Extrinsic and intrinsic factors impacting Cape ground squirrels' hair cortisol

by

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Abstract

Glucocorticoids (GCs) increase during the stress response, so they are commonly studied to measure the chronic stressors an animal experiences. However, GCs can also decrease in response to stimuli they are adapted to, also known as harsh conditions, as a mechanism to conserve energy. Because GCs are closely linked to fitness and survival, understanding how animals' GC levels respond to their environment is crucial. Therefore, this thesis aims to study the changes in hair cortisol, a common GC studied in mammals, of an arid-adapted, egalitarian social species, Cape ground squirrels (*Xerus inauris*), due to extrinsic and intrinsic factors. We also explored the impact of GCs on reproductive success in adult females. We first examined cortisol responses to temperature, rainfall, and group size in adult female Cape ground squirrels across eight years. We found that squirrels illustrate a decrease in cortisol with increasing temperature maximums, suggesting that Cape ground squirrels are adapted to hot temperatures (harsh conditions) that require a metabolic response. There was no significant relationship between hair cortisol and temperature minimums, rainfall, or group size. We then examined hair cortisol and its relationship with intrinsic factors and reproductive success in adult female Cape ground squirrels. Age, body condition, ectoparasite abundance, and prevalence of nits did not have a significant relationship with cortisol. We also found that decreases in cortisol were associated with a lower reproductive success rate, also supporting that cortisol in these squirrels reflects metabolic energy needed for reproduction. Cape ground squirrels are income breeders, so they spend energy on reproduction when it becomes available. Overall, our research supports the hypothesis that hair cortisol in Cape ground squirrels reflects their metabolism and allocation of resources rather than chronic stress. This research is particularly relevant in the context of climate change, as understanding the relationships between GCs, reproduction, and

environmental factors will become increasingly important as Cape ground squirrels' habitats continue to change.

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Contribution of Authors

The thesis is organized as a "sandwich" or grouped manuscript thesis with two research chapters: chapters 2 and 3. Following are the titles of the prepared research chapters, which have not been submitted to any journal, and the authors' contributions:

1. Chapter 2 contains a manuscript called “Extrinsic factors as harsh conditions for Cape ground squirrels: Adaptations of an arid-adapted social species”.

   Author contributions:

   Jenna Williams (Thesis author): Conceptualization, Visualization, Methodology, Software, Writing original draft; **Jane Waterman**: Conceptualization, Methodology, Supervision, Writing - review & editing; **Gary Anderson**: Conceptualization, Writing - editing

2. Chapter 3 includes a manuscript “Hair cortisol and its relationship with intrinsic factors and fitness in Cape ground squirrels”.

   Author contributions:

   Jenna Williams (Thesis author): Conceptualization, Visualization, Methodology, Software, Writing original draft; **Jane Waterman**: Conceptualization, Methodology, Supervision, Writing - review & editing; **Gary Anderson**: Conceptualization, Writing - editing

Chapter 1: Introduction

Glucocorticoids (GCs) are steroid hormones that are crucial to the functioning of reproduction, the immune system, and metabolism (de Guia et al., 2014; Defolie et al., 2020; Eberhardt et al., 2013; Edwards et al., 2019; Rabasa & Dickson, 2016; Reeder & Kramer, 2005). Basal GCs fluctuate throughout the day and seasonally (Reeder & Kramer, 2005). Glucocorticoids are also released above basal functioning after experiencing a stressor, or a disruption to homeostasis that activates the hypothalamic-pituitary-adrenal (HPA) axis, leading to elevated GCs (Reeder & Kramer, 2005). Acute stressors are short-term, such as a predator attack, that activate the fight-or-flight response and increase GCs to help the animal overcome the stressor (Sapolsky, 1992). Chronic stressors are long-term, such as droughts, that impair the HPA axis and lead to elevated GCs for long periods (Bauer et al., 2015). Therefore, GCs are often measured in blood, faeces, urine, and hair to quantify an animal's perception of both chronic and acute stressors (Heimbürge et al., 2019; Santymire et al., 2021). Both extrinsic and intrinsic factors influence GCs. There are some general trends found across species, such as high predation risk, older individuals, and animals with poor body condition illustrating elevated GC levels (Bizon et al., 2001; Boonstra et al., 1998; Dellu et al., 1996; Fokidis et al., 2023; Heimbürge et al., 2019; Robertson et al., 2023; Sapolsky et al., 1983; Wolf et al., 2018; Yin et al., 2017), but the effects of these factors on GCs are also species-specific with some studies producing contrary results (Dettmer et al., 2014; Fourie & Bernstein, 2011; Matzke et al., 2022; van Kampen & Fuchs, 1998).

Extrinsic stressors can include environmental variables such as extreme temperature and rainfall. However, Schradin et al. (2023) and Love and Wagner (2023) argue that environmental variables only increase GCs when an animal is not adapted to those conditions, which are termed stressful conditions. Stressful conditions lead to increases in GCs with the activation of the HPA

axis to increase energetic expenditure to be able to overcome the conditions (Schradin et al., 2023). Schradin et al. (2023) argue that there can be an alternative response of GCs to extreme conditions if the animal is adapted to these conditions, which they describe as harsh conditions. Harsh conditions lead to decreases in GCs with the inhibition of the HPA axis to conserve energy and endure conditions (Schradin et al., 2023). During these conditions, GCs reflect their metabolic functioning and allocation of resources rather than the stress response.

It is important to understand the differences between a response to harsh versus stressful conditions because they can impact reproduction differently. Chronically elevated GCs due to long-term stressors have been correlated with reduced fitness in a variety of species (Boonstra et al., 1998; Edwards et al., 2019; Yin et al., 2017). Alternatively, in response to harsh conditions, GCs decrease below the basal functioning needed for successful reproduction (Edwards et al., 2019; Matzke et al., 2022). Therefore, examining the relationship between GCs and reproduction, and intrinsic and extrinsic factors is needed to gain an understanding of an animal's perception of its environment.

In this thesis, we examined the GCs of Cape ground squirrels (*Xerus inauris*) by studying tail hair cortisol, a type of GC commonly studied in mammals (Sapolsky, 1992). We have been trapping and handling Cape ground squirrels since 2002, adding to a database that allows us to examine long-term changes in intrinsic and extrinsic factors (O'Brien et al., 2021). As Cape ground squirrels are an arid-adapted social species endemic to South Africa (van Heerden & Dauth, 1987), they are an excellent candidate to test the alternative hypotheses of whether hot and dry weather conditions are considered harsh or stressful (Schradin et al., 2023). The objective of this thesis is to understand if hair cortisol in adult female Cape ground squirrels primarily reflects a response to stress or metabolic processes that influence the use of energy.

Due to these squirrels' adaptations to hot and dry environments (van Heerden & Dauth, 1987), we predicted that Cape ground squirrels will support the metabolic perspective on hair cortisol as their adapted environments will be considered harsh. This thesis examines potential extrinsic factors that could influence cortisol (Chapter 2), as well as possible intrinsic factors and cortisol's impact on reproductive success in Cape ground squirrels (Chapter 3).

In Chapter 2, we examined the effects of rainfall and temperature on Cape ground squirrel cortisol levels, specifically exploring alternative hypotheses about whether these environmental factors are experienced as harsh or stressful conditions. Additionally, these squirrels are a highly social species with a unique social structure that has no dominance or aggression (Herzig-Straschil, 1978; Manjerovic et al., 2022; Waterman, 1995). As many studies examining GCs in social species often include a hierarchical structure that can confound the effects of extrinsic and intrinsic factors, like group size (Creel et al., 2013; Dettmer et al., 2014; Kutsukake & Clutton-Brock, 2006; Robertson et al., 2023; Rubenstein, 2007), these squirrels offer a unique opportunity to study GCs in an egalitarian species. Therefore, we also investigated the influence of group size on cortisol, examining how social structure might buffer the impact of environmental conditions on this egalitarian social species.

In Chapter 3, we examined the possible effects of age, body condition, and parasitism on the hair cortisol of Cape ground squirrels. Additionally, female Cape ground squirrels have a low reproductive success rate with no dominance or reproductive suppression occurring in adult females (Waterman, 1995, 1996, 2002). As GCs are related to reproductive success in other rodent species (Charbonnel et al., 2008; Ebensperger et al., 2011, 2013; Matzke et al., 2022), we also analyzed competing hypotheses about how metabolic versus stress-induced responses in cortisol could influence reproductive success in adult female squirrels.

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Chapter 2: Extrinsic factors as harsh conditions for Cape ground squirrels: Adaptations of an arid-adapted social species

Abstract

Glucocorticoids (GCs) can be influenced by factors like rainfall and temperature, making it essential to understand how climate change affects these hormones. However, whether GCs respond to extreme conditions through a stress response—characterized by elevated GCs and increased energy expenditure—or a metabolic response that conserves energy by reducing GCs, is controversial. To explore these competing hypotheses, we studied the influence of environmental factors on GCs in an arid-adapted rodent in South Africa, the Cape ground squirrel (*Xerus inauris*). We hypothesized that if GCs reflect a stress response, higher temperatures and lower rainfall would correlate with increased GCs; conversely if GCs indicate a metabolic response, we anticipated a decrease in GCs under the same conditions. As Cape ground squirrels are social animals, we also investigated the influence of group size on GC levels, predicting an optimal group size that would yield the lowest GCs in intermediate-sized groups. We analyzed hair samples from 272 adult females collected between 2015-2019 and 2021-2023. We employed linear mixed-effects models and found a significant negative correlation between average maximum daily temperatures and hair cortisol levels. Minimum daily temperatures, rainfall, and group size did not significantly affect cortisol levels. Our findings support the hypothesis that increased temperatures are harsh conditions, but not stressors, for these arid-adapted squirrels, leading to decreased cortisol as an energy-saving mechanism. As Cape ground squirrels exhibit low reproductive success, understanding how climate change impacts their GCs and overall fitness is crucial.

2.1 Introduction

Environmental conditions, such as predation risk, food availability, temperature, and rainfall, can act as stressors, or stimuli that disrupt homeostasis (Reeder & Kramer, 2005), for a variety of species (De Bruijn & Romero, 2018). Animals respond and adapt to these stressors behaviourally, morphologically, and physiologically (Wingfield, 2013). However, climate change is impacting the environment by raising temperatures and causing more fluctuations in rainfall patterns (Warrington & Waterman, 2022; Zhang et al., 2024). As climate change accelerates, it is imperative to understand how these changes in environmental conditions are affecting an animal's ability to respond to extrinsic stressors.

An animal's response to a stressor begins in the sympathetic nervous system (SNS). Within seconds, the SNS releases a variety of catecholamine neurohormones leading to increased heart rate, blood pressure, and respiration (Reeder & Kramer, 2005). At the same time, the stressor also initiates the hypothalamic-pituitary-adrenal (HPA) axis (Reeder & Kramer, 2005; Sapolsky, 1992; Sapolsky et al., 2000). The HPA axis begins in the hypothalamus with the paraventricular nucleus producing and releasing the neuropeptide corticotropin-releasing hormone (CRH), which travels through a closed circulatory system to stimulate the pituitary gland to release adrenocorticotropic hormone (ACTH) (Gjerstad et al., 2018; Reeder & Kramer, 2005). ACTH then travels through the bloodstream to the adrenal glands, which sit above the kidneys. ACTH stimulates the melanocortin 2 receptors on the adrenal gland, which initiates steroid synthesis through a cascade of enzyme-mediated processes that convert cholesterol to glucocorticoids (GCs) (Spencer & Deak, 2018). After a few minutes, the adrenal glands release GCs into the bloodstream. Glucocorticoids are steroid hormones that bind to corticosteroid-binding globulin in the bloodstream and perform many roles critical to metabolism, including during the stress response (Reeder & Kramer, 2005; Spencer & Deak, 2018). Glucocorticoids

influence energy metabolism by binding to receptors in the liver, adipose tissue, and muscles to stimulate processes like gluconeogenesis, glycogenolysis, and lipolysis that maintain blood glucose levels (de Guia et al., 2014; Rabasa & Dickson, 2016). The activation of the HPA axis occurs over a few minutes and can remain active for longer periods. Glucocorticoids also bind to receptors in the hypothalamus and pituitary gland to act as a negative feedback loop to down-regulate the production of CRH and ACTH and return to a state of homeostasis (Reeder & Kramer, 2005; Sapolsky et al., 2000).

Glucocorticoids can be measured using an animal's blood, urine, faeces, and hair (Heimbürge et al., 2019; Santymire et al., 2021). Blood, urine, and faeces are common methods of analyzing the effects of acute stressors as the measured GCs reflect a timespan of a few minutes to a few days before collection (Heimbürge et al., 2019). Hair is commonly used to measure long-term stressors as GCs enter the hair through passive diffusion while the hair is growing during the active growth phase (Crill et al., 2019; Heimbürge et al., 2019). The GC levels measured from hair reflect the integrated GCs that the animal was experiencing while the hair was growing.

There is ongoing debate regarding whether the patterns observed in long-term GCs are a response to stressful or harsh environments. Schradin et al. (2023) suggest that when an animal is experiencing a stressful environment, the animal activates the physiological stress response to increase energy expenditure. When the stressor occurs over a long period, it can be categorized as a chronic stressor that has negative consequences on an animal's survival and fitness (Reeder & Kramer, 2005; Sapolsky, 1992). An animal experiencing chronic stressors can have an impaired negative feedback loop due to a decrease in GC receptors in the brain, leading to the HPA axis remaining active for extended periods and elevated GCs (Bauer et al., 2015; Romero,

2004). Chronic stressors can impact the survival of an animal, such as through decreased immune functioning and greater parasite infections (Defolie et al., 2020; Eberhardt et al., 2013). Chronic stressors have also been correlated with decreased reproductive success in a variety of species (Bauer et al., 2015; Ebensperger et al., 2013; Endo et al., 2018). Additionally, long-term changes in environmental conditions can act as a chronic stressor. Changes in temperature, rainfall, food availability, and predation risk have been associated with elevated GCs in a variety of species (De Bruijn & Romero, 2018; Yin et al., 2017). Therefore, increased GC levels due to chronic stressors are important to study in any species as they can impact an animal's survival and fitness.

