ECOLOGICAL CORRELATES OF STRESS IN THE CANADIAN POPULATION OF BLACK-TAILED PRAIRIE DOGS (*Cynomys ludovicianus*)

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ABSTRACT

Black-tailed prairie dogs (*Cynomys ludovicianus*) are a foundational species in the mixed-grass prairie ecosystem whose numbers have been declining in Canada over the past two decades. In predator-prey systems, risk of predation has been shown to increase hormones associated with the physiological stress response, and drive population declines by limiting reproductive success. I investigated whether the perceived risk of predation could be a factor contributing to the local decline of this species.

Cortisol and corticosterone play a key role in the vertebrate stress response. Therefore, I conducted an experiment to manipulate and track the deposition of these hormones in blood, feces and hair in response to a simulated acute and chronic stressor. I determined that cortisol is the primary hormone in the stress response of this species. My experiment elevated cortisol levels in feces and hair, but a variety of influences precluded clear conclusions about the treatment effect of the experiment.

I also investigated how the risk of predation in wild black-tailed prairie dogs is related to cortisol levels and reproductive success. I found that prairie dogs who live on the edge of the colony perceive a higher risk of predation, but this risk does not translate to significant differences in cortisol or reproductive success. Instead, cortisol varies between individuals, but is present at levels that facilitate an adaptive response to environmental challenges, and appears to be repeatable within individuals over time. These results suggest that the risk of predation, whether actual or perceived, should not be considered a limiting factor in the persistence of the Canadian black-tailed prairie dog population.

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DEDICATION

I dedicate this thesis to Jillian Kusch, my prairie dog partner in crime. I would have been lost without your years of support, in academics and beyond.

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CHAPTER 1

Introduction

Global biodiversity is steadily declining. While this is cause for serious general concern, the magnitude of loss varies greatly between regions. The grassland ecosystem, in particular, is at serious risk (Sierra-Corona et al., 2015). Conversion of grasslands to agricultural land and other human uses has resulted in this ecosystem becoming one of the most threatened on the planet, with over 50% of the land degraded or destroyed in North America alone (Hoekstra et al., 2005). The grassland ecosystem is critically endangered based on the dual risks of being heavily converted and only loosely protected, with an 8:1 rate of conversion to conservation (Hoekstra et al., 2005).

Critical to preserving this quickly vanishing ecosystem is maintaining biodiversity within the remaining patches. In Canada, the prairie ecoregion comprises only 5% of the country's total land area, but is home to 10% of its species at risk (Shorthouse, 2010; Environment Canada, 2014; Environment and Climate Change Canada, 2017). Prairie dogs (*Cynomys spp.*) play a vital role in grasslands across North America. They act as ecosystem engineers, altering the ecosystem by their burrowing activities (Van Nimwegen et al., 2008). The physical disturbance and burrow systems created by prairie dogs increase soil aeration, driving changes in vegetation patterns and increasing diversity of small mammal communities on colonies (Van Nimwegen et al., 2008).

There are five species of prairie dogs in North America and, of these, the black-tailed prairie dog (*Cynomys ludovicianus*) is distributed across the widest range (Hoogland, 1995). This range spans over 2,100 km, from southern Saskatchewan in the north to the northern portion of Chihuahua, Mexico in the south (Figure 1.1). Like the grassland ecosystem itself, this species has

suffered a dramatic decline across its range, declining by an estimated 98% in the last two hundred years (Proctor et al., 2006). Conversion of grassland habitat to agricultural and urban land was the main driver of prairie dog declines historically. Today, habitat loss is still the major threat to prairie dogs, though recreational shooting, plague, and poisoning campaigns are also

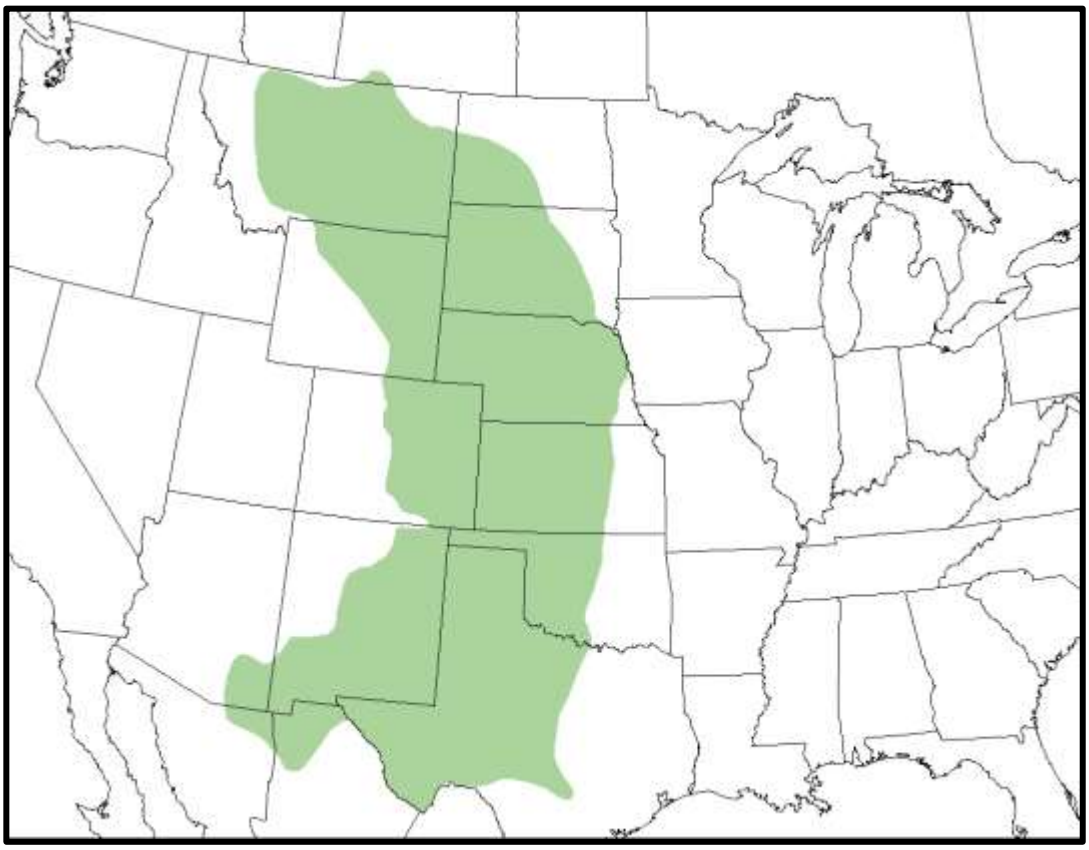

Figure 1.1 Shaded green area represents the black-tailed prairie dog species' range in North America. Geographic data from The IUCN Red List of Threatened Species, version 2017-3, and naturalearthdata.com.

documented drivers of decline.

The Canadian population of black-tailed prairie dogs is limited to 19 colonies over 12 km^2 in southern Saskatchewan, with 17 of the colonies falling within the boundaries of Grasslands National Park (Stephens et al., 2017; COSEWIC, 2011). These colonies are geographically isolated from others, being separated by 20 km from the closest neighbouring colony in Montana, USA (Gummer, 1999). Compounding concerns about this population's

isolation and limited distribution, are that its numbers have been declining in recent decades. Visual counts suggest that the population declined by 22-33% between 2001 and 2011, prompting the Committee on the Status of Endangered Wildlife in Canada to recommend that the species be considered threatened in 2011 (COSEWIC, 2011). Recently, visual count and mark-recapture data (Parks Canada, unpublished data) suggests that numbers have rebounded, but the mechanisms behind these fluctuations in the population remain unknown.

This Canadian population shares some, but not all, of the same risks as populations of prairie dogs continent-wide. The majority of the colonies lie within the boundaries of Grasslands National Park, and are therefore legally protected from recreational shooting, though some illegal shooting does still occur (personal observation). Additionally, while plague poses a substantial threat to black-tailed prairie dogs in more southern latitudes, confirmed animal cases of plague in Canada are rare (Antonation et al., 2014). Though reports of infections are uncommon, plague bacteria (*Yersinia pestis*) is known to be present in the landscape, based on antibody surveys in domestic cats and dogs (Leighton et al., 2001), and PCR of fleas collected from prairie dog burrows (Parks Canada, unpublished data). Outbreaks of plague in the United States result in nearly 100% mortality in this species (Cully and Williams, 2001). While the prevalence of this disease is low, the extremely high consequences prompted COSEWIC to cite plague as one of the two very high impact threats to the current population (COSEWIC, 2011).

The second very high impact threat is the risk of drought (COSEWIC, 2011). Past droughts have decreased Grasslands National Park prairie dog densities dramatically, and was recently shown to be a major factor influencing recent population fluctuations in the Canadian population (Stephens et al., 2017). Further, populations that lie at the edge of a species' range are more likely to occupy marginal habitat, compared to central populations, and thus may be at

greater risk of collapse (Brown, 1984). The compounding influences of more extreme climatic conditions at the range periphery (Gummer, 1999), and the increased sensitivity of poleward locations to climatic changes (Roots, 1989) represent a serious threat to this population.

Predators pose another risk to individuals in this population, and at a broader scale, can be considered a risk to the entire population (COSEWIC, 2011). While, under natural conditions, predators and prey are able to coexist, when these systems become unbalanced the high risk of predation can directly or indirectly reduce the likelihood of persistence of prey. Direct mortality by predation poses obvious risk to prey. The sub-lethal effects of predation, however, pose more subtle risks to prey. The sub-lethal effects of predation on vertebrates act through physiological processes, namely the stress response (Sheriff et al., 2012).

A stress response is the physiological response to a stimulus that an organism perceives as a threat to its survival, henceforth, a stressor. An encounter with a predator is one example of a stressor, though other stimuli, such as harsh weather, loss of a bonded mate, or unpredictable changes in a social system, can also trigger the stress response (Reeder and Kramer, 2005). When a bird or mammal perceives a stressor, its body responds by simultaneously activating the stress response via the hypothalamic-pituitary-adrenal (HPA) axis and via the sympathetic nervous system (SNS). The SNS pathway releases epinephrine from the adrenal medulla and norepinephrine from the spinal nerves, which function to elevate heart rate and mobilize energy by promoting the breakdown of fat and release of glucose from stored glycogen (Reeder and Kramer, 2005). This response is short-lived, and is down regulated by activation of the parasympathetic nervous system within seconds (Reeder and Kramer, 2005). As the SNS is activated, the paraventricular nucleus of the hypothalamus responds by releasing several substances, including corticotrophin-releasing hormone, which travels to the pituitary gland and

stimulates release of adrenocorticotropic hormone (ACTH) into the bloodstream (Reeder and Kramer, 2005). The ACTH activates the adrenal gland, which secretes glucocorticoid (GC) hormones into the bloodstream that facilitate physical changes that enable an individual to respond to the stressor (Reeder and Kramer, 2005). The GC hormones produced by the vertebrate stress response are cortisol and corticosterone. While some vertebrates produce both hormones, one is usually present in higher quantities, which varies by taxa (Touma and Palme, 2005).

It is crucial to understand that GCs are involved in a variety of physiological processes in addition to the stress response, and their function should be interpreted with context and concentration in mind. At low and moderate concentrations, GCs facilitate normal regulation of energy (Busch and Hayward, 2009). At these low and moderate concentrations, GCs regulate circulating levels of glucose and free fatty acids, and play a role in waking and stimulating feeding behaviour (Busch and Hayward, 2009; Reeder and Kramer, 2005). It is only when GCs are present at high concentrations for extended time periods that they predict maladaptive consequences.

When stressors are persistent, and result in high concentrations of circulating GCs for a period of days or weeks, an organism experiences chronic stress, which may be detrimental to health and fitness (Busch and Hayward, 2009). The manifestation and consequences of chronic stress are not uniform across individuals or species, but vary with context (Bonier et al., 2009; Dickens and Romero, 2013). Elevated baseline GC levels, more frequent acute activation of the HPA axis in response to environmental challenges, and increased time required to return to baseline levels are traditionally considered to be the hallmark symptoms of chronic stress (Dantzer et al., 2014).

The tipping point at which low to moderate levels of GCs translate to an emergency response, which could help an individual respond to an acute stressor, or could persist as chronic stress, varies based on a variety of factors. In mammals, baseline and stress-induced GCs vary seasonally (Kenagy and Place, 2000; Romero, 2002), as well as by age (Boonstra et al., 2001), sex (Bosson et al., 2009), reproductive status (Dantzer et al., 2010), social status (Young et al., 2006) and developmental experiences (Mateo, 2006). In addition to the endogenous levels of GCs, an individual's perception of a stimulus being a stressor is also context-dependent. A classic example in two laboratory rats showed that the individual who was able to control the electric shocks that both rats received with a lever had significantly lower GC levels than the rat who had no control over the shocks (Weiss, 1968). Thus, an animal's perception of a noxious stimulus as being predictable or unpredictable influences how they respond to the challenge. Given this variety of circumstantial influences, measures of GCs in free-living species may be best suited to study systems where a considerable amount of individual information is known (Bush and Hayward, 2009).

In a conservation context, GCs have been gaining popularity in recent decades as an indicator of population health (Busch and Hayward, 2009; Macbeth et al., 2010), especially in the context of how wild populations respond to human disturbance (e.g. Pauli and Buskirk, 2007; Gobush et al., 2008; Mastromonaco et al., 2013). Of these, some document a direct relationship between elevated GCs and fitness (e.g. Boonstra et al., 1998; Pride, 2005; Blas et al., 2007). One of the most well-known studies of the effect of elevated GCs on fitness found that snowshoe hares (*Lepus americanus*) suffered from elevated GCs in response to high risk of predation during the apex of their population cycle (Boonstra et al., 1998). These elevated GCs suppressed reproduction of individuals and contributed to the population bust (Boonstra et al., 1998). This

study is a premier example of the complexity of interacting ecological processes, and how measures of physiological responses at the individual level can predict impending changes at the population level.

For Canadian black-tailed prairie dogs, in particular, the risk of predation may be especially relevant, since this population is largely protected from some unpredictable influences that threaten other populations, such as poisoning or recreational shooting. In addition to the traditional predators that have been present on the landscape for many decades, black-footed ferrets (*Mustela nigripes*) were recently reintroduced to Grasslands National Park, after being absent from the landscape for over 70 years (COSEWIC, 2011). Black-footed ferrets rely on prairie dogs for the majority of their diet, and thus represent a potential stressor whose reintroduction could have additive impacts with other predators, though the influences of predation have not yet been investigated in this population.

