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VALIDATION OF A NONINVASIVE TECHNIQUE FOR THE ASSESSMENT OF
PHYSIOLOGICAL STRESS IN COYOTES (*CANIS LATRANS*)

by

Erika T. Stevenson

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Wildlife Biology

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UTAH STATE UNIVERSITY
Logan, Utah

2015

ABSTRACT

Validation of a Noninvasive Technique for the Assessment of Physiological Stress in
Coyotes (*Canis latrans*)

by

Erika T. Stevenson, Master of Science

Utah State University, 2015

Major Professor: Dr. Eric M. Gese
Department: Wildland Resources

Quantifying physiological stress may aid in a better understanding of how animals survive various environmental conditions. One noninvasive technique for assessing physiological stress in animals is to extract steroid hormones (e.g., cortisol, corticosterone) from fecal samples which provide a quantitative value that enables assessment of physiological stress in animals. Therefore, this technique has the potential to aid in wildlife conservation by providing a better understanding of behavior and welfare for a variety of species. The objectives of the study were to (1) determine dose responses in plasma glucocorticoids and fecal GCM concentrations for coyotes (*Canis latrans*), (2) determine the utility of cortisol versus corticosterone for examining physiological stress responses for coyotes when using radioimmunoassays, and (3) determine the longevity of glucocorticoid metabolite concentrations found in coyote scats during 2 different seasons. We first conducted an ACTH challenge in 16 coyotes and examined both plasma and fecal glucocorticoid metabolites. Animals were anesthetized

and intravenously injected with exogenous ACTH with blood samples taken at 5 different time periods. Another 16 coyotes were used as controls and received a saline solution. We also collected fecal samples pre- and post-injection to measure fecal glucocorticoid metabolites. Radioimmunoassays were used to measure concentrations of cortisol in plasma, and fecal cortisol and corticosterone metabolites concentrations. To evaluate if glucocorticoid metabolite concentrations remained in feces for an extended period of time we collected samples from 6 captive coyotes and left the samples in natural environmental conditions for 13 days. Each day a sub-sample was collected, and hormones were extracted and run through radioimmunoassay. We found dose responses after an ACTH challenge in both plasma glucocorticoids and fecal GCMs, validating the use of fecal GCM concentrations as a tool to measure physiological stress in coyotes. We also found there were no significant differences, according to repeated measures, multi-way and one-way ANOVAs, in levels of glucocorticoid metabolite concentrations over 13 days. Our study provides validation for use of fecal glucocorticoid metabolites in coyotes to quantify stress levels and confirms that steroid hormone metabolites are viable up to 13 days post deposition in coyote scat. This noninvasive tool can aid in the evaluation of the abilities of coyotes to adapt and exist in a variety of habitats.

PUBLIC ABSTRACT

Validation of a Noninvasive Technique for the Assessment of Physiological Stress in
Coyotes (*Canis latrans*)

Erika T. Stevenson

Quantifying physiological stress may aid in a better understanding of how animals survive various environmental conditions. One noninvasive technique for assessing physiological stress in animals is to extract steroid hormones from fecal samples. This technique has the potential to aid in wildlife conservation by providing a better understanding of behavior and welfare for a variety of species. The objectives of the study were to (1) determine responses in plasma and fecal steroid hormone concentrations for coyotes (*Canis latrans*), (2) determine which steroid hormone (cortisol or corticosterone) was better for examining physiological stress responses for coyotes, and (3) determine the amount of time steroid hormone metabolites can be found in coyote scats during 2 different seasons. We first conducted an adrenocorticotrophic hormone (ACTH) challenge in 16 coyotes and examined both plasma and fecal steroid hormone concentrations. An ACTH challenge is when there is an externally derived hormone (ACTH) injected into a subject's blood stream, which causes an increase in the subject's circulating steroid hormones associated with physiological stress. We injected 16 treatment animals with ACTH and 16 control animals with a saline solution. We collected blood and fecal samples pre- and post-injection to measure steroid hormone concentrations. Radioimmunoassay, a laboratory method used to measure substances, was used to measure concentrations of steroid hormones in coyote blood and feces. To

evaluate if steroid hormone concentrations remained in feces for an extended period of time we collected samples from 6 captive coyotes and left the samples in natural environmental conditions for 13 days. Each day a sub-sample was collected, and hormones were extracted and run through radioimmunoassay. We found increased steroid hormone concentrations after an ACTH challenge in both blood and feces, validating the use of fecal steroid hormone concentrations as a tool to measure physiological stress in coyotes. We also found there were no differences in levels of steroid hormone concentrations over 13 days. Our study provides validation for use of fecal steroid hormone concentrations in coyotes to quantify stress levels and confirms that steroid hormone metabolites are viable up to 13 days post deposition in coyote scat. This noninvasive tool can aid in the evaluation of the abilities of coyotes to adapt and exist in a variety of habitats.

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Erika T. Stevenson

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CHAPTER 1

INTRODUCTION

The endocrine system plays a vital role in the body's ability to adapt to threatening situations (Boonstra 2004; Sheriff et al. 2011) by altering the physiological and behavioral responses of the organism. Knowledge of physiological stress can be an advantage to better understand the interaction of wildlife and their natural environment (von der Ohe and Servheen 2002; Boonstra 2004; Dalmau et al. 2007). A definition that describes physiological stress well is the following, "in biology and medicine, *stress* refers to the generalized, non-specific response of the body to any factor that overwhelms, or threatens to overwhelm, its compensatory abilities to maintain homeostasis" (Arnemo and Caulkett 2007). The levels of glucocorticoids in free-ranging animals have been used in numerous wildlife species as an index of stress and to identify the environmental factors contributing to stress (Millsbaugh and Washburn 2004; Palme et al. 2005; Keay et al. 2006). Two glucocorticoids, cortisol and corticosterone, are important signaling chemicals of the endocrine system that function to alter physiology and behavior in response to acute stressors in the environment. Elevated glucocorticoid levels allow an animal to maintain homeostasis during exposure to an acute stressor. Most importantly, elevated glucocorticoids promote a short-term increase in the availability of energy to the animal (Boonstra 2004). The mobilization of body energy stores in an animal is important for a flight or fight response to an immediate stressor. Whereas, chronic stress can result in a multitude of negative results including reduced reproduction, prolonged wound healing, and captive animal stereotypies (Möstl and

Palme 2002; Young et al. 2004; Touma and Palme 2005; French et al. 2006; Keay et al. 2006; Ellenberg et al. 2007).

Because of their benefit for coping with environmental stressors, measures of glucocorticoids have become the standard for monitoring the welfare of many wildlife species (Keay et al. 2006). In particular, fecal glucocorticoid metabolite (GCM) analysis has been identified as the most useful measure of exposure to chronic stress in animals (Millspaugh and Washburn 2004; Young et al. 2004). There have been a number of studies conducted using noninvasive methods for testing glucocorticoid levels in animals (McLeod et al. 1996; Wasser et al. 2000; Touma et al. 2003; Young et al. 2004; Creel 2005; Keay et al. 2006). When animals are handled they have a natural stress reaction, therefore a noninvasive means of measurement (e.g., fecal collections) provides more valid information on the physiological state of the animal, instead of a measurement of handling stress (Möstl and Palme 2002; Millspaugh and Washburn 2004; Touma and Palme 2005; Viljoen et al. 2008). This is because the concentrations measured are from metabolized glucocorticoids, meaning that the levels produced in feces will be from a previous time period such as the day before (Young et al. 2004; Touma and Palme 2005; Hulsman et al. 2011). The plasma measurements produce an immediate response to a stressor. This is because glucocorticoids are constantly circulating throughout the body and an increase in the concentration would be detectable in minutes in the plasma (Romero 2002). Therefore, measures derived from scats are representative of an animal's stress response to their environment, whereas levels in the blood would likely indicate the stress response to handling.

Noninvasive fecal collections are also a more feasible means for assessing glucocorticoid levels in wild animals as compared to other methods (Monfort et al. 1998; Mashburn and Atkinson 2004; Millspaugh and Washburn 2004; Young et al. 2004; Palme et al. 2005). However, the use of fecal GCMs should be validated for the species of interest to be sure the results are biologically meaningful (Touma and Palme 2005; Keay et al. 2006). Scat collection in the field has some downfalls such as variability, individual identification, as well as, the cost and time required for collection (Goymann 2012). Therefore, the effects of time and environmental factors of fecal GCM degradation should also be examined. Determining the amount of time that feces obtain viable measurements of GCM concentrations, could assist in determining when scats should be collected, thereby increasing the efficacy and reducing the costs of scat collections.

The overall objective of this study was to validate the use of fecal glucocorticoid metabolites as a means for measuring physiological stress in coyotes. We completed a comprehensive validation through conducting an adrenocorticotrophic hormone challenge and by examining the longevity of glucocorticoid metabolite concentrations in coyote scat. The first part of the study was the adrenocorticotrophic hormone challenge using 32 coyotes and the second part of the study was the degradation test using 6 coyotes.

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CHAPTER 2

**PLASMA AND FECAL GLUCOCORTICOID METABOLITES FOLLOWING
AN ACTH CHALLENGE IN COYOTES: A COMPREHENSIVE
INVESTIGATION**

ABSTRACT

Knowledge of physiological stress can be an advantage to understanding how animals survive in their environment. One technique for assessing physiological stress in animals is to extract steroid hormones (e.g., cortisol, corticosterone) from fecal samples. This procedure provides a means for measuring fecal glucocorticoid metabolite concentrations noninvasively and is thus a useful tool for quantifying stress in animals. However, an adrenocorticotrophic hormone (ACTH) challenge should first be conducted to validate the use of fecal glucocorticoid metabolite concentrations for measuring stress response in animals. We conducted an ACTH challenge using 32 (16 treatment, 16 control) coyotes (*Canis latrans*) and examined the results in both plasma and fecal glucocorticoid metabolites. Treatment and control animals were anesthetized for approximately 90 minutes and intravenously injected with exogenous ACTH and post-injection blood samples were drawn at four different time points; the 16 control animals received injections of saline. We also collected fecal samples pre- and post-injection to measure fecal glucocorticoid metabolites and determine if a physiological stress response could be found in fecal samples. We used radioimmunoassays to measure the concentrations of cortisol in plasma, and the concentrations of cortisol and corticosterone metabolites in feces. We found fecal glucocorticoid metabolite stress responses mirrored

the stress responses of plasma glucocorticoids, validating the use of fecal glucocorticoid metabolite concentrations as a valuable tool to measure physiological stress for coyotes. We also determined that, in the feces, corticosterone appeared to be more responsive to the ACTH challenge and therefore is the more appropriate fecal glucocorticoid metabolite to measure in coyotes.

INTRODUCTION

The endocrine system plays a vital role in the body's ability to acclimatize to threatening situations (Boonstra 2004; Sheriff et al. 2011) by altering the physiological and behavioral responses of the organism. When the endocrine system reacts to a threatening situation or stressor, it mobilizes the energy required to maintain homeostasis and survive (Boonstra 2004; French et al. 2009; Aguilera 2011). This energy mobilization is vital for an immediate stress response, and depending on the stressor, whether it is acute or chronic, changes the energy cost of maintaining homeostasis and surviving (French et al. 2002; Keay et al. 2006). For example, a chronic stressor has a high energy cost which causes the body to pull energy from other life functions, such as reproduction, immune health, and growth (French et al. 2002; Möstl and Palme 2002; Young et al. 2004; Touma and Palme 2005; Keay et al. 2006). An acute stressor, however, quickly mobilizes energy for the flight or fight stress response, increasing catecholamines, coagulation, glucagon stimulation, and breathing, and when all combined, increases the chance of survival (Boonstra 2004; Arnemo and Caulkett 2007).

Knowledge of physiological stress can be an advantage to better understand the interaction of wildlife and their natural environment (von der Ohe and Servheen 2002;

Boonstra 2004; Dalmau et al. 2007). A definition that describes physiological stress well is the following, “in biology and medicine, *stress* refers to the generalized, non-specific response of the body to any factor that overwhelms, or threatens to overwhelm, its compensatory abilities to maintain homeostasis” (Arnemo and Caulkett 2007). Chronic stress can result in a multitude of negative results including reduced reproduction, prolonged wound healing, and captive animal stereotypies (Möstl and Palme 2002; Young et al. 2004; Touma and Palme 2005; French et al. 2006; Keay et al. 2006; Ellenberg et al. 2007). French et al. (2006) found the immune systems of tree lizards (*Urosaurus ornatus*) were suppressed under stress. Wounds of treatment lizards were larger and less healed compared to control lizards. A comparison of yellow-eyed penguins (*Megadyptes antipodes*), showed penguins in a tourist area had higher capture corticosterone levels, as well as lower reproductive success than those in an undisturbed area (Ellenberg et al. 2007). Cabezas et al. (2007) reported a decrease in body mass index found in European wild rabbits (*Oryctolagus cuniculus*) under stress, but interestingly found an increase in survivability of those animals that had higher stress responses once released. A better understanding of physiological stress may also give insight to the capabilities of animals to survive in a variety of habitats, as well as, making improvements for captive animals and their welfare (Touma et al. 2003).

One tool for measuring stress is determining glucocorticoid concentrations (Monfort et al. 1998; Keay et al. 2006; Arnemo and Caulkett 2007; Barja et al. 2008). Glucocorticoids (such as cortisol and corticosterone) are steroid hormones associated with physiological stress (Möstl et al. 1999; von der Ohe and Servheen 2002; Millspaugh and Washburn 2004; Dalmau et al. 2007). One of the useful aspects about glucocorticoids

is they provide a quantitative means for evaluating physiological stress in animals (von der Ohe and Servheen 2002). The release of glucocorticoids is part of a negative feedback loop which starts with the activation of the hypothalamic-pituitary-adrenal axis (HPA-axis) (Creel et al. 1997; Sapolsky et al. 2000; Creel 2005; Arnemo and Caulkett 2007; Aguilera 2011; Sheriff et al. 2011). The HPA-axis is activated when adrenocorticotrophic hormone is released from the anterior pituitary gland which is activated by corticotropin-releasing hormone synthesized in the hypothalamus (Sapolsky et al. 2000; von der Ohe and Servheen 2002; Touma and Palme 2005; Arnemo and Caulkett 2007; Aguilera 2011; Sheriff et al. 2011,). When the glucocorticoids are released, they in turn stop the HPA-axis activation (Creel et al. 1997; Sapolsky et al. 2000; Creel 2005; Aguilera 2011; Sheriff et al. 2011). It is important to note that the main role of glucocorticoids is energy regulation for the maintenance of homeostasis (Busch and Hayward 2009). When there is a high amount of glucocorticoids circulating within the body, an individual is undergoing physiological stress and must re-focus the energy for survival and thus pull the necessary energy from other daily functions (Boonstra 2004; Creel 2005; Busch and Hayward 2009). There are several different means for measuring glucocorticoid concentrations: hair, feathers, saliva, plasma, urine and fecal samples (Sheriff et al. 2011). Some of these methods are more intrusive than others, with the noninvasive methods being more desirable for most studies.