Alternatively, Schradin et al. (2023) suggest that environments can be considered harsh rather than stressful conditions depending on an animal's adaptations. Glucocorticoids have important roles in glucose, lipid, and protein metabolism (de Guia et al., 2014; Rabasa & Dickson, 2016). Increases in GCs are often associated with increases in energetic expenditure outside of the stress response. In response to harsh conditions, animals that are adapted to these conditions will inhibit their physiological stress response to decrease GCs and conserve energy (Love & Wagner, 2023; Schradin et al., 2023). Harsh conditions refer to long-lasting environmental changes, such as drought, that are highly species-specific. Schradin (2008) found that African striped mice (*Rhabdomys pumilio*), a species adapted for long seasonal droughts with decreased food availability, had lower GCs during the dry season compared to the wet season. As climate change is accelerating, especially in habitats that experience extreme conditions, it is imperative to understand how the GCs of species adapted to extreme environments are responding to long-term climatic changes.

Group size is another extrinsic factor that affects GCs in a variety of social species. Large group sizes in great gerbils (*Rhombomys opimus*) are related to increased GCs due to increased competition for resources, such as food or breeding opportunities (Rogovin et al., 2003). Small group sizes, on the other hand, are correlated with fewer vigilance behaviours and higher predation risk, also leading to increased GC levels (Blondel et al., 2016; Quenette, 1990; Roberts, 1996). Therefore, GCs in social species can be affected by social stressors, such as disputes over resources involving agonistic behaviours (Rogovin et al., 2003; Blanchard et al., 2001). Pride (2005) found that ring-tailed lemurs (*Lemur catta*) exhibited an optimal group size or a group size that yielded the maximum fitness of an animal in a given environment. Lemurs in intermediate group sizes showed the lowest GC levels compared to large or small group sizes. As GCs are strongly related to fitness in other species, intermediate group sizes were considered the optimal group size for ring-tailed lemurs (Pride, 2005).

Abiotic factors can also affect the social structure of a species and its relationship with GCs. For example, the optimal group size in ring-tailed lemurs with the lowest GCs is smaller in areas where food is scarce (Pride, 2005). Therefore, group size may represent a potential mechanism affecting social species' resilience to climate change (Blumstein et al., 2023; Komdeur & Ma, 2021). Many studies examining this interaction on GC levels include species with a hierarchical structure, such as dominant and subordinate individuals showing different GCs during varying environmental conditions (Creel et al., 2013; Rubenstein, 2007). This confounding variable makes it difficult to clearly understand how group size and the environment interact to affect GCs. Therefore, investigating the interaction between group size and environmental changes on GCs in social species without a hierarchical structure is crucial to better understanding changing stressors due to environmental factors.

Cape ground squirrels (*Xenus inauris*) are an arid-adapted species living in egalitarian social groups of 2-25 individuals (Herzig-Straschil, 1978; Waterman, 1995). Family groups consist of adult females, subadults (immature animals) of either sex and adult males that have not yet dispersed (Waterman, 1995). Male bands are groups that consist only of dispersed adult males, and bands live independently of female groups. As this species has no dominance structure and aggression is extremely rare (Manjerovic et al., 2022; Waterman, 1995), these squirrels are a good model to study the relationship between GCs and group size without the effects of a hierarchical structure.

Cape ground squirrels in a South African population are adapting morphologically to long-term environmental changes. Hindfoot length has increased and spine length has decreased with increasing ambient temperatures over the past 18 years (Warrington & Waterman, 2022). Additionally, more variable rainfall may impact group sizes and male survival. Adult males are more likely to disperse from their family groups during years with higher annual rainfall (Warrington et al., 2022). Band males also exhibit decreased body condition, decreased white blood cell differentials, and greater ectoparasite abundance during years of low rainfall (O'Brien et al., 2021). Additionally, Cape ground squirrels in Namibia exhibit decreased reproductive success during years of extreme drought (Waterman & Fenton, 2000). These results suggest that Cape ground squirrels are responding to changing environmental conditions, but it is unclear if extreme environmental conditions are chronic stressors for these squirrels, or if they are conditions that squirrels have evolved to tolerate (harsh) (Love & Wagner, 2023; Schradin et al., 2023).

If GCs in Cape ground squirrels represented responses to stressful environments, we expected increased GCs with increasing temperatures and low rainfall. If GCs in Cape ground

squirrels represented responses to harsh conditions, we expected decreased GCs with increasing temperatures and low rainfall. We also hypothesized that large group sizes lead to an increase in competition for resources, and small group sizes are more vulnerable to predation due to lower group vigilance (Edwards & Waterman, 2011; Waterman, 2002). We predicted that we would see intermediate group sizes (4-10) exhibiting the lowest cortisol values compared to small (2-3 individuals) and large group sizes (>10 individuals). Finally, we hypothesized that there would be an interaction between environmental conditions and the optimal group size on cortisol values. We expected that intermediate group sizes would mitigate the effects of extreme long-term changes in temperature and rainfall on cortisol, leading to lower cortisol values during harsh/stressful conditions compared to larger and smaller group sizes.

2.2 Methods

2.2.1 Study Site

We studied squirrels at SA Lombard Nature Reserve in central South Africa (27^{o} 36'S, 25^{o} 28'E), where Cape ground squirrels have been studied since 2002 (O'Brien et al., 2021). We collected group composition using focal animal and scan observations on female social groups using binoculars or spotting scopes (Altmann, 1974). Family groups were defined by who slept in the same social group together. As Cape ground squirrels are diurnal, our observations focused on group emergence in the morning and immergence in the evening (Herzig-Straschil, 1979). We focused our analysis on adult females as females are easier to follow throughout their lifetimes compared to males, who disperse from their families sometime after maturity. Subadults were not included in our analysis because unmatured individuals in a variety of species have different GCs compared to adults, and we wanted to focus on overall patterns due to extrinsic factors (Azevedo et al., 2019; Heimbürge et al., 2019). Female Cape ground squirrels are philopatric and

live with their family group or an adjacent area (Waterman, 1995, 2002). Thus, we knew their age in years, as squirrels were followed annually, except for 2020. Data were not collected in 2020 due to the COVID-19 pandemic.

Daily rainfall (mm) measurements on the site were collected by reserve personnel. We used daily minimum and maximum temperature (°C) data from the South African Weather Service's Bloemhof weather station (15.5 km from SA Lombard Nature Reserve, Warrington & Waterman, 2022). For cases where temperature data was unavailable from the South African Weather Service (7-31 March 2017, 11-24 February 2018, 1-11 January 2023), we used the temperature data collected daily at the reserve using a digital maximum/minimum thermometer (Houkiper, Shenzhen DianLan Technology Co.China).

2.2.2 Trapping and Handling

We trapped squirrels using Tomahawk live traps (Tomahawk Live Trap Inc., Hazelhurst, WI; 15 X 15 X 50 cm) that we baited with peanut butter and bird seed (Waterman, 1995). We deployed traps 2-8 times a day between 08:00 and 17:30 every day of the week, and we checked traps for squirrels every hour (Waterman, 1995). The majority of trap data was collected between May and August during each data collection year.

During handling, we identified each known squirrel using PIT tags and unknown squirrels were given a PIT tag upon first capture (Shenzhen XCC RFID Technology Co., Ltd. China). Each squirrel is also given a dorsal black dye mark (Rodol D dye; Lowenstein and Sons, Inc., New York, NY; Melchior & Iwen, 1965) for their first capture of the season to be able to identify squirrels during behavioural observations. We collected each squirrel's body mass (g) using a spring scale (Pesola AG, Switzerland) and spine length (cm) using a tape measure during every capture. We measured spine length from the base of the neck to the base of the caudal vertebrae. We calculated body condition using the size-mass residuals of the ordinary least

squares regression between log-transformed spine length and log-transformed body mass (Manjerovic & Waterman, 2012; Schulte-Hostedde et al., 2005). Spine length and body mass were log-transformed to achieve linearity and normality of the residuals. Individuals were sexed by visually examining their genitalia. We also placed each squirrel into an age category: subadult, or adult. Adult females were distinguished from subadult females by elongated nipples as nipples swell after the first estrus and remain swollen for the life of the squirrel (Waterman, 1996). All procedures were approved by the University of Manitoba's Animal Care and Use Committee (##F14-032, F18-039, F23-003) and followed the guidelines of the American Mammal Association (Sikes, 2016).

2.2.3 Hair Collection and Cortisol Analysis

Using hair to measure GCs is an easy and non-invasive method compared to other methods, such as blood sampling (Heimbürge et al., 2019; Sergiel et al., 2020). The most common GC for mammal hair is cortisol (Sapolsky, 1992).

On our study site, Cape ground squirrels moult and regrow the hair on their tails between the end of January and March every year (Herzig-Straschil, 1978). Therefore, cortisol levels reflected a timespan of no more than two months during the moulting period. This timeline also coincides with the main rainfall period of the site (Herzig-Straschil, 1978). During this growth period, there were no researchers present on the study site, and the few reserve personnel rarely accessed the area.

All hair samples were collected by trimming hair at the base of the tail with scissors. Tail hair samples were collected annually from every squirrel upon their first capture for the season from 2015 to 2019 and 2021 to 2023. All samples were collected between May and July each year except for hair samples collected in 2021, which were collected in September and October as the field season was delayed due to COVID-19. Hair samples were stored in plastic bags and

frozen at -20°C until prepared for analysis, as storing samples in a dark, dry environment is recommended (Macbeth et al., 2010).

We prepared hair samples for cortisol analysis using protocols described by Macbeth et al. (2010). Samples were first washed three times with 10 mL of methanol per 100 mg of hair to ensure that the hair was free from contaminants. After washing, we set out hair samples to dry on a countertop for three days before grinding the hair into a fine powder using a Retsch ball mill for 0.03 min/mg at 30 Hz. To begin the extraction process, we added 1 mL of methanol to 50 mg of hair powder and set the sample in a shaker table for 24 hours at room temperature with slow rotation. The samples were then centrifuged for 15 minutes at 4500 rpm and the supernatant was collected. Another 1 mL of methanol was added to the hair powder, vortexed and centrifuged, and we collected the supernatant. That process was repeated one more time, resulting in a total of 3 mL of supernatant collected. We evaporated samples using a concentrator (Savant ISS110 SpeedVac Concentrator, Thermo Scientific, Waltham, MA) and samples were frozen at -18°C until radioimmunoassays (RIAs).

We performed RIAs following the methods of Ryan et al. (2012) and using tritium-labelled cortisol and cortisol-specific antibody (IgG-F-2) diluted to 1:15,000. We reconstituted samples with 240 µL of RIA buffer. We added 100 µL of each reconstituted sample (or known dilution of cortisol for standards), 100 µL of antibody, and 100 µL of [3H]-cortisol tracer together and incubated the samples for one hour at room temperature, followed by 12-24 hours at 4°C. After incubation, we added 100 µL of separate buffer (dextran-coated charcoal) and left it on ice for 15 minutes, followed by centrifuging at 2500 rpm for 20 minutes at 4°C. We poured the supernatant into scintillation vials and added 2 mL of scintillation fluid. We measured the radioactivity in counts per minute for 5 minutes per sample using a scintillation counter

(LS6500; Beckman Coulter, Brea, CA). Lastly, we determined the absolute values of cortisol by adding the values calculated by the scintillation counter to a standard curve and adjusting for 76.1% extraction efficiency. Samples were re-analyzed if the sample duplicates had a coefficient of variation (CV) >25%. Interassay variation was 16.2% CV and intra-assay variation was 7.9% CV. Samples demonstrated good parallelism.

2.2.4 Statistical Analysis

We completed RIAs for a total of 272 samples from 147 individuals and 100 social group IDs from 2015-2019 and 2021-2023. We chose samples if they were an adult female with a known age and if there was enough hair to complete the RIA (at least 100 mg of hair).

We analyzed data using linear mixed-effects models in R Studio 4.2.1 using the lme4 package (Bates et al., 2024). We used weighted Akaike information criterion (AICc) values to choose the most parsimonious model from a set of candidate models with log-transformed cortisol values as the response. We included average daily temperature minimum and maximum, daily temperature minimum and maximum variability, total daily rainfall, daily rainfall variability, and group size as fixed effects. The average daily temperature min/max and total rainfall were calculated between the beginning of January and the end of March each year to coincide with the tail hair growth period. The standard deviation of daily rainfall and daily temperature minimum and maximum between January and March measured variability. We also included interactions between group size and total rainfall, rainfall variability, average daily temperature minimum and maximum, and daily temperature minimum and maximum variability. We accounted for age and body condition as fixed effects because previous research has illustrated that age and body condition can impact GC levels (Heimbürge et al., 2019). Random effects included individual ID, social group ID, and year to account for possible variability

between social groups or individuals, or across data collection years. We scaled all continuous variables to have the same units for easier interpretation of the results.

While storage time did not affect cortisol in grizzly bear (*Ursus arctos*) hair samples stored over a year (Macbeth et al. 2010), a longer duration of storage time had a significant relationship with hair cortisol in Egyptian mongoose (*Herpestes ichneumon*), with older samples having lower GCs (Azevedo et al. 2019). We analyzed the effects of storage time on cortisol values by running a linear mixed-effects model. Log-transformed cortisol values were the response with data collection year as the fixed effect. We included squirrel ID and social group ID as random effects. If the year of sample collection significantly influenced cortisol values, we would include it as a random effect in all other models.

We evaluated the homogeneity of variance, linearity, and normality of residuals for the top models visually using residual plots and Q-Q plots. We evaluated the normality of random effects using Q-Q plots and Shapiro-Wilks tests. The normality of the residuals was achieved after log-transforming the cortisol values. To avoid multicollinearity, we sequentially removed variables from the full model by deleting the variables with the highest variance inflation factor (VIF) until all variables had a VIF below 3 (Zuur et al., 2010).

Data are presented as means ± standard error unless otherwise stated. Significance was accepted at $\alpha = 0.05$.

