

To determine if our assay was capable of detecting physiologically significant changes in corticosteroid production and excretion, we compared samples collected during two separate seasons. We chose sampling periods based on commonly accepted wet and dry seasons. The wet season is the time of greatest rainfall and thus supports greater plant growth, which in turn can support larger populations of herbivores. During the dry season decreased rainfall and humidity results in less available forage, forcing animals to migrate greater distances in search of food. We selected 8-10 samples per species from both the wet season (December - February) and the dry season (July-August). Samples were extracted and run at the appropriate sample dilution determined from the laboratory validation (Table 1).

Statistical analyses

Tests for equal slopes (parallelism) were performed, using SAS, to determine if log-transformed curves of serially diluted low and high pool extracts were parallel to log-transformed MP corticosterone I^{125} RIA corticosterone standard curves (Neter et al. 1990). We considered exogenous corticosterone recovery of 90-110% acceptable (Jeffcoate, 1981; O'Fegan, 2000; Harper and Austad 2000) for the biological validation; we compared wet and dry season sample sets for each species using a one-way ANOVA using SPSS. We considered test results significant for $P < 0.05$.

RESULTS

Laboratory Biochemical Validation – Parallelism and Exogenous Recovery

For each species, 10 fecal samples from each season were used for validation; while we were unable to determine the number of individuals represented by these samples, we avoided using samples collected from the same site at the sample time to minimize the likelihood of sampling the same individuals.

For each of the six species, serial dilutions of low and high pool fecal extracts yielded displacement curves that were parallel to the standard corticosterone curve (All $P > 0.55$) (Figure 8A-F). The dilution that provided a percent binding closest to 50% was determined to be the ideal dilution for future assays (Table 2). Exogenous corticosterone sample recovery was within the acceptable range (90-110%) for five of the six species (Table 3). Mean exogenous recovery for pooled kudu samples was 110.860% +/- 3.810 SE.

Biological Validation – Seasonal Differences in FGM Levels

In all species, average dry season sample FGM levels were higher than wet season levels, though only four out of six species showed significant differences. For samples collected in the Thanda Game Reserve, four of the six species studied showed significantly higher FGM levels in May through July than in December through February. The seasonal differences were significant for giraffe (df = 17, F = 5.516, P = 0.030); for kudu (df = 16, F = 5.085. P = 0.040); for nyala (df = 12, F = 6.710, P<0.025); for zebra (df = 18, F = 5.080, P = 0.040). Mean dry season values were higher than wet season for both impala (df = 12, F = 0.545. P = 0.476) and wildebeest (df = 18, F = 0.161, P = 0.693), though the difference was not significant.

DISCUSSION

Our validations suggest the MP Biomedicals I^{125} corticosterone radio-immunoassay is a useful means of detecting the stress response in a wide range of South African herbivores. This study supports the findings of multiple other studies that have used this assay and shown FGM analysis to be an accurate means of quantifying physiological stress in ungulate species (Wasser *et al.,* 2000; Millspaugh *et al.,* 2001; Millspaugh *et al.,* 2002). FGM measurements have been used to monitor stressors in other free-ranging and captive African wildlife, including elephants (*Loxodonta africana*) (Foley *et al.,* 2001; Ganswindt *et al.,* 2003), cheetah (*Acinonyx jubatus*) (Jurke *et al.* 1997; Terio *et al.,* 1998), white rhinoceros (*Ceratotheruim simum*) and black rhinoceros (*Diceros bicornis*) (Turner *et al.,* 2002).

Though FGM analysis has been reliably used in a wide range of species, clear differences occur in fecal composition, diet and glucocorticoid metabolism between taxa,

which can influence the types of metabolites found in the feces (Palme, 2005). As we are not measuring native corticosterone in the feces, it is essential to validate the assay methodology to assure that the suite of metabolites being detected is truly indicative of the level of adrenal activity in the species being studied (Wasser *et al.,* 2000; Touma and Palme, 2005). The MP (then ICN) Biomedicals antibody has been extensively validated; it showed the highest level of cross-reactivity with major fecal metabolites of corticosterone in 11 of 12 species studied, compared to three other antibodies (Wasser *et al.,* 2000). The RIA does not provide information about relative abundance of individual metabolites, but instead provides the concentration of all metabolites in the fecal extract. Identification of individual metabolites would be possible with high-performance liquid chromatography (Palme, 2005; Goymann, 2005); but at this time, we have not determined a biological significance associated with any individual metabolites. Thus, we continue to use net metabolite concentration, to detect the effects of a stressor and identify species differences in stress levels.

In each of the species studied, we found differences in the average FGM levels and in the optimal dilution for sample analysis. While numerous factors, including diet, sex, body size and taxonomy could contribute to the differences in FGM levels, we hypothesized that the changes in FGM levels due to seasonal changes in food abundance would be most evident when comparing browsers and grazers. It is possible that the seasonal changes in plant abundance would affect species differently depending on their primary food source. In general, the browser species had higher overall FGM levels than the grazer and browser/grazer species. Comparison of FGM levels to those found in other studies may not be valid due to slight changes in extraction or analysis protocols.

We cannot determine if this interspecies difference is indicative of different stress levels, because differences in diet, fecal composition, adrenal activity and habitat can affect FGM levels (Wasser, 2000; Palme *et al.,* 2005). Certain dietary nutrients can affect FGM levels by binding hormone, changing gastrointestinal transit time or gut flora (Wasser *et al.,* 1993; Goymann, 2005). Longer GI transit time allows for greater bacterial breakdown of FGMs, and allows metabolites to be reabsorbed and reprocessed by the liver through enterohepatic circulation (Bokkenheuser and Winter, 1980; Palme, 2005). In herbivore species, the level of indigestible fiber in the diet will directly affect fecal bulk and possibly dilute the amount of FGM in a sample (Goymann, 2005). Our study examined a broad range of herbivore species, with different diets and which represent the assortment of herbivore types found in South African parks. Through laboratory validation, using parallelism and exogenous recovery tests, we demonstrated that FGM levels can be accurately measured in each of these species.