In this thesis, I investigated how the risk of predation may affect population growth in Canadian black-tailed prairie dogs. In Chapter 2, I identified the primary GC hormone produced in the black-tailed prairie dog stress response. I conducted a validation experiment with the goal of confirming that GC hormones measured from the plasma, feces and hair of this species are reflective of activation of the HPA axis, as opposed to being a product of other biological processes. In Chapter 3, I tested the hypothesis that variation in perceived risk of predation correlates with GC levels, which are, in turn, associated with variation in reproductive success in this species. My goal in this work was to inform conservation of this at-risk population by examining the physiological impact of predation risk, using individual measures to identify trends that may be driving change at the population level.

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CHAPTER 2

Investigation of the utility of feces and hair as non-invasive measures of glucocorticoids in black-tailed prairie dogs (*Cynomys ludovicianus*)[1]

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. I report the results of a validation on black-tailed prairie dogs (*Cynomys ludovicianus*), an important engineer of grasslands ecosystems that has experienced recent population declines. I conducted an adrenocorticotropic hormone (ACTH) stimulation test in adult female prairie dogs to assess 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. I also gave repeated injections to adult females to assess whether repeated injections of ACTH would result in a chronic increase of hair cortisol concentrations, compared with control individuals injected with saline. A single injection of ACTH at either low or high dose peaked plasma cortisol levels after 30 min, and the cortisol levels declined until 120 min, when 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

[1] This chapter has been submitted to General & Comparative Endocrinology for publication. This chapter is a co-authored manuscript. CC reviewed literature, collaboratively designed the experiment, coordinated logistics, conducted the experiment and collected samples, prepared and analyzed the blood and hair samples, prepared fecal samples for laboratory analysis, and wrote the manuscript.

Crill, C., Janz, D. M., Kusch, J. M., Santymire, R. M., Heyer, G. P., Shury, T. K., Lane, J. E. (In Prep). Investigation of the utility of feces and hair as non-invasive measures of glucocorticoids in black-tailed prairie dogs (*Cynomys ludovicianus*).

treatment groups were not significantly different from those in the control group over the following 12 hours. 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 measured in unmanipulated individuals. These results highlight that while measurement of GCs and their metabolites in feces and hair are potentially valuable conservation tools, these indicators should be interpreted with caution unless additional information about the individual animal is available.

1. Introduction

When vertebrates are confronted by a stressor, their bodies mobilize a stress response via the hypothalamic-pituitary-adrenal (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 can be either short-term and acute, such as an attack by a predator or inclement weather, or longer term and chronic, 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). Each method has its own benefits and drawbacks. Blood samples have been used for decades as the standard for measuring circulating hormone levels, as they provide 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). However, plasma concentrations of GCs can spike within 3 minutes of capture due to the stress of trapping and handling (Romero and Reed, 2005). Therefore, blood samples are often impractical for wildlife studies, and are more suited to laboratory studies, where animals are adjusted to confinement and routine handling. Additionally, since blood samples are representative of hormone levels from a single point in time, they are most suited for studies of an animal's response to an acute stressor.

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, and thus eliminate concern about the collection procedure artificially elevating the results (Mateo and Cavigelli, 2005; Touma and Palme, 2005). Additionally, fecal glucocorticoid metabolites (FGM) represent cumulative hormone levels over the time feces are formed, rather than single moment in time (Touma and Palme, 2005). Thus, FGM represent an attractive, non-invasive option for studying stress in wild populations, especially those that are difficult to access or are endangered (Millspaugh and Washburn, 2004). 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 hours, 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 moulting and hair growth patterns for each species.

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). Typical validation experiments involve stimulation of the HPA axis by evoking an acute stress response through handling the animal, or by administering adrenocorticotropic hormone (ACTH), followed by collection of

samples of the target matrix at several time points post-administration. ACTH administration is the most widely accepted form of physiological validation (Touma and Palme, 2005). While such validations for the use of feces as a tool to measure GCs are fairly common, including multiple examples in sciurid rodents (Boonstra et al., 2001; Bosson et al., 2009; Bosson et al., 2013; Dantzer et al., 2010, Hare et al., 2014; Mateo and Cavigelli, 2005), validations of hair GCs are rare. To date, there are only five published examples of ACTH administration to validate hair as a tool for GC measurement (summarized in Table 2.1). 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 currently considered to be threatened in Canada (COSEWIC, 2011).

Black-tailed prairie dogs are a semi-fossorial, colonial sciurid. They play a vital role in grassland ecosystems by acting as ecosystem engineers, dramatically altering the landscape and providing both shelter and prey for a number of other species (Van Nimwegen et al., 2008). However, due to habitat loss and persecution by humans, prairie dog populations have declined by 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 (Boonstra et al., 1998), though it has not yet been investigated in this population. Black-footed ferrets (*Mustela nigripes*), a specialist predator of prairie dogs, were reintroduced to the area in 2009, re-establishing a potentially severe 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). To date, our ability to test for an effect of these chronic stressors in the population's decline has been hindered because there has never been a validation of feces or hair as non-invasive measures of GCs in this species.

Table 2.1 Published animal studies that used adrenocorticotropic hormone (ACTH) injections to validate that stimulation of the hypothalamic-pituitary-adrenal (HPA) axis increases hair cortisol concentration.

Species	Setting	*n* Treatment	*n* Saline Control	Validated	Reference
Barren ground caribou *(Rangifer tarandus granti)*	Captive	5	1	N	Ashley et al., 2011
Reindeer *(Rangifer tarandus tarandus)*	Domestic	5	1	N	Ashley et al., 2011
Dairy cattle *(Bos taurus)*	Domestic	5	5	Y	González-de-la-Vara et al., 2011
Canadian lynx *(Lynx canadensis)*	Captive	3	0	Y	Terwissen et al., 2013
Eastern chipmunk *(Tamias striatus)*	Wild	5	7	Y	Mastromonaco et al., 2014
Laboratory rat *(Rattus norvegicus)*	Domestic	16	8	Y	Scorrano et al., 2015

The main objectives of this study were to determine the dominant GC hormone in the stress response in black-tailed prairie dogs, and to investigate natural and experimentally stimulated patterns of GC hormone deposition in feces and hair. As this is the first investigation of GC hormones in this species, I first determined whether cortisol or corticosterone was dominant in the stress response for this species, by measuring levels of both in blood collected from free-ranging, unmanipulated individuals. I also examined the levels of cortisol and corticosterone in fecal and hair samples. Then, I simulated both acute and chronic stress stimuli using injections of synthetic ACTH to stimulate an adrenal response in wild-caught individuals. I

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, I 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 demonstrate a 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 (GRA-2014-16101). On March 5-6, 2017, I live-trapped five individuals from a single 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 nonbreeding, and were used to determine the primary circulating glucocorticoid hormone. On March 13-14, 2017, I 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 sex and age-class differences, I restricted the study to adult females. The animals in the colony are part of an ongoing long-term study, where the animal's minimum ages are known, allowing me 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, KY, USA) in both the right and left pinnae for permanent identification.

2.2 Primary Circulating Glucocorticoid

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

I extracted hormones from these plasma samples following the procedures described in section 2.5, 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, USA). 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% (n = 6) and the inter-assay coefficient of variation was 8.1% (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, I investigated the patterns of deposition into feces and hair. I analyzed feces from the acute stress response experiment for both hormones, following the procedures outlined in section 2.6. For hair samples, I randomly selected hair samples collected from five prairie dogs in the long-term study colony and analyzed the cortisol and corticosterone contained in these samples. I prepared the samples according to the procedures detailed in section 2.7, using the Oxford Biomedical Cortisol kit previously described to quantify cortisol, and the Oxford Biomedical Corticosterone kit to quantify corticosterone. The intra-sample coefficient of variation was <15% for all replicates.

2.3 Acute Stress Response Experiment

I randomly assigned the captured individuals to a low-dose treatment (n =5), high-dose treatment (n =4), or control group (n =3). I 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 x 3 cm patch from each animal's right hind leg with a pet hair trimmer (Model #8552, Whal Clipper Co, Sterling, IL, USA). I stored these samples in paper envelopes at room temperature. Following the hair sample collection, I transported the animals, secured in Tomahawk traps, to a nearby field station. I transferred the prairie dogs to polycarbonate rodent cages with wood shaving bedding, provided them with water-rich foods (apples, lettuce) and commercial rodent chow, covered each with a cotton sheet to minimize distress to the animals, and housed them overnight prior to commencing the experiment.

On the following morning, with the help of the long-term project team, I 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. Immediately after collecting the baseline blood sample, I injected each animal intramuscularly with 4 IU/kg of ACTH (Cortrosyn, Amstar Pharmaceuticals, Rancho Cucamonga, California, USA) 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 06:17-07:15 MDT). I collected any feces produced during blood draws and injections as representations of pre-treatment stress levels.

I 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. I centrifuged and stored these blood samples following the same procedures outlined in section 2.2. After each subsequent blood draw, I returned the animals to live traps, provided them with food, and covered the traps with cotton sheets. I 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. I lined the area under the traps with paper towels to absorb urine, and only collected fecal samples that were not visibly contaminated by urine. I collected fecal samples for each animal at each collection period in a whirl pack bag and stored samples at -20°C. I collected a final fecal sample 12 h after the initial injection and then returned animals to the colony, at the location from which they were originally trapped. I stored blood samples and extracted plasma following the procedures outlined in section 2.2.

2.4 Chronic Stress Response Experiment

I initiated the long-term hair glucocorticoid experiment four weeks after the acute stress response experiment, using free-living prairie dogs from the same study colony. The original 12

animals used in the acute stressor experiment remained in the same groups (treatment or control), as subjects in the chronic stress response experiment. Subjects included the original 12 animals, and six additional adult female prairie dogs. I 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 the timing of moult 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, I live-trapped each animal and gave 0-3 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, I gave all animals an additional 2 to 3 intramuscular injections of the same dose and substance. This resulted in 3 to 5 injections total per animal. I 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.

During data analysis, I 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 or saline). I collected samples from this natural population in both the pre-treatment period ($n = 36$) and the post-treatment period ($n = 28$). Due to deaths and disappearances from the colony, I was unable to recapture 8 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

To extract hormones from plasma samples collected for both the determination of the primary glucocorticoid hormone, and during the ACTH challenge, I thawed the samples on ice, vortexed, and then centrifuged them at 4000 rpm at 4 °C for 1 min. I then transferred each sample to a 12×125 mm glass culture tube and diluted each sample with EIA kit buffer to create a total volume of 0.5 ml. After vortexing each sample, I added 2.5 ml of diethyl ether, vortexed the contents again, then let the tubes sit for 3 min to separate the ether and aqueous phases. Once the two layers were visibly separated, I flash froze each tube in liquid nitrogen for 20 seconds, warmed it slightly by hand, and then froze it again for a further 10-15 seconds. Immediately after the second freeze, I decanted the upper, ether phase into a clean test tube. I then evaporated the ether phase from the tubes using a gentle stream of nitrogen gas (4-6 L/min) at 50 °C. I 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, I rinsed the insides of each tube with an additional 1 ml of ether, and evaporated this liquid as above. I reconstituted each sample in 0.25 ml of EIA kit buffer by allowing it to incubate overnight at 4°C. I sealed the tubes with parafilm, and kept them in the dark during incubation. The following morning, I gently vortexed the reconstituted hormone samples, centrifuged the samples at 4000 rpm for 1 min, and stored the samples at -20 °C until running the cortisol and corticosterone assays. I conducted assays using Oxford Biomedical EIA Kits described in section 2.2.

2.6 Extraction and Quantification of Fecal Glucocorticoid Metabolites

I lyophilized fecal samples for 65 h at -90 °C, and shipped them overnight on ice to the Lincoln Park Zoo Endocrinology Laboratory (Chicago, IL, USA) where I processed them following previously published methods (Brown et al., 1994, Santymire et al., 2012). I diluted the samples with dilution buffer to 1:30, vortexed the samples and then analyzed 50 µl aliquots of each sample 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. The cortisol and corticosterone antibody cross-reactivity has been previously reported (Loeding et al., 2011; Santymire and Armstrong, 2010). I 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 ($y = 0.93x + 1.51$; $R^2 > 0.99$). I confirmed the biochemical validity of the corticosterone EIA by demonstrating: (1) parallelism between binding inhibition curves of fecal extracts (1:4-1:1024) dilutions and the corticosterone standard ($R^2 = 0.99$); and (2) significant recovery (>90%) of corticosterone (3.9 - 1000 pg/well) added to fecal 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 cortisol extraction followed a previously validated protocol (Macbeth et al., 2010). Briefly, I removed surface contamination by washing

hairs with methanol (three 3 min washes). Following decontamination, I dried the hair, ground it to a fine powder using a ball mill, and weighed it. I 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, I 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. I 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%, I re-extracted the sample and repeated the assay. I 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 to 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, I 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. I conducted all other statistical analyses using R, version 3.2.3 (R Core Team, 2015), with package nlme (Pinheiro et al., 2017) for mixed modelling. I report all values as ± 1 standard error (SE) unless otherwise noted.

In my investigation of the primary circulating glucocorticoid and patterns of deposition of hormones into each matrix, I used the cor.test function to test for a significant correlation between cortisol and corticosterone. For plasma, the limited number of samples analyzed for

corticosterone (n = 5) precluded meaningful statistical analysis. For feces, I assessed the relationship between metabolites of cortisol and corticosterone using samples collected during the acute stress response experiment. In order to control for individual autocorrelation, I averaged the results across all time points for each subject, resulting in 12 data points from 12 individuals. For hair, I included all 9 data points available for both hormones from 9 individuals.

Blood sample data were normally distributed. I used a general linear mixed-effects model including dose, time, and the time required to collect each sample as fixed effects influencing plasma cortisol. I also included the interaction between dose and time, and the interaction between dose and collection time on plasma cortisol. I also had a time auto-correlation function in place to address the dependence of one blood sample on the previous sample. I chose a final model with both a random slope and random intercept, because it did a better job of accounting for the random effects by allowing the slope to vary within dose groups and 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.

