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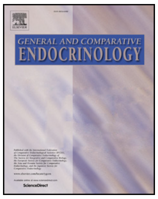

Investigation of the utility of feces and hair as non-invasive measures of glucocorticoids in wild black-tailed prairie dogs (*Cynomys ludovicianus*)

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ABSTRACT

Non-invasive measures of glucocorticoid (GC) hormones and their metabolites, particularly in feces and hair, are gaining popularity as wildlife management tools, but species-specific validations of these tools remain rare. We report the results of a validation on black-tailed prairie dogs (*Cynomys ludovicianus*), a highly social engineer of the grasslands ecosystem that has experienced recent population declines. We captured adult female prairie dogs and brought them into temporary captivity to conduct an adrenocorticotropic hormone (ACTH) stimulation test, assessing the relationship between plasma GC and fecal glucocorticoid metabolite (FGM) levels following a single injection of a low (4 IU/kg) or high dose (12 IU/kg) of ACTH, compared to a single injection of saline. We also gave repeated injections of ACTH to adult females to assess whether this would result in an increase of hair cortisol concentrations, compared with control individuals repeatedly injected with saline. A single injection of ACTH at either low or high dose peaked plasma cortisol levels after 30 min, and thereafter the cortisol levels declined until 120 min, where they returned to pre-treatment levels comparable to those of the saline injected group. Despite the significant elevation of plasma cortisol in the treatment groups following ACTH injection, the elevation of FGM levels in the treatment groups were not significantly different from those in the control group over the following 12 h. Repeated injection of a high dose of ACTH failed to increase hair cortisol concentration in treatment animals. Instead, hair cortisol levels remained comparable to the pre-treatment mean, despite an increase in post-treatment hair cortisol levels seen in the saline-injected group. The magnitude of increase in the saline control group was comparable to natural seasonal variation seen in unmanipulated individuals. These results highlight that while measurement of GCs and their metabolites in feces and hair are potentially valuable conservation tools for black-tailed prairie dogs, further validation work is required before these matrices can be to real-world conservation applications.

1. Introduction

When vertebrates are confronted by a stressor, a physiological stress response is initiated via the hypothalamic-pituitaryadrenal (HPA) axis, which secretes glucocorticoid (GC) hormones, either cortisol or corticosterone, depending on the species (Romero, 2004; Touma and Palme, 2005). These GCs coordinate the physiological response to the stressor, but also play an important role in organizing routine daily and seasonal activities, such as foraging and courtship (Reeder and Kramer, 2005; Touma and Palme, 2005). Common stressors faced by wildlife include both short-term, acute stressors, such as an attack by a predator or inclement weather, and longer term, chronic stressors, such as an unstable social environment (Boonstra et al., 1998; Creel et al., 2013). Although healthy individuals are generally able to cope with these stressors without suffering adverse effects, chronic overstimulation of the HPA axis may result in deleterious effects on health and fitness (Blas et al., 2007; Boonstra et al., 1998; Sheriff et al., 2009). Due to these potential negative consequences, a growing number of researchers are measuring GC levels in wildlife as an indicator of population health (Busch and Hayward, 2009; Dantzer et al., 2014).

There are multiple methods used to study GCs in wildlife. Of these, assessments using blood and feces are the most common, though hair is also gaining popularity (Russell et al., 2012). Blood has been used for decades as the standard for measuring circulating hormone levels, as it

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provides a direct measurement of total GCs in the blood plasma at the time of collection (Sheriff et al., 2011). This method detects the total amount of GCs, both free GCs and those bound to carrier proteins, namely corticosteroid binding globulin (CBG) (Malisch and Breuner, 2010).

Metabolites of free (unbound) GCs can be measured in feces, and are a popular tool for wildlife research because they can be collected with minimal disturbance to the study animal (Mateo and Cavigelli, 2005; Touma and Palme, 2005). The period that fecal samples represent varies greatly based on the metabolism of the study animal and can vary within species based on differences in metabolism between different seasons (Ashley et al., 2011; Touma and Palme, 2005). Studies of rodents in the family Sciuridae, which includes prairie dogs (*Cynomys* spp.), indicate that the rate of metabolism and excretion of exogenous hormones can vary between 4 and 12 h, depending on the species (Bosson et al., 2009; Dantzer et al., 2010; Sheriff et al., 2012).

In contrast to blood and fecal samples, hair samples represent GC levels over a much longer period of time than minutes or hours (Russell et al., 2012). Current research indicates that free circulating GCs diffuse into the hair shaft medulla over the period that hair is growing, and thus represent cumulative GC levels over the course of weeks or months, depending on the species, type of hair, and hair growth cycle (Davenport et al., 2006; Sheriff et al., 2011). Because of this lag time, hair samples can be collected at the time of a stressful event and still provide a pre-stressor baseline sample, when compared to samples taken weeks later (Davenport et al., 2006). Further, hair samples are also advantageous to researchers because they do not require specialized equipment to collect or store (Russell et al., 2012). Finally, since fewer samples per individual are required to gain insight into long-term stress levels, hair is an ideal matrix to study the effects of chronic stressors in wild populations. Just as using fecal samples requires an understanding of gut passage time for each new species, hair samples also require a thorough understanding of the molting and hair growth patterns for each species. For species with seasonal hair growth, the frequency of molt can limit the number of sampling periods, as hair samples only reflect cortisol incorporated during active hair growth. Species with indeterminate hair growth, on the other hand, can provide a continuous, integrated record of HPA activation.

Due to interspecific variation in the amount of time required for GC hormones to be detected in feces or hair, a species-specific validation is required for each new species and each new matrix that is to be studied (Touma and Palme, 2005). Validating these non-invasive methods is especially important for species at risk, since it provides a broader toolset for monitoring the health of these imperiled populations, while reducing the level of human manipulation required. This study is the first to investigate the use of feces and hair to measure GCs in black-tailed prairie dogs (*Cynomys ludovicianus*), which are listed as threatened in Canada (COSEWIC, 2011).

Black-tailed prairie dogs are a highly social, obligately colonial sciurid that play an important role in grassland ecosystems by altering the landscape, and providing both shelter and prey for a number of other species (Hoogland, 1995). However, due to habitat loss and persecution by humans, prairie dog populations have declined by an estimated 98% since the initial European settlement of North America (Proctor et al., 2006). More recently, the Canadian population of prairie dogs, which is restricted to approximately 12 km^2 in southern Saskatchewan, may have declined by 22–33% from 1996 to 2011 (COSEWIC, 2011). Chronic stress due to the risk of predation has been implicated in the decline of other prey species, though it has not yet been investigated in this population (Boonstra et al., 1998). Black-footed ferrets (*Mustela nigripes*), a specialist predator who rely on prairie dogs for the bulk of their diet were reintroduced to the area in 2009, reestablishing a potentially important stressor that had been absent from the landscape for decades (COSEWIC, 2011). Additionally, the Canadian population is threatened by disease and climate, risks that may be exacerbated by their location at the northern limit of the species' range (COSEWIC, 2011).