There have been a number of studies conducted using noninvasive methods for testing glucocorticoid levels in animals (McLeod et al. 1996; Wasser et al. 2000; Touma et al. 2003; Young et al. 2004; Creel 2005; Keay et al. 2006). When animals are handled they have a natural stress reaction, therefore a noninvasive means of measurement (e.g.,

fecal collections) provides more valid information on the physiological state of the animal, instead of a measurement of handling stress (Möstl and Palme 2002; Millspaugh and Washburn 2004; Touma and Palme 2005; Viljoen et al. 2008). This is because the concentrations measured are from metabolized glucocorticoids, meaning that the levels produced in feces will be from a previous time period such as the day before (Young et al. 2004; Touma and Palme 2005; Hulsman et al. 2011). The plasma measurements produce an immediate response to a stressor. This is because glucocorticoids are constantly circulating throughout the body and an increase in the concentration would be detectable in minutes in the plasma (Romero 2002). Therefore, measures derived from scats are representative of an animal's stress response to their environment, whereas levels in the blood would likely indicate the stress response to handling.

Noninvasive fecal collections are also a more feasible means for assessing glucocorticoid levels in wild animals as compared to other methods (Monfort et al. 1998; Mashburn and Atkinson 2004; Millspaugh and Washburn 2004; Young et al. 2004; Palme et al. 2005). Collecting scat is less dangerous for the animals compared to blood sampling (Millspaugh and Washburn 2004; Palme et al. 2005). Blood sampling animals, especially sensitive ones, may cause problems because the animals must be manually or chemically restrained. Capture and handling can also be dangerous to the animal, whether through injury from manual restraint, or animals may have a negative reaction to the chemical immobilization (Creel et al. 1997).

Use of fecal glucocorticoid metabolites (GCM) should be validated to ensure the results are biologically meaningful (Touma and Palme 2005; Keay et al. 2006). The procedure for validating the use of fecal GCMs is to conduct an adrenocorticotrophic

hormone (ACTH) challenge. To properly conduct a fecal ACTH challenge, fecal samples should be collected during multiple points during the day (e.g., morning, noon, and night) for a period before and after an exogenous injection of ACTH. The injection causes the activation of the HPA-axis and therefore an increase in the release of glucocorticoids. Inducing the HPA-axis in this form provides the researcher with the knowledge that there should be an increase in fecal GCMs if they are a valid method for determining glucocorticoid concentrations (Touma and Palme 2005; Keay et al. 2006). When conducting an ACTH challenge it is important to consider the differences between males and females, as well as the diurnal fluctuation of glucocorticoids (Touma et al. 2003; Touma and Palme 2005; Keay et al. 2006; Hoon Son et al. 2011).

Validations of fecal GCMs have been conducted in a number of species from many different taxonomic families (Wasser et al. 2000; Schatz and Palme 2001; Hunt and Wasser 2003; Dloniak et al. 2004; Hunt et al. 2004; Young et al. 2004; Hulsman et al. 2011; Santymire et al. 2012). In the canidae family, ACTH challenges have been conducted in domestic dogs, crab-eating foxes (*Cerdocyon thous*), African wild dogs (*Lycaon pictus*), coyotes (*Canis latrans*), red wolves (*Canis rufus*), maned wolves (*Chrysocyon brachyurus*) and gray wolves (*Canis lupus*) providing information about the physiological stress response (de Villiers et al. 1997; Monfort et al. 1998; Schatz and Palme 2001; Sands and Creel 2004; Young et al. 2004; Vasconcellos et al. 2011; Schell et al. 2013; Rodrigues da Paz et al. 2014). However, none of these studies validated whether the response of fecal GCMs follows the response of blood glucocorticoids.

For this study we conducted an ACTH challenge using 32 (16 M and 16 F) captive coyotes, collecting both blood and fecal samples. The main objectives for this

study were to (1) determine the dose response in plasma and fecal samples collected after an ACTH injection, (2) determine if the response in fecal GCMs resembles blood glucocorticoids, and (3) validate the use of fecal glucocorticoid metabolites through radioimmunoassay, and determine which glucocorticoid (cortisol or corticosterone) is better suited for measuring fecal GCMs in coyotes.

MATERIALS AND METHODS

Study Location. –The experiment was conducted at the U.S. Department of Agriculture, Wildlife Services, National Wildlife Research Center, Predator Research Facility near Millville, UT, U S A. During testing, coyotes were individually housed in outdoor kennels. The coyotes were housed in either a raised or floored kennel (raised floor: 2.4 x 1.2 x 1.8 m; small floored: 3.7 x 0.9 x 2.0 m; large floored: 3.7 x 1.8 x 2.0 m). Due to cold temperatures, floored kennels had pine shavings spread over the floor. Each kennel type was equipped with a den box. Coyotes were moved into kennels one week prior to testing to allow for acclimation. Animals were fasted 24 hours prior to injection; all other days they were provided with their normal ration (650 g) of commercial mink food. Water was provided ad libitum. Kennels were checked every day and cleaned once per day except on the fast day. The experiment ran from 6 November to 18 December 2010.

Study Animals. –We used 32 coyotes (16 males and 16 females) for this experiment, ranging in age from 2 to 5 years. Each individual was randomly assigned as either a treatment or a control animal (8 M and 8 F per group). On the day of the challenge, the coyotes were pushed into their den boxes and manually immobilized with

pin sticks for the intramuscular injection of a 5:1 ketamine to xylazine anesthesia solution (2 animals anesthetized at a time); this type of restraint is routine activity for the coyote colony. The amount of anesthesia drug administered varied between coyotes, based on their body size (approximately 10.71–16.67 mg/kg ketamine, and approximately 1.7–2.77 mg/kg xylazine). Once the animals were anesthetized, we weighed them, and initiated measurements of temperature, respiration, and pulse. The treatment group was given an injection of ACTH (4 IU/kg) (Sigma-Aldrich, Co., St. Louis, MO) and the control group was given an injection of sterile saline solution (4 IU/kg); both were administered intravenously.

Sample Collections. –Fecal collections were initiated 2 days prior to injection and then continued for 2 days post injection: scats were collected 3 times per day with each sample collected by the same person. This collection schedule allowed for a baseline measurement of fecal GCMs while also considering the diurnal fluctuations of the metabolites. To ensure the freshest sample was collected each time, any remaining scat was removed from the kennel. Fecal samples were frozen in a -20° C freezer until extraction.

The blood sampling occurred at 5 different time intervals: pre-injection (first blood draw after anesthetizing), and then at 4 times post injection (10, 30, 60, and 90 minutes post-injection). The amount of blood collected at each interval was approximately 2 mL. The coyotes were kept under anesthesia for approximately 90 minutes and allowed to recover without any drug reversal. Prior to centrifuging, blood clots were removed from the blood tubes. The blood was centrifuged for 20 minutes at

room temperature; the plasma was then collected into a cryovial and frozen at -20°C until extraction.

Plasma and Fecal Hormone Extraction. –Prior to running the radioimmunoassay on the plasma samples, the hormones were extracted. We only examined the cortisol concentrations in the plasma due to a preliminary examination in which we determined that the corticosterone levels were too small to be accurately measured. The methods for extracting the hormones from the plasma followed the protocol in Neuman-Lee and French (2014). The first step in the fecal hormone extraction process was to formulate the phosphate-methanol buffer solution. Distilled water (700 mL) was added to a RIA glass container; 8.75 g NaCl (Sigma-Aldrich, St. Louis, MO), 5.7 g $\text{NaH}_2\text{PO}_4\text{-H}_2\text{O}$ (Sigma-Aldrich), 8.66 g Na_2HPO_4 (Sigma-Aldrich), and 1.0 g sodium azide (Sigma-Aldrich) was added into the water and stirred until dissolved. Next, we added 0.5 mL Tween 20 (Sigma-Aldrich) and gently stirred until mixed. The pH was tested (7.0 was desired) and then distilled water was added for a final volume of 1000 mL. The final step was to slowly dissolve BSA (bovine serum, Sigma-Aldrich) by adding it to the surface of the solution, this stock solution was then refrigerated until used. Just prior to initiating the extraction process, the working buffer was created by mixing stock solution at room temperature and methanol in equal volumes following Shideler et al. (1994), and Bauman and Hardin (1998).

The second step in the fecal extraction process was to homogenize the scat before being weighed; approximately 0.5 g of scat was put into a plastic scintillation vial. We then added 0.5 mL of the working solution to each scintillation vial with our 0.5 g fecal samples. Each sample was vortexed until the solution was homogenized. The samples

were then placed onto a shaker for approximately 16 hours at 200 RPM at room temperature. After shaking we allowed the solution to settle for approximately 1 hour. From the top of the supernatant, 50 μL was pipetted into 12 x 75 mm polypropylene tubes and centrifuged (in a refrigerated centrifuge) for 1 hour at 4,000 RPM. The centrifuged supernatant was decanted into cryovial tubes, then frozen at -80°C until the radioimmunoassay was initiated. The solution left behind in the scintillation tubes were dried overnight in a vented 100°C oven. The dried material was cooled to room temperature and weighed to determine the dry weight of the remaining fecal sample when calculating hormone concentrations.

Radioimmunoassays. –Cortisol concentrations (for both fecal and plasma samples) were determined using radioimmunoassay (Siemens Coat-a-count cortisol RIA kit, Siemens Healthcare Diagnostics Inc., U S A; cross reactivity with cortisol and cortisone is less than 1%). The first step of the process was to turn on a water bath at 37°C , warm all liquids to room temperature, and pull out the cryovial tubes containing the sample supernatant. Two uncoated 12 x 75 mm polypropylene tubes were labeled as total counts (TC) and 2 were labeled as nonspecific binding (NSB). The rest of the tubes used were coated with antibodies to cortisol. The calibrators for creating the standard curve were supplied in the kit; these tubes were labeled A-F and were run in duplicate. The sample tubes were also labeled in duplicate. We then pipetted 25 μL of the zero calibrator (A) into the bottom of the NSB and A tubes as well as 10 μL of the working buffer solution. The remaining calibrators (B-F) were pipetted into the bottom of the appropriately labeled tubes in 25 μL amounts plus 10 μL of the working buffer solution. The samples were pipetted directly to the bottom of their tubes using 10 μL of the

supernatant in addition to 25 μL of the zero (A) calibrator. The next step was adding 1.0 mL of the label (^{125}I) into each tube and vortexing the tubes. The TC tubes were then covered in parafilm and set aside. All of the other tubes were incubated in the water bath for approximately 45 minutes. All of the tubes, except TC, were then decanted and placed upside down in a foam decanting rack for 2-3 minutes. The tubes were then struck on absorbent paper until there was no longer any visible moisture. Each tube was read for 1 minute in a gamma counter. There were some samples that did not fall within the standard curves. These samples were re-run using double the amount of sample supernatant and 15 μL of the zero calibrator solution; we did not have enough material to run these samples in duplicate. For the plasma samples there was no working buffer solution added and the amount of the plasma supernatant added was 50 μL . Unfortunately for 1 of the males on the study there was not enough blood collected to run in the radioimmunoassay for the 30 minute time slot. Each time an assay was run a new standard curve was created.

Corticosterone concentrations (for fecal samples only) were determined using radioimmunoassay (ImmuChemTM Double Antibody RIA kit, MP Biomedicals, Orangeburg, NY; cross reactivity with other metabolites is less than 1 %). The first step of the process was to bring all of the reagents to room temperature, pull out the cryovials with sample supernatant, and add 2.0 mL of distilled water to each of the controls and allow these mixtures to equilibrate to room temperature for 30 minutes. Next we labeled 10 x 75 mm glass test tubes in duplicate. The standard curve made up the first 20 tubes of the assay. We then pipetted 150 μL of the steroid diluent into the NSB tubes. The zero binding tubes had 50 μL of steroid diluent pipetted into them. For the next part of the

standard curve (tubes 5–16) we pipetted 50 μL of calibrator and 10 μL of the working buffer solution into those tubes. Tubes 17–20 were the control tubes and 50 μL of the control solutions provided were pipetted into these tubes. All of the sample tubes received 10 μL of the sample supernatant and 40 μL of the steroid diluent. The next step was to add 100 μL of ^{125}I (blue reagent) to all of the tubes, as well as (except for the NSB tubes) 100 μL of the anti-corticosterone (yellow reagent). All of the samples were vortexed. The tubes were then incubated for 2 hours at room (22–25° C) temperature. Next 250 μL of the precipitant solution (red reagent) was added to all of the tubes and vortexed thoroughly. The tubes were then placed in a refrigerated centrifuge for 15 minutes at 2400 RPM. All tubes were decanted and then blotted, careful to not lose the precipitants, on absorbent paper. Each tube was read for 1 minute in a gamma counter. For the samples that had to be re-run because they did not fall in the standard curves, the amount of sample supernatant was halved and the amount of steroid diluent increased to 45 μL . Each time an assay was run a new standard curve was created.

Calculations. –For both cortisol and corticosterone the method for calculating the concentrations of the metabolites was the same (following procedures provided with radioimmunoassay kits). All samples were run in duplicate. Average sample values were first corrected for non-specific binding (NSB) and then converted to percent bounds by dividing the net counts by the zero bound net count. Percent bound was converted to concentration values using the standard curve. To calculate the final metabolite concentrations we had to do several corrections first. Final concentrations were corrected for dilution factor and for dry fecal mass resulting in a final concentration of hormone per gram of fecal matter. Plasma samples were also corrected via individual recovery values

that were calculated by adding a small amount of radioactivity to all samples prior to extraction. A subsample of each assayed sample was then run through a liquid scintillation counter to correct for any sample loss that happened during the extraction process.

Plasma Sample Statistics. –The first statistical test performed was a multi-way ANOVA to determine the influence of sex (male, female), treatment type (ACTH or control), and the time of the blood draw (0, 10, 30, 60, 90) on concentrations of cortisol in the plasma samples. Next we separated the data for the ACTH and control groups, due to the treatment type being a significant factor. We then ran a one-way ANOVA with a post-hoc Tukey's test for each of these groups (ACTH, control) to determine which times of blood collection were significantly different. In addition, we performed a repeated measures ANOVA to determine whether the individual coyotes responded to treatment differently. We separated the subjects by sex and treatment type to examine if the levels of cortisol were influenced by individual variation (between subjects), versus the time of blood collection (treatment).

Fecal Sample Statistics. – When we conducted the statistical tests for the fecal samples, the samples we used were from the first time period (morning) collection due to the lack of consistent collection during the other 2 time periods. For both the cortisol and corticosterone metabolite levels, we first performed a multi-way ANOVA to determine the influence of sex (male, female), treatment type (ACTH, control) and the day of feces collection before or after injection (2 days before, 1 day before, 1 day after, and 2 days after). Next, due to the sex of the animal being a highly significant factor, we separated the groups by males and females. Then we ran a one-way ANOVA and a post-hoc

Tukey's test to determine which days of fecal collection were significantly different from one another for the glucocorticoid metabolite concentrations. In addition, we also performed a repeated measures ANOVA, for both the cortisol and corticosterone metabolite levels, separated by sex and treatment type, to determine if there was significant individual variation (between the subjects) or significant differences between the fecal collection days (treatment) on the levels of GCM metabolite concentrations in the fecal samples.

RESULTS

We originally planned having 32 coyotes in the experiment. However, we removed 1 male and 3 females from the study due to procedural problems that would influence the accurate and unbiased measurement of cortisol and corticosterone. The male was removed because he recovered from the anesthesia too early and consequently received an additional injection of Telazol to which he reacted poorly. One female was removed due to her becoming hypothermic during anesthesia. The other 2 females were removed due to human error during the ACTH injections (i.e., the animals did not receive the proper ACTH dosage).