Once we performed laboratory validations, it is essential to determine if the assay is capable of detecting biologically significant changes. While proper and thorough validation is ideal, the reality of wildlife health studies often makes it impractical. Ideal validations involve physiologic validations with pharmacological manipulation, in the form of an ACTH challenge (Goymann, 2005; Touma and Palme, 2005; Washburn *et al*., 2000). These manipulations are not possible in free-ranging wildlife, without considerable risk to the animals and investigators. One of our goals with this study was to limit contact with wildlife to minimize experiment-induced stress, thus we employed naturally occurring biological variation for our validation.

Our study demonstrated significantly higher FGM levels in the dry season for four of the six species studied. Dry season FGM levels were higher than wet season levels in the wildebeest and impala samples, though the difference was not significant; individual variation and low sample numbers likely contributed to the lack of significant difference. Further investigation with higher sample numbers is warranted to see if the observed pattern is repeatable in future years. Significant seasonal differences in ungulate FGM levels have been demonstrated in white-tailed deer (*Odocoileus virginianus*) (Millspaugh *et al.,* 2002) and elk (*Cervus elaphus*) (Millspaugh *et al.,* 2001). Distinct seasonal variation in FGM levels has also been documented in birds (Romero and Wingfield, 1999) and rodents (Harper and Austad, 2001). While the mechanism for seasonal variation has yet to be determined, there have been multiple suggested causes; it has been hypothesized that this seasonal variation could be due to changes in reproductive behavior, diet, day length and temperature (Harper and Austad, 2001). In South Africa, seasonal rainfall has a profound impact on plant and animal communities, by determining the amount of plant growth and thus, the available forage for herbivores.

In these parks, it is likely that the variation in seasonal FGM levels could be due to changing food abundance and subsequent increased stress. The browser species (kudu, nyala and giraffe) showed a greater difference in FGM levels between seasons, than did the grazer and browser/grazer species. This may indicate a greater effect of season on leaves and browse than on grasses. Future studies could include year-round sampling and comparison to studies of plant abundance and herbivore resource selection in the area. While we demonstrated that FGM analysis is able to detect seasonal variation in most species, we cannot determine the source of this variation, given the number of

possibilities present. Of critical importance, is acknowledging that this significant variation exists.

These two parks are typical of many similar parks throughout Africa, in which management decisions have to be made based on multiple and often competing interests. Changing natural habitats, including development or species introduction can cause stress in native populations. A single stressor can affect similar species differently (Palme *et al.,* 2005); ecosystem changes that benefit some species may be detrimental to others. For example, introduction of large predators, such as lions, has been an issue in Tembe Elephant Park. While the introduction of lions may detrimentally stress target prey species, it may provide a relative advantage to other herbivores through decreased competition for food and territory. Making management decisions based on data from one or two species may cloud a comprehensive picture of park ecology; even if those species are high profile animals. Our project included a diverse range of browser and grazer species, including: equidae (zebra), giraffidae (giraffe), and multiple bovidae (wildebeest, nyala, impala and kudu). Baseline data from multiple seasons is essential as seasonal variations may be a confounding factor in studies of chronic stress. To determine the effects that seasonal variation will have on stress studies, attempts should be made to sample animals year round, before and after a suspected stressor, or only make comparisons between samples from affected and unaffected parts of a park during the same sampling season.

In each of the species examined, the MP Biomedicals (formerly ICN Biomedicals) radioimmunoassay kit performed well in tests of parallelism and recovery of exogenous corticosterone. The assay was sensitive enough to detect potentially

significant biological changes in seasonal glucocorticoid production. By establishing baseline FGM levels for these six species of herbivores, we can begin other studies directed at assessing the effects of specific stressors. While previous studies of this nature have focused on the effects of a stressor on one species, we hope that future studies in these parks can take a multi-species approach to stress assessment. With a non-invasive technique for quantifying stress, it will be possible to make more educated management decisions for species within African parks and reserves. This validation and baseline data can provide a starting point for research in other parks and as a comparison for captive individuals in zoological institutions. The success of this and previous validation studies support FGM analysis as a robust method of stress assessment, which is applicable to a wide range of species.

Figure 8. Parallelism of fecal glucocorticoid metabolites levels detected in feces using I^{125} corticosterone radioimmunoassay (RIA) kits (MP Biomedicals, Solon, OH, USA). For each species, we plotted the RIA corticosterone standard curve (closed diamonds with thin solid trendline), and serial dilutions of herbivore fecal samples from the dry season (Sample 1, open diamonds with dashed trendline) and wet season (Sample 2, open triangles with thick solid trendline).