Individuals of this species molt twice per year, in mid- to late spring and in late summer to early autumn, with individual variation in molt timing based on age, sex and reproductive status (Hoogland, 1995). Individuals in the best body condition, such as non-breeding yearlings, molt first, while those in the poorest condition, specifically lactating females, molt later in the season (Hoogland, 1995). Lactation appears to constrain the female's ability to molt, as it is such an energetically demanding process that these females typically lose mass in late spring, while other individuals are rapidly regaining mass previously lost over winter (Hoogland, 1995). Molt duration is between 7 and 14 days (Hoogland, 1995), which provides the opportunity to collect samples that represent a one to two-week snapshot of cumulative stress responses from two periods each year. To date, our ability to use hair to test for an effect of chronic stressors in the population's decline has been hindered because there has never been a validation of matrices which can be used as non-invasive measures of GCs in this species.

The main objectives of this experiment were to determine the dominant GC hormone in the stress response in black-tailed prairie dogs, and investigate natural and stimulated patterns of GC hormone deposition in feces and hair. As this is the first investigation of GC hormones in this species, we first determined whether cortisol or corticosterone was dominant in the stress response for this species, by measuring blood collected from free-ranging, unmanipulated individuals. We also examined the levels of cortisol and corticosterone in fecal and hair samples. Then, we simulated both acute and chronic stress stimuli using injections of synthetic ACTH to stimulate an adrenal response in wild-caught individuals. We expected that stimulation with synthetic ACTH would (1) increase plasma and fecal GC levels after a single injection and (2) increase hair GC after multiple injections, compared to animals treated with saline. For the single injection experiment, we divided our treatment into high and low doses of ACTH based on doses used in studies of related species (Hammond et al., 2015; Mateo and Cavigelli, 2005). The goal of this division was to determine which dose would bring subjects closer to their physiological maximum of stimulation, and to determine the dose-dependent relationship between ACTH administration and GC detection.

2. Methods

2.1. Animals

All procedures followed the Canadian Council on Animal Care guidelines for wildlife, and were approved by the University of Saskatchewan Animal Care Committee (AUP 20170012) and Parks Canada (Research and Collection Permit GRA-2014-16101). On March 5–6, 2017 we live-trapped five individuals from a single black-tailed prairie dog colony in Grasslands National Park, Saskatchewan, Canada (49° 3′ 47″N, 107° 21′ 29″W) using Tomahawk live-traps baited with peanut butter and oats. These individuals were a mix of male and female adults and yearlings, both breeding and non-breeding, and were used to determine the dominant circulating glucocorticoid hormone. On March 13–14, 2017 we live-trapped twelve prairie dogs for the acute stress response experiment from the same colony. To reduce individual variation within our sample, therefore maximizing our ability to detect a signal that might otherwise be obscured by differences between sexes and age classes, we restricted the study to adult females. The animals in the colony were part of an ongoing long-term study, where the animal's minimum ages were known, allowing us to exclude females less than 2 years old. These animals were previously marked with unique alphanumeric ear tags (Monel #1, National Band and Tag Co., Newport, Kentucky) in both the right and left pinnae for permanent identification.

As part of the long-term study, we routinely trapped individuals from March through August. Upon capture, we recorded standard measurements including age, sex, reproductive status, body mass, zygomatic arch breadth and length of the right hind foot to the longest toe. We handled each individual no more than once every three days, though some were trapped more frequently and released without handling. Ages were known for animals first captured as juveniles. Males who were not first captured as juveniles were classified as adults. Females who were not first captured as juveniles were classified as yearlings if their nipples were small, and difficult to find, or adults if their nipples were extended and clearly visible. Reproductive status for males was classified as breeding or non-breeding based on the presence or absence of descended testicles, respectively. Reproductive status for females was classified based on nipple condition (lactating or non-lactating), status of the vulva (open or closed), and palpation of the abdomen during the reproductive season. We classified females as lactating if their nipples were long, engorged and the hair around the base of the nipple was worn away, or any of these three conditions were present and we were able to manually express milk. We classified females as non-lactating if their nipples were small or flattened, covered by hair and we could not express milk.

2.2. *Primary circulating glucocorticoid*

Prior to the acute stress response experiment, we collected blood samples to determine whether cortisol or corticosterone is the dominant stress hormone in black-tailed prairie dogs. We collected 0.4–0.6 ml of blood from the medial saphenous vein of five black-tailed prairie dogs. We transferred blood from 3 ml collection syringes into heparinized tubes (BD Microtainer, Becton, Dickinson and Company, Franklin Lakes, NJ) and stored the blood samples at room temperature for 4 h. We centrifuged each sample for 15 min at 4000 rpm, collected the plasma using a transfer pipette, and transferred each plasma sample to a clean 1.5 ml microcentrifuge tubes and stored it at −20 °C until extraction.

We extracted hormones from these plasma samples following the procedures described below, then quantified the amount of cortisol and corticosterone present in each sample using commercial immunoassay kits (EA65 Cortisol and EA66 Corticosterone, Oxford Biomedical, Lansing, Michigan). According to the manufacturer, the cross reactivity for the cortisol EIA is as follows: cortisol (100%), prednisolone (47.72%), cortisone (15.77%), 11-deoxycortisol (15%), prednisone (7.83%), corticosterone (4.81%), 6-β-hydroxycortisol (1.37%), and 17-hydroxyprogesterone (1.36%). All other hormones tested by the manufacturer exhibited cross reactivity of < 1.0%. The cross reactivity of the corticosterone EIA is as follows: corticosterone (100.0%), deoxycorticosterone (38.0%), 6-hydroxycorticosterone (19.0%), progesterone (5.1%), tetrahydrocorticosterone (2.7%), prednisolone (1.5%), and cortisol (1.1%). All other hormones tested by the manufacturer exhibited cross reactivity of < 1.0%. The intra-assay coefficient of variation was 9.2% between wells within a plate ($n = 6$) and the inter-assay coefficient of variation was 8.1% between wells on different plates ($n = 12$) for cortisol assays. For the corticosterone assays, the intra-assay coefficient of variation was 6.4% ($n = 6$) and the inter-assay coefficient of variation was 5.7% ($n = 12$).

In addition to plasma GC levels, we investigated the rates of deposition into feces and hair. Feces from the acute stress response experiment were analyzed for both hormones, following the procedures outlined in Section 2.6. Preparation of hair samples for hormone extraction, detailed in Section 2.6, generally does not result in enough hair to extract both cortisol and corticosterone from the same sample. Therefore, we used 5 hair samples collected from prairie dogs in the long-term study colony and analyzed the cortisol and corticosterone concentrations in these samples. We extracted both hormones from the samples according to the procedures detailed in Section 2.7, and quantified these hormones using the cortisol and corticosterone kits previously described. The intra-sample coefficient of variation was < 15% for all replicates.