I used the Shapiro-Wilk test to assess the FGM data for normality. I calculated the times increase between FGM levels at baseline and peak time points, and used the Kruskal-Wallis to test for differences between the dose groups, following previously published methods (Schell et al., 2013). For the chronic stress experiment, I 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. I used a general liner mixed-effects model with hair cortisol as the dependent variable, season, treatment, and lactation as fixed effects, and individual ID as a random effect to account for repeated measures on each individual.

3. Results

3.1 Primary Circulating Glucocorticoid

Cortisol was the dominant hormone in plasma, present in concentrations more than 21 times that of corticosterone. Cortisol concentrations ranged from 36.00 to 100.87 ng/ml, with an average of 69.15 ± 10.56 ng/ml. Corticosterone concentrations ranged from 3.03 to 3.19 ng/ml, with an average of 3.28 ± 0.11 ng/ml.

We detected both cortisol and corticosterone metabolites in fecal samples. Corticosterone metabolites were present in higher levels than cortisol metabolites ($\bar{x}$ = 197.21 ± 10.81 ng/g corticosterone; $\bar{x}$ = 58.3 ± 3.13 ng/g cortisol). Metabolites of the two hormones were significantly correlated in feces (r = 0.65, t = 2.68, df = 10, p = 0.02) (Figure 2.1).

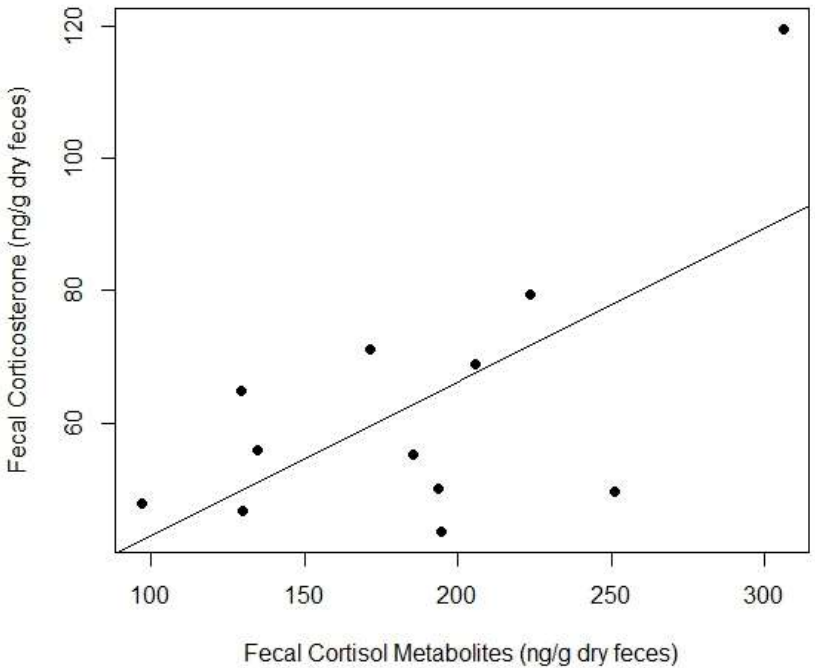

Figure 2.1 Scatter plot and regression line for fecal cortisol metabolites (ng/g) versus fecal corticosterone metabolites (ng/g) measured from wild-caught black-tailed prairie dogs manipulated in the acute stress response experiments. The equation for the regression line fecal cortisol metabolites = 19.48 + 0.23(fecal corticosterone metabolites).

In hair samples, I detected both cortisol and corticosterone. Corticosterone concentrations were higher in hair, ranging from 10.32 to 27.96 pg/mg, with an average of 19.12 ± 2.06 pg/mg. Cortisol concentrations ranged from 3.78 to 9.97 pg/mg, with an average of 6.87 ± 0.78 pg/mg. The two hormones were significantly correlated in hair ($r = 0.81$, $t = 3.66$, $df = 7$, $p < 0.01$) (Figure 2.2).

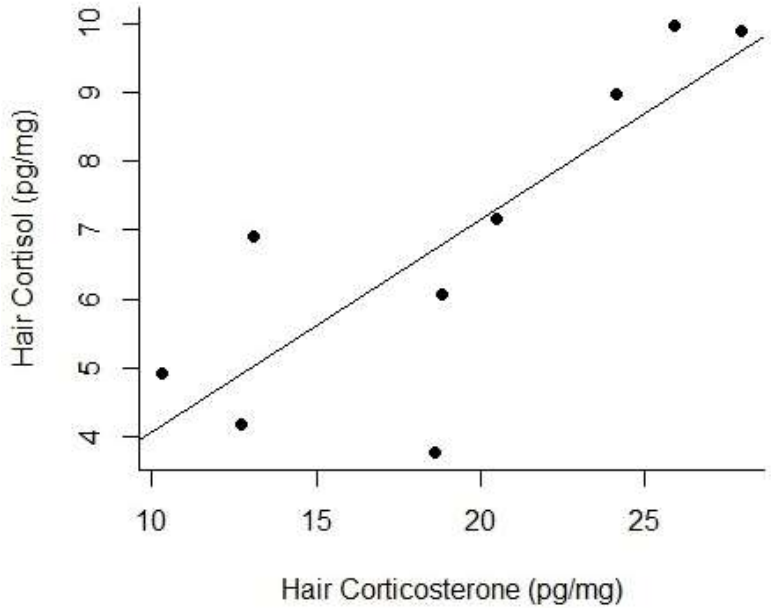

Figure 2.2 Scatter plot and regression line for hair cortisol (pg/mg) versus hair corticosterone (pg/mg) measured from wild-caught black-tailed prairie dogs not manipulated in the acute or chronic stressor experiments. The equation for the regression line hair cortisol = 0.98 + 0.31(hair corticosterone).

3.2 Acute Stress Response Experiment

Plasma cortisol levels were normally distributed, and ranged from 19.65 to 190.50 ng/ml, with an average of 82.92 ± 7.20 ng/ml across all groups and times. There was one outlier value in the low dose group at the 30 minute time point of 381.52 ng/ml, which was excluded from analysis, as it was greater than 2 SD from the mean. Prior to injection (0 min) there was no difference among the groups (Figure 2.3, Table 2.2). The saline group had similar cortisol levels across the 4 times points. For the two treatment groups, plasma cortisol levels were elevated at

30 min and 60 min post-injection (PI) compared to the baseline measure, while 120 min PI was not. At 30 min PI, plasma cortisol levels peaked for both the high and low dose groups, but the high dose group had a higher peak than the low dose group. At 60 min PI, levels for the high and low dose groups declined from 30 min, but were still higher than the baseline measure, but were similar to each other. At 120 min PI, the low dose treatment group was lower than both the high dose and the saline group, while the high dose and saline groups were similar.

Time required to collect blood samples varied by group, and therefore we included 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 at the 30 min time point (p = 0.03). The effect of collection time was not seen in the high dose group (p = 0.34) or the saline group (p = 0.28).

The variance between high dose individuals and saline individuals was low (σ^2_{high} = 2.41 x 10^{-3} and σ^2_{saline} = 0.18 x 10^{-3}), while variance for individuals in the low dose group was high (σ^2_{low} = 23.56). The residual variance was σ^2_{resid} = 18.10.

At the baseline time point, mean fecal cortisol metabolites detected in the feces of the high dose group was 40.8 ± 18.6 ng/g (Figure 2.4). 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 (Figure 2). There was not a significant difference between groups pre-treatment (F = 0.40, df = 2, p = 0.69). Fecal cortisol metabolites for the high dose group peaked at 10 h (76.7 ±11.8 ng/g). Peak 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 an increase of 1.9 times, 1.6 times and 2.0 times from baseline for each group, respectively. These increases were not different between the treatment groups (X^2 = 2.00, df = 2, p = 0.37).

At the baseline time point, mean fecal corticosterone metabolites detected in the feces were higher than the cortisol metabolites for each group: high dose (85.19 ± 13.05 ng/g), low dose (122.14 ± 13.35 ng/g) and saline (203.46 ± 30.75 ng/g). 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 at 10 h (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 an increase of 2.6 times for high, 1.9 times for low and 1.5 times for saline. These increases were not different between treatment groups ($X^2 = 2.00$, $df = 2$, $p = 0.37$).

Figure 2.3 Plasma cortisol concentration (ng/ml) of black-tailed prairie dogs following injection of either a high dose (12IU/kg) of ACTH ($n = 4$), a low dose (4IU/kg) ACTH ($n = 5$) or saline ($n = 3$). Letters (a, b, c) denote significant differences between groups ($p < 0.05$) at each time point, based on a linear mixed effects model. Error bars represent standard error.

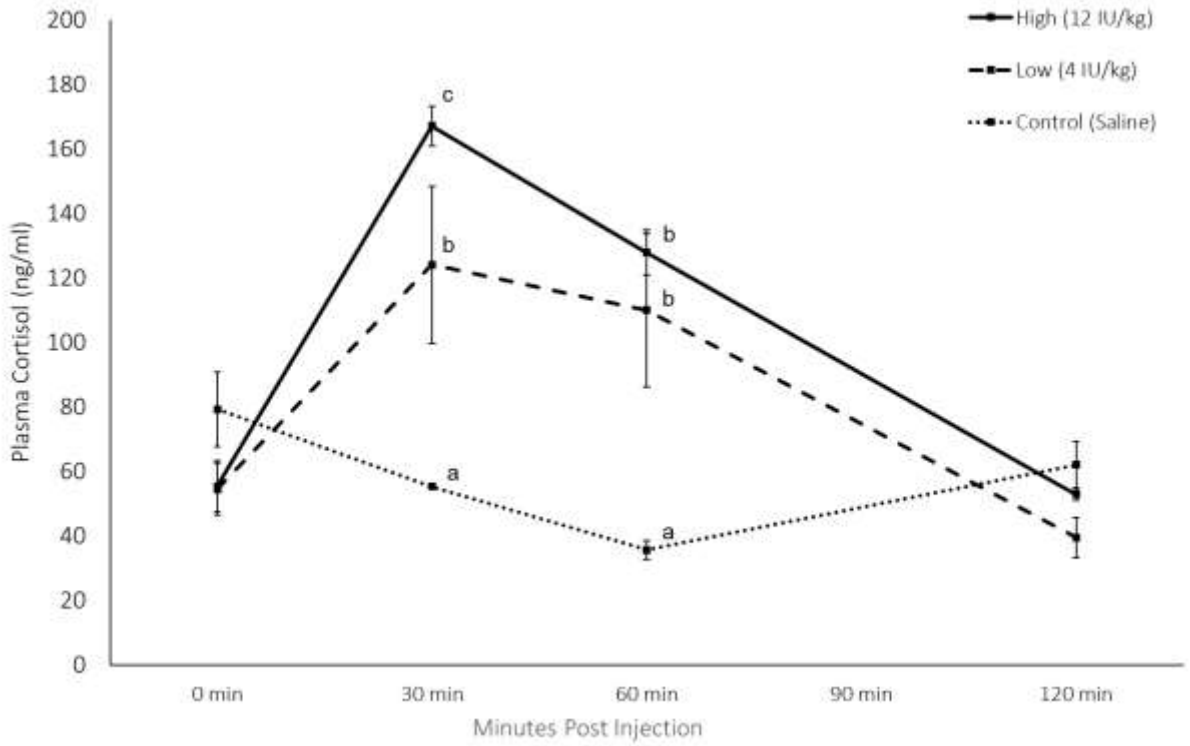

Table 2.2 Estimates, standard error, degrees of freedom, and significance for each fixed effect and interaction in the mixed effects model for plasma cortisol. The results of the full model are shown on the left, while the results of the final model are shown on the right. The full model was fit using a random intercept, while the final model was fit using both a random slope and random intercept. *p* values less than 0.05 are indicated in bold text.

	Full Model				Final Model			
Fixed Effects	Estimate ± SE	*df*	*t*	*P*	Estimate ± SE	*df*	*t*	*P*
(Intercept)	32.06 ± 34.60	20	0.92	0.37	34.80 ± 22.73	20	1.53	0.14
DoseLow	62.15 ± 38.38	9	1.62	0.14	65.26 ± 29.55	9	2.21	0.06
DoseSaline	38.67 ± 41.82	9	0.92	0.38	36.79 ± 27.04	9	1.36	0.21
TimeF30	124.76 ± 23.15	20	5.39	**0.00**	123.23 ± 18.47	20	6.67	**0.00**
TimeF60	82.72 ± 20.13	20	4.11	**<0.01**	81.51 ± 15.42	20	5.29	**0.00**
TimeF120	17.05 ± 30.75	20	0.55	0.59	14.74 ± 21.75	20	0.68	0.51
TimeCollect	3.72 ± 5.20	20	0.72	0.48	3.28 ± 3.34	20	0.98	0.34
DoseL:Time30	-65.52 ± 27.25	20	-2.40	**0.03**	-66.60 ± 23.60	20	-2.82	**0.01**
DoseS:Time30	-138.35 ± 32.25	20	-4.29	**<0.01**	-135.98 ± 26.24	20	-5.18	**0.00**
DoseL:Time60	-28.40 ± 24.94	20	-1.14	0.27	-28.68 ± 20.35	20	-1.41	0.17
DoseS:Time60	-112.49 ± 27.63	20	-4.07	**<0.01**	-111.77 ± 23.24	20	-4.81	**<0.01**
DoseL:Time120	-58.33 ± 34.30	20	-1.70	0.10	-61.44 ± 25.96	20	-2.37	0.03
DoseS:Time120	-23.780 ± 37.24	20	-0.64	0.53	-21.98 ± 27.29	20	-0.81	0.43
DoseL:TimeCollect	-8.94 ± 5.46	20	-1.64	0.12	-9.04 ± 3.77	20	-2.40	**0.03**
DoseS:TimeCollect	-4.58 ± 6.04	20	-0.76	0.46	-4.25 ± 3.81	20	-1.12	0.28
Random Effects	Variance				Variance			
Individual ID	12.86				<0.01			
DoseLow	not fit				23.56			
DoseSaline	not fit				<0.01			
Residual	20.05				18.10			

