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Instability of glucocorticoid metabolites in coyote scats: implications for field sampling

Erika T. Stevenson, U.S. Department of Agriculture, Wildlife Services, National Wildlife Research Center, Department of Wildland Resources, Utah State University, Logan, UT 84322, USA

Eric M. Gese, U.S. Department of Agriculture, Wildlife Services, National Wildlife Research Center, Department of Wildland Resources, Utah State University, Logan, UT 84322, USA *eric.gese@usu.edu*

Lorin A. Neuman-Lee, Department of Biological Sciences, Arkansas State University, Jonesboro, AR 72401, USA

Susannah S. French, Department of Biology, Utah State University, Logan, UT 84322, USA

Abstract: Studying physiologic stress responses can assist in understanding the welfare of animals. One method of measuring the physiologic stress response is evaluating concentrations of glucocorticoid metabolites in feces. Previously, using an adrenocorticotropic hormone challenge, we found fecal glucocorticoid metabolite levels were a reliable indicator of physiologic stress response in coyotes (*Canis latrans*). We determine whether glucocorticoid metabolite concentrations remain stable when collecting feces over a 2-week period, a timeframe commonly used in scat surveys for wild canids. We collected feces from 6 captive coyotes maintained at the U.S. Department of Agriculture, Wildlife Services, National Wildlife Research Center, Predator Research Facility near Millville, Utah, USA, and exposed them to the environment for 13 days during summer (August 26 to September 8, 2011) and winter (January 11–24, 2012). Every 2 days, we collected a sub-sample from each individual scat and then quantified the concentration of fecal glucocorticoid metabolites. We found changes in fecal glucocorticoid metabolite concentrations over the 13-day period, with values increasing 45–79% from day 1 to day 3 of sampling. There was also high variation in fecal glucocorticoid metabolites among individuals over time. We provide evidence that fecal samples collected in the field even 3 days after defecation will not provide reliable measures of fecal glucocorticoid metabolites and thus recommend using only fresh fecal samples. We also recommend that, due to high individual variability in fecal glucocorticoid metabolites, a large number of individuals be sampled when a population-wide assessment is desired.

Key words: *Canis latrans*, coyote, fecal, glucocorticoid metabolites, noninvasive sampling, physiological stress

Levels of glucocorticoid metabolites (GCM) in the feces of free-ranging animals have been used in numerous wildlife species as an index of stress responses (Millspaugh and Washburn 2004, Palme et al. 2005, Keay et al. 2006, Schell et al. 2013, Beehner and Bergman 2017). Two glucocorticoids, cortisol and corticosterone, are important signaling chemicals of the endocrine system functioning to alter physiology and behavior in response to acute and chronic stressors in the environment (Beehner and Bergman 2017, Cockrem et al. 2017, Palme 2019). Fecal GCMs have been identified as a useful measure of physiologic stress in animals, as fecal GCM levels can reflect changes in cortisol and corticosterone (Touma et al. 2003, Dalmau et al. 2007, Cockrem et al. 2017). Additionally, fecal GCM information may be preferred, as collection is a noninvasive procedure that minimizes the impact on, and response of, the study animal to researcher activities, as well as reduces possible injury or harm to the animal during capture (Millspaugh and Washburn 2004, Young et al. 2004, Palme 2019).

Although the noninvasive aspect of scat collection can be of great benefit for monitoring the health and condition of a species, there are some issues with using this method in the field, particularly with collection, storage, and extraction of fecal samples (Palme 2019). An issue when using fecal collections is the time elapsed between fecal deposition and sample collection. In a review, Goymann (2012) pointed out sex, diet, season, metabolic rate, and bacterial degradation could alter fecal GCM concentrations. Additionally, studies remain unclear on the effects of time and environmental factors on the degradation of fecal GCMs in scats.