2.3. *Acute stress response experiment*

We randomly assigned the captured individuals to a low-dose treatment ($n = 5$), high-dose treatment ($n = 4$), or control group ($n = 3$). We collected a pre-treatment hair sample from 10 of the animals (two prairie dogs had already had a hair sample collected 9–10 days previous) by shaving a 3 cm × 3 cm patch from each animal's right hind leg with electric clippers (Model #8552, Whal Clipper Co, Sterling, Illinois). These samples were stored in paper envelopes at room temperature. Following the hair sample collection, we transported the animals, secured in Tomahawk traps, to a nearby field station 3.5 km from the colony (49° 4′ 59″, −107° 23′ 31″). We transferred the prairie dogs to polycarbonate rodent cages with wood shaving bedding, provided them with apples and lettuce and commercial rodent chow, covered with a cotton sheet, and housed them overnight prior to commencing the experiment.

On the following morning, we removed each prairie dog from its cage, and drew 0.4 ml of blood from the lateral saphenous vein of each animal using a sterile 3 ml syringe with a 22 gauge needle. We considered this sample to be a nominal, rather than true, baseline as it was collected after animals had be captured, transported and housed overnight. Immediately after this baseline blood sample, we injected each animal intramuscularly with 4 IU/kg of ACTH (Cortrosyn, Amstar Pharmaceuticals, Rancho Cucamonga, California) for the low-dose treatment, 12 IU/kg ACTH for the high-dose treatment, or 0.48 ml saline for the control animals. All injections occurred within one hour (between 06:17 and 07:15 MDT). We collected any feces produced during blood draws and injections as representations of pre-treatment stress levels.

We collected an additional 0.4 ml of blood at 30, 60 and 120 min after the initial injection, and collected any feces produced at the 120 min collection period. We centrifuged the blood samples and stored them following the same procedures outlined in Section 2.2. After each subsequent blood draw, we returned the animals to live traps, provided them with food, and covered the traps with cotton sheets. We placed traps on an elevated platform, allowing feces and urine to fall through the bars of the trap, and removed the traps every 2 h to collect fecal samples. We lined the area under the traps with paper towels to absorb urine, and only collected fecal samples that were not visibly contaminated by urine. We collected fecal samples for each animal at each collection period in a whirl-pak® bag (Nasco, Fort Atkinson, Wisconsin) and stored samples at −20 °C. Twelve h after the initial injection, we collected a final fecal sample and then returned animals to the colony, at the location from which they were originally trapped.

2.4. *Chronic stress response experiment*

We initiated the long-term hair glucocorticoid experiment four weeks after the acute stress response experiment, using free-living prairie dogs at the same study colony. The original 12 animals used in the acute stressor experiment remained in the same groups (treatment or control), throughout the chronic stress response experiment. To augment our sample size, we included six additional adult female prairie dogs. We collected pre-treatment period hair samples for these six individuals as part of a larger project, then assigned them to the saline treatment group for the hair cortisol experiment (resulting in $n = 9$ control, $n = 9$ treatment).

There was substantial variation in molt timing between the adult females in the study, which proved to be a significant challenge for predicting individual hair growth rates. This resulted in a variable number and timing of injections between individuals. Prior to new hair growth in shaved areas, we gave animals 0–3 intramuscular injections of 12 IU/kg ACTH to the treatment group and 0.48 ml of saline to the control group, spaced over 2–4 days. During the period of hair growth, we gave all animals an additional 2–3 injections of the same dose. This resulted in 3–5 total injections per animal. We waited until hair from the original sample patch visually appeared to be the same length as the surrounding hair, then collected post-treatment hair samples.

We also considered data on natural variation in hair cortisol levels of samples collected from adult female prairie dogs from the same colony that did not receive injections (neither ACTH nor saline). Samples from this natural population were collected in both the pre-treatment period ($n = 36$) and the post-treatment period ($n = 28$). Due to deaths and disappearances from the colony, we were unable to recapture eight of the animals in the natural population for a second hair sample, resulting in a smaller sample size for the post-treatment period.

2.5. *Extraction and quantification of hormones from plasma*

We extracted hormones from plasma samples following previously published methods (McMaster et al., 1992). Briefly, we diluted plasma samples using the EIA kit buffer to create a total volume of 0.5 ml. We added 2.5 ml of diethyl ether and let the tubes sit for 3 min to separate the ether and aqueous phases. We flash froze each tube in liquid nitrogen for 20 s, then decanted the upper, ether phase into a clean test tube. We then evaporated the ether phase from the tubes using a gentle stream of nitrogen gas (4–6 L/min) at 50 °C. We repeated this process for a second collection to ensure maximum recovery of hormones from each sample. This extraction procedure consistently recovers > 95% of plasma GCs, as determined using spike-recovery experiments (McMaster et al., 1992). Following the second ether extraction, we rinsed the insides of each tube with an additional 1 ml of ether and evaporated this liquid as above. We reconstituted each sample in 0.25 ml of EIA kit buffer and stored the samples at −20 °C until we performed cortisol and corticosterone assays. Assays were conducted using Oxford Biomedical EIA Kits described in Section 2.2.

2.6. *Extraction and quantification of fecal glucocorticoid metabolites*

We lyophilized fecal samples for 65 h at −90 °C and shipped them overnight on ice to the Lincoln Park Zoo Endocrinology Laboratory (Chicago, Illinois) where we processed them following previously published methods (Brown et al., 1994, Santymire et al., 2012). We diluted the hormone extracts from fecal samples with dilution buffer to 1:120 for the corticosterone assay, and 1:30 for the cortisol assay, vortexed, and then analyzed 50 µl aliquots in duplicate on both an in-house cortisol [polyclonal cortisol antiserum (R4866) and horseradish peroxidase ligands (HRP) provided by C. Munro (University of California, Davis, California)] and corticosterone [polyclonal corticosterone antiserum (CJM006) and horseradish peroxidase ligands (HRP) provided by C. Munro (University of California, Davis, California)] EIA. Cortisol polyclonal antiserum was raised in rabbits against cortisol-3-carboxymethyloxime linked to bovine serum albumin and cross-reacts with cortisol (100%), prednisolone (9.9%), prednisone (6.3%), cortisone (5%) and < 1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (C. Munro, pers. comm.). Antiserum cross-reactivities for corticosterone were: corticosterone (100%), deoxycorticosterone (14.25%), and < 1% with tetrahydrocorticosterone, 11-deoxycortisol, prednisone, prednisolone, cortisol, cortisone, progesterone, testosterone and 17 β-estradiol (C. Munro, pers. comm.). We biochemically validated the cortisol enzyme immunoassay by demonstrating parallelism between serially diluted fecal extracts (1:256 to 1:1) and the cortisol standard ($R^2 > 0.99$). There was also a significant percent recovery of cortisol (1.95–500 pg/well) added to pooled fecal extracts ($y = 0.93x + 1.51$; $R^2 > 0.99$). We confirmed the biochemical validity of the corticosterone EIA by demonstrating parallelism between binding inhibition curves of fecal extracts (1:1024–1:4) dilutions and the corticosterone standard ($R^2 = 0.99$). There was also significant percent recovery of corticosterone (3.9–1000 pg/well) added to faecal extracts ($y = 0.92x - 1.45$; $R^2 > 0.99$). Cortisol and corticosterone EIA sensitivities were 1.95 and 3.9 pg/well, respectively, and intra- and inter-assay coefficients of variation were < 10%.