2.3 Results

Average cortisol values after adjusting for extraction efficiency were 11.8 ± 0.5 ng cortisol/g hair, with the values ranging from 1.4 ng/g to 52.3 ng/g before log transformation (n = 272). The average age of an adult female was 3.0 ± 0.1 years, with the youngest female being one year old and the oldest being 11 years old. The average daily rainfall from January to March

across the eight data collection years was 3.2 ± 8.5 mm, ranging from 0 mm to 85 mm. The average daily temperature maximum from January to March each year was 32.2 ± 0.2°C, ranging from 18.6°C to 43.7°C, and the average daily temperature minimum was 15.6 ± 0.1°C, ranging from 4.0°C to 25.6°C. After excluding samples with unknown group sizes, the average group size from 50 social groups was 4.8 ± 0.2 individuals, with the smallest group size consisting of 1 squirrel and the largest group size being 12 squirrels.

The linear mixed effect model examining storage time revealed that year did have a significant positive relationship with hair cortisol ($t = 7.45$, $df = 171.72$, $p < 0.001$). Therefore, we included year as a random effect in all subsequent models to account for any variation across years, including storage time.

Of the possible 272 samples, we only knew the family group size of 132 samples. Group size had no significant relationship with hair cortisol ($t = -0.65$, $df = 119.05$, $p = 0.52$). We also found no significant interaction between group size and average daily rainfall ($t = -0.15$, $df = 122.85$, $p = 0.88$), daily rainfall standard deviation ($t = 0.25$, $df = 121.05$, $p = 0.80$), average daily temperature maximum ($t = 0.90$, $df = 126.73$, $p = 0.37$), the standard deviation of daily temperature maximum ($t = 1.50$, $df = 116.12$, $p = 0.14$), average daily temperature minimum ($t = 0.32$, $df = 107.07$, $p = 0.75$), and the standard deviation of daily temperature minimum ($t = 0.91$, $df = 121.22$, $p = 0.37$). Due to the decreased sample size and lack of significant relationships with hair cortisol, we excluded group size and its interactions and generated 21 new models with all 272 samples (Appendix A).

To avoid multicollinearity, we removed daily temperature minimum standard deviation (VIF = 14.06), total daily rainfall (VIF = 5.27), and daily rainfall standard deviation (VIF = 3.48) from all models. The remaining variables had VIF values between 1.1 - 1.7. When examining

these variables in separate models, we found squirrel hair cortisol was not related to total daily rainfall ($t = 0.84$, $df = 5.86$, $p = 0.44$), the standard deviation of daily rainfall ($t = 0.36$, $df = 5.99$, $p = 0.73$), or the standard deviation of temperature minimum ($t = -1.90$, $df = 6.17$, $p = 0.11$).

The best-selected model by AICc was the model only including average daily temperature maximum and random effects (Table 2.1). Average daily temperature maximum has a significant negative relationship with log-transformed cortisol (Table 2.2, Figure 2.1).

The second best-selected model also included the standard deviation of daily temperature maximum. This model had a ΔAICc > 2, but it was ranked higher than the null model (Table 2.1). We found a weak negative trend between temperature maximum standard deviation and log-transformed cortisol (Table 2.3, Figure 2.2). The null model was ranked higher than the remaining 19 models. When examining the full model, we found that the average daily temperature minimum, age, and body condition did not have a significant influence on hair cortisol (Table 2.4).

2.4 Discussion

We found no relationship between daily rainfall and hair cortisol in Cape ground squirrels, partially rejecting both of our alternative hypotheses. In Namibia, Cape ground squirrels had no reproductive output during a two-year drought in the Kalahari (Waterman & Fenton, 2000). Based on these findings and other research linking GCs and reproductive success in other rodent species (Ebensperger et al., 2013), we predicted GCs would be influenced by rainfall. In previous research with *Gracilinanus agilis*, a marsupial living in the tropical climates of Brazil, GCs increased during the dry season, suggesting that low rainfall is a stressful condition for this species as they are not adapted to arid conditions (Hernandez et al., 2018). Alternatively, research with African striped mice, a species adapted to low rainfall conditions,

found that GCs decrease during the dry season as droughts are not unusual conditions for these mice. The hair cortisol in Cape ground squirrels was not associated with rainfall, suggesting that low rainfall is neither a stressful nor harsh condition for these squirrels. Although the average rainfall is 510 mm at our study site, lower annual rainfall amounts are common (Herzig-Straschil, 1979; LaFlèche & Waterman, 2020), and the winter season is a prolonged period of cold, dry weather. As a result, these squirrels do not perceive low rainfall as a stressor, in contrast to *G. agilis*, which inhabits high annual rainfall areas where prolonged dry periods are not the norm (Hernandez et al., 2018; Schradin et al., 2023). However, unlike African striped mice, these squirrels also do not react to low rainfall with a decrease in GCs. The study site for the African striped mice experiences lower annual rainfall than our South African site (Schradin, 2008). Additionally, the Cape ground squirrels from the Kalahari that reduce reproduction during droughts have smaller body sizes and experience significantly lower rainfall than our study site in central South Africa (LaFlèche & Waterman, 2020; Waterman & Fenton, 2000). Therefore, the lack of a relationship between rainfall and hair cortisol in female Cape ground squirrels could be due to our semi-arid study site in South Africa experiencing less extreme low rainfall and droughts compared to other study sites in the Kalahari. Future research can examine how the differences in rainfall across the different study sites affect cortisol in these squirrels.

Previous research with other rodent species has also illustrated that GCs can fluctuate seasonally (Amirat et al., 1980; Matzke et al., 2022; Schradin, 2008). Our rainfall measurements and cortisol values only reflected the period when the hair was actively growing, which occurred during the wet season (Herzig-Straschil, 1979). While our research illustrated no significant differences in cortisol with changes in daily rainfall during the wet season, future research could compare cortisol levels between the wet and dry seasons to gain a better understanding of how

Cape ground squirrels respond to varying rainfall conditions. However, hair only grows during the moulting period, so hair cannot be used to analyze long-term seasonal changes in GCs in these squirrels (Herzig-Straschil, 1979). Other studies often collect GCs multiple times a season with other collection techniques that provide short-term GC values, such as GCs in faeces or blood (Amirat et al., 1980; Heimbürge et al., 2019; Hernandez et al., 2018; Schradin, 2008). This method would have to be used to study the GC differences between the wet and dry seasons in Cape ground squirrels.

Cape ground squirrel hair cortisol is negatively correlated with average daily maximum temperature, which supports our hypothesis that Cape ground squirrels are adapted to harsh conditions in terms of temperature (Schradin et al., 2023). De Bruijn and Romero (2018) found that many species illustrate an increase in GCs with increasing temperatures, especially on a short-term basis, indicating that increasing temperatures can act as a stressor. However, the relationship between long-term temperature increases and GCs in other species is unclear, with results depending on the species and their adapted environment (De Bruijn & Romero, 2018). For example, American pikas (*Ochotona princeps*) in the Rocky Mountains show increased GCs with hotter summer temperatures (Wilkening et al., 2015). These pikas require cold climates, leading to hot temperatures acting as a stressor (Wilkening et al., 2015). Love and Wager (2023) mention that homeostasis-threatening environments must occur repeatedly in an animal's evolutionary history to be considered a harsh condition. Being endemic to southern Africa, Cape ground squirrels are adapted morphologically, behaviourally, and physiologically to hot climates (van Heerden & Dauth, 1987). These squirrels have small bodies and large hind feet to aid in heat dissipation (Warrington & Waterman, 2022). To decrease sun exposure and heat load, these squirrels use their long tails as parasols and frequently return to their burrows during sunny days,

and these behaviours are seen more frequently during the hot summer months (Fick et al., 2009; van Heerden & Dauth, 1987). The kidneys of these squirrels have also evolved to be extremely efficient in conserving water as the medullary thickness of the kidneys is very high and they produce very concentrated urine, especially during the dry season (Marsh et al., 1978). Additionally, January and February are summer months in South Africa, so these squirrels are experiencing seasonal hot temperatures during the moulting period. Therefore, the decrease in hair cortisol with increasing temperatures suggests that cortisol is another adaptive mechanism that conserves energy during hot environments, indicating that high temperatures are considered harsh conditions for Cape ground squirrels.

There is a lack of literature examining increasing temperatures as a harsh condition for arid-adapted species for comparison with our results. Other studies examine seasonal differences in GCs in arid-adapted species between the summer and winter months. For example, male sand rats (*Psammomys obesus*) illustrate decreased GCs during the summer months compared to the winter months (Amirat et al., 1980). African striped mice have decreased GCs and daily energetic expenditure during the hot and dry season (Rimbach et al., 2018, Schradin, 2008). However, female baboons (*Papio cynocephalus*) show an increase in GCs during hotter months, whereas male baboons show no changes, possibly due to sex differences in thermoregulation adaptations or behaviour differences due to their dominance structure (Gesquiere et al., 2008, 2011). Many of these studies include a breeding and non-breeding season and individuals with varying breeding statuses, which can alter GCs responses to changes in environmental conditions across seasons (Amirat et al., 1980; Gesquiere et al., 2008, 2011; Rimbach et al., 2018, Schradin, 2008). Adult Cape ground squirrels breed year-round (Herzig-Straschil, 1978), so future studies

could more clearly examine changes in environmental conditions across seasons without the effects of a breeding season while using other GC collection methods.

There is also a consistent relationship between ambient temperature and metabolic rate across species, specifically a decrease in metabolic rate with increasing temperatures (Clarke et al., 2010). This relationship is also species-specific, with the same metabolic rate occurring in hotter temperatures for animals adapted to warmer climates and occurring in colder temperatures for animals adapted to cooler climates (Clarke et al., 2010; DeLong et al., 2018). In six desert rodent species, Vanjonack et al. (1975) found low plasma GCs and suggested that lower GCs are an adaptive thermoregulatory mechanism to decrease metabolic processes and conserve water. Therefore, integrated cortisol in Cape ground squirrel hair could reflect the metabolic functions of GCs, further supporting the idea that they are adapted to high temperatures (harsh conditions) that require energy saving and decreased metabolism. This relationship could further be studied by comparing metabolic rates with cortisol levels.

Cape ground squirrels are adapted morphologically, behaviourally, and physiologically to high-temperature maximums. Schou and Cornwallis (2024) suggest that adapting to increases in temperature may lead to issues with adaptation to minimum temperatures. For example, the larger hind feet with increasing temperatures in these squirrels aid in heat dissipation (Warrington & Waterman, 2022). Bigger hind feet can also decrease heat retention in colder climates (Schou & Cornwallis, 2024). Based on Schou and Cornwallis' (2024) research and the strong influence of average temperature maximums, we would expect hair cortisol to have a relationship with temperature minimums. However, we found no relationship between cortisol and daily temperature minimums in these squirrels. Temperature minimums reflect the ambient temperature at night when the squirrels are resting in their burrows (Herzig-Straschil, 1979).

Temperatures within the burrows barely fluctuate during the night, possibly explaining why we did not see a relationship between cortisol and minimum ambient temperatures (Herzig-Straschil, 1979). Although we did not find a significant relationship between cortisol and temperature minimums, it is important to continue researching behavioural, morphological, and physiological changes in these squirrels with greater changes in temperature extremes due to climate change.

Our results also illustrated a trend between hair cortisol and variability in daily temperature maximums in the second-best selected model, but it was a weak trend. More variable temperature maximums could also reflect harsh conditions for these squirrels, but the relationship was nonsignificant. Future research across more data collection years is needed to better understand this relationship, especially as temperature variability in extreme environments is increasing due to climate change (Murali et al., 2023).

Schradin et al. (2023) suggest that climate change will increase harshness. Models on climate change in the North West province of South Africa, where our study site is located, predict that average near-surface temperatures will increase by 4-6°C by 2080-2099 (Department of Environmental Affairs, 2018). The number of days above 35°C and the number of heat waves are also expected to increase in the near future (Department of Environmental Affairs, 2018). Therefore, these squirrels' environmental conditions are expected to become harsher, with harsh conditions lasting longer and having greater intensity in the future. This change could have detrimental effects on Cape ground squirrels as GCs are linked with reproductive success in a variety of species (Bauer et al., 2015; Endo et al., 2018; Ebensperger et al., 2011, 2013). Glucocorticoids have many important roles in reproduction (Edwards et al., 2019) and altered GCs due to environmental conditions can decrease reproductive success (Boonstra et al., 1998; MacLeod et al., 2018; Yin et al., 2017). Previous research on Cape ground squirrels found

increased resting metabolic rate and reproductive success rate after removing parasites, but there was no change in body mass, suggesting that these squirrels are income breeders that use their energy as they acquire it (Hillegass et al., 2010; Scantlebury et al., 2007). Therefore, during harsh conditions, these squirrels will decrease cortisol to conserve energy, rather than increase cortisol to spend energy they do not have. If cortisol in these squirrels is lowered as a response to harshness for longer periods, it can decrease the reproductive success rate as they are conserving energy rather than spending it on reproduction. Additionally, as these squirrels are ecosystem engineers, a dramatic decrease in fitness could also dramatically affect surrounding plant and animal communities (Ewacha et al., 2016, 2022).

We also found no relationship between group size and hair cortisol, which rejects the optimal group size hypothesis. Pride (2005) found that optimal group sizes exhibit low GCs in female ring-tailed lemurs, a species that follows a strict dominance structure which influences GCs in this species (Starling et al., 2010). Glucocorticoids differ in group-living versus solitary-living individuals in a variety of species, and the group-living species often also have hierarchical social structures (Robertson et al., 2023; Hill et al., 2021; Woodruff et al., 2013). The relationship between group size and GCs can be affected by the presence of a dominance hierarchy (Creel et al., 2013). Therefore, the lack of a dominance hierarchy in female family groups of Cape ground squirrels may explain the lack of a relationship between group size and hair cortisol. There could also be an influence of sex differences as many studies that found a relationship between group size and GCs include both sexes or focus on males (Blondel et al., 2016; Dantzer et al., 2017; Rogovin et al., 2003; Robertson et al., 2023), whereas we only examined adult females due to the social structure of Cape ground squirrels.