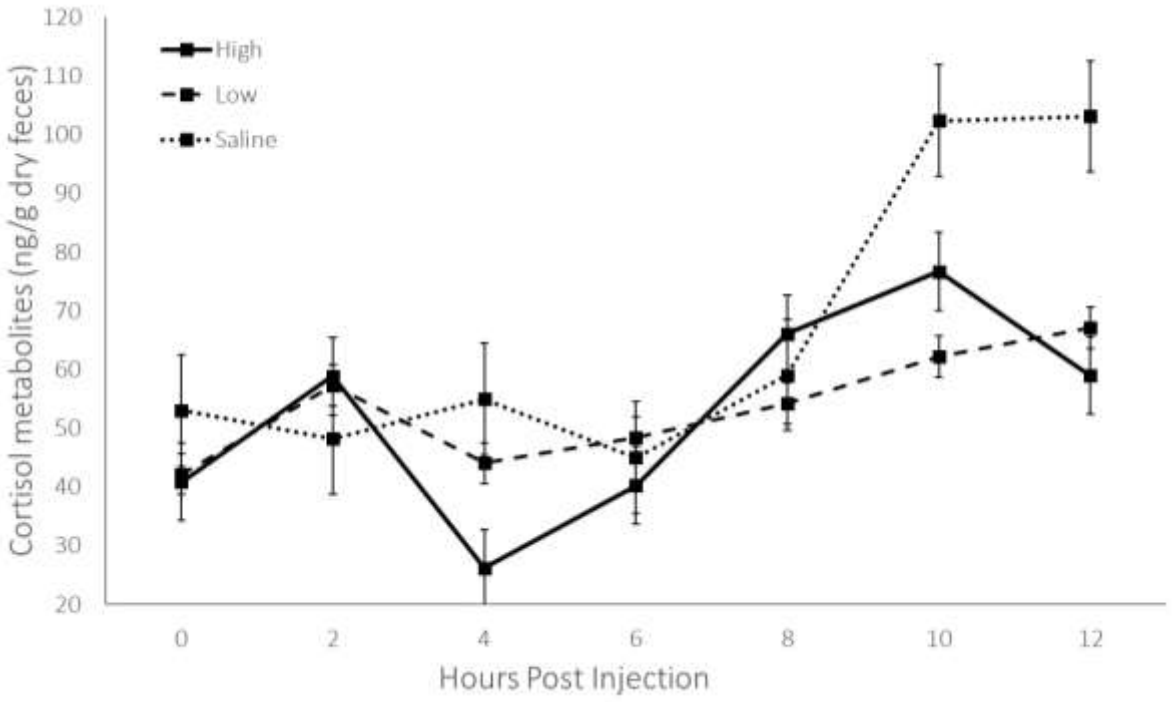

Figure 2.4 Mean (± SE) fecal cortisol metabolites of black-tailed prairie dogs following injection either a high dose (12IU/kg) ACTH, a low dose (4IU/kg) ACTH, or saline.

3.3 Chronic Stress Response Experiment

I collected post-injection hair samples for 17 out of the 18 animals. Due to inter-individual variation in hair regrowth rate, injections spanned 6 to 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 results for each group at the pre-injection and post-injection time periods are shown in Figure 2.3 and Table 2.3. Neither saline nor ACTH treatment significantly affected the post-treatment hair cortisol ($p = 0.06$) (Table 2.3). 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, weight and location on the colony did not significantly affect hair cortisol ($p > 0.05$). The individual variance was $\sigma^2 = 0.33$ and the residual variance was $\sigma^2 = 1.41$.

Table 2.3 Hair cortisol levels of black-tailed prairie dogs prior to, and following repeated injections with 12 IU/kg ACTH, or manipulated controls injected with saline (Saline), compared to unmanipulated controls (Natural)

	Pre-injection			Post-Injection		
	n	Mean	SE	*n*	Mean	SE
ACTH	9	2.35	0.15	8	3.05	0.33
Saline	9	1.48	0.21	9	3.12	0.31
Natural	36	2.22	0.20	28	4.83	0.27

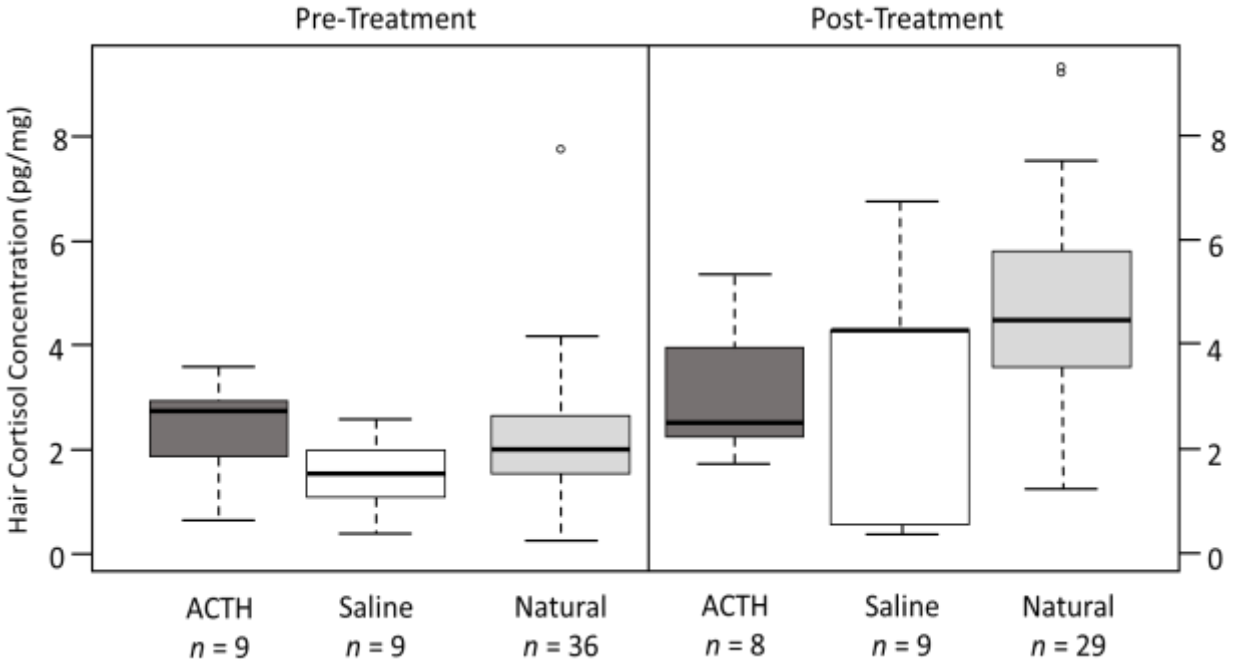

Figure 2.5 Hair cortisol concentration of black-tailed prairie dogs prior to (left) and following (right) repeated injections with either 12IU/kg ACTH (treatment), Saline (control), or Natural (no manipulation). Whiskers represent the range for each group, while the top and bottom edges of the box fall at the 25th and 75th percentiles. The bold line within each box indicates the median, and circles

Table 2.4 Intercept values, standard error, degrees of freedom, and significance for each fixed effect and interaction in the mixed effects model for hair cortisol. The results of the full model are shown on the left, while the results of the final model are shown on the right. p values less than 0.05 are indicated in bold text.

	Full Model				Final Model			
Fixed Effects	Estimate ± SE	df	*t*	*p*	Estimate ± SE	df	*t*	*p*
(Intercept)	4.45 ± 1.41	40	3.15	<**0.01**	1.65 ± 0.56	41	2.95	<**0.01**
DoseNatural	-0.26 ± 0.65	38	-0.40	0.69	-0.77 ± 0.61	40	-1.25	0.22
DoseSaline	-1.27 ± 0.71	38	-1.79	0.08	-1.36 ± 0.71	40	-1.91	0.06
PostTreat	0.64 ± 0.69	40	0.92	0.36	0.64 ± 0.70	41	0.91	0.37
Lactation	1.69 ± 0.45	38	3.73	<**0.01**	1.53 ±0.45	40	3.39	<**0.01**
Age	-0.36 ± 0.24	38	-1.51	0.14	Not fit			
Weight	-0.00 ± 0.00	40	-1.86	0.07	Not fit			
LocationInterior	-0.08 ± 0.36	38	-0.21	0.84	Not fit			
DoseNatural:PostTreat	2.34 ± 0.80	40	2.91	<**0.01**	2.03 ± 0.80	41	2.53	<**0.01**
DoseSaline:PostTreat	1.00 ± 0.95	40	1.06	0.30	1.00 ± 0.97	41	1.04	0.31
Random Effects	Variance				Variance			
Individual ID	0.36				0.33			
Residual	1.38				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. Our findings suggest that while total levels of cortisol are higher in plasma than corticosterone, much of this cortisol is bound to CBG, preventing it from diffusing into the hair shaft as readily

as corticosterone does. Similarly, it appears that corticosterone is more readily metabolized by the liver and excreted in feces in this species.

4.2 Acute Stress Response Experiment

Both low and high doses of ACTH stimulated plasma cortisol concentrations in the expected pattern, peaking at 30 minutes post-injection and returning to approximately pre-injection levels by 120 minutes 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, despite producing increases on par with other studies (Table 2.5). While the high dose ACTH treatment group showed a modest peak at 10 hours, neither the control or low dose treatment groups showed an increase and subsequent decline within the 12 hour collection period. There are two possible explanations for these results. First, while previous research suggests that GC metabolites should appear in the feces within 4-12 hours (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. 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, therefore, could have slowed or entirely suppressed digestion in these individuals, so if a peak in FGM was to be seen in feces, it may have occurred beyond the 10-hour mark for this group, or even beyond the final collection at 12 hours. This interpretation may explain why we still see a

peak in FGM for the control group. Despite not receiving any hormone, handling, blood collection and injection should be 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 peak in FGM, which occurred with the expected time lag since these animals did not receive exogenous ACTH that slowed their gut passage times.

An alternate explanation for the observed results is that, due to the negative feedback regulation of GCs (Reeder and Kramer, 2005; Romero, 2004), the administration of exogenous ACTH actually down-regulated the stress response of the animals in the two treatment groups. Specifically, the introduction of exogenous ACTH, while momentarily leading to the spike in GCs seen in the plasma samples, may have initiated a negative feedback loop, suppressing further expression of endogenous GCs. Consequently, these levels could average out over the time feces are forming in the gut, and thus FGM levels would appear static.

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 (Ashely et al., 2011). The interspecific variation seen across studies reveals the complications and challenges inherent in these sorts of validation studies.

Although the injection of synthetic ACTH is considered the gold standard for assessing adrenal activity, the specifics of this procedure are not standardized. Among studies of sciurid

rodents alone, ACTH preparations, dosages, and injection routes vary, as do post-injection sampling timelines. Table 2.5 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) my use of animals from the at-risk Canadian population resulted in restricted flexibility. In order to limit my impact on the breeding females of this isolated population, my protocols differed from validations of related rodents in two ways. First, I chose to keep wild caught animals for the minimum possible amount of time required to perform the acute stressor experiment in an effort to reduce my impact on their natural behaviour and ecology. In particular, I did not wish to risk study animals losing their social position during their absence from the colony, which is a particular concern for this highly social species (Hoogland, 1995) and I returned them to their burrows immediately after the 12^{th} hour fecal sample collection, to avoid artificially exposing them to nocturnal predators.

These efforts to minimize disruption to the individual study animals could explain the modest difference in increase between the control and treatment groups. If all 9 of the 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 been obscured in the feces by the additional stress of the novel environment. Working within standardized protocols would clarify whether the variation in responses is due to species-specific differences, or a product of study design.

Table 2.5 Validation experiments in sciurid rodents using injections of ACTH (adrenocorticotropic hormone) to stimulate glucocorticoid hormone production and subsequently measure glucocorticoid metabolites excreted in feces. (m), (f) designate values that are specific to males, and females only, respectively. All other values are not differentiated by sex. Parameters that were not specified in publication are noted with n.sp.

Species	*n*	Dose	ACTH Prep	Route	Approx. Lag time	Approx. increase	Citation
Belding's Ground Squirrel *(Urocitellus beldingi)*	5	4IU/kg	Cortrosyn	SQ	18-30 h	1.8	[2]
Belding's Ground Squirrel *(Urocitellus beldingi)*	9	200ug/kg	Cortrosyn	SQ	18-30 h	1.8	[2]
Columbian Ground Squirrel *(Urocitellus columbianus)*	8	4IU/kg	Synethen depot	IM	7 h	8.7 (m) 2.5 (f)	[3]
North American Red Squirrel *(Tamiasciurus hudsonicus)*	8	4IU/kg	Synethen depot	n.sp.	8 h	1.2	[4]
Richardson's Ground Squirrel *(Urocitellus richardsonii)*	8	200ug/kg	n.s.	SQ	n.sp.	1.3 (m) 2.0 (f)	[5]
Alpine Chipmunk *(Tamias alpinus)*	8	12IU/kg	Cortrosyn	IM	24 h	1.9	[6]
Lodgepole Chipmunk (*Tamias speciosus*)	8	12IU/kg	Cortrosyn	IM	24 h	2.9	[6]

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

[2] Mateo and Cavigelli, 2005
[3] Bosson et al., 2009
[4] Dantzer et al., 2010
[5] Hare et al., 2014
[6] Hammond et al., 2015

increased hair cortisol levels, but any effect of treatment was masked by the stronger influence of lactation. Conducting this experiment in male prairie dogs, or during the non-reproductive season with females would eliminate lactation as an influence on hair cortisol concentration and could provide a clearer effect of ACTH treatment.

While I was able to monitor lactation at the individual level, and therefore measure the influence of this variable in the experiment, it is also possible that deviation from the expected result was caused by influences I was unable to measure, such as disease, density or social rank. As previously discussed, 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.

A long-term study of relocation stress in rhesus macaques (*Macaca mulatta*) suggests such an effect (Davenport et al., 2008). Blood and hair samples were collected prior to, one week after and one year after relocating the animals to a new facility (Davenport et al., 2008). Hair cortisol levels increased from the pre-move to the 1 week post-move period, but by one year post-move, hair cortisol levels returned to the pre-move baseline, despite plasma cortisol levels remaining elevated (Davenport et al., 2008). An investigation of free versus bound cortisol revealed that free cortisol levels increased pre-move to post-move, while CBG levels remained constant (Davenport et al., 2008). By one year post-move, CBG levels had increased, resulting in a higher proportion of bound cortisol, though total cortisol levels remained constant (Davenport et al., 2008).