2.7. *Extraction and quantification of hormones from hair*

Preparation of shaved hair samples and hormone extraction followed our previously validated protocol (Macbeth et al., 2010). Briefly, we removed surface contamination by washing hairs with methanol (three 3 min washes). Following decontamination, we dried the hair, ground it to a fine powder using a ball mill, and weighed it. We immersed the ground hair samples (25 mg) in 0.5 ml of high-resolution gas chromatography-grade methanol, gently swirled (10 s), and placed it on a slowly spinning rotator to extract for 24 h. Following extraction, we centrifuged samples for 15 min at 2150*g*, removed the methanol extract, evaporated contents until dryness under nitrogen gas (38 °C), and reconstituted samples in 0.2 ml phosphate buffer, creating an undiluted (1:1) sample for analysis We quantified cortisol in picograms of cortisol per milligram of washed and dried hair (pg/mg) using the Oxford Biomedical Cortisol EIA kit previously described, with samples run in triplicate. If a sample returned triplicate values with a percent coefficient of variation (%CV; SD/mean) > 15%, we re-extracted the sample and repeated the assay. We confirmed the validity of this assay for measuring cortisol in black-tailed prairie dog hair by demonstrating that a concentrated hair hormone extract run in a serial dilution (1:1–1:64) yielded a displacement curve whose slope did not differ from the slope of the linear portion of the cortisol standard curve ($R^2 = 0.99$, $p > 0.99$).

2.8. *Statistical analyses*

In order to demonstrate the validity of the assay for measuring cortisol in prairie dog hair, we used XLSTAT (Addinsoft, Paris, France) 4/5 parameter parallel lines logistic regression to run a Fisher's F test 4 parameter model to assess parallelism between the standard curve and a serially diluted sample. We conducted all other statistical analyses using R, version 3.2.3 (R Core Team, 2015), with package nlme (Pinheiro et al., 2017) for mixed-effects modelling. We report all values as ± 1 standard error (SE) unless otherwise noted.

In our investigation of the primary circulating glucocorticoid and patterns of deposition of hormones into each matrix, we used R to plot the relationship between cortisol and corticosterone, and used the cor.test function to test for a significant correlation. For plasma, the limited number of samples analyzed for corticosterone ($n = 5$) precluded meaningful statistical analysis. For feces, three outlier samples posed excessive influence on the relationship, so we excluded them from the correlation. This resulted in a comparison of 63 data points for each hormone. For hair, we included all 9 data points available for both hormones.

We used the Shapiro-Wilk test to assess the blood sample data for normality. These data were not normally distributed ($W = 0.77$, $p < 0.01$). Therefore, we log transformed these data prior to analysis. We used a general linear mixed-effects model with dose, time, and the time required to collect each sample fitted as fixed effects influencing plasma cortisol. We also included the interaction between dose and time elapsed from injection, and the interaction between dose and collection time on plasma cortisol. We fitted individual ID as a random effect to account for repeatedly sampling the same individual. We also included a time auto-correlation function to address the dependence of one blood sample on the previous sample. We tested models with a random slope, and with both a random slope and intercept. We selected the model based on the log likelihood that was statistically different, resulting in a final model with slightly higher Akaike information criterion (AIC). This model did a better job of accounting for the random effects by allowing the slope to vary within dose groups while allowing for different intercepts for individuals. This was important because the amount of variation seen in the low dose group was orders of magnitude higher than the variation seen within the saline or high dose groups.

We used the Shapiro-Wilk test to assess the FGM data for normality. These data were not normally distributed for fecal cortisol metabolites ($W = 0.82$, $p < 0.01$) nor fecal corticosterone metabolites ($W = 0.93$, $p < 0.01$). Therefore, we calculated the fold increase between FGM levels at baseline and peak time points, and used the Kruskal-Wallis test to test for differences between the dose groups, following previously published methods (Schell et al., 2013).

For the chronic stress experiment, we ran a Kruskal-Wallis test on the time between the first and last injection, and the time between the last injection and the hair collection to ensure all treatment groups received the same injection timeline. We used the Shapiro-Wilk test to assess the hair sample data for normally and found it to be normally distributed ($W = 0.95, p = 0.09$). We used a general liner mixed-effects model to determine the influences on hair cortisol content. Our initial candidate model included hair cortisol as the dependent variable, and season, treatment, age, mass, location on the colony and lactation as fixed effects, as these variables have been shown to influence cortisol levels in other mammalian studies. The effect of lactation was particularly important to control for, as this process is directly tied to timing of the molt. The proportion of females that lactated compared to total individuals in each treatment group varied substantially during the experiment (ACTH 4/8; Saline 7/9; Natural 26/28). We fitted individual ID as a random effect to account for repeated measures of each individual. We used AIC to compare candidate models, and serially excluded non-significant variables ($p < 0.05$) until the most parsimonious model remained.

3. Results

3.1. Primary circulating glucocorticoid

Cortisol was the dominant hormone in plasma, present in concentrations more than 21 times that of corticosterone ($\bar{x} = 69.15 \pm 10.56$ ng/ml cortisol; $\bar{x} = 3.28 \pm 0.11$ ng/ml). We detected both cortisol and corticosterone metabolites in fecal samples. We detected higher levels of corticosterone metabolites than cortisol metabolites, based on both the raw results and the dilution rates required to ensure the results fell within the upper and lower detection limits of the assay (cortisol run at 1:30, $\bar{x} = 58.3 \pm 3.13$ ng/g; corticosterone run at 1:120, $\bar{x} = 197.21 \pm 10.81$ ng/g). Metabolites of the two hormones were significantly correlated in feces ($r = 0.63$, $t = 6.44$, $df = 61$, $p < 0.01$) (Supplemental Fig. S1). We detected both cortisol and corticosterone in hair samples. Corticosterone concentrations were higher than cortisol in hair ($\bar{x} = 6.87 \pm 0.78$ pg/mg cortisol; $\bar{x} = 19.12 \pm 2.06$ pg/mg corticosterone). The two hormones were significantly correlated ($r = 0.81$, $t = 3.66$, $df = 7$, $p < 0.01$) (Supplemental Fig. S2).

