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Hormonal Responses to Seasonal Thermal and Ecological Stressors in Japanese Macaques

(*Macaca fuscata*)

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Abstract

Relative to most primate species, Japanese macaques (*Macaca fuscata*) experience extreme seasonal variation in thermal and ecological stressors that can impact energetic demands. To cope with these environmental changes, levels of metabolic hormones, such as cortisol and triiodothyronine, fluctuate to facilitate energetic adjustments. While previous research in primates has investigated thermal and ecological stressors individually, a combined assessment of these stressors alongside hormone levels can provide a more holistic understanding of the relationship between a primate's thermoregulation, energetic balance, and stress. The goals of this study were to determine the effects of season, temperature and ecological stress on cortisol levels in Japanese macaques, as well as assess the relationship between cortisol and freely circulating triiodothyronine (fT_3). Fecal samples were collected at Kyoto University Primate Research Institute (KUPRI) on semi-free ranging Japanese macaques and tested for cortisol and fT_3 levels using enzyme-linked immunosorbent assay (ELISA). Japanese macaques did not show a significant difference in cortisol levels between seasons, although winter cortisol levels were marginally higher. During summer, there was not a significant effect of daily temperature on cortisol levels. However, in the winter, macaques showed a consistent negative relationship between cortisol levels and daily maximum temperatures. While this indicates that cortisol levels increased as maximum temperatures became colder, there was not a significant correlation with mean or minimum daily temperatures. Using activity levels as a proxy for ecological stress, there were no significant effects of mean daily group activity on cortisol levels during either season. However, there was a significant and strong positive relationship between fT_3 and cortisol levels during both summer and winter seasons, with cortisol levels increasing in conjunction with fT_3. Contrary to predictions, there

was not a seasonal pattern in cortisol levels, although these data provide partial evidence that thermal stress can impact cortisol levels on a daily scale. Animals were provisioned foods, which may have reduced the intensity of ecological stress, possibly dampening seasonal impacts on cortisol levels. Despite these mixed results, the strong relationship between fT_3 and cortisol may suggest that animals modulate metabolic hormones in a coordinated manner to cope with changing energetic needs.

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List of Abbreviations

ANOVA	Analysis of variance
BMR	Basal metabolic rate
CV	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
FGM	Fecal glucocorticoid metabolite
fT_3	Freely circulating triiodothyronine
KUPRI	Kyoto University Primate Research Institute
T_3	Triiodothyronine
T_4	Thyroxine

Chapter 1. Introduction

There are a variety of primate species that experience a broad range of environmental conditions as seasons change. With varying seasons comes abiotic and biotic transformations to a primate's habitat that can result in behavioral and physiological changes. Japanese macaques (*Macaca fuscata*), often referred to as the "snow monkey," experience some of the most significant and extreme seasonal changes in comparison to typically tropical primate species (Hanya, 2010). During the winter, these primates endure mean daily temperatures reaching as low as -1.9°C, and summer daily means as high as 35.6°C (Agetsuma & Nakagawa, 1998; Cozzolino *et al.*, 1992; Thompson *et al*., 2017; Tsuji & Takatsuki, 2009; Tsuji, 2011; Ventura *et al.*, 2005). These extreme temperatures, paired with severe weather conditions such as precipitation, storms and winds, can cause thermal stress that impacts thermoregulatory demands (Beehner & McCann, 2008; Campbell *et al.*, 2018). Also, these seasonal changes in climate can transform the ecological landscape, often altering food availability and distribution, yielding ecological stressors that impact animals' energetic condition. While previous research in primates has investigated the effects of thermal and ecological stressors individually (Garcia *et al.*, 2011; Takeshita *et al.*, 2018), relatively little research has pursued a combined assessment of these stressors. A more holistic approach to investigating seasonal stressors in Japanese macaques may allow for greater understanding of the relationship between a primate's thermoregulatory mechanisms, energetic balance, and stress.

Purpose

To better understand the impact of thermal and ecological seasonal stressors on hormone levels in Japanese macaques.

Scope

Japanese macaques inhabit geographical locations that experience four distinct seasons: winter, spring, summer and fall. With changing season conditions, macaques are subject to various thermal and ecological stressors that impact individuals' behavior and physiology. This study aims to identify the effects of season, temperature, and activity on cortisol levels, in addition to identifying a relationship between freely circulating triiodothyronine (fT_3) and cortisol. I have conducted an analysis that focuses on semi-free-ranging Japanese macaques subject to natural thermal conditions, housed at the Kyoto University Primate Research Institute (KUPRI) in Inuyama, Japan.

Assumptions

1. Japanese macaques experience high levels of seasonal variation, in which they are exposed to thermal and ecological stressors.

2. Cortisol mobilizes energy reserves within body in response to environmental stressors.

Hypotheses

Aim 1. Assess the effect of season on cortisol levels. If seasonal changes are thermally and/or ecologically stressful for Japanese macaques, then cortisol levels should be higher during winter, when animals experience greater energetic costs from thermoregulatory demands and lower food availability.

Aim 2. Assess the effects of daily temperature changes on cortisol levels. If thermal conditions elicit stress, then cortisol levels should be higher when daily temperatures are at their lowest, eliciting greater energetic demands from thermoregulation.

Aim 3. By using activity levels as a proxy for ecological stress (Grueter *et al.*, 2013; Hodges, 2020; Ménard *et al.*, 2013), the effects of macaques' activity levels on cortisol levels will be assessed. During times of greater activity, cortisol levels are expected to increase, as animals are required to expend greater amounts of energy in response to ecological stressors such as changes in food availability.

Aim 4. Assess the relationship between fT_3 and cortisol. If both hormones have corresponding involvement in a primates' energetic condition during times of thermal or ecological stress, then fT_3 and cortisol will show a positive relationship.

Significance

Japanese macaques are one of the few cold-habitat primates that experience extreme seasonal variation. These primates face thermal variability and transformations to the ecological landscape that can cause stress to the animal, resulting in changes in behavior and physiology. This study aims to give insight into the impacts of thermal and ecological stress on primates and provide an understanding of how thermoregulatory mechanisms and energetic balance are utilized to cope with stress.

Chapter 2. Review of Literature

Japanese Macaques and Their Environment

Japanese macaques are endemic to Japan, distributed across the nation's archipelago from the northern Shimokita Peninsula to the southern island of Yakushima (Hanya, 2010). This wide distribution provides roughly four classifications of natural vegetation: 1) warm, temperate evergreen forests, 2) cool deciduous broad-leaved forests, 3) subalpine evergreen coniferous forest, and 4) alpine grassland, with the fundamental difference between these habitats being whether trees shed their leaves in winter (Tsuji, 2010). The climate of Japan exhibits clear seasonal fluctuations in air temperature and rainfall, further subclassifying it into four seasons: spring (March to May), summer (June to August), autumn (September to November), and winter (December to February) (Tsuji, 2010). Climate alone dictates many aspects of a macaque's life, strongly influencing the location and availability of vegetation and the need to maintain energetic condition to keep up with thermoregulatory demands (Agetsuma, 2000; Hanya, 2004). For example, when air temperature decreases from 29°C to 5°C, captive Japanese macaques have been shown to double their heat production in order to meet thermoregulatory needs (Hanya *et al.*, 2007; Hori *et al.*, 1977). Seasonally, there are significant environmental changes in both vegetation and climate that reflect thermal and ecological stressors that influence Japanese macaques' daily lifestyle (Hanya, 2004).