For example, Vynne et al. (2012) showed that fresh samples from maned wolves (*Chrysocyon brachyurus*) improved the accuracy of measured hormone levels. Washburn and Millspaugh (2002) found relative stability in their fecal GCM concentrations for 7 days in varying simulated environmental conditions for fecal pellets from white-tailed deer (*Odocoileus virginianus*) but found an increase in fecal GCM concentration levels for samples exposed to simulated rain. Dloniak et al. (2004) also found stability in fecal GCM concentrations over 48 hours in scat of spotted hyena (*Crocuta crocuta*). In contrast, Muehlenbein et al. (2012) found an increase in variability of the concentration levels within 3 hours after defecation in orangutans (*Pongo pygmaeus morio*), while Möstl et al. (1999) found increases of fecal GCM concentrations after 1 hour in domestic livestock. Conversely, fecal GCM concentrations decreased in scat samples from brown hyenas (*Hyaena brunnea*; Hulsman et al. 2011) and western lowland gorillas (*Gorilla gorilla gorilla*; Shutt et al. 2012) by 6 hours after defecation. It is therefore imperative that fecal GCM consistency be further investigated to elucidate our understanding of changes when fecal samples cannot be collected immediately after defecation.

In canids, scat surveys are typically conducted over 2-week time periods (e.g., coyotes [*Canis latrans*], swift foxes [*Vulpes velox*], kit foxes [*V. marcotis*]; Schauster et al. 2002; Dempsey et al. 2014; Lonsinger et al. 2015, 2018). Therefore, determining the amount of time that fecal GCM concentrations persist consistently in scats could assist in determining when scat collections should occur, thereby increasing the efficacy and reducing the costs of canid scat collections. Although cortisol is the primary circulating glucocorticoid in coyote plasma, it is broken down into various glucocorticoid metabolites in the feces (Palme et al. 2005).

Previously, Stevenson et al. (2018), using an adrenocorticotropic hormone (ACTH) challenge, found that an assay kit with corticosterone antibody more accurately detected fecal glucocorticoid metabolites as an indicator of physiologic stress in coyotes than a kit with a cortisol antibody. Following that work, we wanted to investigate how long glucocorticoid metabolites might persist in fecal samples in the environment. Thus, the objective of this study was to determine the stability of glucocorticoid metabolite concentrations in coyote scats over a 13-day period during 2 different seasons (summer and winter). We predicted that GCM levels during the summer would decrease due to environmental degradation from warmer temperatures, while concentrations during the winter would remain relatively constant over the 13-day period.

Methods

We conducted our research at the U.S. Department of Agriculture, Wildlife Services, National Wildlife Research Center (USDA-NWRC), Predator Research Facility near Millville, Utah, USA. During scat collection, coyotes were housed in male-female pairs in 0.1-ha pens. The animals were fasted 1 day per week; on all other days, they were provided with their normal ration (650 g) of commercial mink food (Fur Breeders Agricultural Cooperative, Logan, Utah, USA). Water was provided ad libitum. The day prior to scat collection, we fed the coyotes glitter (Glitterex Corporation, Cranford, New Jersey, USA) infused into frozen mink food balls (Burns et al. 1995); the males received 1 color and females a different color for individual identification of scats. We collected fecal samples from August 26 to September 8, 2011 for the summer degradation trial and January 11–24, 2012 for the winter trial. The climate during these 2 periods (Figure 1) was reflective of normal temperature and precipitation for that time of year.

We used 6 coyotes (3 male-female pairs), ranging in age from 2–7 years across all sexes; all animals were of reproductive age and had similar history of vaccinations, feeding, and animal care. Our study followed the American Society of Mammalogists guidelines (Sikes et al. 2011), and protocols were approved by the USDA-NWRC Institutional Animal Care and Use Committee (protocol QA-1834). The same coyotes were used for both the summer and winter trials.

To ensure the freshest samples for the study, animals were observed defecating during the collection period. Once the scat was deposited, the observer walked into the pen, collected the scat, and recorded the time and date of collection. Observing the animal defecate further ensured the animal did not urinate on the scat