3.2. Acute stress response experiment

Plasma cortisol levels ranged from 19.65 to 190.50 ng/ml, with a mean of 82.92 ± 7.20 ng/ml across all groups at all times. There was one outlier value of 381.52 ng/ml, which we excluded from analysis, as it was greater than 2 standard deviations from the mean. For the two treatment groups, plasma cortisol levels were elevated at 30 min and 60 min post-injection compared to the baseline measure, while levels at 120 min were similar to the pre-injection cortisol levels (Fig. 1). At 30 min, plasma cortisol levels peaked for both the high and low dose groups, where the high dose group was significantly higher than both the saline group ($p < 0.01$) and the low dose group ($p = 0.02$) (Supplemental Table S2). At 60 min, levels for the high and low dose groups declined from 30 min. The high dose group and low dose group were not significantly different from each other ($p = 0.08$), but still higher than the saline group ($p < 0.01$). At 120 min, the high dose treatment group was similar to the saline group ($p = 0.19$), while the low dose group was significantly lower ($p < 0.01$).

Time required to collect blood samples varied by group, and therefore we fitted collection time as a fixed effect in the statistical model. The longer time required for the low dose group significantly reduced the effect of the ACTH injection ($p < 0.01$). However, this effect was not seen for the other treatment groups ($p = 0.17$) (Supplemental Table S2).

The intercept variance for the high dose and the saline group was low ($\sigma^2_{\mathrm{high}} = 1.54 \times 10^{-6}$ and $\sigma^2_{\mathrm{saline}} = 9.36 \times 10^{-7}$) while the variance for the low dose group was high ($\sigma^2_{\mathrm{low}} = 0.28$). This demonstrates that the low dose group was much more variable in the response to the ACTH injections. The variance not explained by individual was $\sigma^2_{\mathrm{resid}} = 0.15$ (Supplemental Table S2).

We detected both cortisol and corticosterone metabolites in fecal samples following ACTH injection. Corticosterone metabolites were present in higher levels than cortisol metabolites ($\bar{x} = 197.21 \pm 10.81$ ng/g corticosterone; $\bar{x} = 58.3 \pm 3.13$ ng/g cortisol). Across all time points, mean fecal cortisol metabolites detected in the feces of the high dose group was 40.8 ± 18.6 ng/g (Fig. 2). For the low dose treatment group the mean was 42.2 ± 18.8 ng/g, and for the control group the mean was 53.0 ± 17.3 ng/g (Fig. 2). There was not a significant difference between groups pre-treatment ($F = 0.401$, $df = 2$, $p = 0.69$). Fecal cortisol metabolites for the high dose group peaked at 10 h (76.7 ± 11.8 ng/g). The highest elevation for the low dose group (67.1 ± 14.1 ng/g) and the control group (103.1 ± 49.8 ng/g) was at 12 h. These elevations represent a fold increase of 1.9, 1.6 and 2.0 from baseline for each group, respectively. The fold increases were not different between the treatment groups ($X^2 = 0.2$, $df = 2$, $p = 0.90$).

Across all times, mean detected fecal corticosterone metabolites at the pre-injection time point for the high dose group was 85.19 ± 13.05 ng/g, for the low dose group it was 122.14 ± 13.35 ng/g, and for the saline group was 203.46 ± 30.75 ng/g (Fig. 3). There was a significant difference between the treatment groups pre-treatment ($F = 8.49$, $df = 2$, $p = 0.02$). Fecal corticosterone metabolites peaked for the high dose group (220.49 ± 75.00 ng/g) and the saline group (307.65 ± 125.43 ng/g) at 10 h. The low dose group peaked at 12 h (229.04 ± 11.66 ng/g). These elevations represent a fold increase of 2.6 for high, 1.9 for low and 1.5 for saline. These fold increases were not different between treatment groups ($X^2 = 2$, $df = 2$, $p = 0.37$).

3.3. Chronic stress response experiment

We collected post-injection hair samples for 17 out of the 18 animals. Due to inter-individual variation in hair regrowth rate, injections spanned 6–28 days, with an average span of 16 ± 2 days. This span of injections was not significantly different between the treatment groups ($X^2 = 0.34$, $df = 1$, $p = 0.56$). Time between final injection and hair sample collection for both groups combined ranged from 12 to 49 days, with a mean of 33 ± 3 days. There was not a significant difference between groups for time between final injection and collection ($X^2 = 0.28$, $df = 1$, $p = 0.60$). The pre-injection mean for the ACTH treatment group was 2.35 ± 0.15 pg/ml, 1.48 ± 0.21 pg/ml for the saline control group, and 2.22 ± 0.20 pg/ml for the unmanipulated natural group. The post-injection mean for the ACTH treatment group was 3.05 ± 0.33 pg/ml, 3.12 ± 0.31 pg/ml for the saline control group, and 4.83 ± 0.27 pg/ml for the unmanipulated natural group. The distribution of these results for each group at the pre-injection and post-injection time periods are shown in Fig. 4. Neither saline nor ACTH treatment significantly affected the post-treatment hair cortisol ($p = 0.06$) (Supplemental Table S3). Hair cortisol content for the natural treatment was significantly increased from the pre-treatment to post-treatment time period ($p = 0.02$), and lactation significantly increased hair cortisol concentration across all groups ($p < 0.01$). Age, mass, and location on the colony did not significantly affect hair cortisol ($p > 0.05$). The individual variance was $\sigma^2 = 0.33$ and the

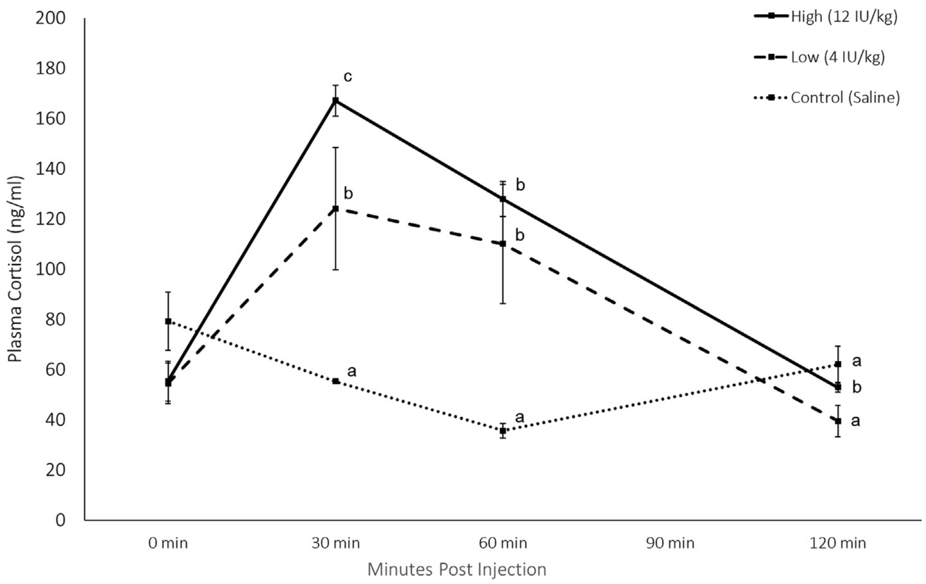

Fig. 1. Plasma cortisol concentration (ng/ml) of black-tailed prairie dogs following injection of either a high dose (12 IU/kg) of ACTH, a low dose (4 IU/kg) ACTH or saline. Letters (a, b, c) denote significant differences between groups ($p < 0.05$) at each time point, based on a linear mixed effects model.