There was also no interaction between group size and the environmental conditions on cortisol in these squirrels. In these squirrels, larger group sizes have warmer body temperatures during cold nights (Scantlebury et al., 2012). Additionally, internal body temperature has been found to have a positive relationship with metabolic processes (Clarke et al., 2010). If there was a thermal benefit to living in larger groups for these squirrels, then we would expect there to be lower cortisol levels in larger groups, especially during lower temperature minimums. We would also expect higher cortisol in smaller groups during lower temperature minimums as individuals would have to generate body heat by increasing metabolic processes (Clarke et al., 2010), such as through increased cortisol (de Guia et al., 2014). However, we found no significant interaction between group size and temperature minimums on cortisol. One possible explanation is that we only examined minimum temperatures in the summer when the tail hair was growing, so perhaps minimums did not get cold enough. Another possibility is that minimum temperatures in burrows are buffered during the night (Herzig-Straschil, 1979). Since group size and temperature minimums are related (Scantlebury et al., 2012), it's logical that neither had an impact on hair cortisol. Therefore, GCs in these squirrels are not influenced by female family group sizes and their interactions with summertime environmental variables.

In conclusion, we found strong support for increases in daily temperature maximums, and no other environmental factors, influencing cortisol in adult female Cape ground squirrels. Due to the negative relationship between cortisol and higher temperatures, hair cortisol in these squirrels may reflect metabolic processes that save energy stores during increasing temperatures. Climate change is predicted to have a strong impact on extreme environments, including deserts, in the coming decades (Murali et al., 2023). Therefore, it is crucial to continue studying the relationship between changes in long-term temperature in arid-adapted species, such as the Cape

ground squirrel. Future research needs to explore the connection between cortisol and other factors affecting these squirrels to gain a better understanding of how this relationship between cortisol and harsh conditions can impact the survival and fitness of these ecosystem engineers.

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2.6 Figures

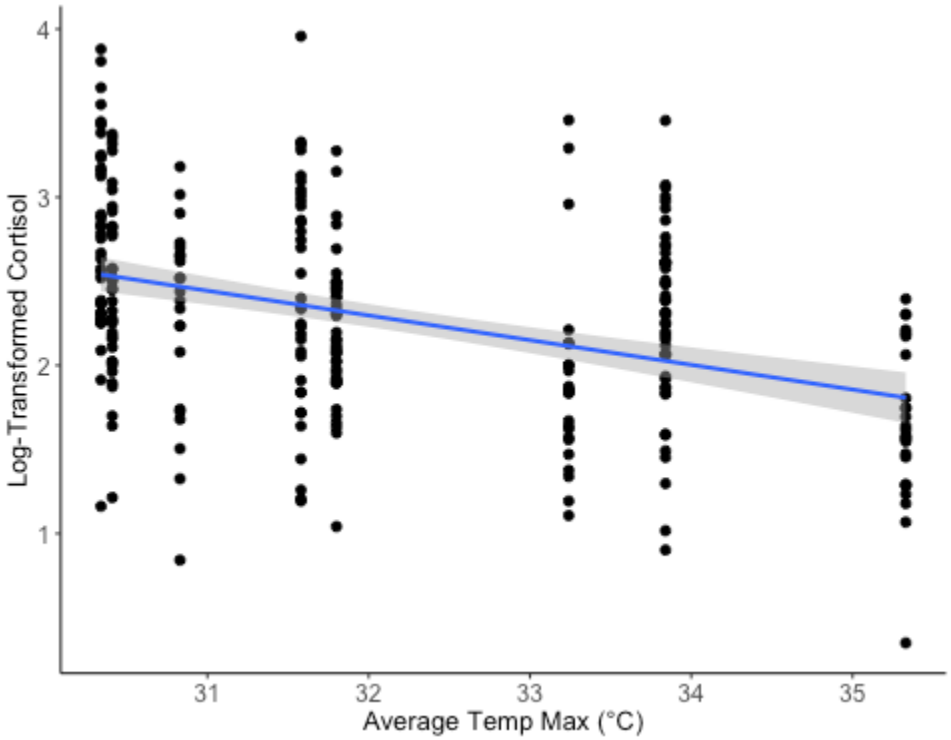


Figure 2.1. Scatterplot of daily temperature maximum averaged between the beginning of January to the end of March every year at S.A. Lombard Nature Reserve, South Africa versus log-transformed cortisol values. The blue line is the trendline and the gray is the 95% area confidence interval.

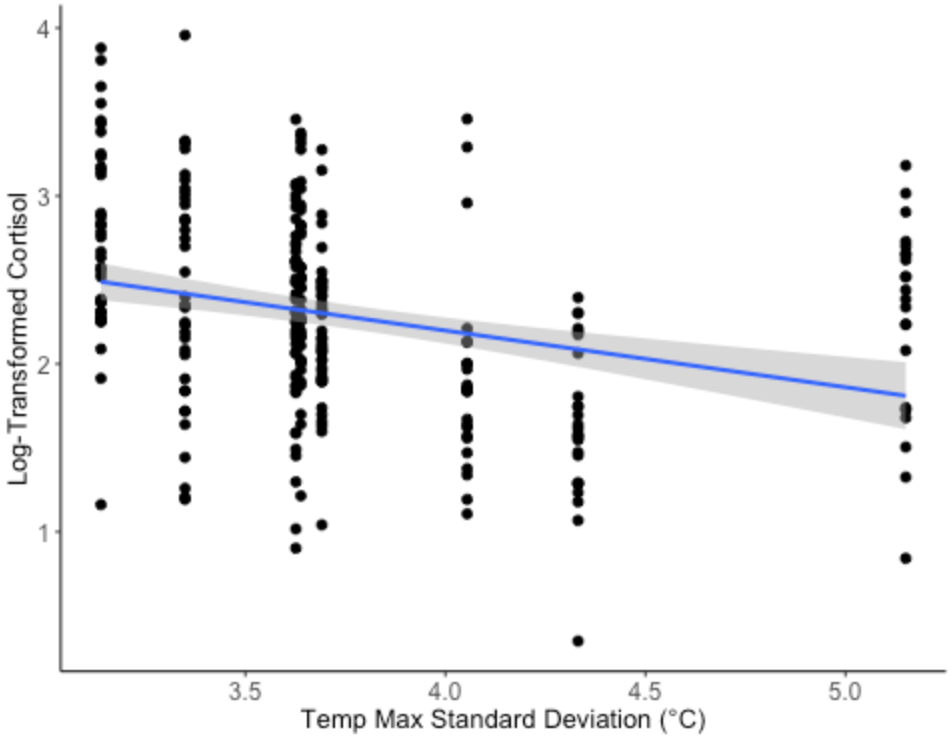


Figure 2.2 Scatterplot of the standard deviation of daily temperature maximum between the beginning of January to the end of March every year (2015-2023 excl. 2020) at S.A. Lombard Nature Reserve, South Africa versus log-transformed cortisol values of Cape ground squirrels. The blue line is the trendline and the gray area is the 95% confidence interval.

2.7 Tables

Table 2.1. Model selection for log-transformed cortisol in female Cape ground squirrels. Avg max refers to the daily temperature maximum averaged from the beginning of January to the end of March each year. Max SD refers to the standard deviation of daily temperature maximum from the beginning of January to the end of March each year. Random effects are squirrel ID, social group ID, and year: 2015-2023 (excl. 2020). Only the models ranked above the null model are shown.

Model	Terms	k	AICc	ΔAICc	AICc Wt
1	Avg Max + ID + Social Group ID + Year	6	472.16	0.00	0.53
2	Avg Max + Max SD + ID + Social Group ID + Year	7	474.65	2.49	0.15
3 (Null Model)	ID + Social Group ID + Year	5	475.15	3.00	0.12

Table 2.2. Estimated linear mixed effects model parameters, standard errors (SE), t-values, and p-values of fixed effects and variances and standard deviations (SD) of random effects for the log-transformed cortisol in adult female Cape ground squirrels from the best-selected model by AICc.

Fixed effects	Estimate	SE	t-value	p-value
Intercept	2.24	0.072	31.05	<0.001
Average Temp Max	-0.26	0.069	-3.77	<0.01

Random effects	Variance	SD
Squirrel ID	0.042	0.21
Social Group ID	3.70E-10	1.90E-05
Year	0.017	0.13
Residual	0.26	0.51

Table 2.3. Estimated linear mixed effects model parameters, standard errors (SE), t-values, and p-values of fixed effects and variances and standard deviations (SD) of random effects for the log-transformed cortisol in adult female Cape ground squirrels from the second-best selected model by AICc.

Fixed effects	Estimate	SE	t-value	p-value
Intercept	2.26	0.06	37.9	<0.001
Average Temp Max	-0.23	0.057	-4.05	<0.01
Max SD	-0.11	0.054	-2.05	0.08

Random effects	Variance	SD
Squirrel ID	0.042	0.21
Social Group ID	1.40E-10	1.20E-05
Year	0.031	0.17
Residual	0.26	0.51

Table 2.4. Estimated linear mixed effects model parameters, standard errors (SE), t-values, and p-values of fixed effects and variances and standard deviations (SD) of random effects for the log-transformed cortisol in adult female Cape ground squirrels from the full model. AICc = 489.75. Significant values are bolded.

Fixed effects	Estimate	SE	t-value	p-value
Intercept	2.27	0.054	41.9	**<0.001**
Average Temp Max	-0.18	0.057	-3.21	**<0.05**
Average Temp Min	-0.066	0.052	-1.28	0.25
Max SD	-0.12	0.050	-2.36	0.057
Age	0.063	0.037	1.71	0.089
Body Condition	0.024	0.035	0.39	0.69

Random Effects	Variance	Standard Deviation
Squirrel ID	0.037	0.19
Social group ID	0.00	0.00
Year	0.012	0.11
Residual	0.26	0.51

Chapter 3: Hair cortisol and its relationship with intrinsic factors and fitness in Cape ground squirrels

Abstract

The relationship between glucocorticoids (GCs) and animal fitness has been widely studied, with GCs playing a complex role in reproduction. Glucocorticoids can aid in reproduction with the allocation of resources, but elevated GCs due to chronic stressors can decrease reproductive success. Therefore, it is important to study the relationship between fitness and GCs, as well as the intrinsic factors that can influence GCs. We analyzed potential intrinsic effects on GCs and explored GC's relationship with reproductive success in Cape ground squirrels, an arid-adapted social species. We studied the effects of age, body condition, and ectoparasites on the hair cortisol of adult female Cape ground squirrels. We also examined the alternative hypotheses of GC's allocation of resources and the stress response on reproductive success, or number of offspring produced per female each year. We found no effects of age, body condition, or parasitism on hair cortisol, possibly due to social buffering or a discrepancy between the collection of intrinsic data and hair growth. Reproductive success did have a significant positive relationship with cortisol, supporting our hypothesis of allocation of energy and metabolism rather than chronic stress. Cape ground squirrels are income breeders, and the role of GCs in metabolism could be allocating energy to reproduction rather than individual survival when resources are available. Therefore, our results provided valuable insights into GCs' role in reproduction for an arid-adapted income breeder, and future research should more closely examine this relationship by studying GCs' effects on physiological reproductive mechanisms, such as sexual hormones.

3.1 Introduction

Glucocorticoids (GC) are steroid hormones that are often measured to analyze an animal's response to a stressor. These hormones are released into the bloodstream as an end product of the hypothalamic-pituitary-adrenal (HPA) axis (Reeder & Kramer, 2005; Sapolsky, 1992; Sapolsky et al., 2000). Glucocorticoids have many crucial roles in the activation and function of protein, lipid, and glucose metabolism (de Guia et al., 2014; Rabasa & Dickson, 2016). The HPA axis also activates in response to a stressor. When experiencing an acute stressor, the fight-or-flight response activates the HPA axis to ensure survival (Sapolsky, 1992). With acute stressors, GCs bind to receptors in the brain and act as a negative feedback loop on the HPA axis, reducing their release (Reeder & Kramer, 2005; Sapolsky et al., 2000). However, chronic stressors keep the HPA axis active for prolonged periods due to an impaired negative feedback loop (Bauer et al., 2015).

Age is a commonly studied intrinsic factor of GCs in a variety of species. The majority of research focuses on the GCs of immature and older adults. Immature animals consistently have greater GCs than adults due to infant hypercortisolism (Azevedo et al., 2019; Fourie & Bernstein, 2011; Fourie et al., 2015; Pryce et al., 2002; van Kampen & Fuchs, 1998). Additionally, older individuals tend to have elevated GCs compared to younger individuals, possibly due to an impaired HPA axis or a longer duration of recovery from a stressor (Bizon et al., 2001; Dellu et al., 1996; Sapolsky et al., 1983). Research examining GCs throughout the intermediate ages of adulthood is more limited and provides mixed results. For example, a variety of baboon species show increases in GCs during adulthood (Fourie et al., 2015; Sapolsky & Altmann, 1991), whereas rhesus monkeys (*Macaca mulatta*) illustrate a decrease in GCs with increasing age (Dettmer et al., 2014). Additionally, research with tree shrews (*Tupaia belangeri*) found no change in GCs across adulthood (van Kampen & Fuchs, 1998). The effects of age on

GCs can also vary within a species, for example, male vervet monkeys (*Chlorocebus aethiops sabaeus*) show an increase in GCs in adulthood, whereas females have a decrease (Fourie & Bernstein, 2011). As the effects of age throughout adulthood on GCs are more species-specific, it is important to understand how intermediate ages impact GCs in a variety of species with different life histories.

Another intrinsic factor influencing GCs is body condition. Glucocorticoids consistently illustrate a negative relationship with body condition in a variety of species (Boonstra et al., 1998; Charbonnel et al., 2008; Fokidis et al., 2023; Heimbürge et al., 2019; Rakotoniaina et al., 2017; Robertson et al., 2023; Wolf et al., 2018). Individuals with low body condition may have a longer response and recovery time from stressors, leading to increased GCs (Heath & Dufty, 1998). Individuals with low body condition can also be more vulnerable to diseases and predation, resulting in a positive relationship between body condition and survival (Rakotoniaina et al., 2017; Robertson et al., 2023; Shave et al., 2019; Wirsing et al., 2002). Given that survival and reproductive success are often linked to GCs (Defolie et al., 2020; Eberhardt et al., 2013), it follows that many species illustrate a negative correlation between GCs and body condition (Heimbürge et al., 2019).