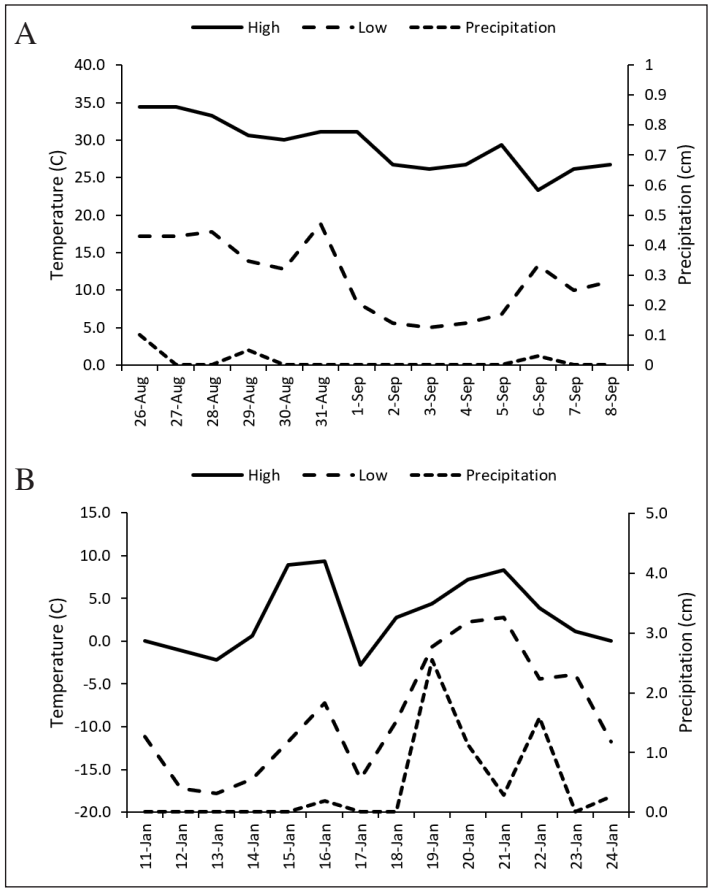

Figure 1. Daily high and low temperatures and amount of precipitation during summer (A) and winter (B) fecal degradation trials, Millville, Utah, USA, 2011–2012.

and contaminate the sample. Each scat was thoroughly mixed together, placed on a plastic-wrapped wooden board, and then placed out in the natural environment on the same day of initial collection (i.e., day 1).

We separated scats from each of the individuals by cardboard dividers. The board was placed in a box trap to prevent small animals from removing the scats, and the trap was elevated off the ground to avoid potential flooding when it rained or snowed (Figure 1). The scat was not covered but exposed to natural decay processes. On each day, for a total of 13 days, we removed a sub-sample from each scat, which was placed into a plastic scintillation vial, and then frozen at -20º C. While we collected scat sub-samples every day during the 13 days, we performed radioimmunoassay (see below) on samples from odd days only.

We previously performed both biological (sex, ACTH challenge time course, blood and fecal comparison) and species (parallelism, non-specific binding, interference) validations for the chosen assay kit (Stevenson et al. 2018). As part of this study, we also tested multiple kits to optimize for antibody yielding the highest concentrations, thereby allowing us to maximize our detectability of GCM in feces, which was most appropriate for our overarching biological questions. We followed the same methods to determine the fecal glucocorticoid concentrations in the current study. The fecal glucocorticoid metabolite was extracted using a 50% phosphate-methanol buffer solution following the methods of Shideler et al. (1994) and Bauman and Hardin (1998). We corrected sample concentrations for the dried weight of the fecal sample. Glucocorticoid metabolite concentrations were determined using a double antibody RIA kit (ImmuChem™ Double Antibody RIA kit, MP Biomedicals, Orangeburg, New York, USA). Kit directions were followed except we used a volume of 10 ul of fecal extract and brought volume up to the kit required volume of 25 ul by adding 15 ul of steroid dilutant provided with the kit. The assay sensitivity was 7.7 ng/ml with the intra-assay coefficient of variation <10% and the inter-assay coefficient of variation <20%.

For statistical analyses, as we had repeated sampling from each coyote over the 13 days, we performed repeated measures analysis of variance (ANOVA) to determine if the level of glucocorticoid metabolite was influenced by the individual animal (between subject effects) and the day the sample was collected since scat deposition (treatment effects). We performed this analysis for each season separately using the statistical program SYSTAT (Wilkinson et al. 1992); data met assumptions of normality and equal variances. Because absolute glucocorti-