Behavioral Responses to Thermal and Ecological Stressors

Thermal and ecological conditions are interconnected, creating a dynamic and ever-changing environment that influences primates. From the selection of microhabitats and posture modulation, to hormone fluctuations resulting in physiological changes, primates employ a

variety of mechanisms to regulate their temperature and reduce energetic costs in response to seasonal stressors.

'Behavioral thermoregulation,' or behaviors displayed by an animal to reduce the physiological costs of thermoregulation, are voluntary actions driven by the goal to conserve or dissipate heat (Hanya *et al.*, 2007; Tan & Knight, 2018). Behavioral changes could include alterations to activity budgets (Kearney *et al*., 2009), the adjustment of posture by changing body shape and direction (Hanya *et al.*, 2007; Morland, 1993; Stelzner & Hausfater, 1986), huddling with group members (Hanya *et al.*, 2007; Schino & Troisis, 1990), or selecting climatically different microhabitats that favor energy conservation and support thermoregulation (Bicca-Marques & Calegaro-Marques, 1998; Hanya *et al.*, 2007; Sargeant *et al.*, 1994; Takemoto, 2004). These adjustments to behavior are often energetically inexpensive and allow an animal to modulate heat loss and respond to changes in thermal environment without more costly, long-term acclimation (Hanya *et al.*, 2007).

Amongst these methods of behavioral thermoregulation, activity budgeting interconnects with both thermal and ecological stressors and can strongly impact an animal's energy stores utilized for thermoregulatory mechanisms (Hodges, 2020). Activity budgeting is the time an animal allocates for various activities and when under stress, an animal's patterns may fluctuate considerably. Changing environmental factors such as harsher climate and food availability are known to drive activity patterns of primates, therefore the analysis of changes in locomotor activity is frequently used as a proxy for ecological stress experienced by primates (Curtis & Rasmussen, 2006; Fernández-Duque *et al.*, 2010; Grueter *et al.*, 2013; Gursky, 2003; Hodges, 2020; Ménard *et al.*, 2013; Rasmussen, 1977; Wright, 1989). Temperature and food-related factors such as food distribution, feeding rate and food abundance, can each impact the amount

and intensity of an animal's activity (Hanya, 2004). For example, during winter seasons when food is of lesser availability and thermoregulatory demands are increased, the amount and intensity of activity and energy spent will be adjusted accordingly to account for thermal and ecological stressors.

Being native to a temperate region, Japanese macaques experience some of the most drastic changes in seasonality in comparison to classically tropical primate species, resulting in the need to balance caloric intake with thermoregulatory and energetic needs. Low temperatures drive thermoregulatory costs, and climatic factors such as precipitation compounds these effects, resulting in macaques decreasing their activity levels during winter seasons to conserve energy (Hanya *et al.*, 2018). Alternatively, the summer season does not require as stringent thermoregulatory demands and activity can be increased. The intensity level of activity fluctuates depending on potential heat stress; a primate avoids generating too much heat through muscle activity during high temperature (Fredericksen, 2014; Hanya *et al.*, 2018 Radford *et al.*, 2010). Also, Hanya (2004) found that both temperature and food availability were significant variables explaining feeding ecology and seasonal diet variation, which can ultimately impact the animal's activity level. During the summer season when highly nutritious fruits are available, Japanese macaques' feeding time decreases, but the distance they must travel increases due to low density of fruit-food trees (Hanya, 2004). During cooler temperatures, macaques feed on herbs, which conserves energy because animals do not have to climb trees or exert additional energy to obtain food. Moreover, herbs are more readily available in sunny areas which may add to energetic savings by reducing thermoregulatory costs (Hanya, 2004). Thus, seasonal changes in food availability and feeding patterns is associated with variation to macaque activity levels.

Physiological Responses to Thermal and Ecological Stress

Physiological responses can also be effective in the generation or dissipation of heat (Tan & Knight, 2018). In preparation for cooler seasons, some primates have been shown to increase body mass, and increase insulation with fat, down and fur to aid in the retention of heat (Génin *et al*., 2003). Also, a primate's body may involuntarily elicit autonomic thermoregulatory responses such as involuntary muscle contraction, shivering, and non-shivering thermogenesis via metabolism and heat production in brown adipose tissue (Chafee & Allen, 1997; Takeshita *et al.*, 2018; Terrien *et al*., 2008). Amongst these physiological changes, variations in hormone levels have shown to elicit both immediate and long-term physiological effects that aid in thermoregulation (Sterling *et al.*, 2013). Hormonal changes are fluid, allowing for the measurement of daily changes that may be used as an assessment tool to decipher quick alternations a primate makes, based on shifts in thermal and ecological conditions.

Hormones play an intricate and multifaceted role in primates, influencing basal metabolic rate (BMR) and thermogenesis while also having an important role in energy allocation in various functions including growth, reproduction and thermoregulation (Davis, 2019; Dias *et al.*, 2017). Hormones that reflect a primate's energetic condition and stress response may allow for greater insight into the impact thermal and ecological stressors can have on a primate's physiology and behavior. Cortisol and thyroid hormones are biomarkers of energetic condition, involved in gluconeogenesis and cellular metabolism (Dias *et al.*, 2017; Kim, 2008; Sapolsky *et al.*, 2000) and excreted in urine and feces (Dias *et al.*, 2017; Norman & Litwack, 1997). By using widely applied non-invasive methods, such as the collection of feces, hormones can be tested via excreta without eliciting unnecessary stress to the animal (Behringer & Deschner, 2017). By performing a more comprehensive assessment of hormones with complementary

roles, such as cortisol and thyroid hormones, information on the impact of both thermal and ecological stressors and their relationship to thermoregulation and energy expenditure may be provided.

Cortisol, a glucocorticoid, is often referred to as the stress hormone and is secreted in response to various stressors, including psychological (Corlatti *et al.*, 2014; Dias *et al.*, 2017; Stocker *et al.*, 2016), environmental (Bourbonnais *et al.*, 2013; Dias *et al.*, 2017; Lewanzik *et al.*, 2012), and physical challenges (de Bruijn & Romero, 2011; Dias *et al.*, 2017; Houser *et al.*, 2011). Researchers have also found that this hormone is critical for energy mobilization, even in the absence of stressful conditions (Dias *et al.*, 2017; Herman *et al.*, 2016). The hypothalamic-pituitary-adrenal axis modulates complex physiological responses by signaling the release of cortisol into the bloodstream, eliciting the mobilization of energy during environmental challenges (Herman *et al.*, 2016; Sapolsky, 2002). This involuntary response engages the autonomic nervous system and drives an array of events that can optimize survival by elevating heart rate, blood pressure and glucose production (Herman *et al.*, 2016). The release of cortisol into the bloodstream is responsible for these rapid physiological changes, allowing an animal to quickly respond to stressors and danger (Herman *et al.*, 2016). While a wide variety of stressors can elicit an influx of cortisol into the bloodstream, I chose to focus on fluctuations in response to seasonal changes that result in thermal and ecological stressors, which are highly relevant to primate species under natural environmental conditions (Weingrill *et al.*, 2004).