Parasites can also be correlated with GCs in a variety of species. A meta-analysis of 65 studies examining endoparasites and ectoparasites on GCs in mammals found an overall positive relationship between parasite infections and GCs in both experimental and observational research (Defolie et al., 2020). Additionally, St. Juliana et al. (2014) examined ectoparasites in eight rodent species and found that, in general, GCs increased after exposure to a pulicid flea species, suggesting that parasite infection can lead to elevated GCs. Eberhardt et al. (2013) found that chronically stressed capybaras (*Hydrochoerus hydrochaeris*) had greater oocyst counts

compared to control capybaras, suggesting that elevated GCs can suppress the immune system and lead to more parasite infections. Therefore, there is usually a positive correlation between GCs and parasitism across mammals as parasite infections can act as a stressor, or chronically elevated GCs can allow for greater parasite infections.

While there are general trends across species in how certain intrinsic factors affect GCs, how GCs respond to different environmental conditions is controversial. Schradin et al. (2023) and Love and Wagner (2023) argue that if an animal is adapted to certain long-term environments, like droughts, individuals will decrease their GCs to conserve energy during those harsh conditions. In this situation, GC values reflect metabolism and allocation of resources, not a response to stressors. If the animal is not adapted to certain environmental conditions, GCs elevate to increase energetic expenditure to overcome the stressor (Love & Wagner, 2023; Schradin et al., 2023). Glucocorticoids reflect the stress response through the activation of the HPA axis under these stressful conditions, and if the stressor cannot be overcome, it can lead to chronic stress (Schradin et al., 2023). It is important to understand the differences in these two responses as they can affect reproduction differently.

Glucocorticoids have many important roles in reproduction (Edwards et al., 2019). For example, acute elevations of GCs have been associated with regulating the production of sexual hormones, protecting ovulatory follicles, and facilitating implantation in mammals (Brann & Mahesh, 1991; Keay et al., 2002; Tetsuka, 2007). Elevated GCs with increased sexual hormones also act as a metabolic response to support the energetic needs during the oestrus cycle in Rasa Aragonesa ewes (*Ovis aries*) and giant pandas (*Ailuropoda melanoleuca*) (Kersey et al., 2011; Sosa et al., 2013). Therefore, decreases in GCs can reduce reproduction, as resources are allocated to mechanisms that can help individuals endure harsh conditions rather than being used

for reproduction (Schradin et al., 2023). In black-tailed prairie dogs (*Cynomys ludovicianus*), individuals with decreased GCs were less likely to produce a litter, suggesting there is a minimum threshold of GCs needed for reproduction (Matzke et al., 2022). These studies illustrate how decreased GCs due to the conservation of energy can have negative effects on reproduction.

However, the GC response to stressful environments can have the opposite effect on reproduction. While acute increases in GCs can aid in reproduction, GCs elevated for long periods due to chronic stressors can decrease reproductive success (Edwards et al., 2019). Increases in GCs due to the activation of the HPA axis have been associated with disrupting ovulation, decreasing implantation success, and early pregnancy failure in females (Edwards et al., 2019). Additionally, increased GCs due to chronic stressors, such as increased predation risk, have been correlated with decreases in the number of offspring, number of adult males with developed testes, and male and female sexual hormones in snowshoe hares (*Lepus americanus*) and plateau pikas (*Ochotona curzoniae*) (Boonstra et al., 1998; Yin et al., 2017). These studies illustrate how elevated GCs due to chronic stressors can negatively impact reproduction.

Cape ground squirrels (*Xenus inauris*) are a social, arid-adapted species that do not demonstrate aggression or dominance (Herzig-Straschil, 1978; Waterman, 1995), and previous research has illustrated that their GCs are affected by extrinsic effects (see Chapter 2). However, the impacts of long-term changes in intrinsic factors on GCs in this species have not been studied. For dispersed (band) males, body condition and age impact reproductive output (Warrington et al., 2024). Previous experiments on female squirrels removed parasites and found that treated individuals increased their resting metabolic rate by about 40%, and the number of weaned offspring was 4 times greater than control individuals (Hillegass et al., 2010; Scantlebury

et al., 2007). These studies illustrate how the fitness of Cape ground squirrels can be impacted by age, body condition, and parasitism. Many studies examining these variables are often confounded by an individual's position in a dominance hierarchy (Creel et al., 2013; Dettmer et al., 2014; Kutsukake & Clutton-Brock, 2006; Robertson et al., 2023). Therefore, examining these relationships in Cape ground squirrels provides a unique insight into how intrinsic factors influence GCs without the presence of a hierarchical structure.

The lack of a change in body mass when reproduction and metabolic functioning increased indicates that these squirrels are income breeders that use energy as they gain it and invest it in reproduction (Hillegass et al., 2010; Scantlebury et al., 2007). Additionally, these squirrels have a low reproductive success rate, with 70% of oestruses failing to wean a litter (Waterman, 1996). This large failure rate is not due to a dominance hierarchy because there is no aggression or dominance in adult females and reproductive skew is not predicted in such non-despotic social systems (Manjerovic et al., 2022; Packer et al., 2001; Waterman, 1995). Additionally, these egalitarian squirrels are plural breeders with no reproductive suppression in adult females (Waterman, 1998, 2002). However, multiparous females wean more offspring compared to primiparous females, suggesting that previous weaning experience is valuable to reproductive success (Pettitt et al., 2008). Therefore, it is important to study how GCs interact with reproductive success and intrinsic factors, such as age in adult females, to provide a greater understanding of such strong reproductive skew in what appears to be an egalitarian species.

Our study aims to analyze the impacts of intrinsic factors on GCs and explore the relationship between GCs and reproductive success in Cape ground squirrels. Based on previous research on these squirrels and common patterns in other species, we hypothesized that GCs in Cape ground squirrels are impacted by intrinsic factors. We expected that squirrels would exhibit

increased GCs with increased ectoparasites, decreased body condition, and increased age in adulthood. If GCs in Cape ground squirrels represented responses to chronic stressors, then we predicted decreased reproductive success with increasing GCs. Alternatively, if GCs in Cape ground squirrels represented the allocation of resources, then we expected a positive relationship between reproductive success and GCs.

3.2 Methods

3.2.1 Study Site

Our study site was the SA Lombard Nature Reserve in central South Africa (27° 36'S, 25° 28'E). We used daily maximum temperature (°C) data from the South African Weather Service's Bloemhof weather station, which is 15.5 km from SA Lombard Nature Reserve. When temperature data was unavailable from the South African Weather Service (7-31 March 2017, 11-24 February 2018, 1-11 January 2023), we used the temperature data collected daily from reserve personnel at the SA Lombard Nature Reserve.

We used binoculars and spotting scopes to collect group composition data on female social groups using scan and focal sampling (Altmann, 1974). Groups were defined by who slept in the same social group together. As Cape ground squirrels are diurnal, we determined group composition by observing groups emerging in the morning and returning to burrows in the evening (Pettitt & Waterman, 2011). Cape ground squirrels live in either female family groups or male bands of no more than 2-25 individuals (Waterman, 1995). Female family groups consist of adult females, juveniles (offspring less than 6 months) and subadults (immature offspring older than 6 months) of either sex and related adult males (natal males) that have not yet dispersed (Waterman, 1995). Female Cape ground squirrels are philopatric and live with their family group or an adjacent area for the duration of their lives, while adult males disperse to join all-male

bands (band males; Waterman, 1995, 2002). We did not use subadults and juveniles in our study because they have different GC levels in a variety of species compared to mature individuals (Azevedo et al., 2019; Heimbürge et al., 2019). Thus, we focused our analysis on adult females, as we knew their specific ages and could follow them throughout their lives.

Cape ground squirrels have no distinct breeding season and can breed year-round (Waterman, 1996; Pettitt et al., 2008). They are promiscuous, with females breeding with multiple males during oestrus (Waterman, 1998; Manjerovic et al., 2022). Females have multiple oestrus periods and can produce up to four litters each year, but the failure rate for raising offspring to weaning is high (Waterman, 1996). The average estimated gestation period for females at our study site is 50.8 days, with lactation lasting around 47.5 days (Pettitt et al., 2008). Females give birth in separate burrow clusters from their family group and rejoin their group after weaning or losing their litter (Waterman, 1996). The average litter size is usually one or two offspring, with the rare maximum litter size being three offspring (Herzig-Straschil, 1978; Waterman, 1996). Reproductive suppression occurs only in subadult females, with subadults in social groups with more than one adult female delaying sexual maturity until about 12 months of age, compared to eight months for subadults living in social groups with one adult female (Waterman, 2002; Pettitt & Waterman, 2011). While the exact birth and death dates are unknown, the lifespan of a squirrel is estimated to be nine years based on long-term on-site research (Warrington et al., 2022).

3.2.2 Trapping and Handling

We trapped squirrels using Tomahawk live traps (Tomahawk Live Trap Inc., Hazelhurst, WI; 15 X 15 X 50 cm) baited with peanut butter and bird seed and deployed 2-8 times a day between 08:00 and 17:30 daily (Waterman, 1995). We checked traps for squirrels every hour. Most trap data is collected between May and August during each data collection year. During

handling, we identified each known squirrel using PIT tags (Shenzhen XCC RFID Technology Co., Ltd. China) and unknown squirrels were given a new PIT tag upon first capture. During the first trap of an individual, we collected a tissue sample from the tip of the tail and during the first capture of each season, we collected a hair sample from the base of the tail. We gave each squirrel a unique and symmetrical dye mark on their back using black hair dye (Rodol D dye; Lowenstein and Sons, Inc., New York, NY; Melchior & Iwen, 1965) for their first capture of the season to be able to identify squirrels during behavioural observations to determine group composition.

We measured spine length (cm) from the base of the neck to the base of the caudal vertebrae using a tape measure. We measured body mass (grams) using a Pesola spring scale (Pesola AG, Switzerland). Size-mass residuals were calculated for body condition using the residuals from an ordinary least squares regression between spine length and body mass (Schulte-Hostedde et al., 2005; Manjerovic & Waterman, 2012). We log-transformed spine length and body mass to achieve normality and linearity of the residuals. Positive values were considered good body condition, while negative values were considered poor body condition.

Individuals were sexed by visually examining their external genitalia. We also determined if squirrels were juveniles, subadults, or adults based on their mass and reproductive maturity. Adult females were distinguished from subadult and juvenile females by the presence of elongated nipples as female nipples remain swollen after the first oestrus (Waterman, 1996). We determined the age in years of adult female squirrels using trap data that has been collected on every squirrel in each group since 2002. The exact date of each squirrel's birth is unknown, but the first year that each squirrel was trapped on the study site as a juvenile was recorded.

Therefore, we approximated the year each squirrel was born to estimate the age in years of each squirrel.

During a squirrel's first handling of the season, we counted the number of adult fleas, ticks, mites, and lice that were seen and collected with a flea comb using three strokes on each plane of the back (left, middle, and right) from the shoulders to the base of the tail (Hillegass et al., 2010; Scantlebury et al. 2007; O'Brien et al. 2021). We also measured the number of nits/eggs seen. Total adult ectoparasite abundance is the total number of parasites that were seen and collected during the first handling. The nits that were measured during the handling period were not present during the hair growth period, so we did not use the total number of nits. However, the presence of nits during the handling period could indicate the prevalence of an adult parasite during the hair-growing period. Therefore, we used the prevalence of nits (1 = nits were present; 0 = nits were absent) instead of the abundance of nits.

All procedures were approved by the University of Manitoba's Animal Care and Use Committee (##F14-032, F18-039, F23-003) and followed the guidelines of the American Mammal Association (Sikes, 2016).

3.2.3 Cortisol Analyses

Measuring GCs with hair has many advantages, such as collection being easy and non-invasive, hair samples can be stored at room temperature, and GC levels can remain accurate for years after collection (Heimbürge et al., 2019; Macbeth et al., 2010). Glucocorticoids are incorporated into the hair shaft through passive diffusion while the hair is growing, so integrated GCs in hair reflect long-term stressors experienced during the active growth phase (Crill et al., 2019; Heimbürge et al., 2019).

The tail hairs of Cape ground squirrels moult and regrow between the end of January and the end of March every year (Herzig-Straschil, 1978), so integrated cortisol levels reflected a

maximum of two months. During this growth period, the only interaction between squirrels and humans outside of the house area is when a few reserve personnel periodically travel throughout the site. We collected samples during 2015-2019 and 2021-2023. Samples were not collected in 2020 due to the COVID-19 pandemic. Well after tail hair growth is completed, all samples were collected from the base of the tail with scissors between May and July each year, except for hair samples collected in 2021, which were collected in September and October as the field season was delayed due to COVID-19. After collection, we placed hair samples in plastic bags, fixed the samples with 70% ethanol, and stored them at -20°C until use.

We used the protocols from Macbeth et al. (2010) to prepare hair samples for cortisol analysis. To clean samples of potential contaminants, we washed samples three times with 10 mL of methanol per 100 mg of hair, and we set samples to dry on a lab countertop at room temperature for three days. We ground hair into a fine powder using a Retsch ball mill at 30 Hz for 0.03 min/mg. To begin the extraction process, we added 1 mL of methanol to 50 mg of hair powder and set samples in a shaker table for 24 hours at room temperature with slow rotation. Samples were then centrifuged for 15 minutes at 4500 rpm. After the supernatant was collected into a separate tube, we added 1 mL of methanol to the hair powder, vortexed, centrifuged, and the supernatant was collected again. We repeated that process one more time, resulting in a total of 3 mL of supernatant collected. We evaporated the supernatant for each sample using a concentrator (Savant ISS110 SpeedVac Concentrator, Thermo Scientific, Waltham, MA, USA), and we froze samples at -18°C until used for radioimmunoassays (RIA).