Similarly, the short interval between injections in this study may have been interpreted as a prolonged, high intensity stressor, prompting the treatment animals to upregulate their CBG levels. The majority of successful hair validation studies to date have used an interval of approximately one week between injections (González-de-la-Vara et al., 2011; Mastromonaco et al., 2014; Terwissen et al., 2013). The short moult duration of black-tailed prairie dogs necessitated a more frequent injection interval and this design may have simulated a more severe stressor, relative to previous studies, and led to upregulated CBG levels. Further research that measures the proportion of free:bound cortisol in the blood of each hair sample animal, would help to test this hypothesis.

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 suggests its use as a matrix for non-invasively monitoring GC levels, I 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. Similarly, the chronic stressor experiment demonstrated 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 highly 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. Keckies et al., 2012). The effects of lactation on hair cortisol concentration further demonstrate that while this matrix is reflective of endogenous physiological processes, I could not replicate these changes with artificial stimulation during the breeding season. My 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.

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CHAPTER 3

The relationships between perceived predation risk, glucocorticoid hormones and reproductive success in black-tailed prairie dogs (*Cynomys ludovicianus*)[7]

Abstract

Activation of the hypothalamic-pituitary-adrenal (HPA) axis in vertebrates stimulates the production of glucocorticoid (GC) hormones, which is the physiological mechanism that facilitates a response to perceived stressors, such as an encounter with a predator. Human biomedical research demonstrates that sustained activation of the HPA axis can lead to detrimental health consequences. This has led to the articulation of the cort-fitness hypothesis, which posits that that chronic stressors elevate GC levels and lead to reduced fitness in wildlife. I predicted that individuals on the edge of a natural colony would perceive increased risk of predation, have elevated GC levels (measured through hair cortisol), and reduced reproductive success. I also investigated other putative influences on GC levels: age, reproductive status, body condition and season of hair growth. Perceived risk of predation appears to vary between edge and interior locations in this colony, based on differences in vigilance behaviour. However, the risk of predation does not appear to represent a chronic, detrimental stressor as evidenced by a lack of a difference in hair cortisol levels between edge and interior individuals. Instead, hair cortisol levels are affected by year, season, lactation and the interaction between age and lactation. Additionally, cortisol measured in hair grown prior to reproduction was not associated

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with reproductive success. Instead, cortisol in hair grown during reproduction was correlated with reproductive success, suggesting that in black-tailed prairie dogs, GC hormones play a role in facilitating physiological processes but are not present in individuals in levels high enough to exert a negative impact on individuals in this population.

1. Introduction

Human and laboratory animal experiments consistently find that chronic stress, defined as long term overstimulation of responses to a noxious stimulus, has negative consequences (Romero, 2004; Touma and Palme, 2005). This idea has been extended to studies of wildlife, in which diminished reproductive success is the measured negative consequence (e.g. Boonstra et al., 1998; Young et al., 2006; Pauli and Buskirk, 2007; Charbonnel et al., 2008).

When an animal encounters the type of noxious stimuli that induces the physiological stress response, such as an encounter with a predator or an unpredictable negative social interaction, the hypothalamic-pituitary-adrenal (HPA) axis is activated, releasing a cascade of hormones (Reeder and Kramer, 2005). The final substances in this pathway are glucocorticoid hormones, which suppress non-essential functions such as digestion and reproduction, while mobilizing glucose to fuel an emergency response to the stressor (Reeder and Kramer, 2005). This association between stressors and GC hormones has led to the articulation of the cort-fitness hypothesis, which is the interpretation that higher GC levels are suggestive of poor condition in individuals, and thus reduce reproductive success (Bonier et al., 2009). The primary support for this hypothesis comes from a study that argued that high risk of predation to snowshoe hares (*Lepus americanus*) during population declines leads to chronic stress, which was negatively

correlated with reproductive success in the hares, driving further reductions in the hare population (Boonstra et al., 1998).

Despite the relationships found by Boonstra et al., (1998), and others (above), there have also been a number of studies returning a mixed or positive relationship between chronic stress and reproductive success (Bonier et al., 2009). In fact, in a review of studies testing this hypothesis, Bonier and colleagues found mixed support for the cort-fitness hypothesis with only approximately 50% of studies providing support (2009).

Further scrutiny of the literature reviewed by Bonier et al., (2009) suggests that one reason for the apparent discrepancies may be the diversity of study protocols. For example, there are a wide variety of measures of reproductive success, and timing of GC measures. Bird studies attempting to address the cort-fitness hypothesis include reproductive success measures as varied as nest abandonment, probability of fledging a single chick, or total number of chicks fledged (Love et al., 2004; Angelier et al., 2006; Cyr and Romero, 2007). Similarly, the timing of GC measures varies widely, from one year prior to the measured reproductive event to thirty hours after parturition in mammal studies (Sheriff et al., 2009; Pauli and Buskirk, 2007). It is not surprising, then, that these varied measures lead to varied outcomes because the relationship between GC hormones and reproductive success is dynamic. For example, chronic exposure to stressors during mammalian pregnancy should elevate GC levels, and can lead to reduced litter sizes or low birth weights (Götz et al., 1998; Diego et al., 2006). After parturition, though, high GC levels in mammals are associated with successful lactation (Voogt et al., 1969), resulting in a positive correlation between GC levels and reproductive success.

These inconsistencies highlight the importance of interpreting examples of the cort-fitness hypothesis within the context in which the data were collected. I deconstructed this hypothesis

and sequentially tested each of its underlying assumptions in black-tailed prairie dogs (*Cynomys ludovicianus*). As a burrowing, colonial species, black-tailed prairie dogs are an ideal subject for studying the effects of predation, since their spatial arrangement naturally provides potential variation in predation risk. Prairie dogs are prey for a variety of terrestrial and avian predators, namely black-footed ferrets (*Mustela negripes*), badgers (*Taxidea taxus*), coyotes (*Canis latrans*), bobcats (*Lynx rufus*), hawks (*Buteo* spp.), and golden eagles (*Aquila chrysaetos*) (Hoogland, 1995). One of prairie dogs' maintain defenses against these predators is alarm calling, which serves to alert conspecifics to the presence of a predator (Hoogland, 1995). The variation in the number of neighbouring alarm callers for prairie dogs living at the edge of the colony, compared to the centre, then, provides a system suited to assessing differing responses to risk, whether actual or perceived.

The first assumption related to the cort-fitness hypothesis is that animals experiencing or perceiving a greater risk of predation should show increased GC levels. Numerous studies in other animals conclude that increased risk of predation leads to increased GC levels (e.g. Sheriff et al., 2011; Monclús et al., 2009; Clinchy et al., 2004). Data from some species, such as Arctic ground squirrels (*Urocitellus parryii*), suggest that increased perceived, rather than actual, risk is enough to elevate GC levels (Sheriff et al., 2012). Further, some studies show evidence that perceived risk alone can decrease reproductive output (Eggers et al., 2006; Zanette et al., 2011), though these studies did not assess physiological drivers of the reduced output. Presently, there are no studies available that connect perceived risk, GC levels and reproductive success. In accordance with the first assumption of the cort-fitness hypothesis, I hypothesize that a correlation between perceived predation risk and GC levels exists in prairie dogs as well. Specifically, prairie dogs who live on the edge of a colony will have higher GC levels, indicative

of chronic activation of the HPA axis due to increased perceived risk of predation, compared to individuals who live on the interior of the colony.

Existing literature lends some support to this hypothesis. A comparison of vigilance time on a prairie dog colony enclosed by trees found that individuals on the periphery spent more time vigilant than their interior conspecifics in 76% of comparisons between the two areas (Hoogland, 1995). It is unclear, however, if this difference in vigilance was a product of the colony being enclosed by trees, or is an inherent property of coloniality. In other systems, position at the centre of a colony provides unique benefits, such as reduced risk of predation for fish positioned at the centre of a school (Pitcher and Parrish, 1993). These examples suggest that the difference in vigilance time between edge and interior is likely an inherent property of colony geography, rather than only an artifact of the physical landscape of the colony, which would have resulted in visual obstruction for the individuals living closer to the surrounding forest. To test the hypothesis that the edge is perceived by prairie dogs as a higher risk area, using the same protocols as Hoogland (1995), I conducted a comparison of vigilance time between edge and interior animals on a colony surrounded by a more typical landscape for the species, open prairie.

If increased perception of risk leads to increased GC levels in black-tailed prairie dogs, the cort-fitness hypothesis predicts that these individuals would have reduced fitness, compared to conspecifics with lower GC levels. There is some evidence for this relationship in black-tailed prairie dogs at the colony level. In a manipulative study of 10 paired colonies, researchers subjected one colony from each pair to several hours of rifle shooting, while leaving the paired colony undisturbed as a control (Pauli and Buskirk, 2007). On the colonies that experienced shooting, individuals increased the amount of time spent vigilant, which forced them to decrease the amount of time foraging by 66% (Pauli and Buskirk, 2007). With this decrease in foraging,

body conditions on the treatment colonies declined and reproductive output in the following year was 82% lower than the previous year, while the control colonies showed equivalent success across the two (Pauli and Buskirk, 2007). Curiously, while juvenile fecal GC metabolites increased by 80% on the shot colonies, adult GC metabolites did not differ between treatment and control colonies after shooting. This suggests that adult prairie dogs may be capable of coping with an intense, acute stressor, despite the fact that behavioural changes in the treatment colonies led to decreased reproduction in the following year.

Pauli and Buskirk's (2007) results suggest that an acute stressor can have detrimental consequences for black tailed prairie dogs. However, the role of GC levels was ambiguous and the relevance for chronic stressors, such as elevated perceived predation risk due to spatial location, was not addressed. Of note, this study used fecal glucocorticoid metabolites (FGM) as the measure of GC hormones. While FGMs are reflective of GC hormones circulating hours previous to excretion (Touma and Palme, 2005), and thus provide a good measure of an individual's response to an acute stressor, Pauli and Buskirk (2007) monitored reproductive success months after the stressor, and thus a longer-term measure would have provided a more appropriate scale of stress measurement. Hair is unique among commonly used matrices for GC measures in that it provides an integrated measure of circulating GC levels throughout the period of hair growth (Russell et al., 2012). In black-tailed prairie dogs, hair grows over 1-2 weeks following the biannual moult (Hoogland, 1995), and thus provides a record of GC levels more suited to investigation of a prolonged, chronic stressor. My focus on predation risk, a chronic stressor, is more relevant in explaining potential population declines over the long-term, and thus better suited for informing conservation strategy in this study system.

Understanding potential contributors to population declines is particularly relevant in Canada, as pronounced declines have been witnessed in recent decades (COSEWIC, 2011), over and above the general decline in black-tailed prairie dogs across North America since European settlement of the West (Proctor et al., 2006). Despite being relatively sheltered from the major threats affecting the species at the continental scale, such as habitat loss, recreational shooting, and poisoning campaigns (Hoogland, 2006*a*; COSEWIC, 2011).

I tested the cort-fitness hypothesis at the individual level in black-tailed prairie dogs to assess whether perceived risk of predation elevates GC levels and reduces reproductive success. I expected that prairie dogs living on the edge of the colony would perceive higher risk of predation, demonstrated by higher time engaged in vigilance behaviours, and that this perception would lead to increased GC levels in edge animals. In addition to predation risk, body condition, age class and reproductive status are known to influence GC levels in related species (Lyons et al., 2017; Mateo, 2006; Boonstra et al., 2001). Therefore, I also included these factors in my investigation.

2. Methods

2.1 Study Area

The Canadian population of black-tailed prairie dogs consists of 19 colonies (Parks Canada unpublished data) located in and around the Frenchman River Valley in Grasslands National Park, in southwest Saskatchewan (COSEWIC, 2011). I studied individuals from one colony (Walker; approximately 119 hectares) in the southern portion of the west block of the park (49° 3' 46.8"N, 107° 21' 28.8"W). The site is mixed-grass prairie, characterized by relatively flat terrain, with several shallow gullies. Grass is the dominant vegetation, with needle and thread

grass (*Hesperostipa comata*), blue gramma grass (*Bouteloua gracilis*) and western wheatgrass (*Pascopyrum smithii*) being the primary species in the area (Stephens et al., 2017).

Opportunistic observations over two years of monitoring of predators included 194 sightings, detailed in Table 3.1. The number of instances of predation recorded by myself and the field crew was very low (3 instances over 10 months), and was likely influenced by our physical presence on the colony. While this observed rate of predators may seem discordant with predator abundance, this rate is actually higher than the 22 instances of predation recorded by Hoogland over 10 years (1995).

2.2 Calculation of Geographic Data

Prairie dogs live in family groups called coteries, in which the entire coterie territory is shared between the members (Hoogland, 1995). Prairie dogs defend this coterie territory from conspecifics, such that neighbouring prairie dogs generally do not cross into the territory of other coteries to forage, including for baited traps (Hoogland, 1995). Therefore, I used trapping location as an indicator of home territory for individual prairie dogs, as the locations where they were repeatedly trapped were generally clustered within one area. I occasionally trapped individuals in locations away from their home territory, but did not include these aberrant locations when determining home territory. Because of the shared nature of coterie territories, I considered the "edge" of the colony to extend inward from the perimeter by the approximate area of one coterie territory, rather than only burrows found only on the perimeter of the colony. Numbers for average territory size were not available for my study colony, so instead, I used the diameter of the average size of coteries from the Hoogland study. This diameter is 62 m based on an average territory size of 0.36 ha (Hoogland, 1995).

Each year, I determined the perimeter of the colony in March and August, with the help of field technicians. We used a GPS device (Garmin e-Trex 20, Garmin Ltd., Olathe, Kansas, USA) to create a track of the outermost active burrows, defined as those that were clear of cobwebs and with fresh feces on the mound. Then, I determined whether territories fell into the edge or interior classification based on these perimeter tracks for each season in which hair samples were grown. For each of the four time periods, I used ArcMap 10.3.1 (ERSI, Redlands, California, USA) to create a buffer 62 m inward from the edge of the colony. I considered waypoints in territories that were further than 62 m from the perimeter to be interior, and those that fell within the 62 m buffer to be edge.