8A. Impala: Standards: $y = -42.107x + 47.256$, $r^2 = 0.9968$; Sample 1:$y = -44.509x + 46.481$ $r^2 = 0.9987$; Sample 2:$-44.898x + 46.129$, $r^2 = 0.9973$

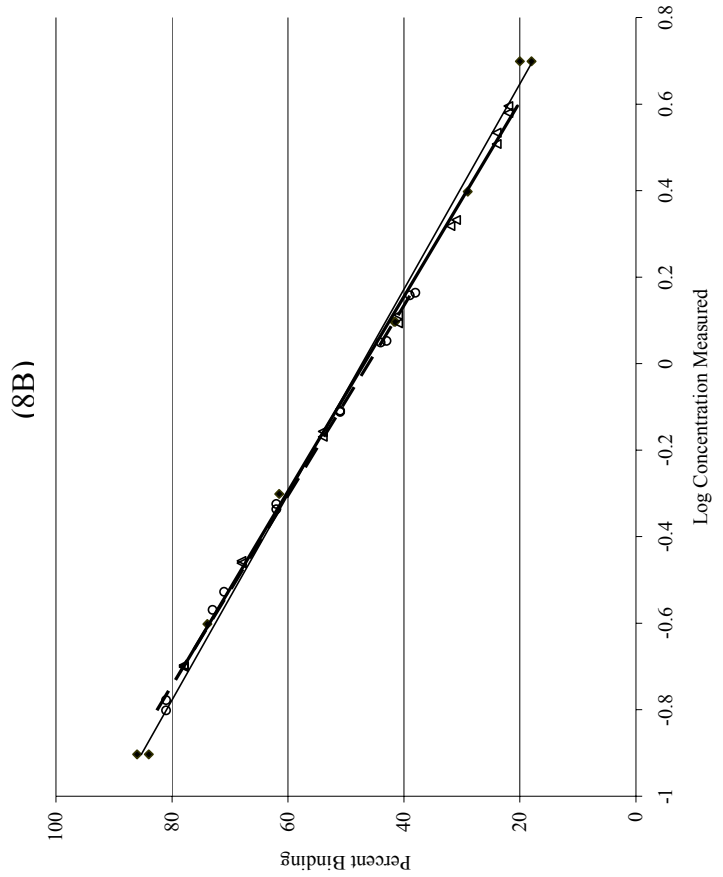
8B. Kudu: Standards: $y = -42.107x + 47.256$, $r^2 = 0.9968$; Sample 1:$y = -45.51x + 46.13$, $r^2 = 0.9974$; Sample 2:$y = -44.381x + 46.871$, $r^2 = 0.9981$

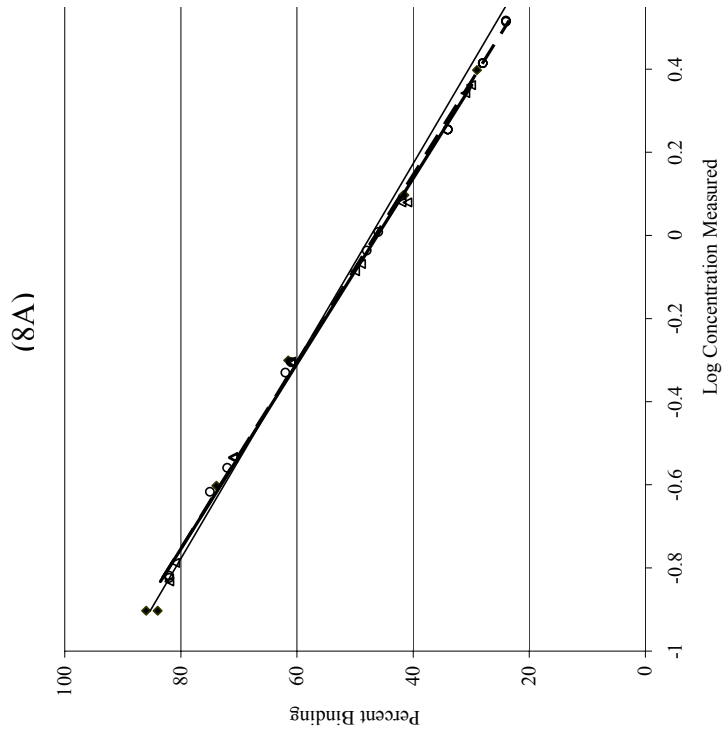
8C. Nyala: Standards: $y = -43.656x + 49.347$, $r^2 = 0.9969$; Sample 1:$y = -44.267x + 48.874$, $r^2 = 0.9975$; Sample 2:$y = -44.933x + 49.334$, $r^2 = 0.9993$

8D. Giraffe: Standards: $y = -41.018x + 48.314$, $r^2 = 0.9956$; Sample 1:$y = -42.922x + 47.87$, $r^2 = 0.9972$; Sample 2:$y = -42.456x + 47.728$, $r^2 = 0.9974$

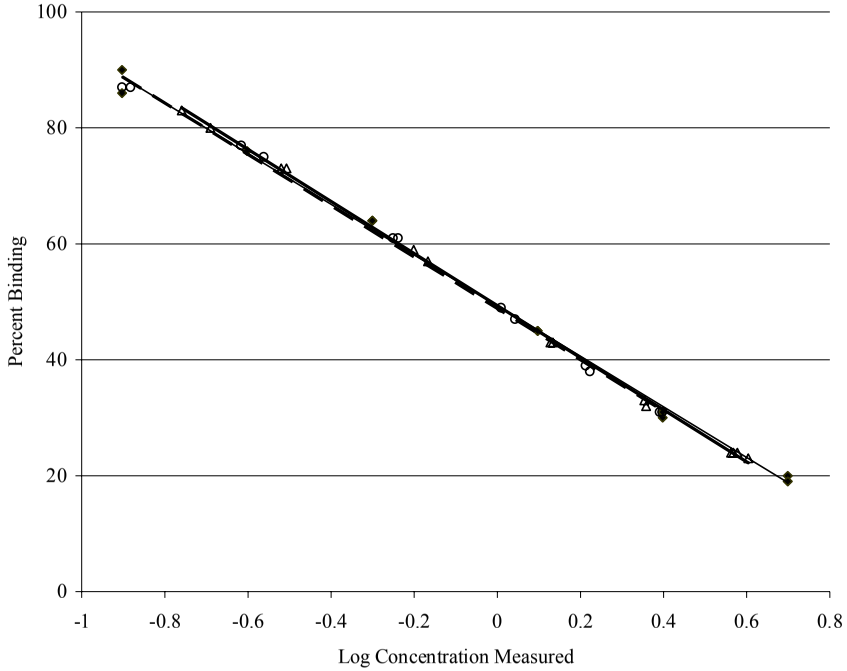
8E. Wildebeest: Standards: $y = -41.018x + 48.314$, $r^2 = 0.9956$; Sample 1:$y = -42.997x + 48.006$, $r^2 = 0.9971$; Sample 2:$y = -42.548x + 47.838$, $r^2 = 0.9971$