We used tritium-labelled cortisol and cortisol-specific antibody (IgG-F-2) diluted to 1:15,000 to perform RIAs following the methods of Ryan et al. (2012). After reconstituting samples with 240 μL of RIA buffer, we added 100 μL of each reconstituted sample (or known

dilution of cortisol for standards in triplicates), 100 µL of antibody, and 100 µL of [3H]-cortisol tracer together in duplicates. We covered samples with aluminum foil and incubated samples for one hour at room temperature, followed by an incubation period of 12-24 hours at 4°C. After incubation, we added 100 µL of separate buffer (dextran-coated charcoal) and let the samples sit on ice for 15 minutes. Samples were centrifuged at 2500 rpm for 20 minutes at 4°C, and the supernatant was poured into scintillation vials. We added 2 mL of scintillation fluid and put samples on a scintillation counter (LS6500; Beckman Coulter, Brea, CA) to measure the radioactivity in counts per minute for 5 minutes. We determined the absolute values of cortisol by adding the values calculated by the scintillation counter to a standard curve and adjusting for 76.1% extraction efficiency. We re-analyzed samples if the sample duplicates had a coefficient of variation (CV) >25%. Interassay variation was 16.2% CV and intra-assay variation was 7.9% CV. Samples demonstrated good parallelism.

We completed RIAs for a total of 277 samples, but we excluded five individuals from this analysis as they did not have spine length measurements needed for body condition calculations. We also had only two samples for ages 10 and 11, both from the same individual, so we excluded those two samples as they were outliers. Of the 270 samples, there are 147 individuals from 100 different social group IDs. We chose samples if they were an adult female with a known age and if there was enough hair to complete the RIAs (>100 mg).

3.2.4 Reproductive Success

We extracted total genomic DNA from all individuals between 2015 and 2023 (excluding 2020) using E.Z.N.A. Tissue DNA kits (Omega Bio-tek Inc, Norcross U.S.A.) and High Pure PCR Template Preparation kits (Roche Diagnostics, Mannheim Germany). DNA from tail tip tissue samples was amplified using the polymerase chain reactions (PCR) with GoTaq® Flexi DNA Polymerase (Promega Corporation, Madison U.S.A.), and 19 fluorescently labelled

forward primer and unlabeled reverse microsatellites primer sets that were developed for this species (Abercrombie et al., 2009; Manjerovic et al., 2009; Manjerovic & Waterman, 2015; Shave & Waterman, 2017). After checking PCR success using a 2% agarose gel, post-PCR products were pooled and diluted. Genotyping was completed at the Centre for Applied Genomics (Hospital for Sick Children, Toronto, Canada). Genotypes were scored with GeneMarker v. 2.6.0 and manually to reduce errors.

After analyzing the genotype results to eliminate genetic matches using Genecap v.1.4. and COANCESTRY v.1.0.1.10 (Wang, 2011), we calculated maternity using MasterBays x.2.57 R package (Hadfield et al., 2006) and CERVUS v. 3.0.7. (Kalinowski et al., 2007) at an 80% confidence level (Wells et al., 2017). Potential mothers with at least 80% confidence were checked for accuracy using trapping and observational data. If potential mothers were alive, an adult, and living in the same social group or area as the offspring, then they were determined to be accurate mothers.

3.2.5 Statistical Analyses

We analyzed intrinsic effects on hair cortisol in R Studio 4.2.1 using the lme4 package (Bates et al., 2024). We created 12 linear mixed-effects models with log-transformed cortisol values as the response and age in years, body condition, total ectoparasite abundance, prevalence of nits, and average daily temperature maximum as fixed effects. We calculated the average temperature maximum from the beginning of January to the end of March as that is the growing period of tail hair of Cape ground squirrels. We included the average temperature maximum as it is significantly related to hair cortisol in Cape ground squirrels (see Chapter 2). We included year, individual ID, and social group ID as random effects to account for variation across years and between individuals and social groups. We scaled all continuous variables to have

consistency across units for easier result interpretation. We compared models using weighted Akaike information criterion (AICc) values to choose the most parsimonious models.

We evaluated the homogeneity of variance, linearity, and normality of residuals for the top models visually using residual plots and Q-Q plots. We evaluated the normality of random effects using Q-Q plots and Shapiro-Wilks tests. The normality of the residuals was achieved after log-transforming the cortisol values. We assessed multicollinearity by checking that the variance inflation factors (VIF) of all variables in the full model were below three (Zuur et al., 2010). All variables had VIF values between 1.02 - 1.08.

Reproductive success was measured by annual individual reproductive output or the number of genetically matched offspring produced by a female each year. This value is not litter size as it could include more than one litter, as litter size is small (mean litter size = 1.45 offspring; Pettitt et al. 2008). We analyzed samples from 2015-2019, excluding 2021-2023, because there was an abnormally low number of offspring from sampled females during those years. We used a total of 165 samples from 90 individuals and 69 social group IDs across the five years.

To analyze the effects of hair cortisol on reproductive success, we ran a mixed-effects model with a Poisson distribution using the glmmTMB package in R Studio 4.2.1 (Brooks et al., 2017). We included the number of offspring as the response with log-transformed cortisol values as the fixed effect. The cortisol values were positively skewed, so we log-transformed the values to give them a Normal distribution for easier interpretation of the results. We included social group ID, individual ID, and year as random effects to account for variation between social groups, individuals, and across data collection years. Assumptions of the model were checked using the DHARMa package (Hartig, 2018). Data was not zero-inflated or over-dispersed, so

zero-inflated models and negative binomial models were not used. The residual plots did not show significant deviations, so the assumptions of the Poisson model were not violated. Results are expressed as mean ± SE unless otherwise stated, and significance was accepted at $\alpha = 0.05$.

3.3 Results

3.3.1 Intrinsic Effects on Cortisol

Overall, the average hair cortisol values were 11.5 ± 0.4 ng cortisol/g hair, ranging from 1.42 ng/g to 45.1 ng/g before log-transformation. The average age of adult female squirrels was 2.9 ± 0.1 years, ranging from one to nine years old. The average total ectoparasite abundance per individual was 1.4 ± 0.1 ectoparasites, ranging from zero to 17 ectoparasites seen and/or collected.

The top model selected by AICc consisted of average daily temperature maximum and random effects (Table 3.1). Average temperature maximum had a significant negative relationship with cortisol values ($t = -3.85$, $p < 0.01$). The next best-selected model was the null model consisting of only random effects and had a ΔAICc > 2. All other models ranked below the null model. We found no relationship between hair cortisol and age (Figure 3.1), body condition (Figure 3.2), total abundance of ectoparasites (Figure 3.3), and prevalence of nits (Figure 3.4) as the top model and null model performed better than all models containing these variables (Table 3.1). When examining the full model, age, body condition, total abundance of ectoparasites, and prevalence of nits were non-significant (Table 3.2).

3.3.2 Reproductive Success and Cortisol

Average hair cortisol values from the 165 samples were 9.6 ± 0.4 ng/g, ranging from 1.4 ng/g to 31.8 ng/g before log-transformation. The average number of genetically matched offspring produced each year was 0.90 ± 0.08 offspring, ranging from zero to five offspring. The

Poisson mixed-effects model found that log-transformed cortisol is a significant predictor of number of offspring produced (Table 3.3). Females that produced more offspring had greater cortisol values than females that produced fewer offspring (Figure 3.5).

3.4 Discussion

We found no relationship between adult age and hair cortisol in Cape ground squirrels. Previous research with other ground squirrel species has found age-related differences in GCs, but these studies included juveniles (Boonstra et al., 2001; Mateo, 2006). In rats (*Rattus norvegicus*), older individuals have elevated basal GCs compared to young animals (Dellu et al., 1996; Sapolsky et al., 1983). Additionally, when experimentally exposed to a stressor, aged rats of different species and rhesus monkeys have higher GC levels for a longer duration than young individuals (Bizon et al., 2001; Dellu et al., 1996; Goncharova & Lapin, 2002; Sapolsky et al., 1983). On the other hand, both adult female and male Cape ground squirrels illustrate decreasing oxidative stress with age (Jacobs et al., 2024). While GCs are correlated with oxidative stress in many vertebrates (Costantini et al., 2011), it is possible that we did not find a relationship between cortisol and age in these squirrels because we examined long-term hair cortisol reflecting the summer season, whereas Jacobs et al. (2024) studied short-term plasma oxidative stress collected in the winter. Oxidative stress can be influenced by acute stressors (Majer et al., 2019), so these differences in sampling period and season could contribute to our varying results. Future research could compare short-term cortisol values with oxidative stress across ages. Additionally, our sample size for older ages is much smaller compared to our younger and intermediate adult ages. Based on these previous findings with other rodent species and Cape ground squirrels, we may see a trend in hair cortisol with age with a larger sample size of older individuals.

The majority of the literature on age-related changes in GCs focuses on changes in very young or very old individuals (Reeder & Kramer, 2005). The research examining GC changes in individuals of intermediate adult ages is lacking, with current research producing mixed species-specific results (van Kampen & Fuchs, 1998; Fokodis et al., 2023; Fourie et al., 2015; Dettmer et al., 2014). Many studies that found a significant difference in GCs with age examined age classes rather than continuous ages (Fourie & Bernstein, 2011; Pryce, 2002; Laudenslager et al., 2012; Dettmer et al., 2014). Perhaps the changes in GCs across individuals close in age are too small to produce consistent findings. Future research could examine changes in cortisol in age categories as we found no association between cortisol and adult ages in years in female Cape ground squirrels.

We also found no relationship between hair cortisol and body condition in adult female Cape ground squirrels. Previous research with other small mammals has found a negative relationship between body condition and GCs (Boonstra et al., 1998; Charbonnel et al., 2008). Animals with low body condition and older age are more susceptible to diseases and less likely to escape predators (Peters et al., 2019; Robertson et al., 2023; Wirsing et al., 2002). Given that female Cape ground squirrels live in stable family groups (Waterman, 1995), it is possible that the dynamics of group living could offset some of the challenges associated with poor body condition and older age. Research with other species has found that individuals in social groups have lower GCs compared to solitary individuals (Robertson et al., 2023; Woodruff et al., 2013), suggesting there are GC benefits to living in social groups. Cape ground squirrels have group vigilance behaviours in social groups, leading to enhanced predator detection (Edwards & Waterman, 2011). Allogrooming in female family groups targets ectoparasites, which can boost the fitness of these squirrels (Hillegass et al., 2008, 2010). Additionally, Arctic ground squirrels

(*Urocitellus parryii*), another social species, have no relationship between GCs and body condition (Zhang et al., 2020). Therefore, living in a female family group may buffer the consequences of low body condition and older age in Cape ground squirrels. Additionally, many studies that found differences in age and body condition with GCs study both sexes or only males (Bizon et al., 2001; Boonstra et al., 1998; Dellu et al., 1996; Fourie et al., 2015; Rakotoniaina et al., 2017; Robertson et al., 2023; Sapolsky et al., 1983), so there could be potential sex differences in hair cortisol on these intrinsic factors that can be studied in the future as we only examined adult female Cape ground squirrels.

Additionally, previous research with these squirrels found no changes in body mass despite fluctuations in metabolic energy, suggesting that these squirrels are income breeders (Hillegass et al., 2010). Available metabolic energy is used for reproduction rather than investing in body condition. Because GCs are tied to metabolic functioning (de Guia et al., 2014; Rabasa & Dickson, 2016), these results may provide an alternative explanation for the lack of a relationship between hair cortisol and body condition. With fluctuations in cortisol due to metabolic functioning, we would not expect changes in individual body condition. Therefore, these findings could also support the hypothesis that hair cortisol in these squirrels represents metabolic functioning rather than the stress response as the energy gained is immediately spent rather than invested into body condition.

Cape ground squirrels have varying metabolic functioning and increases in reproductive success after removing ectoparasites and endoparasites (Hillegass et al., 2010; Scantlebury et al., 2007). As GCs are known to impact metabolic functioning and reproduction in many other species (Bauer et al., 2015; de Guia et al., 2014; Ebensperger et al., 2013; Endo et al., 2018; Rabasa & Dickson, 2016), we predicted that ectoparasite load would impact hair cortisol levels.

However, we found no relationship between ectoparasite abundance or prevalence of nits on hair cortisol in our analyses. As previous experiments with these squirrels found differences in metabolic rate and reproductive success when both endoparasites and ectoparasites were removed (Hillegass et al., 2010; Scantlebury et al., 2007), perhaps future research needs to examine the effects of endoparasites on cortisol. Additionally, Defolie et al. (2020) reviewed 65 mammalian studies and found an overall positive relationship between GCs and parasitism, with protozoans and microparasites having a stronger effect size than macroparasites. Previous research also found that female Cape ground squirrels carry three times more endoparasites than male squirrels (Hillegass et al., 2008). Therefore, these results suggest that examining endoparasites might have a stronger impact on hair cortisol than ectoparasites in these adult female squirrels.

It is also important to consider that the measurements of body condition and ectoparasite load were collected months after the tail hair growth period (Herzig-Straschil, 1978). Therefore, the measurements collected from May to July may not accurately reflect the squirrels' body condition or ectoparasite abundance from January to March, which may contribute to our lack of a relationship between hair cortisol and these intrinsic factors. To close this gap in cortisol and intrinsic measurements, future research could gather the measurements during the hair growth period. Alternatively, short-term cortisol collection methods, such as blood or faeces, collected at the same time as the body condition and parasite measurements may offer greater insight into the relationship of these intrinsic effects with GCs in adult females Cape ground squirrels.

We also found elevated hair cortisol was associated with greater reproductive success. Higher cortisol values were found in females that produced more offspring. Our results support the hypothesis that GCs in adult female Cape ground squirrels' hair cortisol represented

allocation of resources rather than chronic stress. As Cape ground squirrels are income breeders (Hillegass et al., 2010; Scantlebury et al., 2007), the decrease in cortisol with low reproductive success indicates that energy was spent on the individual rather than reproduction. If these squirrels do not have resources, they will not successfully reproduce. For example, Cape ground squirrels in a Kalahari population had no reproductive output during a two-year drought that depleted resources (Waterman & Fenton, 2000). Therefore, the decrease in cortisol could suggest that individuals needed to conserve energy for their survival, leading to no resources available for successful reproduction. Future research can examine what harsh environmental factors are leading to a decrease in cortisol as an energy-saving mechanism and its relationship with reproductive success. Increasing temperatures are considered harsh conditions for these squirrels (see Chapter 2), so future research could examine the interaction between hair cortisol and increasing temperatures on reproductive success.