The 62 m estimate of coterie territory size was the best available estimate for my study. However, it is possible that this dimension was unique to Hoogland's colony, and factors influencing it, such as local density and overall colony size, may not be generalizable to the Walker colony. Therefore, I also considered distance from the edge of the colony as a continuous variable, calculated by measuring the shortest distance in meters from the trap at which a prairie dog was most frequently caught to the colony perimeter. For each time period in which hair was grown, I used the associated perimeter (e.g. hair grown in spring 2016 compared to the spring 2016 perimeter), which resulted in differing distances for each individual at each time point.

Table 3.1 Predators of adult or juvenile black-tailed prairie dogs recorded on the Walker Colony, Grasslands National Park, during March – August 2016 and 2017.

Common Name	Scientific Name	Number of Observations
American badger	*Taxidea taxus*	31
Coyote	*Canis latrans*	4
Least weasel	*Mustela nivalis*	1
Prairie rattlesnake	*Crotalus viridis*	9
Ferruginous hawk	*Buteo regalis*	12
Golden eagle	*Aquila chrysaetos*	8
Northern harrier	*Circus cyaneus*	54
Prairie falcon	*Falco mexicanus*	3
Swainson's hawk	*Buteo swainsoni*	16
Turkey vulture	*Cathartes aura*	4
Rough legged hawk	*Buteo lagopus*	4
Unidentified raptor spp.	*NA*	48
Total Observations		194

2.3 Vigilance Comparison

In May 2017, I classified each GPS waypoint used on the colony as edge or interior using the perimeter of the Walker colony defined in March of 2017. I then assigned a number (001-932) to each waypoint, and used the random number generator function in Microsoft Excel (2016) to select 21 waypoint pairs, with each pair consisting of one edge and one interior waypoint. With the help of field technicians, I visited each waypoint and marked it with a high visibility stake. After a minimum of one hour, I returned to the area, and selected a vantage point at least 150 m from the marked waypoint in any direction. Using a spotting scope while in either a seated or prone position, I scanned to find the closest prairie dog to this waypoint that was actively foraging, indicating that they were no longer considering my presence to be a threat. If no individuals were foraging, I waited until one began to forage to initiate the observation. Given that this work took place on a long-term study colony, where prairie dogs are habituated to human presence, prairie dogs usually returned to foraging within minutes of my approach.

I followed the vigilance comparison outlined in Hirschler et al. (2016). Specifically, once an individual was located foraging, I initiated a 15 min observation session. I used all occurrence sampling of vigilance behaviour and recorded the total amount of time the animal spent being vigilant versus non-vigilant. I based classifications of vigilant or non-vigilant activities provided in Table 3.2 (adapted from Hirschler et al., 2016). If the focal prairie dog was obscured or went underground, I paused the session until the prairie dog returned to view, or 5 min passed. If more than 5 min elapsed before the focal animal returned, I restarted the observation session.

Table 3.2 Behaviours used as indicators of vigilance in black-tailed prairie dogs, adapted from Hirschler et al., 2016.

Non-vigilant	Vigilant
Head down	Standing on hind feet scanning off mound
Eating with all feet on ground	Standing on two or four on mound looking
Interacting with other prairie dogs	

2.4 Hair Sample Collection

This colony is part of an ongoing study (initiated in 2014) and my methods for trapping and handling follow the overall procedures. These trapping and handling procedures follow the Canadian Council on Animal Care guidelines for wildlife, and were approved by the University of Saskatchewan Research Ethics Board (AUP 20140042). Briefly, the field technicians and I baited Tomahawk live-traps (Tomahawk Trap Co, Tomahawk, Wisconsin, USA) with a mixture of oats and peanut butter to trap animals. When an animal was trapped, we removed it from the trap, and weighed it (±5 g) with a spring scale in a cotton handling bag. We tagged new animals in both ears with ear tags (National Band and Tag Co., Newport, Kentucky, USA), and took biopsies of ear tissue for DNA analysis. We then measured zygomatic breadth and right hind foot

length, and recorded sex and reproductive condition for all animals. We dyed new animals with unique symbols using Nyanzol dye for visual identification.

In order to characterize how GC levels vary over the course of the season, I collected, processed and analyzed 151 hair samples from 69 female prairie dogs. I collected hair samples at four distinct periods, which are representative of two different hair growth periods across two different years. These time periods and the growth periods they represent are summarized in Table 3.3, and their growth in comparison to the reproductive season are illustrated in Figure 3.1. I restricted this study to breeding females, because I was able to determine their reproductive success in the field.

While the rump or hind limb are common locations for sampling hair (Macbeth et al., 2010; Mastromonaco et al., 2014), this area is often contaminated with feces in trapped animals (personal observation; Macbeth et al., 2010). Therefore, I collected samples by shaving a 3 cm x 3 cm patch from the right hip — a location that provides cleaner samples. I stored each hair sample in a paper envelope at room temperature until hormone extraction. I cleaned the clippers with a brush between collections, and deconstructed the clippers at the end of each day to remove any hair that became trapped inside. Laboratory procedures for extracting and quantifying hormones from hair samples are detailed in chapter 2, section 2.7.

Table 3.3 Collection and hair growth periods for prairie dog hair samples, compared to reproductive state at the time of hair growth.

Time	Season & Year of Hair Growth	Season & Year Collected	Reproductive Status During Growth
1	Fall 2015	Spring 2016	Non-breeding
2	Spring 2016	Summer 2016	Lactating
3	Fall 2016	Spring 2017	Non-breeding
4	Spring 2017	Summer 2017	Lactating

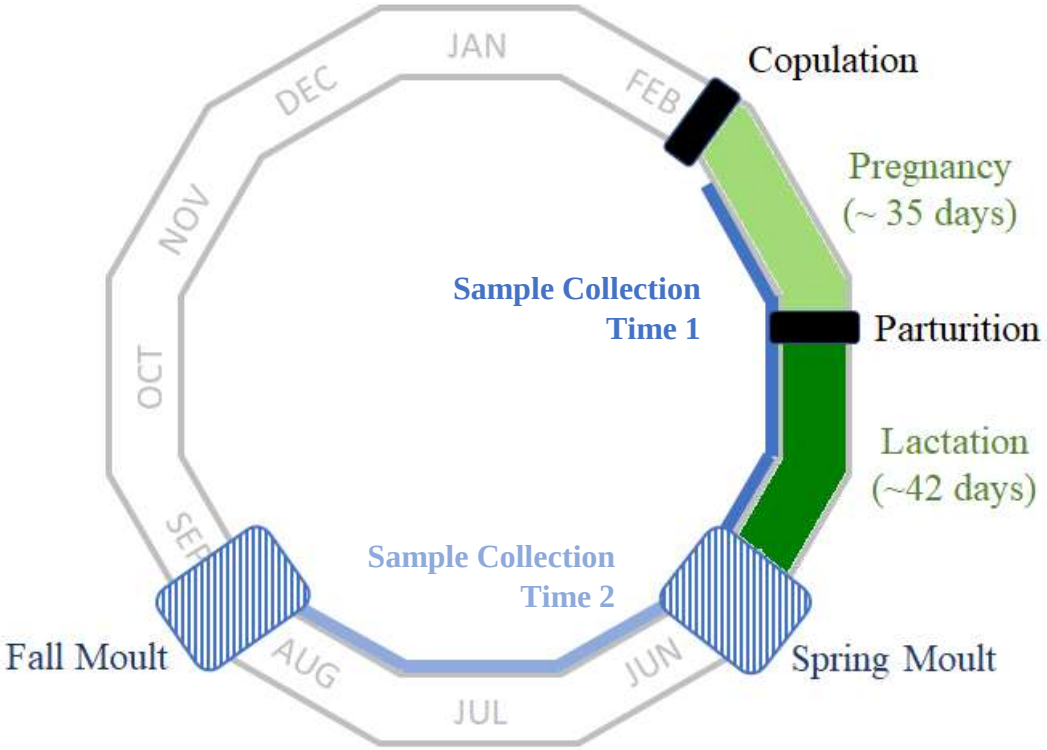

Figure 3.1 Timeline of the annual cycle of reproduction in female black-tailed prairie dogs, along with biannual moult, and hair sample collection times.

2.5 Calculation of Body Condition

Individual body mass varies throughout the year, based on annual changes in food availability and physiological demands associated with reproduction (Hoogland, 1995). Skeletal size measurements for adult animals should be stable throughout the year, resulting in a changing ratio of mass to skeletal size throughout the year. I created a body condition index based on the methods outlined in Schulte-Hostedde et al. (2005). I first calculated each animal's average mass for the time periods directly before each moult, using measurements from March 1 – May 15 for hair samples grown in spring, and June 1 – August 15 for hair samples grown in the fall. I gathered average measurements of foot length and zygomatic arch breadth for the sample time periods then conducted a principle component analysis using these two variables. I used simple linear regression to correlate average individual body mass and principle component one, then used the residuals of this model as an index of body condition.

2.6 Measuring Reproductive Success

In prairie dogs, parturition occurs underground (Hoogland, 1995), therefore, I quantified reproductive success based on counts of the number of juveniles emerging from a natal burrow on the first day of emergence. This method is commonly used as an indicator of reproductive success in this species (e.g., Hoogland, 1995; Shier 2006; Facka et al., 2010). I used data from the same year on frequent trapping locations, as well as behavioural observations, to assign breeding females to natal burrows. Counts of emergent juveniles per natal burrow came from observations, with the assistance of the field technicians on the ongoing study.

2.7 Statistical Analyses

All statistical analyses were performed using R (version 3.2.3, R Core Team, 2015). I conducted preliminary data exploration steps as recommend in Zuur et al., (2010) including checks for normality, outliers, homogeneity of variance, collinearity, interactions between x and y variables and interaction between covariates. Based on these checks, I found that hair cortisol concentration was not normally distributed. Therefore, I square root transformed these data prior to analysis. To test my overall hypothesis that risk on the edge leads to elevated GC levels and maladaptive consequences, I explicitly tested for differences between edge and interior prairie dogs using Welch two sample t-tests to compare body condition and litter size between the groups, which allowed for unequal variances between groups. I also compared the mean vigilance time between edge and interior prairie dogs using a Welch two sample t-test.

To assess what factors influenced hair cortisol content I used a linear mixed effects model, with the package nlme (Pinhero et al., 2017). I predicted that location (categorical: edge or interior, and continuous, see below), reproductive status (categorical: lactating or not), age class (categorical: adult or yearling), year hair was grown (categorical: 2015, 2016 or 2017), season hair was grown (categorical: spring or fall), body condition (continuous), and two-way interactions between these terms would affect hair cortisol levels, and thus included them as fixed effects. Due to the design of the long-term study, and the prairie dog hair cycle, information on some of these predictor variables were unavailable for certain samples. Due to missing data, or manipulation in the experiment described in Chapter 2, 37 records were excluded from the analysis of influences on hair cortisol, resulting in a total sample size of 114 from 69 individuals.

I first fit a general linear model that included these terms, and used the step function to serially drop non-significant terms ($p < 0.05$), resulting in a final model in which all fixed effects were statistically significant at $p = 0.05$. I used an ANOVA to test for significant differences between the serial models. Then I fit linear mixed effects models for both the full and final models, using individual as a random effect with a random slope, and the restricted maximum likelihood method, and used an ANOVA to confirm that there was a significant difference between the full and final models. I tested location as both a categorical and continuous variable in separate models, due to correlation between these variables. The model including continuous distance did not represent a significant improvement over the model using categorical location. Therefore, based on the recommendations in Zuur et al., I selected the categorical variable, because it makes more biological sense, as prairie dogs live within a defined coterie territory, rather that at one distinct point a set distance from the edge (2010).

In testing for an effect of hair cortisol on reproductive success, I used litter size at first emergence as my dependent variable. I predicted that there would be a negative relationship between litter size and cortisol in hair grown in the non-reproductive season (fall), but a positive relationship between these variables in the reproductive season (spring). Therefore, I analyzed samples from spring and fall separately. For the fall dataset, there were 35 measures with no repeated individuals. In the spring dataset there were 37 measures with two entries for two individuals. As this amount of replication is insufficient to fit a linear mixed effects model to avoid pseudoreplication, I randomly selected one of the two measurements for each individual, and excluded these two samples from the analysis. This resulted in a set of 35 litter size measurements from 35 individuals. Litter sizes were not normally distributed, and contained zeros for females who were pregnant but failed to rear a litter to emergence. For both the fall and

spring data, therefore, I fit generalized linear models using the hurdle function in the package pslc (Zeileis et al., 2008) with a truncated Poisson distribution for the non-zero litter size counts and binomial distribution for zero litter size counts compared to non-zero counts.

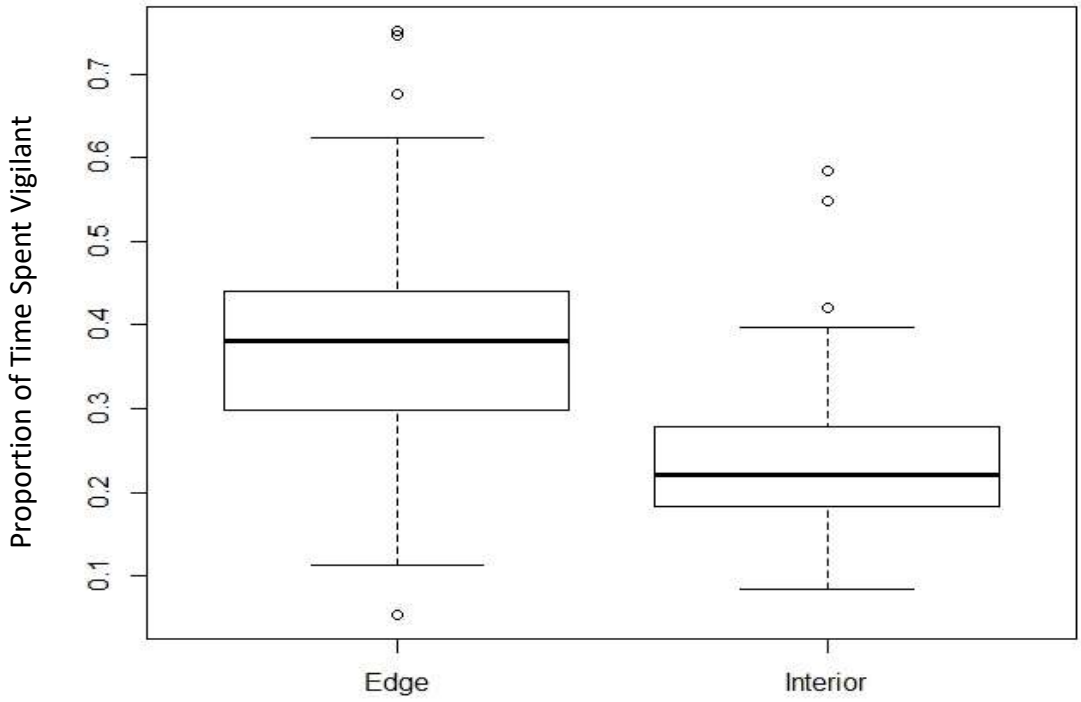

Figure 3.2 Proportion of a 15-minute focal sample that prairie dogs spent being vigilant, by location in the colony.