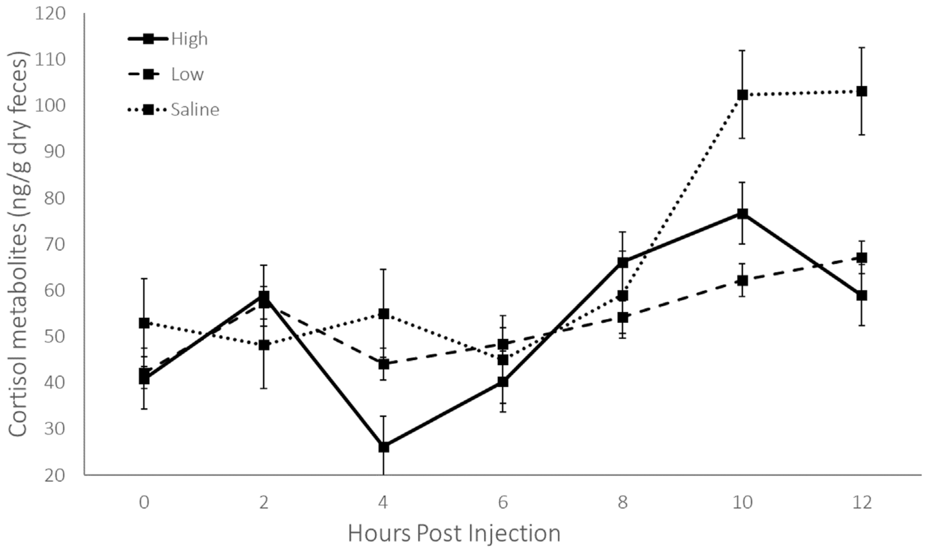

Fig. 2. Mean (± SE) fecal cortisol metabolite concentrations of black-tailed prairie dogs following injection of either a high dose (12 IU/kg) ACTH, a low dose (4 IU/kg) ACTH, or saline.

residual variance was $\sigma^2 = 1.41$.

4. Discussion

4.1. Primary circulating glucocorticoid

Black-tailed prairie dogs produce both cortisol and corticosterone in response to the stress of capture, handling and blood collection. Cortisol is predominant in the plasma, while corticosterone and its metabolites are present in higher levels in feces and hair. These findings align with other studies that have found cortisol to be the primary GC hormone in the plasma of most other sciurid species (Mateo and Cavigelli, 2005). This study is the first to examine the relationship between cortisol and corticosterone in multiple matrices for a single sciurid species.

4.2. Acute stress response experiment

These experiments are the first investigation of black-tailed prairie dog's physiological response to a simulated acute and chronic stressor. Specifically, both low and high doses of ACTH stimulated elevated plasma cortisol concentrations in the expected pattern, peaking at 30 min post-injection and returning to approximately pre-injection levels by 120 min post-injection. Comparison of the responses in the low (4 IU/kg) and high ACTH (12 IU/kg) dose treatment groups reveal that, while the lower dose is sufficient to stimulate an HPA response in black-tailed prairie dogs, the relationship is dose-dependent. As a result, individuals in the low dose treatment exhibited a lower peak and higher variation between individuals, relative to those in the high dose treatment group. As a result, we opted to use the higher dose for the chronic stressor simulation.

The results from the feces did not produce the expected outcome. While the high dose ACTH treatment group showed a modest peak at 10 h, neither the control nor the low dose treatment groups showed an increase and subsequent decline within the 12-h collection period. Unexpectedly, the control group showed the highest elevation of all the groups, with a sustained elevation from 10 to 12 h post-injection. There

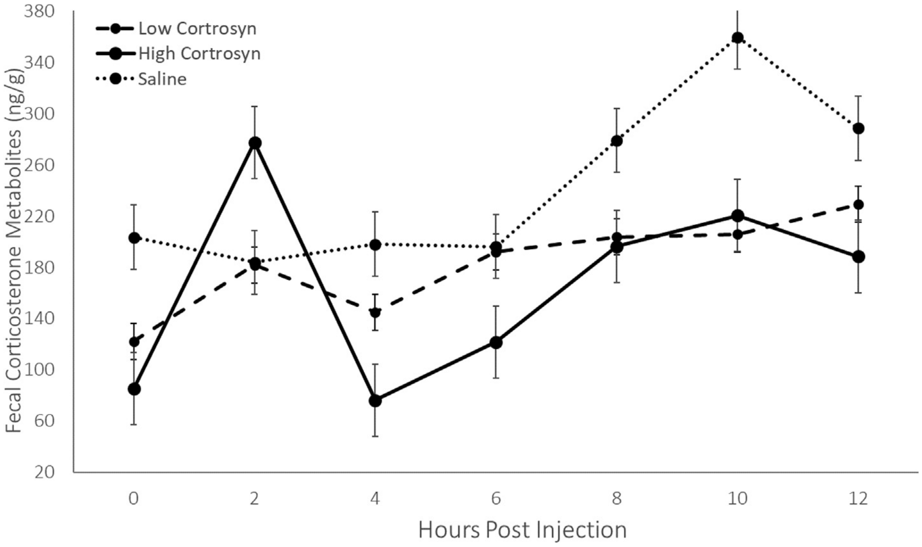

Fig. 3. Mean (± SE) fecal corticosterone metabolite concentrations of black-tailed prairie dogs following injection of either a high dose (12 IU/kg) ACTH, a low dose (4 IU/kg) ACTH, or saline.

are two possible explanations for these results. First, while previous research suggests that GC metabolites should appear in the feces within 4–12 h in other sciurid species (Bosson et al., 2009; Dantzer et al., 2010; Sheriff et al., 2012), it is possible that this period is longer for black-tailed prairie dogs, which could have contributed to our unexpected results. We recommend future investigators seek out existing literature on gut transit time. However, this information may not be available, as was the case for this species, in which case we recommend conducting a pilot study to plan appropriate intervals and duration of fecal sample collection, as well as continuing collection of samples for some time beyond the expected window to preempt this uncertainty. Generally, one function that GCs play in a stress response is suppressing non-essential functions, including digestion (Boonstra et al., 1998). Injecting animals with ACTH elevated blood cortisol levels, and could have slowed or entirely suppressed digestion in these individuals, meaning if a peak in FGM was to be seen in feces, it may have occurred beyond the 10-h mark for this group, or even beyond the final collection at 12 h. This interpretation could explain why we still see a peak in FGM for the control group — despite not receiving any hormone, handling, blood collection and injection was a stressful event for all animals involved, as demonstrated in other experiments with sciurids (Bosson et al., 2013; Hare et al., 2014). This handling stress may account for the elevation in FGM, which occurred with the expected time lag since these animals did not receive exogenous ACTH that slowed their gut passage times.