Japanese macaques experience freezing temperatures, at times undergoing extreme thermal stressors that can result in the rapid increase in gluconeogenesis, mobilizing energy reserves (Eckardt *et al.*, 2016; Sapolsky, 2000).   This influx of glucose into the bloodstream

allows for greater energy expenditure, potentially including production of heat for

thermoregulation. Rapid changes in cortisol levels can occur when seasonal fluctuations lead to

variations in food availability, often associated with a primate traveling further for desirable or

suitable nutrition (Abbott *et al.*, 2003). This may elicit stress, especially when food availability

has decreased, or foods have become more difficult to obtain. In response, changes in cortisol

levels occur allowing the animal to respond to both the stress induced by ecological-based

energetic demands and the thermal stress associated with variation in temperatures (Beehner &

McCann, 2008). Further analysis of cortisol levels in response to stressors will provide insight

into changes that occur seasonally in Japanese macaques.

Energy homeostasis is dependent upon hormonal influence and the balance between

caloric intake and energy expenditure (Rosen & Spiegelman, 2006). The thyroid hormones

triiodothyronine (T_3) and thyroxine (T_4) influence many processes including growth,

reproduction, metabolism and thermoregulation (Silva, 2006; Yen, 2001). These hormones

assist in the regulation of metabolic activity, which aids the body in maintaining energetic

balance, but also plays a critical role in thermogenesis (Kaack *et al.*, 1979; Schaebs *et al.*, 2016).

For example, several mammal species display changes in thyroid levels that are associated with

energy balance; thyroid hormone levels decrease during times of fasting in badgers (Harlow &

Seal, 1981; Schaebs *et al.*, 2016), sheep (Blum *et al.*, 1980; Schaebs *et al.*, 2016) and rabbits

(Mechetti *et al.*, 2015; Schaebs *et al.*, 2016). Also, thyroid levels increase with increasing

energy expenditure in relation to the thermal effect of physical activity (Kim, 2008; Schaebs *et

al.*, 2016) and in response to low seasonal temperatures (Cristóbal-Azkarate *et al.*, 2016; Schaebs

et al., 2016; Silva, 1995). For many mammals, thyroid hormones reach their highest levels

during cooler seasons, causing elevations in BMR in order to produce heat whilst increasing

energy expenditure (Cristóbal-Azkarate *et al.*, 2016; Danforth & Burger, 1984; Dias *et al.*, 2017; Silva *et al.*, 2003). In addition to a response to thermoregulatory changes, thyroid hormones have previously demonstrated fluctuations in response to various stressors. In adult rats, mild stressors can cause a slight increase or relatively no change in thyroid levels (Armario *et al.*, 1984; Helmreich & Tylee, 2011; Turakulov *et al.*, 1994), while more significant, severe stressors can result in decreased thyroid hormone levels (Cizza *et al.*, 1996; Helmreich and Tylee, 2011; Kilburn-Watt *et al.*, 2010; Kondo *et al.*, 1997; Langer *et al.*, 1983).

In wild male Barbary macaques (*Macaca sylvanus*), a seasonal pattern in thyroid hormones has been identified that is consistent with a thermoregulatory role (Cristóbal-Azkarate *et al.*, 2016; Thompson *et al.*, 2017). Freely circulating triiodothyronine (fT_3) increased significantly during cooler seasons, indicating that thyroid hormones are utilized as a mechanism to boost metabolism in response to thermoregulatory pressures. By analyzing fT_3 and cortisol hormones concurrently, we can gain an understanding of the relationship between metabolic hormones and seasonal coping strategies aimed at maintaining energy balance.

Chapter 3. Methodology

Data Collection

Study Site and Subjects

Semi-free ranging Japanese macaques housed at the Kyoto University Primate Research Institute (KUPRI) in Inuyama, Japan (35°23'N, 136°57'E) were utilized for data collection. The animals were exposed to natural thermal conditions in an outdoor enclosure (~900m^2). Provisioned feedings of monkey biscuits and other foods (e.g., sweet potato, carrots, etc.) occurred at roughly the same time each day (09:00 and 16:00 on weekdays; 13:00 on weekends) while water and ground foliage were available ad libitum. The animals were individually identifiable through unique facial tattoos. Sample collection occurred during winter (December) 2014 and summer (July) 2015. During summer data collection, macaques experienced mean daily temperatures of 27.3°C, mean daily lows of 19.2°C and mean daily highs of 35.6°C. For sample collection during winter, macaques experienced mean daily temperatures of 5.7°C with mean daily lows of -1.9°C and mean daily highs of 15.6°C (Thompson *et al.*, 2017).

Fecal Sample Collection

Fecal samples were collected using non-invasive methods in order to measure hormone levels from N=17 individual animals (8 males, 9 females), yielding N=33 samples in total across both seasons (summer: N=17; winter: N=16). Some individual animals were sampled more than once throughout both summer and winter seasons, while others were sampled a single time (range=1-3 samples per individual, x̄=1.9 samples per individual) (**Appendix 1**). Fecal samples were collected by observing defecation, gathering the sample in a sterile bag, and immediately freezing at -80°C.

Temperature Measurement

Ambient temperatures near the animals' enclosure at KUPRI were recorded every ten minutes using an on-site weather station (HOBO U30, Onset Computer Corporation, Bourne, MA). Summer and winter data collection periods were logged (summer=1605.0 sample hours, winter=2155.5 sample hours). The manufacturer states the temperature accuracy to be ±0.2°C with 0.03°C measured resolution.

Activity Data

Previous research by Thompson *et al.* (2017) conducted all occurrence sampling of focal animals from sunrise to sunset and recorded feeding and locomoting. Feeding was defined as any regular grasping, processing, or ingestion of food items, while locomotion was defined as regular movement that did not include any feeding. Both measures were collected continuously, by recording start and stop times, to the nearest minute. Multiple animal observations occurred on any given day, and primate groups collectively coordinate daily activity and movement (Boinski & Garber, 2000). Therefore 'mean daily group activity' was determined per day by calculating the mean number of minutes spent active across all animals on a given day. This was done separately for the number of minutes spent feeding only, locomoting only, and total activity time (combining locomotion and feeding). Data were collected on the daily activity patterns of macaques (N=5 animals) for a total of 620.75 observation hours across all animals (summer= 422.25 hours, winter=198.50 hours). Any animals with <180 minutes of observation time per day were excluded from the data set. Individual macaques were observed in the summer $\bar{x}$=724 minutes per day (range: 315-855 minutes) and in the winter $\bar{x}$=496 minutes per day (range: 270-615 minutes). Due to seasonal changes in day length, less observation time was conducted in the winter.

Measurement of Cortisol

Fecal samples were extracted following established hormone extraction procedures (Hodges & Heisterman, 2011; Wasser *et al*., 2010). In order to standardize hormone levels by dry weight, wet fecal samples were homogenized and lyophilized. Plant matter was eliminated by sifting the material through a 40-mesh sieve (Hodges & Heisterman, 2011; Khan *et al*., 2002). Fecal powder (0.1gm) was added to 70% ethanol (15ml), vortexed for 30 minutes, then centrifuged for 20 minutes at 2200 rpm. Supernatant was then decanted and the original pellet was re-extracted and combined with other extracts to be stored at -20°C. Before performing enzyme linked immunoassay (ELISA) testing, samples were diluted 1:3 using assay buffer. Samples were run in duplicate. A sample control was developed by pooling fecal extracts together and running the sample control across all kits used. The samples control was diluted 1:3 using assay buffer and testing was performed in duplicate.