3. Results

3.1 Vigilance Comparison

Prairie dogs at the edge of the colony spent significantly more time vigilant than those in the interior ($t = 2.85$, $df = 36.64$, $p < 0.01$). Out of a 15 min sample, the mean vigilance time was 6:00 ± 0:36 for edge prairie dogs, compared to 3:45 ± 0:26 for interior prairie dogs (Figure 3.2).

3.2 Correlates of Hair Cortisol Levels

Hair cortisol levels varied between individuals, ranging from 0.26 pg/mg to 9.88 pg/mg with one outlier of 16.02pg/mg, which was excluded from analyses. The median result was 3.74 pg/mg across all samples (n = 114). There was no difference in hair cortisol by location (t = 0.49, df = 90.91, p = 0.63). Significant influences on hair cortisol included season of growth (p = <0.01), year of growth (p = <0.01) (Figure 3.3), reproductive condition (p = <0.01) and the interaction of lactation and age class (p = 0.03) (Figure 3.4). Age class alone did not significantly affect hair cortisol (p = 0.12), but was retained in the model due to its interaction with lactation. Table 3.4 provides full statistical results for this model. There was a nearly significant difference in body condition between edge and interior location (t = -1.89, df = 106.17, p = 0.06), but neither of these influences affected hair cortisol. The individual variance was σ^2 = 0.19 and the residual variance was σ^2= 0.36, producing a repeatability of 35%.

Table 3.4 Comparison of candidate linear mixed effects models used to determine influences on hair cortisol of female black-tailed prairie dogs

	Full model				Final Model			
Fixed Effects	Estimate ± SE	*df*	*t*	*p*	Estimate ± SE	*df*	*t*	*p*
(Intercept)	1243 ± 330	57	3.76	<0.01	1087 ± 198	58	5.48	<0.01
Season (Spring)	-238 ± 386	48	-0.62	0.54	1.09 ± 0.10	50	10.64	**<0.01**
Year	-0.62 ± 0.16	48	-3.76	**<0.01**	-0.54 ± 0.10	50	-5.48	**<0.01**
Lac (Y)	0.66 ± 0.1795	48	3.68	**<0.01**	0.67 ± 0.18	50	3.85	**<0.01**
Age (Yrl)	0.34b± 0.21	48	1.61	0.11	0.32 ± 0.20	50	1.60	0.12
Lac(Y) : Age(Yrl)	-0.48 ± 0.22	48	-2.20	**0.03**	-0.48 ± 0.21	50	-2.22	**0.03**
Location (Interior)	-0.08 ± 0.09	57	-0.82	0.41	Not fit			
BodyCond	0.00 ± 0.00	48	0.59	0.56	Not fit			
Year:Season (Spring)	0.12 ± 0.19	48	0.62	0.54	Not fit			
Random Effects	Standard deviation				Standard deviation			
Individual ID	0.18				0.19			
Residual	0.35				0.36			

3.3 Correlates of Reproductive Success

Litter sizes ranged from 0 to 6 (mean = 3.74 ± 0.20), and were not normally distributed (Figure 3.5). There was no difference in litter size based on location between the edge and interior of the colony (t = 1.00, df = 62.25, p = 0.32). Non-reproductive season hair cortisol did not affect litter size for females who had at least one pup (z = -0.07, p = 0.94), nor in a binary comparison of successful versus unsuccessful litters (z = 1.14, p = 0.26). There was not a significant effect of year for either group (non-zeros: z = -0.49, p = 0.62; binary: z =-0.00, p = 0.62). Reproductive season hair cortisol was correlated with binary reproductive success (z = 2.27, p = 0.02) as was year (z = 2.09, p = 0.04). Reproductive season hair cortisol was not correlated with litter size (z = -0.10, p = 0.92) or year (z = 0.25, p = 0.78) for females who produced at least one pup.

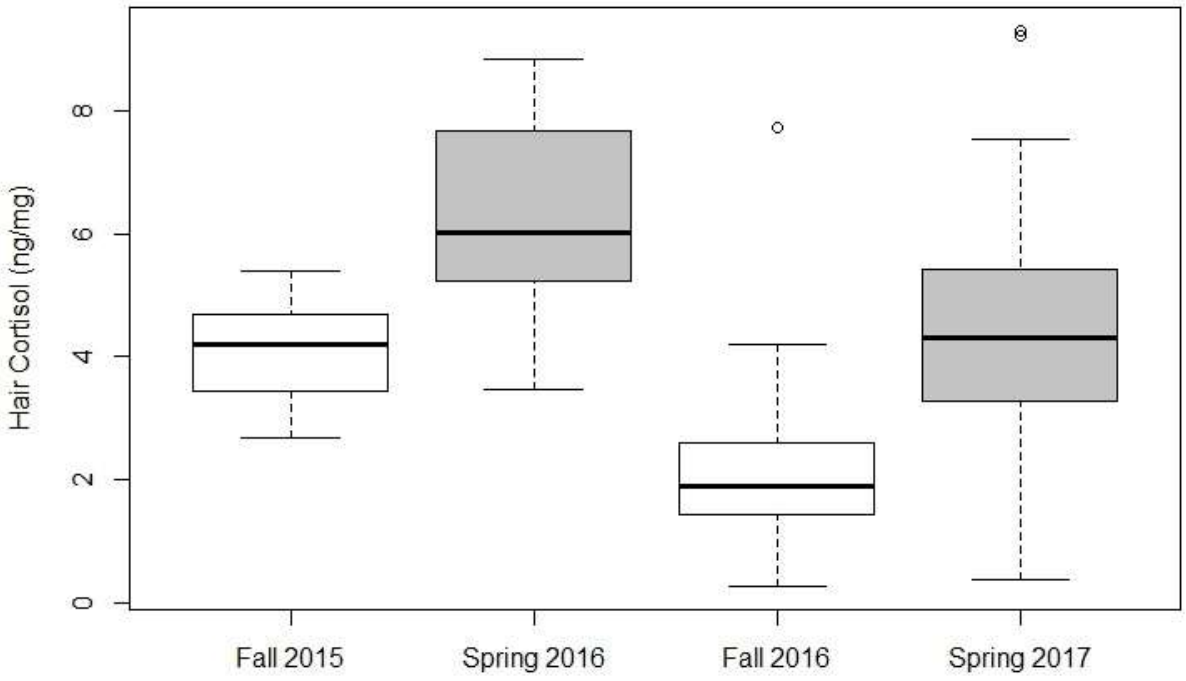

Figure 3.3 Hair cortisol content from female prairie dogs, separated by year and season of growth. Upper and lower bounds of each box represent the interquartile range, the whiskers indicate the range of the data, with outliers indicated as circles, thick horizontal lines indicate the median.

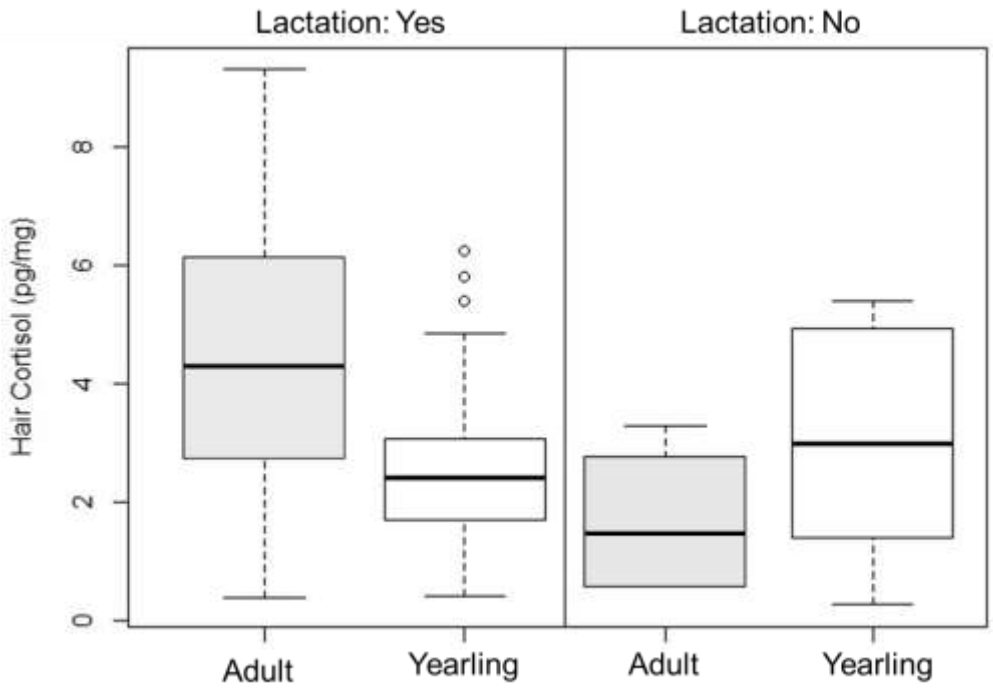

Figure 3.4 Hair cortisol concentration of adult and yearling female black-tailed prairie dogs, separated by lactation status within the year a hair sample was taken. Upper and lower bounds of each box represent the interquartile range, the whiskers indicate the range of the data, with outliers indicated as circles, thick horizontal lines indicate the median.

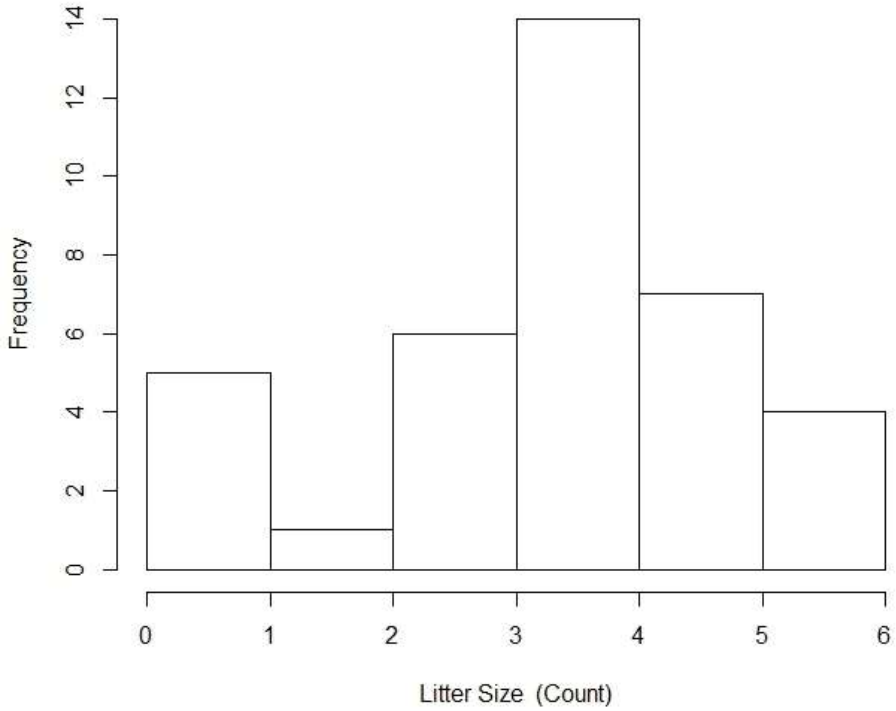

Figure 3.5 Frequency distribution of black-tailed prairie dog litter sizes in 2016 and 2017. Height of bin indicates the frequency of each litter size. n = 35.

4. Discussion

I predicted that prairie dogs who lived on the edge of the colony would perceive an elevated risk of predation, resulting in higher hair cortisol levels, relative to interior prairie dogs, because the latter individuals are surrounded by more conspecifics who could share the burden of threat detection. This was supported by my comparison of vigilance time for individuals at the edge and interior of the colony. I found that animals near the edge spent more time engaged in vigilance compared to their interior counterparts.

There is always some question of the observer's affect on natural vigilance behaviour. I attempted to minimize this influence by working on a long-term study colony, with animals habituated to daily human presence during the active season, limiting my approach distance, and restricting the observations to individuals whose behaviour (non-vigilant foraging) indicated they did not consider my presence to be a threat. My findings align with other comparisons of vigilance by location in black-tailed prairie dogs (Hoogland, 1979; Hoogland, 1995; Kildaw, 1991). Taken together, these finding confirm that prairie dogs perceive the edge to be a riskier location, regardless of the physical landscape surrounding a particular colony.

I predicted animals living on the edge of the colony would have higher levels of hair cortisol than those living in the interior. While the edge animals spent more time engaged in vigilance behaviour, this did not translate to significant differences in hair cortisol. Body condition also did not differ between the groups. While the perceived risk of predation differed between the edge and interior, there may also be variation in forage quality. Intensive grazing in the centre of the colony often depletes the supply of palatable forage in a colony's interior (Garrett et al., 1982). This discrepancy could explain why edge individuals spend more time vigilant, but without the expected elevation in hair cortisol content or body condition. Anecdotal

observation suggests that the edges of the colony have noticeably more vegetation available for foraging prairie dogs, so it may be that prairie dogs on the edge of the colony benefit from increased foraging efficiency, thus enabling them to spend less time foraging and dedicate more time to vigilance. If this interpretation is true, prairie dogs would be disadvantaged and forced to trade off the possible benefits of increased vigilance with the potential cost of not finding enough food in their less nutritious microhabitat. Other studies quantifying forage quality based on representative samples of their colonies would be useful to test this hypothesis.