8F. Zebra: Standards: $y = -41.018x + 48.314$, $r^2 = 0.9956$; Sample 1:$y = -43.763x + 46.178$, r^2; Sample 2:$y = -43.312x + 46.466$, $r^2 = 0.996$

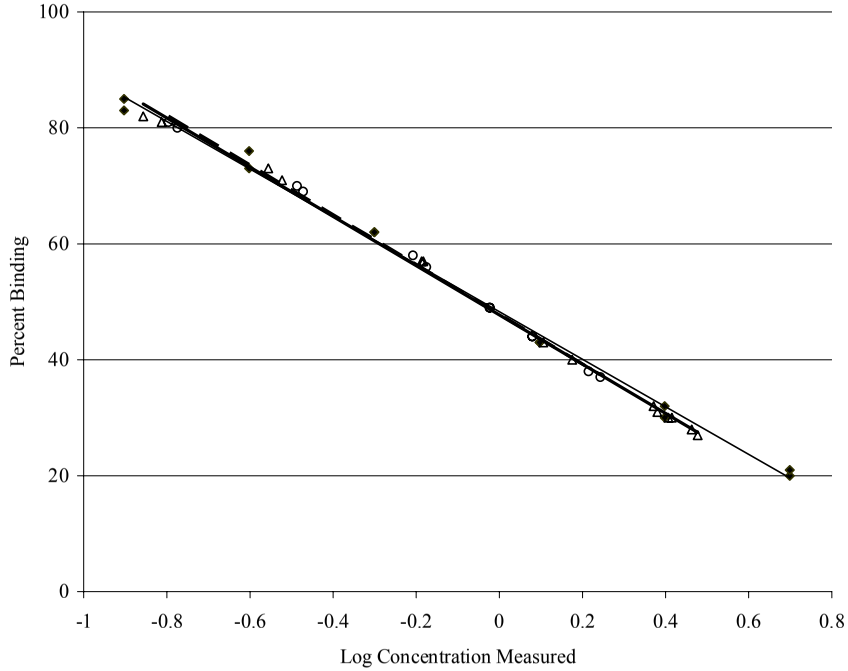

(8B)
Percent Binding
100
80
60
40
20
0
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
Log Concentration Measured


(8A)
Percent Binding
100
80
60
40
20
0
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
Log Concentration Measured

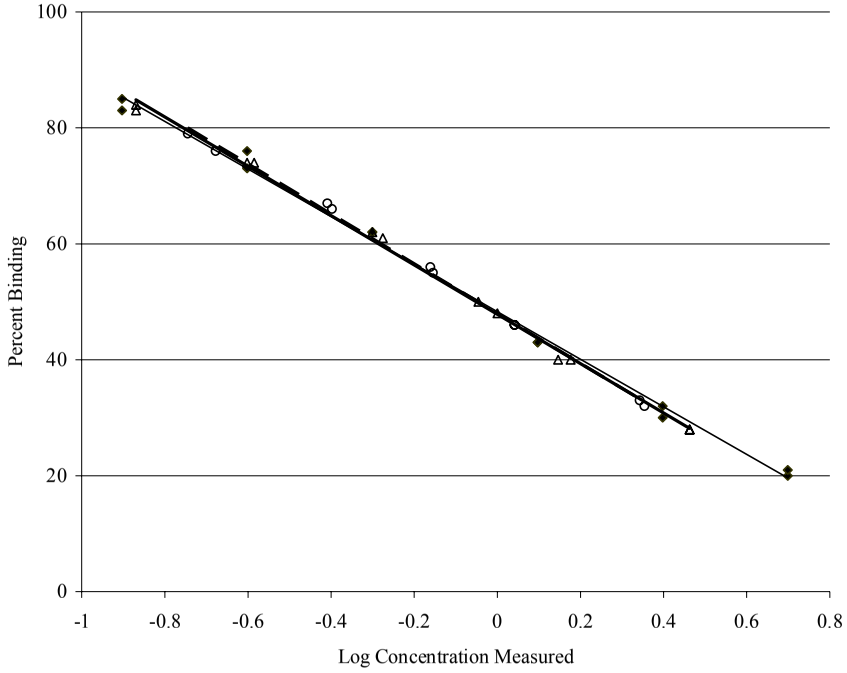
(8C)


100
80
60
40
20
0
Percent Binding
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
Log Concentration Measured