Commercially available, human optimized cortisol ELISA kits (Abnova, Taiyuan City, Taiwan) were utilized to detect levels of cortisol in fecal samples. The use of human adapted kits is a commonly accepted approach when testing primate samples due to the physiological similarities of nonhuman primates and humans (Phillips *et al.*, 2014). A microtiter plate coated with anti-mouse IgG was utilized for the immunological reaction to occur. With the addition of anti-cortisol monoclonal antibody and cortisol-peroxidase, a sample containing cortisol antigen can be determined by analyzing the generated color intensity using a microplate reader at 450nm wavelength.

The intra-assay coefficient of variation (CV) was 7.5% ±3.8%; inter-assay CV was 14.86% over two assays. There was not a significant difference in slope between serial dilutions of samples and the standard curve (t=-0.71, p=0.53), indicating parallelism. A single spike

recovery analysis was performed using a 3,200 pg/mL standard for a spike recovery test, yielding 91.9% recovery.

Measurement of Thyroid Hormone

Thyroid hormone testing was previously conducted by Thompson *et al.* (2017), assessing fT_3 levels from the same fecal samples used in this thesis. Established procedures for hormone extraction were performed as described for cortisol, and fT_3 concentration measurements were evaluated using ELISA kits (Item Code: 1650, Alpha Diagnostics Inc., San Antonio, TX). Validations for inter-assay and intra-assay variation were previously published, along with parallelism and spike recover analyses (Thompson *et al.*, 2017).

Data Analysis

Aim 1: Seasonal Difference in Cortisol

To assess differences in macaques' fecal cortisol levels between seasons, a two-way analysis of variance (ANOVA) was performed with cortisol levels as the dependent variable, season (summer/winter) as a predictor variable. Individual identification was also included as a predictor variable, to control for potential differences between individual animals' baseline hormone levels.

Aim 2: Temperature Effect on Cortisol

To assess the impact of thermal stressors, the effect of mean daily temperatures on Japanese macaque cortisol levels were tested. Lag times were taken into consideration, knowing there is a delay between circulating blood levels of a hormone and the measurement of levels in fecal samples. In Japanese macaques, hormonal excretion of cortisol in feces occurs in 24 to 48 hours (Shimizu, 2005; Takeshita *et al.*, 2014; Takeshita *et al.*, 2018). For this reason, a series of temperature lags were used relating cortisol levels to previous days' temperatures. Using

previous days' successive temperature data, I calculated the mean daily minimum, mean daily maximum, and mean daily mean temperature experienced by animals two, four and six days prior to fecal samples collection. [For example, at the mean daily minimum two day lag, the mean of the previous two days' daily minimum temperatures was calculated.] Multiple lags were used to account for variation between circulating and fecal hormone levels and also, to consider the potential delay between experienced temperature change and the activation of a biological response.

Multiple fecal samples were collected on any given day; therefore a mean daily cortisol level was calculated. On average, $\bar{x}=2.1$ fecal samples were used to generate daily means (range 1-4). No individuals were sampled twice in one day. Although this daily approach limits sample size (summer: N=7 days, winter: N=7 days), it controls for potential pseudoreplication due to the non-independence of repeated daily temperatures in the analysis. One-tailed, bivariate linear regressions were used, separately analyzing each season. To adjust for multiple tests being performed, Bonferroni corrections were applied (within each season) to avoid spurious significant results.

Aim 3: Effect of Activity on Cortisol

Using the amount of activity as a proxy for ecological stress, mean daily group activity and mean daily cortisol levels were used to assess the impact of ecological stress on cortisol levels. Like Aim 2, this daily approach limits sample size (summer: N=7 days, winter: N=8 days), but controls for potential pseudoreplication. Bivariate one-tailed linear regressions were used to assess the effect of mean daily group activity on mean daily cortisol levels, with separate tests for the mean daily time an animal spent feeding, locomoting and total movement. Analyses were performed separately for each season.

Aim 4: Relationship Between fT₃ and Cortisol

A one-tailed Pearson's correlation was used to test for a relationship between fT_3 and cortisol levels separately for each season. This analysis used individual fecal samples for the assessment of fT_3 and cortisol. Individual animals were represented by more than one fecal sample in this analysis (**Appendix 1**), however, analyses from Aim 1 suggest there were not significant effects of individual animal identity on cortisol. Likewise, supplementary analyses excluding repeated sampling of the same animal (by limiting it to the first chronologically collected sample per animal), resulted in similar statistical outcomes as testing all samples.

Statistical outcomes were formally evaluated at α=0.05, except for the Bonferroni corrections mentioned above, which are reported in results. Statistical tests were conducted using SPSS 20.

Chapter 4. Results

Aim 1: Seasonal Difference in Cortisol

Japanese macaques did not show a significant difference in cortisol levels between summer and winter seasons ($\bar{x}$ difference=0.046±0.006µg/mL, $F_{(1,15)}$=2.616, p=0.127), although winter cortisol levels were marginally higher than summer (**Figure 1**). Likewise, season explained 14.8% of the variation in cortisol levels. Cortisol levels did not significantly differ between individual animals ($F_{(1,15)}$=1.084, p=0.536), although individual identity accounted for 53.6% of the variation in cortisol levels.

Figure 1. Fecal cortisol levels in Japanese macaques, by season.
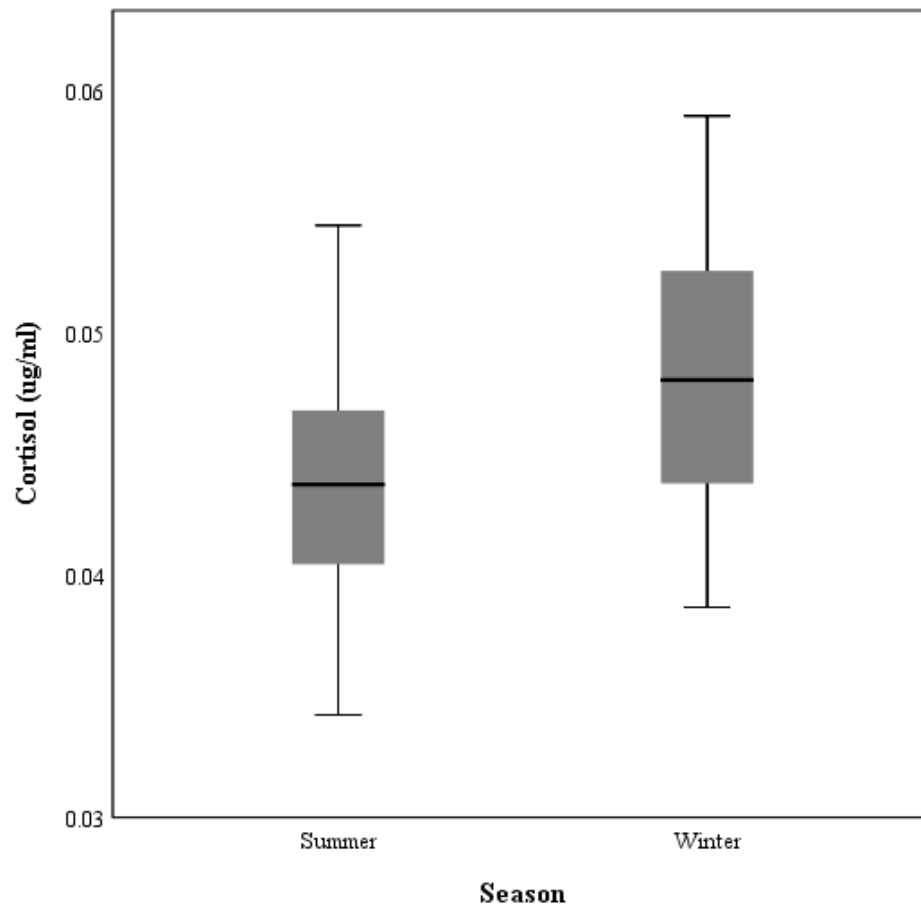
Aim 2: Temperature Effect on Cortisol