I expected that age class would influence hair cortisol, based on previous research on this, and other species, which noted differences in plasma GC levels or fecal glucocorticoid metabolites based on age (e.g. Mateo, 2006; Boonstra et al., 2001; Pauli and Buskirk, 2007). Studies comparing individuals based on hormones measured in hair, however, typically do not find a distinction based on age (e.g. MacBeth et al., 2010; Bechshøft et al., 2011; Carlitz et al., 2014). My results, like those of other hair cortisol studies, indicate that there is no difference in cortisol between adult and yearling prairie dogs. The age-based differences between the study are likely due to methodological differences in the individuals studied, rather than an age-based difference in hormone deposition into difference matrices. Specifically, older literature on HPA function and the associated GC hormones measured from plasma and feces largely focused on either the very young, or very old, with a scarcity of information on middle age classes (Reader and Kramer, 2005). Therefore, an expansion of this research into wider age groups, such as a comparison of juveniles to adults, would likely uncover an age based difference in cortisol.

Like age, body condition, was not significantly correlated with hair cortisol content. This was surprising, given the wealth of literature citing that small mammals in poor body condition have higher GC levels (e.g. Boonstra et al., 1998; Charbonnel et al., 2008; Lyons et al., 2017).

This independence of hair cortisol compared to body condition may be unique based on my study species and design. Specifically, adult black-tailed prairie dogs show dramatic fluctuations in mass over the course of the year, ranging from 500 – 1400 g (Hoogland, 1995). In an extreme example, one female in the study colony increased body mass by 96% over the course of 14 weeks in the spring of 2017. Due to these broad fluctuations, it is possible that body condition calculated from averages of body mass in the season of hair growth may have been a poor representation of this same individual's body condition at the exact time hair was growing.

While body condition was not correlated with hair cortisol content, reproductive status, year and season of growth were associated with variation in individual measures. Hair grown in the spring had a higher cortisol content than hair grown in the fall, and there was a significant difference in hair cortisol across years of growth. Seasonal variation in GC levels has also been shown in other ground squirrels (Nunes et al., 2006; Strauss et al., 2007; Boonstra et al., 2001).

It is likely that the season variable acts as a coarse proxy for a variety of influences. Of these, lactation is likely the largest component, as I found lactation to be a strong predictor of cortisol content, and therefore would expect that it is likely a major source of seasonal variation, as lactation only occurs in spring for this species (Hoogland, 1995). However, weather, food abundance and quality, number of juveniles on the colony, sociality and predation pressure also differ between the two seasons of hair growth. I did not explicitly include these factors in my analysis, and therefore cannot rule out their influence on variation between seasons of hair growth.

Table 3.5 Mean litter sizes of black-tailed prairie dogs in studies using counts of emergent juveniles on the first day of emergence from the natal burrow as an indicator of litter size.

Mean Litter Size	2.7	3.1	3.3	3.3	3.4	4.4
Reference	Garrett et al., 1982	Hoogland 2006*b*	Facka et al., 2010	Garrett et al., 1982	Shier 2006	Knowles 1987

Like seasonal variation in hair cortisol content, my results also demonstrate measurable annual variation in hair cortisol. Overall, cortisol levels across prairie dogs sampled declined over the course of the study. During this time period, reproductive success among adults and yearlings was high. The average litter size of 3.74 pups per female, which is higher than all but one other example in available literature for studies using the same litter size metric (Table 3.5). In conjunction with this high reproductive success, the colony grew over the course of the study, from 93 ha in the fall of 2015 to 118 ha in the fall of 2017. These patterns suggest that the population experienced favourable conditions for growth, rather than being heavily constrained by external pressures, as is often seen in other examples of the cort-fitness hypothesis.

It is also possible the presence of humans on the study colony during the two years of my research affected these results, in two main ways. First, the slight decrease in cortisol levels seen over the course of the two years of the study could be evidence of the animals further acclimatizing to the presence of humans on the colony. Given that the long-term research project was initiated in 2014, though, it is likely that acclimation would have already occurred by the time I began my research. Second, the daily presence of humans at the study colony may have decreased predation pressure, resulting in lower cortisol levels, overall, and more favorable conditions for reproduction. However, the concurrent growth in other prairie dog colonies in the area (Parks Canada, unpublished data) suggests that broader influences, such as climate, were

driving the increased reproductive success seen in the two years of my study. Under less favourable conditions, such as those this population likely experienced during their years of decline, my findings may have been more closely aligned with those predicted by the cort-fitness hypothesis.

In addition to year and season, reproductive status, measured with lactation, was strongly correlated with hair cortisol. If an individual lactated during the year, their hair cortisol content was elevated for both spring and fall samples, compared to those who did not lactate. The effects of lactation on spring hair cortisol samples is expected, as lactation is one of the most energetically expensive physical processes a mammal can undertake (Wade and Schneider, 1992), and spring hair samples are grown during the time when most females are still lactating (Figure 3.1). As lactation only occurs in the spring in this species, it is likely one of the many factors contributing to the seasonal increase in hair cortisol levels. The effects of lactation on hair samples grown in fall, on the other hand, was unexpected. This effect of lactation on hair cortisol, even outside of the breeding season, suggests that the burden of raising offspring extends beyond the physiological demands of lactation, and the mere presence of offspring may elevate cortisol in female prairie dogs.

Overall, lactation was positively correlated with hair cortisol, but this effect differed between the age classes, with lactating yearlings showing lower hair cortisol. If lactation increases hair cortisol, and yearlings have higher hair cortisol, compared to adults, why would yearlings who lactated in a given year have lower hair cortisol? Life history strategies specific to the Canadian population of black-tailed prairie dogs shed light on this seemingly counterintuitive pattern. In other populations, yearling females rarely breed (Hoogland, 1982; Garrett and Franklin, 1988), and those who do are less likely to raise a litter to first emergence, compared to

adults (Hoogland, 1995). In this population, however, breeding in yearling females is relatively commonly, with 29 of 90 yearling females breeding in 2016 and 32 of 57 in 2017, though the trend of yearlings being less likely to successfully carry a litter to parturition compared to adults was also evident in my study population. This suggests that yearlings who are capable of carrying a litter to parturition and then lactating may be in exceptionally good shape, compared to typical yearlings, and may represent a biased sample. This unique situation in the Canadian population is intriguing, and should be investigated further, with an emphasis on uncovering what traits in individual yearlings allow some to successfully reproduce while others do not even breed, and what factors facilitate successful yearling breeding in the Canadian population that are not seen in other prairie dog populations.

I found that reproductive success was affected by hair cortisol in samples grown during the reproductive season, but not during the non-reproductive season. I expected that high levels of cortisol in hair grown before breeding would result in reproductive suppression, based on previous work finding a negative relationship between GCs and reproductive success (Boonstra et al., 1998; Young et al., 2006; Charbonnel et al., 2008). This interpretation posits a direct causal role of GC levels on reproductive ability, but ignores the fact that reproduction itself also elevates GC levels (Wingfield and Sapolsky, 2003). Acknowledging that the relationship between GCs and reproduction success is circular, rather than linear, explains the pattern demonstrated by my results. Prairie dogs in this system seem to have cortisol levels that are adaptive, and facilitate normal functioning. They are positively associated with reproductive success during the appropriate season, but are not present in levels so high as to produce lingering negative effects outside of the reproductive season.

My results also indicate that hair cortisol is repeatable within prairie dog across time points, evidenced by the high proportion of total variance that is explained by individual variance. This suggests that individuals with cortisol levels on the low end of the spectrum tend to have low cortisol in all measures, and vice versa. This repeatability of GC levels within individuals has been demonstrated in birds, fish, and domestic mammals as well (e.g. Rensel and Schoech, 2011; Samaras et al., 2016; Scheidegger et al., 2016). Presently, there is no consensus in the literature for the proximate cause of repeatability, but development of high and low GC responding strains of laboratory animals (Pottinger and Carrick, 1999; Satterlee and Johnson, 1988), suggests at least a partial genetic basis for GC repeatability.

5. Conclusion

I tested the cort-fitness hypothesis in the Canadian population of black-tailed prairie dogs and did not find support for either of its main components. In this scenario of favourable conditions for prairie dog population growth, my results suggest that high risk may be associated with high reward—animals on the edge of the colony undertake an increased burden of vigilance, but without an increase in hair cortisol or detriment to reproductive success that one would expect under the cort-fitness hypothesis. The mechanisms that allow for this acclimation to risk on the edge deserve further investigation. With this future direction, we may be able to uncover precisely how edge individuals, rather than constraining population growth, are well adapted to the challenging conditions in which they live, and maintain reproductive success in the face of increased perceived risk of predation and poorer body condition compared to their interior counterparts.

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CHAPTER 4

Conclusion

4.1 Summary

The goal of this thesis was to investigate the application of hair cortisol as a tool to understand potential drivers of population demographics in the Canadian population of black-tailed prairie dogs. In pursuit of this goal, I first sought to validate hair as a non-invasive indicator of previous stress responses in black-tailed prairie dogs, as is recommended prior to the use of glucocorticoid (GC) hormone measures in any new species (Touma and Palme, 2005). I also included feces in this validation to support future inquiries pertaining to the physiological stress response in this species. I identified cortisol as the dominant hormone in the black-tailed prairie dog plasma, and demonstrated that it can be detected in both feces and hair. The use of wild-caught, reproductively active prairie dogs in my study, however, revealed that the standard procedures for physiological validations of GC hormone measures are better suited to laboratory or captive populations accustomed to human handling.

Given the definitive results of adrenocorticotrophic hormone (ACTH) stimulation of plasma cortisol in prairie dogs, and successful validations presented in other sciurid rodents (e.g. Boonstra et al., 2001; Bosson et al., 2009; Hare et al., 2014; Mastromonaco et al., 2014) measurement of accumulated stress responses from hair and fecal samples should not be discounted. Rather, my research serves as a caution, highlighting the multitude of influences that affect cortisol levels, which may obscure direct observation of cortisol produced in response to exogenous ACTH administration. The factors that likely had a direct impact on my results were the stress of capture, transportation and housing for fecal cortisol metabolites, and the physiological demands of lactation, particularly for cortisol measured in hair.

I used the cort-fitness hypothesis as a framework for my inquiry, as high risk of predation has been shown to increase GC levels in other species and drive a decrease in reproductive success (Boonstra et al., 1998). In my study, I predicted that prairie dogs on the edge of the colony would perceive higher risk than their interior counterparts, demonstrating higher hair cortisol, and that cortisol accumulated in the non-reproductive season would negatively affect reproductive success. My research indicated that while prairie dogs who live on the edge of the colony do spend more time vigilant than interior prairie dogs, ostensibly searching for predators, this increase in vigilance does not translate to a measurable difference in hair cortisol between edge and interior individuals.

In addition to location, I also tested other potentially relevant influencers of hair cortisol, and found that year, season, and lactation, as well as the interaction of age class and lactation impact hair cortisol. My results also show that there is individual repeatability in cortisol levels over time, with some individuals exhibiting consistently higher cortisol levels and some consistently lower across multiple contexts.

4.2 Limitations

I have identified two limitations in my research. First, the use of wild-caught prairie dogs from a protected population likely introduced too many non-target variables for a successful validation, an acknowledgement that is directly supported by the significant effect of lactation on hair cortisol demonstrated in Chapter 3. Second, at the outset of research I expected that prairie dogs would regrow hair from a shaved patch, resulting in a sample from a precise window of time, which I could schedule to coincide with pertinent times in the annual reproductive cycle for this species. However, I discovered that prairie dogs only grow hair during the moulting periods in spring and fall. Although I initially considered this fact to be a limitation, the evidence for

individual repeatability in cortisol levels supports this as positive feature, ultimately allowing me to draw stronger conclusions based on fewer samples.

4.3 Future Directions

This research highlighted three areas of interest that warrant further investigation. First, the high rate of successful reproduction in yearling females is unusual, compared to other populations of this species. Determining whether the difference is simply due to the favourable conditions during which my study took place, or some factor inherent to this population would enhance our understanding of the specific drivers of population growth in prairie dogs range-wide. Despite being considered threatened in Canada (COSEWIC, 2011), this species is regarded as a pest throughout the majority of its range (Protocor et al., 2006). A thorough understanding what drives or limits population growth would be valuable to both those who wish to support and those who wish to restrict it.

Because an understanding of the drivers of prairie dog population dynamics is extremely valuable to conservation of the species, I deliberately structured my research around female prairie dogs to definitively link potential explanatory variables to reproductive success at the individual level. The interaction between risk, cortisol and reproduction in male prairie dogs should also be investigated. Inter-sex difference in cortisol levels are likely, given evidence from other ground squirrel studies (Bosson et al., 2009). The repeatability of cortisol in males compared to females might be a more intriguing direction of research, as at least one study suggests that there may be differences in repeatability between sexes (Adcock et al., 2015), prompting the question of whether this divergence is demonstrated in other species as well, and further, what the adaptive significance of a potential difference might be.

Vulnerability to predation may vary by reproductive status in prairie dogs, with pregnant females less able to run to escape predators, and reproductive males distracted from vigilance (Hoogland et al., 2006). Because I included unmarked individuals in my comparison of vigilance, I was not able to control for these variables. Considering the effects of sex and reproductive status on vigilance would be an interesting future direction that could be explored in this well-marked study colony.

In my study, prairie dogs were resilient to the perceived risk of predation. Given the amount of alarm calls uttered on the study colony in comparison to observations of predators, perceived risk is either higher than actual risk, or alarm calls are an extremely effective defense strategy. It is likely that both are true. To augment my research, investigation of how actual risk affects vigilance, cortisol and reproductive success compared to perceived risk would be valuable. Further knowledge in this direction would allow wildlife managers to determine how this resiliency might change under different levels of actual predation risk. Specifically, this knowledge would be a valuable tool in planning future reintroductions of the extirpated black-footed ferret (Tuckwell and Everest, 2009).

4.4 Broader Relevance

Over the course of my research the Canadian population of black-tailed prairie dogs proved to be resilient. Despite a trend of decline over the decade prior to my work, in the two years of my research this population steadily increased. Further, perceived predation risk does not seem to be a limiting factor, as individuals in the study were adapted to the level of risk in their home territory, and reproductive success was high, overall. Together, these traits suggest a positive future for the continuation of the species in supporting grassland biodiversity. This thesis helps to set a foundation of applying physiology to conservation of black-tailed prairie

dogs, which are themselves a foundation of the grassland ecosystem. With continuing research of the mechanisms that allow this species to ensure under persistent challenges, both anthropogenic and natural, we can efficiently support conservation of the grassland ecosystem as a whole.

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