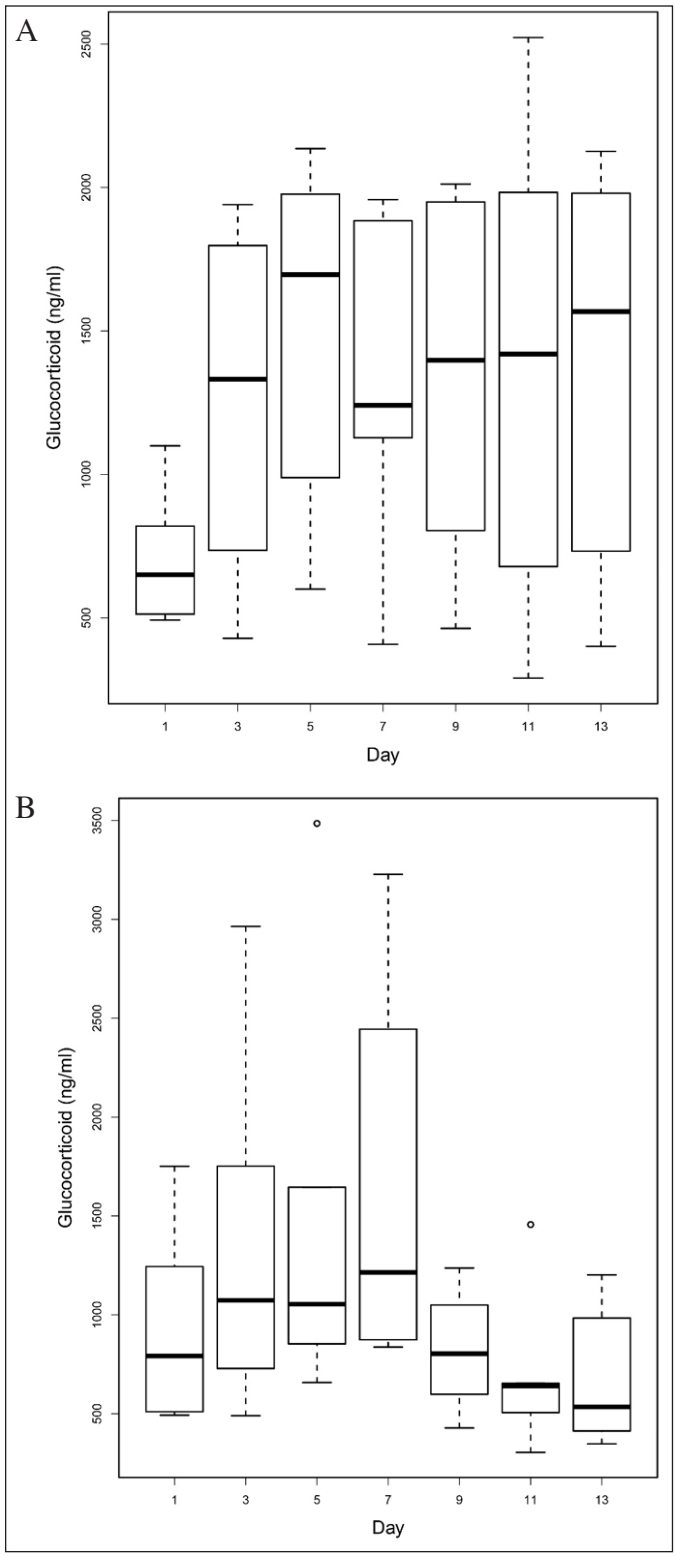

Figure 2. Mean (± SD) fecal glucocorticoid metabolite concentrations measured over 13 days in feces from 3 male-female pairs of coyotes (*Canis latrans*), during summer (A) and winter (B), Millville, Utah, USA, 2011–2012.

coid metabolite values can vary among individuals (Stevenson et al. 2018), we also examined the average percent change across all individuals from 1 day to another as a measure of relative metabolite stability.

Results

For the fecal glucocorticoid metabolite concentrations in the coyote scats during summer, the repeated measures ANOVA showed an influence of days since deposition (treatments: $F_{6,30} = 5.10$, $P = 0.0010$), as well as an influence of individual animals (between subjects: $F_{5,36} = 16.09$, $P = 0.0001$). The differences among individuals explained 69.1% of the variation in glucocorticoid metabolite values, and differences among the days of sampling explained 15.6% of the variation. On day 1, the mean glucocorticoid metabolite value was 704.5 ± 237.7 ng/ml (SD), then increased an average of 73% on day 3, then increased a further average of 26% on day 5, before declining and leveling off to approximately 1,310–1,390 ng/ml during the last 4 sampling points (Figure 2A). Changes in glucocorticoid metabolite values within individual subjects varied widely (Figure 3A), with relative changes ranging from 63–119% between sampling points during summer.