During summer, there was not a significant effect of daily temperature on cortisol levels (**Table 1**). However, during the winter, macaques showed a consistent negative relationship between cortisol levels and the mean daily maximum temperatures experienced, with cortisol levels increasing as maximum temperatures became colder. At α=0.05, two, four and six day mean daily maximum temperatures, the results were significant. However, after taking the Bonferroni correction into account, only two day mean daily maximum temperatures remained significant. Yet, it is worth noting that the relationships between mean daily maximum temperatures and cortisol in winter show consistently larger effect sizes than all other comparisons (**Table 1**).

Table 1: Relationship between Japanese macaque fecal cortisol levels and mean daily minimum, maximum and mean temperatures at two, four, and six days prior.

Days Prior to Fecal Collection	Minimum Temperatures*			Maximum Temperatures*			Mean Temperatures*		
Summer	β	t	p	β	t	p	β	t	p
2	0.098	0.220	0.583	0.601	1.682	0.924	0.416	1.024	0.824
4	0.715	2.286	0.964	0.570	1.552	0.909	0.603	1.690	0.924
6	0.157	0.355	0.631	0.717	2.303	0.965	0.560	1.512	0.905
Winter									
2	-0.443	-1.106	0.160	-0.893	-4.429	***0.004***	-0.667	-2.000	0.051
4	0.291	0.680	0.736	-0.751	-2.545	**0.026**	-0.298	-0.698	0.258
6	0.385	0.834	0.775	-0.836	-3.051	**0.019**	0.112	0.251	0.595

*Mean of daily minimum/maximum/mean temperatures over the two, four, or six days prior to fecal samples collection
All β values are standardized
Bold=significant at α=0.05
Bold and italicized=significant after Bonferroni correction, adjusted α=0.005

Aim 3: Effect of Activity on Cortisol

There were no significant effects of mean daily group activity on mean daily cortisol levels in macaques, when assessing mean time spent feeding, locomoting, or total movement observed, during either season (**Table 2; Figure 2**).

Table 2: Effect of mean daily group activity on cortisol levels, by season.

	Summer (N=7 days)			Winter (N=8 days)		
Time Spent	β	t	p	β	t	p
Feeding only	-0.222	-0.509	0.632	-0.075	-0.185	0.859
Locomoting only	0.053	0.118	0.911	0.384	1.020	0.347
Total movement	-0.029	-0.066	0.950	0.321	0.829	0.439

All β values are standardized

Figure 2. Relationship between mean daily total movement and mean daily cortisol levels, by season.
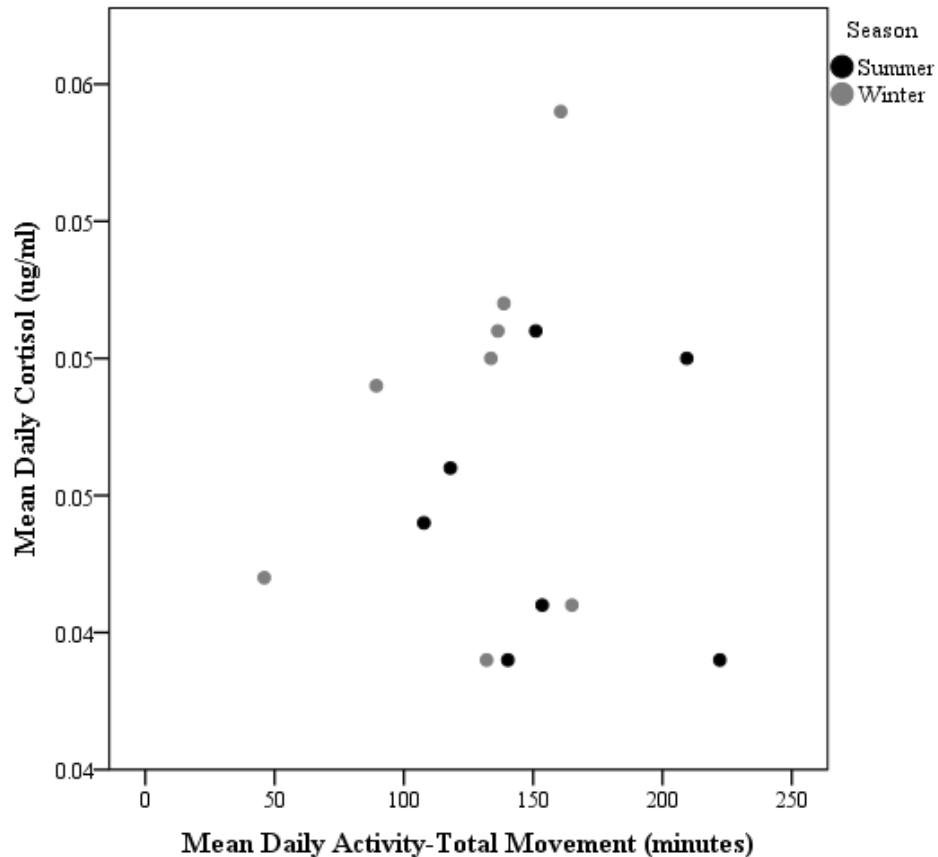
Aim 4: Relationship Between fT$_3$ and Cortisol Levels

There was a significant and strong positive relationship between fT$_3$ and cortisol levels during both summer (r=0.621, p=0.004, N=17) and winter (r=0.700, p=0.005, N=16) seasons.