Plasma Cortisol. –For the cortisol concentrations in the coyote plasma, for both treatment types combined, we found that 71% of the variation in cortisol levels was explained by the sex of the coyote, treatment type, and time of blood draws, and the interactions of these 3 variables. There was significant influence in the multi-way ANOVA of the treatment type, the time of the blood draw, the interaction of the treatment type, and the time of the blood draw (Table 2–1). Our results indicated that the

treatment type of the coyote was a larger influence compared to the time of the blood draw, and the interaction of the treatment type and the time of the blood draw (Table 2–1; Fig. 2–1). According to the results of the one-way ANOVA and post-hoc Tukey’s test, we found that the time of the blood draw was a highly significant factor ($R^2 = 0.312$, $F = 7.385$, $P < 0.001$; Fig. 2–1).

Our results from the repeated measures ANOVA for cortisol concentrations in coyotes showed significant differences for the influence of the time of the blood draw (Table 2–2). This indicates that we have found dose responses for the ACTH injections and handling responses for the control animals. The repeated measures ANOVA also found significant differences between control animals (Table 2–2). This indicates that individual animals have different responses to handling, or rather individual variation. Interestingly, the repeated measures ANOVA model found no significant differences between treatment individuals (Table 2–2). This would indicate that the injection of the ACTH had more of an influence than handling. The assay sensitivity was 2 ng/mL, with the intra-assay coefficient of variation $< 5\%$.

Fecal Cortisol Metabolite. –For the cortisol metabolite concentrations in the coyote scats, we found that only 15% of the variation in the concentrations was explained by the sex of the coyote, the treatment type, the day of fecal collection, and by the interactions of these 3 variables (Table 2–3). From the multi-way ANOVA we found the day of the fecal collection to be a significant factor, which indicates a dose response from the ACTH and a handling response for the control animals. The results from our one-way ANOVA and post-hoc Tukey’s test did not find a significant (at 0.05) influence of the day of collection for the fecal cortisol metabolite concentrations, however, it did appear

to have some influence on male coyotes (males: $R^2 = 0.128$, $F = 2.749$, $P = 0.051$; females: $R^2 = 0.082$, $F = 1.434$, $P = 0.244$).

We found no significant influences, in the one-way ANOVAs, for fecal cortisol metabolite concentrations (Table 2–4). This indicates that there was no influence of the day of the fecal collection for fecal cortisol metabolite concentrations (Fig. 2–2). The assay sensitivity was 2 ng/mL, with the intra-assay coefficient of variation < 10% and the interassay coefficient of variation < 20%.

Fecal Corticosterone Metabolite. –For the fecal corticosterone metabolite concentrations we found 44% of the variation in concentrations was explained by the sex of the coyote, the treatment type, the day of fecal collection, and the interactions of these 3 variables, from the multi-way ANOVA (Table 2–5). Our results indicate that the sex of the coyote was a highly significant variable (Fig. 2–4). The time of the fecal collection was also a significant influence (Fig. 2–3). From the one-way ANOVAs, with post-hoc Tukey’s tests, we found the time of the fecal collection was a significant factor for males but not for females (males: $R^2 = 0.564$, $F = 24.132$, $P = 0.000$; females: $R^2 = 0.138$, $F = 2.558$, $P = 0.066$).

Our results from the repeated measures ANOVAs indicated that the day of the fecal collection influenced control males, ACTH males, and ACTH females (Table 2–6). However, there was not a significant influence of the day of fecal collection for control females. We also found that there was no significant variation between subjects for the fecal corticosterone metabolite concentrations (Table 2–6). These results suggest that there was a dose response of the ACTH injections for both males and females, but only handling response for the control males. Also, there was an indication that individual

variation was not a factor that influenced fecal corticosterone metabolite concentrations. The assay sensitivity was 7.7 ng/mL, with the intra-assay coefficient of variation < 10% and the interassay coefficient of variation < 20%.

We also examined the fecal GCM diurnal fluctuation for both cortisol (Fig. 2–6) and corticosterone (Fig. 2–7) on two different days before the ACTH challenge. We only separated the data by sex for the corticosterone samples due to no sex differences between males and females in the cortisol samples, but we did find differences between the sexes for corticosterone samples. Though there does appear to be some influence of the diurnal fluctuation, the individual variation was a larger influence. The standard deviation error bars overlap for the 3 different time periods on both days indicating no influence of the time of collection on cortisol and corticosterone levels in the fecal samples.

We were also interested in determining if the time of handling on the test day was an influence for the baseline plasma cortisol concentrations. The animals were classified based on the time of handling. The morning group consisted of animals handled before 12 p.m. and the afternoon group was handled after that time. However, due to the overlap of the standard deviation error bars, there does not appear to be any significant differences (Fig. 2–8).

Another variable we examined was age of the coyote. The data was separated by age, and then the baseline measurements for each age were calculated. We also separated the corticosterone fecal data by sex. Result of the analysis does not indicate any significant differences in concentrations of cortisol and corticosterone in the plasma and fecal samples (Figs. 2–9, and 2–10).

DISCUSSION

This study was the first to comprehensively compare the results of plasma glucocorticoids and fecal GCMs in response to an ACTH challenge for a species in the family Canidae. For our plasma samples, we found our exogenous ACTH induced peaks to be between ~30–60 minutes post ACTH injection. Our fecal samples produced clear peaks with the corticosterone metabolites ~1 day (~12 hours) post injection; the cortisol metabolites also peaked ~1 day post injection though the picture was not as clear as the corticosterone metabolites. Our fecal peaks may have occurred sooner, however the study coyotes were fasted the day before the ACTH challenge. Also we were unable to determine the actual time that the fecal samples were deposited in the kennels. Overall, our study results indicated there was a dose response in both the plasma and fecal samples following an ACTH injection, as well as that fecal GCMs do mirror plasma glucocorticoid concentrations and therefore the noninvasive fecal technique to measure physiological stress in coyotes appears to be a valid measurement of stress response.

We also found individual variation of plasma and fecal GCM concentrations. Because our sample size was large, we were able to better interpret our results. Even though there was individual variation we still found dose responses in the treatment animals and handling responses in the control animals. A majority of previous ACTH challenge studies had sample sizes between 1–4 animals, and therefore due to the smaller sample sizes, variation reported in those studies may be misleading. We recommend a large sample size whenever possible to compensate for any individual variation that may occur.

From our data we found that the age of the coyote was not a significant influence on metabolite concentrations in the plasma or fecal samples. Perhaps if we had a larger sample size for each age group we may have found a difference. We also found that the time of handling did not have an influence over the initial plasma glucocorticoid concentrations, probably due to the fact that handling is a stressful event. Though there does appear to be an influence of diurnal fluctuation, we were unable to find the results from the data to be significant. However, we also were unable to regularly collect fecal samples from all of the animals during all 3 time periods. If we had a larger sample size for all 3 time periods the standard deviations would probably be smaller and we would potentially find a significant difference.

Other ACTH challenges involving the comparison of blood glucocorticoids to fecal GCM concentrations also found fecal GCMs to have the same patterns. Similar results were found in snowshoe hares (*Lepus americanus*; Sheriff et al. 2010), Belding's ground squirrels (*Spermophilus beldingi*; Mateo and Cavigelli 2005), and female ring-tailed lemurs (*Lemur catta*; Cavigelli 1999). The noninvasive fecal GCM technique has become an important method for monitoring the welfare of a vast number of species (Wasser et al. 2000; Schatz and Palme 2001; Dloniak et al. 2004; Hunt et al. 2004; Young et al. 2004; Hulsman et al. 2011; Santymire et al. 2012), including those from the Canidae family (de Villiers et al. 1997; Monfort et al. 1998; Schatz and Palme 2001; Sands and Creel 2004; Young et al. 2004; Schell et al. 2013; Rodrigues da Paz et al. 2014).

The method we used to measure the glucocorticoid concentrations was the radioimmunoassay. Our results from this study imply that when fecal GCMs are measured

via radioimmunoassay, corticosterone was the better glucocorticoid to extract and measure (Fig. 2–5). Our findings differ from those reported by Schell et al. (2013), who found cortisol to be the better fecal glucocorticoid metabolite to use for coyotes. However, this difference is explained by the different methods used to determine the concentrations of the fecal GCMs. In our study we used radioimmunoassay as compared to Schell et al. (2013) which used enzyme immunoassay. The discrepancy found between the 2 studies was probably due to the different immunoassay techniques, but could also be due to the different hormone extraction methods and antibody sensitivity. Young et al. (2004) found similar differences between radioimmunoassays and enzyme immunoassays for carnivores as well. Similar to our findings, they reported that fecal cortisol metabolites were better measured using enzyme immunoassays and corticosterone fecal metabolites were better measured using radioimmunoassays (Young et al. 2004).

We also demonstrated that having a noninvasive technique for calculating GCMs better represented the physiological state of the animal. We found that even though our control animals were not induced with ACTH, they still had a stress response from the handling and anesthesia; though the peaks for the control animals were not as high as found in the ACTH animals. The handling stress response was especially observed in the plasma cortisol concentrations. Interestingly, we found handling stress responses in fecal GCMs for the control males but not the control females. Our results indicated sex of the coyote was a significant influence in the corticosterone fecal metabolite levels, with the females having higher concentrations pre- and post-injection. We found no differences between the sexes for plasma and fecal cortisol metabolite concentrations. This further supports the importance of having a large sample size, comparing the use of different

glucocorticoids, and validating the use of fecal GCMs as a tool for assessing physiological stress responses.

In the future, determining the effects that different diets may have on coyote fecal GCMs. Kalliokoski et al. (2015) found that varying the diets of mice had a significant impact on the concentrations of fecal GCMs would be informative. Conducting a study to determine the effects of varying diets would pave the way for future field studies, such as comparing basal physiological stress levels of coyotes in the wild versus urban environments.

In conclusion, we found that measuring the concentrations of fecal GCMs for coyotes is an effective tool for monitoring their physiological stress response. When using radioimmunoassay, we recommend measuring corticosterone from coyote scats. We would also recommend having a large sample size if interested in a measure of the overall population physiological state, to account for any individual variation that may occur. Also collecting scats from either the same time of day or multiple times of day to account for the diurnal fluctuation of fecal corticosterone metabolite concentrations will provide a better measure of overall population response.

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Table 2–1. Results of a multi-way ANOVA on plasma cortisol levels as influenced by sex of the coyote, treatment type (ACTH, control), time (period) of blood draw, and all possible interactions, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

Source	Sum of Squares	df	Mean Sq.	F	P
Sex	0.339	1	0.339	0.023	0.881
Treatment	2783.113	1	2783.113	185.335	0.000
Period	541.922	4	135.480	9.022	0.000
Sex * Treatment	8.271	1	8.271	0.551	0.459
Sex * Period	47.368	4	11.842	0.789	0.535
Treatment * Period	975.916	4	243.979	16.247	0.000
Sex * Treatment * Period	45.515	4	11.379	0.758	0.555
Error	1801.998	120	15.017		

Table 2–2. Results of a repeated measures ANOVA on coyote plasma cortisol levels as influenced by individual animals and the time of blood draw, National Wildlife Research Center, Predator Research Facility, Millville, Utah November – December 2010.

	Control Males		ACTH Males		Control Females		ACTH Females	
	F	P	F	P	F	P	F	P
	Between Subjects	5.27	0.0001	0.74	0.6433	2.72	0.033	0.67
Treatment	8.56	0.0002	6.30	0.001	43.55	0.0001	10.56	0.0001

Table 2–3. Results of a multi-way ANOVA on levels of fecal cortisol metabolites as influenced by sex of the coyote, treatment type (ACTH, control), time (period) of fecal collection, and all possible interactions, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

Source	Sum of Squares	df	Mean Sq.	F	P
Sex	28614.533	1	28614.533	1.758	0.188
Treatment	23.111	1	23.111	0.001	0.970
Period	161012.972	3	53670.991	3.297	0.024
Sex * Treatment	18541.937	1	18541.937	1.139	0.289
Sex * Period	35758.851	3	11919.617	0.732	0.535
Treatment * Period	9021.654	3	3007.218	0.185	0.907
Sex * Treatment * Period	13721.983	3	4573.994	0.281	0.839
Error	1562962.340	96	16280.858		

Table 2–4. Results of a repeated measures ANOVA on levels of coyote fecal cortisol metabolites as influenced by the individual coyote and the time of fecal collection, National Wildlife Research Center, Predator Research Facility, Millville, Utah November – December 2010.

	Control Males		ACTH Males		Control Females		ACTH Females	
	F	P	F	P	F	P	F	P
Between Subjects	1.19	0.351	1.94	0.108	0.64	0.695	0.22	0.947
Day of Collection	2.37	0.105	1.39	0.272	1.34	0.293	0.37	0.778

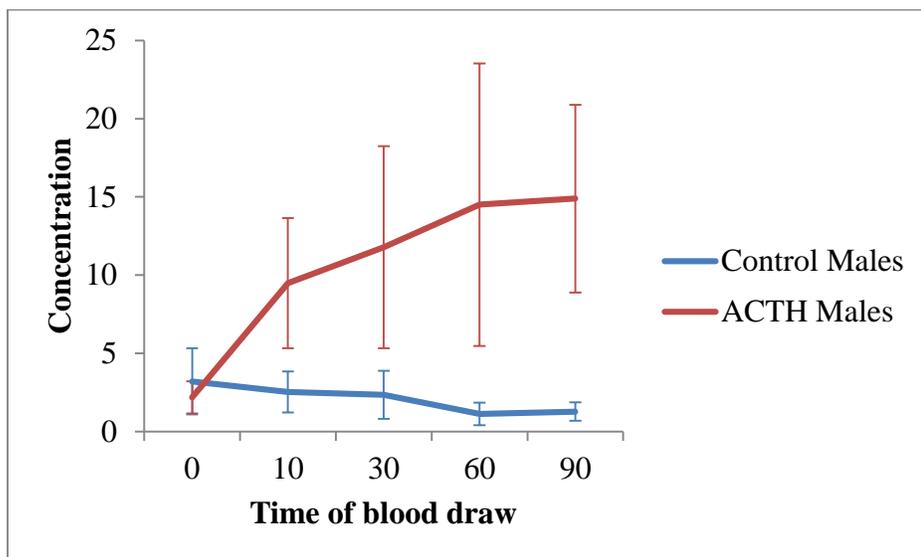
Table 2–5. Results of a multi-way ANOVA on levels of fecal corticosterone metabolites as influenced by sex of the coyote, treatment type (ACTH, control), time (period) of fecal collection, and all possible interactions, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

Source	Sum of Squares	df	Mean Sq.	F	P
Sex	0.605E+09	1	0.605E+09	35.006	<0.001
Treatment	0.230E+08	1	0.230E+08	1.329	0.252
Period	0.251E+09	3	0.835E+08	4.836	0.004
Sex * Treatment	0.210E+08	1	0.210E+08	1.217	0.273
Sex * Period	0.153E+09	3	0.509E+08	2.948	0.037
Treatment * Period	0.186E+09	3	0.619E+08	3.582	0.017
Sex * Treatment * Period	0.184E+09	3	0.612E+08	3.545	0.017
Error	0.166E+10	96	0.173E+08		

Table 2–6. Results of a repeated measures ANOVA on levels of coyote fecal corticosterone metabolites as influenced by the individual coyote and the time of fecal collection, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

	Control Males		ACTH Males		Control Females		ACTH Females	
	F	P	F	P	F	P	F	P
	Between Subjects	0.61	0.717	0.86	0.5501	1.26	0.316	0.90
Day of Collection	18.20	0.0001	12.71	0.0001	0.48	0.702	6.75	0.0042

a.



b.