Given that the results from plasma did not indicate an elevation in cortisol levels in the control group around the time of handling and injection, though, the results do not support this handling stressor explanation. Instead, the elevation seen in the control group may be a result of cortisol elevation associated with daily arousal, generally the highest levels in an animal's unmanipulated diurnal cycle (Coe and Levine, 1995; Mateo and Cavigelli, 2005). As plasma samples were only taken within a two-hour window, plasma cortisol levels elevated by diurnal rhythm may have occurred before the sampling window and resulted in elevated FGM levels approximately 12 h after arousal (i.e. 10-h post injection).

Other ACTH challenge studies in sciurids have found similar discrepancies. In a study conducted in both alpine (*Tamias alpinus*) and lodgepole chipmunks (*Tamias speciosus*), there was a significant difference in FGM levels between the treatment and control groups at peak excretion time for lodgepole chipmunks, but not for alpine chipmunks (Hammond et al., 2015). Additionally, an ACTH challenge study in captive barren ground caribou (*Rangifer tarandus granti*) and reindeer (*R. t. tarandus*) found a significant effect of ACTH treatment in feces for caribou, but not for reindeer (Ashley et al., 2011). The interspecific variation seen across studies reveals the complications and challenges inherent in our validation studies.

Although the injection of synthetic ACTH is considered the gold standard for assessing adrenal activity, the specifics of this procedure

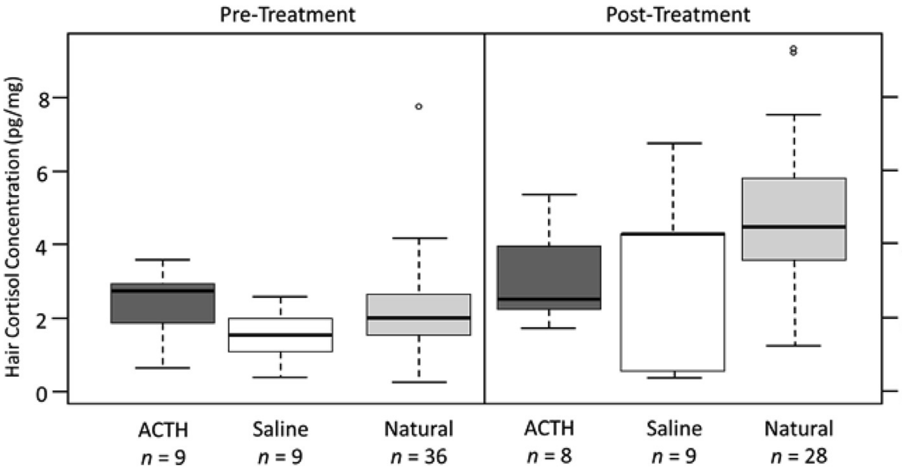

Fig. 4. Hair cortisol concentration of black-tailed prairie dogs prior to (left) and following (right) repeated injections with either 12 IU/kg ACTH (treatment), Saline (control), or Natural (no manipulation). Whiskers represent the range for each group, while the top and bottom edges of the box fall at the 25th and 75th percentiles. The bold line within each box indicates the median, and circles represent outliers.

Table 1

Validation experiments in sciurid rodents using injections of ACTH (adrenocorticotropic hormone) to stimulate glucocorticoid hormone production and subsequently measure glucocorticoid; metabolites excreted in feces.

Species	*n*	Dose	ACTH Prep	Route	Approx. Lag time	Approx. Fold increase	Citation
Belding's Ground Squirrel *(Urocitellus beldingi)*	5	4 IU/kg	Cortrosyn	SQ	18–30 h	1.8	Mateo and Cavigelli (2005)
Belding's Ground Squirrel *(Urocitellus beldingi)*	9	200ug/kg	Cortrosyn	SQ	18–30 h	1.8	Mateo and Cavigelli (2005)
Columbian Ground Squirrel *(Urocitellus columbianus)*	8	4 IU/kg	Synethen depot	IM	7 h	8.7 (m)[1]2.5 (f)	Bosson et al. (2009)
North American Red Squirrel *(Tamiasciurus hudsonicus)*	8	4 IU/kg	Synethen depot	n.s.[2]	8 h	1.2	Dantzer et al. (2010)
Richardson's Ground Squirrel *(Urocitellus richardsonii)*	8	200ug/kg	n.s.	SQ	n.s.	1.3 (m) 2.0 (f)	Hare et al. (2014)
Alpine Chipmunk *(Tamias alpinus)*	8	12 IU/kg	Cortrosyn	IM	24 h	1.9	Hammond et al. (2015)
Lodgepole Chipmunk (*Tamias speciosus*)	8	12 IU/kg	Cortrosyn	IM	24 h	2.9	Hammond et al. (2015)

[1] (m), (f) designate values that are specific to males, and females only, respectively. All other values are not differentiated by sex.

[2] Parameters that were not specified in publication are noted with n.s.

are not standardized. Among studies of sciurid rodents alone, ACTH preparations, dosages, and injection routes vary, as do post-injection sampling timelines. Table 1 highlights the diversity in protocols used in these studies.

Housing of study animals is another source of variation. While other validations conducted with non-threatened species were able to hold the animals for an acclimatization period of days to weeks (e.g. Mateo and Cavigelli, 2005; Hare et al., 2014) our use of animals from the threatened Canadian population restricted our flexibility. In order to limit our impact on the breeding females, our protocols differed from validations of related rodents in two ways. First, we chose to keep wild caught animals for the minimum possible amount of time required to perform the acute stressor experiment to reduce the potential impact of our experimental procedures and limit the risk of study animals losing their social position during their absence from the colony, which is a concern for this highly social species. Second, we returned them to their burrows immediately after the 12th h fecal sample collection, to avoid artificially exposing them to nocturnal predators. These protocols proved to be a major constraint to our validation. We recommend that researchers conducting future validations carefully consider the sociality of a species when planning experiments. Just as removing prairie dogs from their home territory may have affected their social status and influenced their stress response, housing asocial species in close proximity for an experiment could also impact the stress response. Such challenges support the reasoning behind validation experiments typically being conducted in captive populations, where alterations such as confinement, housing, and proximity to conspecifics are often not necessary when individuals are already confined in a zoo or laboratory setting. The use of captive animals maximizes a researchers' ability to detect an effect of ACTH treatment by minimizing confounding effects, but sacrifices ecological realism.