Figure 3. Relationship between an individual's fT$_3$ and cortisol levels with linear trendline, by season.
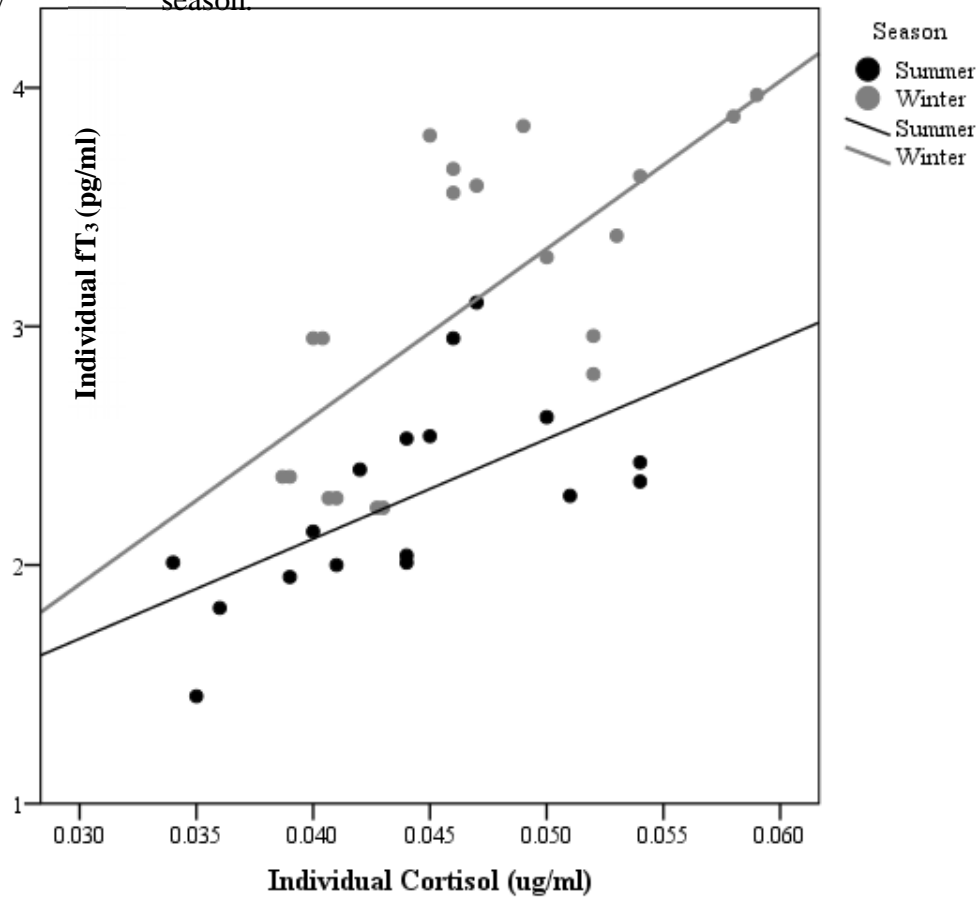
Chapter 5. Discussion

I assessed if Japanese macaques would display seasonal fluctuations in cortisol levels in response to thermal and ecological stressors. The main findings were: 1) macaque cortisol levels did not show significant differences between the summer and winter seasons. 2) During the winter season when temperatures were lower, macaques showed a negative relationship between cortisol levels and two day mean daily maximum temperatures. 3) There was no discernable relationship between cortisol levels and activity levels of the animals. 4) A strong positive relationship between fT_3 and cortisol levels was displayed during both summer and winter seasons.

Aim 1: Seasonal Difference in Cortisol

Japanese macaques experience seasonal alterations in thermal and ecological stressors, and I expected cortisol levels to be lower during the summer and higher during the winter season. My hypothesis was not supported. While winter season cortisol levels were marginally higher than summer, this difference did not reach statistical significance. In this analysis, cortisol levels did show high variation between individual samples (**Appendix 1**), even in the absence of a discernable seasonal difference.

Seasonal variation in glucocorticoid levels has been demonstrated in a variety of species (e.g. ground squirrels (*Spermophilus saturates*): Boswell *et al.*, 1994; humans (*Homo sapiens*): Walker *et al.*, 1997; red deer (*Cervus elaphus*): Ingram *et al.*, 1999; squirrel monkeys (*Saimiri sciureus*): Schiml *et al.*, 1999; rhesus macaques (*Macaca mulatta)*: Vandeleest *et al.*, 2013). Even though most mammal species have demonstrated seasonality in cortisol levels, there is no consistent season when mammals tend to have elevated glucocorticoid levels (Michael Romero, 2002). Of the available literature on cold habitat primates, there is limited

comparative data of a direct relationship between season and cortisol levels. Research conducted by Yang *et al*. (2015) identified that in Sichuan snub-nosed monkeys (*Rhinopithecus roxellana*), variation in fecal glucocorticoid metabolite (FGM) levels were mainly influenced by seasonal change in male subjects, while females FGM levels were more greatly affected by breeding versus non-breeding situations. Takeshita *et al.* (2014) investigated seasonal FGM levels in Japanese macaques at KUPRI and while this study is not directly analogous to mine, it does provide the most relevant comparative data. Takeshita *et al.* (2014) assessed seasonal variation in total FGM levels in reproductive female Japanese macaques, in a sample with N=12 females housed in individual-sized indoor cages with controlled temperature conditions and N=27 semi-free ranging animals exposed to natural thermal conditions. By contrast, my subjects were all semi-free ranging in outdoor enclosures, subject to natural thermal conditions, and included animals of both sexes and differing reproductive states. Both studies used similar collection periods and animals were provisioned food. Takeshita *et al.* (2014) found significantly higher FGM levels in cycling females during October to December than lactating females in May and June, while my study did not find significant seasonal differences in cortisol levels. Though my findings were not significant, both studies found a similar seasonal pattern, with winter cortisol levels being marginally higher than summer levels. A possible explanation for the differences in our findings could be due to variations in housing conditions and study populations. Takeshita *et al.*'s (2014) data were pooled across animals kept in indoor cages and those who were semi-free ranging and exposed to more natural thermal conditions. While the females kept in individual cages displayed higher overall FGM levels than semi-free ranging animals, Takeshita *et al.* (2014) unfortunately did not provide a seasonal comparison between animals in the two housing conditions. Although my results trend in the same direction as those in the Takeshita study, their

larger effect size and significant findings may be attributed to pooling housing conditions and the potential for differential stress experienced by subjects kept in indoor cages compared to the semi-free ranging animals. My study also included a broader population of both male and female subjects at varying reproductive stages which may have displayed more variable levels of stress. This higher variation may have generated a weaker seasonal pattern in cortisol levels, compared to Takeshita *et al.*'s (2014) more uniform sample of only cycling and lactating female Japanese macaques.

Males and female primates also display differences in cortisol levels due to the impacts of seasonal mating practices and dominance rank (Barett *et al.*, 2002; Cavigelli & Caruso, 2015). Japanese macaques are considered seasonal breeders, mating from late autumn to early winter and giving birth from spring to early summer (Garcia *et al.*, 2011). During this time, dominance rank has been shown to influence cortisol levels, with high-ranking males displaying significantly higher levels of cortisol in comparison to low ranking males due to the considerable stress that accompanies mating (Barett *et al.*, 2002). In females, cortisol levels were also shown to be elevated in cycling females during the mating season, leading to potential variation between male and female subjects depending on their dominance rank and cycling status (Takeshita *et al.*, 2014). My statistical test controlled for the effect of individual differences when comparing winter and summer cortisol levels. While there were not significant differences in cortisol between individuals, there was a high amount of individual variation (**Appendix 1**). This variation could be due to differences in dominance rank and/or female subjects who are cycling, leading to an effect on cortisol levels that makes it difficult to discern a seasonal pattern.