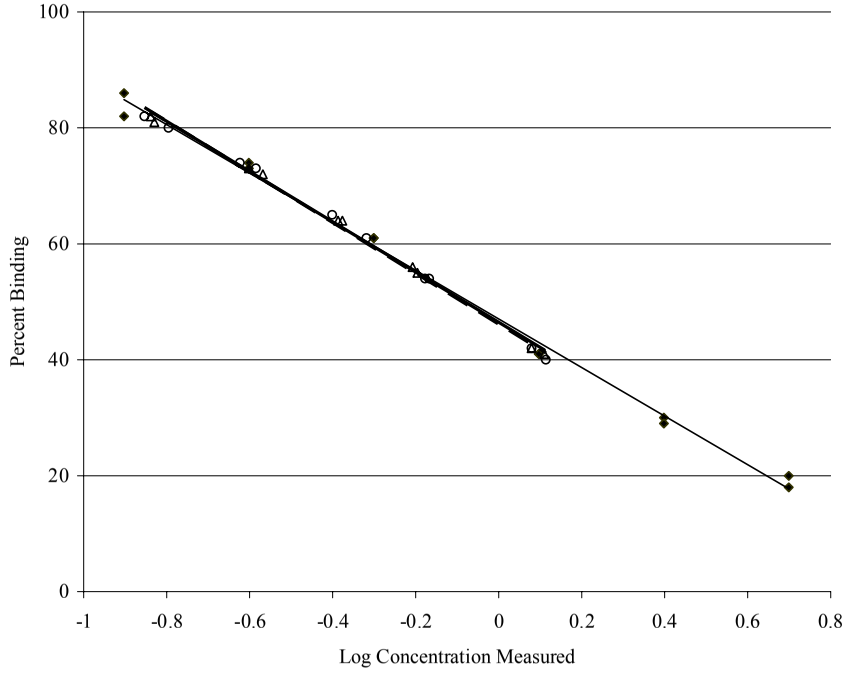
(8D)


100
80
60
40
20
0
Percent Binding
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
Log Concentration Measured

(8E)


100
80
60
40
20
0
Percent Binding
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
Log Concentration Measured

(8F)


100
80
60
40
20
0
Percent Binding
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
Log Concentration Measured

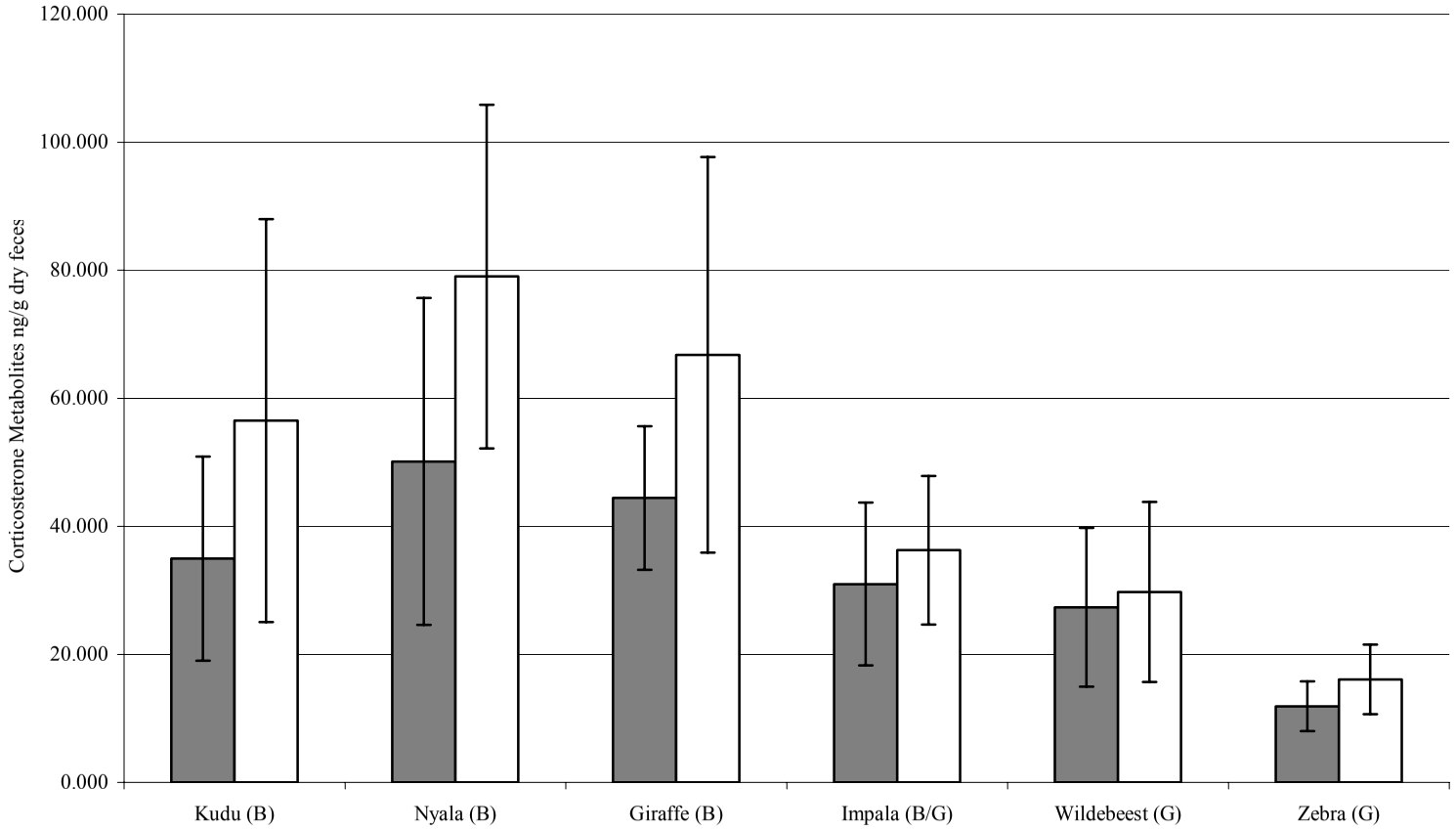


Figure 9. Mean concentration (with standard deviations) of fecal glucocorticoid metabolites (ng/g dry feces) for wet season (December-February, solid gray bar) and dry season (May-July, solid white bars) samples. Browsers are indicated with (B) and grazers with (G); the impala is frequently a browser and grazer (B/G).

Table 2: Optimal sample dilution factors for methanol extracts of fecal glucocorticoid metabolites. The optimal dilution provided the percent binding closest to 50% and thus is most likely to be appropriate for the full range of the assay.