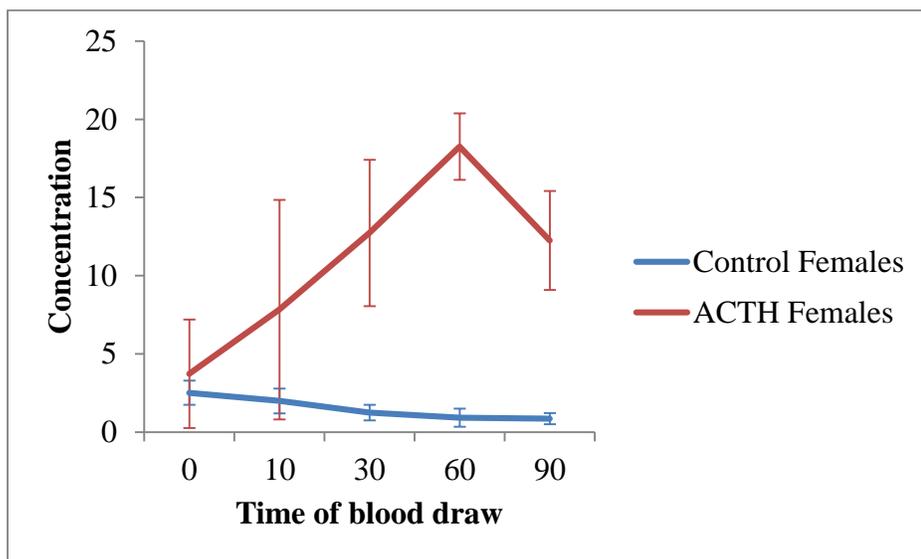
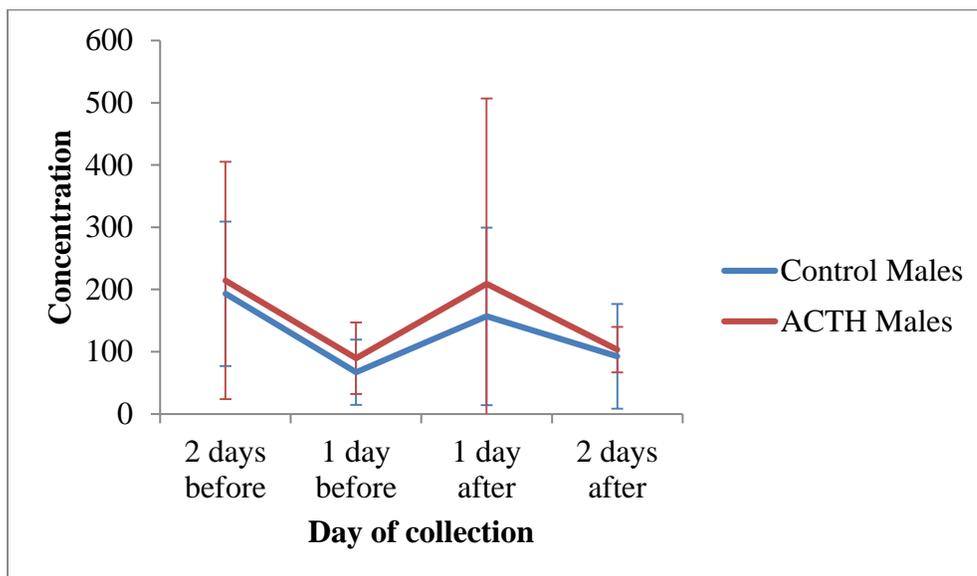


Fig. 2–1. Average cortisol concentrations (ng/mL) in coyote plasma for (a) 7 control males, 8 ACTH males, and (b) 7 control females, 6 ACTH females, at 5 different blood draw times (0, 10, 30, 60, 90 minutes) during an ACTH challenge, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

a.



b.

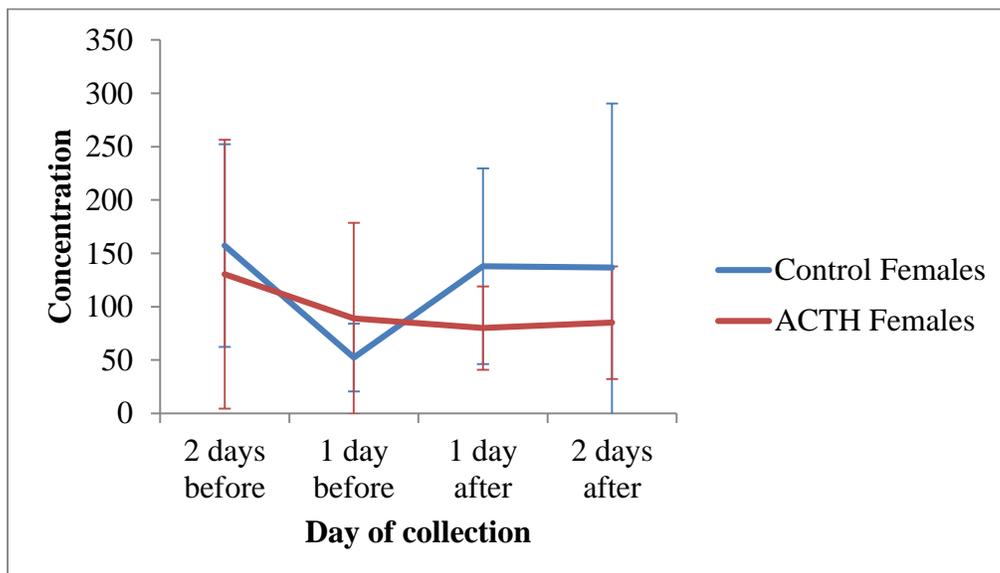
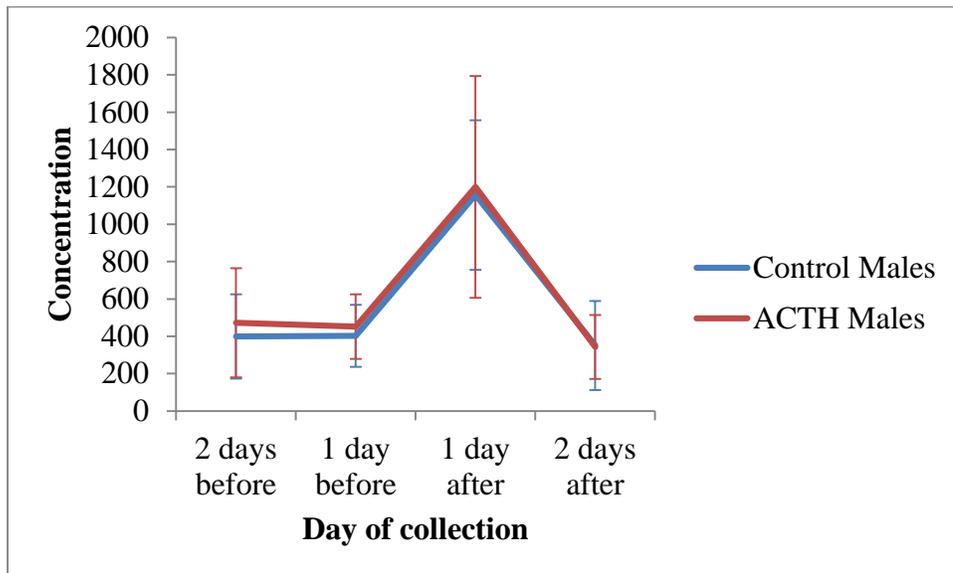


Fig. 2–2. Average cortisol concentrations (ng/g) in coyote feces for (a) 7 control males, 8 ACTH males, and (b) 7 control females, 6 ACTH females, over 4 different fecal collection days before and after an ACTH challenge, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

a.



b.

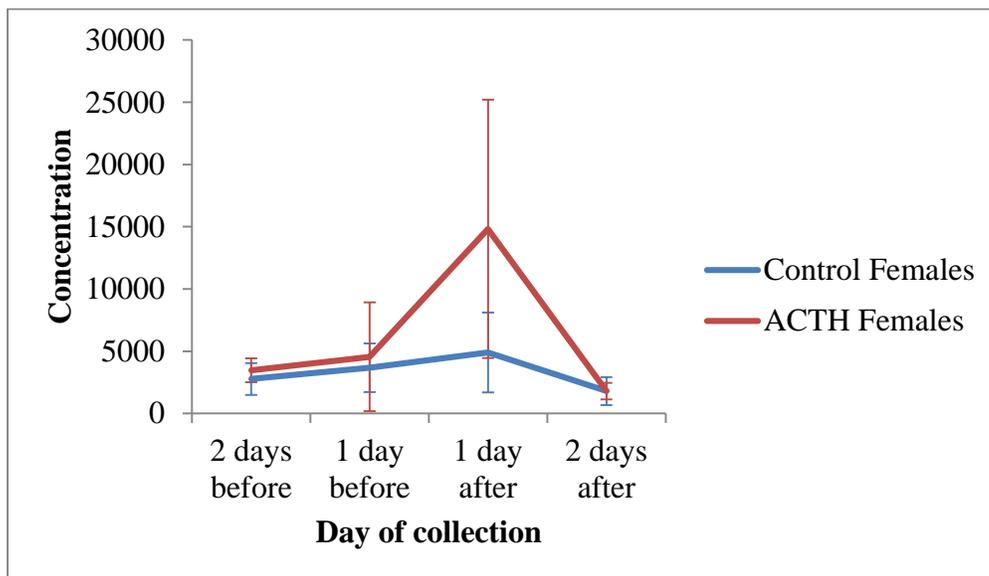


Fig. 2–3. Average corticosterone concentrations (ng/g) in coyote feces for (a) 7 control males, 8 ACTH males, and (b) 7 control females, 6 ACTH females, over 4 different fecal collection days before and after an ACTH challenge, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

a.



b.

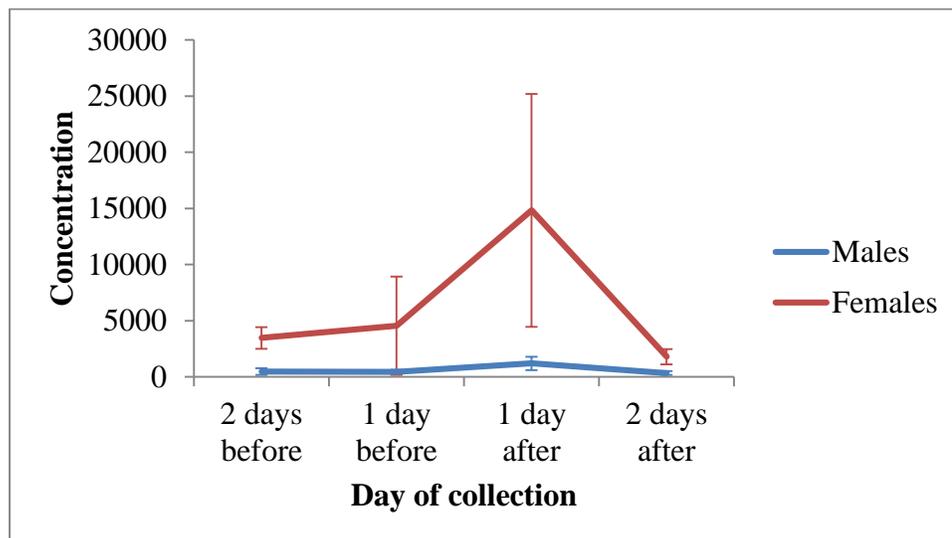
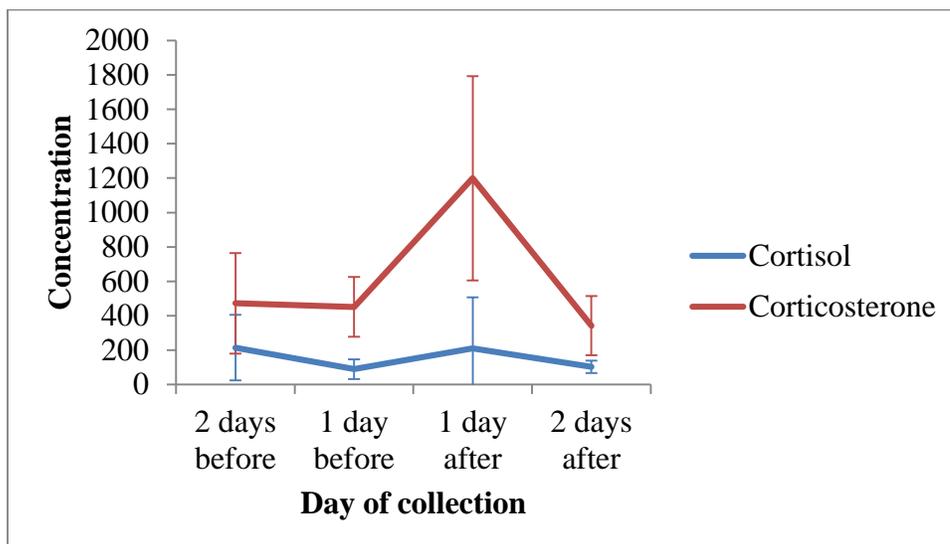


Fig. 2–4. Average corticosterone concentrations (ng/g) in coyote feces comparing males and females for (a) 7 control males, 7 control females, and (b) 8 ACTH males, 8 ACTH females, over 4 different fecal collection days before and after an ACTH challenge, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

a.



b.