Free ranging, captive, or semi-free ranging study subjects experience differences in food availability, some relying heavily on foods that fluctuate seasonally while others are steadily

provisioned foods. In a study performed by Rangel-Negrín *et al.* (2009), free-ranging spider monkeys (*Ateles geoffroyi yucatanensis*), a tropical primate, living in wild conditions displayed seasonal differences in cortisol, while captive spider monkeys did not (Rangel-Negrín *et al.*, 2009; Valero & Byrne, 2007). Free-ranging subjects showed elevated cortisol levels during the dry season due to a decrease in fruit trees and a reduction in food availability, showing that seasonal changes can be stressful even under mild seasonal alterations (Rangel-Negrín *et al.*, 2009).   Captive populations, on the other hand, are artificially provisioned food resources which may alleviate the stressors of fully natural environmental conditions (Morgan & Tromborg, 2007). Typically, winter seasons are more ecologically stressful due to restricted food availability (Hanya, 2004). However, food-related stressors were potentially reduced in my study since subjects were semi-free ranging and provisioned food, with the opportunity to both naturally forage as well as gain food at timed feedings events. Such managed conditions could be considered relatively constant, eliminating additional stressful factors that are experienced seasonally (Sha *et al.*, 2018). This potentially led to a reduction in the intensity of seasonal stress, resulting in low magnitude differences in cortisol levels between seasons.

Aim 2: Temperature Effect on Cortisol

During the summer, there was not a significant relationship between cortisol levels and temperatures experienced by animals, but during the winter, macaques showed a consistent negative relationship between cortisol levels and the mean daily maximum temperatures experienced. These findings weakly confirm my predictions that cortisol levels increase while temperatures become colder, although this was only displayed with two day mean daily maximum temperatures experienced by the macaques and no other temperatures measures, after applying Bonferroni corrections.

Decreased temperatures have been shown to increase FGM levels during cold stress in Japanese macaques (Takeshita *et al.*, 2014), chacma baboons (*Papio ursinus*; Weingrill *et al.*, 2004) and geladas (*Theropithecus gelada*; Beehner & McCann, 2008). Different primate species have shown variable excretion lag times between stressful interactions and detectable glucocorticoid levels in the feces, for example Virunga mountain gorillas (*Gorilla beringei beringei*) exhibited peak levels roughly 60-80 hours post interaction (Eckardt *et al.*, 2016), while Japanese macaques peak levels occur after roughly 24-48 hours (Shimizu, 2005; Takeshita *et al.*, 2014). Takeshita *et al.* (2014) found a significant negative impact of temperature on cortisol levels at two day lag minimum temperatures, while I found significant negative impact of temperature at two day lag maximum temperatures. It is unclear why minimum and maximum temperatures would led to different effects in these studies, but both results nonetheless display a negative relationship between temperature and cortisol levels, in which cortisol increases under cooler conditions. This weakly supports a role of day-to-day temperatures on stress in Japanese macaques. Also, the consistent significance of two day lag times in this study and Takeshita *et al.* (2014) may corroborate a biological pattern in which thermal stress can lead to detectable changes in fecal glucocorticoids within 48 hours.

In addition to temperatures, other weather variables such as wind, precipitation, humidity and solar radiation have shown to be factors that could influence a primate's thermoregulatory responses (Thompson *et al.*, 2017; Weingrill *et al.*, 2004). These variables introduce additional thermal stressors that may be influencing thermoregulatory coping mechanisms beyond what my study assessed.

Aim 3: Effect of Activity on Cortisol

Using the amount of activity as a proxy for ecological stress (Grueter *et al.*, 2013; Hodges, 2020), I predicted that Japanese macaques would increase their activity levels during ecologically stressful times and accordingly display an increase in cortisol levels. My results indicated that there was not an identifiable relationship between cortisol levels and the amount of daily activity performed by macaques. This may potentially be due to semi-free ranging animals experiencing less ecological stress than fully free-ranging animals.

Environmental perturbations can disrupt homeostasis and result in ecological stress that can alter an animal's activity patterns (Rangel-Negrín *et al.*, 2009; Reeder & Kramer, 2005). Fully free-ranging animals are subject to greater environmental variability, experiencing stress due to habitat fragmentation (Franceschini *et al.*, 1997; Romero, 2004; Wikelski & Cooke, 2006) food availability (Cavigelli, 1999; Chapman *et al.*, 2006) and proximity to humans (Davis *et al.*, 2005; Rangel-Negrín *et al.*, 2009), all of which can influence activity patterns (Gaynor *et al.*, 2018). Captive populations, on the other hand, have activity restrictions due to cage or pen size and are subject to additional sources of stress such as restricted feeding and foraging opportunities, routine husbandry and forced proximity to humans (Morgan & Tromborg, 2007). My research subjects were semi-free ranging and hence subject to a combination of stressors experienced by captive and free-ranging animals. I expected ecological stressors such as food availability (of ground foliage available *ad libitum*) to influence my subject's activity patterns, thus effecting cortisol levels, but the overall reduction in ecological stressor in semi-free ranging conditions may have nullified such a pattern.

Previous studies have reported that provisioned primates often regulate their time budgets and activity patterns in response to seasonal food provisioning (Chauhan & Pirta, 2010; Jaman &

Huffman, 2013; Saj *et al.*, 1999; Sussman *et al.*, 2011). Many provisioned animals prefer to forage as this is an inherently rewarding appetitive act and many animals will work to obtain food, even when it is freely available to them (Coulton *et al.*, 1997; Inglis & Ferguson, 1986; Inglis & Shepherd, 1994; Menzel, 1991; Morgan & Tromborg, 2007; Reinhardt, 1994). My research subjects were provisioned and allowed to freely feed on natural vegetation, possibly reducing ecological stress experienced by fully captive animals that are unable to fulfill their natural foraging instincts, or free-ranging animals who may have difficulties in procuring adequate nutrition.

Sha *et al.* (2018) conducted researched on Japanese macaques (at KUPRI, the same as this thesis), where accelerometers and the doubly-labelled water method was used to measure seasonal differences in activity and daily energy expenditure. They found that the total proportion of diurnal activity differed seasonally in Japanese macaques, displaying lower levels of activity in the winter season versus the summer. Though I expected activity to increase due to food foraging efforts during times of reduced food availability (i.e. winter), the intensity of winter temperatures and greater thermoregulatory demands may have more robustly influenced the animals' activity, therefore resulting in less activity in order to conserve energy. For example, Hanya *et al.* (2018) found free-ranging Japanese macaques to be less active when temperatures were at their lowest. Thermal stressors may have played a larger role in shaping an animal's activity pattern in comparison to ecological stressors such as food procurement in the current study, especially since animals were provisioned and may have more heavily relied on foods that were easy to obtain in order to conserve energy. In the Sha *et al.* (2018) study it was also noted that the effect of food resource fluctuation was negligible in the study due to animals being provisioned, which may have influenced activity patterns.

Changing seasons can also lead to variation in the density of natural vegetation, with brush becoming thin or thicker, leading to alterations in the amount of traveling done by Japanese macaques (Hanya, 2004). This further emphasizes that multiple seasonal environmental factors can influence activity and depending on the intensity of weather variables during a given season, the amount and intensity of ecological stress may vary. My broad scope analysis of the effects of activity on cortisol levels may have not been detailed enough to detect the potentially smaller effect size of ecological stressors on semi-free ranging populations.