Species	Optimal Sample Dilution
Impala	1:08
Kudu	1:08
Nyala	1:32
Giraffe	1:16
Wildebeest	1:08
Zebra	1:04

Table 3: Average percent recovery of exogenous corticosterone with standard deviations. ($n = 6$ per species; range of added corticosterone = 0.25-1.25ng/mL)

Species	Average Percent Recovery	Standard Deviation
Impala	109.71	4.57
Kudu	110.86	3.81
Nyala	108.99	1.68
Giraffe	109.76	3.42
Wildebeest	104.24	3.34
Zebra	109.60	3.32

LITERATURE CITED

1. Astheimer, L.B., W.A. Buttemer, and J.C. Wingfield. 1994. Gender and seasonal differences in the adrenocortical response to ACTH challenge in an Arctic passerine, *Zonotrichia leucophrys gambelii*. General and Comparative Endocrinology. 94:33–43.
2. Axelrod, J., and T.D. Reisine. 1984. Stress hormones: their interaction and regulation. Science. 224:454–459.
3. Bokkenheuser, V.D., and J. Winter. 1980. Biotransformation of steroid hormones by gut bacteria. American Journal of Clinical Nutrition. 33:2502–2506.
4. Breuner, C.W., J.C. Wingfield, and L.M. Romero. 1999. Diel rhythms of basal and stress– induced corticosterone in a wild, seasonal vertebrate, Gambel's white–crowned sparrow. Journal of Experimental Zoology. 284:334–342.
5. Cavigelli, S.A. 1999. Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed lemurs, *Lemur catta*. Animal Behaviour. 57:935-944.
6. Cavigelli, S.A., S.L. Monfort, T.K. Whitney, Y.S. Mechref, M. Novotny, and M.K. McClintock. 2005. Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. Journal of Endocrinology. 184:153-163.
7. Cheeke, P.R. 1994. Nutrition and Nutritional Diseases. In: P.J. Manning, D.H. Ringler and C. E. Newcomer, editors. The Biology of the Laboratory Rabbit. 2nd ed. p 321. Academic Press, New York.
8. Cook, C.J., D.J. Mellor, P.J. Harris, J.R. Ingram, and L.R. Matthews. 2000. Hands-on and hands–off measurement of stress. In: G. P. Moberg and J. A. Mench, editors. The biology of animal stress. pp 123–146. CABI Publishing, New York.
9. Creel, S., J.E. Fox, A. Hardy, J. Sands, B. Garrott, and R..O. Peterson. 2002. Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conservation Biology. 16:809–814.
10. Earley, B. and M.A. Crowe. 2002. Effects of ketoprofen alone or in combination with local anesthetic during the castration of bull calves on plasma cortisol, immunological, and inflammatory responses. Journal of Animal Science. 80:1044-1052.
11. Foley, C.A.H., S. Papageorge, and S.K. Wasser. 2001. Noninvasive stress and reproductive measures of social and ecological pressures in free–ranging African elephants. Conservation Biology. 15:1134–1142.
12. Ganswindt, A., R. Palme, M. Heistermann, S. Borragan, and J.K. Hodges. 2003. Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth. General and Comparative Endocrinology. 134:156–166.
13. Goldstein, E.J., J.J. Millspaugh, B.E. Washburn, G.C. Brundige, and K.J. Raedeke. 2005. Relationships among fecal glucocorticoids, lungworm levels, and recruitment in bighorn sheep in South Dakota. Journal of Wildlife Diseases. 41:416–425.
14. Good, T., M.Z. Khan, J.W. Lynch. 2003. Biochemical and physiological validation of corticosteroid radioimmunoassay for plasma and fecal samples in oldfield mice (*Peromyscus polionotus*). Physiology and Behavior. 80:405-411.

15. Goymann, W. 2005. Noninvasive monitoring of hormones in bird droppings: physiological validations, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels. Annals of the New York Academy of Sciences. 1046:35-53.
16. Goymann, W., E. Möstl & E. Gwinner. 2002. Corticosterone metabolites can be measured non-invasively in excreta of European stonechats (*Saxicola torquata rubicola)*. Auk. 119:1167-1173.
17. Graham, L.H., and J.L. Brown. 1996. Cortisol metabolism in the domestic cat and implications for non-invasive monitoring of adrenocortical function in endangered felids. Zoo Biology. 15:71–82.
18. Harper, J.M., and S.N. Austad. 2000. Fecal glucocorticoids: a noninvasive method of measuring adrenal activity in wild and captive rodents. Physiological and Biochemical Zoology. 73:12–22.
19. Harper, J.M., and S.N. Austad. 2001. Effect of capture and season on fecal glucocorticoid levels in deer mice (*Peromyscus maniculatus*) and red–backed voles (*Clethrionomys gapperi*). General and Comparative Endocrinology. 123:337–344.
20. Harvey, S., J.G. Phillips, A. Rees, and T.R. Hall. 1984. Stress and adrenal function. Journal of Experimental Zoology. 232:633–645.
21. Hattingh, J. 1998. Comparative quantitation of the physiological response to acute stress in impala and roan antelope. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology. 89:547-551
22. Herman, J.P. & W.E. Cullinan. 1997. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends in Neurosciences. 20:78-84.
23. Hilton, S.M. 1982. The defense arousal system and its relevance for circulatory and respiratory control. Journal of Experimental Biology. 100:159-174.
24. Huber, S., R. Palme, and W. Arnold. 2003. Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (*Cervus elaphus*). General and Comparative Endocrinology. 130:48–54.
25. Joseph, M. M., and A. H. Meier. 1973. Daily rhythms of plasma corticosterone in the Common Pigeon, (*Columba livia*). General and Comparative Endocrinology. 20:326–330.
26. Jurke, M.H., N.M. Czekala, D.G. Lindburg, and S.E. Millard. 1997. Fecal corticoid metabolite measurement in the cheetah (*Acinonyx jabatus*). Zoo Biology. 16:133–147.
27. Kenagy, G.J., N.J. Place and C. Veloso. 1999. Relation of glucocorticosteroids and testosterone to the annual cycle of free-living degus in semi arid Central Chile. General and Comparative Endocrinology. 115:236-243.
28. Kenagy, G.J., and N.J. Place. 2000. Seasonal changes in plasma glucocorticoids of free-living yellow pine chipmunks; effect of reproduction and capture and handling. General and Comparative Endocrinology. 117:189-199.
29. Le Maho, Y., H. Karmann, D. Briot, Y. Handrich, J.P. Robin, E. Mioskowski, Y. Cherel, and J. Farni. 1992. Stress in birds due to routine handling and a technique to avoid it. American Journal of Physiology. 263:R775–R781.
30. Ludders, J.W., J.A. Langenberg, N.M. Czekala, H.N. Erb. 2001. Fecal corticosterone reflects serum corticosterone in Florida Sandhill Cranes. Journal of Wildlife Diseases. 37:646–652.