Energy could also be allocated to individual growth and development rather than reproduction. Primiparous female Cape ground squirrels are more likely to fail to wean a litter than multiparous females (Pettitt et al., 2008). One possible explanation is that primiparous females allocate their resources to their own development as they are still growing, or they are conserving energy for future reproduction (Descamps, et al., 2007; Pettitt et al., 2008). These results also support our hypothesis that reproduction in these squirrels is impacted by the allocation of energy and metabolism rather than chronic stress. Edwards et al. (2019) explain that GCs play an important role in normal reproductive functioning in mammals, such as influencing the production of sexual hormones, but chronic stress can have detrimental effects. If elevated cortisol values were associated with decreased reproduction, we would expect these values to represent chronic stress. As greater cortisol is associated with increased reproduction, these

cortisol values likely represent normal cortisol functioning that allows for resources to be allocated to reproduction and can even help to facilitate reproduction in these squirrels, but further research needs to be conducted to understand basal cortisol values in these squirrels. Future research can also examine the physiological effects of cortisol on the reproductive system in female Cape ground squirrels.

Research with other social rodent species found similar, but slightly inconsistent results. Matzke et al. (2022) found that increased GCs were correlated with producing offspring in black-tailed prairie dogs, but there was no relationship with litter size. The litter size for black-tailed prairie dogs is larger than the litter size of Cape ground squirrels (Herzig-Straschil, 1978; Waterman, 1996), and the greater variability in litter size might contribute to the researchers' lack of a relationship with GCs. Although it is uncommon, our measurement of the number of offspring produced per year also includes the cases where individuals produced more than one litter, which could also contribute to the differences in our findings (Waterman, 1996). However, both cases represent annual reproductive output as black-tailed prairie dogs produce one litter per year (Matzke et al., 2022). Research on female degu (*Octodon degus*) found that lower GCs during early lactation were correlated with a greater probability that females would produce a second litter (Ebensperger et al., 2013). Additionally, elevated GCs were correlated with increased per capita offspring produced in lactating female degus in two out of the three years examined (Ebensperger et al., 2011). These results suggest that GCs are needed for current reproduction in this species, but these elevations could have negative impacts on future reproductive output. Considering the low probability of weaning many litters per year in Cape ground squirrels and the overall low reproductive success rate (Waterman, 1996), future research could examine the effects of cortisol on the ability to produce a second litter.

In conclusion, adult female Cape ground squirrel hair cortisol is not affected by age, body condition, or parasitism. Decreased hair cortisol values are associated with lower adult female reproductive success, suggesting that GCs represent metabolic functioning and provide resources for the reproduction of these income breeders. Future research can more closely examine the mechanisms of this relationship and its interactions with environmental conditions to better understand the survival and fitness of these squirrels in a changing environment, especially as they are ecosystem engineers that influence surrounding plant and animal communities (Ewacha et al., 2016, 2022).

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3.6 Figures

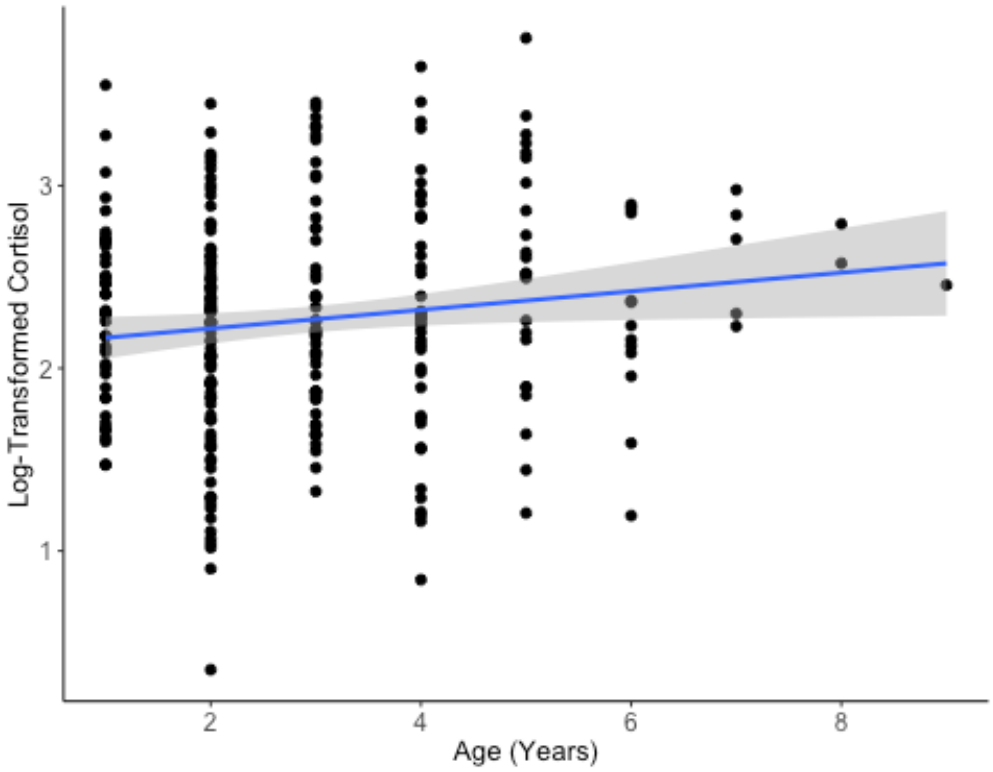


Figure 3.1. Scatterplot of age in years versus log-transformed cortisol values of Cape ground squirrels. The blue line is the trendline and the grey area represents the 95% confidence interval.

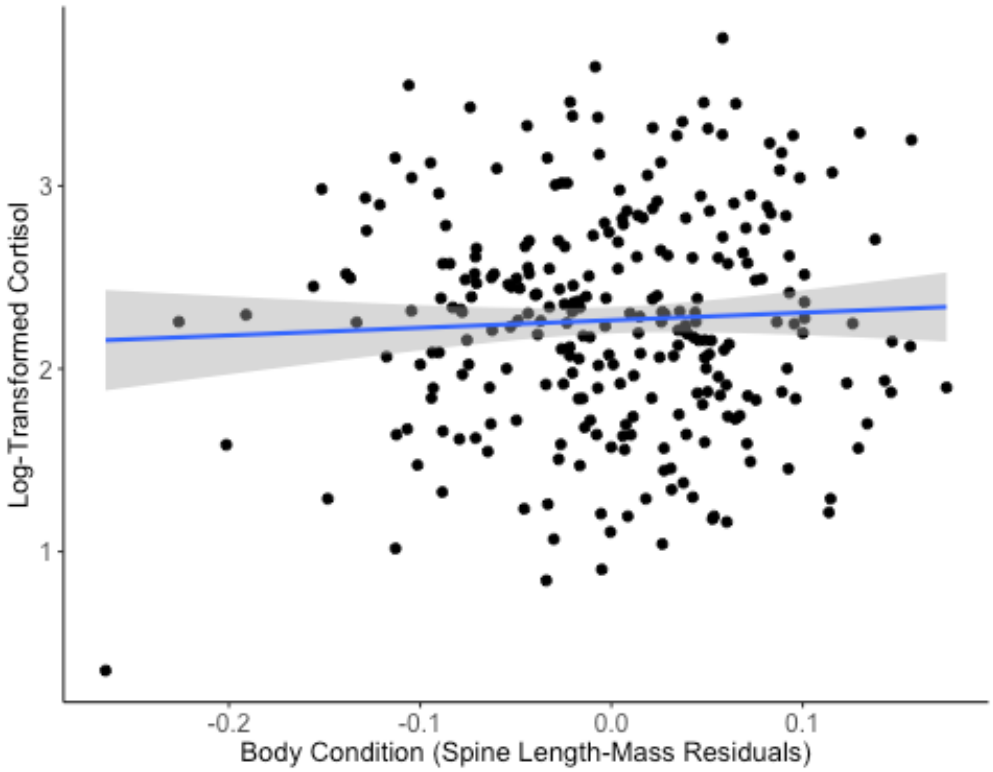


Figure 3.2. Scatterplot of body condition using spine length-mass residuals versus log-transformed hair cortisol values of Cape ground squirrels. The blue line is the trendline and the grey area represents the 95% confidence interval.

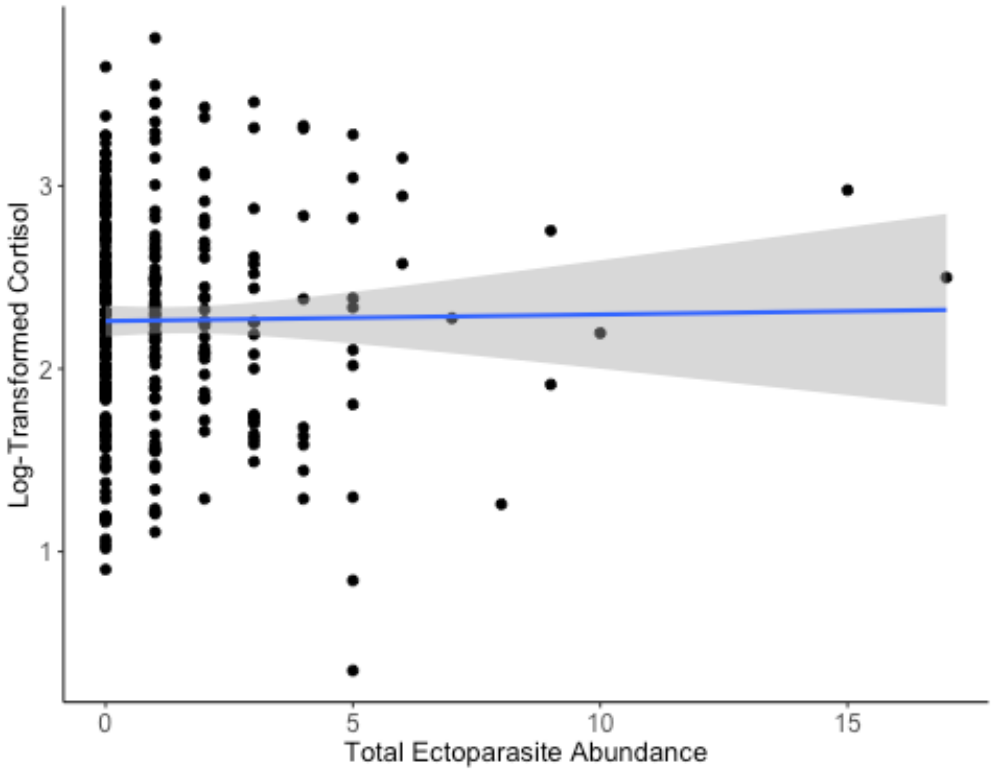


Figure 3.3. Scatterplot of total ectoparasite abundance seen or collected during a Cape ground squirrel's first capture versus log-transformed hair cortisol values. The blue line is the trendline and the grey area represents the 95% confidence interval.

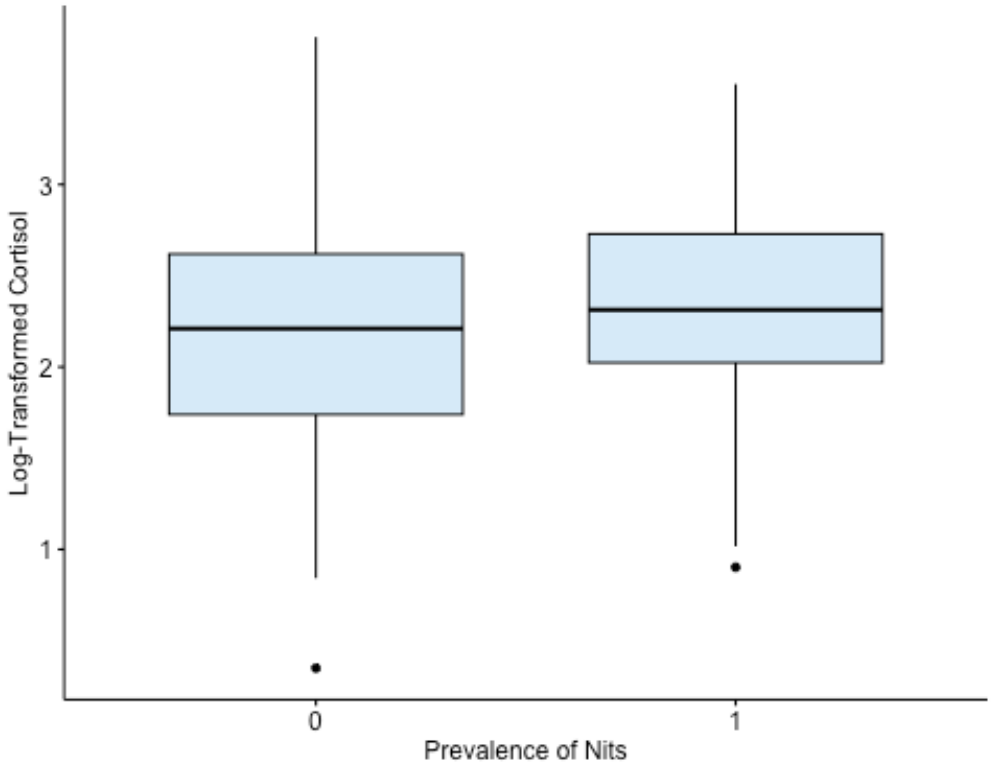


Figure 3.4. Boxplot of the prevalence of nits seen on a Cape ground squirrel versus log-transformed cortisol for Cape ground squirrels. 0 = absence of nits; 1 = presence of nits.