Aim 4: Relationship Between fT$_3$ and Cortisol Levels

My prediction of a relationship between these hormones was supported, showing a significant positive relationship between fT$_3$ and cortisol levels during both seasons. This positive relationship may be attributable to these hormones' corresponding involvement in primates' energetic condition. The relationship between fT$_3$ and cortisol suggests a coordinated metabolic response to meet energetic demands, whether due to thermal, ecological, or other stressors.

Several hormones are involved in the regulation of metabolism (Norman & Litwack, 1997), such as insulin, glucagon, cortisol, and thyroid hormones (Emery Thompson, 2017). These hormones work in concert to facilitate energy storage, regulate energy homeostasis and regulate basal metabolic rate (Emery Thompson, 2017). While tropical mantled howler monkeys (*Alouatta palliata*) do not experience the same intensity of seasonal changes as Japanese macaques, researchers found that glucocorticoids and thyroid hormones reflect variation in energetic condition (Dias *et al.*, 2017). This research suggests that these hormones offer complementary information on the energetic condition of a primate, supporting the positive relationship I found between fT$_3$ and cortisol levels in Japanese macaques. This comprehensive

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metabolic response may be exercised in response to changes in energetic requirements. While my study found only weak support for an effect of thermal stress, and no effect of ecological stress, the relationship between cortisol and fT_3 nonetheless implies that animals fluctuate these metabolic pathways in response to changing stressors experienced in their environment.

Limitations

Some limiting factors should be taken into consideration when assessing my data, including the previously mentioned impacts of food provisioning, as well as dominance rank and length of data collection period. I sampled a small population of semi-free ranging animals whose food was provisioned. While animals were still able to feed freely on natural vegetation, the consistent availability of food may have altered the intensity of ecological stress that free-ranging animals experience when trying to procure food in their natural environment. Also, dominance rank has shown to be a significant stressor experienced by primates, with varying effects on male and female subjects (Cavigelli & Caruso, 2015; Majolo *et al.*, 2005). Dominant males display glucocorticoid levels at or above the levels seen in subordinate males, while the opposite was found in females (Cavigelli & Caruso, 2015). I did control for individual identity in my statistical analysis, but variations due to hierarchical status may still potentially influence cortisol levels (Majolo *et al.*, 2005). Lastly, my research only assessed one summer and winter season. While a larger collection period may not have yielded different results, a long-term assessment may provide additional insight into gradual alterations in season that may not be discernable with only a discrete two-season assessment. Likewise, significant ecological and thermal changes can occur over the course of several years with notable alterations in temperature and precipitation (Graham *et al.*, 2016). These variations may impact an animal's thermoregulatory capabilities and influence energetic condition (Graham *et al.*, 2016; Mitchell *et*

al., 2009). In addition, yearly thermal differences can impact the environmental landscape, altering the quantity and quality of food supply (Graham *et al.*, 2016; King *et al.*, 2005; Pave *et al.*, 2012; Wiederholt & Post, 2010).

Conclusions

Japanese macaques experience significant thermal and ecological stressors that are interconnected and can dynamically influence an animal's behavior and physiology (Hanya *et al.*, 2007; Sha *et al.*, 2018; Takeshita *et al.*, 2018). In this study, cortisol levels did not show a significant difference between summer and winter, but lower maximum temperatures during the winter season resulted in higher cortisol levels, likely related to thermoregulatory and energetic demands. Japanese macaques' activity patterns did not show a relationship with cortisol levels, which may be attributable to reduced ecological stressors under semi-free ranging conditions. Lastly, it appears that a unified metabolic response occurs between fT_3 and cortisol levels, likely responding to energetic demands during both seasons. This highlights the complexity of thermal and ecological stressors experienced by Japanese macaques and the associated physiological changes. Although this study was not able to fully distinguish the detailed effects of seasonal stressors on hormones, my findings suggests that there is a coordinated metabolic response that may aid animals in meeting thermoregulatory and energetic demands.
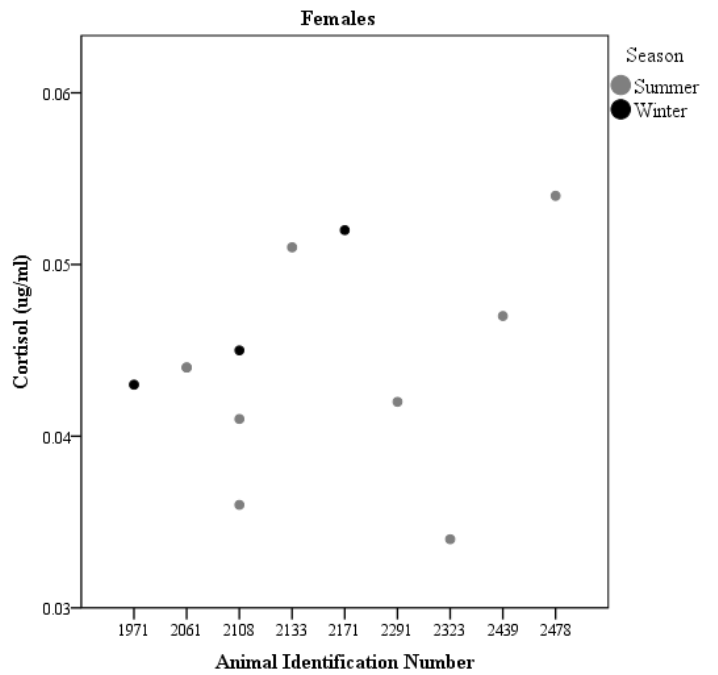
Appendix

Appendix 1. Seasonal cortisol levels by individual animal ID, separated by male (A) and female (B) subjects.

A.)
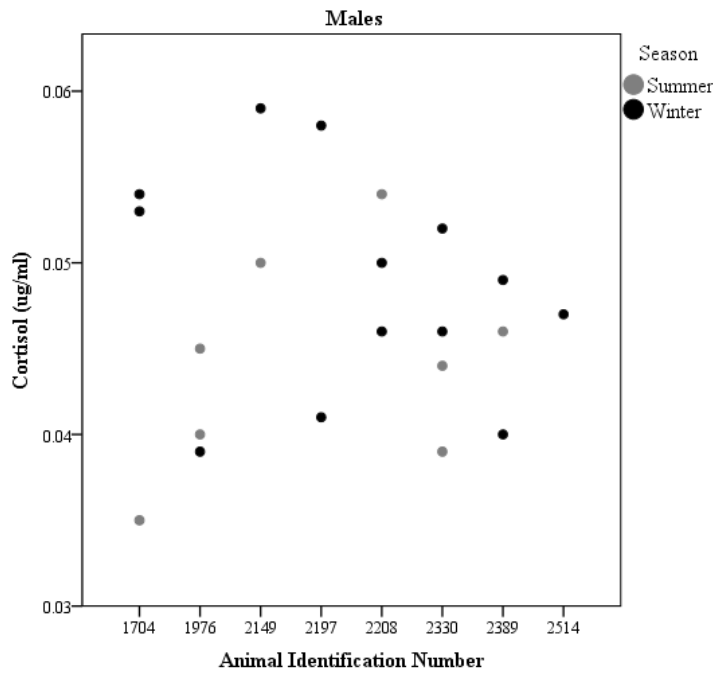
B.)

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