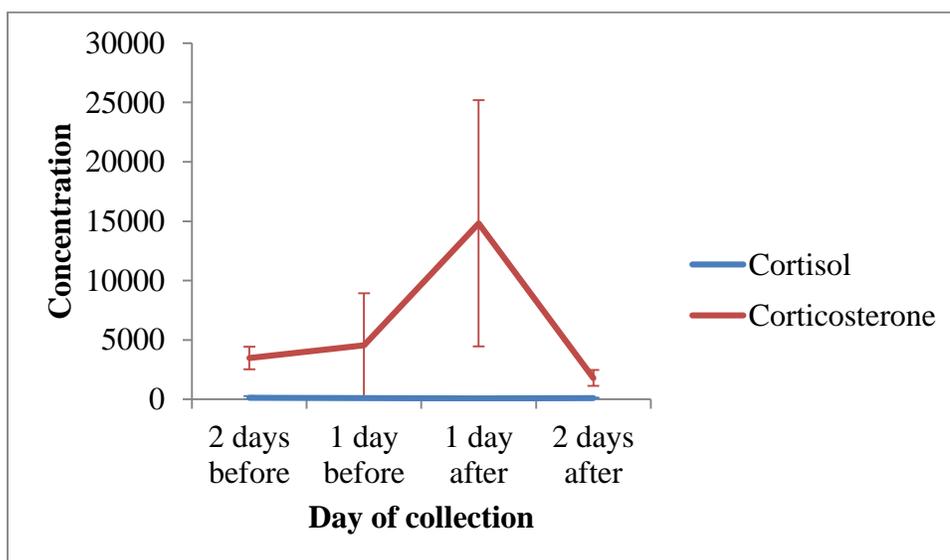


Fig. 2–5. Average corticosterone concentrations (ng/g) compared to average cortisol concentrations in coyote feces for (a) ACTH males, and (b) ACTH females, over 4 different fecal collection days before and after an ACTH challenge, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

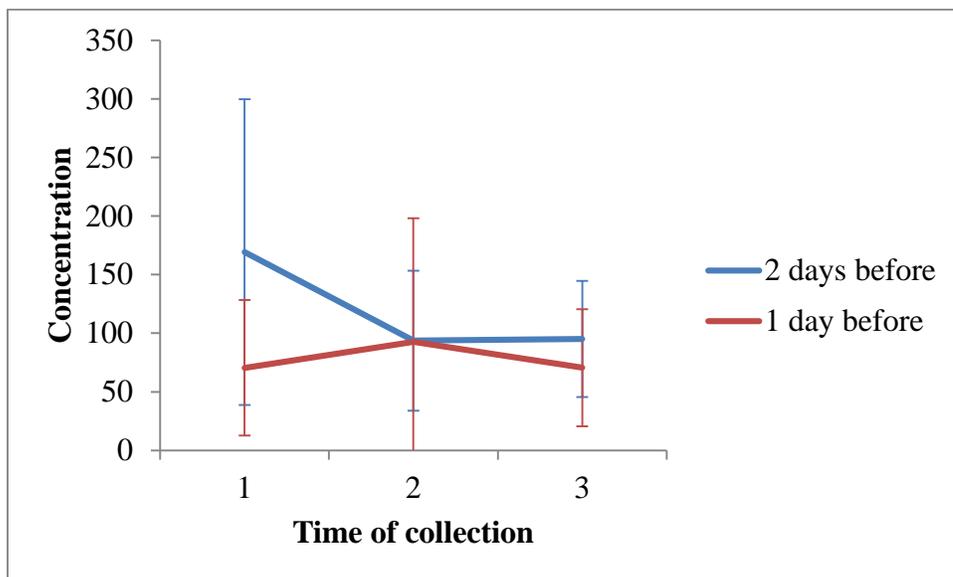
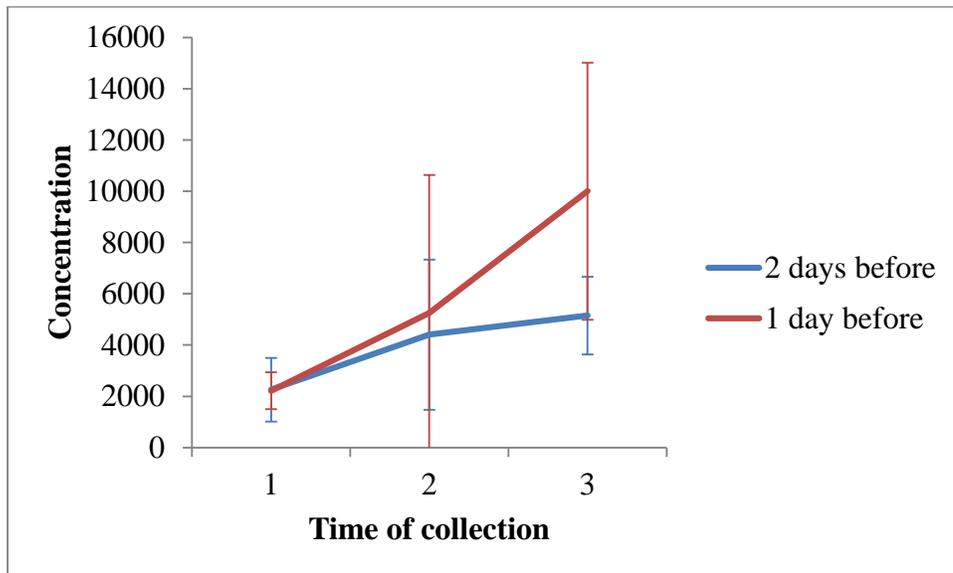


Fig. 2–6. Average cortisol concentrations (ng/g) in coyote feces for 16 males and 16 females, at 3 different time periods on 2 different days, to examine diurnal fluctuation, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

a.



b.

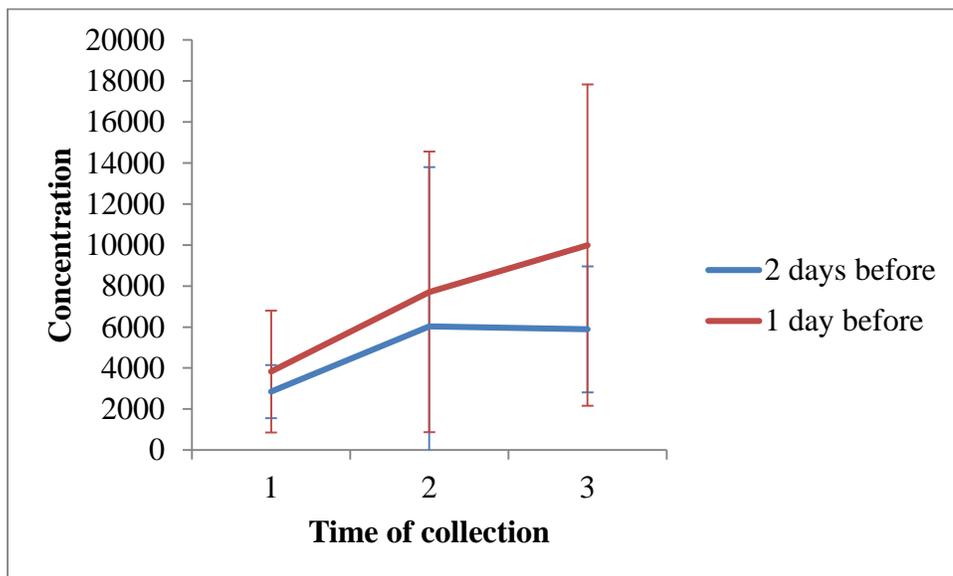


Fig. 2–7. Average corticosterone concentrations (ng/g) in coyote feces for (a) 16 males and (b) 16 females, at 3 different time periods on 2 different days, to examine diurnal fluctuation, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

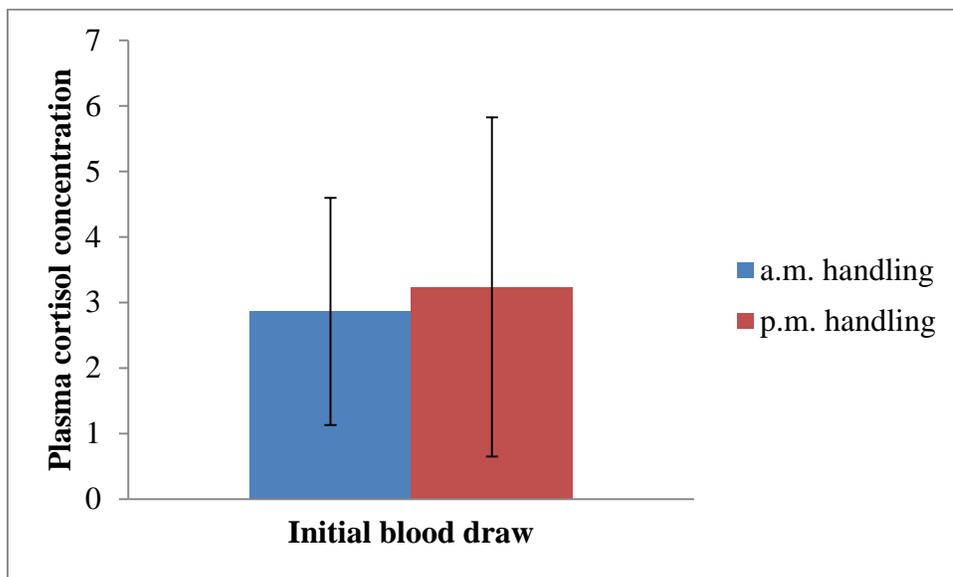
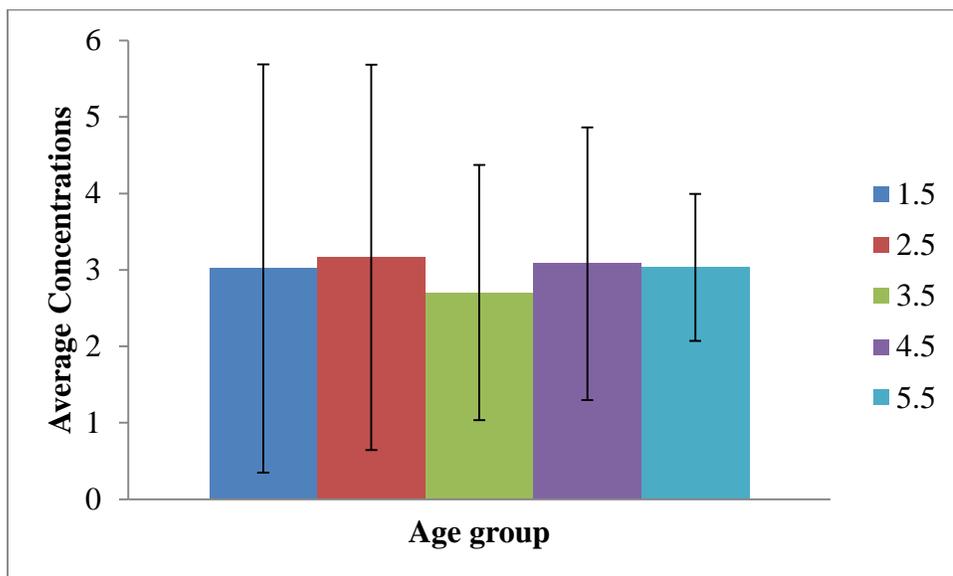


Fig. 2–8. Average cortisol concentrations (ng/mL) in coyote plasma for 16 males and 16 females, separated by handling time (a.m. or p.m.), to examine differences in baseline plasma cortisol concentrations based on time of handling, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

a.



b.

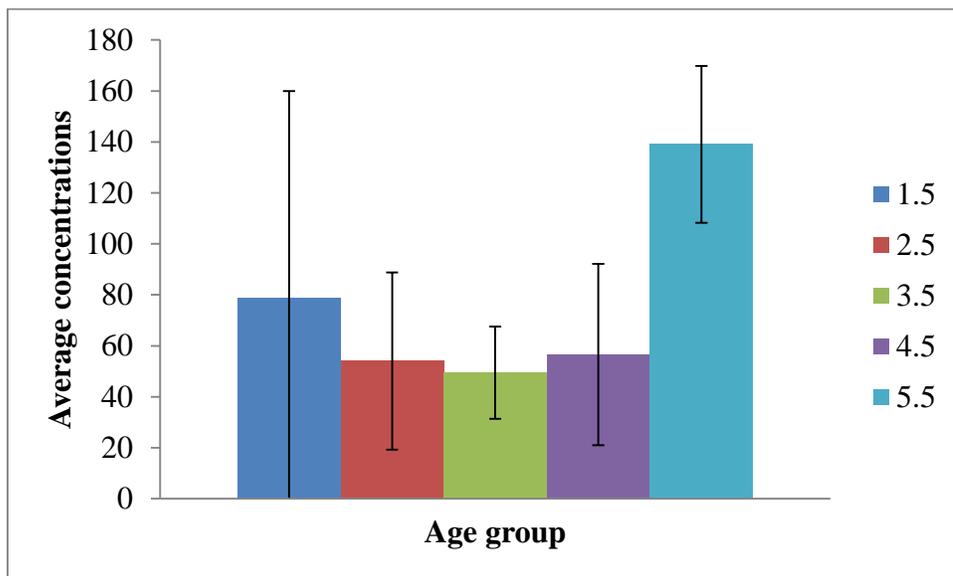
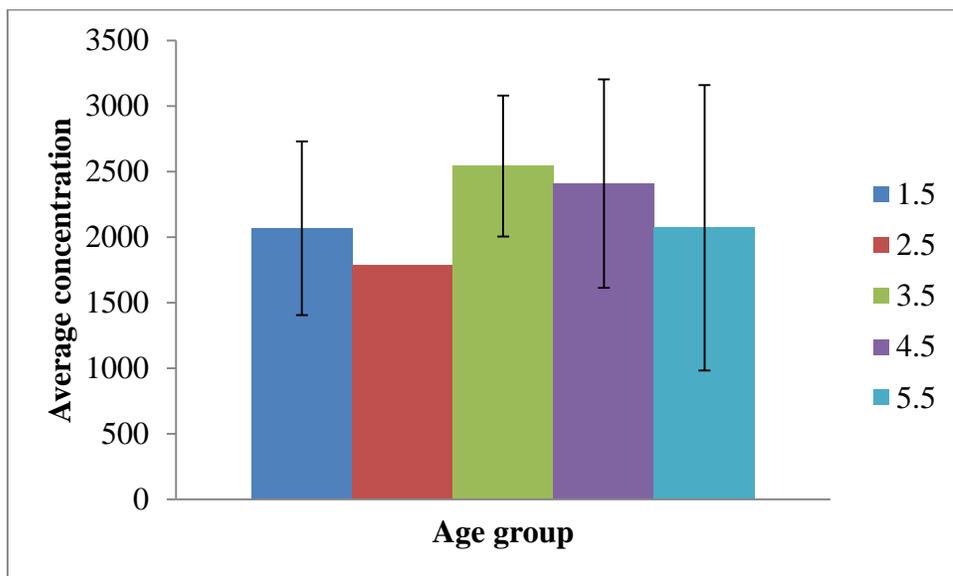


Fig. 2–9. Average baseline cortisol concentrations in coyote (a) plasma and (b) feces for 16 males and 16 females, separated into age groups, to examine influence of age on cortisol concentrations, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

a.



b.

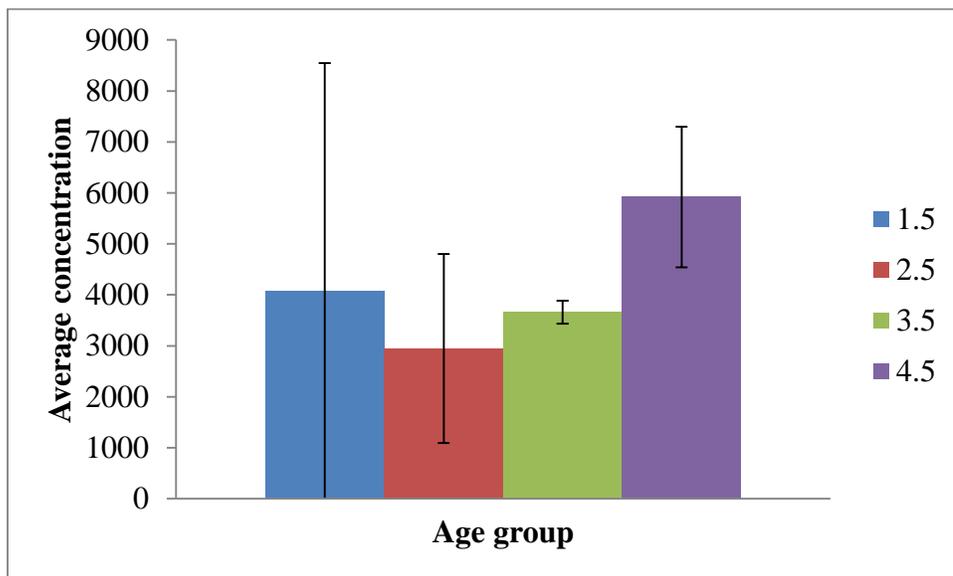


Fig. 2–10. Average baseline corticosterone concentrations in coyote feces for (a) 16 males and (b) 16 females, separated into age groups, to examine influence of age on corticosterone concentrations, National Wildlife Research Center, Predator Research Facility, Millville, Utah, November – December 2010.