Thus, our efforts to minimize disruption while using temporarily captive animals could explain the modest difference in fold increase between the control and treatment groups. If all 9 of the treatment animals in our study were operating at an elevated baseline due to the distress of trapping, transportation and housing in a novel environment, a peak in cortisol due to the injection of ACTH, while evident in the blood, may have averaged out over the hours required for metabolism and excretion, and thus been masked in the feces by the additional stress of the novel environment. Conducting follow up work with a captive population or acclimatization period would reduce the confounding effect of sudden captivity on each individual's stress response.

In addition to the potentially stressful effects of capture, transportation and captivity, a further explanation for our failure to detect a response in the feces of the treatment group animals could be the choice of assay used in our analysis. If the assay was poorly suited to detect the species specific combination of metabolites produced by black-tailed prairie dogs, this would explain why the plasma samples showed a GC peak at the expected time (using an assay designed specifically to detect native hormone in plasma) while the fecal samples did not produce a comparable result (using a more general assay to detect hormone metabolites). The products of hormone metabolism and excretion vary by species, and thus a species-specific assay validation is required for each new species (Touma and Palme, 2005; Palme, 2019). While the FGM assay used in this study has been successfully applied in a host of other mammalian species, including white-tailed prairie dogs (*Cynomys leucurus*) (e.g. Young et al., 2001, 2004; Santymire and Armstrong, 2010; Loeding et al., 2011; Santymire et al., 2012; Schell et al., 2013), this does not preclude the need for assay selection to determine which antibodies produce the clearest detection of variation in FGM levels. At present no such analysis has ever been conducted for black-tailed prairie dogs. As long as this analysis is lacking, our ability to interpret the cause of failure in this validation is limited. An excellent example of an assay selection procedure is given in Bashaw et al. (2016), in which the authors tested both group-specific and hormone-specific antibodies following an ATCH challenge to select one assay best suited to detecting changes in FGM levels over time. We suggest future researchers attempting to validate the use of FGM for any new species consider that study as a guide.

4.3. Chronic stress response experiment

The acute stress response experiment demonstrated that a single injection of ACTH at a dose as low as 4 IU/kg was sufficient to significantly elevate plasma cortisol concentration. However, in the chronic stress response experiment, multiple injections of a high dose of ACTH (12 IU/kg) did not translate to an increase in hair cortisol concentration from the pre-treatment to post-treatment period in hair that was initially shaved (pre-treatment) and then allowed to regrow (post-treatment). Instead, our results showed that the effect of lactation on hair cortisol was stronger than any treatment effect achieved by the dose of ACTH. The treatment may have increased hair cortisol levels, but any effect of treatment was masked by the stronger influence of lactation. The strong influence of lactation on hair cortisol is not surprising, given that lactation is one of the most energetically expensive physical processes a mammal can undertake (Wade and Schneider, 1992). Nonetheless, these results are noteworthy, as the effects of reproductive condition on hair cortisol are rarely discussed in published literature, despite its significant impact. Conducting this experiment in male prairie dogs, or during the non-reproductive season with females would eliminate the natural influence of lactation on hair cortisol concentration, and could provide a clearer effect of ACTH treatment.

While we were able to monitor lactation at the individual level, and therefore measure the influence of this variable on our experiment, it is also possible that deviation from the expected result was caused by influences we were unable to measure, such as disease, social rank, food availability or density. Demographic composition of the colony, which is closely tied to density, is another effect that varies between time periods. The pre-treatment hair samples were grown in late summer of the previous year, at a time when juveniles are fully independent from their mothers, though they remain in their natal coteries. First year survival of emergent juveniles is approximately 50% (Hoogland, 1995), meaning that many juveniles may have already died by the end of their first summer. Hair in the post-treatment time period was grown while females were lactating, shortly after juveniles first emerge from their natal burrows, sharply increasing the number of animals in the colony. This change could influence the stress response in adult prairie dogs in multiple ways. First, through a simple increase in density, but also through more subtle and complex interactions, such as the presence of juveniles attracting additional predators and increased demands on mothers to protect their offspring from infanticide, which is common in this species (Hoogland, 1995).

Plasma cortisol measured by enzyme immunoassay represents a measure of total cortisol, both free and bound. Hair cortisol, regardless of measurement technique, represents only free cortisol, since bound cortisol cannot diffuse into tissues (Malisch and Breuner, 2010). It is possible that the repeated injections of ACTH elevated plasma cortisol in the treatment group animals, but these animals responded by increasing either CBG levels or CBG binding affinity, to reduce the burden of increased free cortisol load. The upregulation of CBG in response to psychologically or physiologically stressful events is demonstrated in studies of other mammals (e.g. Davenport et al., 2008; Chow et al., 2011). We strongly recommend future studies measure plasma CBG, or compare ratios of free:bound cortisol in plasma, before drawing conclusions based on hair cortisol content alone.

5. Conclusion

The results of these experiments identified cortisol as the dominant stress hormone in this species, showed that the HPA axis operated as expected following ACTH injection, and while natural variation in hair suggest its use as a matrix for non-invasively monitoring GC levels, also demonstrated the complexities inherent in validating feces and hair as general tools. The acute stressor experiment suggests that FGM recovered from prairie dog feces may reflect stressful experiences, such as handling and blood collection procedures. However, more investigation of natural and manipulated gut passage times is required before this matrix should be used to definitively link FGM levels to previous experiences with acute stressors. Additionally, further validation would be required to identify the parent hormone responsible for producing each of the FGMs detected. Similarly, the chronic stressor experiment demonstrates that hair cortisol can be measured in prairie dogs and is sensitive enough to show differences between stages of the reproductive season in females.

The natural environment presents a host of variable factors known to influence cortisol levels, such as social status, predation risk, food availability and extreme weather events (Reeder and Kramer, 2005). Some of these factors can be easily quantified and controlled for in analyses, while others cannot. In addition to the known examples listed here, there may be several other influences on the cortisol levels of free-ranging animals which have not yet been studied.

These challenges are typically avoided by conducting validation studies in a controlled laboratory environment. While the benefits of this strategy are clear, researchers in this setting are limited when it comes to applying their findings in free-living wildlife, as laboratory studies often fail to account for key sources of variation in cortisol levels, namely reproduction, which we demonstrate having an even stronger influence on hair cortisol than ACTH treatment.

The significant effect of lactation on hair cortisol supports the argument that cortisol recovered from hair is reflective of endogenous physiological processes, rather than being a product of local cortisol production by the skin or hair follicle, as hypothesized by some other authors who did not detect the expected pattern (e.g. Keckeis et al., 2012). The effects of lactation on hair cortisol concentration further demonstrate that while this matrix is reflective of endogenous physiological processes, we could not replicate these changes with artificial stimulation during the breeding season. Our results, and the work of others, demonstrate that while feces and hair have the potential to act as non-invasive measures of responses to both acute and chronic stressors, the procedures used to validate these measures may not be robust enough to accommodate reproductive individuals, or wild-caught animals unaccustomed to captivity, and may have limited utility outside the controlled environment of a laboratory.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.02.003.

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