31. Mateo, J.M. & Cavigelli, S.A. 2005. A validation of extraction methods for non-invasive sampling of glucocorticoids in free-living ground squirrels. Physiological and Biochemical Zoology. 78:1069-1084.
32. Merl, S. 2000. Pain causes increased concentrations of glucocorticoid metabolites in equine faeces. Journal of Equine Veterinary Science. 20: 586-590.
33. Millspaugh, J.J., and B.E. Washburn. 2003. Within–sample variation of fecal glucocorticoid measurements. General and Comparative Endocrinology. 132:21–26.
34. Millspaugh, J.J., B.E. Washburn, M.A. Milanick, J. Beringer, L. Hansen, and T. Meyer. 2002. Noninvasive techniques for stress assessment in white-tailed deer. Wildlife Society Bulletin. 30:112-120.
35. Millspaugh, J.J., B.E. Washburn, M.A. Milanick, R. Slotow, and G. van Dyk. 2003. Effects of heat and chemical treatments on fecal glucocorticoid measurements: implications for sample transport. Wildlife Society Bulletin. 31:399–406.
36. Millspaugh, J.J., R.J. Woods, K.E. Hunt, K.J. Raedeke, G.C. Brundige, B.E. Washburn, and S.K. Wasser. 2001. Using fecal glucocorticoid assays to study the physiological stress response of elk. Wildlife Society Bulletin. 29:899–907.
37. Millspaugh, J.J. & B.E. Washburn. 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. General and Comparative Endocrinology. 138:189-199.
38. Mirarchi, R.E. 1993. Sexing, aging, and miscellaneous research techniques. In: Ecology and management of the mourning dove (T.S. Baskett, M.W. Sayre, R.E. Tomlinson, and R.E. Mirarchi, Ed). pp 399–408. Wildlife Management Institute, Washington D.C.
39. Moberg, G.P. 2000. Biological response to stress: implication for animal welfare. Pages 1–21 in G. P. Moberg and J. A. Mench (eds). The Biology of Animal Stress, CABI Publishing, New York, New York, USA.
40. Monfort, S.L., C.S. Schwartz, and S.K. Wasser. 1993. Monitoring reproduction in captive moose using urinary and fecal steroid metabolites. Journal of Wildlife Management. 57:400–407.
41. Mong, T.W., J.H. Schulz, and J.J. Millspaugh. *In Press.* Design and construction of mourning dove research pens. Transcripts of Missouri Academy of Science.
42. Möstl, E. & R. Palme. 2002. Hormones as indicators of stress. Domestic Animal Endocrinology. 23:67-74.
43. Möstl, E., S. Rettenbacher & R. Palme. 2005. Measurement of corticosterone metabolites in birds' droppings: an analytical approach. Annals of the New York Academy of Sciences. 1046:17-34.
44. Munck, A., P.M. Guyre, and N.J. Holbrook. 1984. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocrine Reviews. 5:25–44.
45. O'Fegan, P.O. 2000. Validation, p211-238 in J.P. Gosling (ed). Immunoassays. Oxford University Press, New York.
46. Palme, R. & E. Möstl. 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. International Journal of Mammalian Biology. 62(Supplement II):192-197.
47. Palme, R. 2005. Measuring fecal steroids: guidelines for practical application. Annals of the New York Academy of Sciences. 1046:75-80