CHAPTER 3**DEGRADATION OF GLUCOCORTICOID METABOLITES IN COYOTE
SCATS: IMPLICATIONS FOR FIELD STUDIES****ABSTRACT**

The study of physiological stress response in animals can be a tool to help aid in the conservation of species by better understanding the welfare and behavior of a variety of animals, especially when sampled noninvasively. One way to measure physiological stress responses is to evaluate the concentrations of glucocorticoid metabolites, such as cortisol and corticosterone, in fecal samples. We determined the longevity of glucocorticoid metabolite concentrations found in fecal samples of coyotes (*Canis latrans*) during summer and winter. Fecal samples were collected from 6 captive coyotes and exposed to the natural environment for 13 consecutive days during each season. Each day a sub-sample was collected, hormones extracted, and run through a radioimmunoassay. The concentrations of metabolites were then calculated. We found there was no significant decline in concentration levels of cortisol or corticosterone metabolites when sampled up to 13 days, nor did levels differ between sexes or between seasons. This study was the first to determine, for coyotes, if glucocorticoid metabolite concentrations could still be found after an extended period of time. Our studies provided evidence for application to field studies that coyote fecal samples could be collected every 13 days and still obtain viable levels of fecal glucocorticoid metabolite concentrations.

INTRODUCTION

The levels of glucocorticoids in free-ranging animals have been used in numerous wildlife species as an index of stress responses and to identify the environmental factors contributing to physiological stress (Millspaugh and Washburn 2004; Palme et al. 2005; Keay et al. 2006). Two glucocorticoids, cortisol and corticosterone, are important signaling chemicals of the endocrine system that function to alter physiology and behavior in response to acute and chronic stressors in the environment. Elevated glucocorticoid levels allow an animal to maintain homeostasis during exposure to an acute stressor. How glucocorticoids function to maintain homeostasis are diverse (Sapolsky et al. 2000; Boonstra 2004). Most importantly, elevated glucocorticoids promote a short-term increase in the availability of energy to the animal (Boonstra 2004). Elevated glucocorticoids, however, also reduce protein anabolism and increase protein catabolism (von der Ohe and Servheen 2002). The mobilization of body energy stores in an animal is important for a flight or fight response to an immediate stressor.

Because of their benefit for coping with environmental stressors, measures of glucocorticoids have become the standard for monitoring the welfare of many wildlife species (Keay et al. 2006). In particular, fecal glucocorticoid metabolite (GCM) analysis has been identified as the most useful measure of exposure of chronic stress in animals (Millspaugh and Washburn 2004; Young et al. 2004). The primary appeal of fecal GCMs for monitoring adrenocortical activity is that it is a relatively noninvasive procedure that minimizes the impact on and response of the study animal to researcher activities (Millspaugh and Washburn 2004; Young et al. 2004). Hence, the levels of fecal GCMs

should reflect the impacts of environmental factors on the condition of an animal. In captivity, this method has been used to monitor the welfare of animals. Studies have been conducted to determine the effects of environmental enrichment on captive animals such as giant pandas (*A. melanoleuca*: Liu et al. 2006) and black-footed ferrets (*M. nigripes*: Poessel et al. 2011). Another study concerned with the reintroductions of river otters (*L. canadensis*) used this tool to determine if the soft translocation technique (i.e., otters kept in captivity for a period of time) caused the otters to be under chronic stress (Rothschild et al. 2008).

Measuring fecal GCM concentrations can also be used to study animals in the field. Busch and Hayward (2009) summarized the use of fecal GCMs for a variety of reasons such as looking at the effects of predators, food abundance, pollution, human interactions, and habitat changes. They also made suggestions of how this knowledge could be used to support conservation practices. Other studies have looked at the links between behavior, reproductive success, social rank, and fecal GCM concentrations (Creel et al. 1996; de Villiers et al. 1997; Sands and Creel 2004; Weingrill et al. 2004; Creel 2005; Barja et al. 2008).

Although the noninvasive aspect of scat collection is a great benefit for monitoring the health and condition of a species, there are also some downfalls to this method. In a review, Goymann (2012) pointed out there could be differences between sexes, diets, seasons, metabolic rates, and bacterial degradation on fecal GCM concentrations in scats. Another issue is individual identification, where a researcher must observe defecations and then be able to find those scats in the field. As always, time and money are also problems when it comes to fecal collections in the field. Running

transects lines for finding scats requires time and money. An efficient and more accurate way of collecting feces is the use of scat-detection dogs (Smith et al. 2003; Long et al. 2007). However, the cost of using these trained dogs may be prohibitive for some projects. One major issue with using scat is the time between scat deposition and scat collection. Few studies have determined the effects of time and environmental factors on the degradation of fecal GCMs in scats. A study with maned wolves (*C. brachyurus*) determined the importance of having fresher samples for improved accuracy of hormone levels, but they were unable to determine the exact ages of their samples (Vynne et al. 2011). Washburn and Millspaugh (2002) found relative stability in their fecal GCM concentrations over 7 days in varying simulated environmental conditions for fecal pellets from white-tailed deer (*O. virginianus*), but found an increase in fecal GCM concentration levels for the samples exposed to simulated rain. Another study also found stability in fecal GCM concentrations over 48 hours in scat of spotted hyena (*C. crocuta*: Dloniak et al. 2004). In contrast, 2 different studies found an increase in variability in fecal GCM concentrations. Muehlenbein et al. (2012) found an increase in variability of the concentration levels within 3 hours after defecation and Möstl et al. (1999) found increases of fecal GCM concentrations just after 1 hour. Conversely, decreases in fecal GCM concentrations were found in brown hyaenas (*H. brunnea*: Hulsman et al. 2011) and lowland gorillas (*G. gorilla gorilla*: Shutt et al. 2012) between 0–6 hours.

Determining the amount of time that fecal GCM concentrations will persist in scats could assist in determining when scats should be collected, thereby increasing the efficacy and reducing the costs of scat collections. For example, if fecal GCM concentration levels are still viable and accurate in a scat that is 13 days old, then a study

could run transect lines every 13 days instead of every other day and still obtain accurate measures of fecal GCM levels. The objective of our study was to determine the longevity of glucocorticoid metabolite concentrations found in coyote (*C. latrans*) scats during 2 different seasons (summer and winter). We predicted that glucocorticoid concentration levels during the summer would decrease due to degradation over the 13-day time period, while concentrations during the winter would remain relatively constant over the 13-day period.

MATERIALS AND METHODS

The experiment was conducted at the U.S. Department of Agriculture, Wildlife Services, National Wildlife Research Center, Predator Research Facility near Millville, UT, U S A. During testing, coyotes were housed in pairs in 0.1 ha pens. Animals were fasted 1 day per week; on all other days they were provided with their normal ration (650 g) of commercial mink food. Water was provided ad libitum. Routine animal care, such as animal and water checks once per day, and feeding once per day, were done throughout the entire study. During the summer months there was construction in some of the pens; and during the winter months gate shoveling and road plowing for snow removal was done as needed. Coyotes also had access to a den box and were allowed to maintain naturally excavated den holes. The day prior to collection, coyotes were fed glitter (Glitterex Corporation, Cranford, NJ) infused into frozen mink food balls (Burns et al. 1995), such that males received one color and females a different color for individual identification of scats. Scat collections occurred on 26 August 2011 for the summer degradation trial, and 11 January 2012 for the winter degradation trial.

Sample Collection. –We used 6 coyotes (3 males and 3 females) for this experiment, ranging in age from 2 to 7 years. The same coyotes were used for both the summer and winter trials. To ensure the freshest samples for the study, the animals chosen were observed defecating during the collection time. Once the scat was deposited, the observer walked into the pen and collected the scat with the time and date of collection recorded.

Each sample was thoroughly homogenized and placed onto a plastic wrapped wooden board and placed out into the elements. The samples were separated from each other by cardboard dividers. The board was placed in a trap to prevent small animals from taking the scats and the trap was elevated off of the ground to avoid potential flooding. Each day, for a total of 13 days, a sample of approximately 0.5 g was removed from each scat and placed into a plastic scintillation vial and then frozen in a -20° C freezer. A 2-week time period was chosen because the longest degradation study previously performed was 1 week in duration, thus we decided to double that amount of time (Washburn and Millspaugh 2002). While we collected scat sub samples every day during the 13 days, we only ran odd days through the radioimmunoassay for analyses. However, if we found fecal GCMs declined between 2 sample days, we then planned to run the remaining fecal samples through the radioimmunoassay to determine the exact day that GCM degradation began.

Hormone Extraction. –The first step in the extraction process was to formulate the phosphate-methanol buffer solution. Distilled water (700 mL) was added to a RIA glass container; 8.75 g NaCl (Sigma-Aldrich, St. Louis, MO), 5.7 g NaH₂PO₄-H₂O (Sigma-Aldrich), 8.66 g Na₂HPO₄ (Sigma-Aldrich), and 1.0 g sodium azide (Sigma-Aldrich) was

added into the water and stirred until dissolved. Next we added 0.5 mL Tween 20 (Sigma-Aldrich) and gently stirred until mixed. The pH was tested (7.0 was desired) and then distilled water was added for a final volume of 1000 mL. The final step was to slowly dissolve BSA (bovine serum, Sigma-Aldrich) by adding it to the surface of the solution; this stock solution was then refrigerated until used. Just prior to initiating the extraction process, the working buffer was created by mixing stock solution at room temperature and methanol in equal volumes following Shideler et al. (1994), and Bauman and Hardin (1998).

We then added 0.5 mL of the working solution to each scintillation vial with the 0.5 g of fecal sample. Each sample was vortexed until the solution was homogenized. The samples were then placed onto a shaker for approximately 16 hours at 200 RPM. After shaking, we allowed the solution to settle for approximately 1 hour. From the top of the supernatant, 50 μ L was pipetted into 12 x 75 mm polypropylene tubes and centrifuged (in a refrigerated centrifuge) for 1 hour at 4,000 RPM. The centrifuged supernatant was decanted into cryovial tubes, then frozen at -80° C until the radioimmunoassay was initiated. The solution left behind in the scintillation tubes were dried overnight in a vented 100° C oven. The dried material was cooled to room temperature and weighed in order to determine the dry weight of the remaining fecal sample when calculating hormone concentrations.

Radioimmunoassays. –Cortisol concentrations were determined using radioimmunoassay (Siemens Coat-a-count cortisol RIA kit, Siemens Healthcare Diagnostics Inc., U S A; cross reactivity with cortisol and cortisone is less than 1%). The first step of the process was to turn on a water bath at 37° C, warm all liquids to room

temperature, and pull out the cryovial tubes containing the sample supernatant. Two uncoated 12 x 75 mm polypropylene tubes were labeled as total counts (TC) and 2 were labeled as nonspecific binding (NSB). The rest of the tubes used were coated with antibodies to cortisol. The calibrators for creating the standard curve were supplied in the kit; these tubes were labeled A-F and were run in duplicate. The sample tubes were also labeled in duplicate. We then pipetted 25 μL of the zero calibrator (A) into the bottom of the NSB and A tubes, as well as 10 μL of the working buffer solution. The remaining calibrators (B-F) were pipetted into the bottom of the appropriately labeled tubes in 25 μL amounts plus 10 μL of the working buffer solution. The samples were pipetted directly to the bottom of their tubes using 10 μL of the supernatant in addition to 25 μL of the zero (A) calibrator. The next step was adding 1.0 mL of the label (^{125}I) into each tube and vortexing the tubes. The TC tubes were then covered in parafilm and set aside. All of the other tubes were incubated in the water bath for approximately 45 minutes. All of the tubes, except TC, were then decanted and placed upside down in a foam decanting rack for 2-3 minutes. The tubes were then struck on absorbent paper until there was no longer any visible moisture. Each tube was read for 1 minute in a gamma counter. There were some samples that did not fall within the standard curves. These samples were re-run using double the amount of sample supernatant and 15 μL of the zero calibrator solution, we did not have enough material to run these samples in duplicate. Each time an assay was run a new standard curve was created.

Corticosterone concentrations were determined using radioimmunoassay (ImmuChemTM Double Antibody RIA kit, MP Biomedicals, Orangeburg, NY; cross reactivity with other metabolites is less than 1 %). The first step of the process was to

bring all of the reagents to room temperature, pull out the cryovials with sample supernatant, and add 2.0 mL of distilled water to each of the controls and allowing these mixtures to equilibrate to room temperature for 30 minutes. Next we labeled 10 x 75 mm glass test tubes in duplicate. The standard curve made up the first 20 tubes of the assay. We then pipetted 150 μ L of the steroid diluent into the NSB tubes. The zero binding tubes had 50 μ L of steroid diluent pipetted into them. For the next part of the standard curve (tubes 5-16) we pipetted 50 μ L of calibrator and 10 μ L of the working buffer solution into those tubes. Tubes 17-20 were the control tubes and 50 μ L of the control solution provided were pipetted into these tubes. All of the sample tubes received 10 μ L of the sample supernatant and 40 μ L of the steroid diluent. The next step was to add 100 μ L of 125 I (blue reagent) to all of the tubes, as well as (except for the NSB tubes) 100 μ L of the anti-corticosterone (yellow reagent). All of the samples were vortexed. The tubes were then incubated for 2 hours at room (22-25° C) temperature. Next 250 μ L of the precipitant solution (red reagent) was added to all of the tubes and vortexed thoroughly. The tubes were then placed in a refrigerated centrifuge for 15 minutes at 2400 RPM. All tubes were decanted and then blotted, careful to not lose the precipitants, on absorbent paper. Each tube was read for 1 minute in a gamma counter. For the samples that had to be re-run because they did not fall in the standard curves, the amount of sample supernatant was halved and the amount of steroid diluent increased to 45 μ L. Each time an assay was run a new standard curve was created.

Calculations and Statistics. –For both cortisol and corticosterone the method for calculating the concentrations of the metabolites was the same (following procedures provided with radioimmunoassay kits). All samples were run in duplicate. Average

sample values were first corrected for non-specific binding (NSB) and then converted to percent bounds by dividing the net counts by the zero bound net count. Percent bound was converted to concentration values using the standard curve. To calculate the final metabolite concentrations we had to do several corrections first. Final concentrations were corrected for dilution factor and for dry fecal mass resulting in a final concentration of hormone per gram of fecal matter.

We performed repeated measures ANOVA to determine if the levels of cortisol and corticosterone were influenced by the individual animals (between subject effects) and the day the sample was collected since scat deposition, collection (treatment effects). Because we found a significant influence of the individual variation among animals for both cortisol and corticosterone levels, we then performed a multi-way ANOVA to determine the influence of sex, season, and day since scat defecation, and the interactions of these 3 variables. Because we found a significant influence of the day since defecation for the fecal corticosterone metabolite concentrations in the repeated measures ANOVA, we then performed a one-way ANOVA with a post-hoc Tukey's test, for corticosterone only, to analyze the influence of the amount of time since defecation within each season.