During winter, fecal glucocorticoid metabolite concentrations were also influenced by the number of days since deposition (treatments: $F_{6,30} = 3.14$, $P = 0.0165$), as well as the influence of individual animals (between subjects: $F_{5,36} = 4.24$, $P = 0.0039$). The differences among individuals explained 37.1% of the variation in glucocorticoid metabolite values, and differences among the days of sampling explained

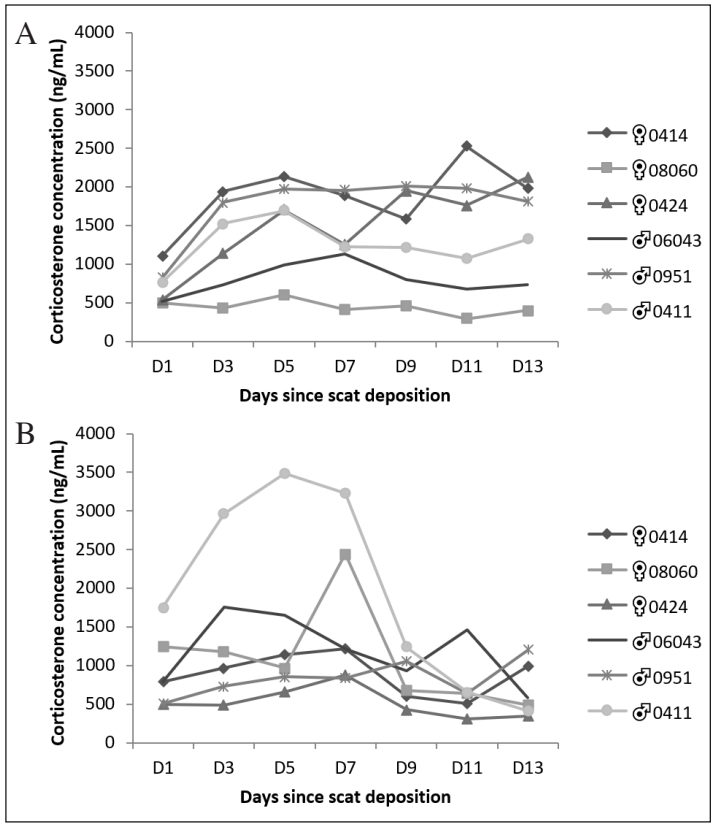

Figure 3. Fecal glucocorticoid metabolite concentrations for 6 individual coyotes (*Canis latrans*) sampled over 13 days in summer (A) and winter (B), Millville, Utah, USA, 2011–2012.

24.3% of the variation. On day 1, the mean glucocorticoid metabolite value was 930.1 ± 485.5 ng/ml (SD), then increased an average of 42% on day 3, then increased an average of 10% on day 5, increased again by an average of 26% on day 7, before declining an average of 39% to 820.0 ± 304.6 ng/ml, then to 700.1 ± 393.8 ng/ml, finally ending at 669.2 ± 344.4 ng/ml (Figure 2B). Changes in glucocorticoid metabolite values within individual subjects again varied widely (Figure 3B), ranging from 27–153% between sampling points during winter.

Discussion

Measuring GCM levels noninvasively through feces provides a means for determining physiological stress response without a handling or anesthesia response and reduces the risk of injury to the subject (e.g., Creel et al. 1997, Möstl and Palme 2002, Viljoen et al. 2008). However, these measures may be difficult to interpret given that they can be influenced by a variety of factors (Palme et al. 2005, Touma and Palme 2005). Our research provided a context for canid fecal samples collected in the field by showing how glucocorticoid levels can fluctuate over time since defecation.

Our results indicated there were large changes in glucocorticoid metabolite values in scats immediately after the first day of collection, with an average 42% increase in winter and an average 73% increase in summer from day 1 to day 3. This trend in increasing glucocorticoid metabolite values continued for the next few days, before leveling off (Figure 1A) in summer, or declining (Figure 1B) in winter. Thus, to acquire accurate glucocorticoid metabolite values from coyote fecal samples, sampling of fresh fecal samples is highly recommended.