48. Palme, R., S. Rettenbacher, C. Touma,S. M. El-Bahr, and E. Mostl. 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Annals of the New York Academy of Sciences. 1040:162-171.
49. Reeves, H.M., A.D. Geis, and F.C. Kniffin. 1968. Mourning dove capture and banding. Special Scientific Report-Wildlife 117, U.S. Fish and Wildlife Service, Washington D.C.
50. Romero L.M., R.C. Romero. 2002. Corticosterone responses in wild birds: the importance of rapid initial sampling. Condor. 104:129-135.
51. Romero, L.M., and J.C. Wingfield. 1998. Seasonal changes in adrenal sensitivity alter corticosterone levels in Gambel's White-crowned Sparrows (*Zonotrichia leucophrys gambelii*). Comparative Biochemistry and Physiology 119C:31–36.
52. Romero, L.M., M. Ramenofsky, and J.C. Wingfield. 1997. Season and migration alters the corticosterone response to capture and handling in an Arctic migrant, the White-crowned Sparrow (*Zonotrichia leucophrys gambelii*). Comparative Biochemistry and Physiology. 116C:171–177.
53. Romero, L.M. & L. Remage-Healey. 2000. Daily and seasonal variation in response to stress in captive starlings (*Sturnus vulgaris*): corticosterone. General and Comparative Endocrinology. 119:52-59.
54. Romero, L.M. 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. General and Comparative Endocrinology. 128:1-24.
55. Sapolsky, R.M., L.M. Romero, and A.U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrinology Reviews. 21:55–89.
56. Schadt, J.C. and E.M. Hasser. 1998. Hemodynamic effects of acute stressors in the conscious rabbit. The American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 272: R814-R821
57. Schadt, J.C. and E.M. Hasser. 2001. The defense reaction alters the response to blood loss in the conscious rabbit. American Journal of Physiology - Regulatory. Integrative and Comparative Physiology. 280:R985-R993
58. Schulz, J.H., A.J. Bermudez, J.L. Tomlinson, J.D. Firman, and Z.He. 2001. Comparison of radiotransmitter attachment techniques using captive mourning doves. Wildlife Society Bulletin. 29:771–782.
59. Schulz, J. H., S.L. Sheriff, Z. He, C. E. Braun, R. D. Drobney, R. E. Tomlinson, D. D. Dolton, and R. A. Montgomery. 1995. Accuracy of techniques used to assign Mourning Dove age and gender. Journal of Wildlife Management. 59:759–765.
60. Schwarzenberger, F., E. Möstl, E. Bamberg, J. Pammer, and O. Schmehlik. 1991. Concentrations of progestagens and oestrogens in the faeces of pregnant Lipizzan, Trotter and Thoroughbred mares. Journal of Reproduction and Fertility Supplement. 44:489–499.
61. Tempel, D.J., and R.J. Gutiérrez. 2004. Factors related to fecal corticosterone levels in California Spotted Owls: implications for assessing chronic stress. Conservation Biology. 18:538–547.
62. Terio, K.A., S.B. Citino, and J.L. Brown. 1999. Fecal cortisol metabolite analysis for non–invasive monitoring of adrenocortical function in the cheetah (*Acinonyx jubatus*). Journal of Zoo and Wildlife Medicine. 30:484–491.

63. Teskey–Gerstl, A., E. Bamberg, T. Steineck, and R. Palme. 2000. Excretion of corticosteroids in urine and faeces of hares. Journal of Comparative Physiology B 170, 163–168.
64. Touma, C. & R. Palme. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of a biological validation. Annals of the New York Academy of Sciences. 1046: 54-74.
65. Touma, C., N. Sachser, E. Möstl, and R. Palme. 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. General and Comparative Endocrinology. 130:267–278.
66. Touma, C., R. Palme and N. Sachser. 2004. Analyzing corticosterone metabolites in the fecal samples of mice: a non-invasive technique to monitor stress hormones. Hormone and Behavior. 45:10-22.
67. Turner W. J., P. Tolson, N. Hamad. 2002. Remote assessment of stress in white rhinoceros (*Ceratotheruim simum*) and black rhinoceros (*Diceros bicornis*) by measurement of adrenal steroids in feces. Journal of Zoo and Wildlife Medicine. 33:214–221
68. Vleck, C. M., N Vertalino, D. Vleck, and T. L. Bucher. 2000. Stress, corticosterone, and heterophil to lymphocyte ratios in free-living Ade′lie Penguins. Condor. 102:392–400.
69. Washburn, B.E., and J.J. Millspaugh. 2002. Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white–tailed deer feces. General and Comparative Endocrinology 127, 217–222.
70. Washburn, B.E., D.L. Morris, J.J. Millspaugh, J. Faaborg, and J.H. Schulz. 2002. Using a commercially available radioimmunoassay to quantify corticosterone in avian plasma. Condor 104, 558–563.
71. Washburn, B.E., J.J. Millspaugh, J.H. Schulz, S.B. Jones, and T.W. Mong. 2003. Using fecal glucocorticoids for stress assessment in mourning doves. Condor 105, 696–706.
72. Wasser, S.K., K. Bevis, G. King, and E. Hanson. 1997. Noninvasive physiological measures of disturbance in the Northern Spotted Owl (*Strix occidentalis caurina*). Conservation Biology. 11:1019–1022.
73. Wasser, S.K., K.E. Hunt, J.L. Brown, K. Cooper, C.M. Crockett, U. Bechert, J.J. Millspaugh, S. Larson, and S.L. Monfort. 2000. A generalized fecal glucocorticoid assay for use in a diverse array of non-domestic mammalian and avian species. General and Comparative Endocrinology. 120:260–275.
74. Whitten, P.L., D.K. Brockman & R. Stavisky. 1998. Recent advances in noninvasive techniques to monitor hormone-behavior interactions. Yearbook of Physical Anthropology. 41:1-23.
75. Wingfield, J.C. 1994. Modulation of the adrenocortical response to stress in birds. Pages 520–528, in Perspectives in Comparative Endocrinology. K. G. Davey, R. E. Peter, and S. S. Tobe (eds). National Research Council of Canada, Ottawa, Canada.
76. Wingfield, J.C., C.M. Vleck, and M.C. Moore. 1992. Seasonal changes in the adrenocortical response to stress in birds of the Sonoran Desert. Journal of Experimental Zoology. 264:419–428.

77. Young, K.M., S.L. Walker, C. Lanthier, W.T. Waddell, S.L. Monfort, J.L. Brown. 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. General and Comparative Endocrinology. 137: 148-165.
78. Yousef, M. K. 1988. Animal stress and strain: definition and measurements. Applied Animal Behavioral Science. 20:119–126.
79. Zar, J.H. 1996. Biostatistical analysis. 3rd edition, Prentice Hall, Upper Saddle River, New Jersey, USA.