RESULTS

Cortisol. –For the cortisol metabolite concentrations in the coyote scats measured over the 13 days since scat deposition, we found there were no significant differences among the days since deposition according to the results of the repeated measures ANOVA (summer: $F = 0.34$, $P = 0.912$; winter: $F = 1.24$, $P = 0.313$). However, there was a significant difference between the individual animal subjects (summer: $F = 5.45$, P

= 0.0008; winter: $F = 7.05$, $P = 0.0001$), indicating that differences between individual coyotes (Fig. 3–1) was a larger influence than the days since scat deposition (Fig. 3–3) on the fecal cortisol metabolite levels in both summer and winter.

For the cortisol metabolite concentrations in the coyote scats measured over 13 days after deposition for both seasons combined, we found that 20% of the variation in cortisol levels was explained by the sex of the coyote, season, and day since deposition, and the interactions of these 3 variables (Table 3–1). There was no significant influence, in the multi-way ANOVA, of sex of the coyote, and days since scat deposition, as well as no significant effects of the interactions of sex*days, season*days, or sex*season*days (Table 3–1). Though not significant at the 0.05 level, season ($P = 0.057$) and the interaction of sex*season ($P = 0.069$) appeared to have some influence on the fecal cortisol metabolite concentrations in coyote scats sampled over the 13 days after scat deposition (Table 3–1). The main result of the ANOVA and repeated measures ANOVA was that the levels of cortisol in the coyote scats did not degrade or decline over the 13 days of sampling after scat deposition either in summer or winter, and differences among individual coyotes was more influential on cortisol metabolite levels measured in the scats (Figs. 3–1, 3–3). The assay sensitivity was 2 ng/mL, with the intra and interassay coefficient of variation < 10%.

Corticosterone. –For the corticosterone metabolite concentrations in the coyote scats measured over 13 days since defecation for both seasons combined, we found a significant influence of the days since defecation according to the results of the repeated measures ANOVA (Summer: $F = 5.10$, $P = 0.001$; Winter: $F = 3.14$, $P = 0.0165$), as well as an influence of individual animals (Summer: $F = 16.09$, $P = 0.0001$; Winter: $F = 4.24$,

$P = 0.0039$). Our results indicated that the amount of days since scat defecation and individual animal variation were influences on the degradation of the corticosterone metabolite concentrations over the 13 days since scat deposition, in both summer and winter.

For the corticosterone metabolite concentrations in the coyote scats measured over 13 days after deposition for both seasons combined, we found that 33% of the variation in corticosterone metabolite levels was explained by the sex of the coyote, season, and days since deposition, and the interactions of these 3 variables. However, results of the multi-way ANOVA showed there were no significant influence of any of the variables, including sex of the coyote, season, and days since deposition, and the interactions of these 3 variables (Table 3–2). These results indicated that the individual variation was the major influence of corticosterone metabolite degradation over the 13 days. The results from the one-way ANOVA and Tukey's post-hoc test examining the effects of day since scat deposition, for each season separately, further supported the results that days since defecation was not an influence on the corticosterone metabolite concentrations in the coyote scats (Summer: $F = 1.078$, $df = 6.35$, $P = 0.394$; Winter: $F = 1.870$, $df = 6.35$, $P = 0.114$). Our main finding showed corticosterone metabolite levels in the coyote scats did not degrade or decline over the 13 days of sampling after scat deposition either in summer or winter (Fig. 3–4), and individual differences among the animals (Fig. 3–2) was the most influential on corticosterone levels in the scats. The assay sensitivity was 7.7 ng/mL, with the intra-assay coefficient of variation < 5%.

DISCUSSION

This study was the first to determine if fecal GCM concentrations remained viable in coyote scat over a 13-day sampling period, and it was the first study to document GCM persistence in scats left out in the environment in the order Carnivora. Up to date, this study also has the longest time period for assessing the degradation of fecal GCMs. Results from this study indicated there was no significant degradation, or increase, of cortisol or corticosterone metabolite levels when sampled over the course of 13 days after scat deposition in summer or winter. Our results were similar to those found by Washburn and Millspaugh (2002) who reported relative linearity in fecal GCM concentrations for white-tailed deer over a sampling period of 1 week (7 days). Our results also indicated that individual variation may influence the fecal GCM concentrations for coyotes. Therefore, if researchers are concerned with overall population glucocorticoid concentrations we recommend collecting scat from a large number of individuals. Overall, we found no changes in the fecal GCM concentrations over the 13 days since scat deposition of coyotes, indicating that in a field study, scat collection could be conducted every 13 days during summer or winter and still obtain viable measurements of fecal GCM concentrations.

The use of fecal GCM concentrations for assessing physiological stress responses in organisms should be validated for each specific species of interest (Touma and Palme 2005; Keay et al. 2006). Determining the rate of degradation has been conducted for the use of fecal DNA (Lonsinger et al. 2015) and we highly recommend the same be done for fecal GCMs. Though we found no significant change in our fecal GCM concentrations

over the 13 day sampling period, other studies have reported changes when samples were not frozen immediately. For example studies with domestic livestock found fecal GCM concentrations to increase between 1-3 hours after collection (Möstl et al. 1999); they reported the increase in fecal GCMs may be due to a physiological process involving the enzyme desmolase, which may increase the GCMs during incubation at room temperature. Muehlenbein et al. (2012) found increased variability of fecal GCMs, in orangutans (*P. pygmaeus pygmaeus*), within 3 hours after scat collection; they assumed that this was due to physiological processes such as bacterial metabolism. The fecal GCM concentrations in brown hyaenas (*H. brunnea*) decreased when the feces were not stored within 5 hours post collection (Hulsman et al. 2011), but fecal GCM concentrations were stable in spotted hyenas (*C. crocuta*) up to 48 hours post collection (Dloniak et al. 2004). These variations between species further supports the need to validate and determine the time of degradation for each species of interest when it comes to using fecal GCM concentrations for assessing physiological stress, and may even need to determine degradation of the GCMs in differing environmental conditions. Some of the discrepancies may also be caused by varying environmental conditions such as higher humidity or cooler temperatures, when using scat it is important to validate the use in the specific environment of the study. Also, the diet of the animal can have an impact. A study with mice stated that varying the fiber content in the diets of the mice causes the fecal GCM concentrations to fluctuate (Kalliokoski et al. 2015). Therefore taking into consideration the diet of the species of interest is also extremely important.

There are a number of benefits from validating and using fecal GCM concentrations for quantifying physiological stress responses, especially when concerned

with the welfare and well-being of both captive and wild animals (Touma et al. 2003). One of the main functions of glucocorticoids in the body is energy regulation for the maintenance of homeostasis (Busch and Hayward 2009). Because of this vital physiological role, glucocorticoids initiate the flight or fight response needed for survival. The flight or fight response is activated by an acute stressor and increases the chance of survival by the organism (Boonstra 2004; Arnemo and Caulkett 2007). When animals are under chronic stress there a number of deleterious effects such as reduced reproduction, slowed growth rates, decreased immune health, and captive animal stereotypies (Möstl and Palme 2002; Young et al. 2004; Touma and Palme 2005; French et al. 2006; Keay et al. 2006; Ellenberg et al. 2007). The capability to measure glucocorticoids noninvasively through feces provides a means for measuring physiological stress without a handling or anesthesia response, and without injury to the subject (Creel et al. 1997; Möstl and Palme 2002; Millspaugh and Washburn 2004; Palme et al. 2005; Touma and Palme 2005; Viljoen et al. 2008). Validating the use of fecal GCMs and determining the amount of degradation that may occur over time will improve our capability to measure GCMs in captive and wild systems alike. Improvement of GCM measurements will thus enhance our knowledge of the physiological stress response and provide a better understanding of the interaction of wildlife and their natural environment (von der Ohe and Servheen 2002; Boonstra 2004; Dalmau et al. 2007).

We concluded that a valid field technique for assessing physiological stress responses in *Canis latrans* would be to use fecal GCM concentrations. We determined that scat collections could be run at least 13 days apart and still obtain viable measurements of cortisol and corticosterone in the scats. However, it should be noted,

that if a study is only concerned with the overall coyote population's physiological stress, a small amount of individuals may skew the results due to large variation among individuals. Thus, an adequate sampling design that samples many individuals should be considered for a population-wide assessment.

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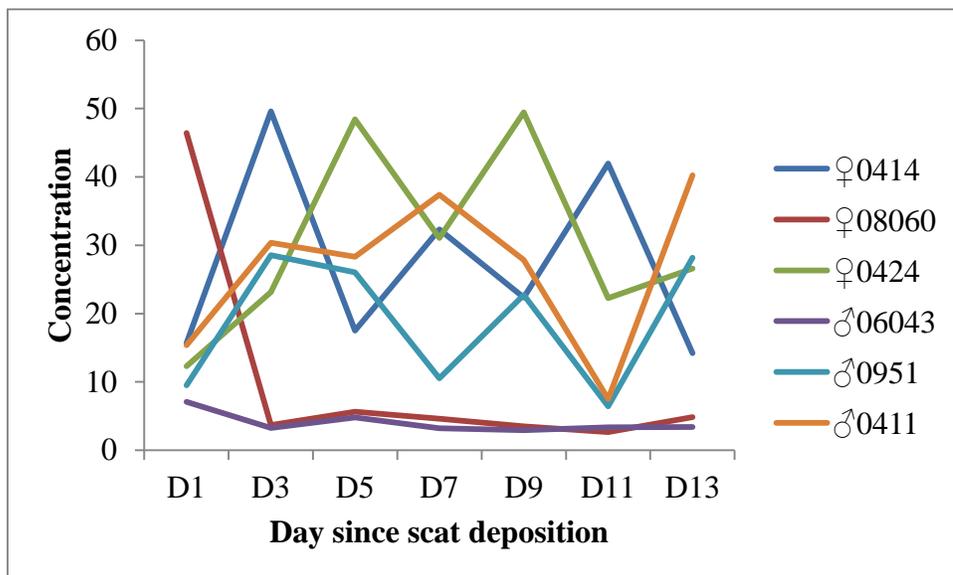
Table 3–1. Results of a multi-way ANOVA on levels of fecal cortisol metabolites, over a 13-day period, as influenced by sex of coyote, season of collection, and days since deposition, and all possible interactions, National Wildlife Research Center, Predator Research Facility, Millville, Utah, 2011–2012.

Source	Sum of Squares	df	Mean Sq	F	P
Sex	11.912	1	11.912	0.065	0.800
Season	699.463	1	699.463	3.790	0.057
Days	399.026	6	66.504	0.360	0.901
Sex*Season	632.448	1	632.448	3.427	0.069
Sex*Days	395.775	6	65.963	0.357	0.903
Season*Days	244.000	6	40.667	0.220	0.969
Sex*Season*Days	286.840	6	47.80	0.259	0.954
Error	10335.838	56	184.569		

Table 3–2. Results of a multi-way ANOVA on levels of fecal corticosterone metabolites, over a 13-day period, as influenced by sex of coyote, season of collection, and days since deposition, and all possible interactions, National Wildlife Research Center, Predator Research Facility, Millville, Utah, 2011–2012.

Source	Sum of Squares	df	Mean Sq	F	P
Sex	1326324.211	1	1326324.211	2.792	0.100
Season	781568.693	1	781568.639	1.645	0.205
Days	4530640.088	6	755106.681	1.589	0.167
Sex*Season	1300866.892	1	1300866.892	2.738	0.104
Sex*Days	1032427.544	6	172071.257	0.362	0.900
Season*Days	3518288.441	6	586381.407	1.234	0.303
Sex*Season*Days	553044.610	6	92174.102	0.194	0.977
Error	0.266067E+08	56	475120.071		

a.



b.

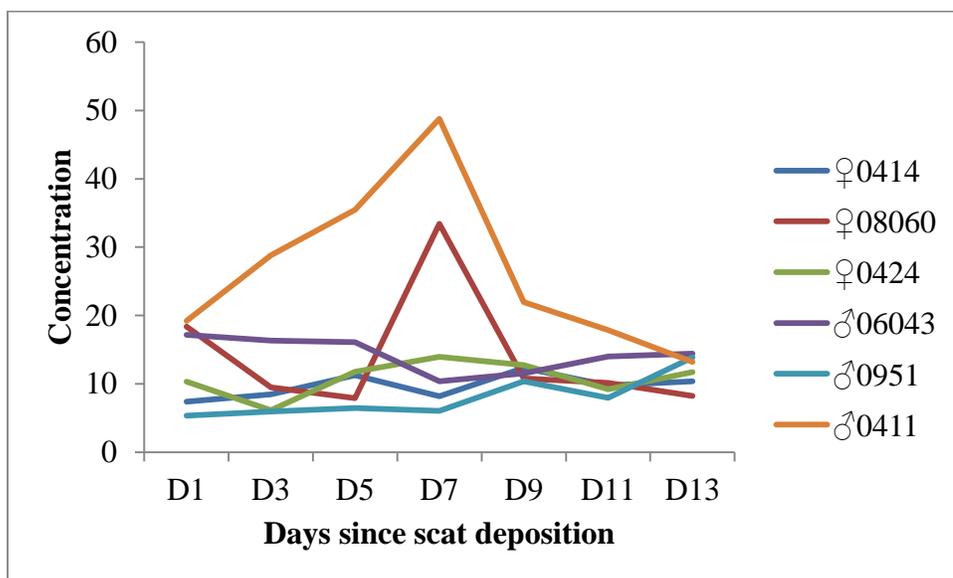
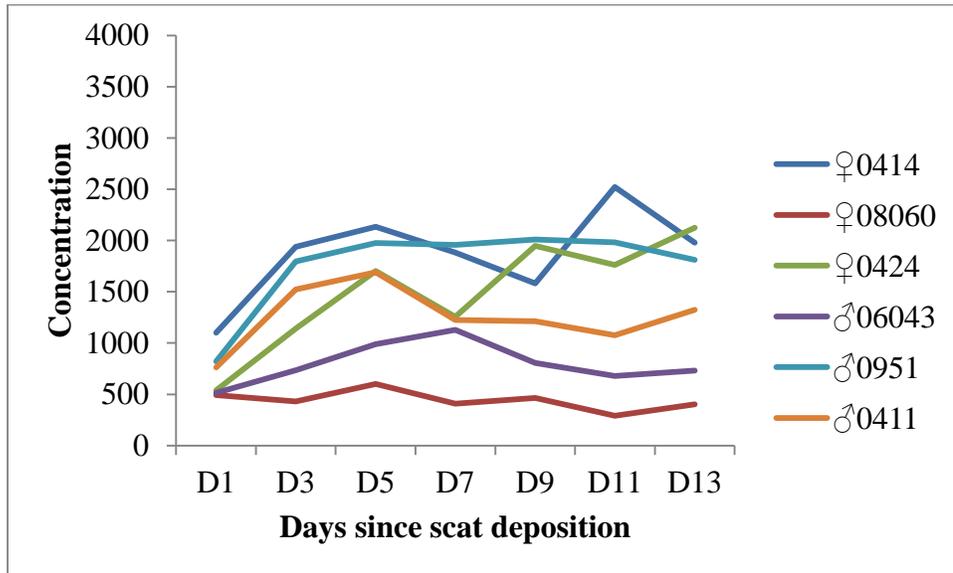


Fig. 3–1. Fecal cortisol metabolite concentrations for 6 individual coyotes sampled daily for 13 days in (a) summer, and (b) winter, National Wildlife Research Center, Predator Research Facility, Millville, Utah, 2011–2012.

a.



b.