We also showed that variability among individual animals greatly influenced the fecal GCM concentrations among coyotes with day 1 samples ranging from 492–1,099 ng/ml during summer and 509–1,751 ng/ml in winter. Stevenson et al. (2018) similarly found large individual variability in coyote fecal GCM values. Cockrem (2013) emphasized the need to understand individual variation when using glucocorticoids to measure stress responses to environmental stimuli, and Goymann (2012) highlighted individualistic characteristics (i.e., sex, diet) that may influence fecal GCM values. Therefore, if researchers are concerned with overall population GCM concentrations, we recommend collecting scat samples from a large number of individuals due to high variability among individual animals.

Determining the rate of degradation has been conducted for canid fecal DNA (Lonsinger et al. 2015), and we strongly recommend the same for fecal GCMs, both across canid species and across taxa. Other studies have reported changes when fecal samples were not frozen immediately. For

example, studies with domestic livestock found fecal GCM concentrations increased from 1–3 hours after collection (Möstl et al. 1999); they reported this increase in fecal GCMs might 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 within 3 hours after scat collection; they assumed this was due to physiological processes such as bacterial metabolism. The fecal GCM concentrations in brown hyenas 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 up to 48 hours post-collection (Dloniak et al. 2004). These variations between species further support the need to determine the rate of degradation for each species of interest and may require determining degradation of the GCMs in differing environmental conditions.

Determining degradation rates will improve our ability to measure GCMs in captive and wild systems alike. Improving our ability to accurately measure GCM concentrations 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, Beehner and Bergman 2017, Palme 2019). There are a number of benefits from validating and using fecal GCM concentrations for quantifying physiologic stress responses, especially when concerned with the welfare and well-being of both captive and wild animals (Touma et al. 2003, Beehner and Bergman 2017). In conclusion, we determined scat collections conducted even 3 days after scat deposition would not provide reliable measurements of fecal glucocorticoid metabolites in the scats of coyotes. We also note that if a study is concerned with a measure of fecal glucocorticoid metabolites, a small number of individuals will skew the results due to the high variability among individuals. Thus, a high number of samples should be considered for population-wide assessments.

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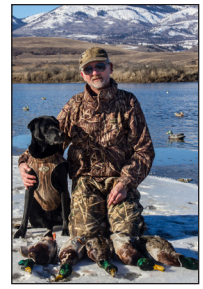
Associate Editor: John Tomeček

Erika T. Stevenson earned her B.S. degree in conservation biology at SUNY Environmental Science and Forestry and her M.S. degree in wildlife biology at Utah State University. Her master's thesis focused on the validation of using coyote fecal samples as a tool to measure glucocorticoid metabolite levels. She is passionate about the importance of public outreach in environmental conservation efforts.

Eric M. Gese is a research wildlife biologist with the U.S. Department of Agriculture's National Wildlife Research Center and a professor in the Department of Wildland Resources at Utah State University. He obtained his B.S. degree in biology from the University of Texas – San Antonio, and M.S. and Ph.D. degrees in wildlife ecology from the University of Wisconsin. He has been fortunate to have conducted research on carnivore behavior, ecology, and management for >35 years examining predator–predator interactions, predator–prey relationships, and wildlife damage management.



Lorin A. Neuman-Lee is an assistant professor of physiology at Arkansas State University. She mostly examines the interactions between stress and immunity in reptiles, although she enjoys applying these techniques and concepts to a wide variety of organisms, including mammals. She runs an active lab and teaches courses related to immunology, endocrinology, and comparative physiology.

Susannah S. French is a professor in the Department of Biology at Utah State University. She received her bachelor's degree from the University of Illinois in 2002 and a Ph.D. degree from Arizona State University in 2006. She was an NIH postdoctoral fellow at Indiana University before she joined the faculty at Utah State University in 2009. Her research group currently includes 5 Ph.D. students and an army of undergraduates, who are invaluable to her work. She has received funding from the National Science Foundation and National Geographic, including the NSF Faculty Early Career Development CAREER Award. She conducts studies of vertebrates in the United States as well as the Bahamas, Galapagos Islands, Honduras, and the Arctic to understand how animals interact with their environments.