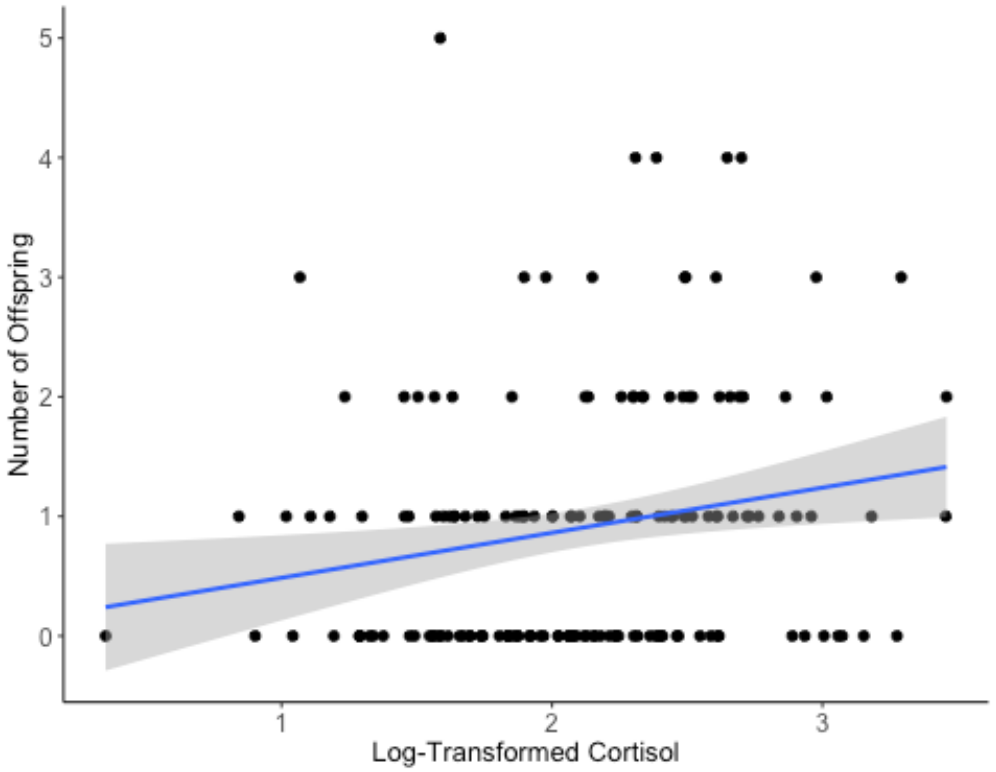


Figure 3.5. Scatterplot of the number of genetically matched offspring produced by an adult female per year versus log-transformed cortisol for Cape ground squirrels. The blue line is the trendline and the grey area represents the 95% confidence interval.

3.7 Tables

Table 3.1. Model selection for log-transformed cortisol in Cape ground squirrels. Avg Max refers to the daily temperature maximum averaged from the beginning of January to the end of March each year. Abun Parasite refers to the total abundance of parasites seen and collected. Prev Nits refers to the prevalence of nits (0/1). Age (in years), body condition (size-mass residuals), squirrel ID (random effect), social group ID (random effect), and year: 2015-2023 (excl. 2020) (random effect).

Model	Terms	k	AICc	ΔAICc	AICC Wt
1	Avg Max + ID + Social Group ID + Year	6	458.92	0.00	0.71
2 (Null Model)	ID + Social Group ID + Year	5	462.05	3.13	0.15
3	BC + Avg Max + ID + Social Group ID + Year	7	464.78	5.87	0.04
4	Prev Nits + Avg Max + ID + Social Group ID + Year	7	464.88	5.97	0.04
5	Abun Parasite + Avg Max + ID + Social Group ID + Year	7	465.23	6.31	0.03
6	Age + Avg Max + ID + Social Group ID + Year	7	465.26	6.35	0.03
7	Abun Parasite + Prev Nits + Avg Max + ID + Social Group ID + Year	8	471.18	12.27	0.00
8	Abun Parasite + BC + Avg Max + ID + Social Group ID + Year	8	471.31	12.39	0.00
9	Age + BC + Avg Max + ID + Social Group ID + Year	8	471.39	12.48	0.00
10	Abun Parasite + Age + Avg Max + ID + Social Group ID + Year	8	471.63	12.71	0.00
11	Abun Parasite + Age + BC + Avg Max + ID + Social Group ID + Year	9	477.93	19.02	0.00
12	Abun Parasite + Age + BC + Prev Nits + Avg Max + ID + Social Group ID + Year	10	483.81	24.9	0.00

Table 3.2. Estimated linear mixed-effects model parameters, standard errors (SE), t-values, and p-values of fixed effects and variances and standard deviations (SD) of random effects for the log-transformed cortisol of Cape ground squirrels from the full model. Bolded p-values are <0.05.

Fixed Effects	Estimate	SE	t-value	p-value
Intercept	2.25	0.067	33.53	**<0.001**
Abun. Ectoparasites	-0.025	0.034	-0.75	0.45
Prev. Nits	0.026	0.057	0.45	0.65
Age	0.020	0.036	0.56	0.58
Body Condition	0.031	0.036	0.88	0.38
Average Temp Max	-0.25	0.063	-3.93	**<0.05**

Random Effects	Variance	SD
Squirrel ID	0.037	0.19
Social group ID	3.70E-10	1.91E-05
Year	0.023	0.15
Residual	0.26	0.51

Table 3.3. Estimated Poisson mixed-effects model parameters, standard errors (SE), t-values, and p-values of fixed effects and variances and standard deviations (SD) of random effects for the total number of genetically matched offspring produced by female Cape ground squirrels.

Fixed effects	Estimate	SE	t-value	p-value
Intercept	-1.35	0.43	-3.14	<0.01
Log Cortisol	0.51	0.18	2.91	<0.01

Random effects	Variance	SD
Squirrel ID	0.11	0.33
Social Group ID	0.13	0.36
Year	1.03E-09	3.21E-05

Chapter 4: Conclusion

We found support across both Chapter 2 and Chapter 3 that hair cortisol in adult female Cape ground squirrels represents metabolism and allocation of resources. Although there was no relationship between rainfall or group size with cortisol, there was a significant negative correlation between average temperature maximum and hair cortisol. We also found no relationship between intrinsic factors, including age, body condition, and ectoparasites, on cortisol. Furthermore, reproductive success did have a significant positive relationship with hair cortisol in adult female squirrels.

The decrease in hair cortisol with increasing temperature maximums suggests that hot climates are considered harsh conditions for which Cape ground squirrels are adapted (Schradin et al., 2023; van Heerden & Dauth, 1987). These squirrels conserve their energy to endure the hot weather. Additionally, we did not find support for our hypothesis that cortisol is influenced by rainfall. While the Kalahari population of Cape ground squirrels illustrate significant decreases in reproductive success during drought (Waterman & Fenton, 2000), we found no effects of low rainfall on cortisol in the South African populations, possibly due to differences in annual rainfall between the two study sites. However, the integrated tail hair cortisol values only represented summer temperatures and the wet season (Herzig-Straschil, 1979), which could explain the lack of a relationship between cortisol and temperature minimums and low rainfall. Future research could examine cortisol with short-term collection methods across seasons to understand how glucocorticoids (GCs) fluctuate across seasons in this species. This study is the first long-term assessment of extrinsic effects on GCs for these squirrels, so further research can explore the impacts of other extrinsic effects, such as predation risk, on the hair cortisol of Cape ground squirrels.

Examining the impacts of extrinsic and intrinsic factors on GCs is crucial to study in arid-adapted species as climate change accelerates, especially in extreme environments. Understanding how an animal's GCs respond to their adapted environments can provide insights into how climate change is going to alter the fitness and survival of these species. Temperatures in South Africa are increasing (Department of Environmental Affairs, 2018), so the duration and intensity of harsh conditions for these squirrels are also going to increase (Schradin et al., 2023), which can affect these squirrels' ability to respond to harsh conditions appropriately. It is possible that an increase in temperature above the temperatures that Cape ground squirrels are adapted to may shift their harsh response to a stress response, and chronically elevated GCs can have detrimental effects on the fitness and survival of this species (Boonstra et al., 1998; MacLeod et al., 2018; Yin et al., 2017). Additionally, these squirrels are ecosystem engineers (Ewacha et al., 2016, 2022), so changes to their fitness and survival due to climate change can impact their surrounding plant and animal communities. Therefore, it is critical to continue monitoring these squirrels' reaction to their changing environment.

Many studies examining social variables and intrinsic factors on GCs include species with a dominance hierarchy (Creel et al., 2013; Dettmer et al., 2014; Hill et al., 2021; Kutsukake & Clutton-Brock, 2006; Robertson et al., 2023; Woodruff et al., 2013). Studying Cape ground squirrels provides a unique perspective on these relationships without the presence of a social hierarchy (Herzig-Straschil, 1978; Waterman, 1995). Therefore, it is possible that with the lack of dominance in female family groups, adult female GCs are not influenced by group size, age, body condition, or ectoparasites. The social living of these squirrels may even provide a buffer for the negative effects of older age, poor body condition, and high ectoparasite abundance on GCs that are usually seen in solitary species or species with a dominance structure. (Edwards &

Waterman, 2011; Hillegass et al., 2008, 2010; Kutsukake & Clutton-Brock, 2006; Robertson et al., 2023; Woodruff et al., 2013). However, our lack of a relationship between hair cortisol and body condition or parasites could also be due to a gap between the hair-growing period and the data-collection period of intrinsic variables.

Similar to other species, hair cortisol in Cape ground squirrels is associated with reproductive success (Bauer et al., 2015; Ebensperger et al., 2011, 2013; Endo et al., 2018). Many studies focus on the negative effects of elevated GCs due to chronic stress on reproductive success (Boonstra et al., 1998; MacLeod et al., 2018; Yin et al., 2017). We found that elevated hair cortisol is correlated with increased reproductive success, supporting our hypothesis that cortisol represents the allocation of resources. Future research can explore how increased cortisol can facilitate female reproduction, such as through examining sexual hormones. Cape ground squirrels live paycheck to paycheck as income breeders, so they will only spend energy on reproduction if it is available (Hillegass et al., 2010; Scantlebury et al., 2007). Therefore, with varying metabolic energy, we would not expect squirrels to invest in body condition, providing another explanation for our lack of a relationship between cortisol and body condition. Future research can expand on our findings by examining potential interactions with cortisol on reproductive success. For example, female Cape ground squirrels do not successfully breed after the age of six (Shofstall, unpublished data), so it may be advantageous to study the potential interactions between cortisol and age, or other intrinsic effects, on reproduction. Further research can examine other measurements of reproduction, such as litter size or the probability of successfully weaning multiple litters, to provide insight into the relationship between hair cortisol and reproductive success in these squirrels.

Examining the interactions between cortisol and extrinsic effects on reproductive success may also be interesting. As increasing temperatures are considered harsh conditions for these squirrels, we may see a significant interaction between temperature maximums and hair cortisol on reproduction. These squirrels also have lower reproductive success with low and high rainfall (Shofstall, unpublished data), suggesting a possible interaction between optimal rainfall and cortisol on reproductive success that might be worth examining. South Africa is predicted to have more variable rainfall and increasing temperatures in the future (Department of Environmental Affairs, 2018), which can deplete resources needed for the successful reproduction of these income breeders. Therefore, understanding the relationships between extrinsic factors, GCs, and the reproduction of female Cape ground squirrels with the acceleration of climate change is essential.

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Appendix A

Table A1. Model selection for log-transformed cortisol in female Cape ground squirrels. Avg max and min refers to the daily temperature maximum or minimum averaged from the beginning of January to the end of March each year. Max SD refers to the standard deviation of daily temperature maximum from the beginning of January to the end of March each year. Age (in years), body condition (size-mass residuals), squirrel ID (random effect), social group ID (random effect), and year: 2015-2023 (excl. 2020) (random effect).

Model	Terms	k	AICc	ΔAICc	AICc Wt
1	Avg Max + ID + Social Group ID + Year	6	472.16	0.00	0.53
2	Avg Max + Max SD + ID + Social Group ID + Year	7	474.65	2.49	0.15
3 (Null Model)	ID + Social Group ID + Year	5	475.15	3.00	0.12
4	Avg Max + Avg Min + ID + Social Group ID + Year	7	477.24	5.09	0.04
5	Max SD + ID + Social Group ID + Year	6	477.70	5.54	0.03
6	Age + ID + Social Group ID + Year	6	478.12	5.96	0.03
7	Avg Max + Max SD + Age + ID + Social Group ID + Year	8	478.15	5.99	0.03
8	Avg Min + ID + Social Group ID + Year	6	478.27	6.11	0.02
9	Avg Max + Avg Min + Max SD + ID + Social Group ID + Year	8	479.10	6.95	0.02
10	Avg Min + Max SD + ID + Social Group ID + Year	7	480.27	8.12	0.01
11	Avg Max + Avg Min + Age + ID + Social Group ID + Year	8	480.86	8.70	0.01
12	Avg Max + Max SD + Body Condition + ID + Social Group ID + Year	8	481.32	9.16	0.01
13	Body Condition + ID + Social Group ID + Year	6	481.80	9.64	0.00

14	Avg Max + Avg Min + Max SD + Age + ID + Social Group ID + Year	9	482.87	10.71	0.00
15	Avg Min + Max SD + Age + ID + Social Group ID + Year	8	483.51	11.35	0.00
16	Avg Max + Avg Min + Body Condition + ID + Social Group ID + Year	8	483.85	11.69	0.00
17	Avg Max + Max SD + Age + Body Condition + ID + Social Group ID + Year	9	485.07	12.91	0.00
18	Avg Max + Avg Min + Max SD + Body Condition + ID + Social Group ID + Year	9	485.69	13.53	0.00
19	Avg Min + Max SD + Body Condition + ID + Social Group ID + Year	8	486.91	14.76	0.00
20	Avg Max + Avg Min + Age + Body Condition + ID + Social Group ID + Year	9	487.75	15.59	0.00
21	Avg Max + Avg Min + Max SD + Age + Body Condition + ID + Social Group ID + Year	10	489.75	17.59	0.00
22	Avg Min + Max SD + Age + Body Condition + ID + Social Group ID + Year	9	490.43	18.27	0.00