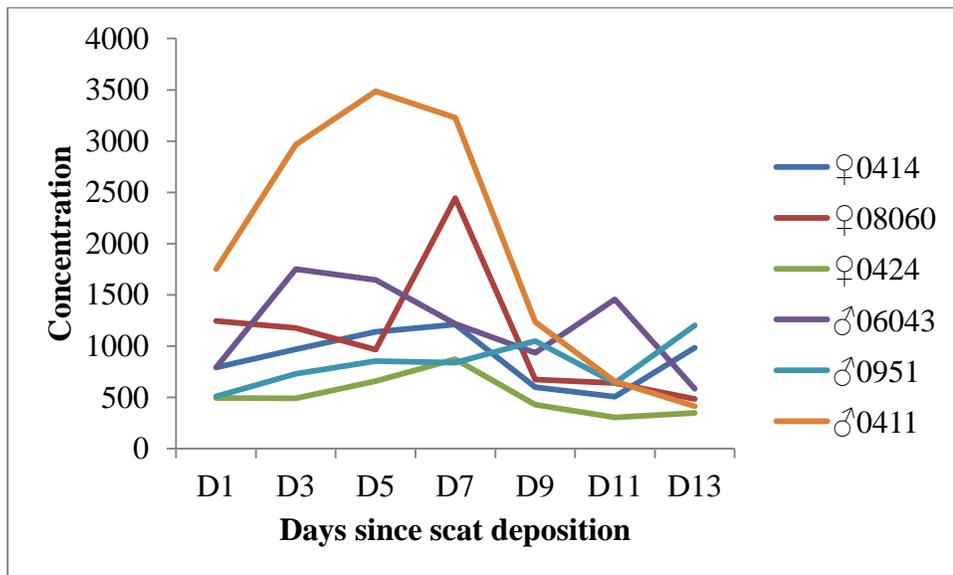
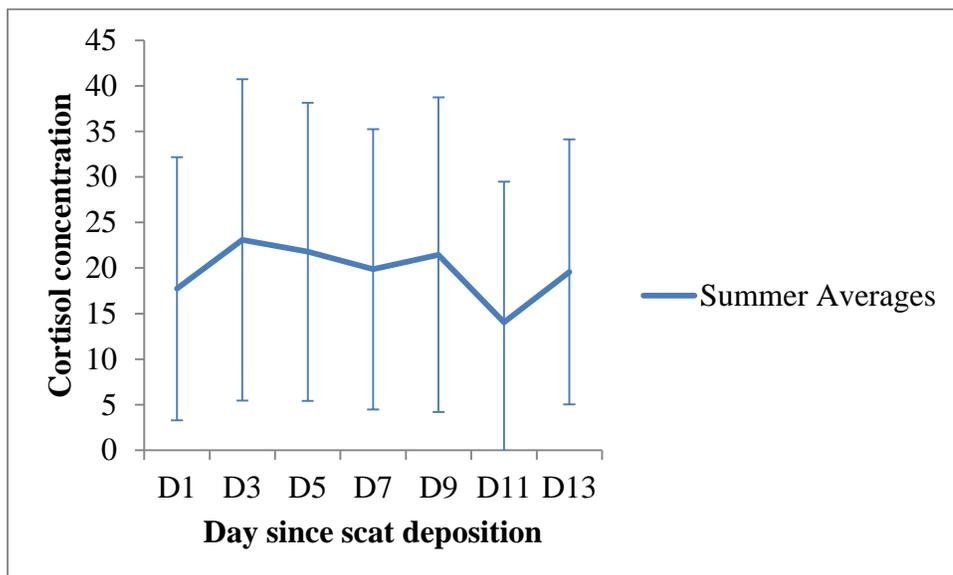


Fig. 3–2. Fecal corticosterone metabolite concentrations for 6 individual coyotes sampled daily for 13 days in (a) summer, and (b) winter, National Wildlife Research Center, Predator Research Facility, Millville, Utah, 2011–2012.

a.



b.

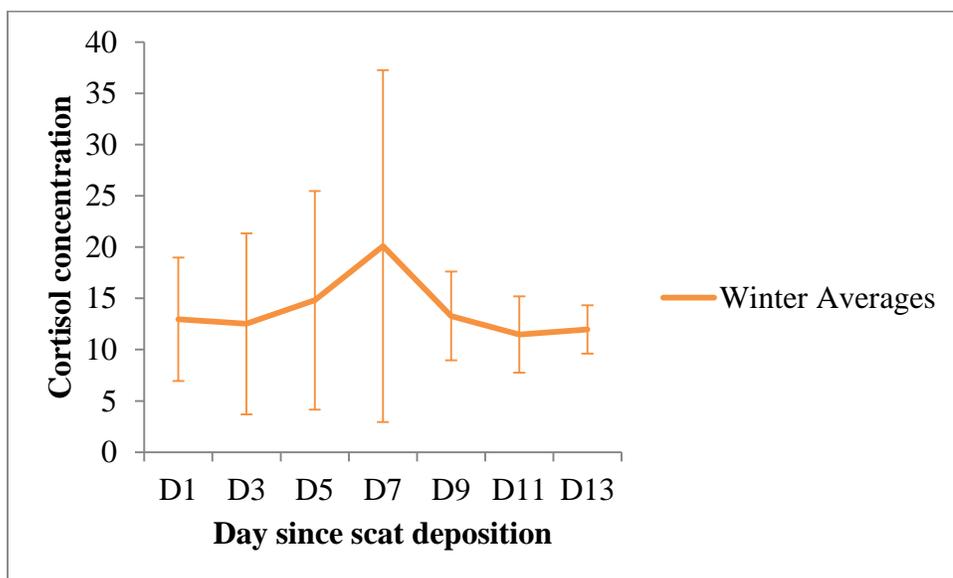
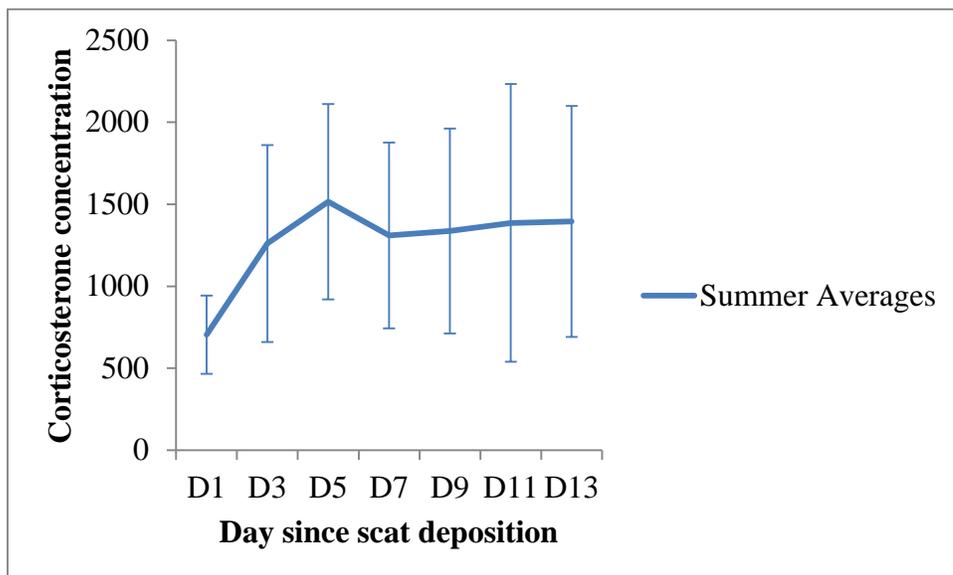


Fig. 3–3. Day averages, 3 males and 3 females, of cortisol metabolite concentrations from coyote feces in (a) summer, and (b) winter, National Wildlife Research Center, Predator Research Facility, Millville, Utah, 2011–2012.

a.



b.

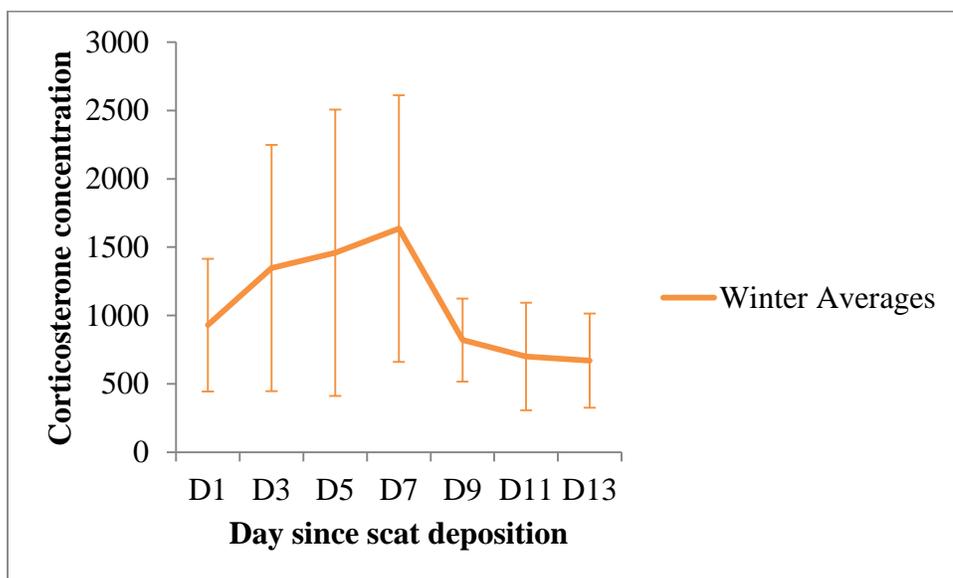


Fig. 3–4. Day averages, 3 males and 3 females, of corticosterone metabolite concentrations from coyote feces in (a) summer, and (b) winter, National Wildlife Research Center, Predator Research Facility, Millville, Utah, 2011–2012.

CHAPTER 4

CONCLUSIONS

This study was the first to comprehensively compare the results of plasma and fecal GCMs in response to an ACTH challenge for a species in the family Canidae. It was also the first to determine if fecal GCM concentrations remained viable in coyote scat over a 13-day sampling period, as well as document GCM persistence in scats left out in the environment in the order Carnivora. Overall, our study results indicated that fecal GCMs do mirror plasma glucocorticoid concentrations and therefore the noninvasive fecal technique to measure physiological stress in coyotes is a valid measurement. We also found no significant degradation, or increase, of cortisol or corticosterone metabolite levels when sampled over the course of 13 days after scat deposition in summer or winter. In both portions of the study our results indicated that individual variation may have an influence on the fecal GCM concentrations for coyotes. Therefore, if researchers are concerned with overall population glucocorticoid concentrations we recommend collecting scat from a large number of individuals. We validated the use of fecal GCM concentrations for coyotes and determined that in the field scat collection could be conducted every 13 days, during summer or winter, and still obtain viable measurements of fecal GCM concentrations. The use of fecal GCM concentrations for assessing physiological stress responses in organisms should be validated for each specific species of interest (Touma and Palme 2005; Keay et al. 2006), and we also recommend as part of the validation process, determining the rate of degradation of fecal GCMs.

When conducting studies to assess physiological stress responses in animals, it is important to take into consideration the differences between males and females, the diurnal fluctuation of glucocorticoids, hormone extraction methods, and immunoassay techniques (Touma et al. 2003; Young et al. 2004; Touma and Palme 2005; Keay et al. 2006; Hoon Son et al. 2011). The method we used to measure the glucocorticoid concentrations was the radioimmunoassay. Our results from this study imply that when fecal GCMs are measured via radioimmunoassay, corticosterone was the better glucocorticoid to extract and measure for coyotes. Our findings differ from those reported by Schell et al. (2013), who found cortisol to be the better fecal glucocorticoid metabolite to use for coyotes. This difference can be explained by the different methods used to determine the concentrations of the fecal GCMs. In our study we used radioimmunoassay as compared to Schell et al. (2013) that used enzyme immunoassay to find their concentrations. The discrepancy found between the 2 studies was probably due to the different immunoassay techniques, but may also be explained by the different hormone extraction methods and antibody sensitivity.

Also from this study we demonstrated that having a noninvasive technique for calculating GCMs better represented the physiological state of the animal. We found that even though our control animals were not induced with ACTH, they still had a stress response from the handling and anesthesia. The handling stress response was especially observed in the plasma cortisol concentrations. Interestingly, we found handling stress responses in fecal GCMs for the control males but not the control females. Our results indicated sex of the coyote was a significant influence in the corticosterone fecal metabolite levels, with the females having higher concentrations pre- and post-injection.

We found no differences between the sexes for plasma and fecal cortisol metabolite concentrations. This further supports the importance of having a large sample size, comparing the use of different glucocorticoids, and validating the use of fecal GCMs as a tool for assessing physiological stress responses.

There are a number of benefits from validating and using fecal GCM concentrations for quantifying physiological stress responses, especially when concerned with the welfare and well-being of both captive and wild animals (Touma et al. 2003). One of the main functions of glucocorticoids in the body is energy regulation for the maintenance of homeostasis (Busch and Hayward 2009). Because of this vital physiological role, glucocorticoids initiate the flight or fight response needed for survival. The flight or fight response is activated by an acute stressor and increases the chance of survival by the organism (Boonstra 2004; Arnemo and Caulkett 2007). When animals are under chronic stress there a number of deleterious effects such as reduced reproduction, slowed growth rates, decreased immune health, and captive animal stereotypies (Möstl and Palme 2002; Young et al. 2004; Touma and Palme 2005; French et al. 2006; Keay et al. 2006; Ellenberg et al. 2007). The capability to measure glucocorticoids noninvasively through feces provides a means for measuring physiological stress without a handling or anesthesia response, and without injury to the subject (Creel et al. 1997; Möstl and Palme 2002; Millspaugh and Washburn 2004; Palme et al. 2005; Touma and Palme 2005; Viljoen et al. 2008). Validating the use of fecal GCMs and determining the amount of degradation that may occur over time will improve our capability to measure GCMs in captive and wild systems alike. Improvement of GCM measurements will thus enhance our knowledge of the physiological stress response and provide a better understanding of

the interaction of wildlife and their natural environment (von der Ohe and Servheen 2002; Boonstra 2004; Dalmau et al. 2007).

In conclusion, we found that measuring the concentrations of fecal GCMs for coyotes is an effective tool for monitoring their physiological stress response. We determined that scat collections could be run at least 13 days apart and still obtain viable measurements of cortisol and corticosterone in the scats. When using radioimmunoassay, we recommend measuring corticosterone from coyote scats. We would also recommend having a large sample size, if interested in the overall population well-being, to account for any individual variation that may occur. Also collecting scats from either the same time of day or multiple times of day to account for the diurnal fluctuation of fecal corticosterone metabolite concentrations will provide a better measure of overall population response. Thus, an adequate sampling design that tests many individuals should be considered for a population-wide assessment. In the future it would be interesting to determine the effects that different diets may have on coyote fecal GCMs. Kalliokoski et al. (2015) found that varying the diets of mice had a significant impact on the concentrations of fecal GCMs. Conducting a study to determine the effects of varying diets would pave the way for future field studies, such as comparing basal physiological stress levels of coyotes in the wild versus urban environments.

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